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Licenciada em Biotecnologia

The role of post-translational modifications on STAT3 interactions

Dissertação para obtenção do Grau de Mestre em

Bioquímica para a Saúde

Orientador: Dr. Federico Herrera, Investigador, ITQB

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Abstract

The JAK/STAT3 pathway is involved in multiple biological phenomena, mostly related to stress or tissue damage, but also development and cancer. A rate-limiting step of the pathway involves STAT3 dimerization, phosphorylation and translocation to the nucleus. Originally, it was thought that STAT3 dimerized upon phosphorylation by JAKs on Y705. However, current evidence indicates that STAT3 exists as a dimer prior to phosphorylation and activation, and that phosphorylation only induces a change in the conformation of the dimer.

One of the aims of this thesis was to explore the importance of post-translational modifications on STAT3 interactions. To this end we used the BiFC system developed in our lab (Venus-STAT3 BiFC) to create STAT3 mutants on several key residues for acetylation, methylation and phosphorylation: K49, K140, K685, Y705 and S727. Through flow cytometry and fluorescence microscopy, we found that none of the mutants interfere with STAT3 dimerization and intracellular localization.

The other objective was to investigate whether the dimerization of unphosphorylated STAT3 is actually spontaneous or regulated by intracellular pathways. For this, a library of 82 kinase inhibitors was screened for their effect on STAT3 dimerization. We found that PLK-1, Aurora and MEK inhibitors prevented STAT3 spontaneous dimerization. The later steps in the screening were done with the PLK-1 family, which were tested at different concentrations and later against the STAT3 mutants. Our preliminary results indicate that HMN-214 could regulate the dimerization of unphosphorylated STAT3 and that S727 may be involved in this effect.

The relevance of our findings stems from the fact that STAT3 is an important molecule in developmental astrogliogenesis, reactive gliosis during neurodegeneration and brain or spinal cord injury, and some types of cancer.

Keywords: JAK/STAT3 pathway, STAT3, post-translational modifications, BiFC system.

Resumo

A via da JAK/STAT3 está envolvida em múltiplos fenómenos biológicos, essencialmente relacionados com stress ou dano tecidular, mas também no desenvolvimento e cancro. Os passos limitantes desta via envolvem a fosforilação, dimerização e translocação para o núcleo do fator de transcrição STAT3. Inicialmente, pensava-se que o STAT3 dimerizava antes de ser fosforilado pelas JAKs no residuo Y705. No entanto, estudos recentes indicam que o STAT3 dimeriza antes de ser fosforilado e ativado e que a fosforilação apenas induz uma alteração conformacional do dímero.

Um dos objetivos da presente tese foi explorar a importância das modificações pós-traducionais nas interações do STAT3. Para este fim, foi usado o sistema BiFC desenvolvido no nosso laboratório (Venus-STAT3 BiFC) para criar mutantes do STAT3 em resíduos-chave para a acetilação, metilação e fosforilação: K49, K140, K685, Y705 e S727. Através de citometria de fluxo e microscopia de fluorescência, observámos que nenhum dos mutantes interfere com a dimerização nem com a localização intracelular do STAT3.

Outro objetivo foi investigar se a dimerização do STAT3 não-fosforilado é espontânea ou regulada por outras vias intracelulares. Para tal foi realizado um rastreio com 82 inibidores de cinases para observar o efeito na dimerização do STAT3, sem que este interfira nos níveis da sua expressão. Através da citometria de fluxo e *Western blot*, as famílias PLK-1, Aurora e MEK foram consideradas promissoras. Dentro destas, a família PLK-1 foi escolhida para testes com diferentes concentrações e mais tarde em mutantes do STAT3. Os nossos resultados preliminares indicaram que o HMN-214 pode regular a dimerização do STAT3 não-fosforilado e que o resíduo S727 poderá estar envolvido nesta regulação.

A relevância dos nossos resultados advém do facto de o STAT3 ser uma molécula importante no desenvolvimento astrogliogénico, na gliose reativa durante a neurodegeneração e em lesões do cérebro e da espinal medula, e em alguns tipos de cancro.

Palavras-chave: Via da JAK/STAT3, STAT3, modificações pós-traducionais, sistema BiFC.

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List of abbreviations

A - Alanine

BiFC - Bimolecular fluorescence complementation

BMP - Bone morphogenetic protein

BSA - Bovine serum albumin

CBP - CREB-binding protein

CCD - Coiled-coil domain

CDK - Cyclin-dependent kinase

CHK - Checkpoint kinase

CIS - Cytokine-inducible SH2-containing protein

CNS - Central Nervous System

CNTF - Ciliary neurotrophic factor

CON - Control

CT-1 - Cardiotrophin-1

DBD - DNA binding domain

DMEM - Dulbecco's Modified Eagle's Medium

EGF - Epidermal growth factor

F - Phenylalanine

FBS - Fetal bovine serum

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GFAP - Glial fibrillary acidic protein

Gp130 - Glycoprotein 130

HEK 293 - Human Embryonic Kidney cells

HepG2 - Liver hepatocellular carcinoma cells

IL - Interleukin

IFN - Interferon

JAK - Janus kinase

JH - Jak homology

LB - Luria Broth

LIF - Leukemia inhibitory factor

LIFR - Leukemia inhibitory factor receptor

K - Lysine

NSC - Neural stem cell

NTD - N-terminal domain

OSM - Oncostatin M

P/S - Penicillin/Streptomycin solution

PBS - Phosphate buffered saline

PC3 - Prostate cancer cells

PCR - Polymerase chain reaction

PI - Propidium iodide

PIAS - Protein inhibitor of activated STAT

PLK-1 - Polo-Like Kinase 1

PPIs - Protein-Protein interactions

R - Arginine

RA - Retinoic acid

RARα - Retinoic acid receptor α

SDS - Sodium dodecyl sulphate

S - Serine

SH2 - Scr homology 2

SHP - SH2 domain-containing phosphatase

STAM - Signal-transducing adaptor molecules

STAT - Signal transducer of activators of transcription

StIP - STAT-interacting protein

SOCS - Suppressor of cytokine signaling

TAD - Transactivation domain

TBS - Tris-HCL buffer saline

TBS-T - Tris-buffered Saline Tween 20

U3A - Human sarcoma cells

V1 - Venus 1

V2 - Venus 2

WT - Wild-type

Y - Tyrosine

1. Introduction

1.1. The JAK/STAT3 pathway

Cytokines play a very important role in the regulation of many biological responses, transducing signals from the outside of the cells to the inside through specific receptors.[1] The Janus Kinase (JAK)/ Signal Transducer and Activator of Transcription (STAT) 3 pathway is one of the pathways which can be activated by cytokines as well as hormones and growth factors. This pathway is a conserved signalling cascade, present from invertebrates such as Drosophila to mammalians.[2,3]

The JAK/STAT3 pathway starts when the ligands associate to their corresponding membrane receptor, promoting a modification on the conformation of the cytoplasmic tail of the receptor, which is coupled to JAKs. These kinases become activated by transphosphorylation, allowing the receptor without intrinsic tyrosine kinase activity to become phosphorylated at specific tyrosine residues on conserved regions.[4] These phosphorylated domains will serve as docking sites for STAT3 dimers. STAT3 dimers are then recruited by means of their own Scr homology 2 (SH2) domains, binding to the receptor and becoming phosphorylated by JAKs on tyrosine (Y) 705. Once activated, phosphorylated STAT3 dimers are released to the cytoplasm and translocate to the nucleus, by a mechanism dependent on importin α-5 and the Ran nuclear import pathway. In the nucleus, STAT3 binds specific sequences in the deoxyribonucleic acid (DNA), named STAT3-responsive elements, and regulates the transcription of target genes (Figure 1.1), such as *gfap, c-fos*, *c-myc*, *sox2*, *mcl-1* and *il-10*.[2,5–8] After activating gene transcription, STAT3 is dephosphorylated and returns to the cytoplasm.[9]

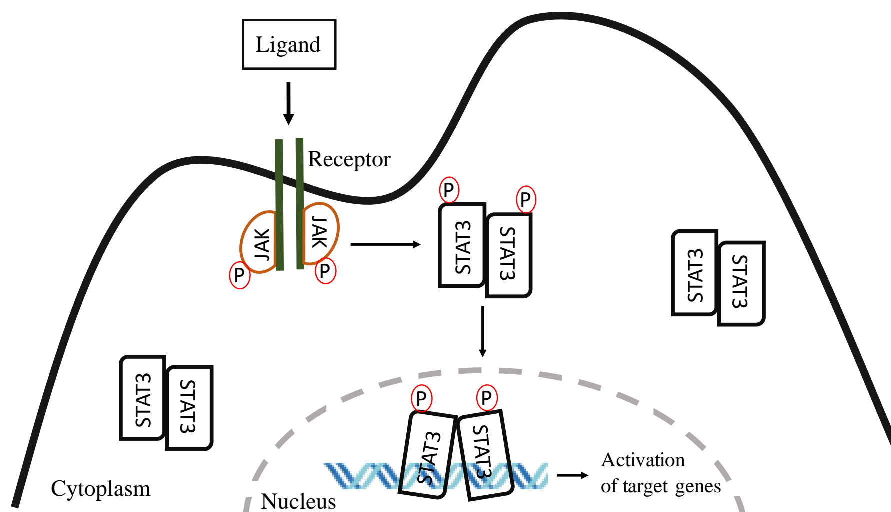

Figure 1.1 – The JAK/STAT3 pathway.
The ligand (cytokine, hormone or growth factor) binds the receptor which is coupled to JAK. JAK phosphorylates specific tyrosine residues in the receptor, providing a docking site for STAT3 dimers. STAT3 is then phosphorylated by JAKs and subsequently translocates to the nucleus to bind the DNA at specific sequences, leading to the transcription of target genes.

This pathway is involved in many biological events and, when its regulation is disrupted, a number of pathological occurrences can appear including immune disorders, cancer and cardiovascular diseases.[2,10]

The JAK/STAT3 pathway is associated with gene regulation during development, hormone release, inflammation and tumorigenesis. STAT3 expression in injured axons is increased as a specific response to initiate neuronal regeneration.[10] Also, STAT3 is required for upregulation of the glial fibrillary acidic protein (GFAP), astrogliosis and glial scar formation after spinal cord injury, as well as for reducing inflammation after central nervous system (CNS) injury.[2,10] Addition of ciliary neurotrophic factor (CNTF) and retinoic acid (RA) to neural precursor cells from embryos in different developmental stages promotes either neurogenesis or astrogliogenesis, both through the activation of the JAK/STAT3 pathway.[11,12]

Recently, JAK/STAT3 has been implicated in several inflammation-related pathologies such as cancer and obesity. This may bring several questions such as: what is really the role of the JAK/STAT3 pathway in inflammation? Is it anti-inflammatory or pro-inflammatory? Although the JAK/STAT3 pathway works towards reducing inflammation following CNS injury, interleukin (IL)-6 (a pro-infammatory cytokine) mediated-activation of JAK/STAT3 seems to work in the opposite direction, promoting inflammation. This is especially true in many types of inflammation-mediated cancers, such as gastric cancers. Increased and persistently activated inflammation mainly mediated by IL-6 and IL-11 in the intestine affects normal proliferation of epithelial cells, eventually leading to tumorigenesis. Leukemia inhibitory factor (LIF) could also be a relevant player in tumorigenesis, as its overactivates the JAK/STAT3 pathway and promotes inflammation in many types of cancer.[13]

1.2. Cytokines and receptors

The STAT3 can be activated through various classes of receptors: receptors with intrinsic protein tyrosine kinase activity (epidermal growth factor (EGF) receptor and fibroblast growth factor receptor, for example); receptors without intrinsic protein tyrosine kinase activity and G-protein-coupled receptors (such as the macrophage inflammatory protein receptor and RANTES receptor).[5] The receptors without intrinsic protein tyrosine kinase activity are especially interesting to us because some of them are part of the canonical activation pathway for JAK/STAT3 in the CNS. They are subdivided in smaller groups according to their structure and their differential affinity for particular cytokines and growth factors: interferon (IFN) family (IFN-α/β, IFN-γ, IL-10, IL-19, IL-20, IL-22), glycoprotein (gp) 130 family (such as IL-6, IL-11, oncostatin M (OSM), LIF, cardiotrophin-1 (CT-1), granulocyte colony-stimulating factor, IL-12, IL-23, leptin), γC family (IL-2, IL-4; IL-7, IL-9, IL-15, IL-21) and single chain family (erythropoietin, growth hormone, prolactin, thrombopoietin).[6]

In this project we focused on the JAK/STAT3 pathway activated by the IL-6 family of cytokines through the gp130 receptor, which are considered very powerful physiological activators of this pathway in a wide range of biological conditions, described above.[7]

The JAK/STAT3 pathway can be activated by various members of the IL-6 family, including IL-6, IL-11, CNTF, LIF, OSM, and CT-1.[14] Here, we are only focused on activation of the JAK/STAT3 pathway by LIF, which was the cytokine that worked better in our cell lines. The activation of this pathway by LIF involves the activation of the gp130 receptor which is known to be the signal transducing subunit in IL-6 cytokine receptors.[4,15] This receptor can homo- or heterodimerize with LIF receptor (LIFR), IL-6 receptor, OSM receptor, IL-11 receptor or CNTF receptor, and transduce different signals depending on the triggering cytokine (Figure 1.2).[16] Gp130-knockout in mice leads to lethality at birth or shortly thereafter. Accordingly, the knockout of most members of this family of receptors leads to severe and mostly lethal phenotypes, such as the LIFR. The lack of LIFR leads to placental defects and loss of motor neurons in LIFR-knockout mice.[17]

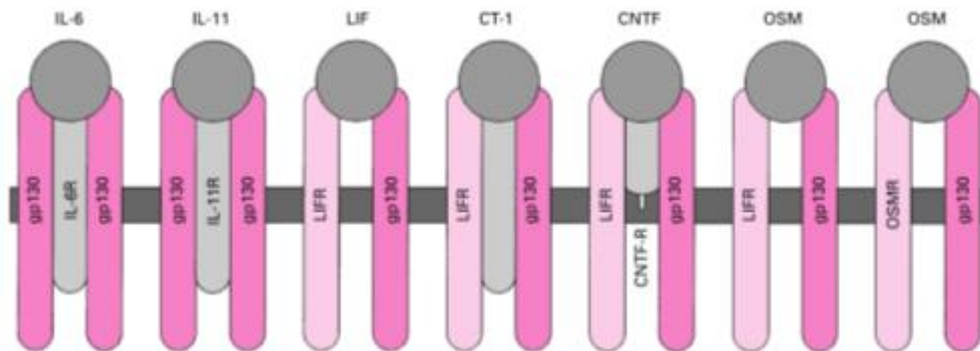

Figure 1.2 – IL-6 family of cytokine receptor complexes.
Gp130 receptor can homo- or heterodimerize with other members of the IL-6 family of cytokine receptors. However, gp130 must always be present for the signal transduction to occur, in this family of receptors. (Heinrich *et al.*, 1998)

