Universidade de Lisboa

Faculdade de Farmácia

VISUALIZATION OF STAT3 DIMERS IN LIVING CELLS

Ana Catarina da Silva Almeida

Dissertação orientada pelo Doutor Federico Herrera e coorientada pela Doutora Joana Amaral

Mestrado em Ciências Biofarmacêuticas

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Resumo

A via de sinalização JAK/STAT3 (do inglês Janus Kinase/ Signal transducer and activator of transcription 3) é uma via intracelular envolvida em múltiplos processos relacionados com crescimento celular, diferenciação, proliferação e apoptose. Esta é também a via canónica envolvida em processos de diferenciação de astrócitos e responsável pela formação da cicatriz glial devido a lesões do sistema nervoso.

A ativação desta via de sinalização inicia-se com a ligação de hormonas, fatores de crescimento ou citoquinas ao seu respetivo recetor, induzindo uma mudança conformacional no mesmo. Para o sinal ser propagado, as subunidades citoplasmáticas do recetor têm de estar ligadas a JAKs. As JAKs possuem domínios cinase no seu C-terminal, que trans-fosforilam o recetor e providenciam locais para a ligação de STAT3. As JAKs fosforilam também STAT3 em resíduos específicos de tirosina (Tirosina705). O paradigma original desta via de sinalização indicava que STAT3 dimerizava apenas quando fosforilado. No entanto, existem evidências recentes de que este fator de transcrição já se encontra na forma de dímeros estáveis no citoplasma. O passo final da via de sinalização é a translocação dos dímeros fosforilados para o núcleo e a sua ligação a sequências específicas em genes alvo, denominadas de SREs (do inglês STAT3-responsive elements). A JAK/STAT3 pode ser ativada pela ligação a diferentes tipos de recetores, incluindo recetores sem atividade tirosina cinase, recetores com atividade tirosina cinase ou recetores acoplados a proteína G. Na via de sinalização clássica, as citoquinas ligam-se a recetores sem atividade tirosina cinase. Entre estes recetores, os recetores da família gp130 (glicoproteína 130) são os de maior interesse para este trabalho, visto que o sinal mediado pela gp130 induz a diferenciação de astrócitos pela JAK/STAT3.

A transdução através da JAK/STAT3 é realizada por uma série de fosforilações e interações entre proteínas, pelo que para o estudo de todos os passos da mesma é necessário a identificação de interações proteína-proteína especificas. Os métodos mais correntes envolvem danos para as células vivas. Deste modo, utilizámos o método de complementação de fluorescência bimolecular (BiFC, das siglas em inglês) para o estudo da via de sinalização JAK/STAT3 em células vivas. O sistema Venus-STAT3 BiFC utilizado baseia-se na ligação de dois fragmentos não-fluorescentes da proteína fluorescente Venus a STAT3. Quando STAT3 dimeriza, as duas metades de Venus ficam suficientemente próximas reconstruindo o fluoróforo, e ocorre fluorescência. Esta fluorescência foi medida por citometria de fluxo e microscopia de fluorescência.

Para aferir a funcionalidade do sistema, testámos o comportamento dos dois construtos do sistema Venus-STAT3 BiFC (V1-STAT3 e V2-STAT3) em células vivas. A fluorescência ocorreu apenas nas células transfetadas com os dois construtos, demonstrando que a dimerização de STAT3 ocorre de forma espontânea. Em trabalhos anteriores a este, foi demonstrado que STAT3 não dimerizou com outras proteínas que continham a metade complementar da proteína Venus. Estes dados sugerem que a fluorescência é específica e indicativa da dimerização espontânea de STAT3 inativo. Estudámos também o comportamento dos dímeros de STAT3 face a estímulos com citocinas. Verificámos que nas células estimuladas, há translocação de toda a fluorescência para o núcleo. No entanto, não se observou nenhum aumento do número de dímeros nas células estimuladas. Estudámos também um inibidor de STAT3, denominado Stattic. Na presença deste inibidor, verificámos uma diminuição do número de dímeros, para além de uma inibição da ativação da proteína (por inibição da fosforilação no resíduo Tirosina705) e da translocação para o núcleo.

Visto que o sistema Venus-STAT3 BiFC conferiu informação direta sobre a dimerização e translocação de STAT3 para o núcleo, decidimos realizar rastreios moleculares. Testámos uma série de complexos dinuclares macrocíclicos, sintetizados pelo grupo da Drª Rita Delgado (ITQB-NOVA, Lisboa). Encontrámos 3 complexos macrocíclicos de zinco, com capacidade de inibir a fosforilação de STAT3 e a sua translocação para o núcleo. Neste momento, estamos ainda a realizar testes para confirmar estes resultados e para melhor compreender qual o mecanismo pelo qual ocorre esta inibição.

Visto que a fosforilação é um evento tão importante na ativação da JAK/STAT3, pretendíamos determinar se outras vias de fosforilação tinham um papel determinante na dimerização espontânea de STAT3. Para isso, realizámos também um rastreio molecular numa libraria de inibidores de cinases. De uma parte desta libraria testada durante a realização deste trabalho, encontrámos um composto que inibiu a dimerização de STAT3. Na restante libraria, existe um outro inibidor que tem a mesma cinase como alvo. Neste momento, existem outros colegas de laboratório a testar este composto.

O sistema Venus-STAT3 BiFC usado neste trabalho mostrou ser uma ferramenta muito útil no estudo da dimerização e translocação nuclear de STAT3. Para além de ser utilizado como uma ferramenta para análise de possíveis moduladores farmacológicos da JAK/STAT3, permite também descobrir novos intervenientes desta via. Fora do contexto desta via de sinalização, é um sistema que pode ser usado para o estudo de interação de inúmeras outras proteínas e em diferentes contextos biológicos.

Palavras-chave: via de sinalização JAK/STAT3; STAT3; astrogliogénese; BiFC.

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Abstract

The JAK/STAT3 pathway is an intracellular signaling pathway involved in many cellular processes, such as cellular growth, differentiation, migration or apoptosis. It is also activated upon astrocyte differentiation and is the main pathway responsible for astrocyte reactivity upon central nervous system (CNS) injury. Signal transduction occurs by a series of phosphorylation and protein-protein interactions, including the rate-limiting step of STAT3 dimerization. Here, we optimized a Bimolecular Fluorescence Complementation (BiFC) assay to study STAT3 dimerization in living cells. This Venus-BiFC system is based on the fusion of two non-fluorescent halves of the Venus fluorescent protein to STAT3. When STAT3 dimerizes, the two halves of the Venus fluorescent protein become close enough to reconstruct the fluorophore, producing fluorescence. This fluorescence was measured by conventional methods such as flow cytometry or fluorescence microscopy. With this Venus-BiFC system, we found that STAT3 dimerizes prior to activation. We also used the system to perform molecular screenings. We found that 3 dinuclear macrocyclic complexes (synthetized by Dr. Rita Delgado group, ITQB-NOVA, Lisbon) were able to inhibit STAT3 phosphorylation and translocation to the nucleus. In another molecular screening, we also identify one possible kinase inhibitor that inhibited STAT3 dimerization. This Venus-BiFC system showed to be a very valuable tool to study STAT3 dimerization and can be used to study other protein-protein interactions and/or to identify novel possible therapeutic targets for human disorders.

Key-words: JAK/STAT3 pathway; STAT3; astrogliogenesis; BiFC.

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List of Abbreviations

JAK	Janus Kinase
STAT	Signal transducer and activator of transcription
SH2	Src homology domain 2
PTK	Protein tyrosine kinase
IFN	Interferon
IL	Interleukin
Gp130	Glycoprotein 130
G-CSF	Granulocyte colony-stimulating factor
CNTF	Ciliary neurotrophic factor
NNT-1/BSF-3	Neurotrophin-1/B cell-stimulating factor-3
Epo	Erythropoietin
GH	Growth hormone
PRL	Prolactin
Tpo	Thrombopoietin
CNS	Central nervous system
LIF	Leukemia inhibitory factor
CT-1	Cardiotrophin-1
OSM	Oncostatin M
CNTF	Ciliary neurotrophic factor
TYK	Tyrosine kinase
ECG	Epidermal growth factor (EGF) receptor
PDGF	Platelet-derived growth factor
CSF-1	Colony stimulating factor-1 (CSF-1)
FGF	Fibroblast growth factor
MIP-1	Macrophage inflammatory protein
PIAS	Protein inhibitor of activated STAT
CCD	Coiled-coil domain
DBD	DNA-binding domain
TAD	Transcriptional activation domain
NPCs	Nuclear pore complexes
NLS	Nuclear-localization signals

NES	Nuclear-export signals
STAMs	Signal-transducing adaptor molecules
CIS	Cytokine-inducible SH_2-containing protein
SOCS	Suppressor of cytokine signaling
JAB	JAK-binding protein
SSI	STAT-induced STAT inhibitor
PTP	Protein tyrosine phosphatase
NSCs	Neural stem cells
NPCs	Neural progenitor cells
GFAP	Glial fibrillary acidic protein
bHLHs	Basic helix-loop-helix
Ngn1	Neurogenin1
SCI	Spinal cord Injury
FRET	Fluorescence resonance energy transfer
BiFC	Bimolecular fluorescence complementation
YFP	Yellow fluorescent protein
CFP	Cyan fluorescent protein
GFP	Green fluorescent protein
APE	A Plasmid Editor
HEK293	Human embryonic kidney cells
PC3	Prostatic cancer cells
DMEM	Dulbecco´s Minimal Essential Medium
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
LDH	Lactate dehydrogenase
PI	Propidium Iodide

Introduction

General description of the JAK/STAT3 pathway

The Janus kinase (JAK)/Signal transducer and activator of transcription 3 (STAT3) pathway is a conserved signaling pathway involved in numerous cellular events such as cell differentiation, migration, proliferation and apoptosis. This pathway is found to be activated in a wide variety of physiological and pathological conditions such as wound healing, cancer, inflammation, and neural development. We are particularly interested in the role of the JAK/STAT3 in astrogliogenesis.

The pathway starts by the binding of a ligand to their membrane receptors, which induces the dimerization/oligomerization of the receptor subunits (Figure 1) [1-3]. For the signal to be propagated, the cytoplasmic domains of the receptor subunits must be bound to JAKs. As JAKs have kinase-homologous domains at the C-terminus, they trans-phosphorylate the receptor when in proximity of each other, providing docking sites for cytoplasmic STAT3s [2, 3]. Phosphorylated JAKs then activate STAT3s in the cytoplasm by tyrosine phosphorylation in a specific tyrosine (Tyr705) residue [4, 5]. The original JAK-STAT3 signaling paradigm was that STAT3s existed as an unphosphorylated, monomeric species in the cytoplasm, and dimerized only when phosphorylated [6-8]. However, current evidence strongly indicates that STAT3 dimerizes prior to phosphorylation and activation [9]. STAT3s dimerizes by reciprocal binding of the Tyr residue of one monomer with the Scr homology 2 (SH2) domain of the other monomer [2]. The final step of the pathway is the translocation of activated dimers to the nucleus where they bind to specific regulatory sequences called STAT3-responsive elements (SREs) in the promoter of target genes and thus regulate transcription [3, 5, 10].

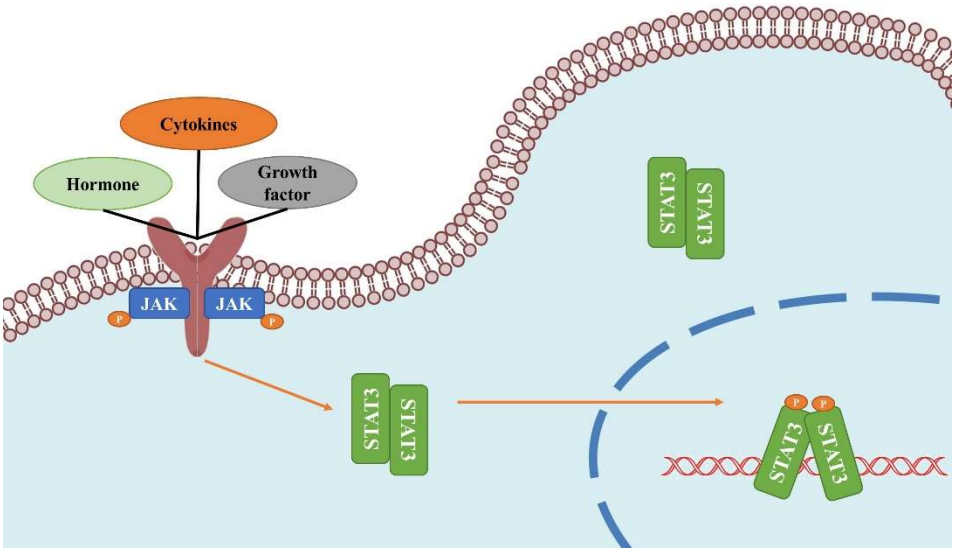

Figure 1: The JAK/STAT3 pathway.
The JAK/STAT3 pathway starts by the binding of a ligand (hormone, cytokines or growth factors) to the receptor. The receptor is connected to JAKs that trans phosphorylate the receptor, providing docking sites for STAT3. STAT3 is also phosphorylated by JAKs and translocate to the nucleus, were it activates transcription of target genes.

Description of main components

Receptors

The JAK/STAT3 pathway is activated by fusion of effectors to different type of receptors, including receptors without protein tyrosine kinase (PTK) activity, receptors with intrinsic PTK activity, G-protein-coupled receptors and non-receptor tyrosine kinases [1]. In the classical JAK/STAT3 pathway, cytokines bind to receptors without PTK activity. These receptors can be divided in four subgroups: interferon (IFN) family [4]; the glycoprotein 130 (gp130) family; the γC family and the single chain family (Table 1) [1, 4, 5, 11].

