UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

ESCUELA NACIONAL DE ESTUDIOS SUPERIORES, UNIDAD JURIQUILLA

EVALUATION OF THE ROLE OF THE KRUPPEL-LIKE TRANSCRIPTION FACTOR 13 (KLF13) ON JAK-STAT PATHWAY IN ADULT MICE HIPPOCAMPAL NEURONS.

T E S I S

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Q U E P A R A O B T E N E R E L T Í T U L O D E

LICENCIADO EN NEUROCIENCIAS

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QUERÉTARO, QUERÉTARO, MÉXICO 2023.

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Abstract

Krüppel-like factor 13 (KLF13) is a transcription factor that functions predominantly as a transcriptional repressor by interacting with GC-rich regions on promoters of its target genes. KLF13 has showed to impact the activity of several signaling pathways with essential roles in the CNS, including the JAK/STAT, which is the canonical mediator of the growth hormone (GH) signaling. To test the hypothesis that KLF13 is a negative regulator of the JAK/STAT signaling pathway, it was analyzed the mRNA levels of genes involved in the pathway after forced expression of KLF13 in an inducible cell line. In a second experiment, the same genes were measured in the HT22 parental and in the HT22-*Klf13*-KO cell lines. On the other hand, to determine whether *Klf13* depletion could enhance GH-dependent JAK/STAT activity, it was analyzed the mRNA levels of target genes of the JAK/STAT signaling pathway in the hippocampus-derived cell line HT22 after treatment with GH.

The HT22-TR/TO-V5Klf13, which was previously engineered to force the expression of KLF13 with doxycycline, was used as a model of KLF13 induction. The parental HT22 cell line which expresses KLF13 constitutively, was used as control. In addition, the HT22-*Klf13*-KO cell line, in which the *Klf13* gene was previously depleted using CRISPR/Cas9 genome editing, was used as a model of loss of function.

The *in vitro* results were validated in an *in vivo* model using both wild type and *Klf13*-KO mice. To assess how KLF13 impacts the activity of JAK-STAT pathway, it was analyzed the mRNA levels of pathway-associated genes in the hippocampus of wild type and *Klf13*-KO mice. To assess how the GH-dependent gene expression of JAK/STAT output genes was impacted by *Klf13* depletion in the *in vivo* model, we performed intracerebroventricular injections of GH in wild type and *Klf13*-KO mice. Our results confirmed that KLF13 negatively regulates the expression of several genes involved in the JAK-STAT pathway and some of these results were recapitulated in the *in vivo* model. These findings support the notion that KLF13 is a negative regulator of the JAK/STAT activity.

Resumen

El factor de transcripción similar a Krüppel (KLF13) es un factor de transcripción que funciona predominantemente como un represor transcripcional al interactuar con regiones ricas en uniones G-C ubicadas en los promotores de sus genes objetivo. KLF13 impacta en la actividad de diversas vías de señalización que tienen roles esenciales en el SNC; por ejemplo, la vía JAK-STAT, la cual es la vía canónica de la señalización de la hormona de crecimiento (GH). Para evaluar la hipótesis de que KLF13 es un regulador negativo de la vía JAK-STAT, se analizaron los niveles de ARNm de genes asociados a la vía en cultivos celulares de una línea celular donde la expresión de KLF13 puede inducirse. Los mismos genes fueron evaluados en cultivos celulares de la línea parental HT22 y en cultivos celulares de una línea HT22 donde *Klf13* está depletado. Por otro lado, para determinar si la depleción de *Klf13* puede potenciar la actividad de la vía JAK-STAT mediada por GH, se analizaron los niveles de ARNm de genes objetivo de la señalización de la vía en cultivos celulares tratados con GH.

La línea celular HT22-TR/TO-V5Klf13 fue previamente diseñada para forzar la expresión de KLF13 añadiendo doxiciclina, y fue usada como modelo inducible de KLF13. La línea parental HT22 derivada de hipocampo de ratón adulto expresa a KLF13 constitutivamente y fue usada como control para comparar los niveles de mRNA de los genes de interés contra la línea HT22-*Klf13*-KO, en la cual *Klf13* está depletado por medio de la técnica de edición genómica CRISPR/Cas9.

Los resultados *in vitro* fueron validados en un modelo *in vivo* usando ratones adultos de tipo salvaje y knockout a *Klf13*. Para evaluar el efecto de KLF13 sobre la vía JAK-STAT en el modelo *in vivo*, se midieron los niveles de ARNm de genes asociados a la vía en hipocampos de ratones tipo salvaje y knockout. Para evaluar si la actividad de la vía JAK-STAT mediada por GH es potenciada en ausencia de KLF13 en neuronas hipocampales para el modelo *in vivo*, se realizaron inyecciones intracerebroventriculares de GH en ratones de tipo salvaje y knockout. Nuestros resultados confimaron que KLF13 regula negativamente la expresión de varios genes asociados a la vía JAK-STAT y

algunos de estos resultados se recapitularon *in vivo*. Estos hallazgos confirman que KLF13 es un regulador negativo de la actividad de la vía JAK-STAT.

Acknowledgments

I would thank to

Universidad Nacional Autónoma de México

Instituto de Neurobiología

Laboratorio Bioquímica de hormonas

Departamento de proteogenómica

Bioterio del Instituto de Neurobiología

Consejo Nacional de Ciencia y Tecnología (CONACYT) (projects CF319930 and CF214971) and Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT) (projects PAPIIT-DGAPA: IA200622; IN21552; IN209621; IN227020) for funding the development of this project and for providing me with a scholarship to complete my degree.

To my thesis advisor Dr. José Ávila Mendoza, thank you for your patience and understanding, for guiding me in the preparation of my thesis and for inspiring me to go for greater things. To me, you are an example of discipline and consistency.

To Dr. Carlos Arámburo de la Hoz, for allowing me to be part of your working group and for giving voice to science in Mexico.

To Dra. Maricela Luna, M. C. Martha Carranza and M. C. Adriana González Gallardo, for sharing with me your knowledge, answering my questions and providing me with technical support.

To laboratory members, Dr. Juan David Olivares, Dra. Valeria Urban, M. C. Jerusa Elienai, L. David Epardo, L. Petry Ticante, Belén Olvera, Sr. Gerardo Curtois and others, for creating an environment of trust and collaboration that made me grow enormously and motivated me to go to the lab every day, for their life advices and good wishes in my new stage.

Dedications

To my parents, María Guadalupe Rueda Acosta y Rogelio Itzamná Delgado Figueroa, who have influenced on me their thirst for self-improvement and their passion for helping people and for giving me all their love and support.

To my brother, Rommel Itzamná Delgado Figueroa, who taught me that discipline leads to consistent results and for accompanying me through the different stages of my life.

To my little brother, Rogelio Alexander Delgado Rueda, for sharing his nobility and affection with me every day.

To my grandmothers Candelaria Rueda Acosta and Elizabeth Figueroa Rojas, that together with my mother are the strongest women I know. The history of our family began with you as examples of courage, leadership and constant struggle. I admire and respect them very much.

To Miguel Latuan Díaz Monroy, for being my confidant and support in all ways.

To all my family for their unconditional love and life lessons.

To all my friends, specially to Martín Morales, that dream of serving the world to make it a better place for everyone.

INDEX

Introduction

In this study the role of Krüppel-like factor 13 (KLF13) in the JAK/STAT signaling pathway in adult mammalian hippocampal neurons was analyzed. The objective is to consider KLF13 as a target to promote neuroprotective and neuroregenerative effects of GH for further studies.

Axonal damage is one of the common features in many types of central nervous system (CNS) injuries. Following the damage, neurons trigger compensatory responses that promote self-repair. However, this mechanism fails to block neurodegenerative signals and thus fails to regenerate, leading to permanent functional deficits. A possible therapy for axonal repair is to enhance the intrinsic ability of neurons to regenerate their axons by activating pro-regenerative signaling pathways, such as the JAK-STAT pathway, which is the canonical mediator of GH signaling. GH has neuroprotective and neuroregenerative effects on axons against CNS damage, although is a mild effect. The intrinsic ability of CNS neurons to regenerate their axons declines upon transitioning from embryonic stages to adulthood. This decline is associated with the increase in the expression of the KLF13 transcription factor, that could be involved in the loss of the ability to elongate neuronal projections.

In this work we investigate the impact of KLF13 on the transcription of genes associated with the JAK-STAT pathway. The results showed that forced expression of *Klf13* with doxycycline in the mouse hippocampus-derived cell line HT22 repressed *Jak1*, *Jak2*, *Jak3*, and *Socs1*, while upregulating *Stat5a* expression. In contrast, KLF13-deficient HT22 neurons showed upregulated expression of *Jak1*, *Stat3*, *Socs1*, *Socs3*, and *Igf1*. Furthermore, it was analyzed whether *Klf13* depletion could enhance GH-dependent JAK/STAT activity. In this regard, the experiment of JAK-STAT activity stimulation using GH demonstrated that GH treatment increased the expression of *Socs3*, *Igf1*, and *Bdnf*, and that this stimulation was strongly enhanced in the absence of KLF13 in the HT22 cell line. Finally, some of these results were recapitulated in the *in viv*o model, supporting the notion that KLF13 is a negative regulator of JAK-STAT activity.

Background

1. Krüppel-like Factors (KLFs) / Specificity Protein (SP) family

Krüppel-like factors (Klfs) belong to a family of transcription factors that activate or repress gene expression by binding to the promoters of their target genes and associating with chromatin-modifying enzymes (Knoedler and Denver, 2014). Along with the specificity protein (SP) family, which was one of the first mammalian transcription factor families to be identified and characterized, they constitute a superfamily characterized by a DNA-binding domain at C-terminal end composed of three zinc finger motifs (C2H2 Znf) (Cook *et al.*, 2006). The DNA-binding domain of SP/KLF members shares a high amino acid sequence identity (Swamynathan, 2010). However, SP factors are characterized by a Buttonhead (BTD) box located N-terminal to the zinc fingers, which distinguishes them from KLFs (Suske *et al.,* 2005).

Twenty-seven genes encoding SP/KLF proteins have been identified in the human genome: 18 are members of the KLF, while 9 belong to the SP family. Genes encoding members of the SP/KLF superfamily are scattered throughout the genome, except for one locus containing two genes: *Klf1* and *Klf2* (Kaczynski *et al.,* 2003; Pei and Grishin, 2013). This superfamily plays essential roles in most living beings, including unicellular and multicellular organisms (Presnell *et al.,* 2015). Specifically, KLFs are involved in a wide range of biological processes, including proliferation, differentiation, apoptosis, development, and regeneration (Pearson *et al.,* 2008; Moore *et al.,* 2009).

The Krüppel-like subfamily received its name due to the shared homology between family members and the Krüppel protein of *Drosophila melanogaster*, which is essential in body segmentation during fly embryogenesis (Schuh *et al.,* 1986). In mammals, the KLFs were first named according to the tissues in which they were initially described, the genetic elements to which they were bound, or their physiological responses. However, they are currently named according to the numerical designation of the Human Gene Nomenclature Committee (HGNC) (Swamynathan, 2010; Bruford *et al.,* 2020).

1.1 KLFs Structure

All members of the KLF family share a characteristic structure. Generally, KLFs contain two main domains: a highly conserved three-zinc finger DNA-binding domain located at the carboxyl terminal end, which enables them to bind to Guanine-Cytosine (GC) and Guanine-Thymine (GT) rich regions found in gene promoters and regulatory elements in the DNA. The other domain is a variable region at the amino terminal end, which allows them to interact with cofactors that mediate their activity as transcriptional repressors or activators (Pearson *et al.,* 2008). Additionally, they have nuclear localization signals adjacent to or outside the zinc finger motif that enable them to enter the nucleus and regulate transcription of different genes (Rodríguez and Martignetti, 2009).

1.1.1 The three zinc finger motif

The DNA-binding domain in the KLF family is 81 amino acids long, consisting of two zinc-fingers, each 23 amino acids in length, and a third one of 21 amino acids. It also contains two conserved linker regions of 7 amino acids (TGEKP(Y/F)X) that space the zinc fingers (Dang *et al.,* 2000). These three fingers make tandem contacts along the DNA and can recognize a wide variety of sequences enriched in GC (Wolfe *et al.,* 2000). The amino acid sequence of each finger folds in the presence of a single zinc ion (Zn^{2+}) to form a β-β-α domain. The Zn^{2+} is coordinated between two cysteines at one end of the β-sheet and two histidine residues in the C-terminal portion of the α-helix (Wolfe *et al.,* 2000). Figure 1 depicts the common structure of Krüppel-like factors (KLFs).

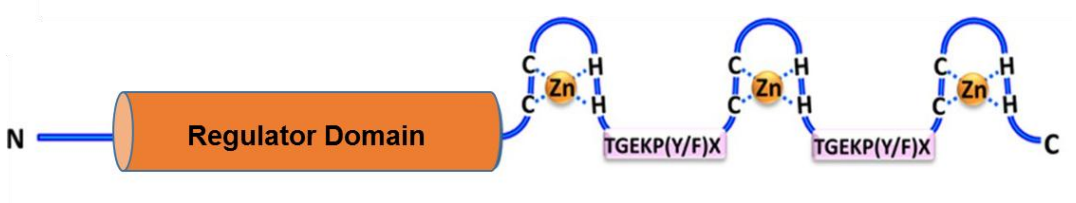

Figure 1. Schematic representation of the common structure of Krüppel-like factors. The C-terminal end of the protein corresponds to the DNA-binding domain, which is composed of three zinc fingers that form a β-β-α arrangement in the presence of a Zn^{2+}. The Zn^{2+} is coordinated by two cysteines and two histidines, and the fingers are spaced by the conserved linker region

TGEKP(Y/F)X. The N-terminal end contains a transcriptional regulator domain consisting of different combinations of activation and repression domains. The figure is adapted from Jain *et al.* (2014).

The three zinc-finger motif is known as the DNA-binding domain that recognizes and binds to GC (GGGCGG) and GT (CACCC) rich regions in the genome, often located within a few hundred base pairs upstream of transcription start sites in many gene promoters, including KLFs themselves (Ávila-Mendoza *et al.,* 2020a). The Zif268 protein is another transcription factor with a zinc-finger structure whose binding to DNA has served as the prototype for understanding DNA recognition by this type of protein. During DNA binding, the α helix of each finger binds to three or four DNA nucleotides in the major groove of DNA. By binding the three fingers, proteins wrap around the DNA, with finger one facing the 3' end while finger 3 interacts with the 5' end of the DNA (Wolfe *et al.,* 2000). In addition to DNA binding, the zinc-finger motifs also function as protein-protein interaction domains that modulate the specificity of DNA binding (Kaczynski *et al.,* 2003). Figure 2 depicts the zinc-finger binding of KLFs to DNA.

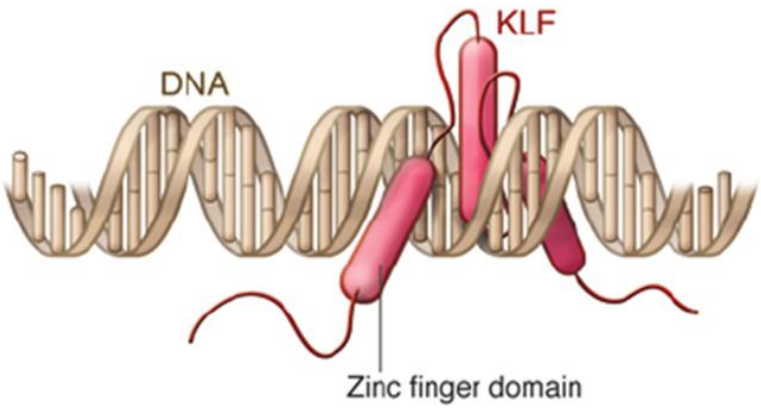

Figure 2. Schematic representation of the KLFs zinc-finger binding to DNA. The three highly conserved zinc fingers of KLFs insert into the major groove of the DNA helix with an arrangement where finger one faces the 3' end and finger 3 interacts with the 5' end of the DNA. By binding the three fingers, proteins wrap around the DNA. The figure is taken from Jeffrey K. (2015).

1.1.2 Amino terminal motif of KLFs

The amino-terminal region of KLFs is highly variable and contains sites of interaction with cofactors, which confer activation/repression properties to KLFs. Based on the structural and functional similarities in this region, KLFs have been clustered into three main groups. The first group includes KLFs 3, 8, and 12. All of these act as transcriptional repressors based on their N-terminal Pro-Val-Asp-Leu-Ser/Thr (PVDLS) motif that promotes their interaction with the C-terminal binding protein (CtBP). CtBP, in turn, recruits chromatin-modifying enzymes that may operate by linking deacetylases to DNA-bound factors or add repressive marks to histones (Dewi *et al.,* 2015; van Vliet *et al.,* 2000; Zhang *et al.,* 2015; Turner and Crossley, 2001).