1.3. JAK family

In mammalians the JAK family is composed by four tyrosine kinases (JAK1, JAK2, JAK3 and tyrosine kinase 2), discovered in the early 1990s.[10,18] The JAK family plays an important role in various signal transduction pathways, involved in cell proliferation, differentiation, survival and apoptosis.[19] This family of proteins can have different sizes, ranging from 120 to 140 kDa and their structure has seven conserved JAK homology (JH) domains (JH1-JH7) (Figure 1.3).[20] The JH1 domain is a functional catalytic domain and the JH2 domain seems to be a docking site for STATs. Both are present on the carboxyl-terminal region. The domains JH3 through JH7 are required for binding to the receptor.[6,21,22]

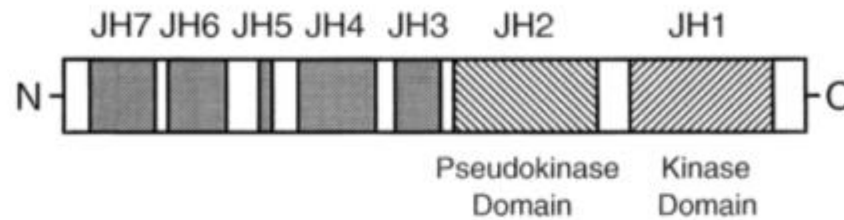

Figure 1.3 - The structure of JAKs.
(Imada & Leonard, 2000)

The regulation of JAK activity must be strict in order to ensure that cytokine- and growth factor-induced responses are normal. Changes in its natural function almost always lead to a pathological event. Its uncontrolled overactivation may lead to tumorigenesis, while the lack of its activity results in immunological deficiencies, such as severe-combined immunodeficiency.[19] Through the study of JAK1-knockout mice it was possible to determine that JAK1 is essential in the mediation of biological responses to cytokine receptors and that it also displays an early postnatal lethal phenotype. JAK2-knockout mice have an embryonically lethal phenotype. These mice died at day 12,5 of the gestation due to the failure in erythropoiesis.[6] In the CNS, studies reveal that JAK1 is involved on astrocyte differentiation and JAK2 in neural stem cell (NSC) proliferation.[15] JAK3's expression is limited to hematopoietic cells and mice without JAK3 can grow normally in pathogen-free environments, even without lymphocytes. Individuals with JAK3 deficiency have a severe combined immunodeficiency, having defects in T cells and normal B cells. Finally, the tyrosine kinase 2, which was the first member of the JAK family to be involved in cytokine signaling, seems to play a very important role in mediating the biological responses to IL-12 and lipopolysaccharides.[6]

JAKs are present in an inactive form before cell stimulation. When a cytokine binds the receptor, JAKs are recruited and then auto- or hetero-phosphorylate with other JAK kinases or other tyrosine kinase family members.[19]

1.4. STAT family

While studying the JAK family and its mechanisms, Darnel *et al.* (1994) described for the first time a group of transcription factors whose activity was induced by IFN. These factors were STATs which are composed by seven transcription factors (STAT 1, 2, 3, 4, 5a, 5b and 6).[10,18] Studies following this one placed STATs in the center of many signal transduction pathways activated by cytokines, growth factors and hormones.[19]

The main function of the STAT family is to transduce external signals to the nucleus leading to the activation of target genes to modulate transcription. STAT proteins are latent in the cytoplasm until their activation and are present ubiquitously among most cell types. Furthermore, the activation of the different members of the STAT family seems to be dependent

on the cell type more than the cytokine or the member of the JAK family.[19,23]

The first STAT genes to be discovered were STAT1 and STAT2. Since then, five more STATs were discovered in mammalians.[5] The different STATs are encoded by different genes: STAT1 and STAT4 genes are localized in chromosome 2; STAT3, STAT5a and 5b genes in chromosome 17; and STAT2 and STAT6 genes in chromosome 12.[5] STATs are composed by around 700-850 amino acids and have six conserved domains (Figure 1.4). The N-terminal domain (N) is involved in dimerization, nuclear translocation and protein-protein interaction (PPI). The coiled coil domain (CC) is essential for receptor binding, Y phosphorylation and nuclear export. The DNA binding domain (DNA) is involved in DNA binding specificity and also in nuclear import and export. The linker domain (LK) is responsible for maintaining the structural integrity of the DNA-binding motif. The SH2 domain (SH2) is involved in the recruitment to the receptor and is fundamental for the dimerization of STAT monomers. Finally, the transactivation domain (TA) holds the regulatory serine (S) residue in most STATs, except in STAT2 and STAT6 (Figure 1.4). The SH2 domain is highly conserved among kinase substrates in many cell pathways and without it STATs cannot be phosphorylated nor activated.[5,9,19,24–28]

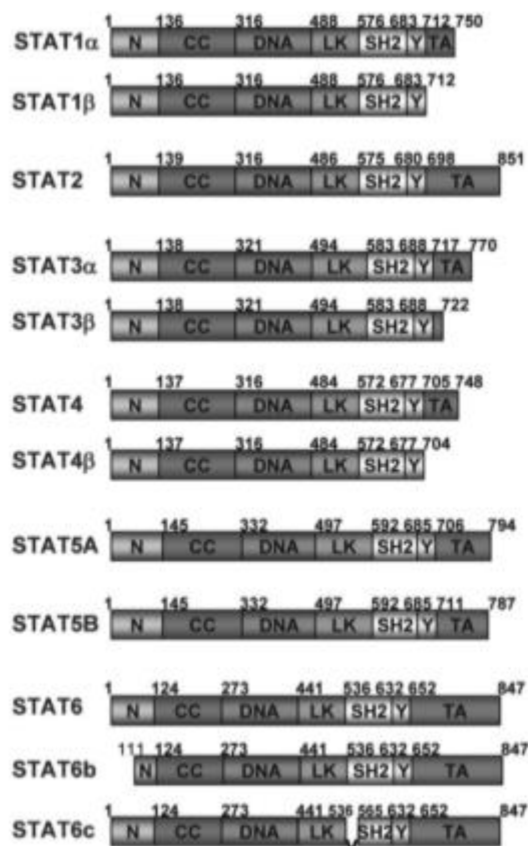

Figure 1.4 – The human STAT protein: functional domains and isoforms.
N = N terminal domain, CC = coiled coil domain, DNA = DNA binding domain, LK = linker domain, SH2 = SH2 domain, Y = phosphotyrosyl tail domain, TA = transactivation domain. (Lim, C. P. & Cao, X., 2006)

STATs can suffer alternative mRNA splicing and post-translational proteolytic processing leading to the formation of shorter isoforms (β, γ and δ). For example, STAT3 can be truncated in its carboxyl-terminal domain generating different isoforms resulting from alternative splicing (STAT3α and STAT3β). The α-isoform contains all naturally-occurring domains but the β-isoform is truncated in the TAD domain, missing the phosphorylation site at S727. Other members of the STAT family can also be subject to this process, such as STAT1, STAT4, STAT5a and STAT5b. This modification can affect S phosphorylation in the TAD domain, attenuating the transcriptional activity, but not the critical Y phosphorylation. This way different proteins may arise with different biological functions and transcriptional activity (Figure 1.4).[5,29,30]

1.4.1.STAT3

The STAT3 member was identified as an acute-phase response factor when activated by IL-6.[31] Actually, STAT3 can be activated by various polypeptide ligands such as IL-7, IL-10, IL-20, leptin, granulocyte colony-stimulating factor, EGF, LIF, CNTF and OSM.[30,32] Its dysregulation or constitutive activation is involved in many carcinomas and many tumors such as leukemias, lymphomas, gastric, brain, prostate, breast, lung, head and neck tumors.[32]

Contrarily to other members of the STAT family (STAT1, STAT2, STAT4, STAT5a, STAT5b and STAT6), STAT3 null mice were embryonically lethal, proving that STAT3 is required for early development.[28,33,34] STAT3 has also been found to play important roles in skin wound healing, liver regeneration and the reduction of mammary glands.[5]

This member of the STAT family has several regulatory residues, but the most important are those involved in phosphorylation, present in the carboxyl-terminal domain: the Y705 which is essential to its activation, and the S727, which is considered to increase STAT3 transcriptional activity, however this is not consensually believed.[31] Most likely, this regulatory activity by S727 is promoter or cell-dependent, much like STAT3 activity itself can have different roles in different cell types.[35]

As mentioned briefly, STAT3 is involved in NSC differentiation. NSCs, when exposed to IL-6, activate STAT3 and decide cell fate. Increasing evidence has been produced towards the understanding that the inhibition of STAT3 can prevent astrogliogenesis and promote neurogenesis. Despite many studies being conducted to elucidate the exact mechanisms by which NSC differentiation is decided, it is still not clear.[36] Some studies, point toward the down-regulation of certain genes such as *notch1* and *notch2* through the deletion of STAT3 in NSC, thus promoting neurogenesis and suppressing astrogliogenesis.[37]

STAT3 plays a protective role in the heart. Heart myocyte-specific STAT3-knockout rendered mice significantly more susceptible to cardiac injury. Although the mechanism by which STAT3 protects the heart is not clear, authors considered its anti-inflammatory effects as

a plausible mechanism.[24]

STAT3 also seems to play an essential role in T-cells, macrophages, neutrophils, dendritic cells as well as bone marrow, as it has been described in the last years through the specific knockout of STAT3 in the immune system of mice. Its absence in macrophages and neutrophils produces mice phenotypically more sensitive to lipoproteins and endotoxins and may increase the susceptibility to chronic inflammatory diseases. The lack of STAT3 in mice dendritic cells and bone marrow tissue leads to an increased chance of development of inflammatory syndromes such as inflammatory bowel disease and Crohn's disease-like pathologies.[38]

In tumors, as well as immune cells, STAT3 also comes to play in the worsening of the disease because of its involvement in the appearance of resistant strains which also have the ability to evade immune cell activity and tumor suppression genes. All this contributes to the problems found in treating STAT3-associated cancers. STAT3 confers a malignant nature to the tumors, promoting phenomena such as the common uncontrolled growth of tumoral cells but also migration to other tissues and the development of metastasis.[8] In healthy brains, STAT3 is not activated, but in human brain tumors phosphorylated STAT3 is overexpressed.[10] Furthermore, STAT3 plays an important role in connective tissue cells, which are involved in the progression of the tumor, making it a promising target in cancer therapy.[13] Moreover, many studies demonstrate that STAT3 can be controlled by means of some drugs, like kinase inhibitors, which prevent STAT3 dimerization, inhibit its translocation to the nucleus and inhibits downstream events without inhibiting upstream targets.[32]

1.4.2. Regulation

The JAK/STAT3 pathway can be regulated at many levels: interfering with the receptor, the JAKs and/or STAT3, or through the activity of different effector or negative-regulating molecules.

Positively, it can be regulated by signal-transducing adaptor molecules (STAM) which enhance the transcriptional efficiency through as yet unknown mechanisms.[7] STAT-interacting proteins (StIP) can also positively regulate STAT3 by acting as scaffolds for the phosphorylation of STAT3 by JAKs.[7]

Bone morphogenetic proteins (BMPs) and LIF can work synergistically to ultimately increase STAT3 transcriptional activity. When both cytokines bind to their corresponding membrane receptors, STAT3 and a heteromeric complex composed by Smad proteins are activated and translocated to the nucleus. STAT3 and the Smad complex work together through the physical interaction with another family of transcriptional coactivators: the CREB-binding protein (CBP)/p300 family. These interactions form a transcriptional complex which promotes the expression of specific genes, such as those related to astrocyte differentiation.[39] The Notch pathway also increases STAT3 phosphorylation and activation. Notch effector proteins, the Hes

proteins, work as scaffolds for the facilitation of the association between JAK2 and STAT3, thus promoting the latter's activity and promotion of astrocyte differentiation.[40] Furthermore, RA and its RA receptor α (RARα) have also been shown to be part of the canonical pathway of astrogliogenesis activation. This is possible through the binding of the RARα to the STAT3-Smad-CBP/p300 complex and the further potentiation of astrocyte differentiation.[41]

Negatively, it can be regulated by protein inhibitors of activated STATs (PIAS), tyrosine phosphatases and supressors of cytokine signaling proteins (SOCS).[20] PIAS3 is a member of the PIAS family which specifically interacts with STAT3. This protein directly interferes with the ability of STAT3 to bind DNA by binding itself to the DNA, attenuating transcriptional activity.[2,5,20] There are some tyrosine phosphatases which are characteristic for having an SH2 domain, which are able to recognize other SH2 domains and dephosphorylate them, leading to their inhibition. The SH2 domain-containing phosphatase (SHP)-1 and SHP-2 are two examples which can interact directly with the receptors, JAKs and STAT3.[2,42] The SOCS family consists of eight members, including the cytokine-inducible SH2 containing protein (CIS), all of them acting upstream of STAT3 activation. They can operate on three different levels: 1) by interacting directly with JAKs, blocking their activity; 2) by coming between the phosphorylated receptor and JAKs; and 3) by competing with STAT3 for the docking site of the phosphorylated receptors (Figure 1.5).[5]

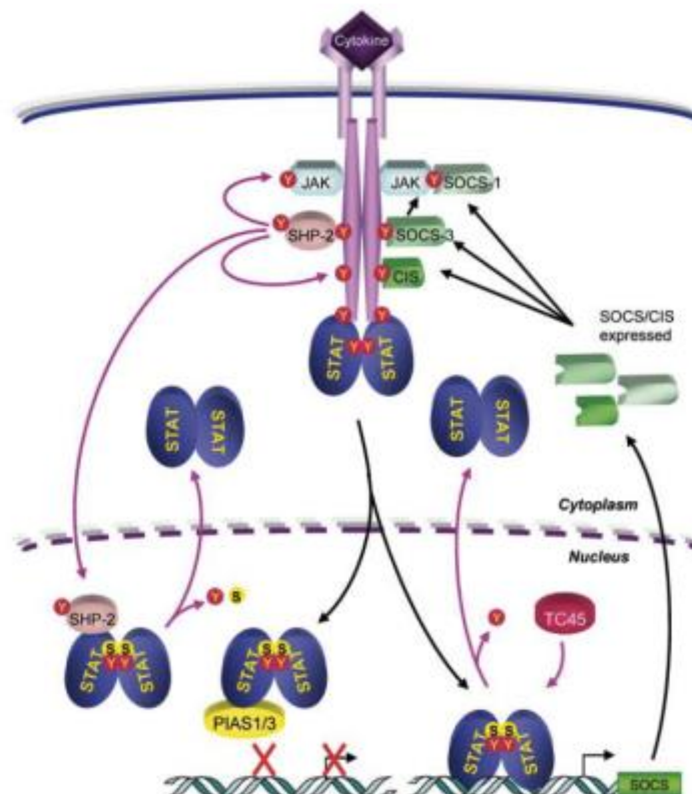

Figure 1.5 – Negative regulation of STATs by PIAS, SOCS and SHP.
(Lim, C. P. & Cao, X., 2006)

1.5. Post-translational modifications

Proteins can be regulated by post-translational modifications (PTM) of the amino acid sequence, including phosphorylation/dephosphorylation (on tyrosines, serines and threonines), methylation/demethylation (on lysines), acetylation/deacetylation (on lysines), isomerization, ubiquitination (on lysines), proteolytic cleavage and others. Some of them are described to play a role in the activity of STATs in general and STAT3 in particular.[2,5] In this project we are especially interested in STAT3 phosphorylation, methylation and acetylation, because these are the most important modifications related to STAT3 activation and regulation.