Family	Receptor
Interferon (IFN)	IFN-α/β; IFN-γ; Interleukin (IL)-10; IL-9; IL-20; IL-22
Glycoprotein 130 (gp130)	IL-6; IL-11; Oncostatin M (OSM); Leukemia inhibitory factor (LIF); Cardiotrophin-1 (CT-1); Granulocyte colony-stimulating factor (G-CSF); IL-12; IL-23; Leptin; Ciliary neurotrophic factor (CNTF); Neurotrophin-1/B cell-stimulating factor-3 (NNT-1/BSF-3)
γC	IL-2; IL-4; IL-7; IL-9; IL-15; IL-21
Single chain	Erythropoietin (Epo); Growth hormone (GH); Prolactin (PRL); Thrombopoietin (Tpo)

Table 1: Receptors without protein tyrosine kinase activity.

Gp130-mediated signaling is especially interesting for us, since it induces astrocytic differentiation through the JAK/STAT3 pathway and is the canonical receptor in the central nervous system (CNS). Knock-out gp130 mice show a lethal phenotype, indicating the biological importance of this receptor [12]. Gp130 is a multichain receptor complex on the cell membrane, constituted by the ligand binding receptor and the non-ligand binding membrane glycoprotein 130 [5]. Its ligands include IL-6, IL-11, leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), Leptin and IL-12 receptors. LIF, CNTF, OSM and CT-1 can only induce the heterodimerization of gp130 with a second signal-transducing receptor subunit, namely LIF receptor or OSM receptor. IL-6 and IL-11 are able to signal trough homodimerization of gp130 [10].

Receptors with PTK activity include the epidermal growth factor (EGF) receptor, the platelet-derived growth factor (PDGF) receptor, the colony stimulating factor-1 (CSF-1) receptor and the fibroblast growth factor (FGF) receptor. G-protein-coupled receptors include chemokines receptors such as receptors for macrophage inflammatory protein (MIP-1) and RANTES. Finally, the JAK/STAT3 can also be activated by non-receptor tyrosine kinases, such as viral oncoproteins (v-src, v-Fps, v-Sis, polyoma virus middle T antigen and v-abl) [2].

JAKs

JAKs represent a family of four non-receptor tyrosine kinases (JAK1, JAK2, JAK3 and TYK2). JAK1, JAK2 and TYK2 are expressed ubiquitously, but JAK3 expression is restricted to cells of the myeloid and lymphoid lineage [13]. JAK1 and JAK2 knock-out mice present lethal phenotypes, due to neurological disorders or lack of correct erythropoiesis, respectively [14, 15]. JAKs have 7 regions of sequence similar between them (JH1-JH7): the region FERM (a band four point one (4.1), ezrin, radixin, moesin), the region SH2, a non-catalytic regulatory domain (JH2) and a tyrosine kinase activity domain (JH1) [1, 3, 4, 16]. Although the functions of each region are not yet fully characterized, it is known that JH1 encodes the kinase, JH2 represents a domain with pseudokinase activity required for JH1 catalytic activity and JH3-JH7 are required for the association to the receptor [1, 3, 4].

STAT3

In mammals, there are 7 types of STATs: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 [16]. Although different STATs can be activated by the same effectors, they have distinct biological roles [17]. Among all STATs, only STAT3 is required for early development since STAT3 null mice are embryonically lethal [18]. Many authors refer that STAT3 is a latent transcription factor that remains as a monomer in the cytoplasm until activated by phosphorylation, and then dimerizes and translocate to the nucleus. However, current evidence indicates that STAT3 already exist in the form of dimers in the cytoplasm [1, 3, 19-21].

All STATs share 6 structurally and functionally conserved domains (Figure 2) [4]. The amino-terminal domain (NH2) is involved in protein-protein interactions between adjacent STAT dimers and DNA, facilitating the formation of STAT tetramers [21]. This domain is also suggested to regulate nuclear translocation and to promote the interaction with the transcriptional activator CBP/p300, the protein inhibitor of activated STAT (PIAS) family and receptor domains. The coiled-coil domain (CCD) is implicated in receptor binding, tyrosine phosphorylation and nuclear export. The DNA-binding domain (DBD) is the domain that recognizes a specific response element in the genome and allows transcription. The linker domain connects de DBD with the SH2 domain. The SH2/tyrosine activation domain binds to specific phosphotyrosine motifs, being essential for STAT recruitment to the receptor and activation. The site for tyrosine phosphorylation in STAT3 has been identified as Tyr^{705} [22]. Finally, the transcriptional activation domain (TAD) is the most divergent domain between

different STATs. TAD has a conserved serine residue, phosphorylated upon cytokine stimulation and important for maximal transcriptional activation [20, 23, 24]. STATs most frequently have this phospho-Serine in the TAD region, with the exception of STAT2 and STAT6 [2]. The site of serine phosphorylation in STAT1 and STAT3 has been identified as Ser^{727} [25].

STAT3 isoforms

STATs can occur in their full-length form (the α isoform) or in shorter isoforms (named β, γ or δ isoforms), due to alternative mRNA splicing or post-translational modifications (Figure 2) [2]. Alternative splicing generates STAT3β isoform, which has a truncated C-terminal transactivation domain. This isoform lacks the 55 C-terminal amino acids of the α isoform but gains a unique region of 7 amino acids in the C-terminal. STAT3β also lacks the serine 727 residue [2]. Proteolytic processing at the C-terminus also generates truncated isoforms of STAT3: STAT3β, STAT3γ isoforms in human neutrophils [26] and STAT3δ isoforms in the early stage of granulocytic differentiation [27].

Like the complete isoform, STAT3β isoform can also be tyrosine phosphorylated, form dimers or bind to DNA [28]. However, both forms are distinctly different in their transcriptional activity and biological functions. In CD34+ bone marrow and HL60 leukemia myeloid cells, G-CSF cytokine only activates STAT3β and not STAT3α [29]. By contrary, STAT3β is unable to activate transcription of IL-6 responsive genes (with the pIRE element) in conditions that STAT3α is [30]. Also, in melanoma cells, STAT3α suppresses transcription of the Fas gene, contrary to STAT3β [31]. Surprisingly, exogenous STAT3β is able to bind to DNA in the absence of ligand, and with greater specific DNA-binding activity that the α isoform [28]. This is attributed to higher dimer stability of STAT3β dimers [32].

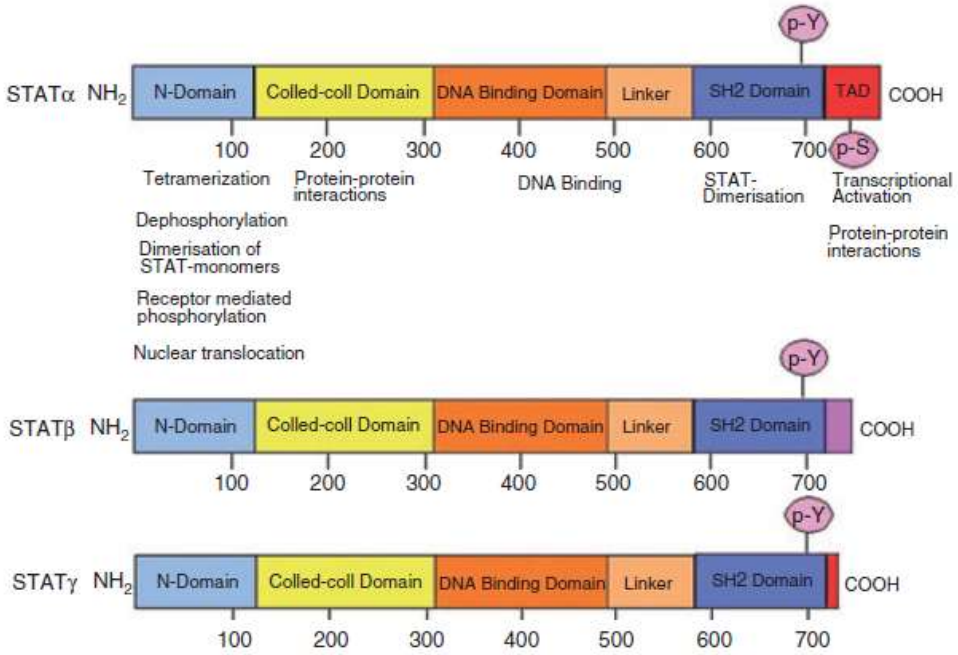

Figure 2 - Modular structure of signal transducer and activator of transcription (STAT) proteins.
All STATs share a common molecular topology and are organized in distinct functional regions. In addition to the full-length form (STATα), STATs can also be present in others forms generated by alternative splicing (STATβ) or proteolytic processing (STATγ) [21].

STAT3 activation

Although STAT3 was originally identified as an acute-phase response factor activated by IL-6, it can be activated by other cytokines, such as IL-10, LIF [4], oncostatin M, CNTF and leptin [33]. Activation of STAT3 is made through phosphorylation of the Tyr705 residue. Additionally, phosphorylation of S727 residue can also regulate STAT3 activation, depending of the promoter and/or cellular context [2, 23].

Tyrosine and serine phosphorylation can be induced and regulated independently, but they can also regulate each other, depending on the stimulus and cellular context. The activation of Raf and MAP kinase pathway induced serine 727 phosphorylation without tyrosine phosphorylation [34]. In EGF-stimulated cells, there was an increase in tyrosine phosphorylation of a transfected STAT3 S727A mutant compared to the wild type protein [34].

Although tyrosine phosphorylation is necessary for the activation of STAT3, unphosphorylated STAT3 is also required for the expression of certain genes [35]. In addition, some of the genes that respond to phosphorylated STAT3 are also regulated by unphosphorylated STAT3 [35]. Activation of hTER-HME1 cells expressing wild type or Y705F STAT3 with IL-6, showed that the response of STAT3-dependent genes occurred in

two phases. The initial phase with the induction of genes with SREs sites that respond to STAT3 dimers and a secondary phase with induction of genes that depend on unphosphorylated STAT3 [35]. Possible mechanisms for the activation of transcription by unphosphorylated STAT3 are proposed. Unphosphorylated STAT3 dimers can bind to a responsive promoter; STAT3 could form heterodimers with other transcription factors and those transcription factor would recognize half of a GAS element [35].

STAT3 nucleocytoplasmic shuttling

The nuclear membrane is composed by passageways designated as nuclear pore complexes (NPCs) (Figure 3). Although smaller molecules can pass freely through these NPCs, larger proteins are restricted or need the assistance of transporters. These transporters recognize specific amino-acid sequences in the proteins. They can function as nuclear-localization signals (NLSs) or nuclear-export signals (NESs). The transporters belong to the karyopherin-β family and can be importins (functioning in nuclear import) or exportins (functioning in nuclear export). These karyopherin-β transporters can bind directly to the proteins or with the assistance of adaptor molecules, known as importin-α family [9].

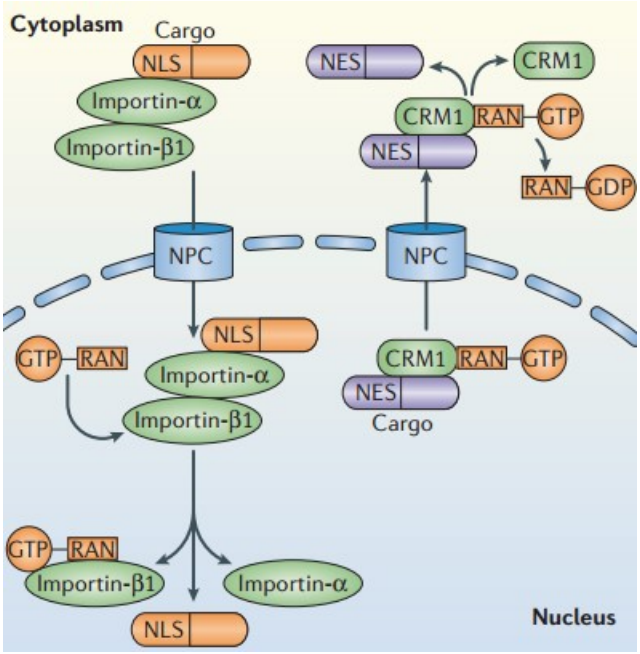

Figure 3: Karyopherin-mediated nuclear trafficking [9].
NLS are recognized in the cytoplasm and transported through the NPCs to the nucleus. NES are recognized in the nucleus and transported through the NPC to the cytoplasm.

Contrary to what is known for other STATs, tyrosine phosphorylation is not required for STAT3 nuclear translocation. In fact, STAT3 is continuously shuttling between the cytoplasm and the nucleus. The nuclear import domain of STAT3 is recognized by importin-

α3 and importin-α6, independently of the phosphorylation state [36]. Silencing importin-α3 inhibits nuclear translocation of STAT3 and leads to aggregation of STAT3 in the cytoplasm [36]. Also, generation of deletions in STAT3 allowed the identification of a constitutive active NLS region (amino-acids 150-162) in the coiled-coil domain, indispensable for nuclear translocation [36]. The presence of unphosphorylated STAT3 dimers in the nucleus raises the question of what is its function in the nucleus. The presence of STAT3 dimers in the nucleus could contribute to a quicker response of transcription activation when needed. Also, it was already mentioned that unphosphorylated STAT3 can also activate gene transcription [35].