The second group consists of KLFs 1, 2, 4, 5, 6, and 7, which share a common acidic activation domain that confer them properties as transcriptional activators. These members can bind to histone acetyl-transferases such as CREB binding protein (CBP), p300, and p300/CREB-binding protein-associated factor (PCAF) that may acetylate KLFs and stimulate their transcriptional activity or acetylate histones to coordinate chromatin opening (Zhang *et al.,* 2001; Evans *et al.,* 2007; Zhang and Teng, 2003).

The third group, which is composed of KLFs 9, 10,11, 13, 14, and 16, shares a Sin3a-interacting domain (SID) that interacts with the transcriptional corepressor Sin3A. Sin3A, in turn, recruits other proteins, such as histone deacetylases (HDACs), to restore histone compaction and inhibit transcription, making them act predominantly as transcriptional repressors (Pollak *et al.,* 2018). However, group 3 is also capable of acting as transactivators, depending on the cellular context, such as the stage of cell differentiation in which the KLFs of this group are expressed (Knoedler and Denver 2014). Finally, as KLF15 and 17 lack defined interaction protein motifs, they are not yet grouped, although their primary amino acid sequences place them closer to group 3 (Presnell *et al.,* 2015; Knoedler and Denver 2014). Figure 3 shows how the KLF family is clustered according to their N-terminal domains.

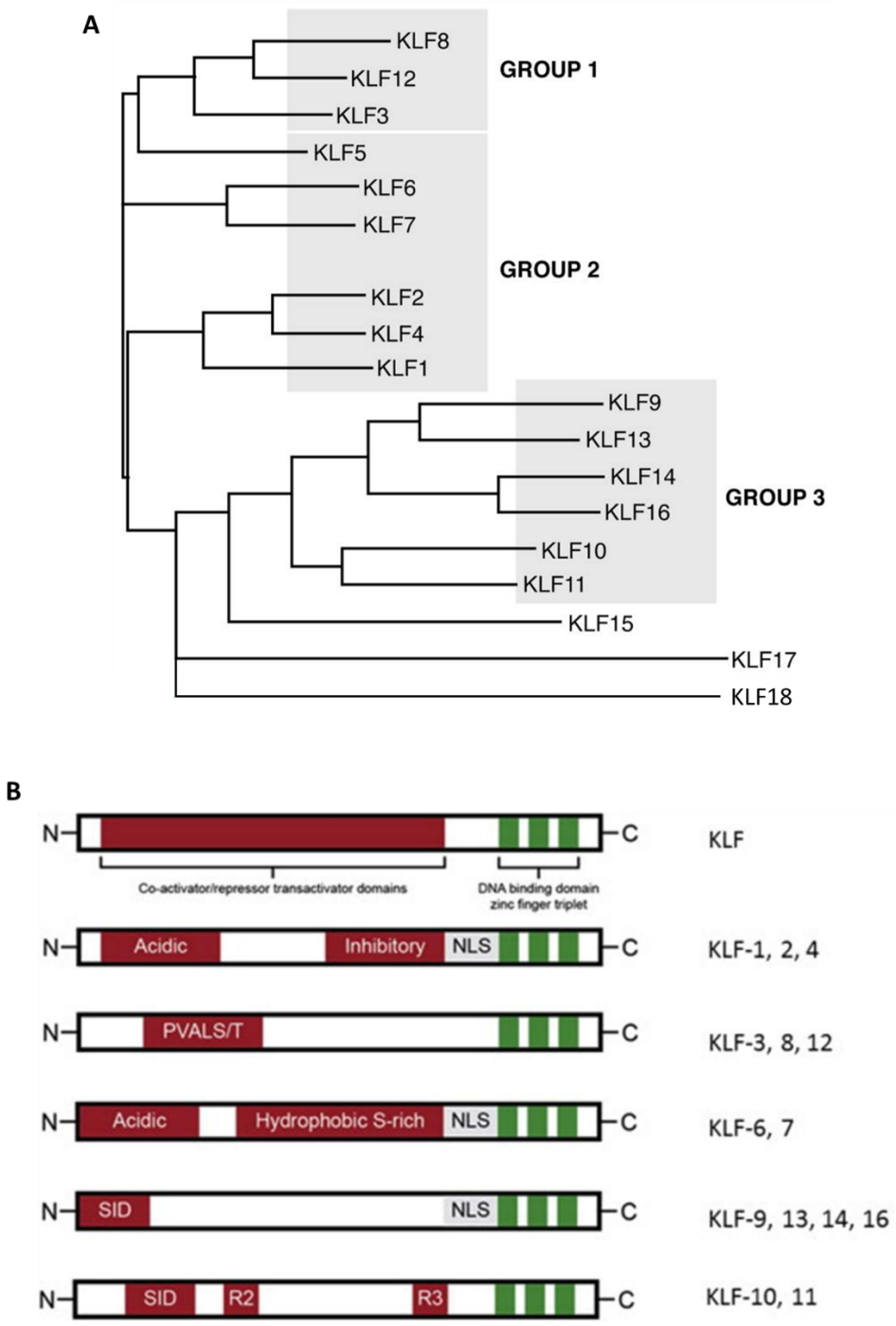

Figure 3. Clustered Krüppel like factors and their functional N-terminal domains. A) Cladogram showing the structural relationships of the Krüppel-like factors derived from human genome. The family members are grouped based on their N-terminal domain similarity. KLFs 15, 17 and 18 are not grouped since little is known about the proteins to which they bind. KLF17 and KLF18 appear together because they are chromosomal neighbors. B) A schematic representation of the functional N-terminal domains of KLFs. Group 1 (KLFs 3, 8 and 12) share a PVDLS motif, which is recognized by the corepressor CtBP protein. Group 2 (KLFs 1, 2, 4, 5, 6, and 7) share an acidic domain where acetylases bind. Group 3 (KLFs 9, 10, 11, 13, 14, and 16) have a Sin3A-binding site (SID) to interact with corepressor Sin3A. Modified from McConnell and Yang, 2010.

1.2 Functions of KLFs

The functions of KLFs vary according to the spatio-temporal contexts and physiological conditions in which they are expressed (Swamynathan, 2010). Some KLFs of the same group can have overlapping or redundant functions to compensate for each other if one is lost or deleted, whereas different KLFs may have antagonistic effects on individual cellular processes (Kaczynski *et al.,* 2003; Swamynathan, 2010).

KLFs 1, 2, 3, 4, 5, 8, 11, 12, and 13 are expressed in erythroid cells (Zhang *et al.,* 2005). Hemoglobin, which is a molecule enriched in erythrocytes, is responsible for transporting oxygen in the body and consists of four subunits. During fetal development, hemoglobin is formed by two alpha-type and two gamma-type subunits, but after birth, it begins to switch to two alpha-type and two beta-type subunits (Sankaran and Orkin, 2013). The switch from gamma to beta type is largely regulated by KLFs. Specifically, KLF1 positively regulates the adult *β-globin* gene and indirectly represses the *γ-globin* gene (Siatecka and Bieker, 2011). KLF2, KLF5, and KLF13 positively regulate the *γ-globin* gene, while KLF4 and KLF8 negatively regulate it in the K562 cell line (a human leukemia cell line) (Kalra *et al.,* 2011; Zhang *et al.,* 2005).

KLFs 2, 3, 4, 9, 10, and 13 have shown a role in the immune system. KLF2 and 4 are expressed in T cells, where both are downregulated upon T cell activation (Hart *et al.,* 2012). Particularly, KLF2 maintains naive T cell quiescence and survival and serves to allow mature T cells to exit the thymus and peripheral lymphoid tissues (Carlson *et al.,* 2006), whereas in B cells, it is involved in differentiation and migration (Winkelmann *et al.,* 2011). KLF4 also maintains naive T cell quiescence and negatively regulates T cell proliferation (Yamada *et al.,* 2009). In addition, KLF4 promotes monocyte differentiation (Feinberg *et al.,* 2007), and together with KLF9, it is downregulated in B memory cells, which makes them proliferate more rapidly than naive cells, thus making the secondary immune response dominant (Good and Tangye, 2007). KLF3 is necessary for B cell normal development and promotes B cell migration from bone marrow to spleen (Vu *et al.,* 2011). KLF10 targets two key genes in CD4+CD25− T cells to turn them into CD4+CD25+ T regulatory cells, thus having a role in CD4+CD25−

T cell activation and T regulatory cell suppressor function (Cao *et al.,* 2009). Finally, KLF13 has a role in homing and migration of effector and memory T cells during acute infections by upregulating the chemokine RANTES in activated T lymphocytes (Zhou *et al.,* 2007).

On the other hand, it has been shown that KLFs 2, 3, 5, 6, and 15 have important roles in metabolism, including adipogenesis. KLF2 and KLF3 inhibit the differentiation of preadipocytes to adipocytes (Sue *et al.,* 2008; Wu *et al.,* 2005), whereas KLF5, KLF6, and KLF15 enhance adipocyte formation through different mechanisms (Oishi *et al.,* 2005; Mori *et al.,* 2005; Li *et al.,* 2005).

Regarding KLF4 and KLF5, it has been described that they are important for maintaining intestinal homeostasis. KLF4 is expressed in differentiated epithelial cells in the upper regions, whereas KLF5 is expressed in actively dividing cells in the bottom of the intestinal epithelium. Both regulate the *Klf4* promoter by directly competing to bind their DNA sequence, where KLF5 negatively regulates while KLF4 positively regulates its transcription (McConnell *et al.,* 2007).

Additionally, each KLF has specific functions. KLF2 is enriched in the lung where it plays a role in normal lung development (Wani *et al.,* 1999). KLF4 is expressed in the intestine, skin, and cornea, where it is involved in epithelial barrier formation. It is also expressed in the testis, kidney, bone, and teeth, where it contributes to cell differentiation (Ghaleb and Yang, 2017). KLF5 is necessary for the development and maintenance of the heart and lung (Salmon, 2020). KLF6 promotes vascular remodeling in response to injury (Gallardo-Vara *et al.,* 2016). KLF7 is highly expressed in the CNS, where it contributes to neuronal differentiation and maturation (Laub *et al.,* 2005). KLF9 is a thyroid hormone-induced protein that is also implicated in postnatal brain development (Denver and Williamson, 2009), and it is necessary for reproductive tissue differentiation (Simmen *et al.,* 2015). KLF10 participates in correct bone development and normal heart function (Salmon, 2020). KLF11 suppresses endothelial cell inflammatory activation (Fan *et al.,* 2012). KLF12 negatively regulates uterine endometrial differentiation during early pregnancy and blocks embryo implantation in the endometrium (Zhang *et al.,* 2015). KLF14 represses transcription of genes in the NF-κB signaling pathway to inhibit inflammation in

endothelial cells (Hu W. *et al.,* 2018). KLF15 switches the use of glucose to fatty acids in response to changes in energy demand in brown adipose tissue by upregulating genes related to fatty acid utilization (Nabatame *et al.,* 2021). KLF16 promotes fatty acid oxidation in the liver by enhancing transcription of peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor involved in lipid metabolism (Sun *et al.,* 2021). Finally, KLF17 upregulates uterine corin (an enzyme that boosts the increase in size of blood vessels) as part of the physiological response in normal pregnancy (Wang *et al.,* 2020).

Overall, some KLFs have restricted tissue expression, whereas others are ubiquitously expressed throughout the body, contributing to transcriptional events that exert various physiological functions, including proliferation, differentiation, and maintenance of cellular identity. These functions, in turn, are involved in complex biological processes such as development. Table 1 summarizes the general functions conferred by KLFs. However, new KLF functions are constantly being discovered.

Table 1. General functions of KLFs.

KLFs	**Functions involved**	**Actions**
KLF1	Hematopoiesis	Promotes adult *β-globin* gene and represses *γ-globin* gene expression.
KLF2	Hematopoiesis T and B cells actions Adipogenesis Maintenance of vascular integrity Normal lung development	Promotes *γ-globin* gene expression. Maintains naive T cell quiescence and facilitates mature T cells exit the thymus. Induces B cell differentiation and migration. Inhibit the differentiation of preadipocytes to adipocytes. Regulates *Nos3* and *Thbd* to confer anti-inflammatory and antithrombotic effects to the vessel wall.
KLF3	Hematopoiesis Adipogenesis	Inhibit the differentiation of preadipocytes to adipocytes.
KLF4	Hematopoiesis T and B memory cells actions Maintenance of vascular integrity. Maintenance of intestinal epithelium homeostasis Epithelial barrier formation	Suppresses *γ-globin* gene expression. Maintains naive T cell quiescence and suppresses T cell proliferation. When is downregulated in B memory cells promotes secondary immune response. Regulates endothelial nitric oxide synthase (*Nos3*) and thrombomodulin (*Thbd*) to confer anti-inflammatory and antithrombotic effects to the vessel wall.

		Suppresses expression of genes that promote cell cycle progression.
KLF5	Hematopoiesis Adipogenesis Maintenance of intestinal epithelium homeostasis Heart and lung development and maintenance	Promotes *γ-globin* gene expression. Enhances adipocyte differentiation. Activates the transcription of several cell cycle promoting genes.
KLF6	Adipogenesis Promotes vascular remodeling	Enhances adipocyte differentiation.
KLF7	Neuronal maturation	It stimulates transcription of the cyclin-dependent kinase inhibitor *p21waf/cip* gene thus promoting differentiation of progenitor cells and phenotype maintenance.
KLF8	Hematopoiesis	Suppresses *γ-globin* gene expression.
KLF9	B memory cells actions Postnatal brain development	When is downregulated in B memory cells promotes secondary immune response.
KLF10	T regulatory cells actions Bone development and normal heart function	Targets two key genes in T cells to induce T regulatory cells activation.
KLF11	Hematopoiesis Inflammation	Suppresses inflammatory response of endothelial cells.
KLF12	Hematopoiesis Embryo implantation	Inhibit uterine endometrial differentiation during early pregnancy.
KLF13	Hematopoiesis T and B cells actions	Promotes *γ-globin* gene expression. Upregulates RANTES in T activated cells during acute infections to induce memory T cells migration. Promotes B cell normal development and B cell migration from bone marrow to spleen.
KLF14	Inflammation	Negative regulator of inflammation of endothelial cells.
KLF15	Adipogenesis Lipid metabolism	Enhances adipocyte differentiation. Promotes fatty acids oxidation by upregulating genes related to lipid utilization in response to changes in energy demand.
KLF16	Lipid metabolism	Promotes fatty acids oxidation in a indirectly manner by activating transcription of PPARα, a nuclear receptor.

KLF17	Development	Upregulates uterine corin (an enzyme that boosts increase size of blood vessels) as part of the physiological response in normal pregnancy.
KLF18	Early embryonic development	Its specific functions are currently unknown but it is thought to being expressed in most of placental mammals.

1.3 Roles of KLFs in axonal regeneration

Axon regeneration after injury is limited in the mammalian CNS. Both intrinsic and extrinsic factors mediate the inability of neurons to regenerate. The extrinsic factors include the presence of mature astrocytes and oligodendrocytes that create an inhibitory environment for axon regeneration. Intrinsic factors, on the other hand, are expressed by neurons during development to promote neuronal differentiation and the establishment of functional synapses, which results in the loss of axonal regeneration capacity (Mahar and Cavalli, 2018).

Extensive research has been done to decipher the mechanisms underlying the loss of regenerative capacity during early development and to find out which developmentally regulated genes are responsible for this switch. Several members of the KLF family have emerged as important candidates for intrinsic regulators that contribute to the developmental loss of axon growth ability. In support of this, the expression of some KLFs is developmentally regulated and correlates with the loss of regenerative capacity of neurons of the CNS, including the retinal ganglion cells (RGCs), cortical neurons, and hippocampal neurons (Mahar and Cavalli, 2018; Moore *et al.,* 2009).

It has been shown in several models that multiple KLF family members differentially affect neurite outgrowth in the CNS. KLF6 and KLF7 increased, whereas KLFS -1, -2, -4, -5, -9, -13, -14, -15, and -16 decreased neurite length in rat cortical neurons from embryonic day 18 (E18) to postnatal day 18 (P18). In this period axon growth capacity decreases due to target innervation (Moore *et al.,* 2009; Goldberg *et al.,* 2002). *Klf6* and *Klf7* are upregulated after injury in regenerative retinal ganglion cells (RGCs) in *Xenopus laevis*, zebrafish, and mice in optic nerve crush models (Whitworth *et al.,* 2017; Veldman *et al.,* 2007; Moore *et al.,* 2009). Conversely, *Klf4* and *Klf9* are down-regulated in mice (Moore *et al.,* 2009), whereas *Klf4* and *Klf12* are down-regulated in regenerative RGCs in

Xenopus laevis (Whitworth *et al.,* 2017). Moreover, it has been shown that overexpression of KLF4 and KLF9 significantly decreases axon growth in RGCs, and further, the inactivation of KLF4 using a Cre/lox platform in developing RGCs promotes axon regeneration in adult mice RGCs *in vivo* after optic nerve crush (Moore *et al.,* 2009).

