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“Analysis of the regulation of the expression of Kv1.2 potassium channel”

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Que cada día te muevas más por inspiración y menos por exigencia…

DEDICATION

To my father, my family, Salvador, Elisa, Simone, Bruno, all the valuable animals and to me.

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I really hope one day, humans can make science in equilibrium with the other species and the environment.

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ABSTRACT

Neuron excitability is one of the most relevant functions in the nervous system and is controlled by voltage-gated ion channels. **Voltage-gated potassium channels 1 (Kv1)** are critical to maintaining membrane potential during resting. Also, Kv1 channels regulate the frequency, duration, and amplitude of action potentials, contributing to the membrane repolarization after the action potential firing. Alterations in the expression, function and subcellular distribution of neuronal Kv1.2 ion channels have been related to epilepsy and neuropathic pain. Even though Kv1.2 ion channels are one of the most characterized Kv1 channels, the regulation of their expression is poorly understood, and there are no transcription factors that regulate its expression.

To determine the putative transcription factors that control the Kv1.2 gene (KCNA2) we did comparative analysis of its promoter region in KCNA2 orthologous. We selected a region of 1500 bp upstream transcription start site (TSS) and the 5´ untranslated region (UTR). To discover conserved regions with a regulatory function in the KCNA2 promoter through evolution, we analyzed the superorder of placental mammals called **"Euarchontoglires"**, which includes Primates, Dermoptera, Scandentia, Rodentia, and Lagomorpha with a more specific focus in species of Primates. We detected an increase in the repertoire of binding sites of transcription factors (TFBS) in the common ancestor of Catarrhini (14 sites), in the common ancestor of Hominoidea (22 sites) and in human (28 sites) KCNA2. By *in silico* analysis using the MAFFT alignment and transcription factor (TF) prediction tools, in the common ancestor of Euarchontoglires we observed four putative TFBS: a conserved site for Sp1 and AP-2, a site for delta factor or Yin Yang 1 (YY1), a site for C/EBPα and a common site for NF-E2, AP-1 and Fra-l transcription factors.

Additionally, using the Phylogenetic Footprinting strategy, we detected ten structured phylogenetic variations in specific groups, such as Catarrhini, Anthropoidea, and Primates suggesting an additional transcriptional regulation through evolution. Among these variations, at 911 bp upstream the TSS we identify a site for YY1 transcription factors with an adjacent change of a T for a C in Catarrhini, which could affect the binding of YY1 to its site in the species belonging to this group.

This work is the first study that identify possible transcription factors regulating KCNA2 expression. Therefore, additional investigations are necessary to probe these findings.

INTRODUCTION

1. Neuronal excitability

The excitability of a neuron refers to the process in which a synaptic input is converted to an axonal input to transmit an information. This operation is dynamic and is determined mainly by the voltage-gated ion channels (VGIC) located in axons. The axons are highly specialized ducts of the neuron that transmits the information in the form of Action Potentials (APs) to the presynaptic terminals to produce the release of a neurotransmitter. The APs are usually brief so they can follow one another in rapid succession. Given the high repertoire of VGIC, the APs may acquire a wide range of shapes, frequencies, and patterns and therefore, a diverse firing behavior in various types of neurons according to the VGIC expressed (Bean 2017). Moreover, changes in VGIC expression may vary in different contexts as development, injury, or pathology, which increases the plasticity in neuron excitability. VGIC plays a key role in axons for transmitting AP. In mammals, the great length of axons may extend from a few micrometers up to several meters in humans, which constitutes a big challenge for the neuronal machinery. Axons possess a high degree of morphological differentiation, reflecting the functional specialization of subdomains in the axon and facilitating the integration and efficient propagation of AP (Waxman 2001; Hille 2001). Among these structural specializations, the axonal initial segment (AIS) plays a critical role in initiating APs and regulates the neuronal polarity by acting as a barrier that separates the somatodendritic domain from the axonal domain. Also, it is characterized by the specific composition of cytoplasmic components and VGICs. We will briefly describe the main characteristics of these channels in axons.

2. Voltage-gated ion channels in axons

The voltage-gated ion channels are macromolecular pores in cell membranes in charge of modulating the electrical properties of membranes, essential for transmitting information through the neurons. They have evolved for millions of years, producing a high diversity of sodium, potassium, and calcium ion channels (Moran *et al.* 2015). They possess different electrophysiological properties according to their structure, location, and interaction with other proteins. After a membrane potential change, specific voltage-gated ion channels are opened or closed (gating). The open pore has a key property of selective permeability, which allows that

restricted class of small flow passively down their electrochemical activity gradients at a rate of 10^6 ions per second. These fluxes produce immediate changes in the membrane potential and therefore, in the excitability of a neuron. VGIC can be modulated at multiple levels: gene expression, post transcriptional and post translational modifications, and localization. Also, the diversity of VGIC in neuronal subtypes and in the combination with auxiliary subunits increase the excitability plasticity of VGIC, which is the base of nervous system function (Rosati & McKinnon 2004; Schulz *et al.* 2008).

The main voltage-gated ion channels in neurons are voltage-gated sodium channels (Na_vs), voltage-gated calcium channels (Ca_vs) and voltage-gated potassium channels (K_vs). Particularly, in myelinated axons, there are subdomains such axonal initial segment (AIS), Node of Ranviers (NoR), Paranode (PN), Juxtaparanode (JXNP) and presynaptic terminal. Each of them is characterized by the presence of specific voltage-gated ion channels. For example, Na_vs channels are enriched at the AIS and NoR where they are responsible for inward Na current. At presynaptic terminals, Ca_vs channels allow the influx of calcium to promote the release of vesicles containing neurotransmitters. At the AIS, Na_vs channels are in charge of starting the APs. At NoR, Na_vs modulate the saltatory conduction of the APs. In the PN there are no voltage-gated ion channels. Next, in the JXPN, K_vs are enriched, particularly K_v1 channels. K_vs channels are also located in the AIS where they determine the properties of APs (Rasband 2010) In general, K_vs channels are recognized for their role in regulating the overall excitability of the neurons (Kaczmarek 2020). In addition, the voltage-gated potassium channels (K_vs) are the most diverse, structurally, and functionally, of all VGIC and K_v1 channels are mostly located in axons. Considering their predominant role in neuronal excitability and their location in axons, where the electric impulse is transmitted, we will focus our study in these channels.

3. Voltage-gated potassium channels 1, K_v1

Voltage-dependent potassium channels (K_v) are the most diverse family of potassium channels (Connors 2009). There are twelve K_v channel subfamilies, from K_v1 to K_v12, each composed of several members, resulting in 40 different K_v channel subunits (Jensen, Rasmussen, and Misonou 2011). K_v channels are expressed in neurons of the central and peripheral nervous systems and their distribution depends on the neuronal type and function. It is worth noting that

K_v1s are almost exclusively located in axons (Rasband 2010). Therefore, the present study will focus on the K_v1 family, which is the product of the human KCNA gene and the orthologue to the *Shaker* potassium channel from *Drosophila melanogaster* (Misonou & Trimmer 2004). Six K_v1 channels (K_v1.1-K_v1.6) are expressed in the mammalian nervous system (McKeown *et al.* 2008). We will briefly describe the main characteristics of this channel family.

3.1. Structure of K_v1 channels

Functional K_v1 channels are constituted by four α subunits, which can form homotetramers or heterotetramers with other K_v1 α subunits. Nevertheless, the abundance of homotetramers at the plasma membrane is low due to trafficking restrictions. Conversely, heterotetrameric K_v1 channels are abundant, providing different trafficking efficiencies and functional specializations (Misonou & Trimmer 2004). Among axonal Kv1 channels, K_v1.1 homotetramers are mainly located in the ER, K_v1.4 homotetramers are located at the cell surface whereas K_v1.2 homotetramers are located both in the ER and at the cell surface. For instance, when K_v1.1 and K_v1.2 are co-assembled with K_v1.4, the cell surface expression of the heterotetramer increases significantly (Manganas & Trimmer 2000).

The main functional domains of each integral α subunit consist of the tetramerization domain (T1), six transmembrane (TM) domains (S1-S6), extracellular domains, a voltage-sensing domain, a pore-forming domain, and an intrinsically disordered C-terminus. Notably, the N and C-terminal tails are orientated to the cytoplasm and are involved in protein-protein interactions with one another, with portions of the core domain, with auxiliary subunits, and with different channel interacting proteins that contributes to their membrane specific anchorage (Rasband & Trimmer 2006). The tetramerization domain (T1) is critical for the interaction with auxiliary subunit, the Kvβ subunit, while the consensus N-linked glycosylation (NLG) sites and other post-translational modifications modulate the trafficking of Kv1 channels (de Souza & Simon 2002; Watanabe *et al.* 2015; Winklhofer *et al.* 2003). Each subunit also possesses a membrane reentering P-loop between S4 and S5 that forms the bulk of the conduction pathway (Misonou & Trimmer 2004; Robbins & Tempel 2012). An ER-retention (ERR) signal in the external face of the channel pore domain, only present in K_v1.1, K_v1.2, and K_v1.6, is one of the major determinants of channel trafficking. Finally, a cytoplasmic C-terminal PDZ-binding domain (PDZ-BD) is involved in the

interaction with PDZ-domain scaffolding proteins, which regulate the clustering of K_v1 to specific surface membrane microdomains (Misonou & Trimmer 2004) (Figure 1).

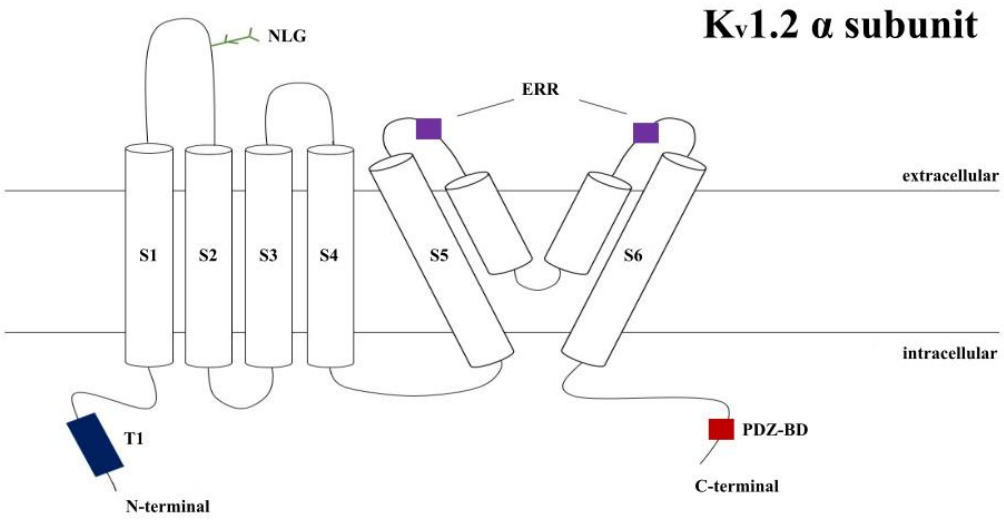

Figure 1. **Structure of the Kv1.2 α subunit**. The α subunit is composed of six transmembrane segments (S1-S6). The pore is located between S5 and S6. The N and C-terminal domains are faced to the intracellular side. At the N-terminal a tetramerization domain (T1) is located. Between S1 and S2, in the extracellular side, is located a N-linked glycosylation region (NLG). An extracellular ER-retention domain is between S5 and S6. At the C-terminal a PDZ-binding domain (PDZ-BD) is in the intracellular side.

The interaction between the heterotetramers and auxiliary $K_v\beta$ subunits also modifies the plasma membrane expression of the channel and provides additional folding and gating characteristics (Manganas & Trimmer 2000). Subunits α and β are assembled in the ER early during biosynthesis and they are subsequently trafficked toward axonal subdomains (Nagaya & Papazian 1997). The most abundant K_v1 α subunit in the mammalian neurons is the $K_v1.2\alpha$ subunit, where approximately 85% of K_v1 heterotetramers are conformed by this component (Ovsepian *et al*. 2016; Utsunomiya *et al*. 2008). For this reason, we will focus our study on $K_v1.2$ potassium channels.

3.2. Function of $K_v1.2$

The main function of $K_v1.2$ channels is controlling neuronal excitability. The $K_v1.2$ channels produce a fast activated and low inactivation threshold delayed rectifier current, which means that they open a few milliseconds after the membrane potential changes. They are mainly located in the axonal initial segment (AIS), the juxtaparanodes, and the pre-terminal segment of

the axon. Due to a combination of their fast, low-threshold activation and their subcellular localization, K_v1 channels significantly impact the axonal action potential by regulating its threshold, waveform, and frequency (Rasmussen & Trimmer 2019). At the AIS, $K_v1.1$ and $K_v1.2$ α subunits play leading roles in determining the properties of action potentials (AP), such as (i) AP timing and firing patterns, (ii) repolarizing the distal AIS, and (iii) establishing the site of spike initiation after an AP has been triggered (Trimmer 2015). On the other hand, juxtaparanodal K_v1 channels prevent repetitive firing and contribute to internodal resting potential (Poliak & Peles 2003; Rasband 2004). They further regulate the release of neurotransmitters from presynaptic terminals (Yang *et al*. 2014). Mutations in the human $K_v1.2$ gene are associated with early infantile epileptic encephalopathy-32 (EIEE32) (Pena & Coimbra 2015) and in mice cause ataxia and convulsions (Xie *et al*. 2010).

Several studies suggest that $K_v1.2$ decrease contributes to hyperexcitability and peripheral sensitization observed in several neuropathic pain animal models (Ishikawa *et al*. 1999; Kim *et al*. 2002; Zhao *et al.* 2013; Fan *et al*. 2014). No pain studies have been performed in $K_v1.2$ *knocked out* mice because they died within 2-3 weeks after birth because of several seizures, indicating a non-redundant and unique role of this channel (Brew *et al*. 2007). Conversely, a slight reduction in auxiliary $K_v\beta2$ subunit has been observed in sensory neurons after nerve damage (Park *et al*. 2003; Rasband *et al*. 2001).

While the regulation of the K_vs function is very well described (Rasmussen & Trimmer 2019), there is scarce evidence about the molecular mechanisms controlling $K_v1.2$ mRNA expression. The KCNA2 gene encodes for $K_v1.2$ channel and needs to be transcribed to an mRNA for their later translation and axonal localization. However, the mechanisms underlying the regulation of the transcription are scarce. Particularly, there are no studies focusing on the transcription factors regulating the expression of KCNA2 gene.

3.3. Transcriptional control of KCNA2 gene expression

The transcription of KCNA2 is performed by RNA polymerase II with many proteins called general transcription factors (TFs), which help to position RNA polymerase II correctly at the promoter, aid in pulling apart the two strands of DNA to allow transcription to begin. The TFIID is the protein that starts the transcription by binding to a short double-helical DNA sequence

primarily composed of T and A nucleotides (TATA box) and is typically located 25 nucleotides upstream from the transcription start site (TSS) (Alberts *et al*. 2007). Not all promoters have a TATA box, but the transcription occurs anyway (Dusing & Wiginton 1994; Singh *et al*. 2012; Emami 1998). In fact, the KCNA2 promoter region does not contain a TATA-box or other consensus elements near the TSS. Transcriptional activators or transcription factors (TFs) recognized specific sequences of DNA (typically 5 – 10 nucleotides pairs in length) that are often called *cis*-regulatory sequences because they must be on the same chromosome (that is, in *cis*) as the genes they control. According to their structural motifs, there are different types of TFs, which include helix-turn-helix protein, homeodomain proteins, leucine zipper proteins, β sheet DNA recognition proteins, zinc finger proteins, and helix-loop-helix proteins. Many TFs usually act as homodimers or heterodimers, depending on their interaction motifs (Alberts *et al*. 2007).

As mentioned above, there is scarce evidence about TFs regulating KCNA2 expression. However, some studies have been focusing on other voltage-gated potassium channels. For example, K^+ channels belonging to the K_v1 family such as $K_v1.3$ (KCNA3 gene), $K_v1.4$ (KCNA4 gene), and $K_v1.7$ (KCNA7 gene) have been controlled by Specific Protein 1 (Sp1) transcription factor. Sp1 is a ubiquitously expressed protein belonging to the zinc finger family of transcription factors that bind GC-rich elements in the promoter of many housekeeping and tissue-specific genes. Sp1 contains a highly conserved DNA-binding domain consisting of three zinc fingers (Philipsen & Suske 1999), which interacts with GC boxes to stimulate transcription initiation through the recruitment of the TBP-TFIID complex in TATA-less promoters (Wierstra 2008). The promoter of KCNA3, expressed in T cells, macrophages, osteoclasts, platelets, and in the brain, contains a GC-rich, TATA-less region and is regulated by enhancers (Simon *et al*. 1997). In cancer cells, Sp1 regulates the nuclear expression of the Kv1.3 potassium channel, which has a role in modulating nuclear membrane potential and activation of other TFs such as CREB (Jang *et al*. 2015). Sp1 transcription factor has also been described as a regulator of murine $K_v1.5$ potassium channel in vascular smooth muscle during physiology and pathology (Fountain *et al*. 2007). Additionally, the human $K_v1.7$ promoter has a binding sequence for Sp1 in skeletal muscle, however, no functional experiments have been developed (Kashuba *et al*. 2001). Members of K_v3 (Gan *et al.* 1996), K_v4 (Li *et al*. 2012), K_v7 (Mucha *et al*. 2010; Masuda *et al*. 2018; Lee *et al* 2014), K_v8 (Ebihara *et al*. 2004), and K_v9 (Lee *et al*. 2015) families seem also to be regulated by

Sp1. For example, even if, four Sp1 binding sequences have been described in the K_v3.1 promoter, only a CRE element has been probed to modulate K_v3.1 expression in PC12 cells, suggesting that the expression of this channel could be regulated by cAMP levels in these cells (Gan *et al.* 1996). Regarding K_v7 family members, interestingly, the decrease in the expression of K_v7.2 and K_v7.3 potassium channels was associated with hyperexcitability in sensory neurons. The promoter region of these channels contains a binding sequence for Sp1 which corresponds to a GC box region that is evolutionarily conserved. Experiments in vitro confirm that this TF binds to K_v7.2 and K_v7.3 promoters enhancing their expression. On another side, the repressor REST diminished K_v7.2/7.3 expression promoting the hyperexcitability state observed in epilepsy and pain (Mucha *et al*. 2010). K_v7.1 and K_v7.5 potassium channels have also been regulated by Sp1 in excitable cells (Masuda *et al*. 2018; Lee *et al.* 2014).

While there are many studies about transcription factor regulation of other K_v channels, K_v1.2 does not have known transcription factors. Importantly, we cannot assume that the TFs that regulate other K_v´s regulates K_v1.2. Thus, how can we study the regulation by TFs of a gene?

3.4 Phylogenetic Footprinting as a strategy to identify binding sites for transcription factors

One of the refined strategies to study gene expression regulation is by comparing our interest gene among related species. This, since we can assume that regions with a regulatory function in a gene are most probable conserved. Therefore, we can analyze them and search specific conserved regions through evolution. To study the human K_v1.2 we could study the orthologues in other primates and other related species. Comparing current species (*i.e.*, human, chimpanzee, macaques, lemurs, mouse and rat), it is possible to infer what happened in the past since few sequences are conserved. This strategy, called "Phylogenetic Footprinting" (Tagle *et al.* 1988), allows us to infer the regulatory elements in a common ancestor of interest. While the coding sequence of the KCNA and their corresponding amino acid sequence of the Kv1.2 potassium channel is studied; the promoter region has not been studied yet. So, we can expect that the differential regulation of KCNA2 gene is not given by changes in coding sequence, but by regulatory sequences. With this strategy, we can infer a stock of regulatory elements in the common ancestors of each group included in Euarchontoglires.