After STAT3 translocation to the nucleus and the following gene transcription activation, counterregulatory pathways must be activated in order to ensure that this signal is transient. Therefore, STAT3 needs to be exported to the cytoplasm. Three NES elements were identified as being regulators of STAT3 nuclear export (residues 306-318, 404-414 and 524-535) [37]. The first NES (residues 306-318) is located in the coiled-coil domain which means that it is completely accessible, and that its function could be affected by structural changes in STAT3 [37]. The second NES element (residues 404-414) is localized in the DNA-binding domain, which is buried inside the 3D structure of the protein when bound to DNA. This suggests that this NES element only regulates STAT3 export after dissociation from DNA [38]. The third NES element (residues 524-535) is located in the linker domain, making it more accessible but still partially exposed.

JAK/STAT3 regulation

In addition to the main components of the JAK/STAT3 pathway, there are also other contributors to the main events, such as effectors and negative regulators. Signal-transducing adaptor molecules (STAMs) are adaptor molecules that facilitate the transcriptional activation of specific JAK/STAT target genes by a still poorly understood mechanism. STAT-interacting protein (StIP) is another facilitator that associates with JAKs and unphosphorylated STATs, serving as possible scaffold to facilitate their phosphorylation. Finally, SH2B/LnK/APS family, are also effectors of the pathway, serving as substrates for JAK phosphorylation [3].

Many negative regulators of the JAK/STAT pathway are known: cytokine-inducible SH2-containing protein (CIS); suppressor of cytokine signaling (SOCS); JAK-binding protein (JAB); STAT-induced STAT inhibitor (SSI), protein inhibitor of activated STAT (PIAS); protein tyrosine phosphatases (PTP) or protein degradation by the ubiquitin-proteasome pathway (Figure 4) [2].

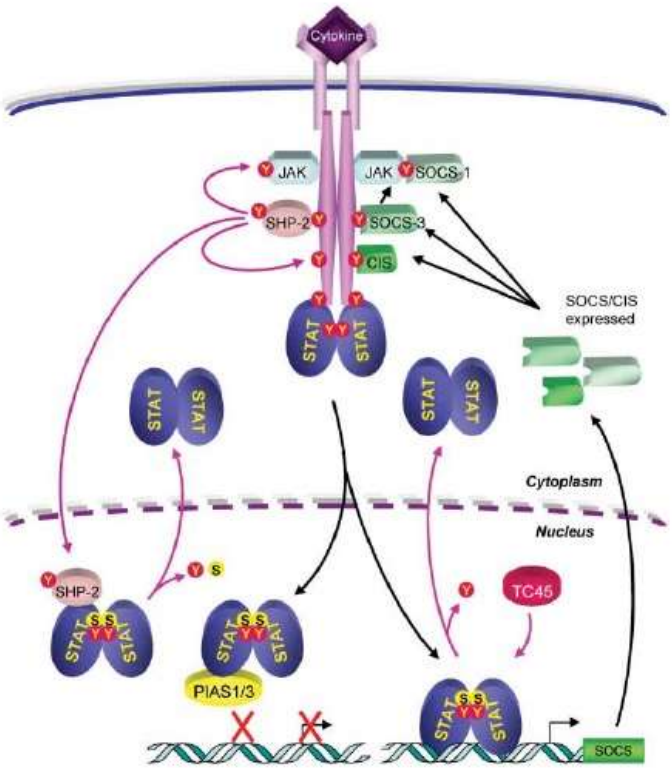

Figure 4: STATs regulation.
Schematics of STATs regulation by SOCS, PIAS or SHP-2. Adapted from [2].

SOCS expression is regulated by ligand and inhibits STAT proteins by JAK suppression or by receptor competition. PIAS, unlike SOCS, are constitutively expressed and interact directly with phosphorylated STATs. From all PIAS proteins, PIAS3 is more specific towards STAT3, and it blocks its binding to DNA [2]. The major place of phosphorylation and dephosphorylation in all STATs is the tyrosine residue. A SH2 domain-containing PTP (SHP2) inhibits phosphorylation and activation of STAT3 in various biological contexts [39]. In addition to SHP2, other PTPs such as CD45, PTP1B, TC-PTP, PTPRT and PTP-BL also regulate the JAK/STAT pathway by dephosphorylation of either JAKs or STATs [39].

Physiological and pathological roles of STAT3

Many cellular processes involve STAT3 activation, such as cell proliferation, differentiation and apoptosis [40]. The biological significance of STAT3 is further demonstrated by the fact that all knockout models for proteins of the STAT family are viable, except for STAT3 [1, 18]. This loss of viability, together with the fact that even in embryonic stem cells loss of STAT3 is lethal, expresses the importance of STAT3 from early

embryogenesis [41]. However, despite the fact that STAT3 has a crucial role in embryogenesis, the mechanism by which this happens remains unknown.

In the development of the mammalian nervous system, STAT3 controls the switch between the neurogenic phase to astrocyte differentiation. One of the epigenetic changes by which this happens is the silencing of genes necessary for astrocyte formation through DNA methylation and chromatin modifications [42-45]. During the neurogenic phase, newly born neurons release gliogenic cytokines, enabling the cortical precursors to generate astrocytes. This way, astrocytes are generated only after neurons [46, 47]. This cytokine-mediated differentiation involves the activation of the JAK/STAT3 pathway mainly through CNTF, LIF and CT-1 [47]. Furthermore, the absence of these cytokine´s receptors is responsible for deficits in astrogliogenesis [48-50].

JAK/STAT3 pathway is also the main pathway leading to astrocyte reactivity in response to pathological conditions that affect the CNS. This reactivity is characterized by morphological alterations in astrocytes and by transcriptional and functional changes. Since astrocytes are responsible for several physiological functions, this astrocytic reactivity leads to serious consequences in other cells and in their environment [10]. After spinal cord injury (SCI), for example, NSCs and NPCs proliferate to the lesion where they differentiate in astrocytes, leading to the formation of a astrogliotic scar. In an acute phase of the lesion, this scar separates healthy tissue from the injury, allowing the restoration of the bool-spinal cord barrier and preventing further inflammatory processes. However, in chronic phases, this scar can inhibit axonal regrowth which prejudices lesion recovery. The role of JAK/STAT3 on astrocyte reactivity is definitely proved. However, further studies are required to elucidate the mechanisms involved in this process [5].

The importance of STAT3 in cancer is also very well described [51]. Besides the fact that STAT3 is crucial for the transduction of signals from many tyrosine kinases, he also regulates the expression of genes that contribute to tumor progression. Furthermore, the role of STAT3 in immune responses makes it an important frontier for the regulation of antitumor immune responses [52, 53].

Although JAK/STAT3 is usually associated to mechanisms of cell proliferation and differentiation, it can also regulate apoptosis. STAT3 can have either a pro or an anti-apoptotic. In STAT3-deficient T cells, STAT3 is required for the IL-6-mediated prevention of apoptosis [54]. However, the STAT3β isoform attenuated MHC II induced T cell apoptosis, suggesting a pro-apoptotic effect of the complete STAT3 isoform [55].

The absence of functional STAT3 in the epidermis leads to a phenotype of sparse hair and ulcer development pronounced with age. This indicates an important role of STAT3 in the skin, by mechanisms that could be initiated by growth factor like EGF and TGF-α, already mentioned as effectors of the JAK/STAT3 pathway [56].

STAT3 is also critical to myeloid development, as in macrophages and neutrophils, STAT3 gene ablation leads to a susceptibility to endotoxic shock and chronic enterocolitis [57].

During the mammary development, STAT3 activation leads to a delay in the mammary involution, a process by which the mammary gland returns to its non-lactating state. In this case, STAT3 seems to have a pro-apoptotic effect over the involuting mammary gland [58].

BiFC system

The JAK/STAT3 pathway depends on specific protein-protein interactions. Protein-protein interactions can be studied using many approaches, such as co-precipitation, fluorescence spectroscopy and image correlation spectroscopy, among others. However, these methods imply direct or indirect consequences to cells, often involving cell lysis and biochemical methods to extract the proteins. There are only two methods to observe protein-protein interactions in living cells with minimal or no perturbation [59]. These are fluorescence resonance energy transfer (FRET) and bimolecular fluorescence complementation (BiFC) [60].

In FRET, the proteins of interest are fused to fluorophores, generally variants of the green fluorescent protein (GFP) [61]. The two different fluorophores have overlapping emission/absorption spectra, being one the donor and other the acceptor. FRET is observed by exciting the sample at the wavelength of the donor and measuring the emitted fluorescence intensity at the wavelength of the acceptor. If both the donor and the acceptor are at a favorable distance and orientation, the donor emission intensity decreases and the acceptor emission intensity increases [62].

BiFC consists in the fusion of two halves of a fluorescent protein with each of two proteins of interest. When the proteins of interest interact, the two halves of the fluorescent proteins are close enough to reconstitute the fluorophore and produce fluorescence (Figure 5). Fluorescence is therefore proportional to the amount of dimers and it can be measured by conventional methods such as flow cytometry and fluorescence microscopy [63].

In comparison to FRET, BiFC has the benefit of detecting interactions at lower levels and being less susceptible to culture conditions. Also, FRET requires that the fluorophores exist

in close proximity (less then 100Å), while in BiFC the fragments of the fluorescent protein can be farther apart, since they have flexibility to associate in the complex [60].

A limitation of BiFC systems is that the maturation of the fluorophore requires some time, which prevents the detection of rapid real-time changes in interactions [64]. Another disadvantage of BiFC is that it is mostly irreversible, although in some conditions dissociation of the complex is observed. Furthermore, fluorescent fragments can associate without interaction between the proteins fused to them, producing unspecific fluorescence. This background can be alleviated by expressing the fluorescent protein in levels similar to those of the endogenous interest proteins, by specific mutations on the reporter protein or by combining different lengths of the reporter fragments [60, 64].

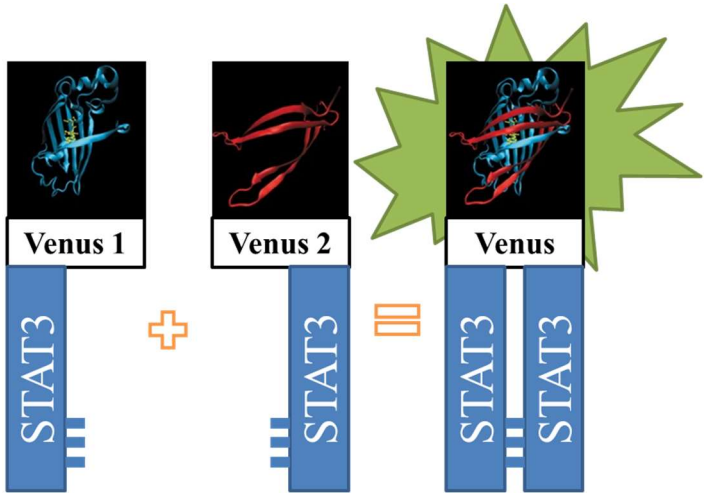

Figure 5: Schematic representation of the BiFC system.
The Venus-BiFC system consists in the fusion of two non-fluorescent halves of the Venus fluorescent protein, to STAT3. When STAT3 dimerizes, the two Venus-halves became close enough to reconstruct the fluorophore, producing fluorescence.

BiFC has been previously applied by Dr Herrera and other authors to visualize protein-protein interactions in living cells, giving new insights into the functions and localization of protein complexes associated to neurodegeneration [59, 65-67]. Venus, a third-generation yellow fluorescent protein, is the most frequent choice, since it produces brighter fluorescence and it can reconstruct the fluorophore at 37ºC, a physiological temperature [68]. Other fluorophores need to be incubated at 30ºC to reach higher levels of fluorescence, which can damage or perturb most cell types [63]. However, Venus fragments can produce higher background and lower signal-to-noise ratios, comparatively to the original proteins [63].

Methods

Materials and reagents

HEK293 human embryonic kidney cells (ATCC reference CRL-1573) were a kind gift from Dr. Julia Costa (ITQB-NOVA, Portugal). PC-3 cells (ATCC reference 1435) were a kind gift from Dr. Juan Carlos Mayo (Universidad de Oviedo, Spain). Dulbecco´s Modified Eagle´s Medium (DMEM), DMEM F12 and phosphate buffered saline (PBS) were purchased from Lonza (Basel, Switzerland). Fetal Bovine Serum (FBS) was obtained from Biowest (Nuaillé, France). Penicillin/Streptomycin was acquired from Life Technologies (NY, USA). Glutamine was acquired from Thermo Scientific (MA, USA). Trypsin was purchased from GE Healthcare Life Sciences (Buckinghamshire, United Kingdom). Lipofectamine® 2000, IL-6 and Propidium Iodade (PI) were acquired from Invitrogen (MA, USA). LIF was acquired from R&D systems (Minneapolis, USA). DAPI was acquired from PanReac Appli-Chem (Barcelona, Spain). Stattic was purchased from Selleckchem (TX, USA). LDH assay kit was purchased from TAKARA (Tokyo, Japan). 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Calbiochem (San Diego, USA).

The NE-PER™ Nuclear and Cytoplasm Extraction kit was acquired from Thermo Scientific (MA, USA). Bradford was acquired from PanReac Appli-Chem (Barcelona, Spain). Anti-STAT3 (total and phosphorylated) monoclonal antibodies were obtained from Cell Signaling (MA, USA). Anti-Lamin B antibody was obtained from Santa Cruz Technologies (TX, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody was purchased from Ambion (CA, USA). Alexa Fluor 594 chicken anti-rabbit IgG antibody was acquired from Life Technologies (NY, USA). Secondary horseradish peroxidase-conjugated antibodies (sheep anti mouse IgG and donkey anti-rabbit IgG) were purchased from GE Healthcare Life Sciences (Buckinghamshire, United Kingdom). Chemiluminescent HRP substrate Immobilon™ Western was obtained from Milipore (Billerica, USA).