Furthermore, the gene expression profile of rats RGCs from E17 through postnatal day 21 (P21) showed that *Klf6* and *Klf7* decreased their expression, while *Klf4* and *Klf9* increased postnatally, with *Klf9* exhibiting higher expression levels after birth compared to *Klf4* (Moore *et al.,* 2009). Additionally, when overexpressing combinations of KLFs (growth suppressive - KLF4 or -9 - and growth-enhancing - KLF6 or -7 - KLFs) in rats P5 cortical neurons, it was found that the effect of suppressors dominated over enhancers. Specifically, KLF6 and -7 did not enhance growth in the presence of KLF4 or -9, whereas KLF4 suppressed neurite growth when co-overexpressed with KLF6 or -7 (Moore *et al.,* 2009).

Due to KLF9's higher expression compared to KLF4 after birth, and their similar effect on axon growth suppression, it has garnered attention for studying the molecular mechanisms governing the loss of regenerative ability in neurons (Apara and Goldberg, 2014). In this regard, both KLF9 and its paralog, KLF13, which are members of group 3, inhibit neurite outgrowth in hippocampal cells. In these cells, they act as transcriptional repressors by associating with chromatin within proximal promoters of genes involved in the cyclic adenosine monophosphate (cAMP) pathway (Ávila-Mendoza et al., 2020b). This pathway plays a crucial role in axon growth by inducing a proregenerative gene program (Hannila and Filbin, 2008). Additionally, it has been found that KLF13 has a stronger suppressive effect in this pathway compared to KLF9 (Ávila-Mendoza *et al.,* 2020b). Moreover, both KLF9 and KLF13 have been shown to affect other signaling pathways involved in the dynamics of axon growth, such as the actin cytoskeleton and axon guidance, and the JAK-STAT signaling pathway (Ávila-Mendoza *et al.,* 2020b). However, the transcriptional role of these KLFs in these pathways has not been elucidated.

2. Krüppel-like factor 13

KLF13 was originally named by three independent studies as RANTES factor of late activated T lymphocytes-1 (RFLAT-1) (Song *et al.,* 1999), basic transcription element binding protein-3 (BTEB-3) (Martin *et al.,* 2000), and fetal Krüppel-like factor-2 (FKLF-2) (Asano *et al.,* 2000). It has been found in several embryonic and adult mouse tissues, such as the heart, kidney, spleen, liver, uterus, stomach, intestine, skeletal muscle and brain (Martin *et al.,* 2000; Lavallée *et al.,* 2006).

KLF13 is a 289-amino-acid protein with a molecular weight between 31.1 and 38 kDa (Asano *et al.,* 2000; Song *et al.,* 1999). Its structure consists of three zinc fingers at the C-terminus, two independent nuclear localization signals, one located immediately upstream of the zinc-finger DNA-binding domain and the other within the zinc-fingers. It also has combined activation/repression domains at the N-terminus (Song *et al.,* 2002). The zinc-finger of KLF13 also contains specific domains that allow its interaction with coactivators such as CBP/p300 and p300/CBP-associated factor (PCAF) (Song *et al.,* 2002). These acetylate KLF13 and enhance its DNA binding and transcriptional activity (Song *et al.,* 2002). On the other hand, the KLF13 repression domain is distributed in three separated regions at the N-terminal: R1, R2, and R3, which are at positions 1–24, 55–74, and 75–114, respectively (Kaczynski *et al.,* 2001). These interact with the Sin3A corepressor, which recruits HDAC-1 to modify histone structure and block transcription (Kaczynski *et al.,* 2001). Figure 4 represents the structure of KLF13.

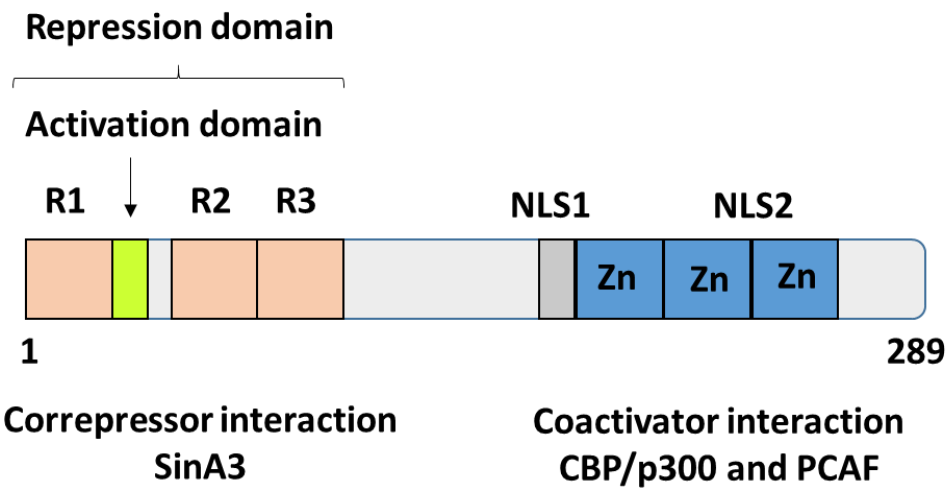

Figure 4. KLF13 structure and functional domains. KLF13 contains three zinc finger motif, two independent nuclear localization signals and a transactivation domain at its C-terminal, whereas it has a combination of transactivation and repression domains at its N terminal. The repression domain comprises R1, R2 and R3. KLF13 repression domain recruits corepressor factors as SinA3 whereas transactivation domain recruits coactivator proteins such as CBP/p300 and PCAF.

KLF13 interacts with promoters of *Sv40*, *Sm22α*, *γ-globin*, and *Rantes* genes to activate their gene transcription. On the other hand, it binds to promoter to repress the transcription of *Bcl-xl* and cytochrome P450 *Cyp1a1* genes (Martin et al., 2000; Asano et al., 2000; Song et al., 1999; Zhou et al., 2007). Some of these promoters contain GC-rich sequence elements recognized by KLF13 through its DNA binding domain. For example, the basic transcription element (BTE), a GC-rich region, is located in the *Sv40* promoter (Martin *et al.,* 2000). In summary, this indicates that the regulatory activity of KLF13 depends on the promoter to which it binds and the cofactors that it recruits.

It is worth noting that RANTES also plays a crucial role in neuroprotection. RANTES is a chemokine that induces the recruitment and activation of immune cells in inflammatory sites (Appay and Rowland-Jones, 2001). Moreover, RANTES contributes to the protective action of PACAP, a neuroprotective peptide in CNS injury and diseases such as cerebral ischemia, Parkinson's disease, and trauma (Sanchez *et al.,* 2009). These findings suggest that KLF13 may have a potential role in the CNS during neuroprotection and neuroregeneration.

2.1 KLF13: a dual transcriptional regulator of signaling pathways in cellular function and disease

KLF13 exhibits a dual role as a repressor or activator of transcription (Avila-Mendoza, et al., 2020b), depending on cellular context. In some of these actions, KLF13 works as a downstream mediator of various receptor signaling pathways (Knoedler et al., 2014), significantly impacting multiple cellular functions. For instance, in cardiomyocytes, *Klf13* is directly targeted by glucocorticoid nuclear receptor signaling (GR). In this context, the expression of *Klf13* promotes the activation of genes involved in pathways crucial for cardiomyocyte survival (Cruz-Topete et al., 2016).

On the other hand, KLF13 regulates the expression of membrane proteins in B and T cells during their maturation. For instance, it can repress the expression of CD21 and CD23 proteins, thereby inhibiting the transition to mature B cells. Furthermore, KLF13 is suggested to play a role in regulating signaling intermediates of B and T cell receptors (Outram et al., 2008).

The dual role of KLF13 in transcription and cellular actions becomes more prominent in cancer cells. In prostate carcinoma cells, KLF13 suppresses cancer cell proliferation by modulating the AKT signaling pathway (Wang Q., et al., 2018). In contrast, KLF13 acts as a promoter of oncogenes and promotes the proliferation of oral cancer cells (Henson and Gollin, 2010).

In the CNS, KLF13 inhibits proliferation in glioma cell lines, and these suppression effects are abrogated by AKT activation (Wu R., 2019). However, KLF13 promotes the differentiation of oligodendrocytes by binding to regulatory regions of myelin genes and acts in synergy with other transcription factors to boost the activation of myelin gene expression. These actions also apply to KLF9, a closely related transcription factor (Bernhardt *et al.,* 2022). In this regard, the redundant actions of both KLFs have been studied in axon regeneration of hippocampal neurons, where both inhibit axon regeneration by suppressing the gene expression of genes associated with the cAMP pathway, with KLF13 showing a stronger suppressive role (Ávila-Mendoza *et al.,* 2020b). Other signaling pathways that are suggested to be affected by KLF13 include the JAK-

STAT pathway, which is known to promote regeneration (Ávila-Mendoza *et al.,* 2020a).

In summary, KLF13 may impact different signaling pathways by regulating gene expression, which can result in the promotion or inhibition of proliferation and cell differentiation. In the CNS, KLF13 has been shown to have a beneficial effect on glioma tumor suppression and oligodendrocyte myelination but inhibits axon regeneration. KLF13's actions in the CNS are mediated through its regulation of various genes associated with different signaling pathways.

3. Janus Kinase -Signal Transducer and Activator of Transcription (JAK-STAT) signaling pathway

3.1 Generalities

The JAK-STAT pathway involves several proteins from the JAK and STAT families, which are recruited based on the tissue and receptor type. The pathway is initiated by the binding of an extracellular ligand to its receptor, leading to the activation of cytokine receptor-associated kinases (JAKs) in the cytosol. JAKs are transphosphorylated and then phosphorylate signal transducers and activators of transcription (STATs) at specific tyrosine residues. Once STATs are activated through phosphorylation, they can homodimerize or heterodimerize via their SH2 domain, translocate to the nucleus, bind to DNA, and modify the gene expression profile (Schindler and Darnell, 1995; Hu *et al.,* 2021). The pathway is regulated by a set of regulatory proteins that control the timing and duration of the signaling cascades (Seif *et al.,* 2017). The JAK-STAT pathway can be activated by a variety of extracellular messengers, including cytokines, growth factors, and interferons (IFNs), which bind to their specific cell-membrane receptors. Cytokines and growth factors bind to cytokine class I receptor, while class II receptors specifically recognize interferons and IL-10 family cytokines (Morris *et al.,* 2018).

3.1.1 Janus Kinases

Janus kinases (JAKs) were first described as signal transducers in the interferon alpha/beta signaling pathway (Velazquez *et al.,* 1992). JAK proteins are ~1100 amino acids in length with molecular weights ranging from 120 to 140 kDa (Yamaoka *et al.,* 2004). They are constitutively associated with the cytoplasmic side of type I and type II cytokine receptor subunits (Rane *et al.,* 2000). JAKs are enzymes that modify other proteins by catalyzing the covalent transfer of a phosphate group from adenosine triphosphate (ATP) to the tyrosine residues of their target proteins, which function as intracellular effectors of the signaling pathway (Enjalbert and Pechon-Vallee, 2003). Therefore, JAKs play a key role as signal transducers downstream of cytokine receptor activation (Babon *et al.,* 2014).

In mammals, including humans, the JAK protein family consists of four subtypes: JAK1, JAK2, JAK3, and TYK2 (Tyrosine kinase 2). These subtypes are evolutionarily conserved, with four members also found in birds and fish (Yamaoka *et al.,* 2004). Each subtype binds to a different set of cytokine receptor subtypes (Haan *et al.,* 2006). Class I cytokine receptors are preferentially bound to JAK1, JAK2, and JAK3, whereas class II is bound to JAK1 and TYK2 (Morris *et al.,* 2018).

Each JAK protein shares a common structure, consisting of seven JAK homology (JH) domains numbered from the carboxyl to the amino terminal as JH1-JH7. These domains form the kinase (JH1), pseudokinase (JH2), Src homology 2 (SH2) (JH3-JH4), and the four-point-one, ezrin, radixin, moesin (FERM) domains (JH5, JH6, JH7) (Bousoik and Montazeri, 2018). The kinase domain is responsible for protein phosphorylation. The pseudokinase domain is catalytically inactive and acts as a suppressor of the catalytic activity of the adjacent tyrosine kinase domain (Yamaoka *et al.,* 2004). The FERM and SH2 domains mediate the interaction of JAK with specific receptors by binding with the proline-rich "Box1" and hydrophobic "Box2" motifs, respectively, which are present in the intracellular domain of cytokine receptors (Ferrao and Lupardus, 2017). These domains positively regulate kinase activity (Zhou *et al.,* 2001).

Upon stimulation by a ligand, the cytokine receptor subunits dimerize, allowing the JAKs to come closer together, inducing transphosphorylation of the JAKs and subsequent phosphorylation of the intracellular tails of the receptor at tyrosine residues. These phosphorylated tyrosine residues then serve as docking sites for signaling molecules, especially members of the signal transducer and activator of transcription (STAT) family (Morris *et al.,* 2018). STAT proteins bind to the receptor through their SH2 domains, and once bound, JAKs phosphorylate the C-terminal tyrosine residues of STATs (Morris *et al.,* 2018). Figure 5 provides an illustration of the JAK structure and the conformational changes that occur upon receptor activation through the binding of its ligand.

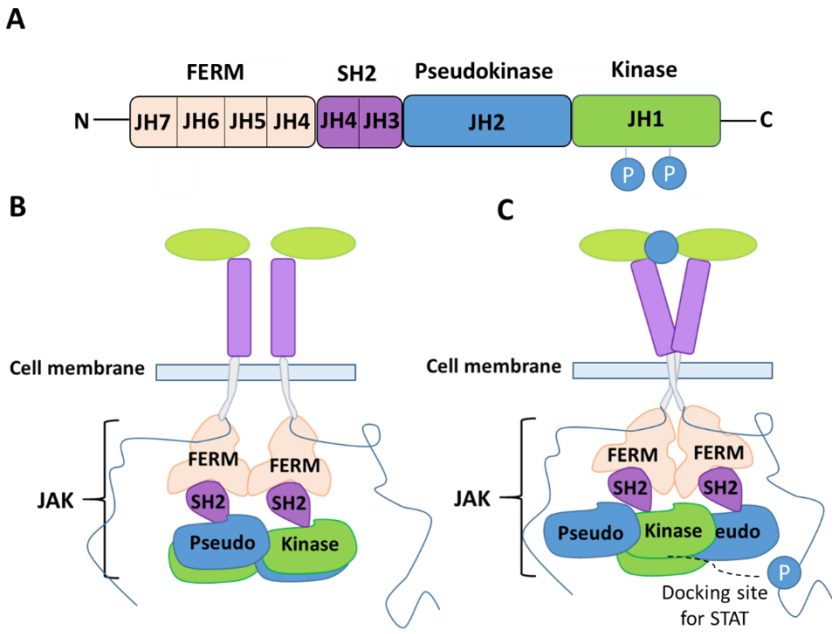

Figure 5. Structure and functional domains of Janus Kinases. A) JAK domains include: FERM, SH2, pseudokinase and kinase domains. FERM and SH2 mediate the interaction of JAK with its receptor through Box1 and Box2 motifs respectively; the pseudokinase domain suppresses catalytic activity of the adjacent kinase domain which in turn is responsible for protein phosphorylation. B) Upon receptor activation, all proteins involved undergo a conformational change that allows for the transphosphorylation of JAKs, which in turn phosphorylate tyrosine receptor tails to leave docking sites for STAT recruitment. Modified from Hu *et al.,* 2021 and Dehkoda *et al.,* 2018.

3.1.2 Signal transducers and activators of transcription

Signal Transducers and Activators of Transcription (STATs) were discovered in 1998 as a protein capable of stimulating type I interferon transcription by interacting with response elements in its gene promoter (Darnell et al., 1994). These transcription factors are between 750 and 800 amino acid residues in length (Akira, 1999) and occur as dimers in both their active and inactive forms (Braunstein *et al.,* 2003), although they can also be found as monomers. STATs reside in the cytoplasm while inactive and are primarily activated by membrane receptor-associated JAKs to promote the transcription of specific genes (Darnell, 1997), involved in cell growth, suppression of apoptosis, and cell motility among others (Akira, 1999).

There are seven STAT proteins in mammals: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6, involved in a wide variety of downstream signaling cascades. In mammals, there is a single variant of each STAT, except for STAT5, which has two isoforms: STAT5a and STAT5b. They are encoded in two separate genes located on the same chromosome and share about 94% identity, with the C-terminal region being the most variable between them (Able *et al.,* 2017). STATs are recruited by different JAKs according to specific receptors; class I cytokine receptors signal downstream by STAT3, STAT4, STAT5, and STAT6, whereas class II signals through STAT1, STAT2, and STAT3 (Morris *et al.,* 2018).