1.5.1. Phosphorylation, methylation and acetylation of STAT3

The phosphorylation of STAT3 on Y705 is the most well documented PTM. Mohr *et al.* (2013) replaced Y705 by a phenylalanine (F) (STAT3 Y705F) on Human Embryonic Kidney (HEK) cells. This mutant cannot be phosphorylated in this residue and has a dominant-negative activity. When cells were stimulated with IL-6, STAT3 Y705F did not translocate to the nucleus.[43] On the same note, earlier, Kaptein *et al.* (1995) also demonstrated on liver hepatocellular carcinoma (HepG2) cells that STAT3 Y705F mutants were not able to activate and showed low levels of reporter gene expression following stimulation by IL-6.[44]

The role of S727 phosphorylation remains controversial. It has been demonstrated to be necessary for STAT3 transcriptional activity, but also that it negatively regulates STAT3 activity. Wen *et al.* (1995) described that S727 phosphorylation is essential for maximal transcriptional activity in human sarcoma U3A cells. This was demonstrated by mutating this residue and replacing it with an alanine (A) (STAT3 S727A). This mutant showed impaired transcriptional activity in luciferase assays, although it seems this residue was not necessary for binding DNA and it enhances STAT3 activity only once it is bound to DNA.[45,46] Interestingly, Hazan-Halevy *et al.* (2010) have described that chronic lymphocytic leukemia B-cells constitutively phosphorylate S727 and this alone is enough to allow DNA binding, without need for Y705 phosphorylation.[47] On the other hand, S727 also seems to be responsible for the regulation of the duration of STAT3 activity. In HepG2-STAT3-knockdown cells later reconstituted with STAT3 mutants, STAT3 activity is prolonged when S727 was mutated to A and therefore could not be phosphorylated. This process involves the nuclear phosphatase TC45, which dephosphorylates Y705.[48]

Interestingly, it seems that even unphosphorylated STAT3 has importance in regulation of gene expression. While phosphorylated STAT3, which is constitutively activated in many cancers, activates proliferation, angiogenesis, invasion and suppression of apoptosis, unphosphorylated STAT3 has been related with DNA architectural roles and maintenance of heterochromatin stability. From different recent studies, monomers and dimers of

unphosphorylated STAT3 have been found bound to AT-rich DNA sequence sites within negatively supercoiled plasmid DNA, suggesting that unphosphorylated STAT3 may function as a transcriptional activator and a chromatin/genomic organizer.[49,50]

STAT3 acetylation can occur on the K685 residue and the dimethylation on the K140 residue.[51–53] The K49 residue can be either acetylated and dimethylated.[54] K685 acetylation is essential for the dimerization of STAT3 upon stimulation by OSM.[55] However, data were not conclusive and were later disputed by Dasgupta *et al.* (2014), who specified that this acetylation is only critical for the transcriptional activity of unphosphorylated, but not for Y705-phosphorylated STAT3.[52,55] This PTM is mediated by histone acetyltransferase CBP/p300.[55] The dimethylation of the K140 residue by H3K4 methyl transferase SET9 occurs in the nucleus when STAT3 is bound to the promoter of target genes, and it plays mainly an inhibitory role in response to IL-6 stimulation.[53] Ray *et al.* (2005) demonstrated that acetylation of K49 is required for IL-6-dependent STAT3 activation, a process mediated by CBP/p300.[51] Later in 2015, Dasgupta *et al.* added that this residue can also be dimethylated by the histone methyl transferase EZH2, as a response to IL-6-dependent STAT3 activation. This dimethylation is crucial for the expression of most STAT3-regulated genes, at least when STAT3 is activated by IL-6.[54]

2. Aims

Our main goal in this thesis is to study the role of PTM on STAT3 phosphorylation and dimerization. To address this question, we have two specific aims:

1- Elucidate the role of specific residues susceptible of PTMs (i.e. K49, K140, K685, Y705 and S727) on STAT3 phosphorylation, dimerization and intracellular localization; and

2- Screen a library of 82 kinase inhibitors to identify the intracellular pathways involved in STAT3 dimerization and activation.

3. Methods

3.1. Material and reagents

HEK293 cells were purchased from ATCC (Barcelona, Spain). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Lonza (Basel, Switzerland), fetal bovine serum (FBS) from Biowest (Nuaillé, France) and Penicillin/streptomycin (P/S) solution from Thermo Fisher Scientific (MA, USA).

The Venus-STAT3 bimolecular fluorescence complementation (BiFC) assay was designed by Ana Maia Rocha (MSc) and optimized by Catarina Almeida (MSc) at our laboratory (Cell Structure and Dynamics, ITQB-NOVA, Oeiras, Portugal) (Figure 3.1). This system includes two constructs carrying STAT3 fused to two complementary fragments of the Venus fluorescent reporter, Venus1(V1)-STAT3 and Venus 2 (V2)-STAT3, which were synthesized by Invitrogen (MA, USA) (Figure 3.2). The Venus halves were fused to the N-terminal region of STAT3 to guarantee that possible post-translational cleavage of the protein would not interfere with the technique. Fluorescence is due to the reconstitution of the fluorophore and is therefore proportional to the amount of dimers. It can be analysed quantitative and qualitatively by flow cytometry and fluorescence microscopy.[56]

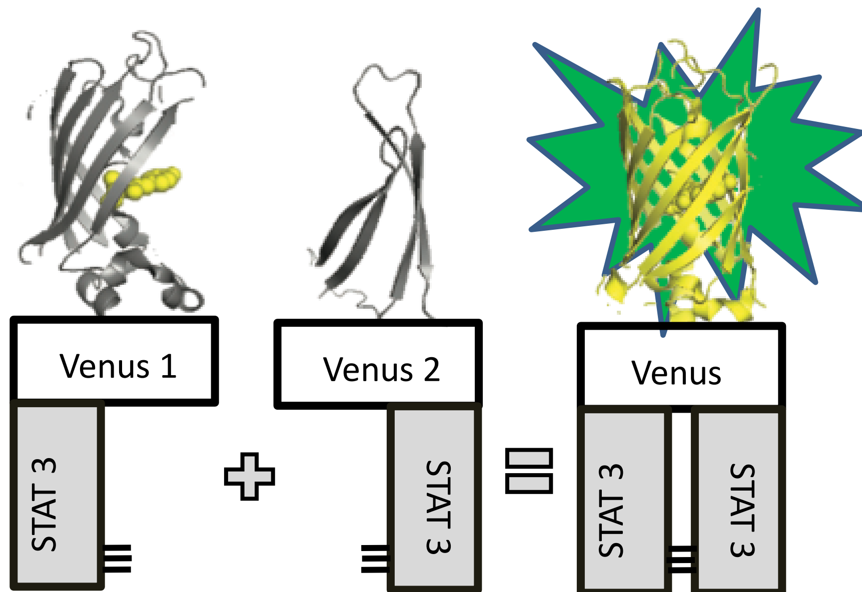

Figure 3.1 – A BiFC celular model for the visualization of STAT3 dimers in living cells.

Lipofectamine 2000 was acquired from Invitrogen (MA, USA), LIF from R&D systems (Minneapolis, USA) and the library of kinase inhibitors from Selleckchem (Houston, USA).

Trypsin was purchased from GE Healthcare Life Sciences (Buckinghasmshire, United Kingdom) and phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} from Lonza (Basel, Switzerland).

The NE-PER™ Nuclear and Cytoplasm Extraction kit was obtained from Thermo Fisher Scientific (MA, USA) and Bradford from Applichem Panreac (Darmstadt, Germany). The anti-STAT3 rabbit monoclonal antibody, the anti-Phospho-STAT3 (Y705) rabbit polyclonal antibody and the anti-Phospho-STAT3 (S727) rabbit polyclonal antibody were acquired from Cell Signalling (MA, USA). The anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody was purchased from Ambion (CA, USA) and the Lamin-B goat polyclonal antibody from Santa Cruz Biotechnology (Dallas, USA). Secondary antibodies ECL™ sheep anti-mouse IgG HRP and donkey anti-rabbit IgG HRP were obtained from GE Healthcare Life Sciences (Buckinghasmshire, United Kingdom) and the donkey anti-goat IgG HRP from Santa Cruz Biotechnology (Dallas, USA). The chemiluminescent HRP substrate was purchased from Millipore (Billerica, USA).

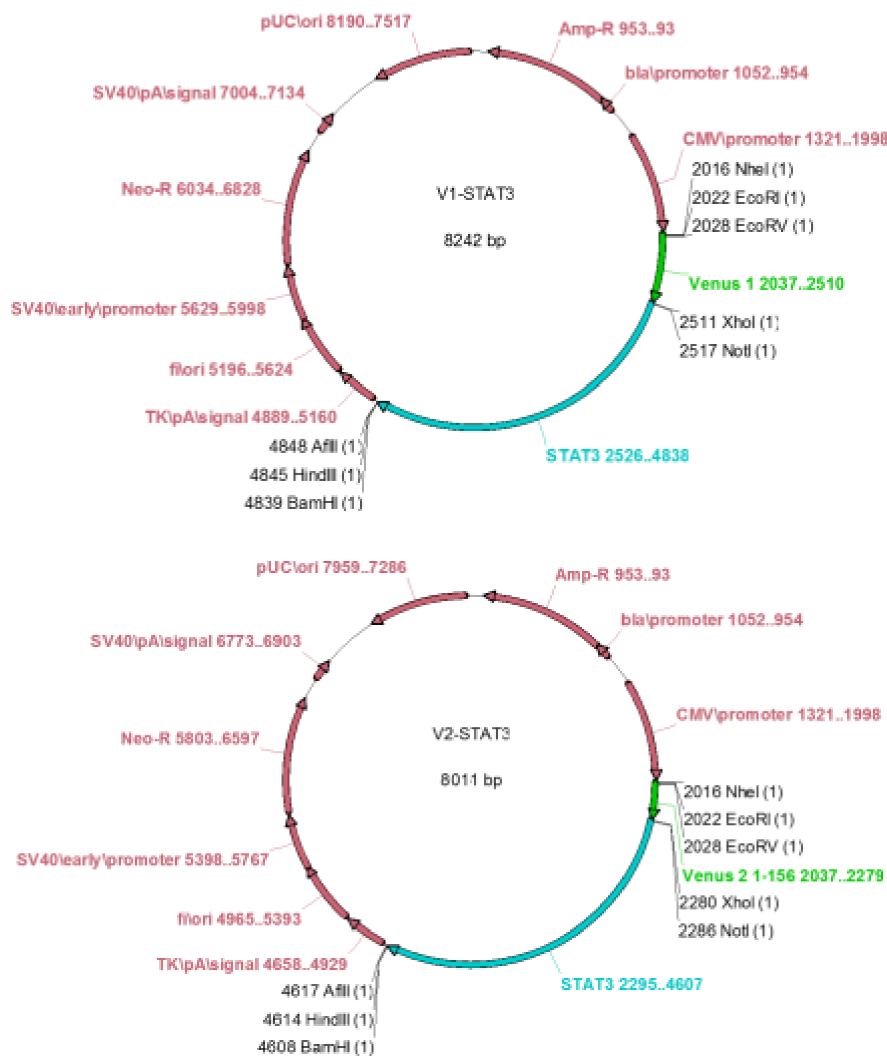

Figure 3.2 – Plasmid maps of the Venus-STAT3 BiFC constructs.
Top image: Venus1-STAT3 and lower image: Venus2-STAT3.

3.2. Generation of STAT3 mutants

The original Venus-STAT3 BiFC system carrying wild-type (WT) STAT3 was used as a template for Polymerase chain reaction (PCR)-based site-directed mutagenesis. We generated five different STAT3 mutant constructs fused to Venus1 (amino acids 1-158) and another five fused to Venus2 (amino acids 159-238). Figure 3.3 outlines the procedure used to perform the mutants.

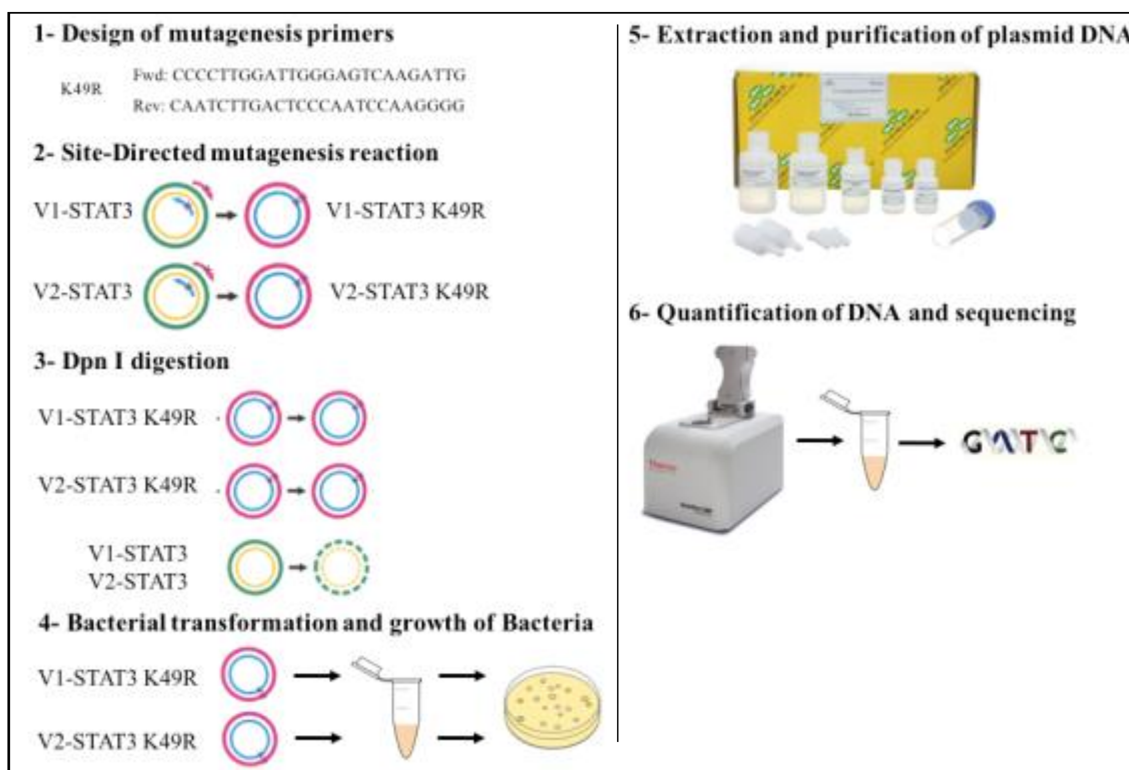

Figure 3.3 – Schematic representation for the generation of the STAT3 K49R mutant construct. Different primers were designed and then all of the Venus-STAT3 BiFC mutants (K140R, K685R, Y705F and S727A) were made using the same protocol described in the schematic.