Dinuclear complexes were synthesized by the group of Dr. Rita Delgado (ITQB-NOVA, Portugal). The kinase inhibitor library was purchased from Selleck Bio (TX, USA).

Plasmid construction

Briefly, the V1-STAT3 and V2-STAT3 BiFC constructs were designed using the A Plasmid Editor (APE) freeware and manufactured by Life Technologies on a pcDNA 3.1(-) backbone. The Venus tags were located in the N-termini of STAT3 in order to avoid their

possible post-translational cleavage, while preserving STAT3 capability to shuttle in and out of the nucleus (Figure 6). These constructs were successfully expressed in several mammalian cell lines, as determined by flow cytometry and western blot analyses with total protein extracts.

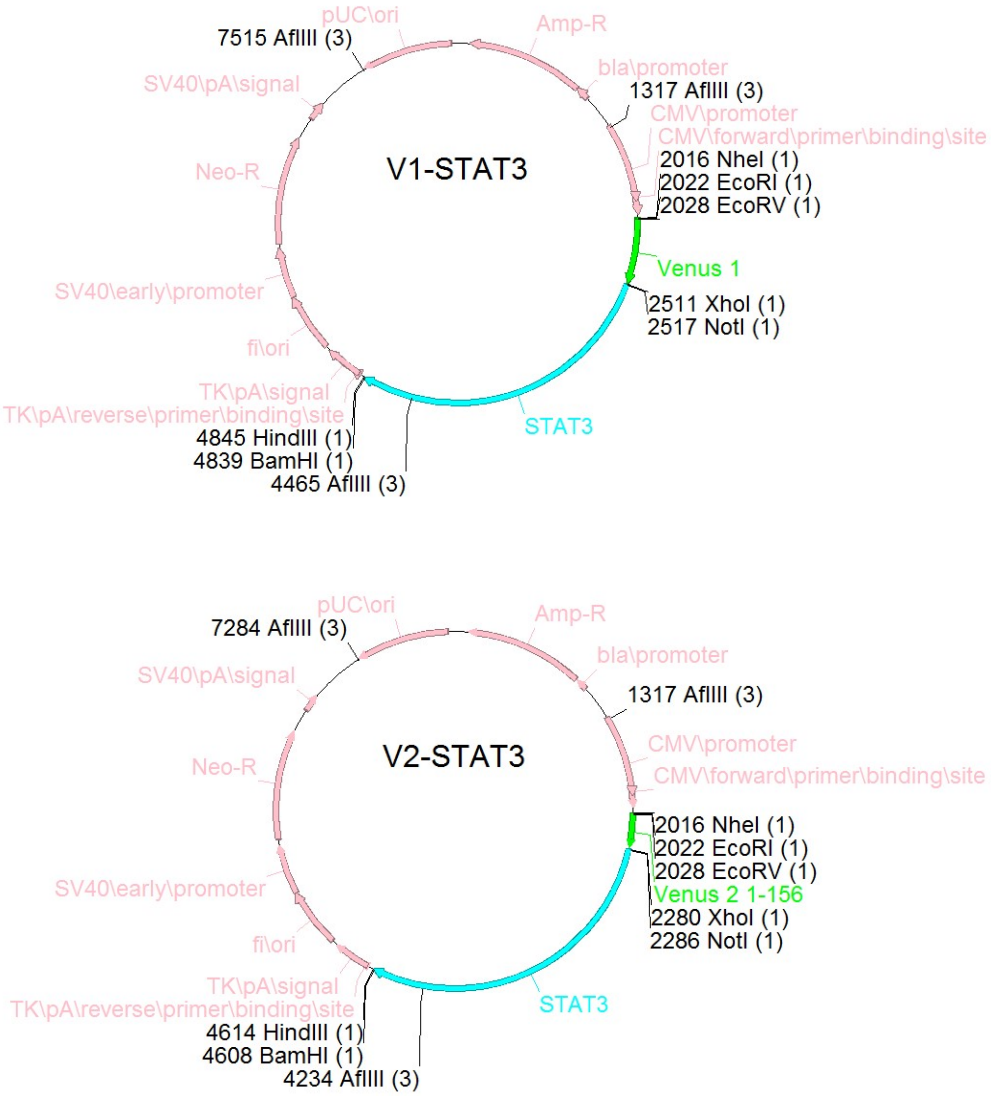

Figure 6: Plasmid maps of the BiFC constructs.
Left: V1-STAT3 BiFC construct. Right: V2-STAT3 BiFC construct.

Cell culture, transfections and treatments

All experiments were carried out in human embryonic kidney (HEK293) cells and human prostatic cancer (PC3) cells, maintained in DMEM or DMEM:F12 respectively, and supplemented with 10% FBS and 1X Penicillin/Streptomycin mixture, at 37ºC in a humidified atmosphere of 5% CO_2. For flow cytometry experiments, cells were seeded at $8x10^5$ cells/well (6-well plates). For microscopy, cells were seeded at $8x10^5$ cells/dish in 35mm glass-bottom

dishes coated with poly-L-lysine (20µg/mL) for better adherence. For western blotting, cells were seeded at $2x10^6$ cells/dish in 60mm dishes. For MTT viability assays, cells were seeded at $3{,}25x10^3$ cells/well in 96 well dishes. For LDH assay, 100µL of medium were taken from the plates where the cells were seeded (flow cytometry, microscopy or western blotting).

Transfections were carried out 24 h later, using Lipofectamine ® 2000, in a ratio DNA:Lipofectamine of 1µg:4µL. Culture medium was replaced 19 h after transfection with serum-free culture medium for 2 h, followed by incubation with LIF or IL-6 (100 ng/mL) for another 2 h in serum-free medium. Treatments with drugs or inhibitors were performed in two possible experimental conditions, as specified in each case: either after changing serum-containing medium for serum-free medium for a total of 4 h before samples were collected or from the moment of transfection (19 h).

Fluorescent Microscopy

Living or fixed cells were examined in a custom-built Nikon Eclipse TE2000-S inverted fluorescence microscope equipped with a Hamamatsu Flash 2.8 sCMOS camera. Photos were taken using a 100x objective and handled with ImageJ free software.

For immunocytochemistry cells were rinsed in PBS once, permeabilized in ice-cold methanol for 10 min and washed in PBS (3x 5 min). Samples were then blocked in 1% BSA in PBS for 1 h and incubated with primary anti-STAT3 antibody (1:1000 in 1% BSA) at 4ºC overnight. After washing samples again with PBS (3 x 5 min), they were incubated with Alexa Fluor 594 secondary antibody (1:1000 in 1% BSA) for 1 h at room temperature in the dark. Nuclei were stained with DAPI (1µg/mL) for 10 min, washed (3 x 5 min) and observed under a fluorescent microscope.

Flow cytometry

Cells were washed once in PBS, trypsinized (0.05% w/v) for 5 min at 37ºC and collected in microcentrifuge tubes. After centrifugation (300xg, 5 min at RT), supernatant was discarded and the cell pellet resuspended in 1%PFA in PBS. Fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) equipped with a low-power air cooler 15mW blue (488nm) argon laser and a red (635nm) diode laser (band-pass filter 530/30nm). For each experimental group 10000 events were analyzed. Data were analyzed by means of FlowJo (Tree Star Inc.,OR,USA) and Flowing free software (Turku Centre for Biotechnology, Finland).

The procedures were similar in the case of the kinase inhibitor screening, but we did not fix the cells to be able to identify dead cells by means of propidium iodide (PI). PI is not cell-permeable, and will enter only those cells with a damaged membrane. PI (1mg/mL) was added immediately prior analyses, and BiFC and PI fluorescence were determined simultaneously. The percentage of PI-positive cells was considered as a relative measure of cell death.

Western blotting

For nuclear and cytoplasmic protein extraction, cells were washed in PBS, trypsinized (0.05%w/v) for 5 min at 37ºC and collected in microcentrifuge tubes. After centrifugation, the supernatant was discarded and the pellet resuspended in cytoplasmic extraction reagent I (plus protease inhibitors) following the instructions of the NE-PER nuclear protein extraction kit (Thermo Scientific, MA, USA). The suspension was homogenized by vortex and incubated in ice for 10 min. We then added cytoplasmic extraction reagent II (Thermo Scientific, MA, USA), and the mixture was vortexed and centrifuged. The resulting supernatant, which is the cytoplasmic protein extract, was collected and stored at -20ºC. The pellet was resuspended in nuclear extraction reagent plus protease inhibitors (Thermo Scientific, MA, USA) and homogenized using vortex (4x in 10-min intervals). The suspension was then centrifuged and the supernatant (nuclear protein extract) was collected and stored at -80ºC.

Protein concentrations were determined using Bradford solution and a standard curve with known protein concentrations (Bovine serum albumin from 0.125 to 2 µg/µL). Samples were incubated for 10 min with Bradford reagent in a microplate and their absorbance read at 595nm in a microplate reader.

Loading buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 mM Tris-HCl, pH6.8) was added to 20µg of protein extract per sample and boiled for 5 min. Protein samples were separated on 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S (0.1% w/v) to verify protein transfer efficiency. To remove Ponceau, membranes were washed in Tris-buffered saline Tween 20 (TBST) (150mM NaCl, 50 mM Tris pH 7.4, 0.05 % Tween-20). Blocking was performed in 5% (w/v) non-fat dry milk in TBS for 1 h at room temperature. After another washing step with TBST (3x 5 min), membranes were incubated with primary antibodies diluted in 5% BSA in TBS 1 x and 0.05% w/v of sodium azide overnight at 4ºC. Primary antibodies used were anti-STAT3 (1:1000, rabbit monoclonal), anti-Phospho-STAT3 (Tyr705, 1:1000, rabbit polyclonal), anti-Lamin B (1:1000,

goat polyclonal) and anti-GAPDH (1:1000, mouse monoclonal). Lamin B is a nuclear protein and GAPDH is a cytosolic protein that served as loading controls for nuclear and cytosolic extracts, respectively. After the incubation period, membranes were washed with TBST (3 x 5 min) and incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (HRP) (1:10000 in 5% w/v non-fat dry-milk) for 1 h at 4ºC. Membranes were washed with TBST (3x 5 min), incubated for 1 min with chemiluminescent HRP substrate and processed for protein detection using a Chemidoc XRS + device (Biorad, CA, USA).

MTT assay

MTT is a viability assay based on a readout of cellular metabolic activity. Intracellular NAD(P)H-dependent oxidoreductases in viable cells, are capable of reducing MTT into formazan precipitates. Formazan is then solubilized by adding DMSO or other solvents and the absorbance of the solution at 570 nm is quantified. In principle, absorbance is directly proportional to the proportion of metabolically active, viable cells.

HEK293 cells were seeded in 96 well plates. Twenty-four hours later the cells were incubated with the different drugs for a period of 19 h. After this period, 10 µL of MTT was added to each well and incubated for a period of 2 h (when a purple formazan precipitate was visible). Finally, 100 µL of DMSO 100% v/v was added to each well. The plate was left at room temperature in the dark for 2 h. Absorbance was read at 570 nm in a microplate reader (Thermo Fisher (MA, USA), against adequate control groups (blank wells with only medium and untreated cells).

LDH assay

LDH (lactate dehydrogenase) is a cytoplasmic oxidoreductase that converts lactate to pyruvate, and is released to the medium in the case of damage to the cell membrane. The amount of LDH released to the medium is proportional to cell damage and can be quantified by an enzymatic reaction. In a first step, NAD+ is reduced to NADH/H+ by LDH-mediated conversion of lactate to pyruvate. In a second step, a catalyst (diaphorase) transfers the H/H+ from NADH/H+ to a yellow-colored tetrazolium salt, reducing it to formazan. The amount of formazan produced is then directly proportional to LDH activity and therefore to cell toxicity, exactly the opposite to MTT assays.

For LDH assay, a sample of 100µL of medium was removed from each experimental plate and placed in triplicate in a 96 well plate. According to the manufacturing protocol, 100µL

of solution C was added in each well and incubated for 20 min at room temperature, protected from light. Absorbance was read at 490 nm, against adequate controls.

Statistical Analysis

Statistical analysis was performed using SigmaPlot software (San Jose, USA). Experiments were done in triplicate, unless otherwise indicated. Results are shown as the average ± standard deviation and were analyzed by a one-way ANOVA test followed by a Tukey´s Multiples Comparison Test for comparison of averages. Results were considered significant when the p value was lower than 0.05. Some of the results presented are preliminary (n=1) and this is indicated in the corresponding figures and sections of the manuscript.

Results

STAT3 dimerizes prior to activation and is mainly localized in the cytoplasm

To test the functionality of our BiFC system in living cells, PC3 cells (STAT3 null) were transfected with the two Venus-STAT3 BiFC plasmids (V1-STAT3 and V2-STAT3) separately or together. Only cells transfected with both plasmids showed fluorescence, as assessed by flow cytometry (Figure 7). Previous results from our laboratory indicate that STAT3 does not dimerize with other proteins tagged with Venus BiFC fragments (Ana Maia Rocha, MSc thesis, Faculdade de Ciências da Universidade de Lisboa, 2015), suggesting that this fluorescence is specific and indicative of spontaneous dimerization of non-activated STAT3.