Each member of the STAT family contains an N-terminal domain (NTD) followed by a coiled-coil domain, a DNA binding domain, a linker region, an SH2 domain, a conserved tyrosine residue, and a C-terminal transactivation domain (TAD) (Morris *et al.,* 2018). The N-terminal domain allows for the formation of dimers between non-phosphorylated STAT monomers, as well as STAT tetramers between adjacent STAT dimers on DNA. It also recruits phosphatases for some STATs (Mitchell and John, 2005; Meyer *et al.,* 2003). The coiled-coil domain is required for importing STATs to the nucleus (Reich, 2013). The next three domains, the DNA binding domain, linker region, and SH2 domain, allow STATs to effectively bind to DNA (Mertens *et al.,* 2015). Additionally, the SH2 domain allows STATs to recognize phosphorylated cytokine receptors and

facilitates STAT dimerization. The conserved tyrosine residue is the phosphorylation site for JAK enzymes (Morris *et al.,* 2018). Finally, the C-terminal transactivation domain recruits cofactors that influence STATs' transcriptional activity (Parrini *et al.,* 2018).

Upon activation of JAKs due to receptor activation, unphosphorylated STATs residing in the cytoplasm are phosphorylated by JAKs and form stable dimers. Most STATs work primarily as homodimers, but some can also form heterodimers (Hu *et al.,* 2021). Phosphorylated STATs leave docking sites on the receptors and translocate to the nucleus using metabolic energy and the importin complex. Once in the nucleus, they bind to specific DNA sequences as dimers or as tetramers to regulate gene transcription (Levy and Marié, 2012). STATs usually bind to gamma-activated sites (GAS) located in the promoters of cytokine-inducible genes to induce transcriptional activation (Mitchell and John, 2005). Maximal transcriptional activation by STATs is achieved by their interaction with chromatin remodeling molecules. Therefore, they recruit histone acetyltransferase (HAT) coactivator proteins and CBP/p300 through their transactivation domains (Paulson *et al.,* 1999).

The canonical pathway for activating STATs involves ligand binding to the receptor, followed by JAK activation, which enables STATs to cross the nuclear membrane and reach their gene targets in DNA to regulate transcription (Hu *et al.,* 2021). However, in the non-canonical pathway, non-phosphorylated STAT proteins (dimers or monomers) constantly shuttle between the cytoplasm and nucleus. Unlike non-phosphorylated STATs, phosphorylated STATs are retained in the nucleus and are only released upon dephosphorylation by nuclear phosphatases (Böhmer and Friedrich, 2014). The duration and magnitude of STAT activation are critically regulated by several different mechanisms at the cytoplasmic and nuclear levels, which together ensure that these proteins function in a tightly controlled manner in normal cells (Hu *et al.,* 2021). Figure 6 illustrates STATs' functional domains and their canonical activation.

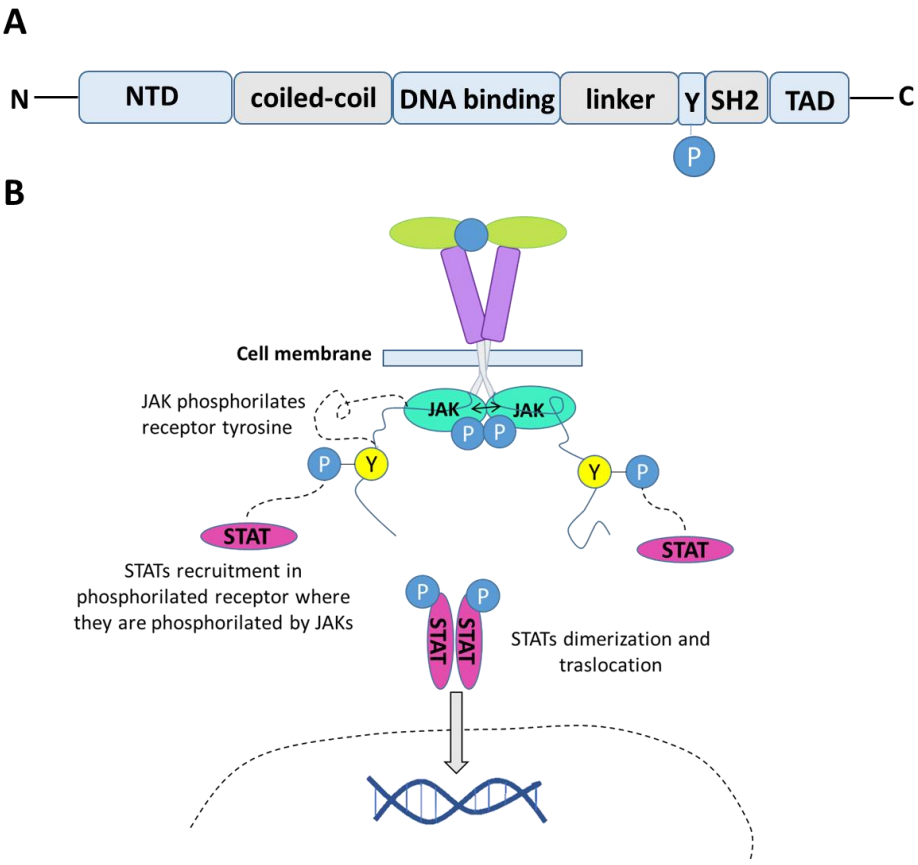

Figure 6. STAT functional domains and canonical JAK-STAT activation. A) Illustration of the structure of STAT proteins, which comprise N-terminal domain (NTD), coiled-coil domain, DNA-binding domain, linker region, tyrosine residue, SH2 domain, and transactivation domain (TAD). The NTD mediates STAT tetramerization on DNA, the coiled-coil domain is necessary for STAT import to the nucleus, the tyrosine residue is the site for JAK phosphorylation, the SH2 domain mediates STAT dimerization and recognition of phosphorylated receptors, and the TAD mediates STAT cofactor binding. B) Illustration of the canonical JAK-STAT signaling pathway upon ligand binding to the receptor. Modified from Hu *et al.*, 2021.

3.2 JAK-STAT pathway inhibition

3.2.1 Protein Tyrosine Phosphatases (PTPs)

Tyrosine phosphorylation is the mechanism by which protein tyrosine kinases (such as JAKs) activate molecules in the JAK-STAT pathway. Protein tyrosine phosphatases (PTPs) are responsible for dephosphorylating the signaling components to regulate the pathway (Xu and Qu, 2008). PTPs can be divided into four subgroups: non-receptor PTPs (such as SHP1 and SHP2),

receptor tyrosine phosphatases (such as CD45), dual-specificity phosphatases, and low molecular weight phosphatases. All of these, except for dual-specificity phosphatases, recognize serine/threonine residues in addition to phosphotyrosine residues (Xu and Qu, 2008).

SHP1 and SHP2 are cytosolic tyrosine phosphatases that contain two SH2 domains (N-SH2 and C-SH2), followed by a protein-tyrosine-phosphatase (PTP) domain and two regulatory tyrosine residues at their C-terminal. The N-SH2 domain recognizes phosphorylated tyrosine residues on other molecules, while the PTP domain dephosphorylates these molecules (Böhmer and Friedrich, 2014). Generally, PTPs are located on the membrane or in the cytoplasm act on JAKs, whereas those in the nucleus act on STATs (Dehkhoda *et al.,* 2018). In their inactive state, the N-terminal SH2 and PTP domains interact, blocking PTP domain activity (Fiebelkorn *et al.,* 2021). While SHP1 is expressed in hematopoietic cell lineages, SHP2 is ubiquitously expressed (Dehkhoda *et al.,* 2018).

SHP2 can interact with phosphorylated STATs both in the cytoplasm and nucleus. It can also be recruited to the cell membrane to dephosphorylate receptor tyrosine kinases in their cytoplasmic domains, where the STAT binding sites are located (Niogret et al., 2019). In general, different phosphorylated proteins involved in the JAK-STAT signaling pathway will be regulated by different SHP phosphatases. For example, SHP1 dephosphorylates JAK2 activated by the Erythropoietin receptor (Xu and Qu, 2008).

3.2.2 Protein Inhibitors of Activated STATs (PIAS)

The PIAS family comprises four members: PIAS1, PIASx (or PIAS2), PIAS3, and PIASy (or PIAS4), which are constitutively expressed (Seif *et al.,* 2017). Different PIAS proteins regulate different STATs, with PIAS1 and PIAS4 inhibiting STAT1, PIAS2 inhibiting STAT4, and PIAS3 inhibiting STAT3 (Murphy *et al.,* 2010). However, despite their name, they do not only regulate STATs.

PIAS proteins exhibit their inhibitory effects through several mechanisms. Firstly, they can bind to STATs and mask their DNA binding domain or prevent

STAT dimerization (Wójcik *et al.,* 2018). Secondly, these proteins have a domain called SIM that allows them to interact with SUMO (small ubiquitin-like modifier), a protein that functions as a post-translational modification. PIAS proteins bind to SUMO and attach it to a target protein in order to alter its localization, stability, and/or function, leading to the inhibition of STAT activity (Kotaja *et al.,* 2002).

3.2.3 Suppressor of cytokine signalling (SOCS) family

The SOCS family comprises eight members: SOCS1-SOCS7, and cytokine-inducible SH2 domain protein (CIS or CISH). All of them act as physiological regulators of cytokine responses (Wang *et al.,* 2019), and their expression is induced by cytokine stimulation in a STAT-dependent manner (Hu *et al.,* 2021). SOCS proteins contain the SOCS box at their C-terminal and a central SH2 domain (Seif *et al.,* 2017). This SH2 domain directly binds to phosphorylated tyrosine residues of activated JAKs, consequently blocking both the kinase activity of JAKs and recruitment of STATs (Starr *et al.,* 1997; Yasukawa *et al.,* 1999). In addition, SOCS proteins also interact with phosphotyrosine residues located within receptor subunits (Hu *et al.,* 2021). On the other hand, the SOCS box recognizes substrates for polyubiquitination, a post-translational modification that mediates the degradation of substrate proteins by proteasome. One substrate protein is JAK, and therefore, SOCS can mediate JAK degradation (Linossi and Nicholson, 2012).

This family is implicated in the regulation of over 30 cytokines, including GH. Particularly, GH up-regulates CIS, SOCS-1, SOCS-2, and SOCS3. Each of them interacts with the GH receptor (GHR), and when overexpressed, they interfere with the JAK2-STAT5b pathway (Ram and Waxman, 1999). Although GH seems to induce *Socs3* expression preferentially (Adams *et al.,* 1998), it is thought that SOCS2 plays an important physiological role in the regulation of GH action. SOCS1 and CIS, besides binding to receptor phosphotyrosine residues, also inhibit signaling by competing with STAT in binding to recruitment docking sites in the receptor complex. SOCS3, on the other hand, preferentially acts by binding to phosphotyrosine residues in the receptor with high affinity (Hu X. *et al.,*

2021). Figure 7 depicts how these regulator proteins interrupt signaling of JAK-STAT pathway.

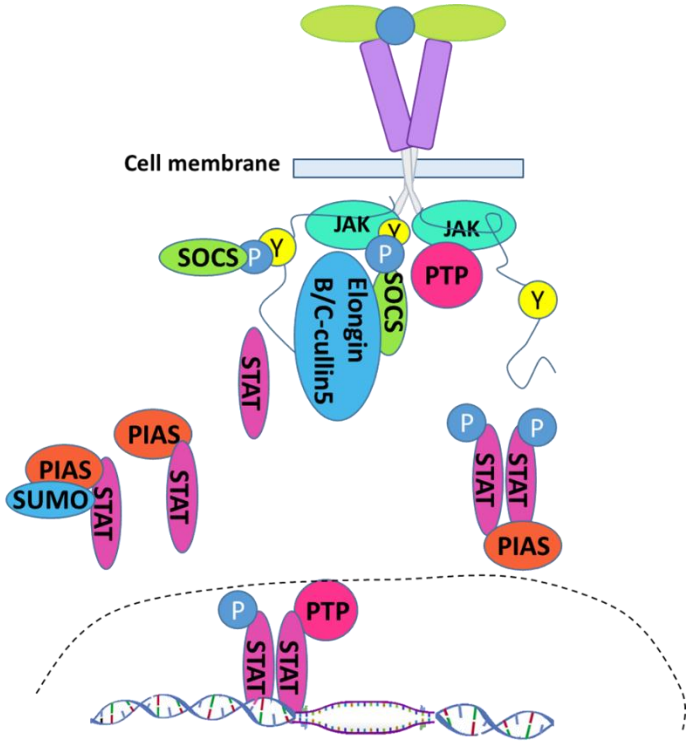

Figure 7. Mechanisms of action of regulatory proteins in the JAK-STAT Pathway. PTPs, specifically the SHPs motif, possess an N-terminal SH2 domain that recognizes phosphorylated tyrosine residues on other molecules. Their C-terminal PTP domain then dephosphorylates these molecules. SHPs located in the cytoplasm dephosphorylate JAKs, while those in the nucleus act on STATs. PIAS proteins bind to STATs and exert inhibitory effects through two mechanisms. Firstly, they prevent the dimerization and phosphorylation of STATs. Secondly, they can mask the DNA binding domain of STATs, hindering their translocation to the nucleus. Additionally, PIAS proteins can bind to SUMO through their SIM domain, facilitating the attachment of SUMO to STATs and altering their stability. SOCS proteins play a crucial role in regulating the JAK-STAT pathway. They possess a central SH2 domain that directly binds to phosphorylated tyrosine residues on activated JAKs or within the receptor subunits. They block the kinase activity of JAKs and inhibit the recruitment of STATs. SOCS proteins also contain a C-terminal SOCS box that recognizes substrates to be polyubiquitinated. This process leads to the formation of a protein complex that promotes degradation, ultimately mediating JAK degradation.PTPs: protein tyrosine phosphatases; PIAS: protein inhibitors of activated STATs; SOCS: suppressor of cytokine signaling (SOCS). Modified from Hu *et al.,* 2021.

3.3 JAK-STAT pathway has a role in axonal regeneration in the CNS

In the CNS, the JAK-STAT pathway is an efficient and highly regulated system that is involved in the proliferation of neural stem cells, glial and neuronal differentiation, synaptic plasticity, glial scar formation after central nervous system injury, as well as in neuronal regeneration (Nicolas *et al.*, 2013).

Although the expression of JAK and STAT proteins in the CNS is weaker than in other systems (Nicolas *et al.,* 2013), members of the JAK-STAT pathway are expressed in different regions of the brain and are differentially regulated depending on the stages of brain development. Some of these regions include the striatum, cerebral cortex, hypothalamus, cerebellum and hippocampus (De-Fraja *et al.,* 1998; Nicolas *et al.,* 2013). Particularly, JAK2 and STAT3 are highly expressed in the brain (Nicolas *et al.,* 2012). It has been also proven that two JAK-STAT signaling inhibitors, SOCS1 and SOCS3, are expressed in the CNS (Baker *et al.* 2009).

Cumulative evidence has reported a role of JAK-STAT pathway signaling in restorative processes after an injury to the CNS, including axonal regeneration. This has been evidenced in retinal ganglion cells of zebra fish (Elsaeidi *et al.,* 2014) and in adult mice and rats after an optic nerve crush injury (Pernet *et al.,* 2013; Vigneswara *et al.,* 2014). These effects have been achieved in part by deleting or inhibiting SOCS3 or other JAK-STAT endogenous inhibitors, including members of the KLFs, such as KLF4 (Qin *et al.,* 2013), as well as by inducing the JAK-STAT activity with cytokines administration, such as CNTF (Pernet *et al.,* 2013; Vigneswara *et al.,* 2014). In addition, activation of the JAK-STAT pathway is involved in the transduction of several cytokines and growth factors, including GH, which accelerates axonal regeneration after peripheral nerve injures (Tuffaha *et al.,* 2016).

Therefore, the JAK-STAT pathway plays a crucial role in axonal regeneration, and inhibiting its inhibitors may be a promising approach to stimulate regeneration. Some of the endogenous inhibitors of this pathway belong to the KLF family.

4. Growth Hormone

4.1 Synthesis and structure

In humans, the *Gh* gene belongs to a multigene locus located on chromosome 17 that harbors five genes: the pituitary growth hormone (*Gh*-N), placental growth hormone (*Gh*-V), and three chorionic somatomammotropin (*Csh*) genes. *Gh*-N is mainly expressed in somatotrophs of the anterior pituitary gland, whereas the 4 remaining genes are exclusively expressed in the placenta of women during pregnancy (Pérez-Ibave *et al.,* 2014). In addition to its pituitary expression, Gh-N is also locally expressed in several extrapituitary tissues, including various regions of the brain (Yoshizato et al., 1998; Donahue et al., 2006), as well as in other neural tissues such as the retina (Harvey, 2010). GH is also expressed in bones, muscles, ovaries and testes, salivary glands, pancreas, liver, kidney, colon, stomach, lung, heart, and immune system (thymus, spleen, and lymph nodes) (Pérez-Ibave *et al.,* 2014). The locally expressed GH may act in an autocrine/paracrine manner since its receptor is also expressed in these tissues (Harvey, 2010).