Five pairs of mutagenesis primers were designed by means of PrimerX free software (http://www.bioinformatics.org/primerx/) (Table 3.1). The original lysine (K) residues on positions 49, 140 and 685 were replaced by arginine (R) residues, the tyrosine (Y) residue on position 705 by phenylalanine (F) and the serine (S) residue on position 727 by alanine (A). These mutations prevent ubiquitinylation/acetylation/methylation/SUMOylation or phosphorylation of the corresponding residues but preserve the basic structural function of the original amino acids. PCR was carried out using PfuTurbo DNA polymerase from Stratagene (CA, USA) (1.25U), using venus-STAT3 BiFC constructs as templates (10 ng) and the corresponding mutagenesis primers (125 ng) (Table 3.1). PCR conditions were 30 sec at 95°C, 16x [30 sec at 95°C, 1 min at 55°C, 8 min at 68°C] and finally 10 min at 68°C.

Table 3.1 – Primers for site-directed mutagenesis.

Mutation	Primers
K49R	Fwd: CCCCTTGGATTGGGAGTCAAGATTG Rev: CAATCTTGACTCCCAATCCAAGGGG
K140R	Fwd: GGTGACGGAGACACAGCAGATGCTG Rev: CAGCATCTGCTGTCTCTCCGTCACC
K685R	Fwd: GAGGCATTCGGAAGGTATTGTCGGCC Rev: GGCCGACAATACCTTCCGAATGCCTC
Y705F	Fwd: CAGGTAGCGCTGCCCCATTCCTGAAGACCAAGTTTATC Rev: GATAAACTTGGTCTTCAGGAATGGGGCAGCGCTACCTG
S727A	Fwd: CATTGACCTGCCGATGGCACCCCGCACTTTAGATTC Rev: GAATCTAAAGTGCGGGGTGCCATCGGCAGGTCAATG

Mutagenesis reactions were incubated with DpnI from Promega (WI, USA) for 2 h at 37°C. DpnI is an endonuclease which cleaves only methylated DNA. The plasmid templates have been amplified in bacteria, where they were methylated, but the in vitro PCR generates a new double-stranded DNA molecule without methylation. Therefore, DpnI only digests the template DNA, in principle leaving exclusively the new copies with the desired mutations. Reactions were transformed into thermocompetent *Escherichia coli* from NZYtech (Lisbon, Portugal). Competent bacteria were mixed with each construct and incubated for 30 min in ice. Then bacteria were heat-shocked for 45 seconds at 42ºC and incubated for 2 min in ice. Three-hundred µL of Luria Broth (LB) medium (1% w/v Tryptone; 0.5% w/v Yeast extract; 171mM NaCl) without antibiotics were added to the bacteria suspension and incubated in agitation at 120 rpm, 37ºC for 1h. Transformed bacteria were seeded on LB agar 1x (1% w/v Tryptone; 0.5% w/v Yeast extract; 171 mM NaCl; 1.5% w/v Agar) petri dishes containing 100 µg/mL of ampicillin and incubated overnight at 37ºC. In order to obtain enough plasmid DNA for extraction, purification and sequencing, each colony was grown in 3 mL of liquid LB medium containing 100 µg/mL of ampicillin at 37ºC, in agitation at 180 rpm overnight. DNA was then extracted and purified by means of the ZymoPURE™ Mini Prep Kit from Zymo Research (CA, USA). Glycerol stocks were also done by adding 500 µL of glycerol from AppliChem Panreac (Darmstadt, Germany) on 500 µL of the bacteria suspension in LB liquid medium, and the stocks were stored at -80ºC. DNA was quantified by means of a Nanodrop 2000c from Thermo Fisher Scientific Inc. (Waltham, United States), and ultrapure water was used as blank. The

DNA was then sent for sequencing to GATC (GATC Biotech AG, Germany) to confirm the mutations.

3.3. Cell cultures

HEK293 cells were grown in DMEM medium supplemented with 10% FBS, 2 mM glutamine and 1x P/S mixture, under controlled conditions of temperature (37ºC) and CO_2 (5%). Medium was changed every other day and cells were passed once a week by trypsinization (Trypsin 0.25% w/v) for 5 min at 37ºC. For all the experiments, cells were counted using a Neubauer Chamber from Assistent (Sondheim, Germany) and seeded in different types of dishes according to the type of assay. Transfections were carried out by means of Lipofectamine 2000 in a 1:3 proportion (1 µg of DNA: 3 µL of Lipofectamine), 24 h after seeding. After transfection, STAT3 dimerization and cell death were evaluated by flow cytometry, STAT3 expression and phosphorylation by Western blot and intracellular STAT3 localization by fluorescence microscopy (Figure 3.4).

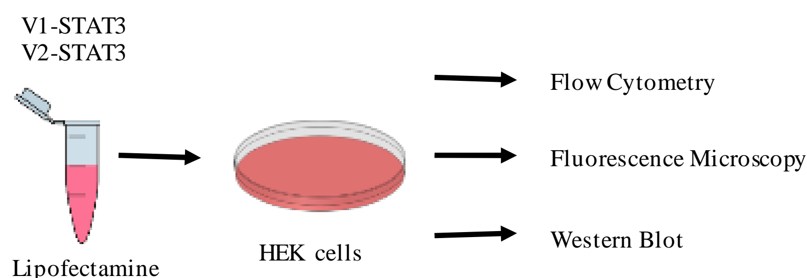

Figure 3.4 - Schematic representation of cell transfection and different types of analyses. The transfection mixture was prepared in a microcentrifuge tube and then added to HEK cells. Cells were seeded twenty-four hours earlier in different plates depending on the type of assay. The results were then analyzed by flow cytometry, fluorescence microscopy and Western blot.

3.4. Flow cytometry

Fluorescence intensity of transfected cells was determined by flow cytometry. For this assay, $8x10^5$ cells per well were seeded in 6-well plates from TPP (Trasadingen, Switzerland). Cells were washed once with PBS and trypsinized (0.05% w/v) at 37ºC for 5 min. Trypsin was neutralized with complete DMEM medium and cells were collected into sterile microcentrifuge tubes. Cells were centrifuged at 300x*g* for 5 min (VWR micro star 17R centrifuge), the supernatant was discarded and the pellet was resuspended in 500 µL of PBS. Immediately before acquisition, propidium iodide (PI) (1 mg/mL) was added to cells to quantify cell death in our samples, since this staining can cross the membrane of dead cells only.

Ten thousand cells per experimental group were analysed by means of a Cyan ADP flow cytometer from Beckman Coulter (Brea, CA, USA) equipped with a 25mW solid state blue

(488nm) laser, a 50mW solid state violet (405nm) laser, and a 60mW red (643nm) diode. For data analysis and representation, the FlowJo software (Tree Star Inc., Ashland, OR, USA) was used.

3.5. Protein extraction

For Western Blot, 2 x 10^6 cells were seeded on 60 mm dishes from Thermo Scientific (MA, USA) and two different protocols were used for protein extraction, depending on the type of sample under analysis.

For total protein extraction, cells were washed once with PBS, lysed with NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-Cl pH 8.0, 1% NP-40) containing cocktail tablets of protease and phosphatase inhibitors from Roche (Basel, Switzerland). Cells were scrapped directly from the plates into microcentrifuge tubes and incubated 10 min in ice. A W-450 D sonicator (Emerson, Danbury, USA) was used for 5 sec at 10% of amplitude. Sonication was essential to disrupt cell membranes and release intracellular proteins, permitting their isolation and detection by Western blot. Cells were then centrifuged at 10000x*g* for 10 min at 4ºC and the supernatant containing the proteins was collected into a new microcentrifuge tube. Samples were always kept on ice to avoid protein degradation during extraction protocols.

For nuclear and cytoplasmic protein extraction, the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit from Thermo Scientific (MA, USA) was used. Cells were washed once with PBS and trypsinized (0.25% w/v) at 37ºC for 5 min. Trypsin was neutralized with complete DMEM medium and cells were collected into a microcentrifuge tube. Cells were then centrifuged at 300x*g* for 5 min at room temperature, the supernatant was discarded and the pellet was resuspended in cold cytoplasmic extraction reagent I with protease inhibitors. The suspension was homogenized by vortex (15 seconds) and incubated in ice for 10 min. Cold cytoplasmic extraction reagent II was then added to the mixture, homogenized by vortex and incubated for 1 minute in ice. The mixture was once again vortexed and centrifuged at 16000x*g* for 5 min. The supernatant, corresponding to the cytoplasmic protein extract, was collected into a new sterile microcentrifuge tube and stored at –20°C. The pellet was resuspended in nuclear extraction reagent with protease inhibitors and homogenized by vortex 4 times every 10 min. Then the sample was centrifuged at 16000x*g* for 10 min and the supernatant, corresponding to the nuclear protein fraction, was collected into a new sterile microcentrifuge tube and stored at -80°C.

Protein concentration was quantified on a microplate reader from Thermo Scientific (MA, USA) by means of the Bradford method. A standard curve with known concentrations of bovine serum albumin (BSA, 0.125 to 2 µg/µL) was used to determine protein concentration. Samples were incubated with 200 µL of Bradford solution for 5 min and read at 595 nm.

3.6. Western blot

The same amount of protein (15-20 µg) from each sample was mixed with 4x denaturing loading buffer (0.125 mM Tris pH 6.8; 4% sodium dodecyl sulphate (SDS), 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue) and boiled for 5 min at 100ºC. Protein samples were loaded and run on 10% w/v SDS-polyacrylamide gel electrophoresis made with Protogel reagents from National Diagnostics (Atlanta, United States). Samples were run in running buffer (25 mM Tris-Base; 3.5 mM SDS; 0.2 M Glycine) at 120V for 1 h and transferred to a nitrocellulose membrane from GE Life Technologies (Boston, USA) at 100V for 1 h in transfer buffer (25 mM Tris-Base; 0.2 M Glycine; 20% methanol).

After electrophoretic transfer, membranes were stained with Ponceau S (0.1% w/v) from Amresco (Solon, United States) to check if protein transfer was efficient. Ponceau solution was removed from the membranes by washing them with Tris-buffered saline Tween 20 (TBS-T) (150 mM NaCl, 50 mM Tris pH 7.4, 0.5% Tween 20). Membranes were then blocked for 1 h in 5% (w/v) non-fat dry milk in TBS at room temperature, washed with TBS-T (10 min, 3 times) and incubated overnight with primary antibodies diluted in 5% BSA in TBS 1x and 0.05% of sodium azide. The primary antibodies used were: anti-phospho-STAT3 Y705 (1:1000, mouse monoclonal), anti-phospho-STAT3 S727 (1:1000, rabbit monoclonal), anti-STAT3 (1:1000, rabbit monoclonal), anti-GAPDH (1:1000 mouse monoclonal) and anti-Lamin-B (1:1000, goat monoclonal). After incubation with primary antibodies, membranes were washed with TBS-T (10 min, 3 times) and incubated for 2 h at 4°C with the appropriate secondary antibody (1:1000 in 5% of non-fat dry milk in PBS), washed with TBS-T (10 min, 3 times) and incubated 1 min with chemiluminescent HRP substrate before imaging in a Chemidoc XRS device from Biorad (CA, USA). GAPDH was used as loading control for cytoplasmic protein extracts and total proteins, and Lamin-B for nuclear protein extracts.

3.7. Fluorescence microscopy

All images of fluorescent living cells were acquired using a Leica DMI6000 fluorescence microscope equipped with a Hamamatsu Flash 4.0 LT sCMOS camera and DAPI + GFP fluorescence filter sets, and controlled by means of the Las X software (MN, USA). For microscopy, $8x10^5$ cells per well were seeded in 35 mm dishes from Ibidi (Planegg, Germany), coated with poly-L-lysine (20µg/mL) for 1 h at room temperature, for a better adherence of cells. Nuclei were stained with Hoechst 33342 (10µg/mL) from Thermo Fisher Scientific (MA, USA) for 1 min at room temperature. Cells were washed once with PBS and 1 mL of PBS was added to the cells. Pictures of 25-30 cells per experimental group were taken using the 100x objective and then analyzed by means of Fiji free online software (http://fiji.sc/).

4. Results

4.1. JAK/STAT3 pathway is activated by LIF in HEK cells

To verify that LIF activates the JAK/STAT3 pathway in HEK cells, these cells were seeded and 19 h later the medium was changed to medium without FBS to allow the activation by LIF. Two hours later, HEK cells were treated with LIF (100 ng/mL) and 2 h later nuclear and cytoplasmic proteins were extracted.

Nuclear proteins were analysed by Western blot to observe endogenous STAT3 phosphorylation. The membrane was incubated with anti-STAT3 phosphorylated on Y705 (pSTAT3 Y705), anti-STAT3 and anti-Lamin-B antibodies (Figure 4.1). Lamin-B levels were used as loading control to make sure that we use the same amount of protein in all of the samples. On the figure below we have cells non-stimulated and stimulated with LIF and we observe that the band of pSTAT3 Y705 only appears when they were stimulated with LIF. The levels of total STAT3 are the same in non-stimulated and stimulated cell.

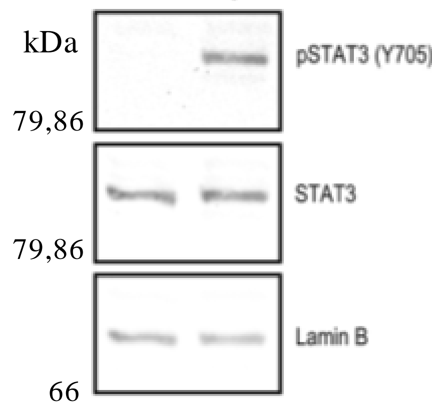

Figure 4.1 – JAK/STAT3 patway is activated by LIF in HEK cells.
HEK cells were incubated with LIF (100 ng/mL) or the vehicle. Nuclear protein extracts were analysed by Western blot using specific antibodies (pSTAT3 Y705, STAT3 and Lamin-B).