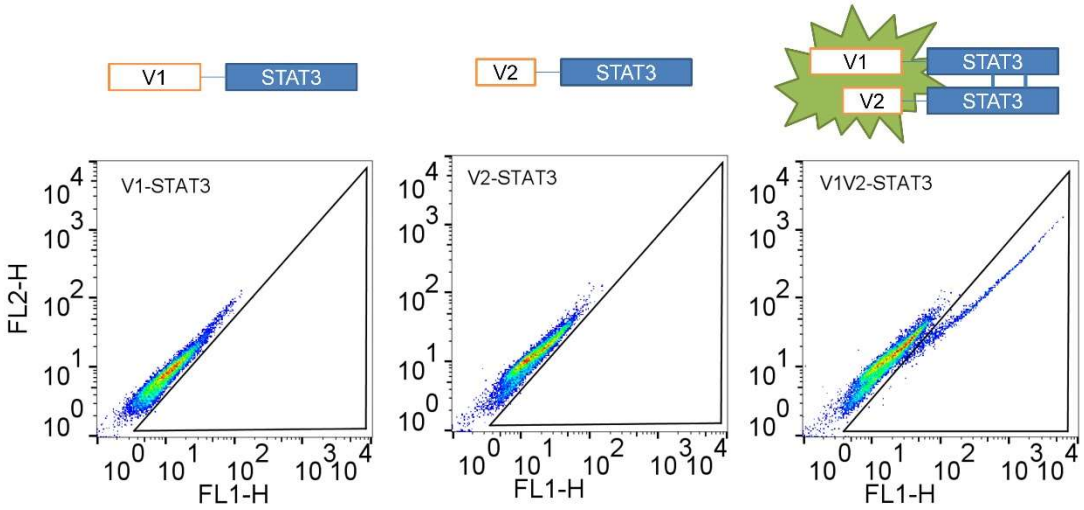

Figure 7: Flow cytometry results show the presence of fluorescent cells only in the cells transfected with both plasmids.
PC3 cells (STAT3 null) were transfected with the two plasmids (V1-STAT1 and V2-STAT3), separately or together. After 19 hours, 10000 events were analyzed by flow cytometry. The presence of fluorescence only in the group of cells transfected with both plasmids is an indicator of STAT3 dimerization. Venus fluorescence is mainly detected in FL1. FL2 is used to normalize the signal and distinguish between specific and unspecific fluorescence.

To confirm the presence and distribution of Venus-STAT3 constructs, cytoplasmic cell extracts prepared from these cells were separated by SDS/PAGE and immunoblotted using an antibody specific for STAT3 (Figure 8). Since PC3 cells are STAT3 null, there was no presence of endogenous STAT3 in the first lane, corresponding to untransfected cells. The presence of one or two bands in the other lanes confirmed the presence of the two constructs.

To analyze the subcellular localization of STAT3 dimers, HEK293 and PC3 cells were transfected with both plasmids and visualized by fluorescence microscopy (Figure 9). The presence of fluorescence confirms the spontaneous dimerization of STAT3 dimers. In HEK293 cells, fluorescence was localized predominantly in the cytoplasm, while in PC3 cells, fluorescence was localized both in the nucleus and cytoplasm.

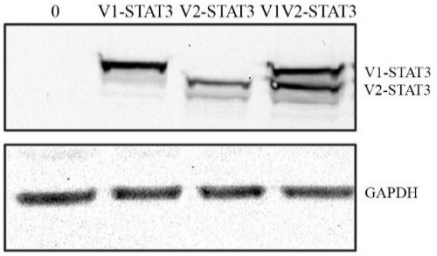

Figure 8: Venus-STAT3 BiFC fusion proteins are effectively expressed in mammalian cells.
PC3 cells (STAT3 null) were transfected with the two BiFC plasmids (V1-STAT1 and V2-STAT3) for 19 hours, separately or together. The cytoplasmic cell extracts were separated by SDS-PAGE and immunoblotted with a specific STAT3 antibody. Since PC3 cells are STAT3 null, there is no presence of STAT3 in the first lane.

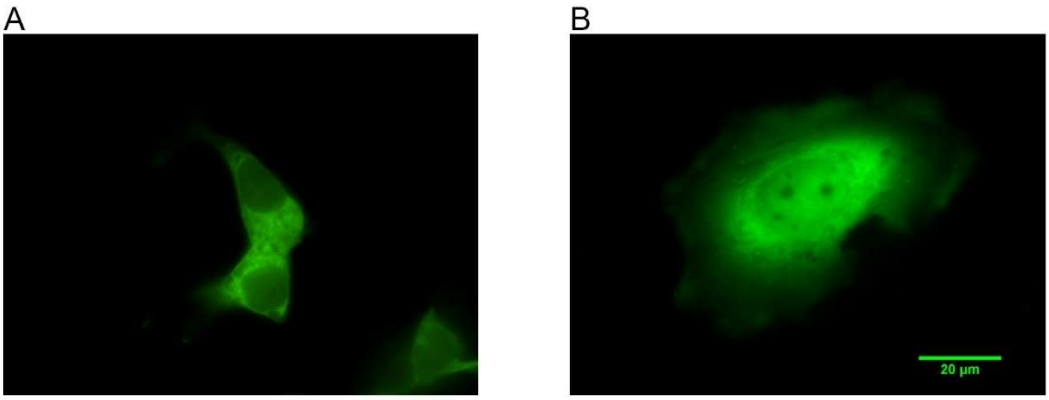

Figure 9: STAT3 dimers have a different location in HEK293 or PC3 cell lines in the absence of stimuli.
A: In HEK293 cells, non-activated STAT3 dimers are located mainly in the cytoplasm. **B:** In PC3 cells, fluorescence is localized both in the nucleus and cytoplasm. Scale bar, 20µm.

Nuclear translocation is dependent on STAT3 activation

To verify that the Venus-STAT3 constructs were fully functional, we stimulated HEK293 and PC3 cells with LIF 100ng/mL from 2 to 6 h, but we show here only the results at 2 h, when the peak of STAT3 phosphorylation and translocation was observed (Figure 10). Cells stimulated with LIF were analyzed by flow cytometry to quantify the amount of dimers

and visualized by microscopy to confirm nuclear translocation. The activation state of STAT3 through Tyr705 phosphorylation was evaluated by western blotting.

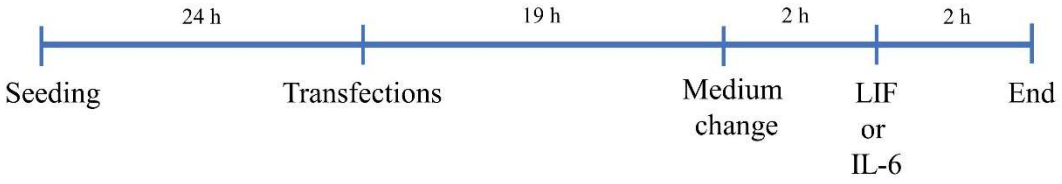

Figure 10: Schematic representation of experimental design.
Cells were seeded, allowed to adhere and grow for 24 h and then transfected with the BiFC constructs. Medium was replaced for serum-free medium 19 h after transfection. LIF or IL-6 (100ng/mL) were added 2 h later. Samples were collected after 2 hours of stimulation with cytokines.

Flow cytometry data from HEK293 and PC3 cells showed that there was no difference in the amount of dimers between control and LIF-stimulated cells (Figure 11). This is consistent with a previous report indicating that STAT3 does not dimerize upon activation, but exists as a dimer prior to activation and only changes conformation when dimers are phosphorylated [20]. Nuclear translocation induced by stimulation with LIF was visible by microscopy in HEK293 but not in PC3 cells (Figure 12). Fluorescence moved from the cytoplasm to the nucleus upon LIF stimulation in 45% of HEK293 cells (Figure 13). IL-6, another member of the IL-6 family of cytokines, was also unable to induce nuclear translocation in PC3 cells (Figure 14).

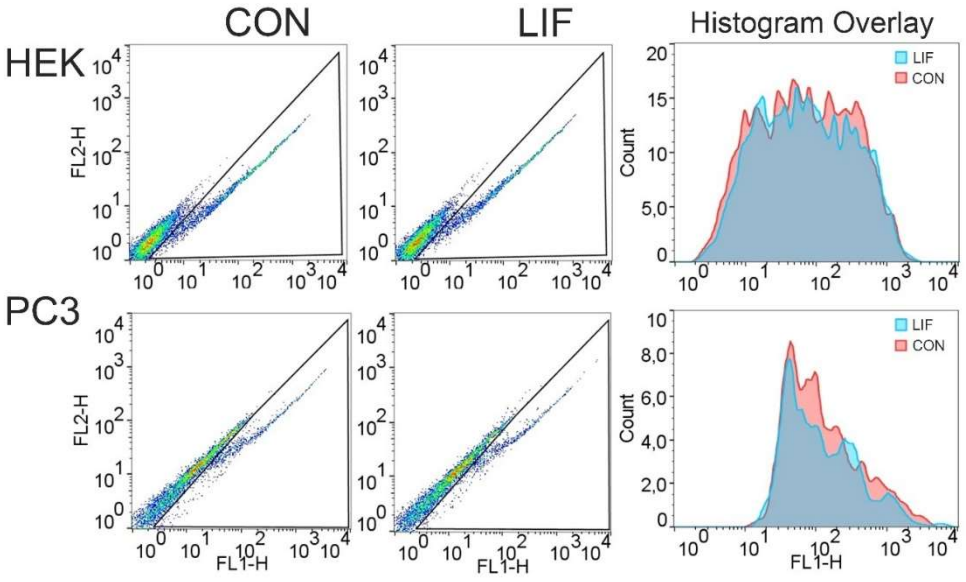

Figure 11: LIF does not induce STAT3 dimerization.
HEK293 and PC3 cells were stimulated with LIF (100ng/mL) for 2 hours after 19 hours of transfection. Ten thousand events were analyzed by flow cytometry to quantify the percentage of fluorescent cells.

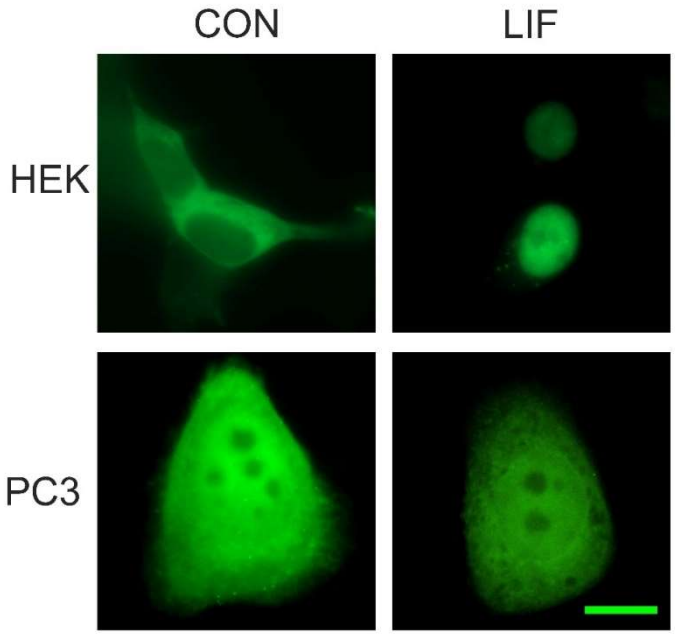

Figure 12: STAT3 translocated to the nucleus of HEK293 cells upon stimulation with LIF.
HEK293 and PC3 cells were transfected for 19 h. Medium was replaced for serum-free medium and after 2 h cells were stimulated with LIF (100ng/mL) for another 2 h. Cells were analyzed by microscopy to visualize nuclear translocation. In HEK293 cells control group, fluorescence is localized mainly in the cytoplasm. After activation of cells with LIF, fluorescence is localized in the nucleus. PC3 cells do not show differences between the control group and cells treated with LIF. Scale bar, 20µm.

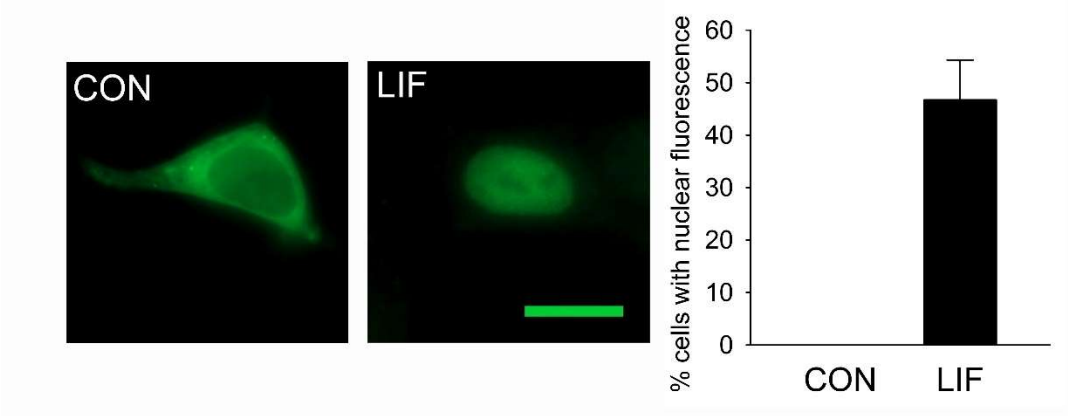

Figure 13: Quantification of STAT3 nuclear translocation upon stimulation of HEK293 cells with LIF.
HEK293 cells were transfected for 19 h. Medium was replaced for serum-free medium and after 2 h cells were stimulated with LIF (100ng/mL) for another 2 hours. After stimulation with LIF approximately 45 % of cells show fluorescence only in the nucleus. Scale bar - 20µm.