The GH protein secreted by somatotrophs is a 191 amino acid single-chain protein with a molecular weight of 22 kDa. It is secreted in a pulsatile manner with diurnal variation (Lim and Khoo, 2020) and represents around 90% of the total GH in circulation; the remaining 10% belong to the 20 kDa isoform along with other less studied isoforms derivated from the 22 kDa isoform (Ribeiro de Oliveira et al., 2018). Pituiary GH has four helical regions and two disulfide bonds (Ribeiro de Oliveira *et al.,* 2018) and also has two binding sites, namely site I and site II, which allow it to interact with the GH receptor (Bidlingmaier and Strasburger, 2010).

4.2 Biological actions

The GH is best known for its growth-related functions, including the increase in bone length, bone density, and muscle mass during early developmental stages (Dehkhoda *et al.,* 2018). However, it also plays a role in

reproduction as it is necessary for the induction of sexual maturation and optimal female and male fertility (Hull and Harvey, 2014). Additionally, it has metabolic effects mainly in the liver, adipose tissue, and skeletal muscle. In the liver, GH stimulates hepatic glucose production and fatty acid oxidation (Vijayakumar *et al.,* 2011). In adipose tissue, GH inhibits adipocyte differentiation and increases lipolysis (Kopchick *et al.,* 2020), and in skeletal muscle, it stimulates protein synthesis and the induction of free fatty acid (FFA) uptake, thereby promoting lipid utilization (Chikani and Ken, 2014; Vijayakumar *et al.,* 2011).

GH perform its actions directly through binding to its specific receptor (GHR) by inducing the activation of GHR-associated tyrosine kinases (JAK), or indirectly by inducing liver or peripheral IGF1 production (known as GH-IGF1 axis) (Ranke and Witt, 2018). The GH-IGF1 axis is primarily regulated by two hypothalamus-derived modulating factors: it is positively regulated by the Growth Hormone Releasing Hormone (GHRH) while somatostatin (SST) has a negative control on it (Ranke and Witt, 2018).

4.3 GH signaling through GHR

The GHR is a homodimeric receptor of 638 amino acids in length, comprising an extracellular domain (ECD), a single-pass transmembrane domain, and a cytoplasmic intracellular domain (ICD). It belongs to the class I cytokine receptor family, which includes more than 30 receptors such as the prolactin receptor (PRLR) and erythropoietin receptor (EPOR) (Dehkhoda *et al.,* 2018). The GHR is ubiquitously distributed in most tissues of the body (Brooks and Waters*,* 2010).

Each monomer of the GHR comprises two fibronectin III beta domains in their extracellular domain, located above a single transmembrane sequence. Each fibronectin III domain contributes a tryptophan residue that allows GH binding (Brooks and Waters, 2010). The ICD consists of the Box1 and Box2 motifs. Box1 is a proline-rich motif located near the cell membrane and is conserved among all class I cytokine receptor members. This motif acts as a binding site for JAKs (Janus Kinases). Box2 is composed of acidic and aromatic

residues, located near the C-terminal of Box1 and is less conserved (Usacheva *et al.,* 2002).

As mentioned previously, cytokine receptors lack intrinsic protein tyrosine kinase (PTK) activity, so they are coupled to non-receptor PTKs for signal transduction. For the GHR, the only JAK family member that binds to the receptor is JAK2 (Waters, 2016). Classical JAK-STAT pathway activation through GHR activates STAT5, which is the predominant transcription factor that mediates GH-induced cell proliferation and actions. GHR activation and signaling also involves activation of STAT1 and STAT3 via JAK2, but these appear not to require binding to the phosphorylated receptor (Dehkhoda *et al.,* 2018).

In the inactive homodimeric GHR, the JAK2 kinase domain is inhibited by interaction with the pseudokinase domain from the JAK2 bound to the opposing receptor. GH initially binds to a single receptor monomer through site 1 and subsequently to the second receptor monomer through site 2, causing receptor dimerization and intracellular signal transduction. This binding induces conformational changes that allow the JAK2 kinase domain of both JAK2 to interact and transphosphorylate each other. Then, JAK2 phosphorylates multiple tyrosine residues on the ICD of the receptor, leading them to be uncoupled from the receptor. This allows STAT5 to bind to the receptor, where they are phosphorylated by JAK2 (Brooks and Waters, 2010; Dehkhoda *et al.,* 2018). Activated STAT5 forms homodimers, whereas active STAT1 and STAT3 form both homodimers and STAT1-STAT3 heterodimers (Brooks and Waters*,* 2010).

The activation of GHR triggers other signaling pathways in addition to the JAK-STAT pathway, including the PI3K-AKT and MAP kinase pathways (Dehkhoda *et al.,* 2018).

4.4 Actions of GH in the CNS

In addition to its effects on growth and metabolism, GH also plays an important role in the CNS. GH-responsive neurons are located in various regions of the brain, including the hypothalamus, septum, thalamus, amygdala, and hippocampus (Wasinski *et al.,* 2019).

It has been shown that GH signaling in the brain is involved in regulating a variety of processes, including cognition, behavior, sleeping patterns, neuroendocrine changes, and neuroprotection (Arámburo *et al,* 2014; Wasinski *et al.,* 2019). GH-deficient individuals may experience symptoms such as poor memory, fatigue, sleep disturbances, decreased well-being and mood, and attention-deficit disorder. GH therapy has been found to improve these symptoms (Nyberg and Hallberg, 2013). One of the ways that GH may affect cognition is by enhancing excitatory synaptic transmission in the hippocampus (Nyberg and Hallberg, 2013). GH is secreted in a pulsatile manner during the day, with the highest amplitude occurring during slow-wave sleep (SWS) at night. This promotes sleep (Gohil and Eugster, 2019), and individuals with GH deficiency may experience excessive SWS (Copinschi, *et al.,* 2010).

Finally, GH also has an effect on the central nervous system (CNS) control of metabolism. Intracerebroventricular administration of GH to mice has been shown to induce food intake by directly activating hypothalamic nuclei, including the arcuate nucleus, thereby exerting an orexigenic effect on the CNS (Donato *et al.,* 2021).

4.5 GH and axonal regeneration in the CNS

Cumulative evidence suggest that GH acts as a trophic factor in the CNS after an injury, providing neuroprotective and regenerative effects. These include preventing apoptosis (Alba-Betancourt *et al.,* 2013), enhancing synaptogenesis and promoting neurite outgrowth during the repair process (Olivares *et al.,* 2021). These effects are partially attributable to GH through the activation of the GH receptor (GHR), and indirectly using IGF-1 as a mediator (Baltazar-Lara *et al.,* 2020). GH and IGF1 have received attention since both hormones have therapeutic potential due to their role in accelerating axonal regeneration (Akram *et al.,* 2022). GH has shown to promote axon regeneration after a hypoxia-ischemia injury (Olivares-Hernández *et al.,* 2022) and an excitotoxicity damage (Martinez-Moreno *et al.,* 2019).

Brain ischemia refers to the interruption of the blood supply, whereas hypoxia refers to the decrease of oxygen supply, in some cases because of ischemia. These processes promote apoptosis and excitotoxicity (among others), resulting in cell death (Sekerdag *et al.,* 2018). During these events, GH and IGF-1 are upregulated in several brain areas as an endogenous mechanism to provide neuroprotection after the CNS has been exposed to hypoxia-ischemia (Baltazar-Lara *et al.,* 2020). Two brain structures more susceptible to this type of insult are the cortex (some areas include the insular and parietal cortex) and hippocampus among others (Olivares-Hernández *et al.,* 2022), where neuroregenerative effects of GH have been observed (Olivares-Hernández *et al.,* 2022).

Following a hypoxic−ischemic (HI) insult, treatment with GH stimulates neurite length and branching in primary pallial and hippocampal cell cultures of chicken (Olivares-Hernández *et al.,* 2022; Olivares-Hernández *et al.,* 2021) as well as in the cerebral pallium (a brain region analogous to the mammalian brain cortex) of chick embryos (Olivares-Hernández et al., 2022) and the hippocampus of young adult mice (Sanchez-Bezanilla *et al.,* 2020). These effects are thought to occur via the upregulation of axonal and synaptic markers including NRX1, NRX3, NLG1 and GAP-43, as well as the expression of neurotrophins involved in axon growth, such as NT-3, BMP4, BDNF and IGF-1 (Olivares-Hernández *et al.,* 2021; Olivares-Hernández *et al.,* 2022; Zhu *et al.,* 2010). Interestingly, GH treatment also induces the expression of GH itself and its receptor (GHR) in these pallial and hippocampal neurons (Olivares-Hernández *et al.,* 2021; Olivares-Hernández *et al.,* 2022), suggesting that GHR signaling may play a role in these regenerative effects.

On the other hand, excitotoxicity refers to the neuronal overactivation of glutamate receptors, mainly through the N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainic acid (KA) receptors, that can lead to neuronal death (Dong *et al.,* 2009). The neuroregenerative effects of GH after an excitotoxicity insult induced by kainic acid (KA) have been assessed in the retina and hippocampus, which are particularly susceptible to this type of insult (Candelario-Jalil *et al.,* 2001). Upregulation of GH in hippocampus is an endogenous neuroprotective mechanism against excitotoxicity in lactating female rats (Arellanes-Licea *et al.,*

2018). Also, upregulation of IGF1 in astrocytes protects neurons against excitotoxicity (Chen *et al.,* 2019).

After inducing excitotoxicity with kainic acid (KA), exogenous administration of GH induced neuronal outgrowth in cultures of chicken primary neuroretinal cells of the inner nuclear and inner plexiform layers (Martínez-Moreno *et al.,* 2018) as well as in ganglion cell, inner plexiform and inner nuclear layers in postnatal chicken retinas (Martínez-Moreno *et al.,* 2019). The suggested mechanisms for this effect include the induction of the expression of axonal/synaptic markers such as DLG1, NRXN1 and GAP-43 as well as neurotrophins such as BDNF and NT-3 (Martínez-Moreno *et al.,* 2019). Furthermore, it is known that hippocampal cells express GH under basal conditions. GH treatment upregulates GH in hippocampus of young adult rats in response to KA administration, where its effect observed is on stimulating proliferation of hippocampal precursors (Devesa *et al.,* 2011). Table 2 sumarizes the neuroregenerative effects of GH described above.

Table 2. Neuroregenerative effects of GH in the CNS

Brain damage	**In vitro/ in vivo approach**	**Neuroregenerative effects of GH administration**	**Neuroregenerative processes associated**
Hypoxia-ischemia damage	Chicken embryonic primary pallial cell cultures and cerebral pallium of chicken embryos	Induced expression of: -**Presynaptic markers involved in neurotransmission:** **NRXN1** (Neurexin 1) **NRXN3** (Neurexin 3) **NGL1** (Netrin-G1) -**The molecular marker of the growth cone in regenerating axons:** **GAP-43** (Growth-associated protein 43) -**Neurotrophins associated to axonal growth and other regenerating processes:** **BDNF** (Brain derived neurotrophic factor) **NT-3** (Neurotrophin 3) **BMP4** (Bone morphogenetic protein 4) **IGF-1** (Insulin-like growth factor 1)	Synaptogenesis Neuronal growth GHR activation

		-**GH** and **GHR** (Growth Hormone and its receptor)	
	Chicken embryonic primary hippocampal cell cultures	Induced expression of: -**Presynaptic markers involved in neutransmission:** **NRXN1** (Neurexin 1) **NRXN3** (Neurexin 3) **NGL1** (Netrin-G1) -**The molecular marker of the growth cone in regenerating axons**: **GAP-43** (Growth-associated protein 43) - **A neurotrophin associated to axonal growth and other regenerating processes:** **BDNF** (Brain derived neurotrophic factor) - **GHR** (Growh Hormone Receptor)	Synaptogenesis Neuronal growth GHR activation
	Hippocampus of young adult rats	Induced expression of: -**A synaptic receptor that plays a major role in excitatory synaptic transmission:** **GluR1** (Glutamate receptor subunit 1) **Improved performance on Visual Discrimination task** **Promoted cell proliferation in dentate gyrus**	Synaptogenesis Neurogenesis Improving of cognitive functions
Kainic acid (KA)-induced excitotoxicity damage	Chicken embryonic primary neuroretinal cell cultures	Induced expression of: -**Neurotrophins associated to axonal growth and other regenerating processes:** **BDNF** (Brain derived neurotrophic factor) **NT-3** (Neurotrophin-3)	Neuronal growth
	Postnatal chicken retinas	Induced expression of: **- Presynaptic and postsynaptic markers involved in neutransmission:** **NRXN1** (Neurexin 1) **DLG1** (Discs large homolog 1)	Synaptogenesis Neuronal outgrowth Neuroprotection

		-**The molecular marker of the growth cone in regenerating axons**: **GAP-43** (Growth-associated protein 43) **-A neurotrophin associated to axonal growth and other regenerating processes:** **BDNF** (Brain derived neurotrophic factor) -**Glutamate receptors previously described in healing process:** **NR1** (NMDA-R subunit) **GRIK4** (KA-R subunit) -**Notch signaling molecules**: **Notch1** **Hes5**	Notch pathway signaling
	Hippocampus of young adult rats	-**Enhanced the proliferation of hippocampal precursors** -**Upregulation of GH expression**	Proliferation of hippocampal precursors

5. The Hippocampus

5.1 Anatomy and Physiology

The hippocampus is a subcortical brain structure located at the medial level of the temporal lobe (Fogwe *et al.,* 2022). It is found below the neocortex, in a subregion of the allocortex known as archicortex, which, in contrast to the 6 layers of the neocortex, comprises three neuronal cell layers (Strominger *et al.,* 2012).

The hippocampus is part of an entire set called hippocampal formation, which includes Cornu ammonis (CA), hippocampus proper, which consists of four regions: CA1, CA2, CA3, and CA4 (the later is also known as hilus), the dentate gyrus (DG), subiculum and entorhinal cortex (EC), which is the anterior portion of the parahippocampal gyrus (Schultz and Engelhardt, 2014).

There is a classical connectivity in the hippocampus known as the 'trisynaptic circuitry', which comprises unidirectional axonal projections that are

mostly glutamatergic or excitatory. These projections include: from the entorhinal cortex to the dentate gyrus through the perforant path, from the dentate gyrus to the CA3 region via the mossy fiber pathway, and from the CA3 region to the CA1 region via the Schaffer collateral pathway. The circuit is completed when the CA1 region projects back to the entorhinal cortex (Knierim, 2015).

The axons of the perforant path that project to the dentate gyrus mainly arise from layer II of the entorhinal cortex and terminate in the granule cells of the dentate gyrus. Mossy fibers are axons of dentate gyrus granule cells that extend to CA3 pyramidal cells, and the Schaffer collateral pathway comprises axons from CA3 pyramidal cells to CA1 pyramidal cells (Yeckel and Berger, 1990).

5.2 Clinical relevance of axonal regeneration in hippocampus

Axonal regeneration has been extensively studied as a therapy to restore function after severe spinal cord injuries, where long tracts of axons are interrupted (Tuszynski and Steward, 2012). However, other regions of the CNS also present axonal degeneration after certain types of damage, such as ischemic brain injury (Hinman, 2014) and traumatic brain injury (Hill *et al.,* 2016), where progressive axonal loss increases the risk of suffering adverse long-term outcomes.

After ischemic brain injury, three phases can be recognized: the acute phase, subacute phase, and the degenerative/regenerative phase. The acute phase is characterized by a rapid loss of axons due to aberrant calcium signaling that triggers proteolysis in the axonal cytoskeleton. The subacute phase represents a progressive secondary damage, where surviving axons from the acute phase undergo delayed axonal degeneration. Finally, in the degenerative/regenerative phase, disconnected axons undergo Wallerian degeneration (the neuronal anterograde degeneration), which worsens the clinical deficits. On the other hand, plastic mechanisms such as neurogenesis may be triggered to ameliorate the damage (Hinman, 2014; Nakatomi *et al.,* 2002).

The hippocampus is a target therapeutic area due to its vulnerability to ischemic insult. It is frequently affected as a secondary area after focal cerebral cortex or striatum ischemia (Butler *et al.,* 2002). Additionally, it is one of the first brain areas affected in global ischemia (Neumann *et al.,* 2013), where hippocampal CA1 pyramidal neurons die even after reperfusion (blood flow recovery) (Wang *et al.,* 2011; Bartsch *et al.,* 2015).