4.2. The Venus-STAT3 BiFC system

Along this project, we are using a BiFC system to study STAT3 dimerization and the effect of its PTM (phosphorylation, acetylation and methylation) in living cells. The BiFC system is composed by two plasmids (V1-STAT3 and V2-STAT3), which are fused to the two halves of the Venus fluorescent protein. When STAT3 dimerizes, a complementation of the two halves of Venus occurs, leading to fluorescence (Figure 4.2A). Protein extracts from HEK cells transfected with the Venus-STAT3 system show three distinct bands upon exposure to STAT3 antibodies. These correspond to V1-STAT3, V2-STAT3 and endogenous STAT3, from the

highest to the lowest molecular weight (Figure 4.2B). In this system, STAT3 effectively dimerizes and produces fluorescence, which is predominantly located in the cytoplasm of HEK cells, as demonstrated by microscopy and flow cytometry analyses (Fig. 4.2C and 4.2D).

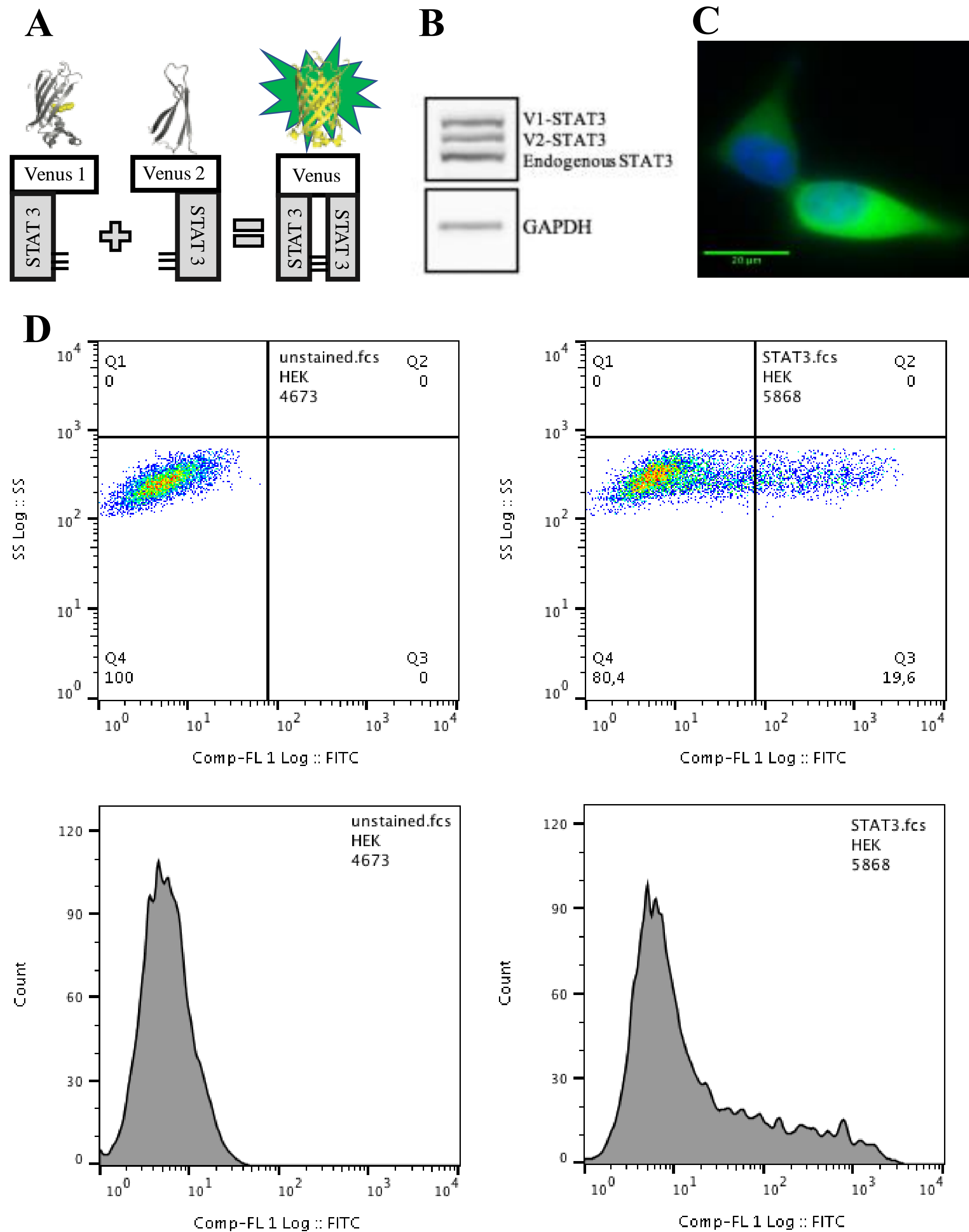

Figure 4.2 – **The Venus-STAT3 BiFC system in living HEK cells.**
A) Representation of the Venus-STAT3 BiFC system. HEK cells were transfected with both Venus-STAT3 BiFC plasmids (V1-STAT3 and V2-STAT3) and were analyzed 19 h later. B) total protein extracts were analysed by Western blot, and show 3 bands corresponding to V1-STAT3, V2-STAT3 and STAT3 endogenous from top to bottom. C) cells were observed by fluorescence microscopy. Venus-STAT3 is predominantly localized on the cytoplasm (green) and nuclei were stained with Hoechst 33342 (blue). Scale bar: 20 µm. D)10000 cells were analysed by flow cytometry, showing a clear signal in the green spectrum (FL-1) in cells transfected with the Venus-STAT3 BiFC constructs.

As described in section 3.2 (Materials and Methods), using our BiFC constructs as templates, we made 5 different point mutations on STAT3 residues involved in acetylation, methylation and phosphorylation of the protein. Figure 4.3 represents the STAT3 structure with the 5 residues of interest (K49, K140, K685, Y705, S727).

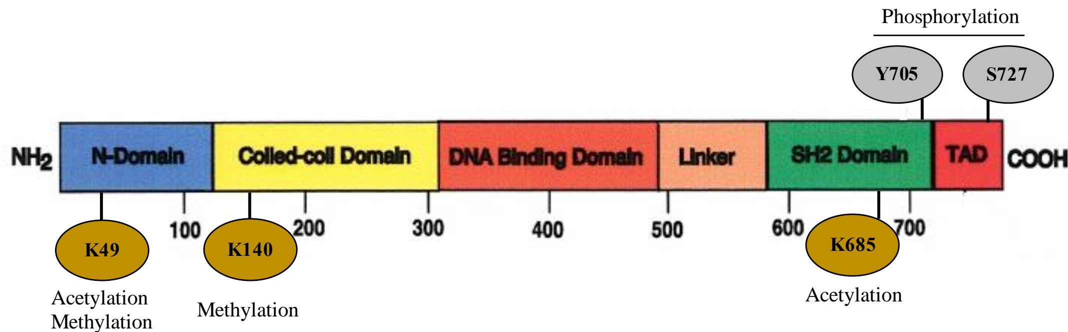

Figure 4.3 – Structure of STAT3 with our target phosphorylation, acetylation and methylation sites.
Several regulation residues in STAT3 were targeted with point mutations to study their purpose in the dimerization and therefore in PPI. (Hendry, L. *et al.*, 2004)

In order to confirm the effects of the different mutations on LIF-stimulated STAT3 phosphorylation on Y705 and S727 residues, nuclear protein extracts were analysed by Western blot by using specific antibodies to phosphorylated STAT3 on Y705 and S727 (Figure 4.4). The reader must be mindful, however, that the results shown herein are not optimal since this Western blot was only done once.

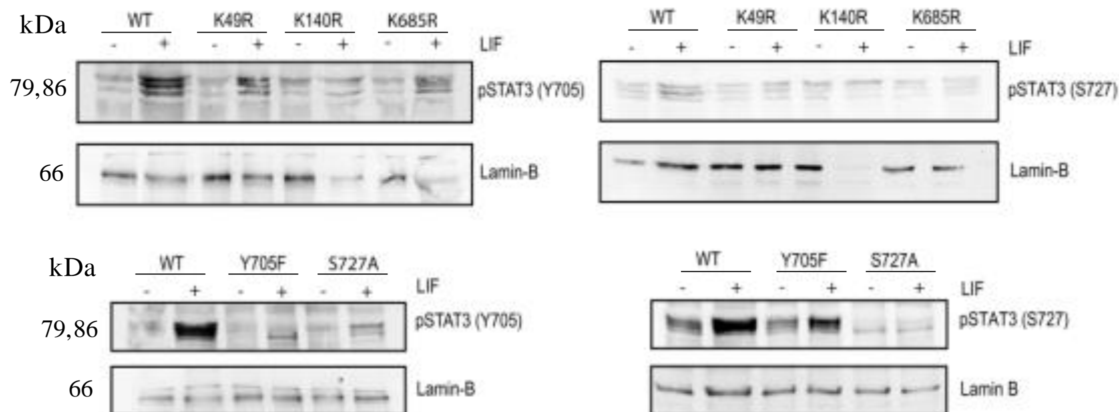

Figure 4.4 – Besides the phosphorylation residues, others are also important for the correct phosphorylation and activation of STAT3.
HEK cells were transfected with mutated or non-mutated Venus-STAT3 BiFC constructs (V1-STAT3 and V2-STAT3). After 19 h medium was changed by medium without FBS and 2 h later cells were stimulated with LIF (100 ng/mL) for 2 h. Nuclear and cytoplasmic protein were extracted and analysed by Western blot. Anti-pSTAT3 Y705, anti-pSTAT3 S727 were used to detect STAT3 phosphorylation. Lamin-B levels were used as loading control.

What is visible in Figure 4.4 is that, firstly, LIF leads to an increase in p-STAT3 levels at both Y705 and S727, confirming once more what was described in section 4.1 (Results).

Regarding the effects of mutation K49R on the phosphorylation of these two residues, it seems that it does not alter the phosphorylation of Y705, but, on the contrary, seems to slightly reduce the phosphorylation of S727. The increase induced by LIF is so slight it is barely noticeable. This suggests that K49 regulates the phosphorylation of S727, but not Y705.

As for the mutation K140R it appears that not only it affects the phosphorylation of both Y705 and S727 but also seems to interfere with the expression of Lamin-B when cells were stimulated with LIF. In both Western blots, loading controls appeared at considerable lower levels comparing to the control. The combination of the mutation and the activation by LIF should be tested further to assess if K140R does indeed interfere with the phosphorylation, once the loading control problem is solved.

The mutation K685R seems to reduce slightly the phosphorylation of Y705 but significantly reduces S727 phosphorylation. However, it must be taken into account, that the loading controls in the p-STAT3 Y705 gel for the K685R mutation with and without LIF are not very clear, so further studies must be made to confirm these results. Moreover, on the p-STAT3 S727 membrane, the control itself did not show a significant increase in its phosphorylation levels, when cells were stimulated with LIF. This too, adds to the need for more studies and the confirmation of these preliminary results.

Concerning the Y705F mutation, as expected, we see no phosphorylation of this residue. This is evident since tyrosine was replaced by a phenylalanine, a residue which cannot be phosphorylated.[43] However, it is still visible that there is phosphorylation of endogenous STAT3 on Y705, corresponding to the third band on the LIF-stimulated lane. This mutation, on the other hand, did not seem to interfere with the S727 phosphorylation.

Finally, regarding the S727A mutation, it seems it is necessary for the correct phosphorylation of Y705. The difference between non-stimulated and stimulated cells seems to be minimal when comparing to the control (WT). As for the phosphorylation levels of S727 itself, once again as expected, they did not increase when serine was replaced by alanine. Interestingly, endogenous STAT3 (lowest band) shows phosphorylation of S727 with and without LIF stimulation.

4.3. STAT3 mutants do not affect STAT3 dimerization

To determine the relative role of the different residues on STAT3 dimerization, HEK cells were transfected with different combinations of mutated and non-mutated Venus-STAT3 BiFC constructs (Table 4.1). Twenty four hours later the samples were analysed by flow cytometry (Figure 4.5) and fluorescence microscopy (Figure 4.6).

Table 4.1 – Combination of the Venus-STAT3 BiFC constructs.

		V1					
		K49R	K140R	K685R	Y705F	S727A	WT
V2	K49R	1					
	K140R	2	7				
	K685R	3	8	12			
	Y705F	4	9	13	16		
	S727A	5	10	14	17	19	
	WT	6	11	15	18	20	21

The results from flow cytometry show that no combination of the Venus-STAT3 constructs alters significantly the percentage of fluorescence, suggesting that the tested residues do not affect STAT3 dimerization (Figure 4.5).

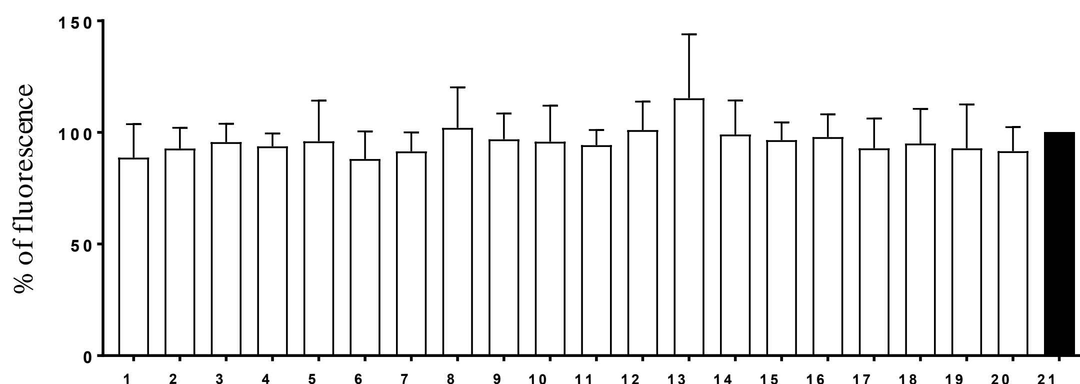

Figure 4.5 - STAT3 mutants have no effect on fluorescence levels in HEK cells.
HEK cells were treated with the mutants of the Venus-STAT3 BiFC plasmids conjugated with each other. Ten thousand events were analysed by flow cytometry. Percentage of fluorescence of the STAT3 mutants were then compared with the STAT3.

Figure 4.6 shows that STAT3 dimers are predominantly localized on the cytoplasm in all the combinations of STAT3 mutants. However, 4 out of 6 combinations containing the K49R construct (K49R/K49R, K140R/K49R, S727A/K49R, WT/K49R) and also 4 out of 6 combinations with the S727A construct (S727A/K49R, S727A/K140R, S727A/K685R and WT/S727A) show some aggregation. K685R/K140R also showed some aggregates. Given the high incidence of aggregates when K49R and the S727A mutants are present, it is possible that these residues could be relevant for either STAT3 degradation or stability.