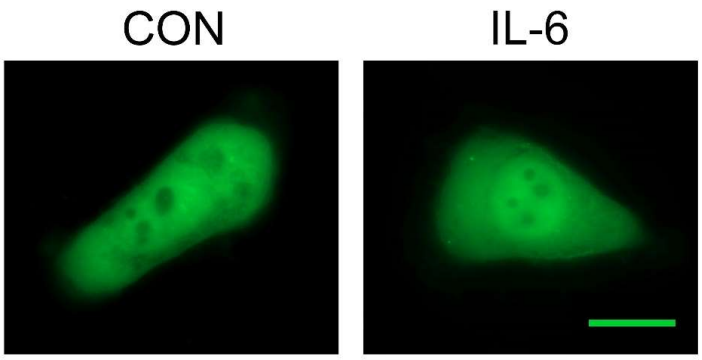

Figure 14: IL-6 does not induce STAT3 nuclear translocation in PC3 cells.
PC3 cells were transfected for 19 h. Complete medium was changed for serum-free medium and after 2 h, cells were stimulated with IL-6 (100ng/mL) over a period of 2 h. Scale bar, 20µm.

Nuclear protein extracts of HEK293 and PC3 cells treated with LIF and/or IL-6 were separated by SDS/PAGE and immunoblotted against specific P-STAT3(Y705) and STAT3 antibodies (Figure 15). In HEK293 cells, there was an accumulation of phosphorylated and total STAT3 in the nucleus, while in PC3 cells neither LIF nor IL-6 were able to phosphorylate STAT3 or increase its nuclear levels significantly.

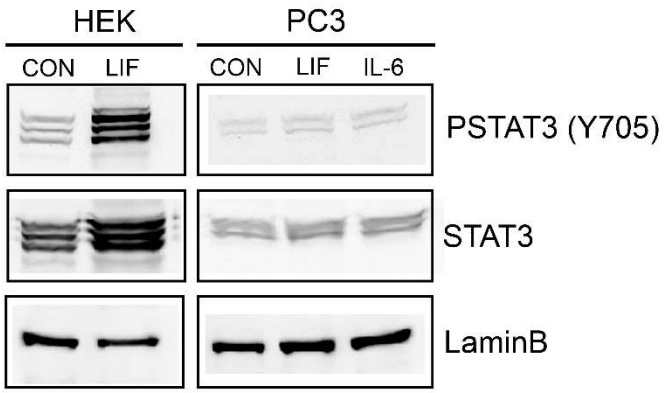

Figure 15: LIF induces STAT3 phosphorylation in HEK293 cells.
Cells were transfected for 19 h. Medium was replaced for serum-free medium and after 2 h, cells were stimulated with LIF and/or IL-6 (100ng/mL) for another 2 h. Nuclear protein extracts of control or cytokine-treated cells were separated by SDS-PAGE and immunoblotted against P-STAT3(y705) and STAT3. LaminB levels were used as a loading control.

Stattic inhibits dimerization and nuclear translocation of STAT3

To further assess the functionality of the Venus-STAT3 BiFC system, we used a STAT3 specific inhibitor. Stattic is a non-peptide small molecule that inhibits the function of the STAT3 SH2 domain, inhibiting STAT3 activation, dimerization and nuclear translocation [69]. HEK293 cells were incubated with Stattic (0.5 or 1μM) from the moment of transfection with Venus-STAT3 BiFC constructs, in order to study its effect on STAT3 spontaneous dimerization. Flow cytometry results showed a decrease in the amount of fluorescent cells with increasing concentrations of Stattic (Figure 16). The same result was not verified when Stattic was introduced to the cells after 19 hours of transfection, in a total of 4 hours (Figure 17). Immunoblotting of total protein extracts with a specific antibody against STAT3 showed no significant decrease in the expression of the protein (Figure 18), indicating that the decrease in fluorescence was not due to an unspecific effect of Stattic on STAT3 expression.

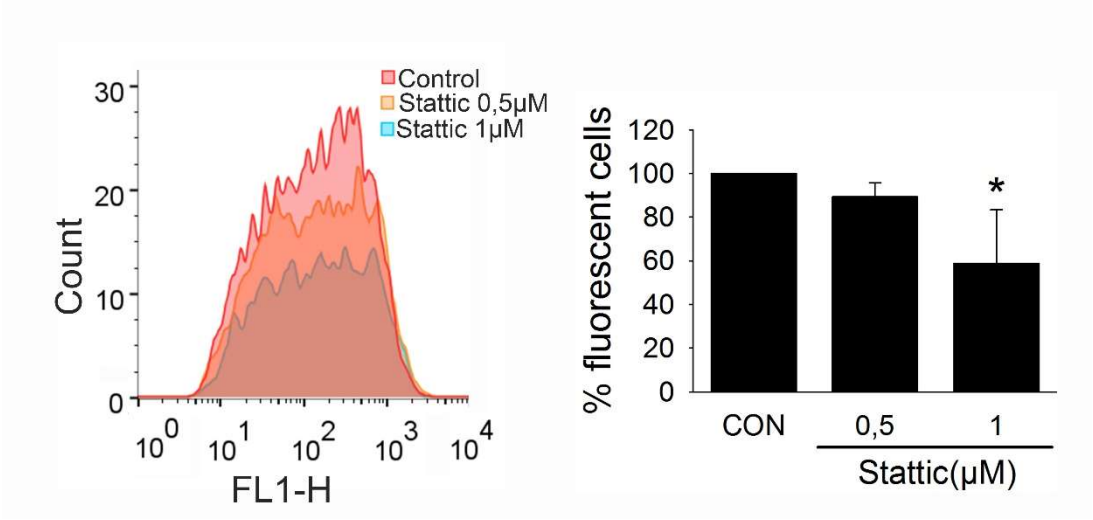

Figure 16: Stattic inhibits STAT3 dimerization in HEK293 cells.
HEK293 cells were incubated with Stattic from transfection (19 h), to study the effect of the inhibitor over STAT3 spontaneous dimerization. A total of 10000 events were analyzed. Flow cytometry results show a decrease in the amount of fluorescent cells, with increasing concentration of Stattic (* $p<0.05$).

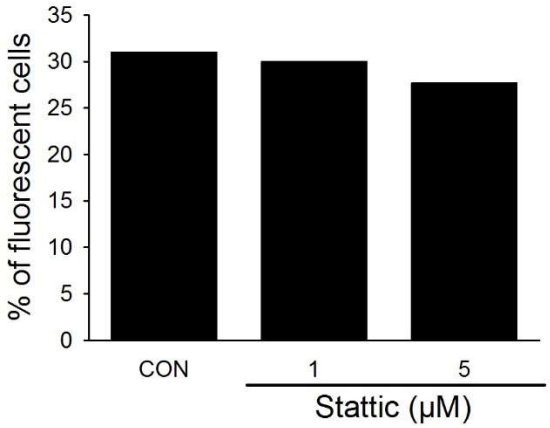

Figure 17: Lower exposure times with Stattic do not inhibit STAT3 dimerization.
HEK293 cells were incubated with Stattic for 4 hours after 19 hours of transfection. A total of 10000 events were analyzed. Flow cytometry results do not show a decrease in the amount of fluorescent cells.

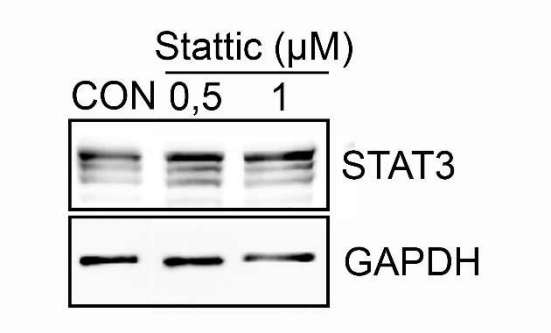

Figure 18: Stattic does not decrease the expression of STAT3.
HEK293 cells were incubated with Stattic from the moment of transfection (19 h). Total protein extracts were separated by SDS-PAGE and immunoblotted with STAT3 specific antibody. GAPDH levels were used as a loading control.

To study the effect of Stattic over nuclear translocation, cells were treated with Stattic (1 and 5 μM) for a total period of 4 h, in the presence or absence of LIF for 2 h (Figure 19). In unstimulated control cells, the localization of STAT3 dimers is predominantly cytoplasmic. After stimulation with LIF, STAT3 dimers translocate to the nucleus. The presence of Stattic inhibits STAT3 translocation to the nucleus in a dose dependent manner. However, Stattic 5μM is clearly toxic to cells and alters STAT3 subcellular distribution in both unstimulated and stimulated cells.

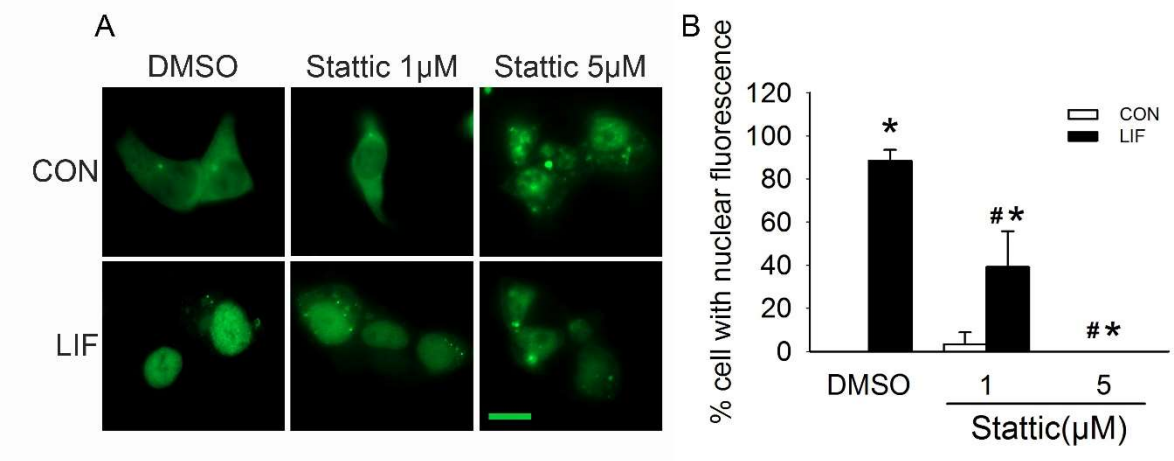

Figure 19: Stattic inhibits STAT3 nuclear translocation.
A: Cells were transfected for 19 h. Medium was replaced for serum-free medium and cells were treated with Stattic for a total period of 4 h in the presence or absence of LIF (100ng/mL) for 2 h. In unstimulated cells, dimers are located predominantly in the cytoplasm. Stimulation with LIF induces nuclear translocation of STAT3 dimers. Scale bar, 20μm. **B:** Stattic reverses nuclear translocation in a dose-dependent manner (*, significant vs CON, $p<0.05$; #, significant vs DMSO plus LIF, $p<0.05$).

In a small number of cells, we observed an accumulation of fluorescence in vesicles/aggregates mostly in the cytoplasm. The amount of aggregates increased upon incubation with LIF (100ng/mL) and/or Stattic 5µM (Figure 20).

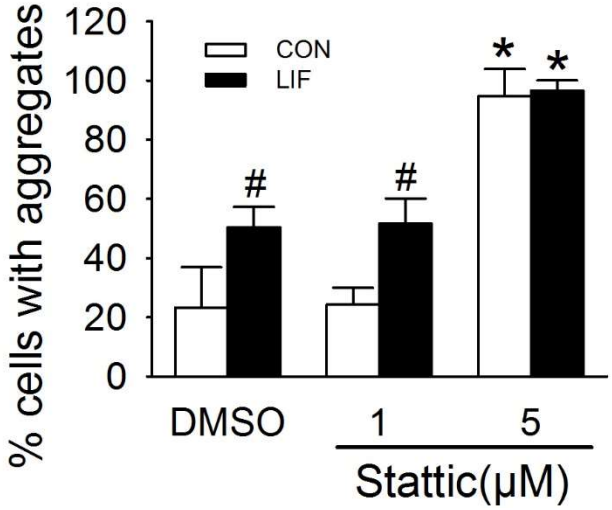

Figure 20: The presence of Stattic and LIF increases the formation of STAT3 aggregates.
Cells were transfected for 19 h. Medium was replaced for serum-free medium and cells were treated with Stattic for a total period of 4 h in the presence or absence of LIF (100ng/mL) for 2 h. The amount of aggregates increased when LIF or Stattic were introduced to the cell (*, significant vs CON plus DMSO, $p<0.05$; #, significant vs CON, $p<0.05$).

A molecular screening to identify new drugs that inhibit STAT3 dimerization and/or nuclear translocation

We used the BiFC system to test the ability of a group of dinuclear macrocyclic complexes to inhibit STAT3 dimerization and translocation to the nucleus, numbered 1 to 8 for the sake of simplicity. These complexes were produced by Dr. Rita Delgado´s group (ITQB-NOVA, Portugal). We had previously shown that the parental molecule of these dinuclear macrocyclic complexes effectively inhibited STAT3 activity (Figure 21) [70].

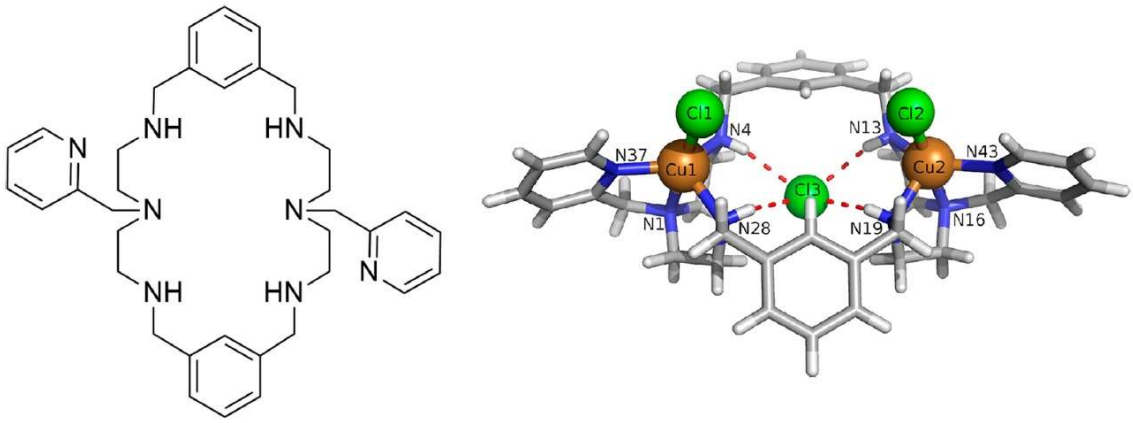

Figure 21: Parental structure of our dinuclear complexes.
Left: Structure of the [Cu2L(Cl)2⊃Cl]+ complex cation. Right: Cristal structure of the samel molecule, determined by single crystal X ray diffraction data [70].