Traumatic or diffuse axonal injury is a subtype of traumatic brain injury, which refers to brain damage caused by an external force acting directly on the skull and brain (Johnson *et al.,* 2013; Peng and Bonaguidi, 2018). Traumatic axonal injury primarily affects white matter tracts (Bruggeman *et al.,* 2021) and involves two main components: the primary mechanical breaking of the axon due to shearing or stretching caused by inertial forces, and the secondary axon degeneration resulting from the activation of deleterious molecular mechanisms in the cytoskeleton triggered by excessive calcium influx as a consequence of the primary damage (Hill *et al.,* 2016).

Similar to ischemic brain injury, the damage caused by traumatic axonal injury is not limited to the acute and subacute phases following trauma. Reports indicate that axonal loss can progress for days or even years post-injury, leading to adverse long-term outcomes and an increased risk of neurodegenerative diseases, including Alzheimer's disease (Peng and Bonaguidi, 2018). The hippocampus, which is associated with verbal memory deficits, also exhibits neuronal loss following traumatic brain injury in TBI subjects (Ariza *et al.,* 2006).

In summary, ischemic and traumatic brain injuries are acquired conditions that can result in hippocampal disconnection by causing axonal damage. However, they present distinct time windows during their pathological progression, which offer therapeutic opportunities for axonal rescue to restore neuronal network connectivity and improve the deficits caused by the disconnection. One potential therapeutic approach for axonal rescue in these injuries involves identifying the endogenous inhibitors of pro-regenerative signaling pathways, which has been explored in this study.

Justification

The Krüppel-like factors have emerged as important regulators of several biological processes in the CNS, including proliferation, differentiation, and regeneration. Among them, KLF13, a member of group 3 of the KLF family, has been demonstrated to play a role in promoting and maintaining neuronal differentiation. KLF13 may also be involved in the loss of the neurons' regenerative capacity, as the intrinsic ability of neurons to elongate axons is reduced during the transition from embryonic stages to adulthood, coinciding with increased expression of KLF13. Acting as a transcriptional regulator, KLF13 controls gene transcription, predominantly functioning as a transcriptional repressor, although it can also induce the expression of certain target genes. It has been proposed that several target genes of KLF13 are involved in signaling pathways with important functions in the CNS, including the JAK-STAT signaling pathway. This pathway is regarded as one of the central communication nodes in cell function and is activated by cytokines and growth factors, including GH. In the CNS, it has been widely demonstrated that GH, in part by activating the JAK/STAT signaling pathway, has important actions as a neurotrophic factor, providing neuroprotective effects on neurons against several insults, such as hypoxia-ischemia and excitotoxicity. However, the activity of the JAK/STAT pathway induced by GH declines rapidly, likely due to the presence of intrinsic inhibitors, including KLF13. Therefore, studying the effect of KLF13 on the regulation of JAK/STAT pathway activity becomes relevant as it could offer insights into enhancing the neurotrophic effects of GH and other activators of the JAK/STAT pathway. Accordingly, the regulation of KLF13 on JAK-STAT activity was studied here using the HT22 cell line derived from the hippocampus of mice.

Hypothesis

KLF13 will negatively regulate the activity of the JAK-STAT signaling pathway by regulating the expression of genes associated with the pathway in cultured mouse hippocampal neurons. Therefore, the depletion of *Klf13* will

enhance the activity of the pathway, which in turn can be enhanced by adding a pathway activator.

Objectives

General objective

Investigate the impact of the transcription factor KLF13 on the JAK-STAT pathway in hippocampal neurons.

Specific objectives

1. To investigate the effects of KLF13 on gene regulation of genes involved in the JAK/STAT pathway.
2. To analyze the impact of KLF13 deficiency on the mRNA levels of genes involved in the JAK-STAT signaling pathway.
3. To investigate the effect of GH on the mRNA levels of JAK-STAT pathway output genes in KLF13-deficient neurons.
4. To evaluate whether the findings observed *in vitro* are recapitulated in an *in vivo* model.

Materials and methods

Animals

Adult wild-type (control) and Klf13-knockout (*Klf13*$^{-/-}$) mice of the C57BL/6J strain were used. They were provided by the animal facility of the Institute of Neurobiology, UNAM. The mice received food and water *ad libitum* and were maintained on a 12:12 light-dark cycle. All mice were anesthetized by inhalation of 4% isoflurane before being euthanized by rapid decapitation. The experiments involving the use of mice were performed following the protocol 126.A approved by the Bioethics Committee of the Institute of Neurobiology, UNAM.

Hippocampus-derived HT22 cell lines

Previously, the CRISPR/Cas9 genome editing technique was used to deplete the *Klf13* gene in the HT22 cell line (HT22-Klf13-KO). In addition, the HT22 cell line was engineered to express KLF13 under the control of doxycycline (HT22-TR/TO-V5Klf13, Ávila-Mendoza*, et al.,* 2020). The parental HT22 line with normal KLF13 expression was used as a control. All HT22 cell lines were cultured in high glucose DMEM culture medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin (100 U/ml) in a 5% CO_2 atmosphere at 37°C and maintained in T75 flasks containing 10 ml of complete DMEM media. Once they reached approximately 90 % confluency, the cells were sub-cultured in 12-or 6-well plates at a density of 1 X 10^5 or 2.5 X 10^5, respectively, based on the experimental strategy. These subcultures were stabilized for 24 h before adding treatments or harvesting. Four replicates (wells) were used per treatment and per experiment.

Experimental strategy in HT22 cell lines

To investigate the effect of KLF13 induction over time on the mRNA levels of genes involved in the JAK-STAT signaling pathway (*Jak1, Jak2 Jak3, Stat3, Stat5a, Stat5b, Socs1* and *Socs3)*, we used the HT22-TR/TO-Klf13 cell line. This cell line contains the V5Klf13 transgene, which expression can be induced by the addition of doxycycline. Doxycycline arrests the transcriptional repressor TET and inhibits the binding of TET Repressor to the TET Operator sequence located upstream the promoter sequence of V5Klf13, allowing V5Klf13 transcription (Ávila-Mendoza, *et al*., 2020a). For our experiments, the *V5Klf13* transgene was induced using doxycycline (dox; 1 µg/ml) for 1, 2, 4, 8 and 16 hours. In a previous study, the expression of the V5Klf13 transgene was time-dependently induced by doxycycline treatment. After 2 hours of doxycycline treatment, a peak fold-change was observed, which continued to increase until 24 hours. Additionally, the Western blot analysis of V5Klf13 protein concentration showed a similar trend (Ávila-Mendoza et al., 2020a). These findings are represented in figure 8.

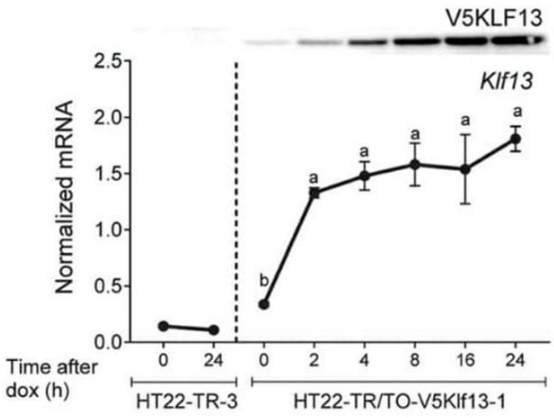

Figure 8. Forced expression of the V5Klf13 transgene was performed in the HT22-TR/TO-Klf13 cell line using 1 µg/ml of doxycycline at various time points (Ávila-Mendoza *et al.,* 2020a). Treatment with doxycycline resulted in a time-dependent expression of V5Klf13, as determined by RT-qPCR and Western blot analysis. Notably, the control cell line HT22-TR did not show induction of V5Klf13 expression in response to doxycycline.

After induction of the V5KLF13 expression by doxycycline in our experiments, total RNA was extracted and purified as described below.

To assess the impact of *Klf13* depletion on the expression of genes involved in the JAK/STAT signaling pathway, the HT22-*Klf13*-KO cell line was used. This cell line was cultured as described above and after 24 h of stabilization, cells were processed to extract and purify total RNA as described below.

To investigate the impact of *Klf13* depletion on the GH-dependent JAK-STAT pathway activation, a time course experiment with GH treatments was performed in parental and *Klf13*-KO HT22 cell lines analyzing the expression of four JAK-STAT output genes (*Socs1*, *Socs3*, *Igf1* and *Bdnf*). After a 24-hour period of culture stabilization, eight groups were formed for each cell line, with four replicates per group. One group per genotype served as the control and received no treatment. The remaining groups were treated with 1 nM of bovine GH for specific time durations: 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, and 16 hours. After the treatments, cells were washed in PBS and then processed to extract and purify total RNA.

Experimental strategy in an *in vivo* model

The GH-dependent activation of the JAK-STAT signaling pathway was analyzed in the hippocampus of both wild-type and *Klf13*$^{-/-}$ mice. To perform intracerebroventricular treatments, adult wild-type and 12 week *Klf13* knockout mice were used. Animals were sorted into two groups per genotype, with four animals per group. The first group (vehicle) was injected intraventricularly with 2 µl of saline solution. The second group was injected intraventricularly with 2 µl of bovine GH solution (1 ng). After one hour following injection, all mice were anesthetized with 4% isoflurane, sacrificed by rapid decapitation, and their hippocampi from both sides were harvested, immediately frozen, and stored at -70 °C until use.

Intracerebroventricular injections

The animals were deeply anesthetized by inhalation of 4% isoflurane and placed in a stereotaxic frame. A small incision was made in the scalp to locate the bregma. Taken bregma as reference, the injection site was located at coordinates anteroposterior (AP) = -0.5 mm and mediolateral (ML) = +1 mm, where a small hole was made with a micro drill. A 34 G needle coupled to a Hamilton syringe was then inserted into the hole to a depth of dorsoventral (DV) = -2 mm, and 2 µl of the bovine GH solution was injected. After incubation for 5 minutes, the needle was withdrawn, and the animals were kept under observation until they recovered and then they were euthanized.

Total RNA extraction and cDNA synthesis

For experiments involving RNA extraction, cell cultures were performed in 6-well plates. After treatment, the cells were rinsed with PBS and lysed in 600 µl of Trizol Reagent (Invitrogen). Total RNA was extracted using the Direct-zol RNA Miniprep Plus kit (Zymo Research). Briefly, an equal volume of absolute alcohol was added to RNA preparation, wich was then passed through columns supplied

in the kit. DNA contamination was removed by DNase treatment on the column, followed by washing with the appropriate buffers. Total RNA was eluted in 50 µl of RNAase-free water, quantified using a Nanodrop spectrophotometer, and stored at -70°C until use. For the *in vivo* model, one hippocampus was used per sample and RNA extraction was conducted following the same protocol.

The cDNA was synthesized from 1 µg total RNA per sample using the High-Capacity cDNA Reverse Transcription Kit (Appied Biosystems) following the manufacturer's instructions. Briefly, RNA was retro transcribed in presence of dNTPs, random hexamers and the MultiScribe reverse transcriptase for 2 h at 37°C followed by inactivation of transcriptase at 65 °C for 5 min. The synthesized cDNA was then used to quantify gene expression of several targets.

Quantitative Polymerase Chain Reaction (qPCR).

To quantify the mRNA levels of the different genes of interest, the qPCR technique was conducted using a Quantum Studio machine (Applied Biosystems) and the PowerUp SYBR Green Mix (Thermo-Fisher) reagent. First, standard curves were performed to validate the efficiency of the different oligonucleotides used (listed in Table 3) by pooling cDNA samples and preparing serial dilutions with a 1:5 dilution factor. After validating efficiency, qPCR reactions were performed containing 0.5 µM oligonucleotides, 1X master mix and 2 µl of cDNA from 1:5 dilution. The program in the thermocycler was as follows: initial denaturalization at 50 °C for 2 min and 95 °C for 10 min; 40 cycles of 95 °C for 15 sec, 60 °C for 15 sec and 72 °C for 30 sec; a melting curve was performed to analyze the specificity of the reaction.

To calculate changes in the expression of mRNAs, a relative quantification was conducted using the $2^{-(\Delta\Delta Ct)}$ method and taking the control group (or the vehicle group in the *in vivo* model) as calibrator. All genes were normalized to the geometric mean of the mRNA levels of the constitutive genes *Gapdh* and *Ppia*, whose mRNAs were unaffected by the treatments.

Table 3. Oligonucleotides used for qPCR.

Oligonucleotides	Forward (5'-3)	Reverse (5'-3)
Ghr	AAGTACAGCGAGTTCAGCGA	GGACTGGGGGTAAAATCAGCA
Gapdh	TGTGTCCGTCGTGGATCTGA	CTTCACCACCTTCTTGATGTCACT
Ppia	GGTTCCTCCTTTCACAGAAT	AATTTCTCTCCGTAGATGGAC
Jak1	CAAGTCTAGTGACCCTGGCA	CAGATTTCCCAGAGCGTGGT
Jak2	TTGGGCAAGCTGAAGGAGAG	CATGCCTGGTTGACTCGTCT
Jak3	GAACCTGGGTCACGGTTCTT	GCGGGTAGGATACTTGGCTC
Stat3	TGGATGCGACCAACATCCTG	CAATGGTATTGCTGCAGGTCG
Stat5a	CACTCCTGTACTTGGTTCGTCA	CCAGGTCAAACTCGCCATCT
Stat5b	GTACTACACACCGGTCCCCT	ATGCATTTGCAAACTCGGGG
Socs1	GATTCTGCGTGCCGCTCTC	CGGGGAGATCGCATTGTCG
Socs3	CTACGCATCCAGTGTGAGGG	TGAGTACACAGTCGAAGCGG
Igf1	TGGATGCTCTTCAGTTCGTG	GTGGGGCACAGTACATCTCC

Statistical analysis

In all graphs, values are expressed as mean ± SEM. For statistical differences between two groups, an unpaired Student's t-test was used. For differences between experimental groups compared with the control group, one-way ANOVA followed by a Dunnett's or Turkey's post hoc test was performed. P-values of less than 0.05 were used to reject the null hypothesis ($P < 0.05$; $P < 0.01$; $P < 0.001$). Statistical analysis and graphs were performed using the software Graphpad Prism 9 (Graphpad software, USA).

Results

1. KLF13 induction in HT22-TR/TO-*Klf13* cell line impacts mRNA levels of several genes involved in the JAK/STAT signaling pathway.

The expression of KLF13 was induced by doxycycline in the HT22-TR/TO-*Klf13* cell line. After 1, 2, 4, 8 and 16 hours of *Klf13* induction, the expression of *Jak1, Jak2, Jak3, Stat3, Stat5a, Stat5b, Socs1* and *Socs3* genes was analyzed. Each group treated with doxycycline was compared against the control group, which received no treatment.

The results showed that induced expression of KLF13 repressed the expression of *Jak1*, *Jak2*, *Jak3* and *Socs1*. *Jak1* mRNA levels decreased by 50 ± 11 % after 4 hours of doxycycline treatments and remained repressed until 16 hours after KLF13 induction. The expression of *Jak2* was reduced from the first hour (30 ± 9 %) to 16 hours of doxycyline treatment (90 ± 10 %). *Jak3* mRNA levels reduced by 50 ± 10% at 4 hours and remained lower (70 ± 30 %) until 16 hours, compared to the control group. The mRNA levels of *Socs1* were lower after 2 (45 ± 4 %), 8 (80 ± 6 %) and 16 hours (70 ± 4 %) of KLF13 induction (Fig. 9).

In contrast, the induction of KLF13 increased the expression of *Stat5a*. The mRNA levels of Stat5a were higher than the control group by 2.4- and 2.7-fold in HT22-TR/TO-Klf13 cells treated with doxycycline for 2 and 4 hours, respectively. The *Stat5a* mRNA levels returned to baseline after 8 hours of doxycycline treatment. The mRNA levels of Stat3, Stat5b and Socs3 were unaffected by the KLF13 inductions (Fig. 9).