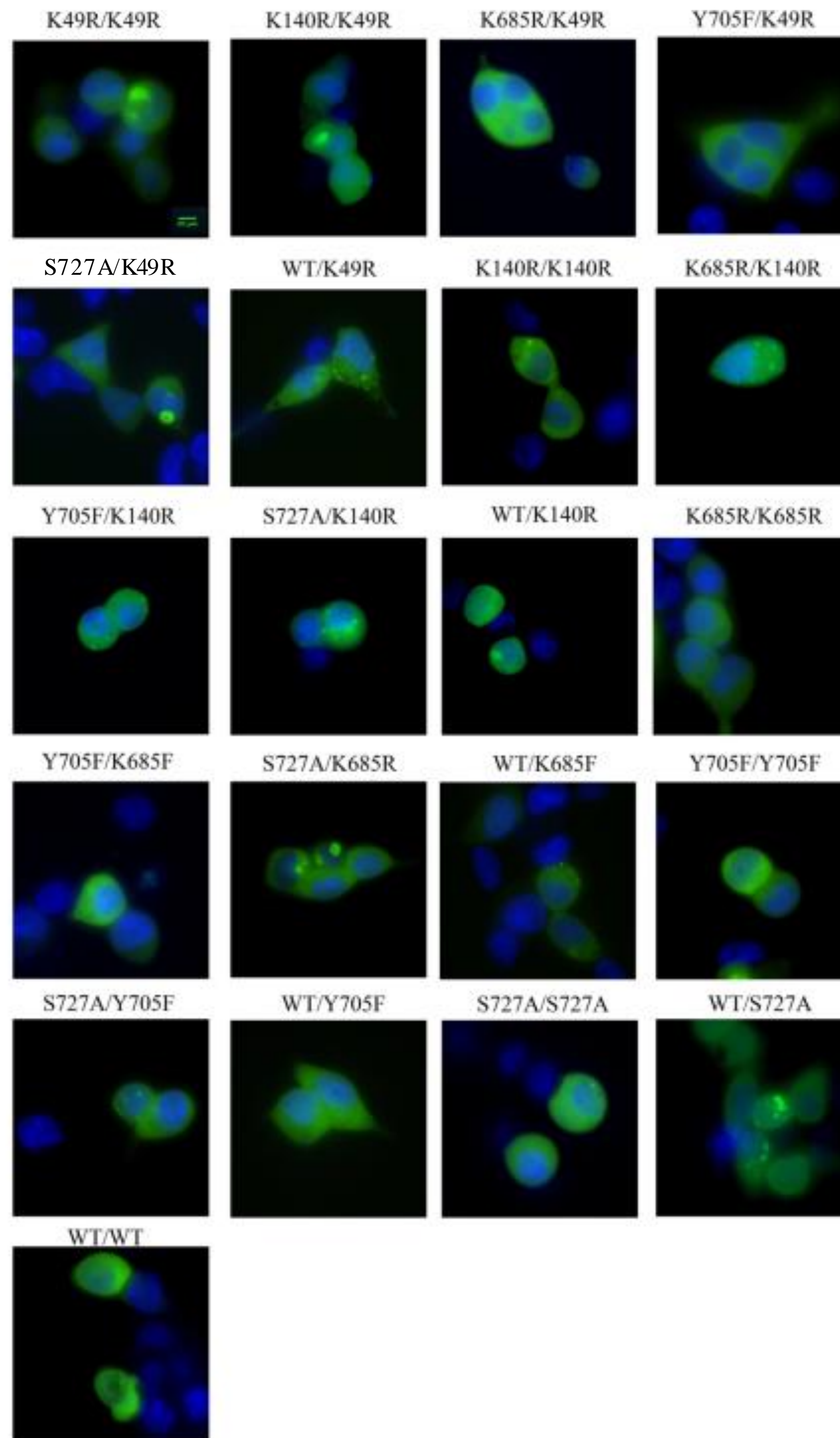

Figure 4.6 – Mutations of PTM residues have no effect on the intracellular localization of STAT3 dimers.
HEK cells were transfected with Venus-STAT3 BiFC mutants plasmids conjugated with each other and the samples were analysed by fluorescence microscopy. Aggregates are visible as the brighter green spots found in the cytoplasm of the cells. STAT3 was used as control (WT/WT). Scale bar: 20µm.

4.4. Polo-like kinase (PLK)-1 inhibitors reduce spontaneous STAT3 dimerization

Since STAT3 is involved in many cellular events and its dysregulation leads to diverse pathologies, targeting this protein could be a promising therapeutic strategy. Phosphorylation pathways are involved in the regulation of STAT3 activity, and kinases are part of the "druggable genome". On the other hand, STAT3 dimerization occurs prior to phosphorylation of Y705 or S727.[57] So, we next asked whether phosphorylation pathways were in any way involved in spontaneous STAT3 dimerization. To address this question we used the BiFC system to carry out a pharmacological screening with a library of 194 kinase inhibitors, of which 82 were tested in this work (Figure 4.7). We established stringent criteria to accept a hit as positive. First, it had to produce a fold-change in the fluorescence levels lower than 0.5 or higher than 2. Second, the inhibitor should not be significantly toxic to cells nor modify STAT3 levels. Third, several different inhibitors for the same kinase -when available- should show similar results.

To perform this experiment, HEK cells were transfected with Venus-STAT3 BiFC plasmids and treated with the different kinase inhibitors at a concentration of 1 μM. After 19 h, samples were analysed by flow cytometry to quantify the percentage of fluorescent cells. Cell death was determined simultaneously by means of PI (1 mg/mL). Total protein extracts were obtained in a paralel experiment and analysed by Western blot to determine the levels of STAT3 expression.

It should be mentioned, however, that these results were not confirmed through repetition because the tests were only done once.

Some kinase inhibitors decreased the percentage of fluorescence (Figure 4.7, black bars), namely Hesperadin (73), PHA-680632 (75) and SNS-314 Mesylate (76) (Figure 4.7 B), BI 2536 (57), GSK 461364 (58), HMN-214 (59), ON-01910 (60), Flavopiridol (Alvocidib) (62), AZD7762 (68) and AS703026 (79) (Figure 4.7 C). However, in most cases they were isolated cases. Only four PLK-1 kinase inhibitors seemed to have a consistent pattern, decreasing the percentage of fluorescence by more than 50% (57, 58, 59 and 60, Fig. 4.7 C, black bars). PLK-1 inhibitors were not significantly toxic for cells (Figure 4.7 C, white bars).

Aside from the PLK-1 family, the Aurora (3 out of 12), the cyclin-dependent kinase (CDK) (1 out of 7) and the MEK (1 out of 4) families, as well as the only checkpoint kinase (CHK) inhibitor of the library showed a significant decrease in the levels of STAT3 dimerization. Further work will be done with these families, except for the CHK inhibitor (68) which showed high levels of cell death.

Only one of the tested inhibitors increased the percentage of fluorescence more than 2-fold: Sunitinib Malate (28) (Figure 4.7 A, black bars). Regarding the profile obtained by flow cytometry it appeared that this inhibitor is autofluorescent. On Figure 4.8 we can confirm that there is a shift of the whole population towards the right, indicating autofluorescence. Because

of this, Sunitinib Malate has been excluded from the list of promising inhibitors. Of the c-met family of inhibitors, 4 out of 6 showed a tendency towards the increase of percentage of fluorescence, but the most noteworthy were PF-2341066 (49) and SU11274(PKI-SU11274) (51). These two, however, did not overcome the threshold set for the needed increase in fluorescence and so were excluded from further experiments.

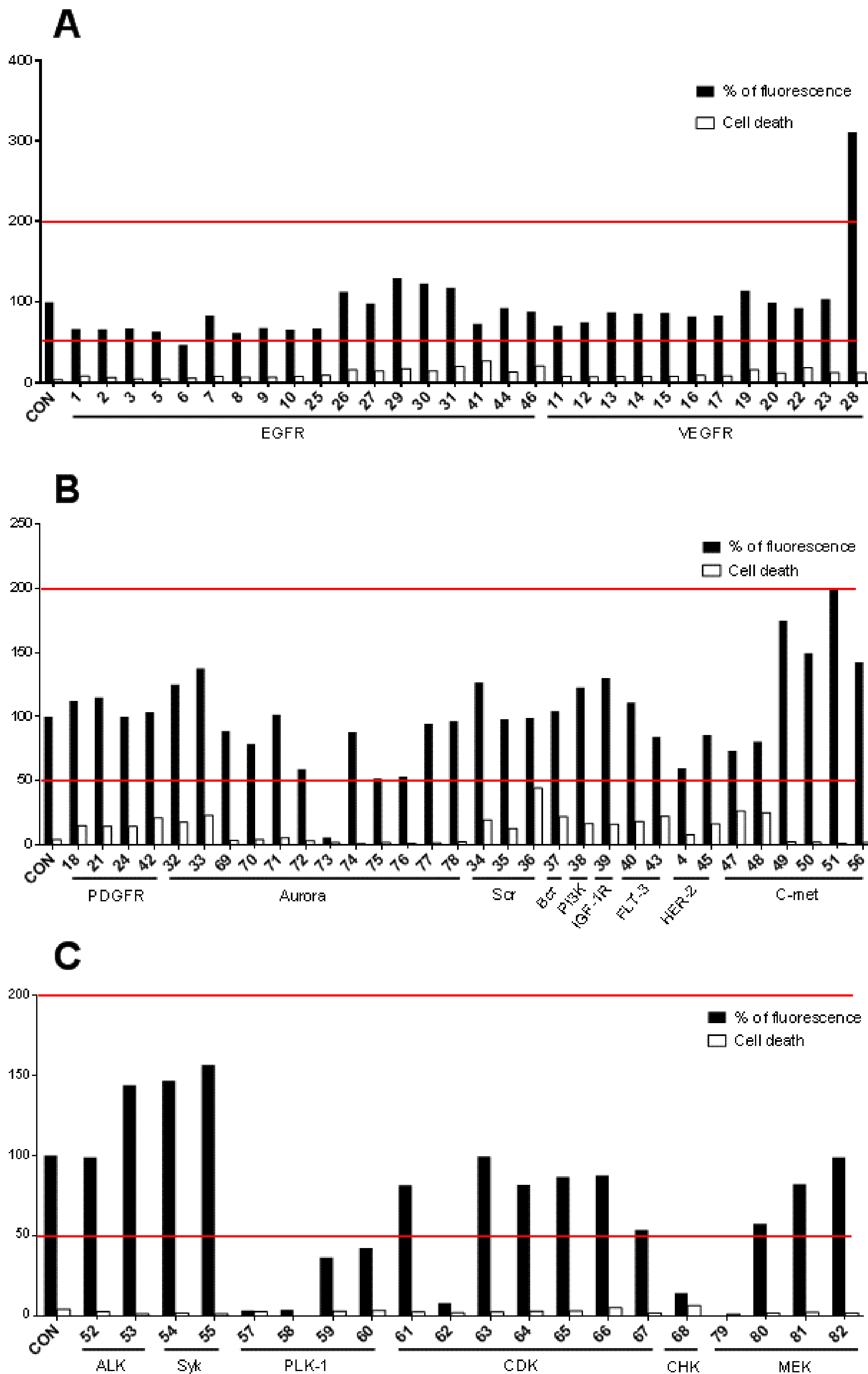

Figure 4.7 - Screening with a library of kinase inhibitors.
HEK cells were transfected with both Venus-STAT3 BiFC plasmids (V1-STAT3 and V2-STAT3) and treated with a library of kinase inhibitors (1 µM). Flow cytometry results show the amount of STAT3 dimers in the presence of the inhibitors (black bars) and also the toxicity of the inhibitors to cells (white bars). Venus-STAT3 BiFC construct was used as control (CON).

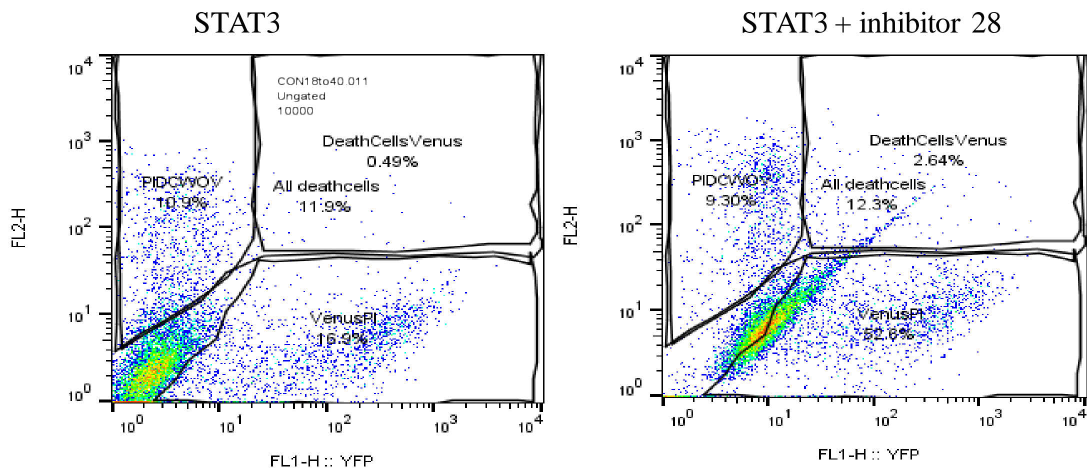

Figure 4.8 –Kinase inhibitor 28 is autofluorescent.
HEK cells were transfected with both Venus-STAT3 BiFC plasmids (V1-STAT3 and V2-STAT3) and treated kinase inhibitor 28 (1 µM). Flow cytometry results show a shift to the right when the cells were exposed to the inhibitor. STAT3 without the inhibitor was used as control.

To make sure that the decrease in the percentage of fluorescence is not due to a decrease in STAT3 expression levels, total protein extracts were analysed by Western blot using a specific STAT3 antibody (Figure 4.9).

Relatively to the PLK-1 inhibitors, only 59 and 60 did not reduce STAT3 expression at 1µM. The other two members of this family (57 and 58) need to be tested at a lower concentration to understand if they too can reduce the dimerization without interfering with the expression.

Taking into account the Aurora members 73, 75 and 76, all the members seem to be able to decrease STAT3 spontaneous dimerization independently from the expression. On the same note, the MEK inhibitor 79 seems to be able to reduce the levels of fluorescence without altering STAT3 expression. This furthers the interest in these promising inhibitors. More experiments shall be made to explore these inhibitors in the future.

CDK inhibitor 62 apparently reduces STAT3 expression, when comparing to the control. This hints at the need to test this inhibitor at lower concentrations than 1 µM, since it showed a promising reduction of the levels of fluorescence.