Phylogenetic Footprinting has been widely used to identify possible binding sites for transcriptional factors with the posterior *in situ* experiments to validate the initial predictions. For example, a comparative study in the sestrin3 promoter region of primates and rodents was used to detect 20 conserved motifs and identify two transcription factors regulating the expression of sestrin3 gene: SOX2 and FOXO3 transcriptionally regulate the expression of this gene (Srivastava *et al.* 2016).

For this thesis, we selected the Primates since in this group there are the most human-related species. Also, we included Dermoptera and Scandentia order because of their evolutive nearness to Primates. As most of the experiments to study $K_v1.2$ channels are performed in rodents, we also included species of the order Rodentia. The Lagomorpha order is the closest relative to Rodentia, so we included it in the analysis too. All these mammalian orders constitute the superorder Euarchontoglires, so therefore we analyzed representative species belonging to this, with a special emphasis in Primates. Their phylogenetic relationships, common ancestors of each group and their estimated age is detailed in Figure 2.

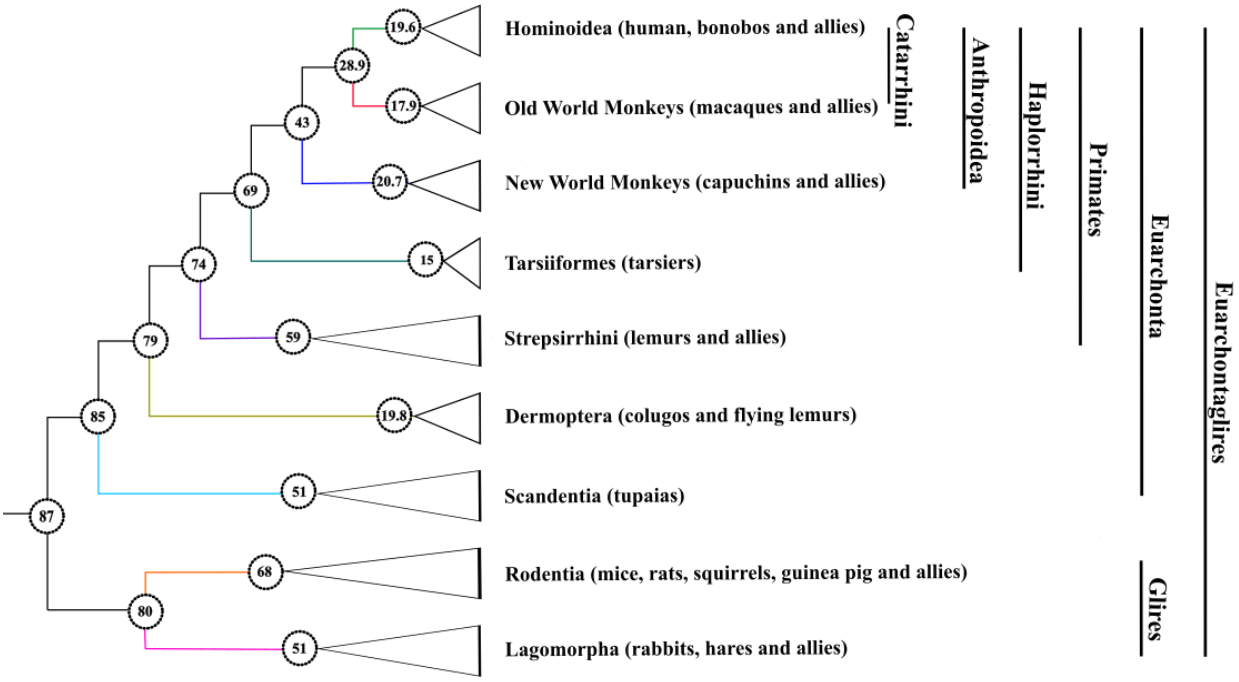

Figure 2: Phylogenetic relationships among *Euarchontoglires*. The phylogenetic tree shows the studied taxa. The main orders are Primates, Scandentia, Dermoptera, Rodentia and Lagomorpha (Perelman *et al.* 2011; Blanga-Kanfi

et al. 2009). The numbers in circles indicate the date of divergence expressed as million years (my). For details of each group see Table 2. The phylogenetic relationships were obtained from most recent literature (Perelman *et al*. 2011; Lang *et al*. 2022; Zhang *et al*. 2022; Cano-Sánchez *et al*. 2022; Fleagle & Seiffert 2020) and the divergence time from TimeTree 5 (Kumar *et al*. 2022)**.**

3.5 Other mechanisms controlling Kv1.2 mRNA expression

While no clear transcription factors have been described for KCNA2, some proteins involved in epigenetic changes have been studied. In neuropathic pain, where K^+ current decreases, increased activity of euchromatic histone-lysine N-methyltransferase-2 (G9a) histone was observed. G9a promoted the demethylation of Lys9 on histone H3 (H3K9me2) at the promoter of KCNA4 (K_v1.4), KCND2 (K_v4.2), KCNQ2 (K_v7.2), and KCNMA (KCa1.1) genes, which then, induced a decrease in K^+ current. The effect on K_v channels was reversed by G9a inhibition (Laumet et al., 2015). Also, G9a overexpression reduces the K_v1.2 mRNA levels in sensory neurons (Liang et al., 2016). Additionally, the acetylation state of the histone controls the gene expression of K_v potassium channels. Li *et al.*, (2019) described that Histone Deacetylase 2 (HDAC2) is involved in neuropathic pain after nerve injury by decreasing K_v1.2 expression (Li *et al.*, 2019). In the same line, nerve injury provoked an increment in the expression of DNA methyltransferase DNMT3a, a repressor of gene expression. The increase in DNMT3a was induced by the activation of the Octamer transcription factor 1 (Oct-1). Conversely, the promoter of the KCNA2 gene was methylated by Oct-1/DNMT3a activation after injury, promoting gene repression of the K_v1.2 potassium channel. These findings suggest that DNMT3a may contribute to neuropathic pain by KCNA2 repression in sensory neurons (Zhao *et al*., 2017). Another regulator of DNMT3a seems to be the epigenetic repressor methyl-CgG-binding domain protein (MBD1) by recruitment of DNMT3a to the KCNA2 promoter region during neuropathic pain development and therefore producing a decrease in K^+ current conducting to hyperexcitability in sensory neurons (Mo *et al*., 2018).

Once the mRNA exists the nucleus, it may be subject to post-transcriptional mechanisms. Long non-coding RNA (lncRNA) have been demonstrated to regulate the expression of the channel. The Kcna2 antisense RNA is expressed in DRG neurons and increased after peripheral nerve injury through activation of myeloid zinc finger protein 1, a transcription factor that binds to Kcna2 antisense RNA gene promoter. This RNA downregulates K_v1.2 potassium channel and

reduces total K^+ current, incrementing excitability in DRG neurons and producing neuropathic pain symptoms (Zhao *et al*., 2013). This lncRNA also contributes to ventricular arrhythmias by $K_v1.2$ silencing (Q. Long et al., 2017). By other side, the miRNA mi-R-17-92 seems to downregulate the expression of $K_v1.1$ and $K_v1.4$ channels and the K^+ currents after nerve injury in peripheral neurons (Sakai et al., 2017).

The localization of Kv1.2 mRNA in axons could be another control point of the channel expression. Previous studies have been detected ion channels mRNAs in axons (Thakor *et al*., 2009; Ruangsri *et al*., 2011) and axonal Kv1.2 mRNA has been described in hippocampal neurons (Biever *et al*., 2020). The transport of mRNAs could be mediated by cytoskeletal motors, random diffusion through the cytosol or by a combination of random diffusion and protein complex that protects the mRNA from degradation (Lipshitz & Smibert 2000). The axonal mRNA transport is performed by RNA-Binding Proteins (RBPs) associated with Ribonucleoparticles (RNPs). RBPs usually recognize specific nucleotides signals in the 5´ or 3´UTR and/or secondary/tertiary structures of a mRNA. Most RBPs assemble on mRNAs at the moment of transcription and shepherd it to its destination (Di Liegro *et al*., 2014). Regulation of mRNA localization has a special role in neurons because local translation of pre-localized mRNA in axons has been implicated in development and injury response

4. Biomedical relevance

After peripheral nerve injury, a spontaneous regenerative process called Wallerian Degeneration occurred (Dubový, 2011) which is necessary to promote the regeneration of the proximal stump. The action of the injured axon, activated-Schwann Cells (SCs), immune cells and target tissue combined with multiple factors such as neurotrophic factors (NGF, BDNF and NT-3), inflammatory mediators (prostaglandins, IL-6, among others) and extracellular matrix (ECM) proteins may intervene in the proper axonal regeneration. It is interesting to note that, the damage-induced changes are thought to recapitulate some of the developmental processes that occur before synapse formation; that is, switching the neuron from an active electrically transmitting state, back to an electrically silent, growth-competent state (Mahan & Cavalli, 2018). After peripheral nerve damage, there are changes in the injured/regenerating nerve; the soma of injured nerves in the DRG and similarly; in neighboring intact afferents, defined as peripheral sensitization. To make

sense of this, a co-evolution of damage repair mechanisms and nociceptive sensibility has been proposed (Melemedjian & Khoutorsky, 2015). Alterations in electrophysiological discharge patterns are observed in type-C fibers, in type-Aβ fibers and in new branches formed after the lesion in animals. Spontaneous activity and a decrease in activation threshold in these fibers were observed, which means that their action potentials were triggered with minor stimuli. Consequently, these two alterations produce an hyperexcitability state in the fibers, which increase the susceptibility and the frequency of discharge (Campbell & Meyer, 2006); Costigan et al., 2009; Meachan *et al.*, 2017). This state is promoted by a decrease in the levels of Kv1.2 potassium channel (Ishikawa *et al*., 1999; Kim *et al*., 2002; Park *et al*., 2003; Zhao *et al*. 2013; Fan *et al*., 2014; Li *et al*., 2015; Laumet *et al*., 2015; Liang *et al.*, 2016; Li *et al*., 2017; Li *et al*., 2019; Mo *et al*., 2018; Yuan *et al*., 2020; Zhang *et al*., 2020).

Even though peripheral nerves regenerate after nerve injury, functional recovery does not always occur. Most regenerative studies have been performed in mouse models of nerve injury. However, in humans, the prospect is less optimistic given the great length of peripheral nerves (*e.g.*, the sciatic nerve). Therefore, the regeneration is usually incomplete or maladaptive, leading to inadequate recovery of sensory and/or motor functions. Consequently, one of the possible alterations after peripheral nerve injury in the somatosensorial system is the neuropathic pain, a pathology where in the absence of nociceptive stimulus, a painful stimulus is transmitted from peripheral nerves to central nervous system. The establishment of peripheral sensitization is the first step in the development of the neuropathic pain. Therapeutic strategies to reverse this condition are key in preventing the progression of the disease as well as its symptoms such as spontaneous pain, allodynia (pain in absence of stimulus) and/or hyperalgesia (Gordon, 2016; Silva *et al*., 2017)

The identification of possible transcription factors regulating Kv1.2 expression could show us new therapeutic targets to nerve injury treatment and neuropathic pain prevention.

Summary

The main ideas are:

- Plasticity in neuronal excitability is mainly regulated by voltage-gated ion channels.

- Voltage-gated potassium channels are fundamental in the axonal transmission of the action potential, where $K_v1.2$ has the main role in regulating neuronal basal excitability.
- $K_v1.2$ mRNA levels can be modulated at multiple levels, however there are not information about their regulation by transcription factors.
- The comparison of promoter region of an interest gene in related species to human (Phylogenetic Footprinting) represents a powerful strategy to detect conserved regions with a possible regulatory function such as binding sites for transcription factors.

Considering these, we propose that:

HYPOTHESES

1. *"*Using an evolutionary strategy by comparing upstream sequences in a phylogenetic framework we will find *conserved regions in KCNA2 promoter that are putative binding sequences for transcription factors in species belonging to Euarchontoglires"*

General aim

1. To identify binding sequences for transcription factors in the promoter region of the KCNA2 gene in Euarchontoglires using an evolutionary strategy.

Specific aims

1. Recognize binding sites for transcription factors in the promoter region of the human KCNA2 gene that putatively regulate its expression.

2. Identify putative binding sites for transcription factor in the promoter region of KCNA2 gene in species representative of all main groups of Primates.

3. Detect conserved regions in the promoter of KCNA2 gene, that putatively are binding sites for transcription factors, in the last common ancestor of Euarchontoglires.

4. Identify phylogenetically structured variations in the promoter region of KCNA2 gene that may be regulatory elements for $K_v1.2$ potassium channel expression.

MATERIALS AND METHODS

1.Identifying transcription binding sites for transcription factors of KCNA2 gene

1.1 Annotation process

We obtained the human KCNA2 gene sequence from Ensembl v107 (Cunningham *et al*. 2022). This was selected as our referenced sequence: transcript ID *ENST00000316361.10* (*NM_004974.4* accession number), which corresponded to a sequence of 11829 bp (only exons) located in reverse strand on chromosome *1:110,593,580 - 110,606,358*. The transcript has 3 exons and encodes for a protein of 499 amino acids. To minimize errors in the annotation of the sequences, using Geneious Prime software (Geneious Prime® 2022.1.1) we manually annotated the KCNA2 gene in representative species of the following groups: Hominoidea (i.e. human, chimpanzee, among others), Old World Monkeys (i.e. olive baboon, macaque, among others), New World Monkeys (Bolivian squirrel monkey, capuchin, among others), Tarsiiformes (tarsier), Dermoptera (sunda flying lemur), Scandentia (chinese tree shrew), Strepsirrhini (Coquerel's sifaka, bushbaby, among others), Rodentia (mouse, rat, among others) and Lagomorpha (rabbit) (Table 1). We verified the KCNA2 sequence of all species annotated. We did this because we figured it out that even though, multiple genomes are available, the KCNA2 exons and coding sequence were not well annotated. First, we identified the location of KCNA2 genes in Ensembl v107 (Cunningham *et al*. 2022). Once identified, genomic pieces were extracted including 2500 bp toward 5´ and 3´ from KCNA2 gene. After sequence extraction we annotated the upstream region (we considered 1500 bp upstream the transcription start site, TSS) and the 5´ Untranslated Region (5´UTR). To annotate the exons and coding sequence of KCNA2 orthologues, we compared the sequence of the human (query sequence) channel to the species of which the genomic piece (subject sequence) is being annotated using the program Blast2seq v2.5 from the NCBI website (Altschul *et al*. 1990) with default parameters. We used the human KCNA2 sequence for annotation of all the species belonging to the Euarchonta clade. We performed the same procedure for species belonging to the Glires clade using the mouse (*Mus musculus*) KCNA2 sequence as a query sequence.

1.2. Alignment procedure

We align the selected sequences of *Euarchontoglires* using the software MAFFT v7.450 (Katoh and Standley 2013; Katoh et al. 2002). The specifications for MAFFT alignment were *algorithm= auto* (FFT-NS-2 strategy, according to data size); *scoring matrix= 200 PAM/k=2*; *gap open penalty= 1.53* and *offset value = 0.123.* We did multiple alignments considering 1500 bp upstream the TSS and 5´untranslated region (5´UTR) regions. We decided to do sequential alignments including one group at a time: *i.e.*, alignment 1 corresponds to superfamily Hominoidea, alignment 2 corresponds to the parvorder Catarrhini (Hominoidea + Cercopithecoidea (Old World Monkeys) superfamilies), alignment 3 corresponds to infraorder Anthropoidea (Catarrhine + Platyrrhini (New World Monkeys)). Alignment 4 includes Tarsiiformes forming the suborder Haplorrhini (Anthropoidea + Tarsiiformes). Alignment 5 includes both suborders Haplorrhini and StrepTYO97sirrhini forming the order Primates. Alignment 6 includes the orders Primates, Dermoptera and Scandentia constituting the Euarchonta group. The final alignment includes Rodentia and Lagomorpha (Euarchontoglires group). We did not include those species with Ns in their sequences (showed alignments), except tarsier (*Carlito syrichta*), sunda flying lemur (*Galeopterus variegatus*), and chinese tree shrew (*Tupaia belangeri*), because they are the unique species in the groups Tarsiiformes, Dermoptera, and Scandentia respectively.

1.3. Selection of conserved sequences

After the alignment procedure, we searched transcription binding sites for transcription factors using two strategies: a) prediction based on a bioinformatic tool and b) an evolutionary approach based on the Phylogenetic Footprinting.

a) We use the *EMBOSS 6.5.7 program tfscan* based in the Transfac database (Wingender et al. 1997). We considered a minimum length of 7 bp for the prediction of binding sites for transcription factors of vertebrate class. No mismatches were allowed in these analyses.
b) Based on the principle of phylogenetic footprinting described by Tagle (Tagle *et al.* 1988) we proposed searching binding sites for transcription factors. Briefly, this approach is used to identify transcription factor binding sites within a non-coding region of DNA of interest by comparing this region to the orthologous sequences in different species. These regions

are usually short in length and can exhibit sequence variation. In our case, we compared species of the Euarchontoglires clade (Figure 2 and Table 1). The principles of Phylogenetic Footprinting are two: 1) the function and DNA binding preferences of transcription factors are well-conserved and 2) Non-coding DNA sequences that have crucial functions such as for the regulation of gene expression should not show changes or present minimal changes in different species. And a slower rate of change occurs in regions with key functions, such as transcription factor binding sites, than in other regions of the non-coding genome. With this information, we selected those conserved regions with equal or more than identical 7 bp.

RESULTS

Specific aim 1. Recognize binding sites for transcription factors in the promoter region of the human KCNA2 gene that putatively regulate its expression.

According to the literature (Ebihara *et al.* 2004; Mucha *et al*. 2010; Q. Li *et al.* 2012; Jang *et al.* 2015; Lee *et al.* 2014), we selected 1500 bp upstream of the transcription start site (TSS) (the main transcription factors that control K_v expression are located up to 1500 bp upstream of TSS) and also the 5´untranslated region (5´UTR) to analyze putative binding sites for transcription factors. We repeated the same procedure in representative species of all mammalian orders belonging to Euarchontoglires (Figure 2), which includes Primates, Dermoptera, Scandentia, Rodentia, and Lagomorpha. For details, see Table 1 (Appendix 1).

First, we search binding sites for transcription factors using the *EMBOSS 6.5.7 program tfscan* considering a minimum length of 7 bp in the human KCNA2 sequence. We detected a total of 28 binding sites for transcription factors. In the 1,500 bp upstream the TSS, we observed 8 putative binding sites for Sp1 transcription factor, 2 sites for GATA-1 transcription factor, and 1 site for MAZ, CAC-binding protein, NF-Zc, delta factor or YY1, GATA-1, C/EBPα and LyF-1 transcription factors in the indicated positions (in blue the position relative to TSS and in black the position relative to start codon ATG). In the 5´UTR we observed 3 putative binding sites for CTF transcription factor, 2 sites for C/EBPα and AP-1 transcription factors and 1 site for Sp1, Oct-1c, Pit-1a, CIIIB1, NF-E2, FraI, Pu/box binding factor, URTF, AP-2 and MEP-1 transcription factors (Figure 3).