Incubation of HEK293 cells with the different dinuclear complexes (16.25-130µM) for a period of 19 h showed no evidence of toxicity as determined by the MTT viability assay (Figure 22). We therefore used a concentration of 130µM for the following experiments.

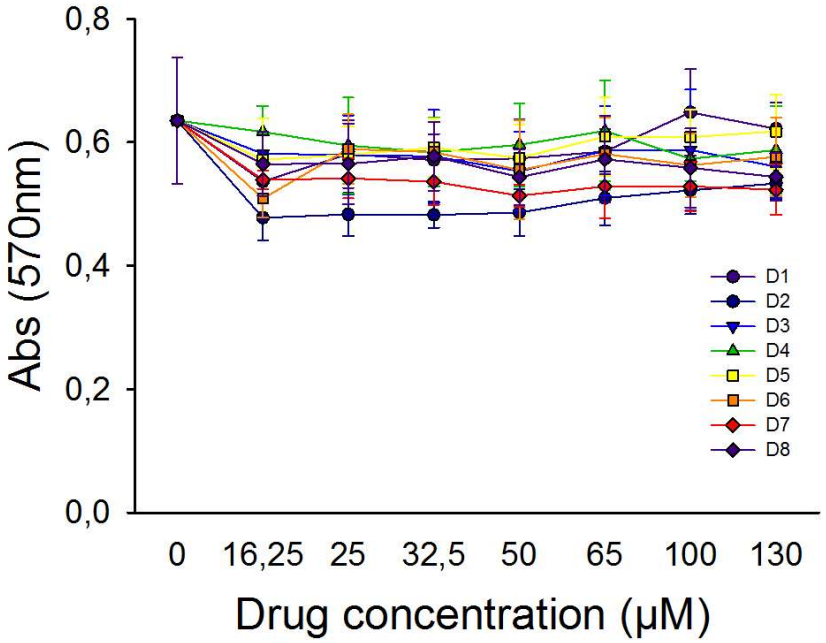

Figure 22: Dinuclear complexes are not toxic.
HEK293 cells were seeded in a 96 well plate and treated with drugs for 19 h. Concentration used ranged from 16.25 to 130µM. Higher absorbance values indicate higher cell viability.

To test the effect of these dinuclear complexes on STAT3 dimerization, HEK293 cells were transfected with the BiFC constructs, and 19 h later were incubated with the dinuclear

complexes for 4 h. Only drug 2 seemed to decrease slightly but not significantly the percentage of fluorescent cells transfected with our Venus-STAT3 BiFC system (Figure 23-A). None of the dinuclear complexes produced significant changes in mean fluorescence either (Figure 23-B). Since the dinuclear complexes were synthetized to bind specifically to phosphorylated substrates, we decided to test their effect in cells stimulated by LIF, where STAT3 is phosphorylated (Figure 23-C). However, the results were also negative. These results indicate that these dinuclear complexes, in principle, do not influence the dimerization of STAT3, although we have ongoing experiments to test if the presence of the drugs from the moment of transfection can modulate STAT3 dimerization (see Discussion).

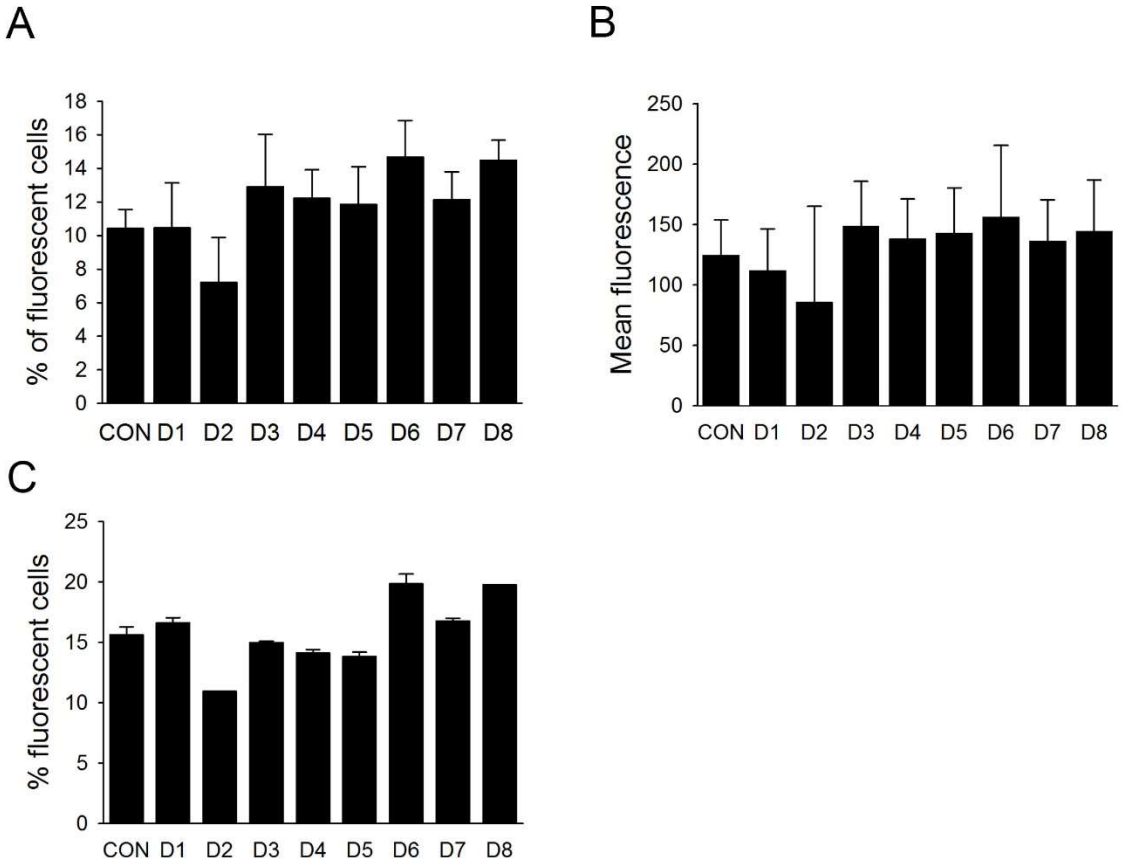

Figure 23: Dinuclear complexes do not prevent STAT3 dimerization.
After 19 h of transfection, HEK293 cells were incubated with the different dinuclear complexes at a concentration of 130µM for a period of 4 h. A: Percentage of fluorescent cells. B: Mean fluorescence. C: After the period of 19 h of transfection, the medium was changed for serum-deprived medium, and cells were treated with the different dinuclear complexes (130 µM). LIF (100ng/mL) was added 2 h later, and samples were collected after 2 h of incubation with LIF.

We proceeded to investigate if these dinuclear complexes inhibited STAT3 nuclear translocation. A representative picture of cells incubated with each dinuclear complex is presented in Figure 24. For a quantitative result, the number of cells with fluorescence in the

nucleus was quantified for each group of cells (Figure 25-A). The number of cells with aggregates was also quantified (Figure 25-B). When performing these experiments, we noted that in the presence of drug number 2, there was a larger number of cells detached from the plate. Despite the fact that MTT results indicated that this drug was not toxic at this concentration, we decided to perform LDH assays to confirm our results. In fact, LDH assay indicated that cells treated with drug number 2 have a significant increase ($p<0.05$) in released LDH activity, indicating toxicity.

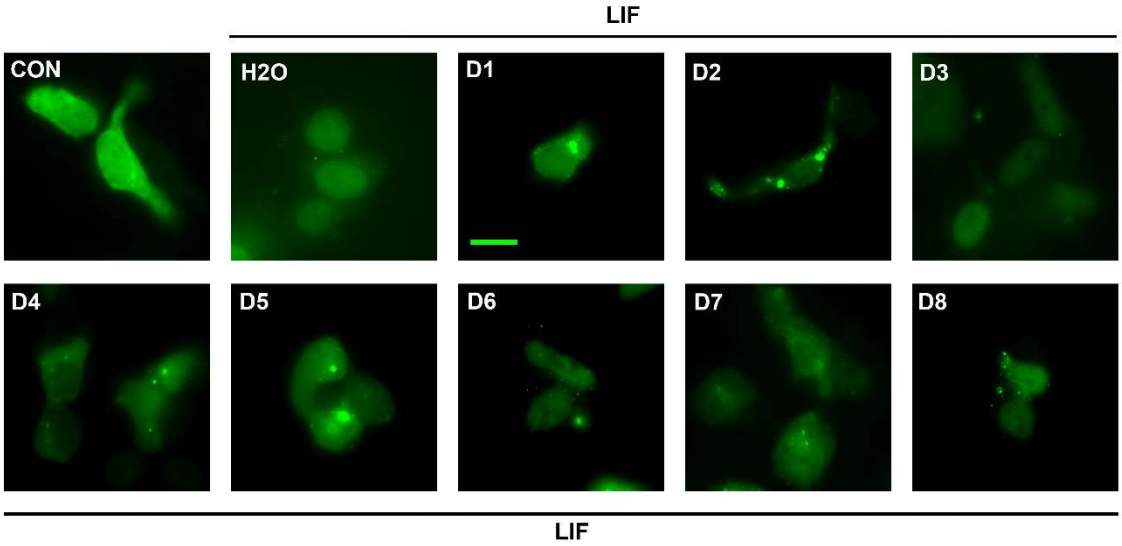

Figure 24: Dinuclear complexes inhibit STAT3 nuclear translocation.
After 19 h transfection, medium was changed to non-serum medium. HEK293 cells were incubated with the different dinuclear complexes (130µM) for a total period of 4 h and 2 h with LIF (100ng/mL). Scale bar -20µm.

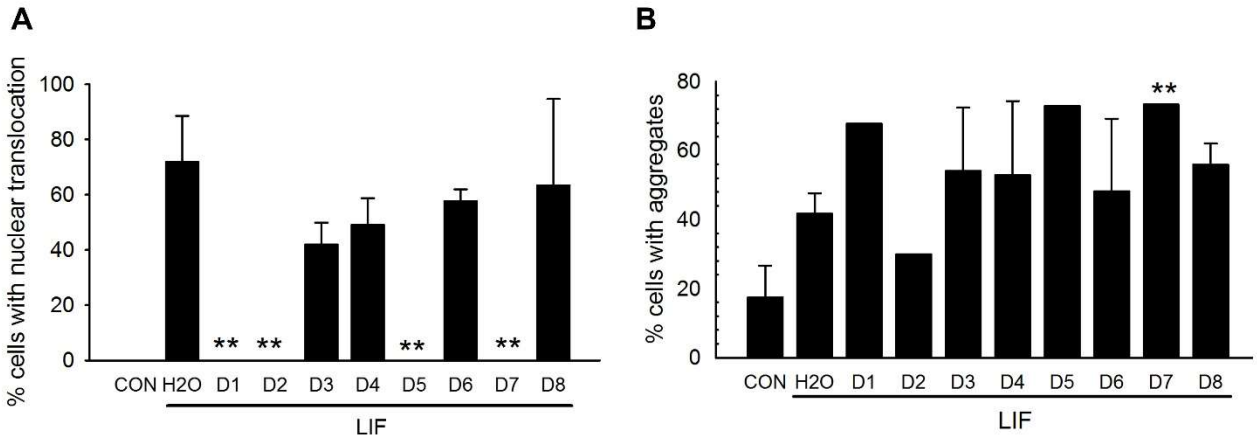

Figure 25: Quantification of microscopy results on STAT3 nuclear translocation.
After 19 h transfection, medium was changed to non-serum medium. HEK293 cells were incubated with the different dinuclear complexes (130µM) for a period of 4 h total and with LIF (100ng/mL) for 2 h. A: The graph represents the percentage of cells with fluorescence in the nucleus. B: % of cells with aggregates. (**, significant vs H2O, $p<0.05$)

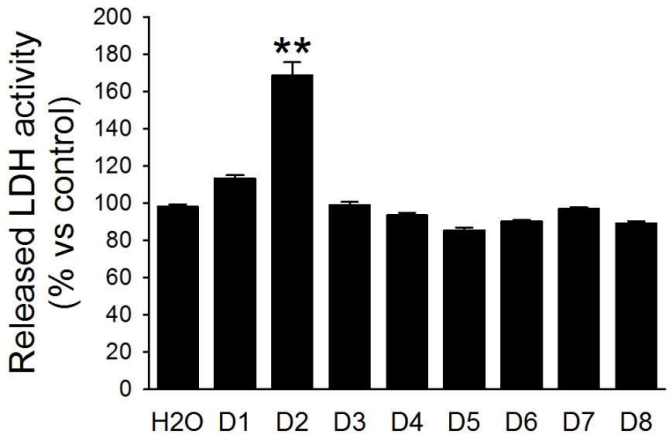

Figure 26: Dinuclear complex number 2 induces toxicity as determined by the LDH assay.
Medium samples removed from the plates containing HEK293 cells treated with drugs in the presence of LIF were used to perform LDH assay (**, significant versus control group (H_2O), $p<0.05$).