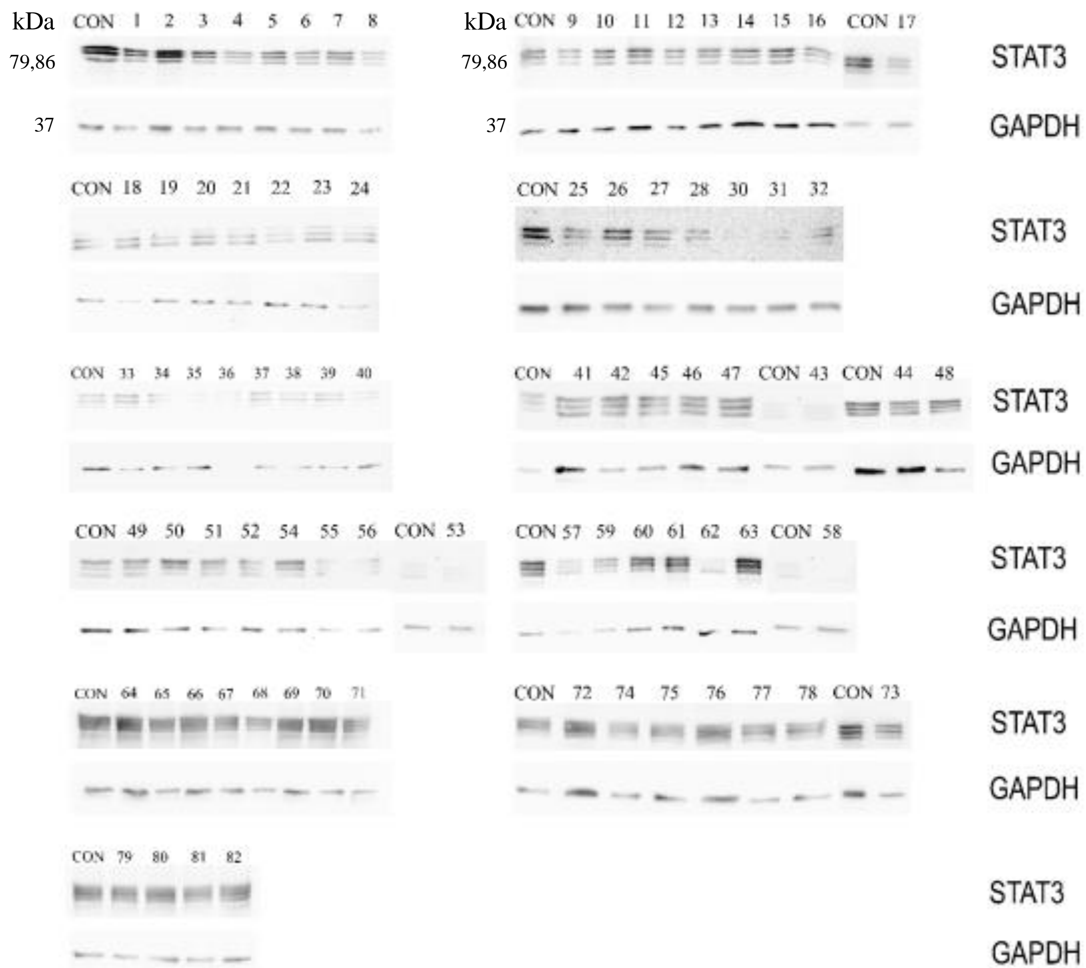

Figure 4.9 - Effect of a library of kinase inhibitors on STAT3 expression.
HEK cells were transfected with both STAT3 BiFC plasmids (V1-STAT3 and V2-STAT3) and treated with a library of kinase inhibitors (1 μM). Total protein extracts were analysed by Western blot using specific antibodies (anti-STAT3 and anti-GAPDH). Venus-STAT3 BiFC construct was used as control (CON) and GAPDH levels as a loading control.

4.4.1. HMN-214 (PLK-1 inhibitor 59) reduces spontaneous STAT3 dimerization

Since four PLK-1 inhibitors apparently decreased STAT3 dimerization, but some of them also decreased STAT3 expression, we focused on this family and tested their effects at different concentrations. Inhibitors 57, 58 and 59 were tested at 0.1, 0.2 and 0.5 μM and inhibitor 60 was tested at 0.1 and 1 μM (Figure 4.10). These tests, it must be said, were only performed once, so the results were not confirmed.

As the concentration increased, the percentage of fluorescence decreased for 57 and 58 (Figure 4.10, black bars). Inhibitors 57 and 58 seemed the most potent inhibitors. However, when analyzing the Western blot results done simultaneously, it is possible to see that these

inhibitors interfered with STAT3 expression, providing a plausible explanation for the decrease in fluorescence.

Regarding inhibitors 59 and 60, the results showed a significant decrease in fluorescence only when using 0.5 and 1 μM, respectively. Inhibitor 59 appears to be the most promising, demonstrating no alterations on STAT3 expression at 0.5 μM. Inhibitor 60, on the other hand, reduced fluorescence but also STAT3 expression (Figure 4.11).

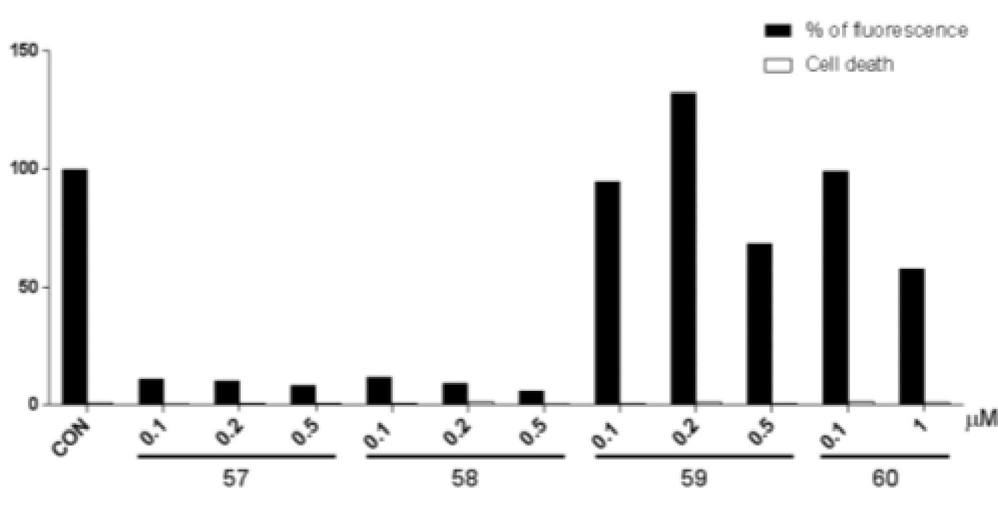

Figure 4.10 – PLK-1 inhibitors apparently decrease STAT3 dimerization.
HEK cells were transfected with both Venus-STAT3 BiFC plasmids (V1-STAT3 and V2-STAT3) and treated with 57, 58, 59 and 60 kinase inhibitors at different concentrations (0.1, 0.2, 0.5 and 1 μM). Flow cytometry results show the amount of STAT3 dimers in the presence of the inhibitors (black bars) and also the toxicity of the inhibitors to cells (white bars). Cells transfected with the Venus-STAT3 BiFC constructs and treated with the corresponding amount of DMSO (100%) were used as control (CON).

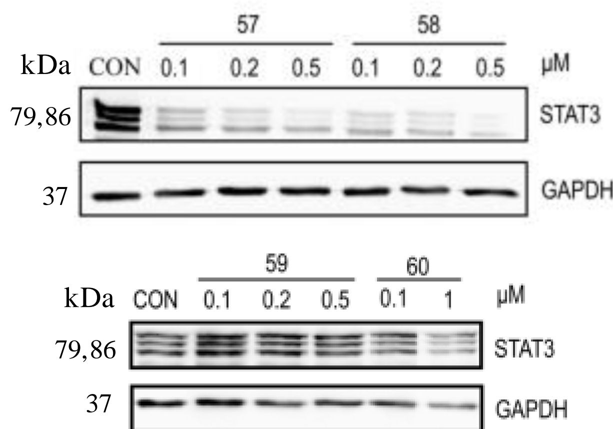

Figure 4.11 – Inhibitor 59 (HMN-214) at concentration of 0.5 μM does not decrease STAT3 expression.
HEK cells were transfected with both Venus-STAT3 BiFC plasmids (V1-STAT3 and V2-STAT3) and treated with 57, 58, 59 and 60 kinase inhibitors at different concentrations (0.1, 0.2, 0.5 and 1 μM). Total protein extracts were analysed by Western blot and specific antibodies were used (anti-STAT3 and anti-GAPDH). GAPDH levels as loading control. Cells transfected with the Venus-STAT3 BiFC constructs and treated with the corresponding amount of DMSO (100%) were used as control (CON).

4.4.2. HMN-214 prevents spontaneous STAT3 dimerization on most STAT3 mutants

HMN-214 was also tested in STAT3 mutants to investigate if any of the residues could be involved in the effects caused by the PLK-1 inhibitor.

To achieve this, HEK cells were transfected with the five mutants of the Venus-STAT3 BiFC plasmids and treated with HMN-214 at a concentration of 0.5 µM. Nineteen hours later samples were analysed by flow cytometry and total protein extract was analysed by Western blot. Again, these tests were not repeated three times, so the results were not confirmed.

Flow cytometry results (Figure 4.12) were in agreement with Figure 4.5 where the STAT3 mutants did not have a significant alteration on STAT3 dimerization.

When exposed to HMN-214, the control's fluorescence levels decreased to almost half the value when not exposed to the inhibitor. Figure 4.12 also demonstrated that the mutants have only a slight decrease on STAT3 dimerization when cells were treated with HMN-214. On the contrary, the mutant S727A almost had no changes in fluorescence, suggesting that this mutation could prevent HMN-214 effects and also that S727 could be essential for STAT3 dimerization.

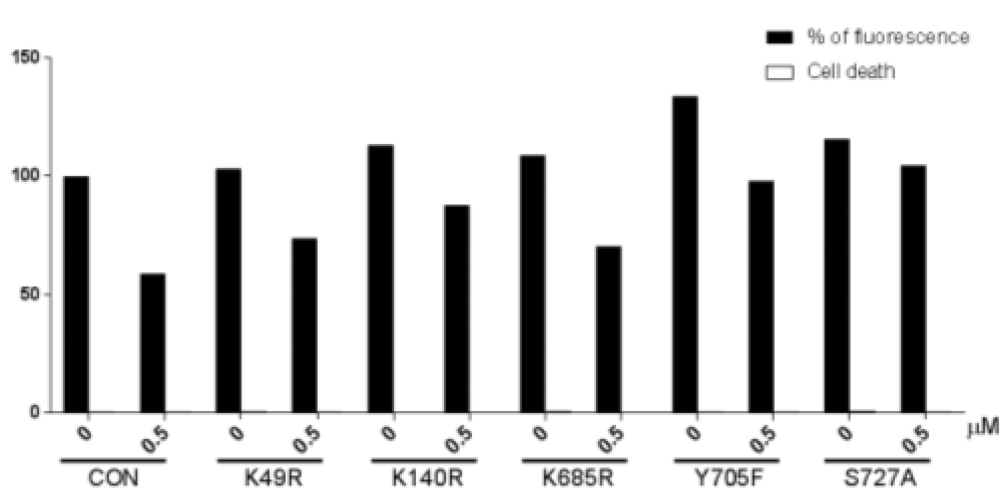

Figure 4.12 – HMN-214 decrease the dimerization of STAT3.
HEK cells were transfected with both Venus-STAT3 BiFC plasmids (STAT3 and STAT3 mutants) and treated with the HMN-214 (0.5 µM). Black bars correspond to the percentage of fluorescence and the white bars to the population of cell death. Venus-STAT3 BiFC construct was used as control (CON).

Western blot analyses came against what was shown previously (Figure 4.13). When exposed to HMN-214 we expected no changes in expression as seen in Figure 4.11. However, even the upper membrane's control had its expression altered by the inhibitor. The same

happened on all K mutants (K49R, K140R and K685R) where a slight decrease in STAT3 expression was also found.

Regarding the lower membrane, the same situation was found for the control, its expression reduced unexpectedly by the presence of the inhibitor. On the contrary, the mutation Y705F did not show changes in STAT3 expression. Curiously, S727A showed an increase in STAT3 expression levels when exposed to the inhibitor. Further studies will be made to better understand the role of these residues in STAT3 dimerization.

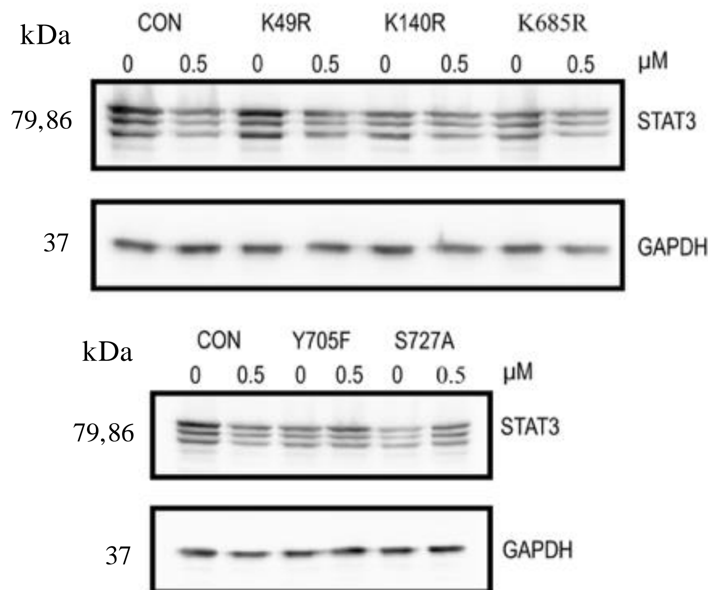

Figure 4.13 – Expression of STAT3 is affected by HMN-214.
HEK cells were transfected with both Venus-STAT3 BiFC plasmids (STAT3 and STAT3 mutants) and treated with the HMN-214 (0.5 µM). Anti-STAT3 and anti-GAPDH were used as specific antibodies. Venus-STAT3 BiFC construct was used as control (CON) and GAPDH levels as loading control.

5. Discussion

In the present thesis, we explored STAT3 phosphorylation and dimerization by means of a BiFC system developed in our laboratory. The role of different STAT3 residues (K49R, K140R, K685R, Y705F and S727A) on the phosphorylation of Y705 and S727 was investigated. We found that K140R reduced STAT3 phosphorylation on both residues, although the loading control was not very reliable. K685R affects S727 phosphorylation and reduces slightly Y705 phosphorylation. Expectedly, Y705F completely inhibited STAT3 phosphorylation on Y705, but not on endogenous STAT3. Interestingly, S727A reduces phosphorylation on Y705 and seems to be constitutively phosphorylated on endogenous STAT3.

Furthermore, we investigated the STAT3 mutants K49R, K140R, K685R, Y705F and S727A and their involvement in PTM and its effects on STAT3 phosphorylation and dimerization. We showed that no combination of these mutants affects STAT3 dimerization nor its intracellular localization. We also tested a library of 82 kinase inhibitors to find suitable candidates for the control of STAT3 dimerization and found that inhibitors for PLK-1 (4 out of 4), Aurora (3 out of 12), CDK (1 out of 7) and MEK (1 out of 4) were promising candidates to regulate spontaneous STAT3 dimerization.