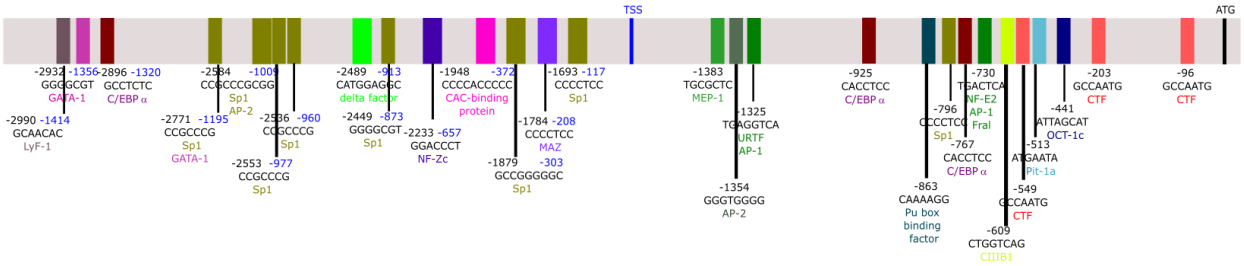

Figure 3*:* Predicted binding sites for transcriptional factors in human KCNA2. The image shows 1,500 bp upstream of the transcription start site of the KCNA2 gene and its 5´UTR. Predicted binding sites for different transcription factors are represented by color code. The corresponding sequence and location represent each binding site with respect to the TSS (blue) and/or the ATG (black). TSS: Transcription Start Site. ATG: first codon

Specific aim 2. Identify putative binding sites for transcription factor in the promoter region of KCNA2 gene in species representative of all main groups of Primates.

Using the above alignment, we applied the *EMBOSS 6.5.7 tool tfscan* for transcription factor prediction in the common ancestor of each group of Primate order, *i.e.*, Hominini, Hominoidea, Catarrhini, Anthropoidea, Haplorrhini and Primates) (Kumar et al. 2017).

First, we consider human and chimpanzee to identify putative BSTF in the common ancestor of Hominini (alignment 1). A total of 26 BSTF were inferred, one less that in human KCNA2 promoter (Figure 4), which suggests a common regulation in the expression of KCNA2 in these species. Next, we included species such as gorilla, orangutan, and gibbon (alignment 2). In the common ancestor of Hominoidea we identify a total of 22 BSTF, 10 of them are located 1500 bp upstream of the TSS and 12 sites are in the 5´UTR. Most of these sites are conserved to those observed in the human and common ancestor of Hominoidea prediction, suggesting that similar transcription factors could regulate the expression of KCNA2 in humans and other hominids.

The Old World Monkeys with Hominoidea group are included in Catarrhini group. Before to estimate the conserved sequences in Catarrhini ancestor, we identified the BSTF in the ancestor of Old World Monkeys. We did this to detect if there are BSTF that are unique for hominid KCNA2. We identified a total of 21 BSTF, 12 of the located in the 1500 bp upstream the TSS and

the other 9 in the 5´UTR. To note, only in this ancestor appeared two sited for myogenin transcription factor, one site for MEP-1 in a different position compared to ancestor of Hominoidea, and an additional site for both AP-2 and MAZ. In Hominoidea, the exclusive BSTF were a CTF site located 203 bp upstream the start codon ATG, a CIIIB1 site, the site for C/EBPα located at -925 and at -2896 respect to start codon ATG, a CAC-binding protein site, a MEP-1 site and and additional Sp1 site. These results suggest a differential regulation in both groups, Hominoidea and Old World Monkeys.

Next, we included in the analysis the Old World Monkeys with Hominoidea, forming the Catarrhini group (alignment 4). In these, we detected 6 binding sites for transcription factors in the 1500 bp upstream of the TSS and 8 binding sites in the 5´UTR (Figure 7), a total of 14 BSTF. Going back in time, the New World Monkeys was the next group included which with Catarrhini formed the Anthropoidea group (alignment 5). Only 3 binding sites were predicted in the upstream region of the TSS and 5 binding sites in the 5´ UTR (Figure 8), a total of 8 BSTF. The Haplorrhini group includes Anthropoidea plus Tarsiiformes (alignment 6); owing to the available information, only one species was included in this taxon: the tarsier. The sequence of tarsier was not completely sequenced, a segment of N´s is located between -997 bp and -553 bp upstream of the TSS (black box named a), so therefore in these segments we considered the binding sequences conserved in the previous group (Anthropoidea). The sequence of the other region is available, so in summary, the binding sites for transcription factors for this group are seven, three located upstream of the TSS and four in the 5´ UTR (Figure 9). Strepsirrhini is the last group included in the Primates (alignment 5). In this, only five BSTF were conserved, two in the upstream region of the TSS and three in the 5´UTR (Figure 10).

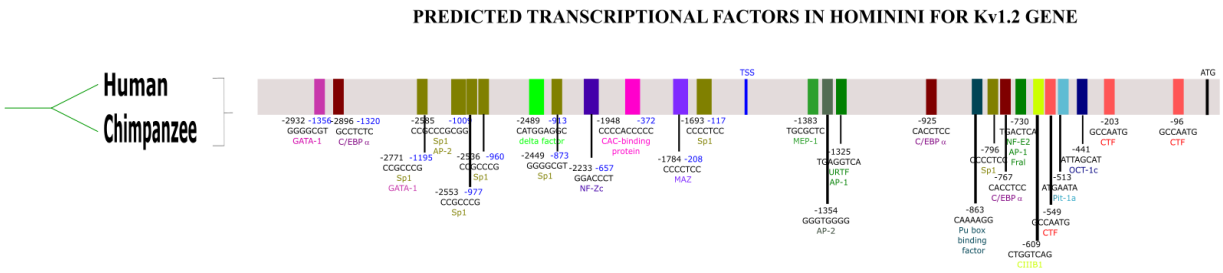

Figure 4. Predicted binding sites for transcription factor of KCNA2 in the common ancestor of Hominini (alignment 1). The image illustrates the predicted binding sites for transcriptional factors in the Hominini group composed of the human and chimpanzee. These predictions were obtained by the alignment of the region of 1500 bp

upstream of the TSS and the 5´UTR of KCNA2 genes of the analyzed species. Each binding site is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). TSS: Transcription Start Site. ATG: first codon.

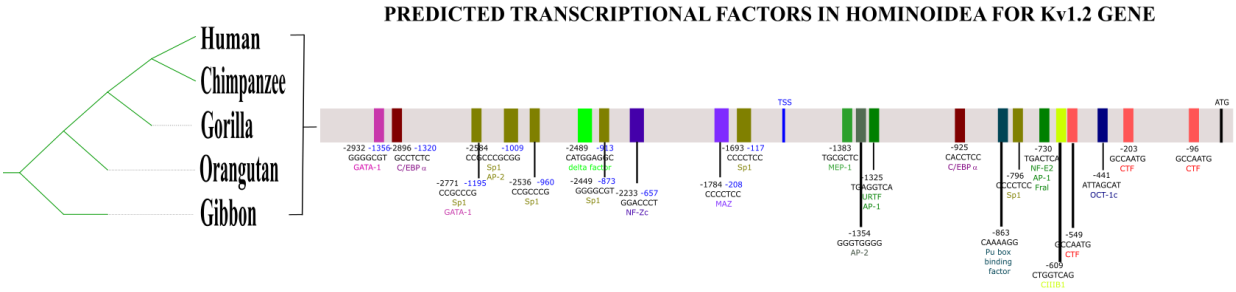

Figure 5. Predicted binding sites for transcription factors of KCNA2 in the common ancestor of Hominoidea (alignment 2). The image illustrates the predicted binding sites for transcriptional factors in the Hominoidea group composed of the human, chimpanzee, gorilla, orangutan, and gibbon. These predictions were obtained by the alignment of the region of 1500 bp upstream of the TSS and the 5´UTR of KCNA2 genes of the analyzed species. Each binding site is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). TSS: Transcription Start Site. ATG: first codon.

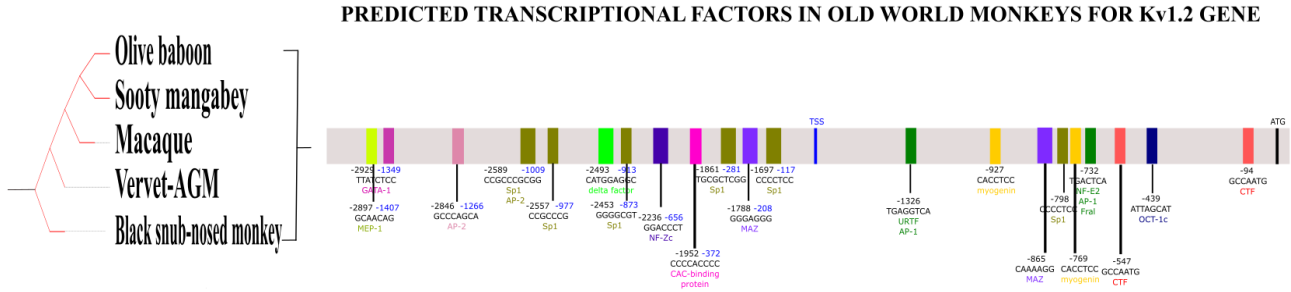

Figure 6. Predicted binding sites for transcription factors of KCNA2 in the common ancestor of Old World Monkeys (alignment 3). The image illustrates the predicted binding sites for transcriptional factors in the Old World Monkeys group composed of the olive baboon, sooty mangabey, macaque, vervet AGM and black snub-nosed monkey. These predictions were obtained by the alignment of the region of 1500 bp upstream of the TSS and the 5´UTR of KCNA2 genes of the analyzed species. Each binding site is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). TSS: Transcription Start Site. ATG: first codon. AGM: African Green Monkey

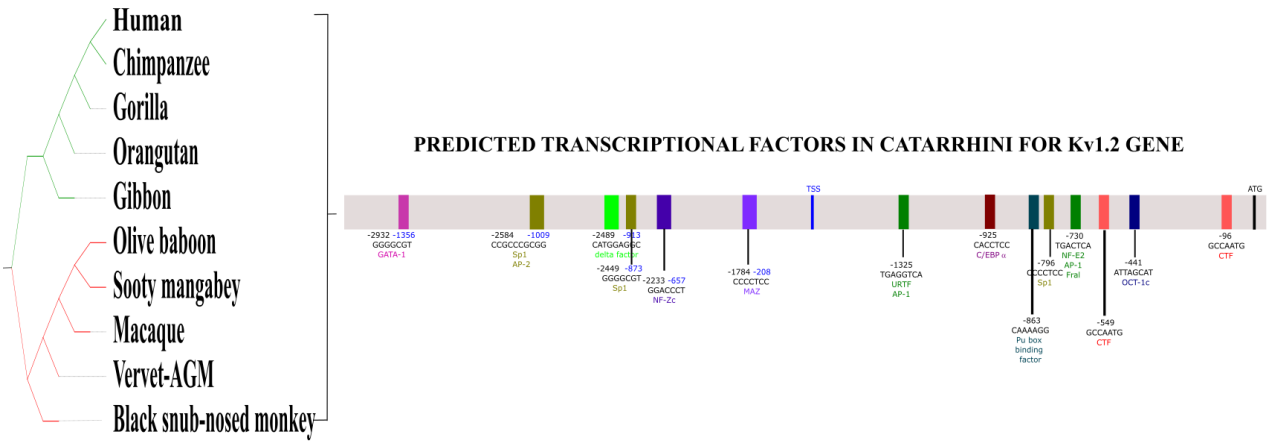

Figure 7. Predicted binding sites for transcription factors of KCNA2 in the common ancestor of Catarrhini (alignment 4). The image illustrates the predicted binding sites for transcriptional factors in the Hominoidea (green lines) and the Old World Monkeys (red lines) groups. These predictions were obtained by the alignment of the region of 1,500 bp upstream of the TSS and the 5´UTR of KCNA2 genes of the analyzed species. Each binding site is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). AGM: African Green Monkey; TSS: Transcription Start Site; ATG: first codon.

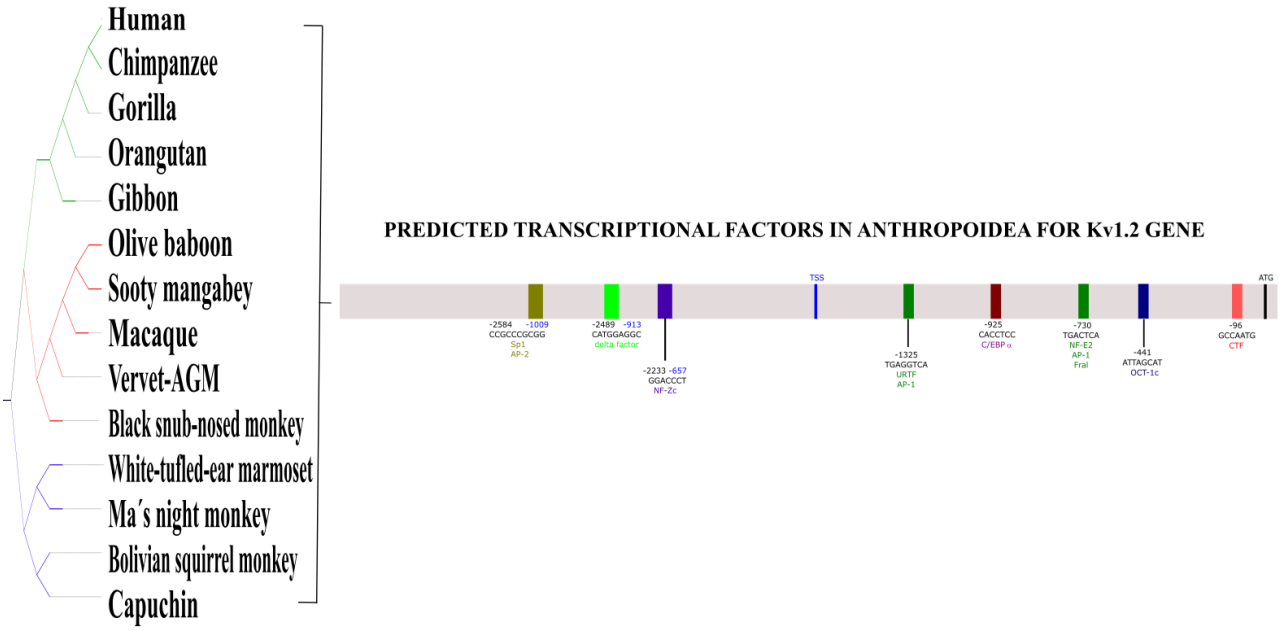

Figure 8. Predicted binding sites for transcription factors of KCNA2 in the common ancestor of Anthropoidea (alignment 5). The image illustrates the predicted binding sites for transcriptional factors in the Hominoidea (green lines), Old World Monkeys (red lines), and New World Monkey (blue lines) groups. These predictions were obtained by the alignment of the region of 1,500 bp upstream of the TSS and the 5´UTR of KCNA2 genes of the analyzed species. Each binding site is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). AGM: African Green Monkey; TSS: Transcription Start Site; ATG: first codon.

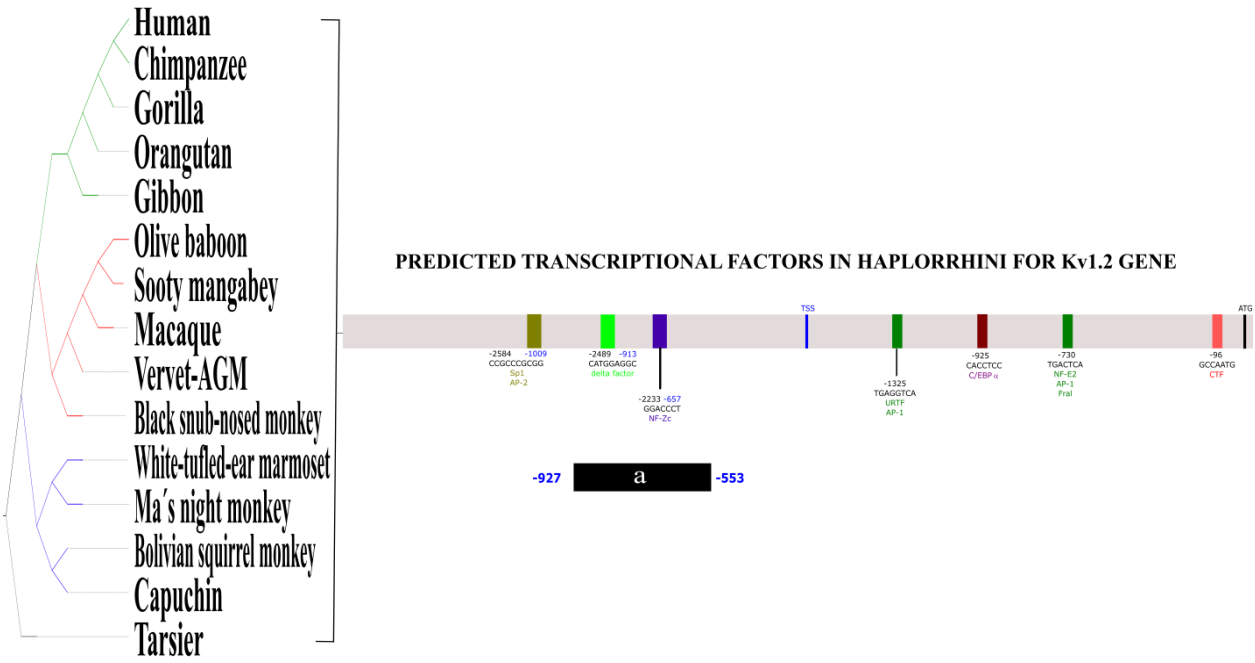

Figure 9. Predicted binding sites for transcription factors of KCNA2 in the common ancestor of Haplorrhini (alignment 6). The image illustrates the predicted binding sites for transcriptional factors in the Hominoidea (green lines), Old World Monkeys (red lines), New World Monkey (blue lines), and Tarsiiformes (turquoise lines) groups. These predictions were obtained by the alignment of the region of 1,500 bp upstream of the TSS and the 5´UTR of KCNA2 genes of the analyzed species. Each binding site is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). The black box represents regions without information in Tarsiiformes. AGM: African Green Monkey, TSS: Transcription Start Site; ATG: first codon.

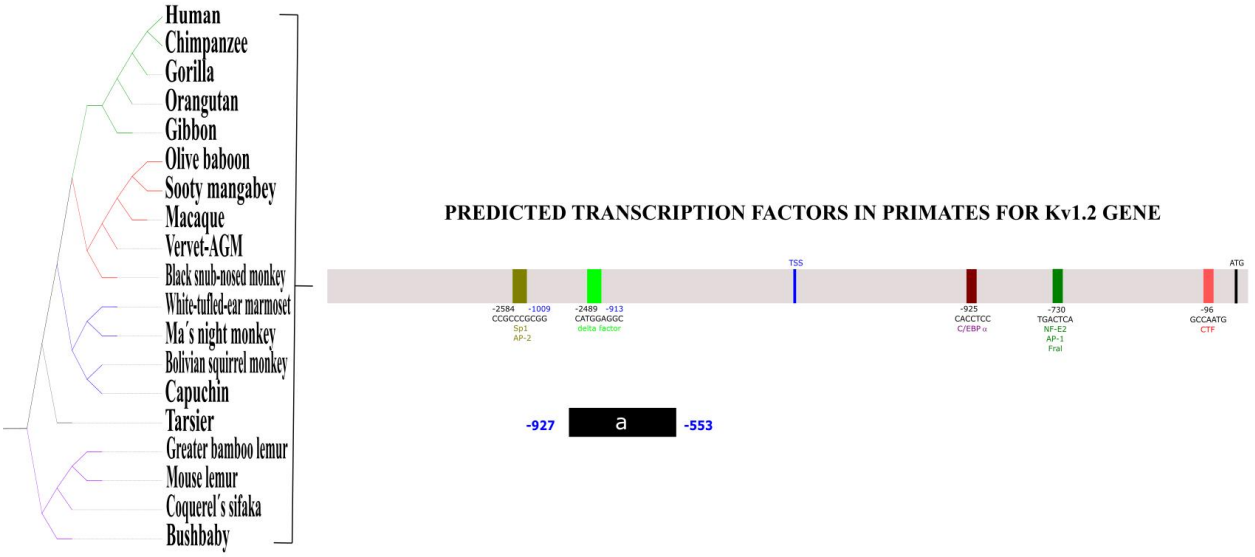

Figure 10. Predicted binding sites for transcription factors of KCNA2 in the common ancestor of Primates (alignment 7). The image illustrates the predicted binding sites for transcriptional factors in the Hominoidea (green lines), Old World Monkeys (red lines), New World Monkey (blue lines), Tarsiiformes (turquoise lines), and Strepsirrhini (purple lines) groups. These predictions were obtained by the alignment of the region of 1,500 bp upstream of the TSS and the 5´UTR of KCNA2 genes of the analyzed species. Each binding site is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). The black box

represents unsequenced regions in Tarsiiformes. AGM: African Green Monkey; TSS: Transcription Start Site; ATG: first codon.