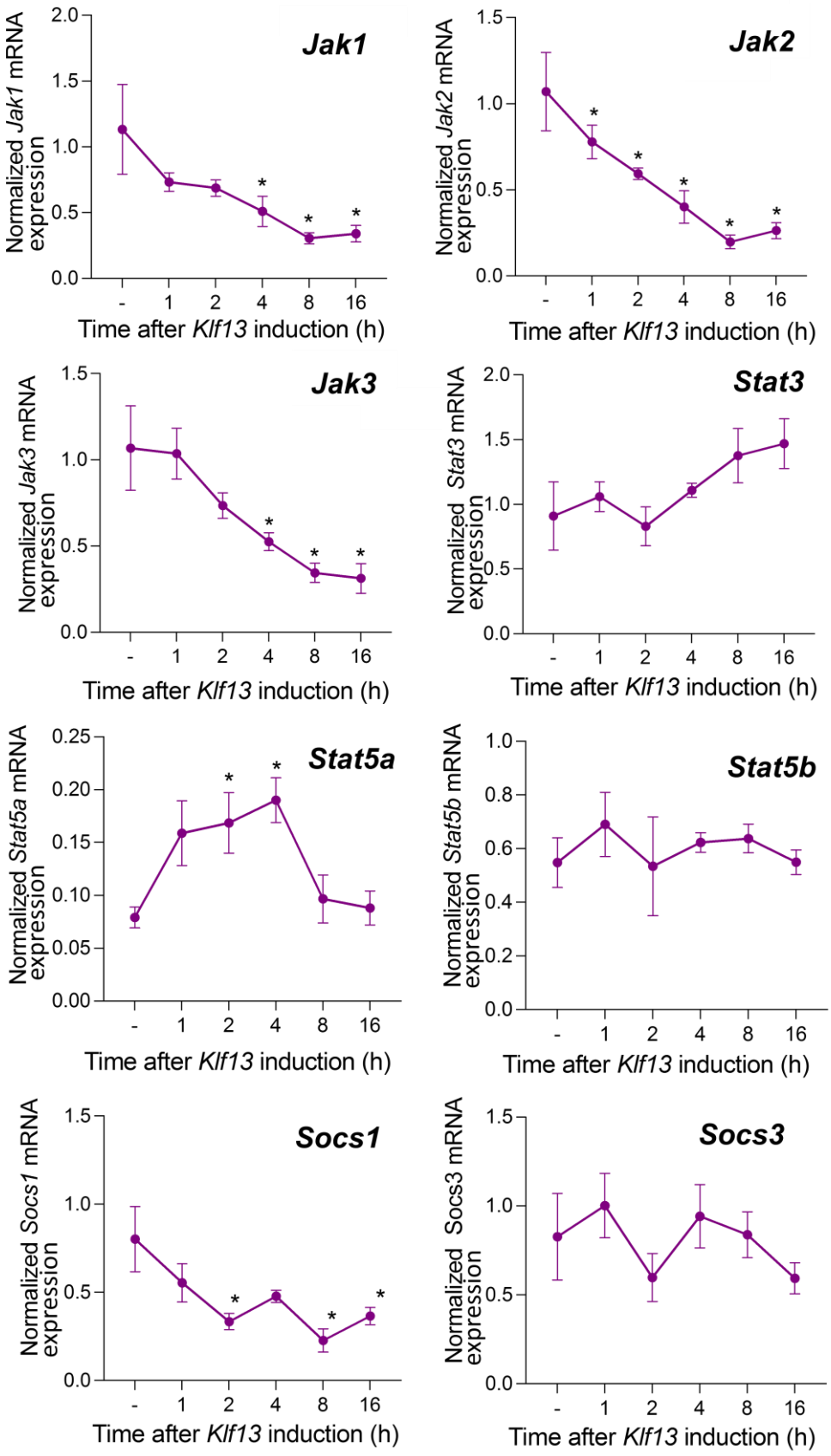
Jak1
Normalized Jak1 mRNA expression
Time after Klf13 induction (h)
Jak2
Normalized Jak2 mRNA expression
Time after Klf13 induction (h)
Jak3
Normalized Jak3 mRNA expression
Time after Klf13 induction (h)
Stat3
Normalized Stat3 mRNA expression
Time after Klf13 induction (h)
Stat5a
Normalized Stat5a mRNA expression
Time after Klf13 induction (h)
Stat5b
Normalized Stat5b mRNA expression
Time after Klf13 induction (h)
Socs1
Normalized Socs1 mRNA expression
Time after Klf13 induction (h)
Socs3
Normalized Socs3 mRNA expression
Time after Klf13 induction (h)

Figure 9. Effect of induced expression of KLF13 in the mRNA levels of genes involved in the JAK-STAT signaling pathway. The KLF13 was induced with doxycycline in the HT22-TR/TO-V5Klf13 cell line. After 1, 2, 4, 8 and 16 hours of treatment, cells were harvest, RNA isolated, and mRNA quantified by RT-qPCR. Each group was compared against the control group. Treatment of HT22-TR/TO-V5Klf13 cells with doxycycline caused a time-dependent reduction of *Jak1* [F(5,17) = 6.96, p = 0.001], *Jak2* [F(5,17) = 13.50, p < 0.0001], *Jak3* [F(5,17) = 10.03, p = 0.0001] and *Socs1* [F(5,17) = 5.1, p = 0.0049], while induces the *Stat5a* gene [F(5,18) = 4.588, p = 0.0071]; one way ANOVA followed by Dunnet's post-hoc test. Data of each gene was normalized to the geometric mean of *Gapdh* and *Ppia* mRNA levels. Points represent the mean ± SEM (n = 4 / time point).

2. The absence of KLF13 in the HT22 cell line impacts the mRNA levels of some genes associated with the JAK-STAT pathway.

Using real-time PCR (qPCR), mRNA levels of *Ghr, Jak1, Jak2, Jak3, Stat3, Stat5a, Stat5b, Socs1, Socs3* and *Igf1* genes were analyzed in both HT22 parental and HT22 *Klf13*-KO cell lines. The results showed that *Klf13* depletion increased the expression of *Jak1, Stat3, Socs1, Socs3* and *Igf1*. *Jak1* increased by 0.5-fold, *Stat3* by 1.3-fold, *Socs3* by 2-fold and *Igf1* by 2.5-fold (Fig 10).

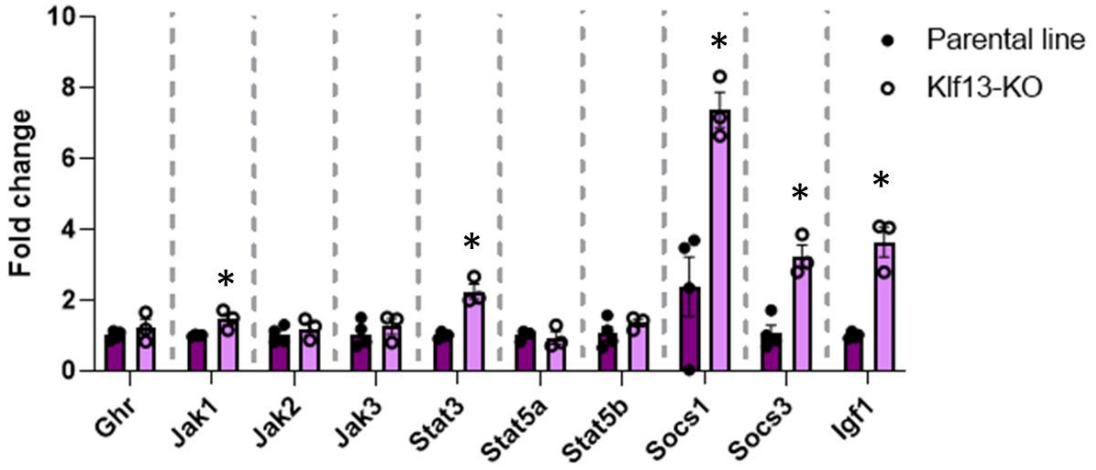

Figure 10. Effect of the absence of KLF13 on the expression of genes associated with the JAK-STAT pathway in HT22 *Klf13* knockout cell line compared to HT22 parental cell line. Cells were harvest, RNA isolated, and mRNA quantified by RT-qPCR. Absence of *Klf13* caused the increase of *Jak1* [F(2,3) = 115.9, p = 0.0219], *Stat3* [F(2,3) = 18.02, p = 0.0011], *Socs1* [F(2,2) =1.416 , p =0.0029], *Socs3* [F(2,3) =1.486 , p =0.0023], and *Igf1* [F(2,3) = 66.29 , p =0.0007]; data of each gene was normalized to the geometric mean of *Gapdh* and *Ppia* mRNA levels and separately analyzed by t-student test. Bars indicate the standard error of the mean ± SEM (n = 4).

3. The absence of KLF13 in the hippocampus of mice affects the mRNA levels of some genes involved in the JAK-STAT pathway.

The hippocampus of wild type and *Klf13*-KO mice was extracted to quantify the mRNA levels of genes involved in the JAK-STAT signaling pathway by real-time PCR (qPCR). The results showed that in the hippocampus of *Klf13*-KO mice, the mRNA levels of *Jak3* and *Socs1* increased by 1.5- and 6.4-fold, respectively, whereas the levels of *Stat5a* decreased by 40% (Fig 11).

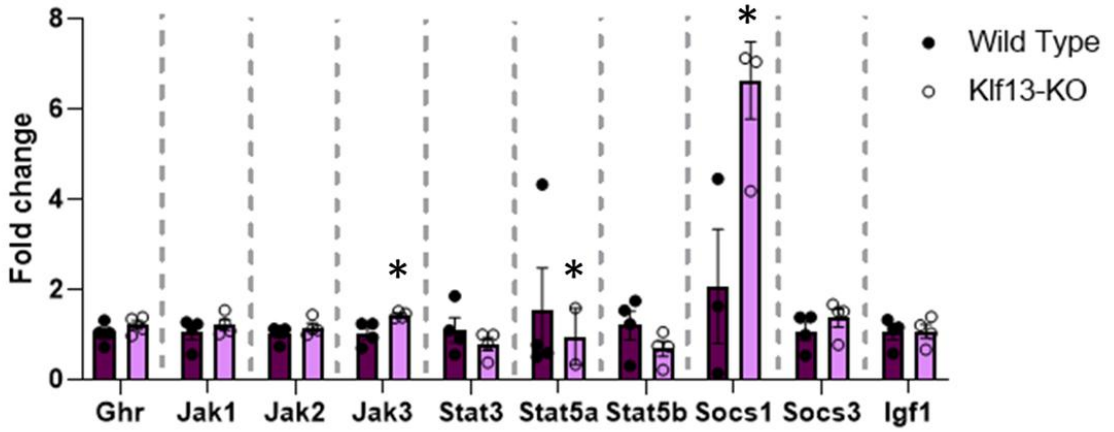

Figure 11. Effect of the absence of KLF13 on the expression of genes associated with the JAK STAT pathway in left hippocampus of wild type and *Klf13* knockout mice. The hippocampi were harvest, RNA isolated, and mRNA quantified by RT-qPCR. Absence of KLF13 induced the increase of *Jak3* [F(3,3) = 13.02, p = 0.0253] and *Socs1* [F(2,3) = 1.637 p = 0.0264]; data of each gene was normalized to the geometric mean of *Gapdh* and *Ppia* mRNA levels and separately analyzed by t-student test. Bars indicate the standard error of the mean ±SEM (n = 4).

4. The GH-dependent expression of JAK-STAT target genes is strongly enhanced in KLF13 deficient HT22 cells.

To assess the impact of KLF13's absence on the JAK-STAT pathway activity induced by GH, the expression levels of four target genes of the pathway (*Igf1, Socs1, Socs3,* and *Bdnf*) were quantified in both the HT22 parental and knockout cell lines following GH treatment for 15 minutes, 30 minutes, 1, 2, 4, 8 and 16 hours. Each group treated with GH was compared against the control group, which received no treatment.

The results showed that mRNA levels of *Igf1* in the parental cell line increased from 2 to 8 hours, and high levels were maintained until 16 hours of GH treatment. The increase of mRNA levels was 1.8-fold at 2 and 4 hours, 2.8-fold at 8 hours, and 2.3-fold at 16 hours compared to the control group. However, in the *Klf13*-KO cell line, *Igf1* mRNA levels increased by 3.5-fold at 8 hours, and high levels were maintained 1-fold at 16 hours (Fig. 12). Intriguingly, the absolute levels of *Igf1* mRNA were higher in KLF13-deficient cells compared with the parental line.

The mRNA levels of *Socs1* in parental cell line decreased by 50% at 4 and 16 hours compared to the control group. Conversely, mRNA levels of *Socs1* increased in *Klf13*-KO cell line by 1.8-fold at 1 hour and 2-fold at 8 hours after GH treatment (Fig 12). The absolute levels of *Socs1* mRNA were strongly increased in HT22-*Klf13*-KO cell line compared with parental line.

The mRNA levels of *Socs3* in the parental cell line increased after GH treatment by 9-fold at 1 hour and 5-fold at 8 hours after treatment. In comparison, in the *Klf13*-KO cell line, the mRNA levels of Socs3 increased 5.5-fold at 30 minutes, 10.5-fold at 1 hour and 7-fold at 8 hours. (Fig 12). Interestingly, *Socs3* was the only gene whose mRNA levels increased similarly in both the parental and knockout lines at certain time points with GH treatment (particularly at 1 and 8 hours), although absolute levels were higher in HT22-*Klf13*-KO cells.

In the parental cell line, the mRNA levels of *Bdnf* increased by 1-fold after 1 hour of GH treatment compared to the control group. However, this gene increased its expression by 4-fold at 1 hour, 2-fold at 2 hours, and 2.5-fold at 8 hours after GH treatment in KLF13-deficient HT22 cells (Fig. 12).

In general, between the four target genes measured in the parental cell line, GH treatment showed the greatest effect on *Igf1* and the lowest on *Bdnf*. Whereas for the *Klf13* knockout cell line, GH treatment showed the greatest effect on *Socs3* and the lowest on *Igf1* (Fig.12).

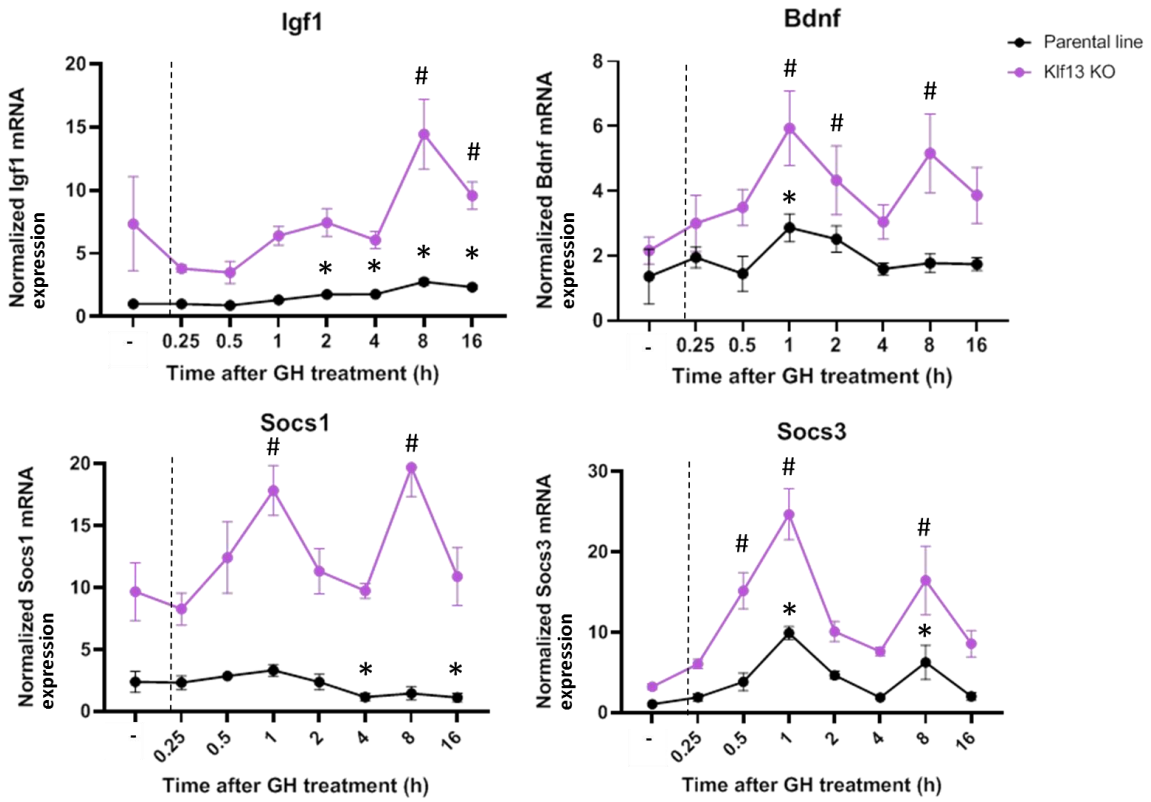

Figure 12. Effect of GH over *Igf1, Socs1, Socs3* and *Bdnf* expression on HT22 parental and *Klf13*-KO cell lines. In the *x* axis, the (-) represents the control group. Experimental groups were treated with 1 nM GH for 15 minutes, 30 minutes, 1, 2, 4, 8 and 16 hours. After treatments, cells were harvested and RNA extracted to quantify the expression of each gene by RT-qPCR. Each group was compared against the control group. GH treatment caused a time-dependent increase of mRNA *Igf1* levels in parental cell line at **2** (p= 0.0009), **4** (p=0.0008), **8** (p<0.0001) and **16** hours (p<0.0001); for Klf13-KO cell line, GH treatment increased mRNA *Igf1* levels at **8** (p<0.0001) and **16** hours (p=0.0241). For *Socs1,* GH treatment caused a decrease of *Socs1* mRNA levels in parental cell line at **4** (p= 0.0380) and **16** hours (p= 0.0335) whereas it caused an increase on Klf13-KO cell line at **1** (p= 0.0080) and **8** hours (p=0.0033). For *Socs3*, GH treatment caused an increase of *Socs3* mRNA levels in parental cell line at **1** (p<0.0001) and **8** hours (p= 0.0113); by comparison, GH treatment caused an increase of mRNA *Socs3* on Klf13-KO cell line at **.5** hours (p= 0.0103) as well as the same time points than in the parental line at **1** (p<0.0001) and **8** hours (p= 0.0042). Finally, for *Bdnf*, GH treatment caused an increase of mRNA *Bdnf* levels in parental cell line only at **1** hour (p= 0.0031) whereas it caused an increase of *Bdnf* mRNA levels on Klf13-KO cell line at **1** (p<0.0001), **2** (p=0.0226) and **8** hours (p=0.0013). The data of each gene was normalized to the geometric mean of *Gapdh* and *Ppia* mRNA levels. One-way ANOVA followed by Dunnet's post-hoc test. Two-way ANOVA followed by Tukey's post-hoc test yield that GH treatment had a significant effect on mRNA levels [F(7,8) = 5.232 p = 0.0002], [F(7,47)= 4.135; p=0.0013], [F(7, 47) = 16.14; p<0.0001], [F (7, 48) = 11.39; P<0.0001], as well as the absence of Klf13 [F(1,48) = 81.18 p<0.0001], [F(1,47) = 196.1; p<0.0001], [F (1, 47) = 76.94; p<0.0001], [F

(1, 48) = 127.0; p<0.0001] in *Igf1, Socs1, Socs3* and *Bdnf* genes respectively. Points represent the mean ± SEM (n = 4 / time point).