The PLK-1 family of inhibitors was studied in more detail and so will other families in the future. Inhibitors 57, 58, 59 and 60 were tested at different concentrations and we found that inhibitor 59 (HMN-214) was the most promising of the four. HMN-214 was then tested on cells transfected with the STAT3 mutants and we unexpectedly found that the inhibitor reduced control expression. On the other hand, the mutant S727A protected STAT3 expression from being reduced. Future work will be undertaken to better understand the role of S727 in STAT3 dimerization and phosphorylation and additional studies must be made to explore the other promising kinase inhibitors mentioned here.

Firstly, in the present project, we demonstrate that LIF is able to stimulate the JAK/STAT3 pathway in HEK cells (Figure 4.1). This result was expected since LIF is part of the IL-6 family, which are the most common activators of the JAK/STAT3 pathway. Our results are in agreement with the previous study by Dey *et al.* (2011) where they show that HEK cells stimulated by LIF (10 ng/ml) led to the activation of STAT3, represented by the increase in phosphorylation.[58]

Furthermore, we also demonstrate that mutation on some of the acetylation and metylation residues (i.e., K140 and K685) can affect STAT3 phosphorylation on Y705 and S727 (Figure 4.4).

The K140R mutation affected both Y705 and S727 phosphorylation. This is not consistent with studies found in the literature. Dasgupta *et al.* (2015) demonstrated that this mutation does not interfere with STAT3 Y705 phosphorylation in A4 cells stimulated with IL-6.[54]

Accordingly, phosphorylation on S727 should not be expected to be changed by this mutation as well. Since in our own preliminary results the loading control lamin-B was not appearing at consistent levels of expression, further studies must be made to confirm our results.

In our hands, the STAT3 K685R mutation showed slightly lower levels of phosphorylation on Y705 and a significantly reduced S727 phosphorylation. In the literature, the K685 residue has been found to be relevant for STAT3 dimerization. Yuan *et al.* (2005) demonstrated that histone acetyltransferase p300-mediated STAT3 acetylation is required for the formation of stable dimers, using prostate cancer (PC3) cells lacking STAT3, PC3 cells expressing STAT3 WT and the mutant STAT3 K685R. Treating cells with OSM, they found that cytokine-stimulated STAT3 DNA binding activity and transcriptional regulation only happened when stable dimers were formed, which only happened when STAT3 showed acetylation on this residue.[55] Furthermore, in the same study, these authors showed that the phosphorylation of Y705 and S727 were not altered by this mutation. This was not what we obtained in our results. However, it should be mentioned that our results are only preliminary and that the loading controls were not very clear.

Regarding the Y705F mutation the effect on the phosphorylation of the tyrosine was expected, as the phenylalanine residue could not be phosphorylated. Since endogenous STAT3 is not mutated, it still shows phosphorylation when the membrane is incubated with an anti-p-STAT3 Y705. Mohr *et al.* (2013) show in their study of this particular mutant's effect on endogenous STAT3 activity after stimulation with IL-6 in HEK cells that Y705F STAT3 cannot be phosphorylated on this residue.[43]

Expectedly, the S727A mutation abolished S727 phosphorylation, since the alanine residue cannot be phosphorylated. Moreover, our results suggest that Y705 phosphorylation is lower than WT suggesting that S727 is required for increasing STAT3 phosphorylation on Y705. The same is mentioned by Wen *et al.* (1997), who, in their studies using COS cells stimulated by epidermal growth factor (100 ng/ml), found that for maximal activation of STAT3 to occur, both Y705 and S727 residues must be phosphorylated.[45]

Furthermore, in our results we unexpectedly found that endogenous STAT3 appeared to be constitutively phosphorylated on S727. This occurrence was previously discussed by Hazan-Halevy *et al.* (2010) when in chronic lymphocytic leukemia B-cells they found that S727 is constitutively phosphorylated and this alone is enough for STAT3 activation, migration to the nucleus, DNA binding and activation of target genes.[47]

In our study of the effects of the Venus-STAT3 BiFC mutant constructs combinations, we found that they do not change the intracellular localization nor STAT3's ability to dimerize. This is evident from the existence of fluorescence inside transfected cells. However, some of the combinations, namely 4 out of 6 containing K49R and also 4 out of 6 containing S727A, revealed considerable aggregates in the cytoplasm. The exact reason for these aggregates is not

clear, although it is possible that these residues can be implicated in the natural degradation of STAT3 as well as its stability. In the literature, it has been reported that lysine modification by acetylation can be protective of STAT3 degradation by ubiquitination and that there can be a sort of competition between histone acetyltranferases and the ubiquitin-proteasome effectors (for a review see: [59]). Therefore, when there is a mutation on these residues (such as K49R, K140R and K685R) it is possible that more stable aggregates form due to the inability to mark these for proteasome degradation through ubiquitination.

Regarding the stability conferred by S727 it seems that a similar situation was reported by Wakahara *et al.* (2012). In HepG2 cells transfected with the STAT3 mutant S727A, the duration of STAT3 activity was prolonged, because of the inability of nuclear phosphatase TC45 to remove the phosphorylation on S727, since in this mutant there is none.[48] In our own results we showed that STAT3 dimers are apparently more stable in the combinations with S727A. This may suggest a role other than regulation of the duration of STAT3 activity for this phosphorylation residue. Further work must be done along these lines.

Several studies have reported that STAT3 exists as stable dimers prior to its phosphorylation.[57,60,61] Because of this and due to STAT3 importance in many physiological events such as cell proliferation and differentiation, it is of great relevance to study ways to regulate its activity. This could be achieved through the control of its spontaneous dimerization.

In the library of 82 kinase inhibitors we tested, we gave special significance to PLK-1, Aurora, CDK and MEK inhibitors because of their promising effects on the inhibition of STAT3 dimerization.

PLK-1, Aurora and CDK are all kinases involved in cell mitotic events (for a review see: [62]). Since STAT3 is involved in cell proliferation it is predictable that these kinases can be involved in the STAT3 pathway. However, it has been shown by Vultur *et al.* (2015) that in various melanoma cell lines where STAT3 is overactivated, MEK inhibition leads to induction of STAT3 signaling.[63] This is contrary to our results shown here, although it should be clear that our results are still preliminary. Furthermore, the cells used were HEK cells and not melanoma cells. Further studies must be made to clarify this matter.

The PLK-1 family has been implicated in cell division, in the maintenance of genome stability during DNA replication and also in the response to DNA damage. Recently, a connection between PLK-1 and STAT3 has been found, namely that there is a reciprocal activation between the two. Zhang *et al.* (2012) showed in esophageal cancer cells that PLK-1 is dysregulated and upregulated in the majority of cancer types. The same can be said for STAT3. In this study, these authors described that PLK-1 increases STAT3 phosphorylation and expression and that when PLK-1 expression is inhibited through the use of silencing RNAs, STAT3 phosphorylation and expression is also impaired. Inversely, when silencing RNAs were

used to suppress STAT3 expression, expectedly STAT3 expression and phosphorylation were reduced but also PLK-1 expression was considerably decreased.[64]

Our own results showed that PLK-1 inhibitors are able to decrease by more than 50% the percentage of STAT3 dimerization in HEK cells. The toxicity of these 4 inhibitors was also tested and none were toxic to cells. Regarding the expression of STAT3 only 59 and 60 did not decrease STAT3 expression. Yet, since inhibitor 60 required a greater concentration for our desired effects on dimerization and at greater concentrations it affects STAT3 expression, it was left out. Inhibitor 59, HMN-214, was more closely followed for the rest of the screening since it was initially shown to decrease STAT3 dimerization at 0.5 μM.

Later in the screening, we tested HMN-214 against the STAT3 mutants previously generated. Apparently, this inhibitor affects all mutants' spontaneous dimerization levels except for STAT3 S727A, which only decreases very slightly. It may seem that this residue could play a protective role against PLK-1 inhibitors. More studies will be made to confirm this. Yet, when the Western blots were analyzed, we saw that, contrarily to what was found previously, HMN-214 reduces STAT3 expression levels on the lysine mutants as well as the control cells. Because of this unexpected finding, more work must be made to either confirm or contest this, since in earlier parts of the screening this inhibitor was shown to be quite promising in the control of STAT3 spontaneous dimerization. The STAT3 Y705F mutant did not show an apparent decrease in the levels of STAT3 expression but, unfortunately, since the controls were not very clear, sound conclusions cannot be made at this point. More studies with this inhibitor will be made to strengthen our claims. As for the STAT3 S727A mutant we found that the levels of STAT3 expression were actually increased after the exposure to HMN-214. This finding can explain the resistance found against the decrease in STAT3 dimerization but again we cannot confirm this result at this point.

6. Conclusions

In this thesis, we found that residues susceptible to undergo PTMs do not interfere with STAT3 spontaneous dimerization.

Furthermore, PLK-1, Aurora and MEK kinases are possibly involved in the regulation of STAT3 spontaneous dimerization. In this line, more studies will be made with these inhibitors to eventually elucidate their suitability for the treatment of STAT3-related pathologies, such as neurodegenerative diseases and cancer.

HMN-214 is a possible candidate for regulating STAT3 spontaneous dimerization and its effect could be mediated by the phosphorylation of S727.

These preliminary results were important to find ways that can regulate STAT3 dimerization, phosphorylation and activation, which could be central to many pathological phenomena.

7. Future work

The studies made in the present thesis allowed a first insight into the role of some of STAT3 residues that can be phosphorylated (Y and S), methylated or acetylated (K) in STAT3 dimerization and phosphorylation. However, these results are mostly preliminary, and more work should be done to confirm them. On the same note, several kinase inhibitors of the screening seemed to be promising, but we only had time to explore one of them in more detail. Therefore, some of our future goals are:

- Further investigate the importance of each residue, especially the role of acetylation and methylation on STAT3 regulation, as these seemed to be unclear;
- Confirm the results of the screening, namely by running more Western blot and flow cytometry tests;
    - With the possible candidates, perform tests at different concentrations, like what was shown here for the PLK-1 inhibitors.
- Determine the importance of S727 phosphorylation on the effects of PLK-1 inhibitors on STAT3 expression and dimerization.

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Appendix

Tabela 2 – Library of the kinase inhibitors with their respective numbers.

Number	Inhibitor	Number	Inhibitor
1	BMS-599626	43	KW 2449
2	Erlotinib Hydrochloride	44	CI-1033(Canertinib)
3	Gefitinib(Iressa)	45	CP-724714
4	Neratinib	46	BAY 73-4506(Regorafenib)
5	PD153035 hydrochloride	47	JNJ-38877605
6	Pelitinib	48	PF-04217903
7	Vandetanib	49	PF-2341066
8	WZ3146	50	SGX-523
9	WZ4002	51	SU11274(PKI-SU11274)
10	WZ8040	52	NVP-TAE684
11	AV-951(Tivozanib)	53	SB 525334
12	Axitinib	54	R406
13	BIBF1120(Vargatef)	55	R406(free base)
14	BMS 794833	56	XL184
15	Cediranib(AZD2171)	57	BI 2536
16	CYC116	58	GSK461364
17	Imatinib(STI571)	59	HMN-214
18	Imatinib Mesylate	60	ON-01910
19	Ki8751	61	AT7519
20	KRN 633	62	Flavopiridol(Alvocidib)
21	Masitinib(AB1010)	63	BS-181 hydrochloride
22	MGCD-265	64	PD0332991
23	Motesanib Diphosphate	65	PHA-793887
24	MP-470 (Amuvatinib)	66	Roscovitine(CYC202)
25	OSI-930	67	SNS-032(BMS-387032)
26	Pazopanib Hydrochloride	68	AZD7762
27	Sorafenib Tosylate	69	Aurora A Inhibitor I
28	Sunitinib Malate	70	AZD1152-HQPA(Barasertib)
29	TSU-68	71	CCT129202
30	Vatalanib	72	ENMD-2076
31	XL880(GSK1363089)	73	Hesperadin
32	PHA-739358(Danusertib)	74	MLN8237
33	AT9283	75	PHA-680632
34	AZD0530(Saracatinib)	76	SNS-314 Mesylate
35	Bosutinib(SKI-606)	77	VX-680
36	Dasatinib	78	ZM-447439
37	Nilotinib	79	AS703026
38	Quercetin(Sophoretin)	80	AZD6244(Selumetinib)
39	NVP-ADW742	81	AZD8330
40	AC-220	82	BIX 02188
41	AP24534	83	BIX 02189
42	Tandutinib (MLN518)	84	BMS 777607

Number	Inhibitor	Number	Inhibitor
85	CI-1040 (PD184352)	127	AT7867
86	PD318088	128	AZD1480
87	PD0325901	129	LY2784544
88	PD98059	130	Enzastaurin
89	U0126-EtOH	131	SB 431542
90	LY2228820	132	ABT-869(Linifanib)
91	BIRB 796	133	AEE788
92	SB 202190	134	BIBW2992(Tovok)
93	SB 203580	135	Lapatinib Ditosylate
94	Vinorelbine(Navelbine)	136	JNJ-7706621
95	VX-702	137	BEZ235
96	VX-745	138	GSK1059615
97	GDC-0879	139	PI-103
98	PLX-4720	140	AG-490
99	RAF265	141	CP-690550(Tofacitinib)
100	SP600125	142	Crenolanib (CP-868569)
101	AS-605240	143	GSK1838705A
102	GDC-0941	144	KX2-391
103	IC-87114 (PIK-293)	145	NVP-BSK805
104	LY294002	146	PCI-32765
105	PIK-293	147	PF-562271
106	PIK-90	148	DCC-2036
107	PIK-93	149	LDN193189
108	TG100-115	150	AZD8931
109	TGX-221	151	Raf265 derivative
110	XL147	152	NVP-BHG712
111	XL765	153	OSI-420
112	ZSTK474	154	R935788
113	AZD8055	155	AZ 960
114	Deforolimus(MK-8669)	156	Mubritinib
115	Everolimus(RAD001)	157	PP242
116	MK-2206	158	Cyt387
117	Rapamycin(Sirolimus)	159	Apatinib
118	Temsirolimus	160	CAL-101
119	WYE-354	161	PIK-294
120	PIK-75 Hydrochloride	162	VX-765
121	CHIR-99021	163	Telatinib
122	Indirubin	164	BI6727
123	SB 216763	165	WP1130
124	KU-55933	166	BKM-120
125	KU-60019	167	CX-4945
126	KU-0063794	168	Phenformin hydrochloride

Number	Inhibitor
169	TAK-733
170	AZD5438
171	PP121
172	OSI027
173	LY2603618
174	PKI587
175	CCT128930
176	A66
177	NU7441
178	GSK2126458
179	WYE-125132
180	WYE-687
181	A-674563
182	AS252424
183	GSK1120212
184	Flavopiridol hydrochlori
185	AS604850
186	WAY-600
187	TG101209
188	GDC-0980
189	A-769662
190	TAK-901
191	AMG 900
192	ZM 336372
193	PH-797804
194	PF-04691502