Specific aim 3. Detect conserved regions in the promoter of KCNA2 gene, that putatively are binding sites for transcription factors, in the last common ancestor of Euarchontoglires.

In addition to Primates, Dermoptera, Scandentia, Rodentia, and Lagomorpha orders are also included in Euarchontoglires.

The last common ancestor of Primates and Dermoptera (Primatomorpha) lived 79 mya. The conserved regions identified in this ancestor are illustrated in Figure 11 (alignment 8). Since we found N´s in Dermoptera between 1,044 - 809 bp upstream of the TSS, this segment was not included in the analysis. A total of 5 putative binding sequences for transcription factors were detected.

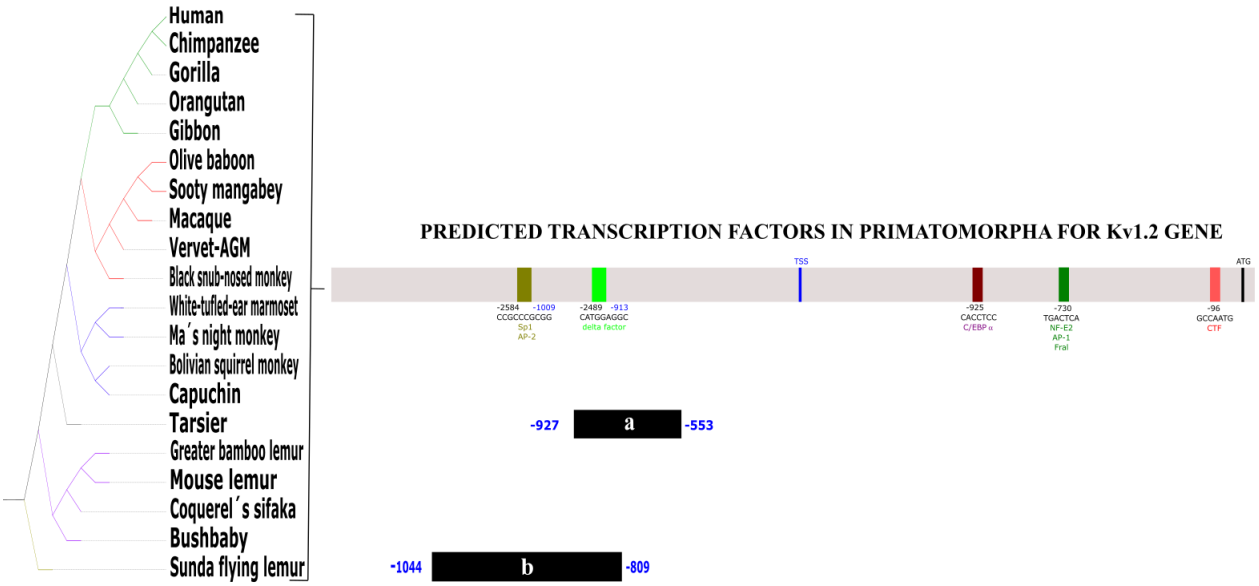

Figure 11. Predicted binding sites for transcription factors of KCNA2 in the common ancestor of Primatomorpha (alignment 8). The image illustrates the predicted binding sites for transcriptional factors in Primates: Hominoidea (green lines), Old World Monkeys (red lines), New World Monkey (blue lines), Tarsiiformes (turquoise lines), and Strepsirrhini (purple lines) groups and Dermoptera (olive green line). These predictions were obtained by the alignment of the region of 1,500 bp upstream of the TSS and the 5´UTR of KCNA2 genes of the analyzed species. Each binding site is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). Black boxes represent regions without information in a: Tarsiiformes, b: Dermoptera. These regions were not included in the prediction of binding sites for transcription factors. TSS: Transcription Start Site. ATG: first codon. AGM: African Green Monkey.

Next, we included in the analysis the Chinese tree shrew, order Scandentia, to obtain the putative binding sites in the common ancestor or Euarchonta. We observed N´s in Scandentia between 1,054 - 831 bp upstream of the TSS. We did not include this segment in the analysis. Therefore, we observed the same binding sequences as in the Primates and Primatomorpha (Figure 12).

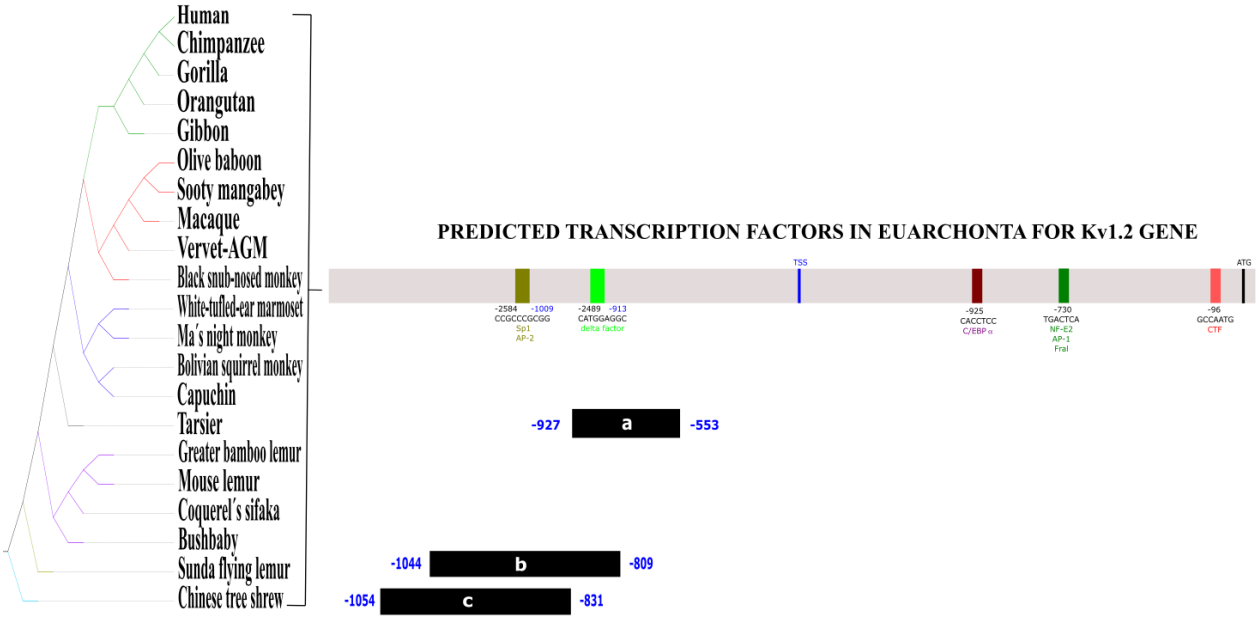

Figure 12. Predicted binding sites for transcription factors of KCNA2 in the common ancestor of Euarchonta (alignment 9). The image illustrates the predicted binding sites for transcriptional factors in Primates: Hominoidea (green lines), Old World Monkeys (red lines), New World Monkey (blue lines), Tarsiiformes (turquoise lines), and Strepsirrhini (purple lines) groups; Dermoptera (olive green line) and Scandentia orders (calypso line). These predictions were obtained by the alignment of the region of 1,500 bp upstream of the TSS and the 5´UTR of KCNA2 genes of the analyzed species. Each binding site is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). Black boxes represent unsequenced regions in a: Tarsiiformes, b: Dermoptera, and c: Scandentia. These regions were not included in the prediction of binding sites for transcription factors. TSS: Transcription Start Site. ATG: first codon. AGM: African Green Monkey.

Finally, we included Rodentia and Lagomorpha orders in the analysis (final alignment) (Figure 13). At this point, we are located 87 mya in the Euarchontoglires clade. Here, only 2 binding sites for transcription factors were predicted in the 1,500 upstream of the TSS: an Sp1 and a delta factor site for transcription factors. Additionally, 2 sites were predicted in the 5´UTR: a C/EBP**α** site and a common site for NF-E2, AP-1, and FraI transcription factors. We will consider these predicted binding sites for posterior discussion.

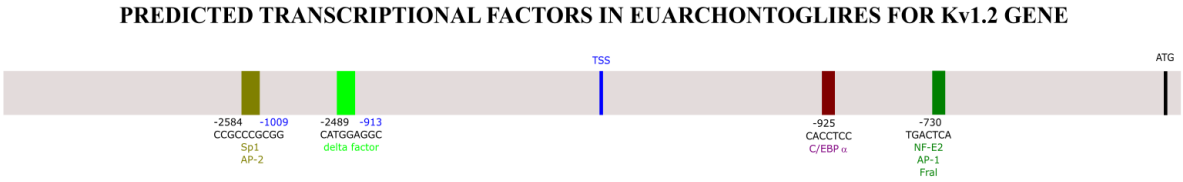

Figure 13. Predicted binding sites for transcription factors of KCNA2 in the common ancestor of Euarchontoglires (final alignment). The image illustrates the predicted binding sites for transcriptional factors in Euarchonta composed of Primates (Hominoidea, Old World Monkeys, New World Monkey, Tarsiiformes, and Strepsirrhini groups), Dermoptera and Scandentia, and Glires composed of Rodentia and Lagomorpha. These predictions were obtained by the alignment of the region of 1,500 bp upstream of the TSS and the 5´UTR of KCNA2 genes of the analyzed species. Each binding site is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). Unsequenced regions in Tarsiiformes, Dermoptera, and Scandentia were not included in the prediction of binding sites for transcription factors. TSS: Transcription Start Site. ATG: first codon.

To obtain a reconstruction of the conserved sites in the common ancestor of each group, we illustrated the repertoire of binding sites for transcription factors (BSTF) in Figure 14. We observed a total of 28 BSTF in the human KCNA2, in the common ancestor of Hominini (human + chimpanzee) 26 binding BSTF remained conserved, in the common ancestor of Hominoidea 22 BSTF, in the common ancestor Catarrhini 14 BSTF were conserved, 8 BSTF remained conserved in the common ancestor of both, Anthropoidea and Haplorrhini. In the common ancestor of Primates only 5 BSTF were conserved. Next, in the common ancestor of Primatomorpha and Euarchonta there was not change in the conserved BSTF. Finally, when we included Rodentia and Lagomorpha in the analysis, only 4 BSTF were conserved in the common ancestor of Euarchontoglires (details in Figure 13).

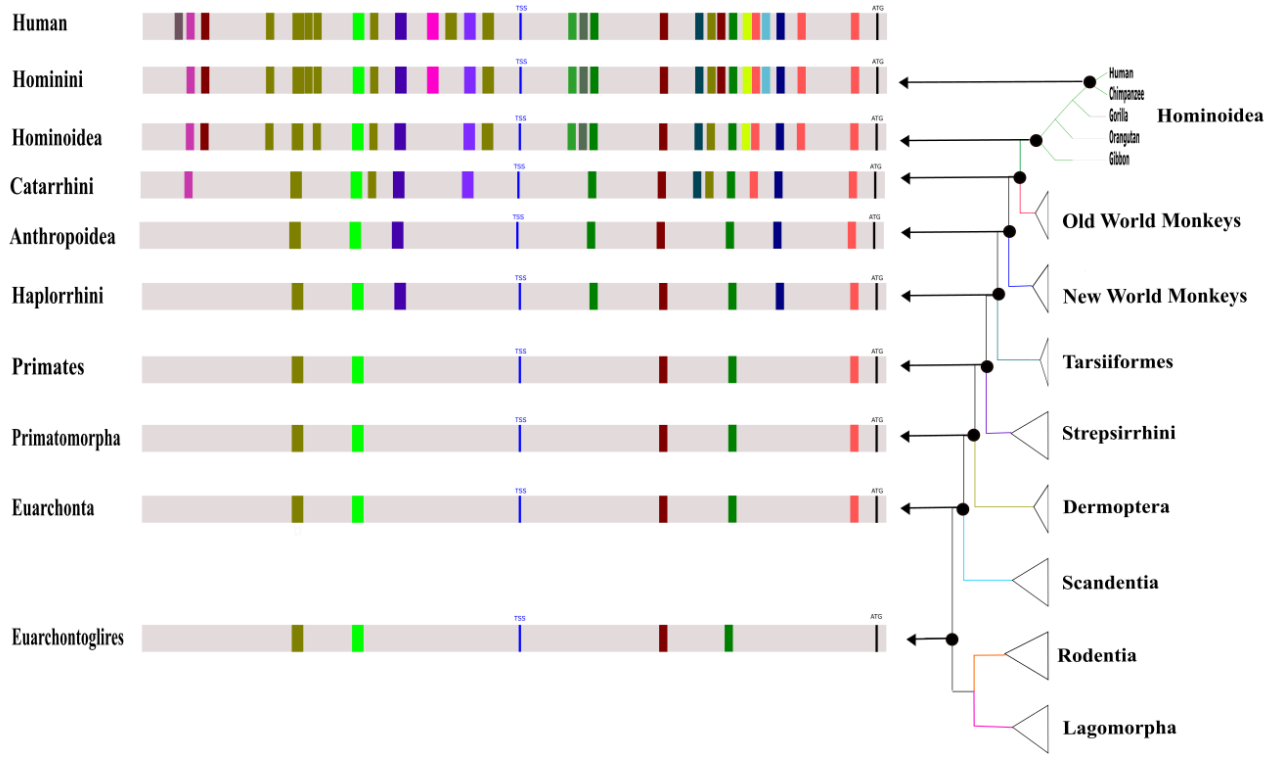

Figure 14: Summary of predicted binding sites for transcription factors for KCNA2 in Euarchontoglires. The 1,500 bp upstream TSS and the 5´UTR of KCNA2 gene from organisms belonging to the Euarchontoglires clade (see Table 1) were aligned and analyzed for binding sites of transcription factors represented by color boxes. TSS: Transcription Start Site. ATG: first codon.

To obtain more information about possible regulatory regions that were not predicted previously, we identify all the conserved regions in the common ancestor of Euarchontoglires, which include the four putatively binding sites for transcription factors previously detected.

We observed a total of 29 conserved regions of at least 5 bp, 15 of them located in the 5´UTR and 14 of them in the 1,500 bp upstream of the TSS (Figure 15). Additional studies are necessary to identify the role of these regions in the expression of KCNA2 gene.

CONSERVED SEQUENCES IN EUARCHONTOGLIRES PROMOTOR AND 5´UTR OF Kv1.2 GENE

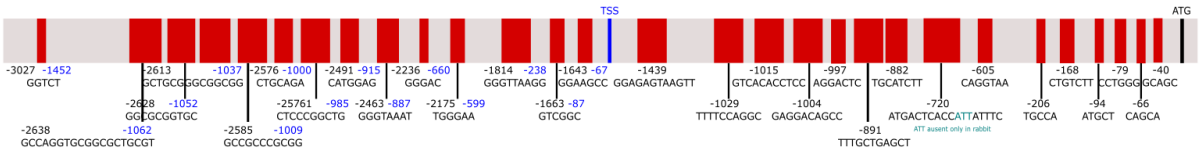

Figure 15*:* Conserved regions in KCNA2 promoter in Euarchontoglires. The 1,500 bp upstream of the TSS of the KCNA2 gene and its 5´UTR of organisms belonging to the Euarchontoglires clade (see Table 1) were aligned. In red boxes are illustrated the conserved sequences between all these organisms. Each box is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). TSS: Transcription Start Site. ATG: first codon

Specific aim 4. Identify phylogenetically structured variations in the promoter region of KCNA2 gene that may be regulatory elements for K_v1.2 potassium channel expression.

We determined ten regions containing phylogenetically structured variations (Figure 16) in the KCNA2 promoter region: four of them are located 1,500 bp upstream the TSS and the other six located in the 5´UTR. First, we identified a region of 20 bp at 1,063 bp upstream the start codon: the sequence GAGCCAGGTGCGGCGCTGCT is conserved in Catarrhini (Hominoidea + Old World Monkeys) and a single variation GGCCAGGTGCGGCGCTGCT was detected in New World Monkeys, Strepsirrhini, Dermoptera, Scandentia, Rodentia and Lagomorpha. Also, we identified a conserved sequence of 9 pb CGGGACGTC conserved at -1027 bp upstream the start codon in Catarrhini. In New World Monkeys, Strepsirhini, Dermoptera, Scandentia, Rodentia and Lagomorpha, a single variation was found in the same region: CGGGCCGTC. This sequence was not present in Tarsiiformes (Figure 16A). Next, two conserved regions were identified in Anthropoidea, and a single variation of these regions found in Strepsirrhini, Dermoptera, Scandentia, Rodentia and Lagomorpha. At -2487 bp upstream the start codon the sequence of 11 bp CATGGAGGCCC was conserved in Anthropoidea and at -1643 bp upstream the start codon ATG the sequence AGTCCGGAAGCC. In the other groups, the sequence presents a single variation at -2,487 bp upstream the start codon: CATGGAGGCTC and at -1643 bp upstream the start codon ATG also a single variation was detected: AGCCCGGAAGCC (Figure 16A). Surprisingly, a binding sequence for transcription factor was previously predicted at -2489 bp upstream the start codon in Euarchontoglires: a binding sequence for the delta factor (CATGGAGGC) (Figure 16). This sequence is included in the non-variable region (9 bp) of the whole conserved region (11 bp). In Tarsiiformes, the sequence located at -2489 bp upstream the start codon is not sequenced (N´s) and the sequence located at -1643 bp upstream the start codon was not observed). At -925 bp upstream the start codon we observed a long 27 bp region that is

highly conserved in Hominoidea, Old World Monkeys, New World Monkeys, Strepsirhini and Lagomorpha: TTTTTTCCAGGCAACGTCACACCTCC. Only in Rodentia this region presents four variations: TGTTTTCCAGGCCGGGTCACACCTCC. This sequence was not observed in Tarsiiformes, Dermoptera and Scandentia (Figure 16A). At -881 bp upstream the start codon, we observed a sequence of 21 bp conserved in Primates (except Tarsiiformes): TTTTGCTGAGCTTGCATCTT. Only in Rodentia and Lagomorpha, this sequence presents a single variation: TTTTGCTGAGCTCTGCATCTT (Figure 16B). At -604, -75 and -33 bp upstream the start codon ATG, three regions are conserved in Anthropoidea (Hominoidea, Old and New World Monkeys): a region of 11 bp (GGTCAGGTAAA), 23 bp (ATGCTCCTGTGCTTCCTGGGGCC) and 17 bp (ACCAGGCAGCTGAAAGG) respectively.