It is worth mentioning that for *Socs1, Socs3* and *Bdnf* genes in the *Klf13* knockout cell line, there were two peaks at 1 hour and 8 hours of GH treatment, whereas for *Igf1* there was only one peak at 8 hours. In addition, there was a peak in the mRNA levels of *Socs3* and *Bdnf* at 1 hour of GH treatment in the parental cell line. Interestingly, in the parental cell line, *Igf1* was the only gene that constantly increases its mRNA levels from 2 hours to 16 hours of GH treatment, whereas *Socs1* was the only one that decreases with GH treatment. Furthermore, *Socs3* showed two peaks at 1 and 8 hours of GH treatment, as observed in *Klf13*-KO cell line.

5. The *in vitro* results are not recapitulated in an *in vivo* model of intraventricular injection of GH.

To confirm the *in vitro* results showing a significant increase in mRNA levels of 3 target genes of the JAK-STAT pathway after 1 hour of GH treatment in the knockout cell line, we conducted an experiment to determine the effects of a 1-hour GH treatment on the hippocampus of mice. Wild type and *Klf13*-KO mice were given intracerebroventricular GH injections for this purpose, and we evaluated the same target genes (*Igf1, Socs1, Socs3,* and *Bdnf*) as in the in vitro study. The results showed a 1.6-fold increase in the baseline of *Socs1* mRNA levels in Klf13-KO mice compared with wild type mice. Conversely, the basal levels of *Socs3* mRNA decreased by 69.3% in the hippocampus of Klf13-KO mice. However, the GH treatment did not induce the same gene expression response in compare with HT22 cells (Fig. 13).

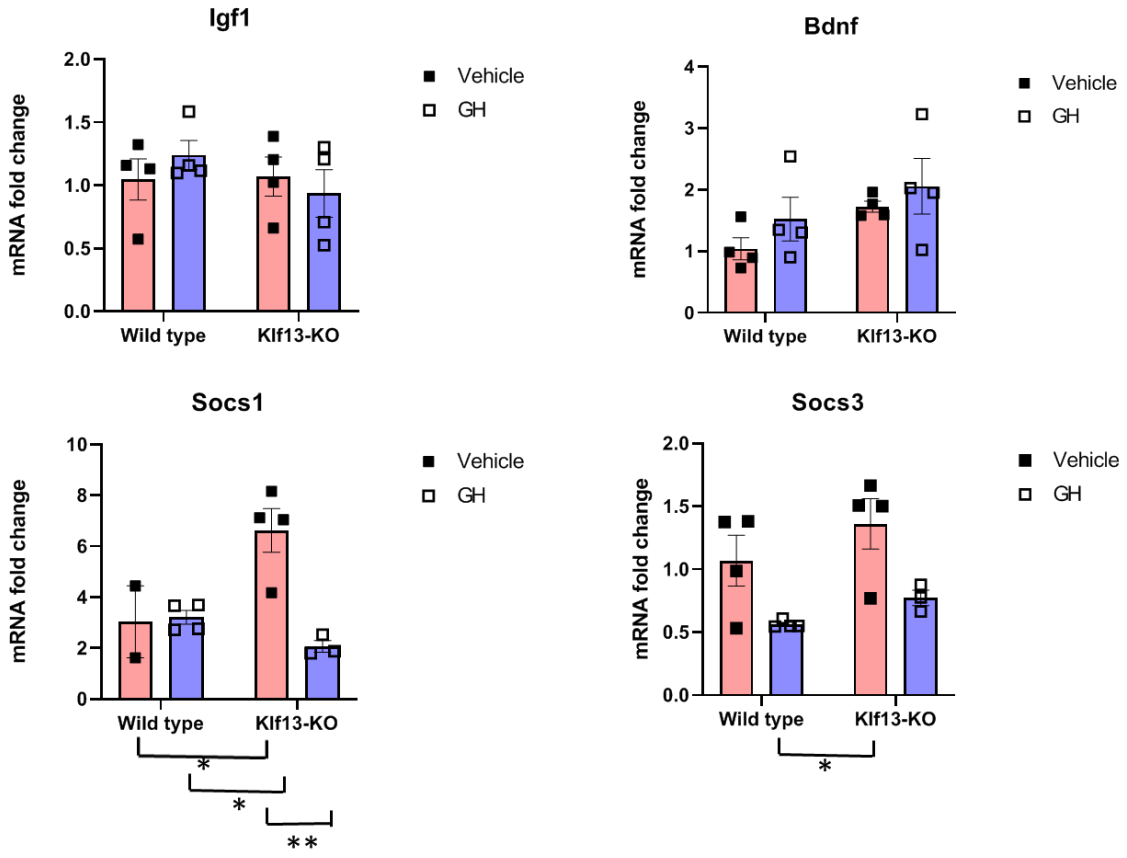

Figure 13. Effect of GH on mRNA levels of *Igf1, Socs1, Socs3* and *Bdnf* in wild type and Klf13-KO mice hippocampi. The animals received an intracerebroventricular injection of 2 µl of GH solution. Mice were sacrificed at 1 hour after the injection. For *Socs1*, there was an increase in Wild type-Vehicle vs. Klf13-KO-Vehicle (p= 0.0367) and Wild type-GH vs. Klf13-KO-Vehicle (p= 0.0162), whereas there was a decrease in Klf13-KO-Vehicle vs. Klf13-KO-GH (p= 0.0045). For *Socs3*, there was an increase in Wild type-GH vs. Klf13-KO-Vehicle (p= 0.0146). The data of each gene was normalized to the geometric mean of *Gapdh* and *Ppia* mRNA levels measured in mice hippocampus. Two-way ANOVA followed by Tukey's post-hoc test. The GH factor had an effect on mRNA levels for *Socs1* [F (1, 9) = 9.272; p= 0.0139] and *Socs3* [F (1, 11) = 12.19; p= P=0.0050]. Bars represent the mean ± SEM (n = 4 / time point).

Discussion

This work aimed to elucidate the effects of KLF13 on transcriptional expression of genes involved in the JAK-STAT signaling pathway. In addition, the effects of *Klf13* depletion on the GH-induced expression of some JAK-STAT target genes were analyzed as a measure of its activity in hippocampal neurons of mice.

Previous studies have described that KLF13 predominantly works as a transcriptional repressor by interacting with the promoters of its target genes (Ávila-Mendoza *et al.*, 2020a). These genome-wide studies showed that genes with differential expression induced by KLF13 in hippocampal neurons could impact several signaling pathways essential to neuronal physiology, including the JAK-STAT signaling pathway. In agreement with previous results, here we showed that KLF13 regulates the expression of several genes whose encoded proteins constitute the core of the JAK-STAT signaling pathway, impacting, in consequence, the activity of the pathway when it is activated by the GH. This suggest that KLF13 works as an endogenous inhibitor of the JAK-STAT signaling.

The induction of KLF13 expression in HT22-TR/TO-V5Klf13 cells repressed the three Jaks evaluated (*Jak1, Jak2* and *Jak3*), with a strong effect observed on *Jak2*, since its mRNA levels were repressed at all time points assessed. Interestingly, *Socs1*, an endogenous inhibitor of JAK-STAT signaling, was also repressed, while the only gene up-regulated by the forced expression of KLF13 was *Stat5a*. This upregulation could be a compensatory mechanism against the interruption of JAK signaling, as well as the down-regulation of *Socs1,* to allow the pathway to continue signaling.

Compared to the parental line, which expresses KLF13 constitutively, in HT22 *Klf13*-KO cells (lacking *Klf13*), *Jak1, Stat3, Socs1, Socs3* and *Igf1* are up-regulated. In particular, *Jak1* and *Socs1* have an inverse expression pattern confirmed by the previous results, since the induced expression of KLF13 represses them, but the absence of KLF13 up-regulates them. JAK1 and SOCS1 are widely expressed in various tissues. JAK1 is known to phosphorylate all STATs, as reported by Hu *et al.* (2021), while SOCS1 directly inhibits both JAK1 and JAK2, as demonstrated by Liau *et al.* (2018). Notably, the inhibition of JAK2 by SOCS1 also leads to the inhibition of STAT3, as discussed in Wang *et al.* (2017). In the context of axon regeneration, JAK1 has been found to play a role in promoting axon regeneration in retinal ganglion cells (RGCs) through the activation of the interferon gamma receptor (IFNGR) by IFNγ in adult *Ptpn2*-KO mice (Wang et al., 2023). Conversely, SOCS1, as well as SOCS3 (Smith *et al.,* 2009), have inhibitory roles in axon regeneration, as observed in adult mice retinal ganglion cells (Park et al., 2009). Among the members of the STAT family, STAT3, which along with *Jak1*, *Socs1*, *Socs3*, and *Igf1*, is upregulated in HT22

Klf13-KO cells. STAT3 is a signaling mediator of the JAK-STAT pathway and is closely associated with axonal regeneration, as highlighted by Mehta *et al.* (2016). Additionally, STAT3 can be activated following central nervous system injury (Dziennis and Alkayed, 2008).

On the other hand, *in vivo* experiments showed that *Jak3* and *Socs1* are up-regulated in the hippocampus of *Klf13-KO* mice, whereas *Stat5a* is down-regulated. JAK3 is predominately expressed on hematopoietic cells where it modulates cell signaling in response to cytokines, but it is also found in neurons (De Mars *et al.,* 2017), where through JAK3-STAT3 activation mediated by CNTF promotes long-distance axon regeneration of retinal ganglion cells after axon injury (Pernet *et al.,* 2013). Table 4 summarizes the results *in vitro* and *in vivo*.

Table 4. Summary of mRNA changes of JAK-STAT associated genes measured by qPCR in the *in vitro* and in the *in vivo* models.

	Jak1	**Jak2**	**Jak3**	**Stat3**	**Stat5a**	**Stat5b**	**Socs1**	**Socs3**	**Ghr**	**Igf1**
HT22 inducible cell line	↓	↓	↓		↑		↓			
HT22 parental cell line	↓			↓			↓	↓		↓
HT22 Klf13-KO cell line	↑			↑			↑	↑		↑
WT mice hipp neurons			↓		↑		↓			
Klf13-KO mice hipp neurons			↑		↓		↑			

Overall, the analysis of JAK-STAT pathway-associated genes *in vitro* and *in vivo* showed that KLF13 has a predominantly repressive role in the transcription of some of these genes. The results in the three approaches were consistent with the repression effect of KLF13 over different *Jaks* and *Socs*

genes. However, the *in vitro* and *in vivo* approaches differ in the regulation of *Stat3*, *Stat5a* and *Igf1* genes by KLF13. One possible explanation is that in the *in vitro* approach, *Klf13* was knocked out in HT22 differentiated neurons (Ávila-Mendoza *et al.,* 2020a). Although compensatory mechanisms by other KLFs have been described, these mechanisms may not be enough to restore the actions of KLF13. On the other hand, the *in vivo* approach implies that KLF13 is absent in mice from the beginning of growth. Therefore, actions of KLF13 could be better compensated by other KLFs during early development, as was suggested previously (Ávila-Mendoza *et al.,* 2020a; Knoedler *et al* 2019). We must also consider that in the *in vivo* approach, neurons interact with other cell types, which could be another source of compensatory mechanisms. These variables may trigger different mechanisms that impact the gene expression.

Treatment with GH in HT22 *Klf13*-KO cells showed that the JAK-STAT pathway target genes *Socs1, Socs3* and *Bdnf* had two peaks in their mRNA levels, one at 1 hour and the other one at 8 hours of GH treatment. This oscillation pattern of *Socs1, Socs3* and *Bdnf* mRNA levels in the HT22 *Klf13* knock out cells showed a ultradian rhythmicity, and this might be due to the fact that in absence of KLF13, its paralog KLF9, with which it has partially overlapping functions, exerts compensatory functions. Additionally, KLF9 has shown to exhibit circadian rhythmicity in its association in chromatin in HT22 cells and mouse hippocampus. This circadian rhythmicity is caused by CLOCK+BMAL1 binding to canonical E-box (CACGTG) motifs upstream of the *Klf9* transcription start site (TSS), and that KLF9 itself is able to regulate transcription of the clock-output gene *Dbp*, which is part of an auxiliary loop that stabilizes the main regulatory clock loop (Knoedler *et al.,* 2020). Conversely, KLF13, which is expressed constitutively in HT22 parental cells and is also a clock-output gene, exhibits circadian oscillation to a lesser extent than KLF9 (Knoedler *et al.,* 2020).

It is worth nothing that *Socs1* is up-regulated in HT22 *Klf13*-KO cells, whereas it is repressed in HT22 parental cells. This supports the notion that KLF13 may act as an endogenous inhibitor of the JAK-STAT pathway, since its constitutive expression represses *Socs1* expression even when the pathway is activated.

Igf1 is a GH-responsive gene showing increased levels of its mRNA after GH treatment in both cell lines, *Klf13*-KO and parental cells. Its highest levels

were found in KLF13-deficient HT22 cells. This increase might be due to over-activation of the pathway by GH treatment in combination with the KLF13 depletion, further supporting the notion that KLF13 is an endogenous inhibitor of this pathway. Although *Igf1* expression has some circadian control (Chaudhari *et al.,* 2017), it did not show a remarkable circadian rhythm in its transcription like the other three genes assessed. This may be due to the fact that GH/GHR signaling itself is poorly activated despite GH treatment due to upregulation of *Socs1* and *Socs3* within the first few hours of treatment.

Due to the fact that most of the pathway target genes assessed were responsive 1 hour after GH treatment, wild type and *Klf13*-KO mice were intracerebroventricularly injected with 20 ng of GH and euthanized 1 hour after injection to analyze the expression of JAK-STAT target genes. However, the results did not recapitulate the *in vitro* results. Instead, only *Bdnf* showed an increase in its mRNA levels after GH treatment in wild type mice, an effect that was enhanced in KLF13-deficient mice. The absence of KLF13 by itself also increased *Bdnf* basal levels, although these results did not show significant differences. One possible explanation for the lack of changes in *Igf1* expression in the *in vivo* model may be that the treatment duration was only 1 hour, whereas in the cell line the *Igf1* response was observed up to 2 hours after treatment in the parental line. On the other hand, for *Socs1* and *Socs3*, genotype had an effect on transcription, although the results are inconclusive because their mRNA levels decreased with GH treatment. It should be noted that these samples showed extensive variance among them; therefore, this experiment should be repeated to obtain more accurate data.

Conclusion

The results of the present research suggest that KLF13 plays a crucial role in regulating the activity of the JAK-STAT signaling pathway in HT22 hippocampal neurons by regulating the expression of genes involved in the pathway. Additionally, the depletion of KLF13 leads to increased JAK-STAT pathway activity induced by GH. These findings suggest that targeting KLF13 could be a

potential strategy to enhance the effects of GH on neurons, including its neurotrophic effects.

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