The region located at -604 bp upstream the start codon ATG presents a single variation in Strepsirhini, Rodentia and Lagomorpha: GGCCAGGTAAA. At -75 bp upstream the start codon we observed a single variation in Strepsirhini and Rodentia (ATGCTTCTGTGCTTCCTGGGGCC). However, this region in Lagomorpha presents three variants compared to the region identified in Anthropoidea (ATGCTTCTGCGTTACCTGGGGCC). Next, the region of 17 bp located -33 bp upstream the start codon presents two variations in Strepsirrhini (GCCAGGCAGCTGGAAGG), two variations and the loss of a base in Rodentia (G-CAGGCAGCTGGAAGG) and only one variation in Lagomorpha (ACCAGGCAGCTGGAAGG). These three regions were not observed in Tarsiiformes, Dermoptera and Scandentia. Finally, we identified a region of 13 bp located at -1 upstream the start codon ATG (CTCAGTCCCAATT) conserved in Primates (except Tarsiiformes because they do not possess this region). Rodentia presents a single variation in this region CTCAGCCCCAATT and in Lagomorpha we observed two variations in this region (CTCAGCCCCAATA). Also, this region was not observed in Dermoptera and Scandentia (Figure 16B).

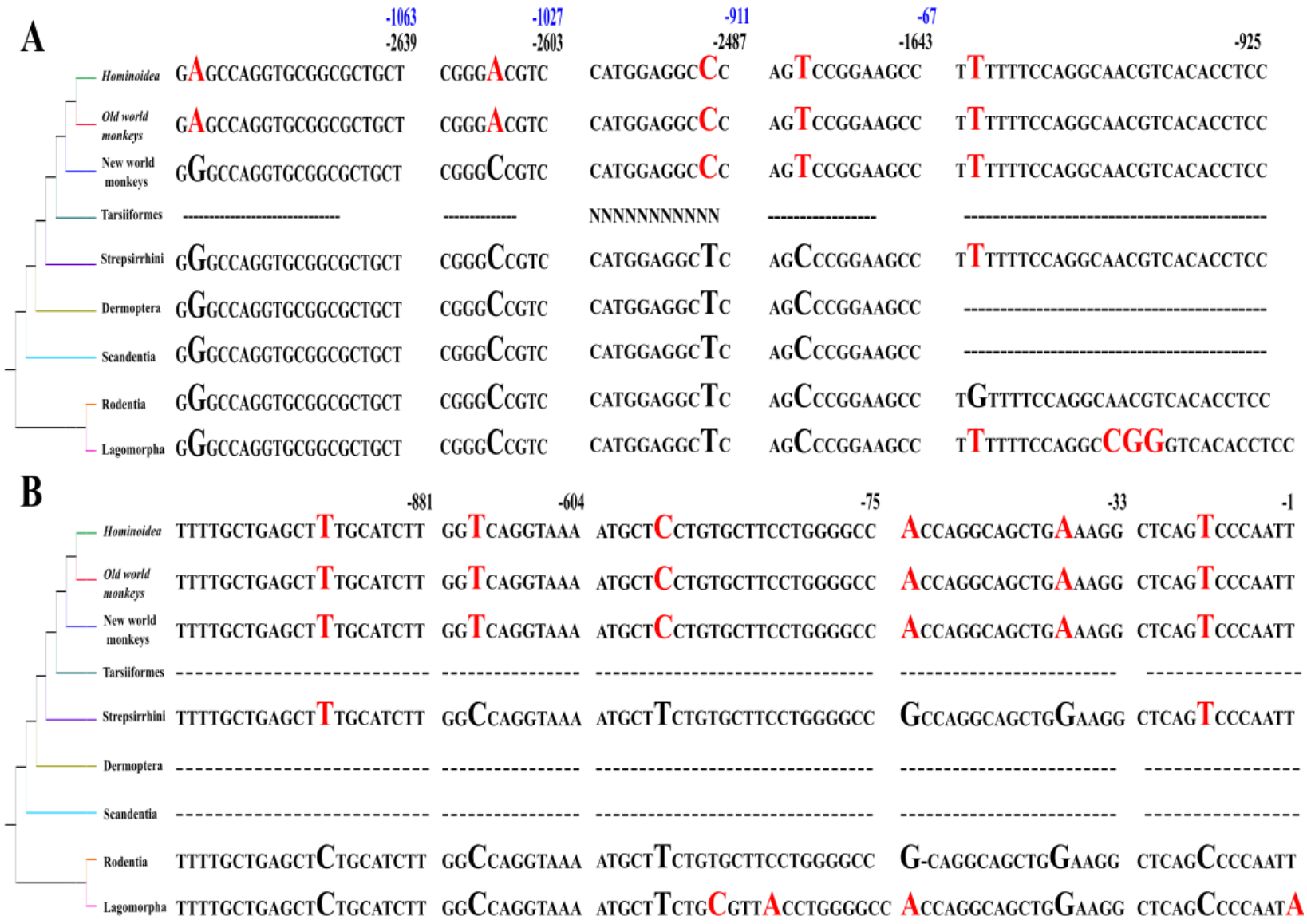
A
-1063
-2639
-1027
-2603
-911
-2487
-67
-1643
-925
Hominoidea
Old world monkeys
New world monkeys
Tarsiiformes
Strepsirrhini
Dermoptera
Scandentia
Rodentia
Lagomorpha
GAGCCAGGTGCGGCGCTGCT CGGGACGTC CATGGAGGCCC AGTCCGGAAGCC TTTTTCCAGGCAACGTCACACCTCC
GAGCCAGGTGCGGCGCTGCT CGGGACGTC CATGGAGGCCC AGTCCGGAAGCC TTTTTCCAGGCAACGTCACACCTCC
GGGCCAGGTGCGGCGCTGCT CGGGCCGTC CATGGAGGCCC AGTCCGGAAGCC TTTTTCCAGGCAACGTCACACCTCC
---------- ---------- NNNNNNNNNN ---------- ----------
GGGCCAGGTGCGGCGCTGCT CGGGCCGTC CATGGAGGCTC AGCCCGGAAGCC TTTTTCCAGGCAACGTCACACCTCC
GGGCCAGGTGCGGCGCTGCT CGGGCCGTC CATGGAGGCTC AGCCCGGAAGCC ----------
GGGCCAGGTGCGGCGCTGCT CGGGCCGTC CATGGAGGCTC AGCCCGGAAGCC ----------
GGGCCAGGTGCGGCGCTGCT CGGGCCGTC CATGGAGGCTC AGCCCGGAAGCC TGTTTTCCAGGCAACGTCACACCTCC
GGGCCAGGTGCGGCGCTGCT CGGGCCGTC CATGGAGGCTC AGCCCGGAAGCC TTTTTCCAGGCCGGGTCACACCTCC
B
-881
-604
-75
-33
-1
Hominoidea
Old world monkeys
New world monkeys
Tarsiiformes
Strepsirrhini
Dermoptera
Scandentia
Rodentia
Lagomorpha
TTTTGCTGAGCTTTGCATCTT GGTCAGGTAAA ATGCTCCTGTGCTTCCTGGGGCC ACCAGGCAGCTGAAAGG CTCAGTCCCAATT
TTTTGCTGAGCTTTGCATCTT GGTCAGGTAAA ATGCTCCTGTGCTTCCTGGGGCC ACCAGGCAGCTGAAAGG CTCAGTCCCAATT
TTTTGCTGAGCTTTGCATCTT GGTCAGGTAAA ATGCTCCTGTGCTTCCTGGGGCC ACCAGGCAGCTGAAAGG CTCAGTCCCAATT
---------- ---------- ---------- ---------- ----------
TTTTGCTGAGCTTTGCATCTT GGCCAGGTAAA ATGCTTCTGTGCTTCCTGGGGCC GCCAGGCAGCTGGAAGG CTCAGTCCCAATT
---------- ---------- ---------- ---------- ----------
---------- ---------- ---------- ---------- ----------
TTTTGCTGAGCTCTGCATCTT GGCCAGGTAAA ATGCTTCTGTGCTTCCTGGGGCC G-CAGGCAGCTGGAAGG CTCAGCCCCAATT
TTTTGCTGAGCTCTGCATCTT GGCCAGGTAAA ATGCTTCTGCGTTACCTGGGGCC ACCAGGCAGCTGGAAGG CTCAGCCCCAATA

Figure 16. Structurally phylogenetic variations. The 1500 bp upstream of the TSS and the 5´UTR of KCNA gene of the species belonging to the Euarchontoglires clade (see Table 1) were aligned and analyzed in search of single variations phylogenetically structured in conserved regions. Ten regions were identified in the analysis indicated by larger letters. In A the regions between -2639 and -925 with respect to the ATG. In B the regions between -881 and -1 with respect to the ATG. Each region is represented by its corresponding sequence and its location with respect to the TSS (blue) and/or the ATG (black). The segmented lines represent gaps in the sequence in the alignment and N´s represents unsequenced regions. Numbers in blue indicate the position relative to the TSS: Transcription Start Site. Numbers in black indicate the position relative to ATG: first codon.

DISCUSSION

The literature regarding $K_v1.2$ regulation has been mainly focused on classical mechanisms such as trafficking and biophysical characteristics of the channel (Arnold 2007; Gu & Barry 2011; Jan & Jan 2012; Khalili-Araghi *et al*. 2006; Watanabe *et al*. 2007). However, to our knowledge, no previous studies have studied binding sequences for transcriptional factors regulating $K_v1.2$ channel expression. Therefore, this investigation is one of the first in studying these mechanisms.

The conserved regions through evolution are more probable to correlate with regulatory regions of the gene (King & Wilson 1975; Tagle *et al*. 1988). When we look at human ancestors, we identified an increasing number of binding sites for transcription factors to the extent that they are more contemporary to modern humans. The analysis of non-coding regions in the promoter region of KCNA2 gene of Euarchontoglires ancestor demonstrates that four regions are conserved, suggesting that they are putative binding sites for transcription factors (Figure 13). Additional conserved sequences in Euarchontoglires and phylogenetically structured variations may also be regulatory elements in $K_v1.2$ expression.

First, we identify 28 putative binding sites for transcriptional factors in the human KCNA2 promoter. There are some repeated binding sites for the same transcription factors, *i.e.*, there are eight sites for Sp1, three sites for CTF and three sites for C/EBPα, which suggests a strong regulation by these transcription factors, especially Sp1. We also observed that in the common ancestors of Hominini, Hominoidea and Catarrhini 26, 22 and 14 sites were identified respectively, which suggests that in these groups, compared to the other analyzed, a finest transcriptional regulation for $K_v1.2$ mRNA.

The common ancestor of humans and chimpanzees conserved 26 of 28 sites predicted in human KCNA2 promoter. This implies that this ancestor has a very similar $K_v1.2$ regulation to humans; maybe the time at was Hominini ancestor lived (6.4 mya) compared with the time that human arose was not enough to accumulate mutations in the regulatory regions of KCNA2 gene.

In the common ancestor of Hominoidea, there are six conserved sites for Sp1, two less than the human KCNA2 promoter and one less than Hominini ancestor. The sites for CTF are conserved, while one site for C/EBPα was lost compared to the human sites, which suggests that in Hominoidea ancestor still there was a strong regulation by Sp1. In Catarrhini ancestor, there are

only three sites for Sp1, which suggest an intermedial regulation compared with the ancestors mentioned above. However, since the Anthropoidea ancestor onwards, only one site for Sp1 is conserved. The same is observed for CTF and C/EBPα. Only one site for C/EBPα, as Sp1, is conserved in Euarchontoglires ancestor. However, CTF is only present in the Euarchonta ancestor and not in the Euarchontoglires ancestor. In summary, we observed that as more species were incorporated into the analysis, fewer binding sequences for transcription factors are conserved, which suggests that the conserved regions identified in Euarchontoglires ancestors were fundamental in the expression of $K_v1.2$ potassium channels in the evolution. With this information, we can propose a minimal stock of regulatory regions for $K_v1.2$ expression in the ancestors of every group.

With the arise of Anthropoidea, the brain volume started to increase substantially, which makes us think that new regulatory systems emerged in the nervous system function. One of them was the control of the neuron excitability to orchestrate coordinated transmission of information in the form of Action Potential. The VGIC should have a key role in this process. It has been proposed that changes in non-coding sequences are the main responsible for regulatory changes in the nervous system, called as regulatory evolution (Sousa *et al*. 2017). Therefore, the increment of putative binding sequences for KCNA2 gene expression as we conduct the Hominoidea ancestor could explain a more complex regulation in this gene, impacting neuron excitability and information transmission in the nervous system. The most species in Anthropoidea have diurnal activities, which is accompanied with visual acuity and color vision, which supposed a maturation of visual systems. Additionally, the increase in neocortex size was also key for information processing (Rosales-Reynoso *et al*. 2015). The increment of non-coding cis regulatory sequences in the expression of KCNA2 gene could supposed changes in the Action Potential at the level of threshold, waveform and frequency, the main points where $K_v1.2$ channels act. Moreover, the specific localization of $K_v1.2$ channels at AIS and JXPN would be an important step in the visual complexification and neocortex size during evolution.

Possible transcription factors regulating KCNA2 expression

In the last common ancestor of Euarchontoglires we identified four conserved sequences. We will discuss the possible transcription factors that could bind to these regions to regulate KCNA2 gene expression.

Sp1 or Specific Protein 1 is a transcription factor belonging to the family of zinc finger transcription factors. Sp1 also belongs to the Sp1-like family, which are distinguished by their ability to bind GC and/or GT-rich DNA regions within promoter regions and to activate gene transcription. These also share structural features: they contain a highly conserved DNA-binding domain composed of three zinc fingers close to the C-terminus, and Ser/Thr domains and glutamine-rich domain in their N-terminal. Despite their similarity, Sp2, Sp3 and Sp4 are not functional equivalents of Sp1, being this last one, unique in their function (Suske 1999; Wierstra 2008). For example, Sp3 has been described as a repressor of Sp1-mediated transcription (Hagen et al. 1994). It has been proposed that there is a synergistic activation of promoters by Sp1 through multiple GC-boxes (Courey *et al*. 1989). Promoters that are Sp1-responsive often contain multiple binding sites, and the most important site is usually the one closest to the TSS. As mentioned above, in the human KCNA2 promoter there are multiple binding sites for Sp1, suggesting a synergistic activation. The closest Sp1 site from the TSS is located 117 bp upstream the TSS, suggesting that this site could be key for Sp1 regulation. This could also be possible for species belonging to Catarrhini (Hominoidea and Old World Monkeys) according to our results. However, only one site for Sp1 was conserved in Anthropoidea ancestor, which suggests that 43 mya (divergence point of Catarrhini and New World Monkeys, Figure 2), the species belonging to Catarrhini group, started to acquire a more complex regulation by Sp1.

While the presence of several binding sites for Sp1 suggests its possible synergically transactivation, there is evidence that this transcription factor can transactivate with other transcription factors as GATA-1, AP-2 and Oct1, among others (Wierstra 2008). We mentioned these because *i.e.,* a site for GATA-1 is conserved in Catarrhini, one site for AP-2 site is also the site for Sp1 conserved in Euarchontoglires and there is evidence that Oct-1 regulates K_v1.2 expression. To our knowledge, no evidence for GATA-1 regulation of K_v channels has been described yet. Nevertheless, GATA-4 regulates K_v4.2 expression in cardiomyocytes (Jia &

Takimoto 2003) and the cardiac sodium channel $Na_v1.5$ has in its promoter a binding site for GATA-1, suggesting that GATA-1 and GATA-4 could have a role in regulation the expression of cardiac ion channels. Members of the family GATA transcription factors have been described as tissue-specific where GATA-1 seems to regulate the gene expression in cells of the erythroid lineage and hematopoietic lineages (Merika & Orkin 1993) and its expression is very low in neurons such as DRG neurons (Lee *et al*. 2017), which suggests that, while a binding site was observed in KCNA2 promoter, probably this transcription factor does not regulate KCNA2 expression. Additionally, the location of this site is so far from the TSS and from the initial codon ATG, decreasing even more the possibility of regulating KCNA2 expression.

Also, it has been suggested that Sp1 with MAZ transcription factor can function in conjunction with a downstream TATA-box, but they can also participate in transcription from a TATA-less promoter (Song *et al*. 2003). In other genes, Sp1 and MAZ bind to the same cis-elements in their promoters (Song et al. 2003) because the consensus sequence of Sp1 binding sites is very similar to that of MAZ-binding sites. Sp1 and MAZ might have dual function in gene expression regulation. For instance, Sp1 increases and MAZ inhibits the expression of the eNOS gene (Karantzoulis-Fegaras *et al*. 1999). On the other hand, both Sp1 and MAZ promote the expression of AdMLP gene (Karantzoulis-Fegaras *et al*. 1999; Parks & Shenk 1997). In the human KCNA2 promoter, besides the Sp1 site located 117 bp upstream the TSS, there is a MAZ site located 208 bp upstream the TSS (Figure 2). Considering that KCNA2 promoter is a TATA-less promoter and the proximity of Sp1 and MAZ sites in KCNA2 promoter of Catarrhini ancestor, we can suggest that these transcription factors could act in sync in the regulation of KCNA2 gene expression. However, we cannot predict if this regulation could be synergistic, competitive, or otherwise. Additional experiments are needed to reveal this kind of regulation.

In the Anthropoidea ancestor, a conserved binding site for Octamer binding protein 1c (Oct1c) was observed. Synergistic activation between Sp1 and Oct1 can mediate gene expression. In this line, the human small nuclear U2 RNA gene (U2 snRNA) contains a binding site for Oct-1 and three binding sites for Sp1 where both transcription factors bind cooperatively to regulate U2 snRNA gene expression. While both sites in KCNA2 promoter of Anthropoidea are separated for 570 bp approximately, the DNA can fold approaching two distant sites, therefore it would be alluring to study if these transcription factors can act synergistically in regulating KCNA2

expression. Also, it is important to mention that Oct-1 induces the expression of DNMT3a, methylating the KCNA2 promoter and then inhibiting its expression during neuropathic pain development in rats (Zhao *et al.* 2017), which makes us think that Oct-1 can have dual functions in regulating KCNA2 expression during physiological and pathological circumstances and could also depend on species. As our results showed that Oct-1 regulation emerged in the ancestor of Anthropoidea 43 mya it could be possible that Oct-1 regulates KCNA2 expression directly in the species belonging to this group. An indirect regulation mediated by DNMT3a could exist before Anthropoidea arises in the evolution.

The CAAT box transcription factor (CTF), also known as Nuclear Factor I (NF-1) regulates gene expression during development and in adulthood (Gronostajski 2000). In the human promoter of KCNA2 and in Hominoidea, three binding sites for this transcription factor were detected. Then, in Catarrhini, only two sites, and in Anthropoidea and the rest of groups, only one site for CTF was detected, being a conserved region in Euarchontoglires clade, which think us that there is a possible regulation of KCNA2 transcription by this transcription factor. This data, also lead us to think that the regulation of KCNA2 gene by NF-1 started to be more determinant since Catarrhini origin, this is 28.9 mya because the duplication of the binding sequence for this transcription factor. A little is known about ion channel regulation by NF-1. Some studies have linked a Sp1 and NF-1 co-regulation of the β1 subunit of the voltage-dependent and Ca^{2+}-activated large-conductance K^+ channel (BKβ1) (Zhang *et al.* 2009), the membrane cation channels gated by extracellular ATP, such as P2X1 (Zhao & Ennion 2006) and the serotonin gated channel, 5-HT_3R (Bedford *et al.* 1998), which requires the formation of NF-1 complexes for their neuronal expression in sensory ganglia. Also, NF-1 regulates the expression of other ligand-gated ion channels such as the $GABA_A$-α6 receptor subunit (Stock 2002). Importantly, both 5-HT_3R and $GABA_A$-α6 gene possess a TATA-less promoter, such as KCNA2 gene. However, there is scarce evidence that links NF-1 regulation to voltage-gated ion channels; thus, experimental evidence is necessary to definitively probe if NF-1 controls the expression of KCNA2 gene.

Delta (∂) transcription factor or Yin Yang 1 (YY1) is a transcription factor belonging to the zinc fingers family of transcription factors. In nervous system, its main role has been described in promoting the axon myelination by oligodendrocytes (He *et al*. 2007; Emery 2010) and Schwann cells (He *et al*. 2010; Pereira *et al*. 2012), this function could be important for the

establishment of axonal subdomains such the node of Ranvier and its adjacent regions as paranode and juxtaparanode. As mentioned above, in myelinated axons, Kv1.2 is mainly located at juxtaparanodes and the expression of YY1 in oligodendrocytes and Schwann cells should be necessary for a proper Kv1.2 location in axons. Interestingly, it has been described that after a nerve injury, a re-myelinating process is necessary to heal the damaged nerve (Makwana & Raivich 2005; Gordon & Sulaiman 2013; Smith *et al*. 2020) and recover the electrical features of the regenerating axon (Shim & Ming 2010), therefore, the expression of YY1 could be relevant for the recovery of axonal function after nerve injury. Regarding ion channels, SCNA8 gene (Nav1.6) is positively regulated by YY1 through the binding to its promoter. Importantly, a decrease in Nav1.6 is involved in sensory deficits in critical illness neuropathy (Drews *et al*. 2007; Li *et al.* 2015). After all, YY1 could be more relevant in non-neuronal cells during nerve injury and modulating other ion channels as Nv1.6 during neuropathies. However, as this binding site is conserved in Euarchontoglires, it would be interesting to investigate if it is associated with KCNA2 promoter in neurons of species such as human, mouse and rat.

Another binding site in the KCNA2 promoter identified in our study was CCAAT/enhancer binding protein α (C/EBPα). This belongs to the C/EBP family of basic region-leucine zipper proteins (Nerlov 2007). There is no direct evidence that links C/EBP members to K_v regulation. Some studies have shown the involvement of a binding site for C/EBP in the c-AMP dependent regulation of Inward Rectifier Potassium Channel 7.1 (Kir7.1) (Nakamura *et al*. 2000), that SCN1A (Nav1.1) promoter possess a binding site for C/EBP (Long *et al*. 2008) and a conserved binding site for C/EBP in mouse and rat for the voltage-dependent anion channel 1 (Vdac1) (Kfoury & Kapatos 2009). Hence, experimental studies are needed to elucidate if C/EBP controls the expression of KCNA2 gene.

The sequence TGACTCA is the conserved region closest to the start codon ATG, this sequence was predicted as a binding site for three transcription factors: NF-E2, Activator protein 1 (AP-1) and Fra-1. The transcription factor AP-1 consists of a variety of dimers composed of members of the Fos and Jun families of proteins (Jochum *et al*. 2001). While the Fos proteins (c-Fos, FosB, Fra-1, and Fra-2) can only heterodimerize with members of the Jun family, the Jun proteins (c-Jun, JunB, and JunD) can both homodimerize and heterodimerize with other Jun or Fos members to form transcriptionally active complexes (Angel & Karin 1991; Jochum *et al.* 2001).

c-Jun is a major component of the AP-1 transcription factor complex and together with JunB and JunD forms the family of mammalian Jun proteins (Mechta-Grigoriou *et al.* 2001). Curiously, the AP-1 transcription factor c-Jun has been implicated in axonal regeneration; the increase of c-Jun is necessary for an efficient regeneration of the axon after injury in the central nervous system (Raivich *et al.* 2004). Also, an increase in c-Jun expression after peripheral nerve injury has been described in sensory and motor neurons (Herdegen & Zimmermann 1994) until two months after the damage, suggesting an important role of c-Jun in regenerating axons. An important step in regeneration after axonal injury is the recovery of the electrical properties of the axons, which suggest that c-Jun may have a relationship with ion channels changes after axonal injury. In other cell types such as smooth muscle cells (SMC), an increase in c-Jun is related to decrease in $K_v1.5$ channels and hence, K^+ currents in these cells (Yu *et al*. 2001). A possible mechanism for c-Jun regulation of KCNA2 expression could be through Sp1 interaction since it has been studied that Sp1 can act as an anchor protein to recruit transcription factors as c-Jun (Chang & Chen 2005). In fact, a protein-protein interaction between Sp1 and c-Jun has been demonstrated to regulate the expression of the neurotransmitter-gated ion channel, the neuronal nicotinic acetylcholine receptor (Melnikova & Gardner 2001). Another member of the AP-1 family is the Fos-related antigen1 (Fra-1), whose role in the central and peripheral nervous system is poorly understood. Some studies have shown that Fra-1 expression is mainly restricted to the hippocampus. However, its immunoreactivity can increase after damage to the central nervous system (Beer *et al*. 1998; Pozas *et al.* 1999). As mentioned above, Fra-1 can heterodimerize with c-Jun, it would be alluring to study their role in KCNA2 expression.

As mentioned above, our predictions suggest that the Nuclear Factor Nuclear Factor Erythroid-derived 2 (NF-E2) could bind to KCNA2 promoter. NF-E2 belongs to the basic-leucine zipper family of dimeric transcription factors. It is composed of p45 and p18 subunits. NF-E2 is found almost exclusively in hematopoietic progenitors and cells of the erythroid/mega/mast cell trilineage where it acts as a transcriptional activator (Su *et al*. 2012; Andrews 1998). Therefore, even though we detected a conserved binding site for this transcription factor in KCNA2 promoter, considering its mainly location in cells where KCNA2 is not expressed, it is unlikely that NF-E2 regulates its expression.

Additional conserved regions and phylogenetic variations suggest extra regulatory elements controlling K_v1.2 expression

In the Euarchontoglires ancestor we identified 29 conserved sequences (Figure 15), some of them are predicted to be bindings sites for transcription factors and others not. Therefore, these regions could also play a regulatory function in KCNA2 transcription. Additional experiments are necessary to unravel the role of these sites.

Using a Phylogenetic Footprinting approach, we detected ten conserved regions with phylogenetic variations in Euarchontoglires (Figure 16). Four of them are located upstream the TSS and the other six in the 5´UTR of KCNA2 gene. The two regions furthest from the TSS are conserved only in Catarrhini, which suggest that these regions could diverge in their regulation approximately 28.9 mya. In this group, the region located 1,063 bp upstream the TSS presents a change of a guanine nucleotide by an adenine nucleotide. Structurally, both nucleotides present the same heterocyclic ring, but are different in their radicals which may impact in the binding of other proteins such as transcription factors. The region located 1,027 bp upstream the TSS presents a change of a cytosine nucleotide by an adenine nucleotide. This implies a change from a pyrimidine to a purine nucleotide, which may impact profoundly in the binding of transcription factors. Next, two regions diverge in the Anthropoidea group, which means a change in the regulation of KCNA2 promoter 43 mya approximately. In these, the changes are between pyrimidines, which may have a lower impact. The region located at 911 bp upstream the TSS contains the binding sequence for YY1 transcription factor. However, the variation is just located adjacent to the star of the binding sequence. This could affect the binding of this protein to the region. However, it would be interesting to study the contribution of these regions to the putative binding of YY1 to the KNCA2 promoter. As mentioned above, YY1 is important for the recovery of nodes of Ranvier after axon injury, therefore small variations in adjacent regions to the binding sequence of YY1 could have a great impact on the axonal subdomains after damage. It would be interesting to study if YY1 affects in the same way a species belonging to Anthropoidea (*i.e.*, human) than species belonging to the other analyzed groups, such as Rodentia. This could lead us to think that these single variations could impact the axonal location of K_v1.2 channels.

In general, the main possible regulatory sequences are in the 5´region upstream of the TSS, which suggests that the above-mentioned four sites are candidates for transcription regulating sequences. However, also in the 5´UTR has been described regulatory regions. In the 5´UTR of KCNA gene, there are six putative regions for regulating transcription of the gene, some of them are highly conserved and present divergences in Catarrhini and Primates, which suggest an evolutionary regulation in the KCNA2 gene expression. However, it is necessary to prove in situ if these sequences regulate or not the expression of KCNA2 gene. Therefore, more experiments by site directed mutations are necessary. Interestingly, the Lagomorpha, which includes rabbits, showed several single mutations compared with the other group, which suggest that maybe this group evolved in a different way in the regulation of KCNA2 expression.

Other mechanisms regulating KCNA expression: epigenetics and repressors

It has been described that deacetylation and methylation might also contribute to the regulation of the expression of individual genes, with zinc-finger proteins as Sp1 and MAZ (Song *et al*. 2003). For example, the histone deacetylases HDAC1, HDAC2 HDAC3 could interact with MAZ and, DNA methyltransferase 1 (DNMT1) could interacts with Sp1 in a transcription complex (Song *et al*. 2001; Wierstra 2008). Considering that it has been demonstrated that Sp1 binds directly to HDAC1 and DNMT1 (Doetzlhofer *et al*. 1999; Song *et al*. 2001; Wierstra 2008) this could be relevant for K_v1.2 gene expression since HDAC2 is involved in K_v1.2 decrease after nerve injury in sensory neurons, promoting neuropathic pain (Li *et al*. 2019). Moreover, in the same pathology, DNMT3a acts as a repressor of K_v1.2 expression by Oct-1 transcription factor (Zhao *et al*. 2017) and an increased activity of euchromatic histone-lysine N-methyltransferase-2 (G9a) histone reduces the K_v1.2 mRNA levels in sensory neurons (Liang *et al*. 2016). Also in neuropathic pain, a pathology characterized by a hyperexcitability state where there is a decrease in K_v1.2 potassium channel, other potassium channels seem to contribute to hyperexcitability. G9a promoted the demethylation of H3K9 at the promoter of KCNA4 (K_v1.4), KCND2 (K_v4.2), KCNQ2 (K_v7.2), and KCNMA (K_{Ca}1.1) genes, which then, induced a decrease in K^+ current. The effect on K_v channels was reversed by G9a inhibition (Laumet *et al*. 2015). Moreover, the Mucha group demonstrated that K_v7.2 and K_v7.3 decrease their expression in hyperexcitability conditions such as neuropathic pain and epilepsy. In this work, there were identified two mechanisms for regulating K_v expression in DRG neurons: Sp1 as an activator of the K_v7.2 and K_v7.3 expression

and REST as a repressor of their expression. In the promoter of these genes a Sp1 conserved consensus site, between human, mouse, and rat genomes, was identified for both K_v7.2 and K_v7.3 genes, near the 250 bp and 500 pb upstream the TSS, respectively. A similar regulation was observed for the REST repressor (Mucha *et al*. 2010).

These data suggest that human K_v1.2 expression could be regulated by the Sp1 transcription factor including complex mechanisms involving other transcription factors such as MAZ, histone acetylation and histone methylation. However, additional experiments are necessary to unravel the transcriptional mechanisms in human neurons.

K_v channel regulation in other excitable cells

Interestingly, Sp1 regulation of K_v channels is not restricted to neurons. In other excitable cells such as cardiomyocytes, it has been shown that Sp1 regulates the expression of the K_v7.1 potassium channel in rat cardiac cells (Masuda *et al*. 2018). Additionally, potassium channels belonging to the K_v1 family have also been shown to be regulated by Sp1. For example, the promoter of K_v1.5 in murine smooth muscle cells possess three binding sites for Sp1. The experiments showed that the expression of K_v1.5 is dependent on Sp1 regulation via CACC box motifs, and this channel could be involved in physiological or pathological conditions in vascular smooth muscle (Fountain *et al*. 2007). K_v1 potassium channels are usually located at plasma membranes, however a K_v1.3 nuclear channel was detected and regulates nuclear membrane potential and activates the expression of transcription factors. In this investigation, binding of Sp1 to the promoter of K_v1.3 gene was observed in cancer cells and human brain types (Jang *et al*. 2015). These data suggest that Sp1 could be a master regulator of the expression of K_v1 potassium channels, especially important in Catarrhini group because of the presence of multiple binding sites that act synergistically, nevertheless this is an uncharted field of study.

Transcriptional regulation of other channels

Binding sites for Sp1 have also been present in the promoter of other K channels as MaxiK channels, a calcium-activated potassium channel. These are composed by a pore-forming α subunit which shares high homology with the α subunit of K_v channels. The promoter of this gene is TATA-less and presents multiple GC rich regions and therefore, binding sites for Sp1. Additionally, this promoter also presents a binding site for C/EBP, however, since there are

C/EBPα and β transcription, the authors do not indicate the type of C/EBP detected (Dhulipala & Kotlikoff 1999). It seems that Sp1 controls the expression of several ion channels, since it has been describe that this transcription factor also regulates the expression of ion channels as transient receptor potential vanilloid type 1 (TRPV1) (Chu *et al*. 2011), hyperpolarization activated channels 2 and 4 (HCN2 and HCN4) (Lin *et al*. 2009), voltage-gated calcium channels type 3.1 ($Ca_v3.1$) and its auxiliary subunits (α2δ-1) (González-Ramírez *et al*. 2014; Gómez *et al*. 2019; Martínez-Hernández *et al*. 2013), and nicotinic acetylcholine receptor (nAChR) (Melnikova & Gardner 2001).

Sp1 and AP-2 binding sites have also been detected in kainic acid receptor 2 (KA2), a kind of glutamate receptor. However, no functional experiments have been developed (Myers *et al.* 1999). Accordingly, it would be interesting to study the role of these two transcription factors in regulating the expression of Kv1.2 channels.

Localization of Kv1.2 mRNA in peripheral axons opens the field of local synthesis of ion channels

All the evidence about K_v1 changes in neuropathic pain was obtained in the dorsal root ganglia (DRG), that is, in the neuronal soma. Interestingly, $K_v1.2$ expression decreased in myelinated axons after nerve injury at distal sites from injury (close to DRG) and there is a redistribution of Kv1 channels from the juxtaparanodal towards the paranodal zone. Electrophysiological and behavioral pain experiments suggest that local changes in K_v1 α subunit expression and its redistribution would act as a brake on the hyperexcitable state that arises in myelinated axons following traumatic nerve injury, which might act as a "protective" mechanism to prevent neuropathic pain (Calvo *et al*. 2016). However, little is known about the local mechanisms contributing to the supply of $K_v1.2$ channels in the axons.

The transport of the channel from the soma to specific axonal subdomains is the main pathway to provide axons with $K_v1.2$ potassium channels by a direct pathway of vesicular transport from the soma (Arnold 2007; Rivera *et al*. 2007; Vacher et al. 2008; Gu & Barry 2011; Jensen et al. 2011;Su *et al.* 2013; Jensen et al. 2014; Misonou 2018) These imply that the mRNA is translated in ribosomes and the nascent peptide imported into the endoplasmic reticulum (ER). Given this is a co-translational process, the ribosomes attached to ER membrane form regions called rough ER (RER). The participation of the subunit G2 of the eukaryotic initiation factor (eIF4FG2) regulates

the expression of $K_v1.2$. eIF4F is one the protein complexes involved in the initiation phase of protein translation and is an important effector of post-transcriptional gene regulation. It is composed of three subunits, where the eIF4FG subunit contains three isoforms (Prévôt *et al.* 2003). During axonal injury, the expression of eIF4FG2 increased in the injured soma downregulating the $K_v1.2$ expression and then, contributing to the development of neuropathic pain (Zhang *et al.* 2021). After exit the RER, the channels move through the secretory route for proper folding and adquistion of post-traductional modifications. Moreover, the co-assemble with auxiliary subunits helps the ionic channels to reach the right destination (Deutsch 2003). Although it is not fully clear, the axonal targeting of ion channels is dependent on the neuronal type, axonal microdomain, channel type, and importantly, cellular context. Specific mechanisms in the axonal compartment and targeting sequences in the mRNA or peptide sequence likewise contribute to a precise axonal localization and membrane insertion (Misonou 2018). Nevertheless, a model based solely on transport from the soma may not account for a rapid and efficient response during development, plasticity, or damage, which suggests that complementary mechanisms could operate to regulate the axonal ion channels.

In the last few years, local mechanisms in the axons have been described to be necessary for the axonal regeneration after nerve injury (Zheng *et al*. 2001; Bradke *et al*. 2012; D. Willis *et al*. 2005; Gumy *et al.* 2010) and also during development (Campbell & Holt 2001; Cox *et al*. 2008; Rodrigues Batista & Hengst 2016). While, the local synthesis of multiple cytoplasmic proteins has been widely described in axons (Satkauskas & Bagnard 2007; Eng *et al*. 1999; Rodrigues Batista *et al*. 2017), only a few studies have shown the axonal synthesis of membrane proteins (Hanus *et al*. 2016; Hafner *et al*. 2019; Cornejo *et al.* 2017). Among them, the synthesis of potassium channels has been described only in central neurons (Biever *et al.* 2020). The scenery in peripheral axons is less established. For synthesis of transmembrane proteins, are necessary secretory organelles, as REL, ERGIC, Golgi apparatus and secretory vesicles. In peripheral axons, while the whole organelle has not been described, functional components of a secretory pathway have been detected (González *et al*. 2016; Wu *et al*. 2017; Cioni *et al*. 2019), suggesting a non-conventional secretory route. In fact, the results of our collaborators showed that the β2 subunit of Na_v channel is capable of trafficking through peripheral axons (González et al. 2016) and also the TRPM8 channel trafficking is controlled by non-conventional axonal organelles (Cornejo *et al*. 2020). The

same group has proposed a Golgi bypass for local delivery of axonal proteins through post-endoplasmic reticulum pathways that could replace the canonical Golgi apparatus (González *et al.* 2018). Interestingly, the local synthesis of functional transmembrane proteins in dendrites that may totally or partially bypass the Golgi apparatus and result in immature glycosylated proteins in the plasma membrane (Hanus *et al*. 2016; Bowen *et al*. 2017). These observations suggest that trafficking and modification in the Golgi apparatus may be not necessary to produce functional membrane proteins in dendrites, posing the question if the same holds true for axons.

Regarding voltage-gated ion channels, an interesting observation in central neurons indicates that other families of K_vs, such as K_v2.1 channels are targeted to axons by a non-conventional trafficking pathway independent of Golgi apparatus, which seems to be regulated by post-translational modifications (Jensen *et al*. 2017). Local synthesis of translational regulators (Jiménez-Díaz *et al*. 2008; Géranton *et al.* 2009) and ion channels such as Na_v and TRP, are involved in nociceptive plasticity and contribute to hyperexcitability, suggesting that local synthesis of ion channels might play a role in the processes (Thakor *et al*. 2009; Ruangsri *et al*. 2011). In a squid model, an invertebrate, axonal local synthesis of functional K_v1.1 channels have been demonstrated (Mathur *et al.* 2018). In mammals, local translation of K_v1.2, K_v1.3, K_v1.4 mRNA together with other synaptic proteins have been recently described in monosomes within neuropile of hippocampal neurons. It seems clear that the preferential axonal translation in monosomes plays a role in the synaptic function (Cajigas *et al*. 2012; Biever *et al*. 2020). In addition, the suppression of local synthesis of K_v1.1 is necessary for synaptic excitation in dendrites of hippocampal neurons, suggesting a role in neuronal excitability (Raab-Graham *et al*. 2006).

To our knowledge, only one study has observed local changes of K_v1.2 channels in sciatic nerve. The group of Calvo described a reduction of K_v1.2 protein levels in sciatic nerve after injury with an hyperexcitability of the nerves, suggesting a pathological context similar to neuropathic pain (Calvo *et al*. 2016). We observed a tendency to decrease in K_v1.2 mRNA in the sciatic nerve after injury (Figure 18, Appendix 2), which suggests a possible cooperative mechanism between local synthesis and K_v1.2 transport from the soma to the injured axons in the initial phase of regeneration. However, another mechanisms could contribute to axonal availability of K_v1.2

channels. $K_v1.1$ and $K_v1.2$ mRNA have been found in the material transferred through ribonucleoparticles (RNPs) from Schwann cells to injured nerves (Canclini *et al*. 2020). To solve this, we developed an *in vitro* injury model in embryonic DRG neurons to isolate axons. Our results showed that axons by themselves have $K_v1.2$ mRNA in these neurons (Figure 19, Appendix 2). The detection of $K_v1.2$ mRNA in adult axons it would be confident to confirm the sources of this mRNA.

The embryonic neurons are an interesting model for studying axonal mechanisms, because as mentioned above, the axonal injury response seems to resemble the axonal development mechanisms, because the formed growth cone expands through the surrounding environment and reacts to growth and guidance cues. The results observed in these neurons (Figure 19, Appendix 3) suggest that soma are the main determinants of $K_v1.2$ supply in regenerating axons with a time dependent expression of this mRNA. The tendency to downregulates $K_v1.2$ mRNA levels at axons could be explained because the axotomy induces a drastic reduction in axonal mass. To regenerate the axonal channel expression and the electrical properties of axonal membranes, the soma activates an increase in $K_v1.2$ mRNA levels to support their synthesis and axonal transport. We also observed a tendency to increment $K_v\beta2$ mRNA in somas. This partial increase in this auxiliary subunit could help in the channel folding and to promote axonal transport of newly synthesized Kv1.2α subunits.

Collectively, these data suggest that the axonal availability of Kv1.2 potassium channels is mainly controlled by conventional synthesis and axonal transport. The possible local synthesis of these channels could be a mechanism to support channel exchange in normal conditions, with less magnitude after axonal injury.

The levels of mRNA in axons are very low. Therefore, this is a limitation of this study. Low levels of mRNA could affect the sensibility of their detection by qPCR. This could impact in the lack of statistically significant differences. An option to solve this difficult could be incrementing the starting material.

The evidence presented here suggests the presence of $K_v1.2$ and $K_v\beta2$ mRNA in sciatic nerve (Figure 17, Appendix 2) and only $K_v1.2$ mRNA in pure axons (Figure 19C, Appendix 2).

However, this is not a probe of axonal synthesis. For this, in collaboration with Couve lab, we developed a strategy to study axonal synthesis known as Ribotag mice described by (Shigeoka *et al*. 2018). This technique consists in the isolation of tagged ribosome-bound mRNA from a specific tissue. The ribosome tagging was made to mice that had a targeted mutation of the ribosomal protein L22 (Rpl22) locus harboring a loxP-flanked wildtype C-terminal exon 4 that was tagged with three copies of the hemagglutinin (HA). When these mice were bred to Cre-expressing mice, their offspring had the floxed wildtype exon 4 deleted in the cre-expressing tissue and subsequent use of the downstream HA-epitope-tagged exon 4 as the terminal exon. The 23 kDa HA-epitope-tagged ribosomal protein (Rpl22-HA) was incorporated into actively translating polyribosomes. These Ribotag mice allowed Cre-mediated HA epitope tagging of ribosomes from user-defined cell types and immunoprecipitation of ribosomes bound to mRNA from those specific cell types (Shigeoka *et al*. 2016). We isolated ribosome-bound mRNA from axons of $Na_v1.8$ neurons, which are mainly sensitive peripheral axons (we called Cre^+ mouse to those expressing HA-tag in Cre-$Na_v1.8$ neurons). Cre^+ genotype was confirmed by conventional PCR (data not shown). First, to confirm the HA-expression in $Na_v1.8$ neurons we extracted peripheral trigeminal ganglia and DRG from both Cre^- and Cre^+ mice. We collected the tissue from three animals per genotype and performed histological cuts for posterior immunofluorescence staining with anti-HA and anti-neurofilament antibodies. We observed positive HA staining in Cre^+ trigeminal ganglia and DRG, and only background staining in Cre^- ganglia (Figure 20A, appendix 3). We could not detect the HA epitope in axons from trigeminal and sciatic nerves of Cre^+ mice, probably because the technique was not sensitive enough (data not shown). To validate axonal HA expression, we lysed the trigeminal nerves and performed immunoprecipitation with an anti-HA antibody. In Figure 20C (Appendix 3) we observed HA expression only in Cre^+ nerves, suggesting that only axons of Cre^+ nerves expressed Rpl22-HA ribosomal protein. To obtain actively transcribed mRNA, first we lysed the sciatic nerves and then immunoprecipitated the ribosomal complex with an anti-HA antibody. The extracted mRNA was retro-transcribed to cDNA and then $K_v1.2$ expression was detected by qPCR. In sciatic nerves, we observed an increase in $K_v1.2$ expression approximately 8-fold over Cre^-. β-actin was amplified too (Figure 20D, appendix 3). The ribosome associated transcripts suggested their active local translation on peripheral axons. However, it is necessary to increase the replicates of this experiment to confirm the axonal synthesis of $K_v1.2$ mRNA.

PROJECTIONS

As we propose many regulatory regions and transcription factors for regulating KCNA2 expression, it would be interesting to probe in the lab if these predictions are true for central and peripheral neurons in normal and adaptive conditions as injury.

We could start by cloning the promoter region (including 1500 bp upstream the TSS and 5´UTR) of human KCNA2 gene and make sitio directed mutations in the phylogenetic conserved regions with a focus in the single variations. We also could clone mouse and/or rat promoters for comparisons. We could express these mutants in heterologous systems, such as HEK293 or HeLa cells, and measure K^+ currents to determine what mutants promote changes in the total current. To determine the role of these mutations in neurons, we could perform these experiments in neuron-derived cell lines as PC12 (rat) or SH-SY5Y (human) cells, and in hippocampal or cortical neurons from murine primary cultures.

One of the main candidates for regulating KCNA2 expression is the Sp1 transcription factor. We could start working in a heterologous system by co-transfecting Sp1 and $K_v1.2$ plasmid and measure K^+ currents, $K_v1.2$ mRNA and its protein expression. Next, in peripheral neurons as DRG culture or central neurons as hippocampal or cortical cultures, we could transfect a Sp1 plasmid and measure if there are changes in $K_v1.2$ mRNA and $K_v1.2$ protein levels. Also, in this system we can measure K^+ currents. To determine if axonal injury promote changes in a possible KCNA2-Sp1 regulation, we can damage central or peripheral neurons and determine $K_v1.2$ expression or K^+ currents.

In the case that we prove that Sp1 regulates KCNA2 expression we could use a pharmacological strategy to confirm the participation of this transcription factor. Mithramycin A is a pharmacological inhibitor of Sp1 widely used. We could incubate central and/or peripheral neurons and measure the expression of mRNA and protein levels of the $K_v1.2$ potassium channel.

To determine if Sp1 and/or other predicted transcription factors in this work interact with KCNA2 promoter, we could realize a DNase I footprinting assay. Briefly, this assay detects DNA-protein interactions using the fact that a protein bound to DNA will often protect the DNA from enzymatic degradation. This method uses the enzyme deoxyribonuclease (DNase) to cut the radioactively end-labeled DNA, followed by gel electrophoresis to determine the resulting cleavage pattern. This

assay could allow us to detect what proteins are bonded to possible binding sequences for transcription factors and the binding sequences as well. After this, we could study separately each bounded protein as previously described for Sp1.

As mentioned in discussion, there is evidence that Sp1 may interact with MAZ. As we also detect a MAZ binding site in species belonging to the Catarrhini group, we could study if the co-transfection of Sp1 and MAZ affects $K_v1.2$ expression and K^+ currents in neuron culture.

If we confirm that one or more of the predicted transcription factors regulate KCNA2 expression, it would be interesting to investigate if treatment with this or these TFs influence neuropathic pain development, where there is a decrease in $K_v1.2$ expression promoting a hyperexcitability state. We may work at cellular level on axonal injury models or in murine models by behavioral experiments that measure neuropathic pain symptoms. This could have an important impact on biomedicine since no successful therapies for neuropathic pain have been developed.

It is important to mention that the strategy used for prediction of binding sequences for transcription factors could be used for other ion channels, since the mechanisms regulating VGIC expression are scarce and many channelopathies have emerged in the last years.

We could not prove strongly that the $K_v1.2$ potassium channel is axonally synthesized. Therefore, it would be alluring to continue with the experiments in Ribotag mice to confirm that Kv1.2 channels are locally synthesized in peripheral axons. This animal model could be also used to study the axonal synthesis of other voltage-gated ion channels during normal and injury conditions.

Appendix 1

Super order	Order	Clade	Infraorder	Parvorder	Family	Specie	Name	Common name (spanish)	Gene ID
Euarchonta	Primates	Haplorrhini	Anthropoidea / Simiiformes	Catarrhini	Hominoidea (Superfamily)	*Homo sapiens*	Human	Humano	ENSG00000177301
						Pan troglodytes	Chimpanzee	Chimpancé común	ENSPTRG00000051151
						Gorilla gorilla gorilla	Gorilla	Gorila occidental de llanura/planicie	ENSGGOG00000011711
						Pongo abelii	Sumatran Orangutan	Orangután de Sumatra	ENSPPYG00000001046
						Nomascus leucogenys	Gibbon	Gibón de mejillas blancas del norte	ENSNLEG00000003647
					Cercopithecidae / Old world monkeys (Superfamily)	*Papio anubis*	Olive baboon	Papión oliva/babuno de Anubis	ENSPANG00000007311
						Cercocebus atys	Sooty Mangabey	Mangabey gris	ENSCATG00000034501
						Macaca fascicularis	Crab-eating macaque	Macaco cangrejero	(ENSMFAG00000000561
						Chlorocebys sabaeus	Vervet-African Green Monkey	Mono africano verde	ENSCSAG00000000168
						Rhinopithecus bieti	Black snub-nosed	Langur negro de nariz chata	ENSRBIG00000038695

							monkey		
				Platyrrhini / New world monkeys	Cebidae	*Callithrix jaccus*	White-tufled-ear marmoset	Tití común	ENSCJAG00000047659
						Aotus nancymaae	Ma's night monkey	Mono nocturno de Nancy Ma	ENSANAG00000021269
						Saimiri boliviensis boliviensis	Bolivian squirrel monkey	Mono ardilla boliviano	ENSSBOG00000023407
						Cebus capucinis imitator	Capuchin	Mono carablanca/capuchino	ENSCCAG00000030589
			Tarsiiformes	-	Tarsidae	*Carlito syrichta*	Tarsier	Tarsero filipino	ENSTSYG00000007240
		Strepssirhini	Lemuriformes		Cheirogalidae	*Microcebus murinus*	Mouse lemur	Lemur ratón gris	ENSMICG00000035462
					Lemuridae	*Prolemur simus*	Greater bamboo lemur	Lemur grande del bambú	ENSPSMG00000010845
					Indridae	*Propithecus coquereli*	Coquerel´s sifaka	Sifaca de Coquerel	ENSPCOG00000019803
			Lorisiformes	-	Galagidae	*Otolemur garnetti*	Bushbaby	Gálago de Garnet	ENSOGAG00000009201

	Dermoptera	-	-	-	Cynocephalidae	*Galeopterus vaeriegatus*	Sunda flying lemur	Colugo	103590903 (NCBI)
	Scandentia	-	-	-	Tupaiidae	*Tupaia belangeri*	Chinese tree shrew	Tupaya de Belanger	102497389 (NCBI)
Glires	Rodentia	Mouse-related clade	Myodonta	Muriudea	Muridae	*Mus musculus*	Mouse	Ratón casero	16490 (NCBI)
						Mus caroli	Ryukyu mouse	-	MGP_CAROLIEiJ_G0025494
						Mus pahari	Shrew mouse	-	MGP_PahariEiJ_G0026940
						Mus spicilegus	Sleppe mouse	Ratón de las estepas	ENSMSIG00000032163
						Rattus novergicus	Rat	Rata gris/parda	ENSRNOG00000018285
					Cricetidae	*Mesocricetus auratus*	Golden hámster	Hámster dorado	ENSMAUG00000016476
		Squirrel-related clade	-	Sciuriodiea	Sciuridae	*Sciurus vulgaris*	Eurasian red squirrel	Ardilla roja	ENSSVLG00005016685
		Ctenohystrica	Hystricognathi	Caviomorpha	Cavioidea	*Cavia porcellus*	Guinea pig	Cuy/cobayo	ENSCPOG00000039886
	Lagomorpha	-	-	-	Leporidae	*Oryctologus cuniculus*	Rabbit	Conejo	ENSOCUG00000008908

Table 2: List of species analyzed and Ensemble (or NCBI) gene ID. The organisms belong to Domain: Eukarya Kingdom: Animalia Phylum: Chordata Class: Mammalia Magnorder: Boreoeutheria Superorder: Euarchontoglires.

Appendix 2

Detection of Kv1.2 mRNA in axons

A) Methodology

1. Animals

C57/B6XSJL mice were obtained from the Animal Maintenance Unit in the Laboratory of Cellular and Molecular Neurobiology at Facultad de Medicina, Universidad de Chile and euthanized according to National Institutes of Health and Comisión Nacional de Investigación Científica y Tecnología (CONICYT) (Chile) guidelines. The animals were maintained under 12:12 hour light / dark cycles and with free access to food and water, unless treatment requires otherwise. The experimental protocol was approved by the Institutional Bioethics Committee (Universidad de Chile, CBA 0925 FMUCH, Andrés Couve, Appendix 3)

2. Sciatic nerve transection

For this, every animal was anesthetized with isoflurane inhalation and injected subdermal with ketoprofen 1%. The left sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through biceps femoris. Proximal to the sciatic trifurcation the nerve was transected. The incision was closed in layers and the animal was left in a heated surface until he regained consciousness. In each animal, an identical surgery was performed on the opposite side, except that the nerve was not transected (sham operated). Fourteen days after the surgery, 1 cm of the proximal section to the neuroma was extracted. An equivalent segment of sciatic nerve was extracted from the right hind paw.

3. Embryonic DRGs culture

WT E14 mouse embryos were decapitated, and the vertebral column removed. The spinal cord with DRGs was dissected and placed on coverslips coated with rat tail collagen (Invitrogen). DRGs was maintained in 24-well dishes containing 400 µL of Neurobasal Medium (Invitrogen), 2% B27 (Invitrogen), 0.3% L-glutamine, 1% streptomycin/penicillin, 4 µM aphidicolin, 7.5 µg/mL 5-fluoro-2-deoxyuridine and 50 ng/mL NGF 2.5S. The mixture of aphidicolin and 5-fluoro-2-deoxyuridine inhibits proliferation of Schwann cells by inhibition of DNA polymerase, and DRGs cultivated in these conditions rarely contain Schwann cells. DRGs were cultured in microfluidic chambers for up to 10 days at 37°C and 5% CO2. On day 5, axons were removed by vacuum

aspiration. This procedure eliminates all axons from the axonal compartment and the soma remains intact.

3.1. Microfluidic chambers preparation for compartmentalized culture

The day previous to culture, the microfluidic chambers were washed with Micro-90 and sonicated to eliminate residues. Then, both microfluidic chambers and coverslips were treated with 100% ethanol for 15 m and exposed to UV light for 30 min. The day of culture, we assembled the microfluidic chamber on the coverslip and added 100 µL of poly-L-lysine for 1 h at 37°C. Next, we washed 3 times with water and incubated with 2 µg/mL laminin for 1 h at 37°C. Finally, we added 120 µL of collagen 3% and incubated at room temperature until the seed. DRG neurons were seeded in the somatodendritic compartment with more volume of medium in this compartment. The volume difference favors the axonal growth to the axonal compartment. These devices allowed us to culture somas in one side of the chamber (somatic compartment) and due to hydrostatic pressure gradient given by volume differences, only the growing axons crossed to the other side of the chambers. We fabricated our own microfluidic chambers based on the model of the group of Taylor (Taylor et al. 2005). The microgrooves of our chamber are 15 µm width and 750 µm long, somas are bigger than 15 µm, therefore they could not cross. Cells were maintained until axons crossed the microgrooves (5-7 DIV).

3.2. In vitro injury model

In compartmentalized embryonic DRG neurons, after 5 DIV axons of the axonal compartment were removed mechanically (axotomy) through a vacuum aspiration. To avoid soma removement, the somatic compartment was filled with culture medium. After the axotomy, we added a new culture medium in the axonal compartment. Refill of each compartment was done for the next few days keeping the volume differences. The culture was maintained until mRNA extraction (2, 4 or 7 days post axotomy).

4. RNA extraction

Extraction of RNA from sciatic nerves was achieved using Trizol reagent (Invitrogen, catalog #15596026). Briefly, the tissue was triturated with 1 mL of Trizol using a tissue homogenizer and then 200 µL of chloroform was added for posterior vortex until we observed the mixture completely homogenous. Next, we centrifuged the tubes at 12,000 g for 30 min at 4°C.

We collected the aqueous phase and added it to isopropanol, shook and incubated on ice for 30 min. After this, the mixture was centrifuged at 12,000 g for 15 min at 4°C and the isopropanol was discarded. One mililiter of 70% ethanol was added and centrifuged at 12,000 g for 10 min at 4°C. Finally, the supernatant was discarded, and we left the tubes upside down for evaporating the remaining ethanol. We added 20 µL of free nuclease water to resuspend the RNA. To eliminate possible DNA contamination, we realized a DNase treatment (Sigma Aldrich, catalog #AMPD1) following the instructions of the manufacturer

Extraction of RNA from DRG cultures was achieved using RNeasy mini kit (Qiagen, catalog #74104) according to the instructions of the manufacturer.

The RNA concentration was determined using a nanophotometer (Fermelo biotec, Nano-400) and the purity of the RNA extraction was checked measuring the 260/280 ratio. A 1.8–2 ratio was considered optimal. Equal quantities of RNA of each sample were immediately retrotranscribed to cDNA by RT-PCR.

5. RT-PCR and quantitative PCR

The RT reaction was done with the High-Capacity cDNA RT kit (ThermoFisher, catalog #4368814) to obtain 10 µL of cDNA. Then, the cDNA was amplified by qPCR reaction with the Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies, catalog #600828) following the instructions of the manufacturer. A total of 20 µL of reaction was prepared using a 1:10 dilution of cDNA and 100 mM of each primer. The reaction involves 40 cycles of amplification. The used primers are detailed in Table 2. The experiments were done using the AriaMx Real-time PCR System (Agilent, #G8830A).

Primer		Sequence	Tm (°C)	Product size
Kv1.2	**Forward** **Reverse**	**5´AGAGGCAGACCATGAATGCTGT 3´** **5´ TGGGAACTGGGCTAAGGTCTTT 3´**	**62.1** **62.1**	**91 bp**
Kvβ2	**Forward** **Reverse**	**5´ TGCCAGAGCTGTTCCACAAGATAG 3´** **5´ TTCAACCACTGGTAGCCCTTCA 3´**	**58.8** **58.4**	**156 bp**
β-actin	**Forward** **Reverse**	**5´ CTCAGGAGGAGCAATGATCTTGAT 3´** **5´ TACCACCATGTACCCAGGCA 3´**	**60.2** **60.5**	**97 bp**

Table 2: List of primers used for qPCR.

6. Statistical analysis

For comparison of mRNA relative levels in sciatic nerve, we used the non-parametric test Mann-Whitney. For regenerating somas and axons of embryonic DRG neurons, we used the non-parametric test Kruskal-Wallis. Dunn´s multiple comparisons test was applied from each time of axotomy vs basal condition in embryonic DRG. A p value < 0.05 was considered as statistically significative.

B) Results

We used two different approaches to identify the presence of $K_v1.2$ mRNA in uninjured and injured axons of mouse peripheral neurons: an *ex-vivo* and *in vitro* strategies. *Ex vivo* strategy includes the detection of $K_v1.2$ mRNA in the sciatic nerve of adult mice, a peripheral nerve containing sensory and motor, unmyelinated and myelinated axons. The *in vitro* strategy involves the detection of $K_v1.2$ mRNA in pure axons obtained from dorsal root ganglia (DRG) neurons from embryonic mice in a compartmentalized culture for isolation of soma and axons. Both strategies include a model of injury.

1. mRNA in normal and injured sciatic nerve

The largest neurons and axons in the mammal peripheral nervous system are present in DRGs and sciatic nerve respectively. DRGs contain clusters of cell bodies of sensory neurons. These neurons extend their axons into the sciatic nerve. Therefore, we detected $K_v1.2\alpha$ subunit and $K_v\beta2$ subunit mRNA in both tissues by qPCR (Figure 17) using specific primers (Table 2). Since differential activity of K_v1 has been associated with different stages of remyelination and may modulate excitability following injury, we also detected $K_v1.2$ and $K_v\beta2$ subunits mRNA following sciatic nerve transection (Figure 18). For this, the left sciatic nerve of wild type mice was exposed at the level of notch and transected (trans) with a micro-spring scissor. The right sciatic nerve was used as a surgical control (sham). Fourteen days post injury (DPI) we extracted 5 mm segments of sciatic nerve proximal to the transected area.

We found that $K_v1.2$ and $K_v\beta2$ mRNAs are present in the sciatic nerve tissue from naive animals (not submitted to any surgery) (Figure 17)

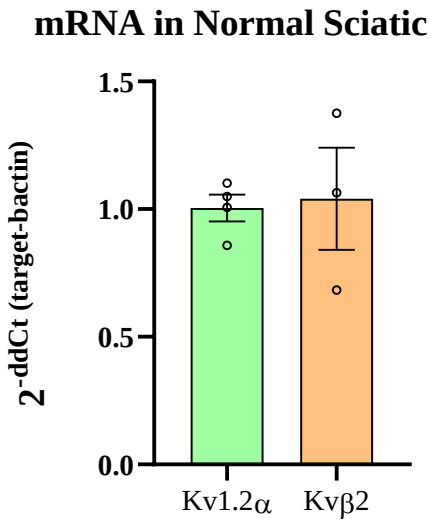

Figure 17: Basal mRNA of $K_v1.2$ and $K_v\beta2$ subunits are present in uninjured sciatic nerve. mRNA from 1 mm of sciatic nerve of both hind paws was extracted with Trizol reagent and then transcribed to cDNA to perform real time qPCR using corresponding primers. Data are expressed as fold change over WT using β-actin as a loading control. Values are shown as mean + SEM (n = 4 animals for $K_v1.2$ and n=3 for $K_v\beta2$).

Following transection, $K_v1.2$ mRNA in injured sciatic nerve shows a tendency to decrease compared to uninjured nerve $K_v\beta2$ mRNA decreases in injured nerves compared to sham condition. However, we did not observe significant changes. Additional experiments are necessary to confirm this decrease.

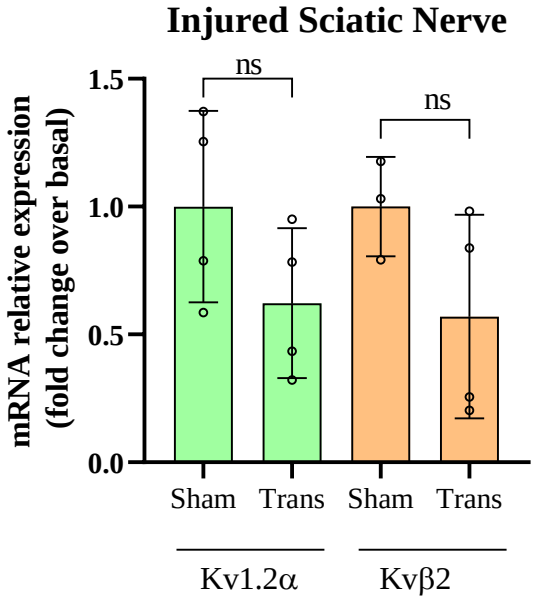

Figure 18: mRNA expression in injured sciatic nerve after 14 days post injury. Left sciatic nerves of wild-type mice were injured and right sciatic nerves were used as surgical controls (sham). After 14 days of injury a 10 mm segment of the sciatic nerve was removed in the proximal injured segment of damaged nerves. An equal segment of nerve was removed from sham mice. mRNA extraction was performed using Trizol reagent and then transcribed to cDNA to perform real time qPCR using corresponding primers. Each data is expressed as fold change over sham value. Normalization was performed using *dd*Ct method using β -actin was used as a loading control. Values are shown as mean + SEM, ns: not statistically significant (Mann-Whitney test) (n = 4 animals per genotype).

Because the sciatic nerve also contains other non-neuronal cell types, as Schwann Cells, fibroblast, and Immune System Cells, we decided to distinguish if $K_v1.2$ and $K_v\beta2$ mRNAs are in axons of or maybe in other cells present in nerves. Thus, we used an in vitro strategy to determine if these mRNA are detected in pure axons.

2. mRNA in pure axons

As mentioned above, a compartmentalized culture of embryonic DRG neurons allows us to physically isolate somas from axonal compartments, keeping their intracellular communication. The advantage of embryonic DRG neurons is their increased capacity to regenerate after an in-vitro axonal injury (Chierzi *et al*. 2005).

We used a compartment microfluidic chamber of 750-µm long and 15-µm width microchannel array. DRG were seeded in the somatic compartment and after 1 DIV axons started to cross to the axonal compartment. Axotomy was performed at 5 DIV and we collected mRNA from both compartments before axotomy (5 DIV) and 2, 4 and 7 days post axotomy (Figure 19A).

After axotomy, neurons regenerated their axons. Therefore, we established an *in vitro* injury model of DRG neurons.

In the somatic compartment the $K_v1.2$ mRNA tend to increase after 2 and 4 days post axotomy (dpa). At 7 dpa we observed an increase near of three times compared to basal expression of $K_v1.2$ mRNA levels ($p = 0.0369$) (Figure 19B). In regenerated axons, a slight decrease was detected at 2, 4 and 7 dpa however, not significant changes were observed.

$K_v\beta2$ mRNA was only detected in somas with a tendency to increase after axotomy, nevertheless no statistically significant changes were observed. In axons, $K_v\beta2$ mRNA was not detected neither, before nor after axotomy, suggesting this mRNA is not present in embryonic DRG axons (Figure 19C). Only in one case, the $K_v\beta2$ mRNA amplified at Ct=33.9 in one sample of 2 dpa. However, in the rest of samples, no amplification for this gene was detected. H1 and P0 genes were used as a control of axonal purity. Both genes were not detected in axonal compartment, only in somatic compartment.

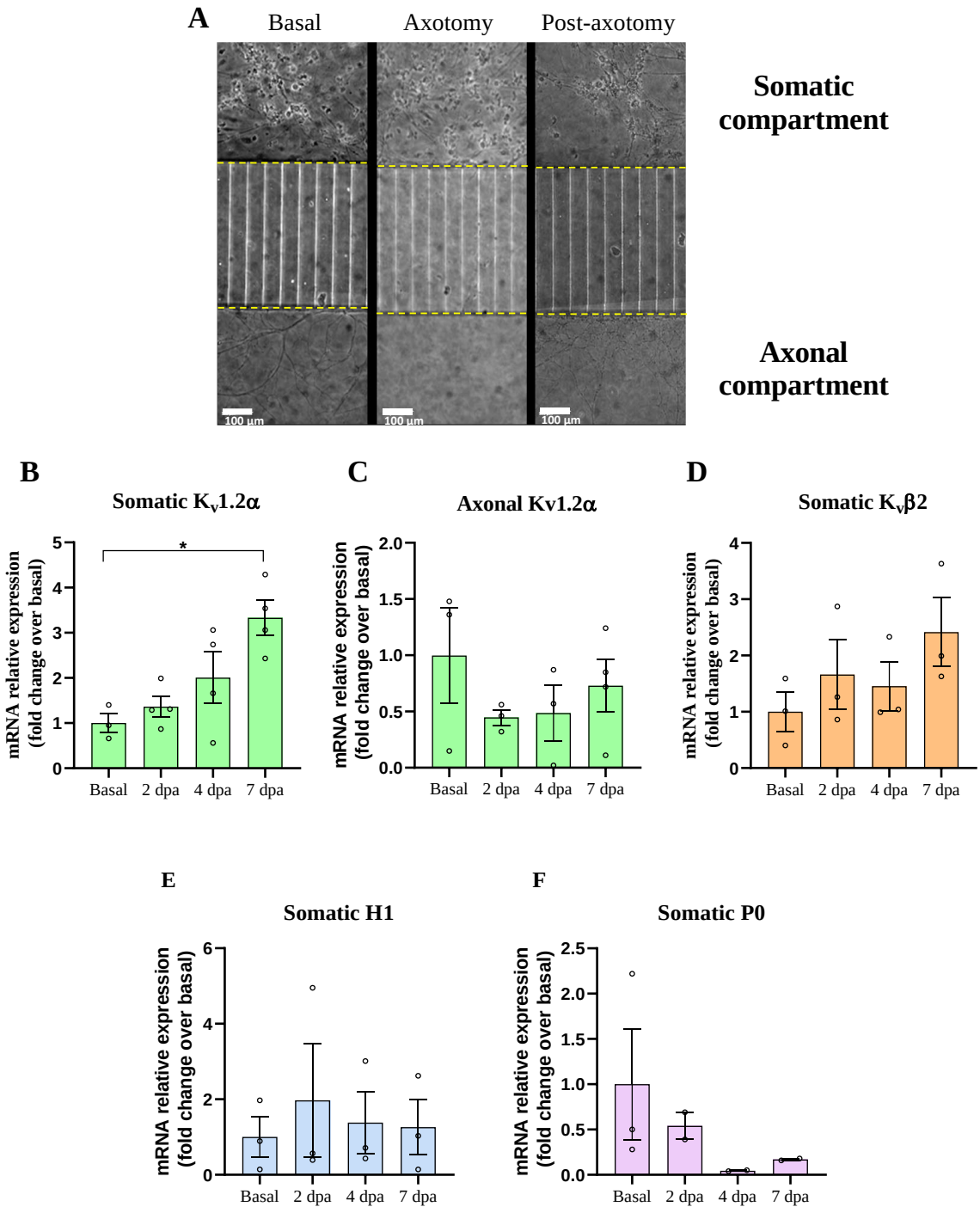

Figure 19. mRNA expression in somas and pure axons of embryonic DRG neurons after in vitro axotomy. Cultures were prepared from E14 embryonic DRG. Neurons were seeded in the somatic compartment of microfluidic chambers until axons crossed the microgrooves and reached the axonal compartment. Axotomy or in vitro injury was done at 5 DIV and axons regenerated after this procedure in the indicated days. B, D, E and F mRNAs from soma and C mRNA from axonal compartment were extracted using RNeasy mini kit and then transcribed to cDNA to perform real time qPCR using the corresponding primers. Kvβ2, H1 and P0 was not detected in axons neither basal nor post

axotomy. H1 and P0 correspond to somatic and Schwann cells controls respectively, these genes were not detected in axonal mRNAs. β-actin was used as a loading control. Data were normalized with basal levels using *dd*Ct method and were expressed as mean + SEM, * p= 0.0369. Kruskal-Wallis test and Dunn´s multiple comparisons test were applied (n = 4 cultures, each culture is prepared from 6 - 10 embryos). dpa: days post axotomy. H1: histone 1. P0: myelin protein 0.

Appendix 3

RiboTag experiments

A) Methodology

1.Animals

A heterozygotic Ribotag mouse was crossed with a $Na_v1.8$ Cre-expressing mouse (gently donated by Utrera's lab). Animals were weaned off at 21 days and then genotyped for experiments. $Na_v1.8$ Cre-Ribotag mice were named Cre+ animals and correspond to mice expressing the Ribotag in $Na_v1.8$ positive tissue (peripheral sensitive neurons). Cre- littermates were used as control and correspond to Ribotag mice that did not express Cre recombinase.

2. Lysis and pre-clearing of tissue of Ribotag mice

The protocol was done as previously described (Shigeoka et al. 2018, 2016). Briefly, the DRG and sciatic nerve was extracted from 3 Cre+ and 3 Cre- Ribotag littermates and immediately frozen in dry ice or at -80°C to prevent RNA degradation. The tissue was lysed with 500 ul of CHX lysis buffer (lysis buffer with cycloheximide) in a homogenizer on ice. Then, we collected the lysate and centrifuged it at 16,000 g at 4°C for 10 min. The supernatant was taken and transferred to a pre-chilled tube. The supernatant contains tagged ribosome-mRNA complexes. 5 ul of supernatant was stored as the "input". During this step, the magnetic Protein G beads (Invitrogen, #10003D) was prepared by placing a tube with 40 ul of beads on the magnet to separate beads from the solution and remove the supernatant. Then the beads were resuspended in 40 µL of CHX-lysis buffer and placed on the magnet to remove the supernatant as before. The supernatant that contains the ribosome-mRNA complex was transferred to the washed beads and the tube was rotated (head-to-toe rotation) for 1h at 4°C (pre-clearing step to minimize non-specific binding of mRNAs to the Protein G beads). After this, the tube containing the lysate and the beads was placed on the magnet and the supernatant was transferred to a new pre-chilled tube on ice (pre-cleared lysate).

3. Ribosome Immunoprecipitation and RNA purification

The protocol was done following the previously described (Shigeoka et al. 2016, 2018). We added 2.5 ul of rabbit anti-HA antibody (Abcam, #AB9110) to the pre-cleared lysate and the tube was rotated (head-to-toe rotation) at 4°C over-night. Then, the beads were washed with 40

µL of CHX-lysis buffer as described above and added to the lysate containing the antibody (the beads must be wet). After 4 h of head-to-toe rotation at 4°C, the tube was placed on a pre-chilled magnet on ice and the supernatant was removed (the beads must be wet because they contain tagged ribosome-mRNA complexes). At this point, the supernatant can be saved as the "unbound fraction". Later, 500 µL of wash buffer was added to the beads and rotated the tube for 5 min at 4°C. The washing step was repeated at least three times more. Then, 500 µL of wash buffer was added to the beads and transferred the buffer and the beads to a new pre-chilled tube. After, the wash buffer was removed from the beads using a DynaMag magnet and the beads were resuspended in 100 µL of CHX-lysis buffer. For RNA purification, first, 350 ul of RLT buffer of RNeasy mini kit (Qiagen, catalog #74104) containing 2-mercaptoethanol was added vortexed and incubated for 5 min at RT. During this step, mRNAs were dissociated from the tagged ribosome. Then, the mixture was centrifuged for 30 sec using a bench-top centrifuge and the supernatant was collected using a DynaMag magnet at RT. The supernatant contains axon-TRAPed mRNAs. After, we added 250 µL of 100% ethanol to the supernatant and the RNA was purified using RNeasy mini kit (Qiagen, catalog #74104) according to the instructions. To remove potential DNA contamination, an on-column DNase digestion was performed. Finally, the RNA was eluted in 14 µL of RNA-free water and cDNA was synthesized as described before. Quantitative RT-PCR was done using Kv1.1, Kv1.2 primers and β-actin primers as control.

B) Results

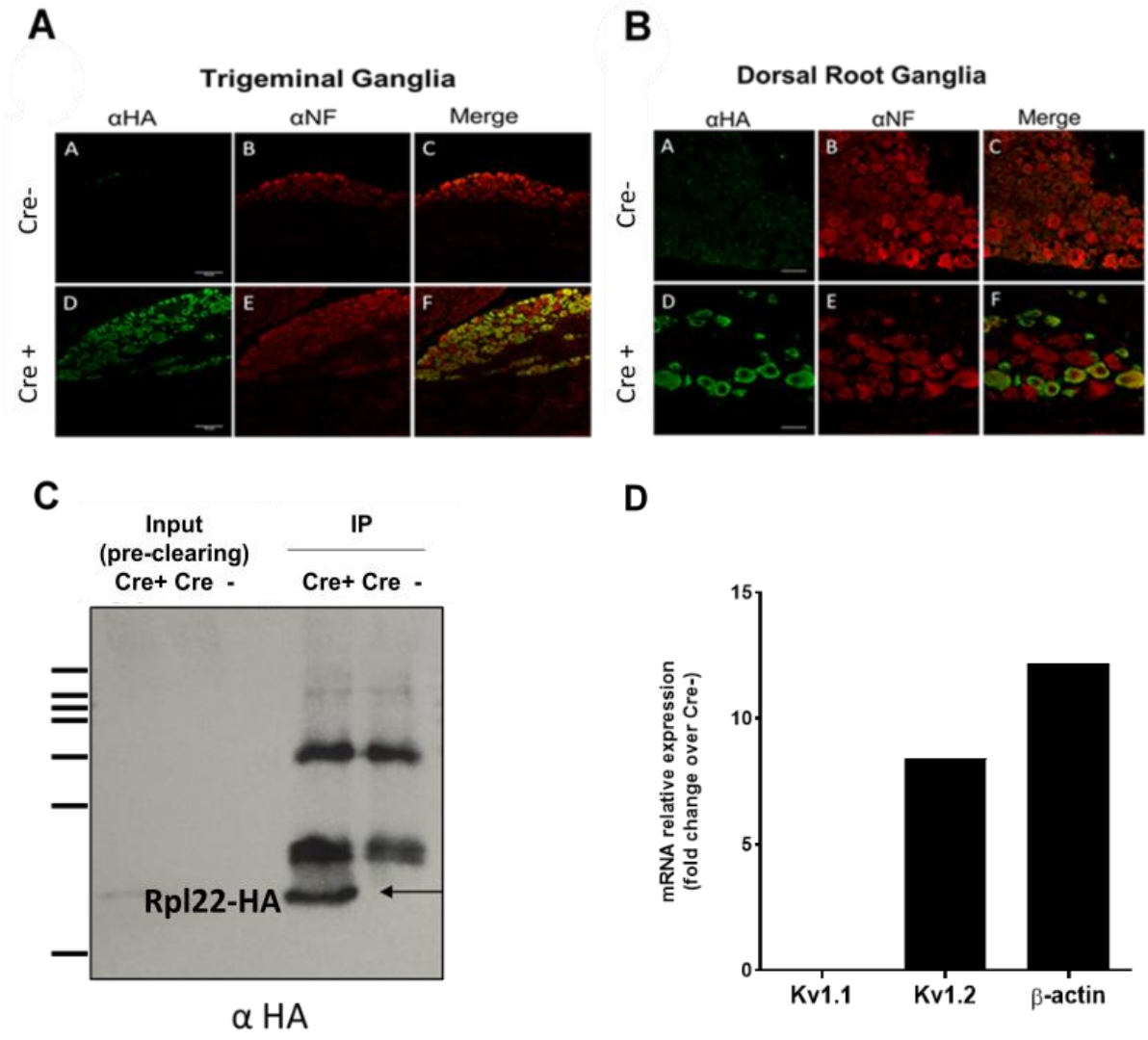

Figure 20. Expression of Rpl22-HA in peripheral tissue and gene expression in Ribotag mice. Immunofluorescence with anti-HA antibody in Trigeminal Ganglia (A) and Dorsal Root Ganglia (B) of Cre- and Cre+ mice (Scale bar = 100 μm). C Immunoprecipitation of the hemagglutinin protein (HA) in Cre- and Cre+ mice. D Sciatic nerve Kv1.2 mRNA detection by qPCR. The results are expressed as fold over Cre- mice. n=1, each n was prepared from 2 animals per genotype.

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