The phosphorylation state of STAT3 was analyzed by Western blotting. HEK293 cells were treated with the dinuclear complexes for a total of 4 h, in the presence of LIF (100ng/mL) for 2 h. Nuclear protein extracts were immunoblotted with P-STAT3(Y705) and STAT3 (Figure 27). The lanes corresponding to complexes 1, 5 and 7 show a striking decrease or absence in P-STAT3 (Y705).

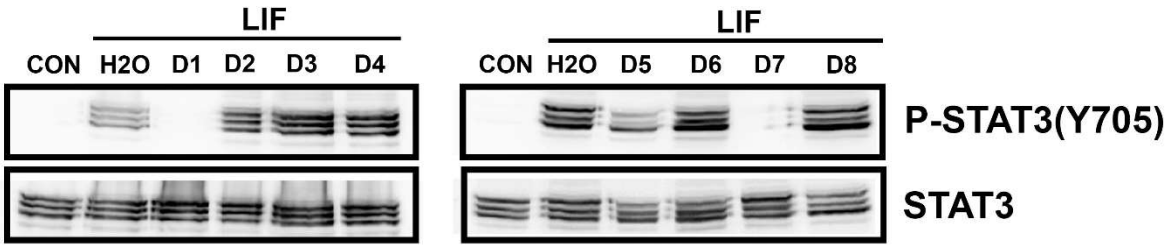

Figure 27: Dinuclear complexes 1, 5 and 7 inhibit STAT3 phosphorylation.
Nuclear cellular extracts of HEK293 cells treated with the different dinuclear complexes for 4 h and in the presence of LIF (100 ng/mL) for 2 h were immunoblotted with specific P-STAT3(Y705) and STAT3 antibodies.

Effect of kinase inhibitors on spontaneous dimerization of non-active STAT3

Another molecular screening was performed using a kinase inhibitor library. The main goal was to find a kinase inhibitor that inhibits spontaneous dimerization of non-activated STAT3. For this, HEK293 cells were transfected with the BiFC constructs and treated with a library of kinase inhibitors at a concentration of 1μM. After 19 h, the amount of fluorescent cells was quantified by flow cytometry. In order to assess the toxicity of the inhibitors, the samples were stained with PI right before analyses. The expression of STAT3 was also verified by western blotting of total protein extracts. Since the library contained 194 compounds in a total of three 96 well plates, the work was divided between me and my labmates. Here, I present the results for a total of 67 kinase inhibitors, and the reader is advised that these results are only preliminary (n=1).

Flow cytometry results indicate that some of the inhibitors tested are able to decrease the percentage of fluorescent cells, indicating that they inhibit STAT3 spontaneous dimerization (Figure 28). From all the inhibitors, three of them (D8, G6 and G10) decreased the percentage of fluorescent cells by more than 50%.

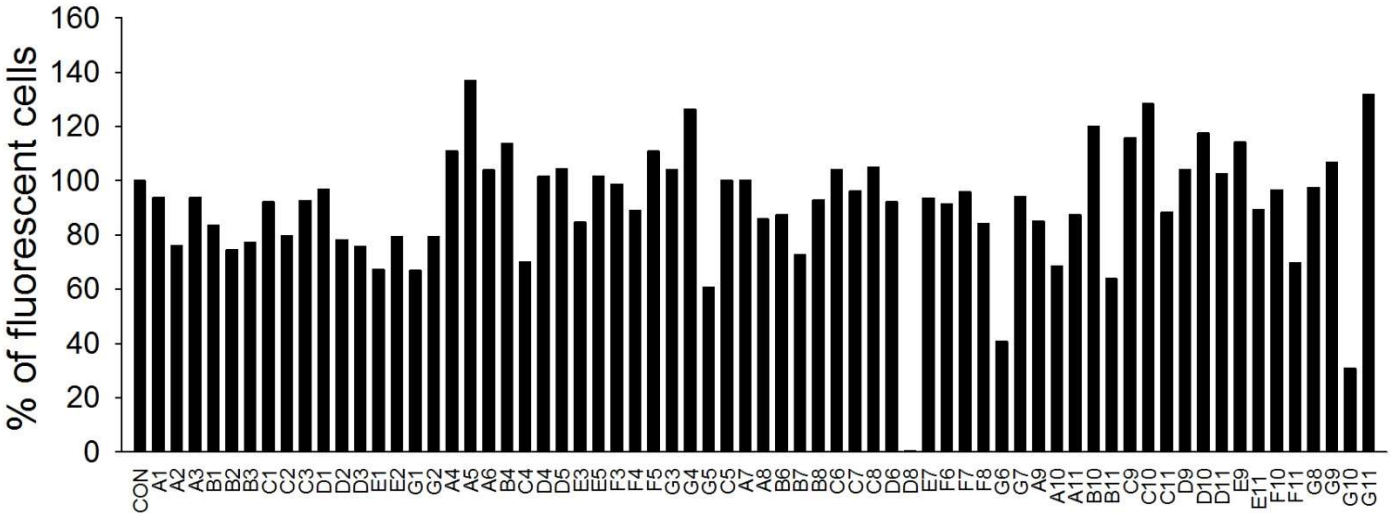

Figure 28: Kinase inhibitors decrease spontaneous dimerization of non-activated STAT3.
HEK293 cells treated with the different kinase inhibitors from the time of transfection. Flow cytometry results indicate a range of inhibitors able to decrease the percentage of fluorescent cells.

In order to rule out the possibility that the changes observed in fluorescence were due to toxicity, the population of PI-positive dead cells was quantified in each group (Figure 29). From the three potential inhibitors presented above, only G10 does not increase toxicity significantly.

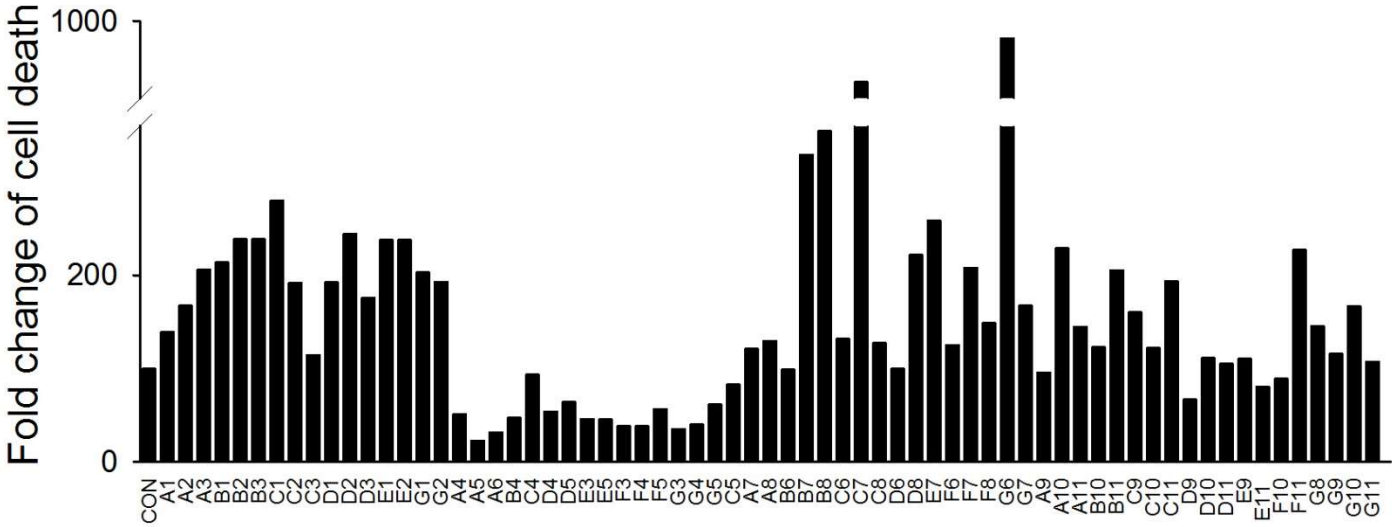

Figure 29: Toxicity of kinase inhibitors.
HEK293 cells were treated with the different kinase inhibitors at the time of transfection. After 19 h, cells were stained with PI and analyzed by flow cytometry. The results indicate the percentage of dead cells versus control (CON), which was incubated with the vehicle (DMSO 0.1 % v/v).

To rule out that our hits were due to a decreased expression of STAT3 BiFC constructs, total protein extracts were immunoblotted with a specific STAT3 antibody (Figure 30). D8 induces a striking decrease in STAT3 levels and G10 seem to slightly decrease the expression of STAT3. However, in the case of G10 the band corresponding to GAPDH is also slightly decreased, so we still have to confirm and determine what is the effect of this compound on STAT3 dimerization. These results are still preliminary, and we are now carrying out further experiments to confirm or reject them.

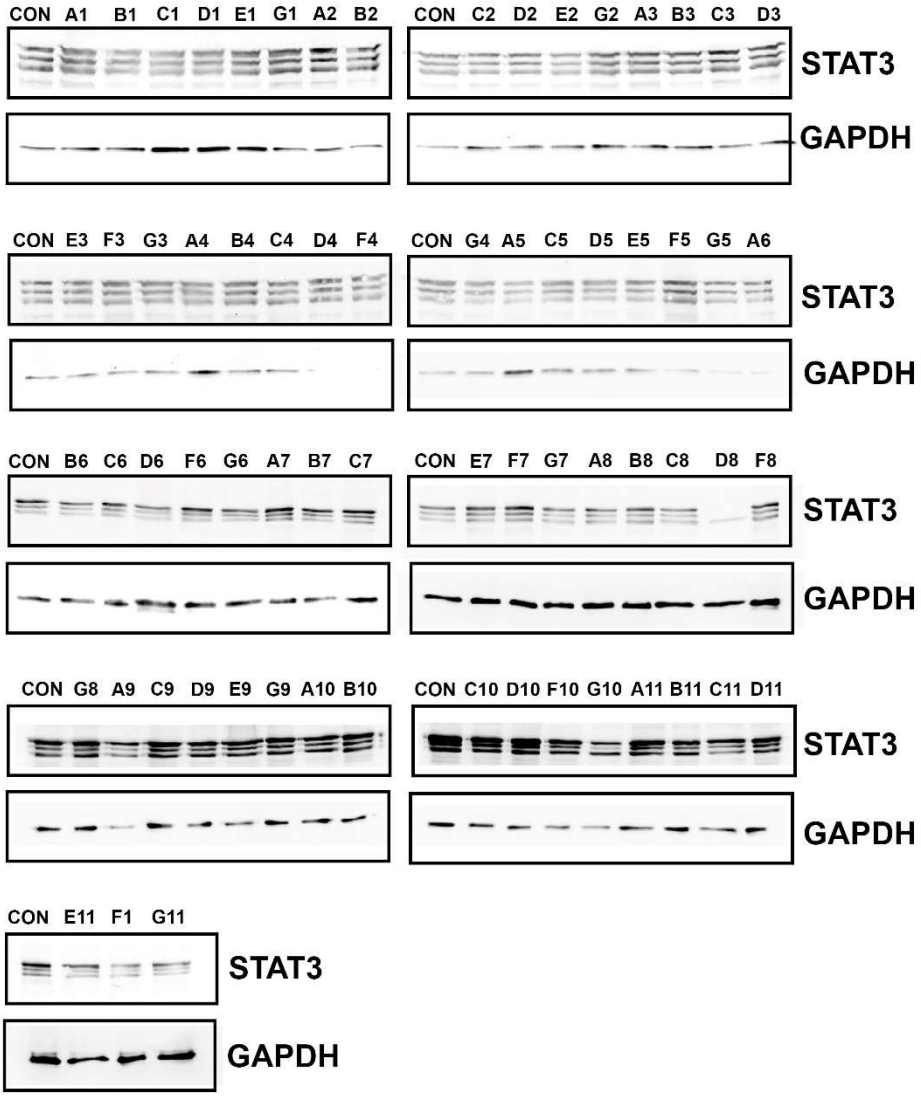

Figure 30: Effects of kinase inhibitors on STAT3 expression.
Total protein extracts from HEK293 cells treated with the different kinase inhibitors (1μM) for 19 h were immunoblotted against STAT3 specific antibody. GAPDH levels were used as a loading control.

Discussion

STAT3 is a transcription factor that plays a key role in numerous cellular events, both in physiological and pathological conditions. Its dimerization, phosphorylation and nuclear translocation are critical steps that lead to the transcription of specific sets of genes. Despite the original assumption that STAT3 existed as monomers and dimerized only when phosphorylated, recent evidence indicate that STAT3 is able to associate prior to activation [19, 20, 61, 71]. Our results with the Venus-STAT3 BiFC system are consistent with the literature, as they indicate STAT3 dimerization in the absence of stimuli. Venus-based BiFC systems have some tendency to spontaneous binding of Venus halves without the mediation of the interaction of the proteins of interest [63, 72]. However, previous results from our group indicated that Venus-STAT3 fusion proteins did not complement with other BiFC fusion proteins (Ana Maia Rocha, MSc thesis, Faculdade de Ciências da Universidade de Lisboa, 2015), indicating that our signal in the STAT3 BiFC system is at least partially specific. This is also supported by the facts that 1) a STAT3 inhibitor partially prevented the formation of functional fluorophore (Figure 16 and Figure 19); 2) LIF induced STAT3 phosphorylation but did not increase the amount of dimers (Figure 15 and Figure 11); and 3) LIF promoted the translocation of all fluorescence to the nucleus (Figure 12).

Most current methods to study STAT3 protein interactions involve direct or indirect damage to living cells. This question was partially addressed by the use of the FRET technique, that allowed the study of STAT3 interactions in living cells [61]. These authors demonstrated visually that STAT3 actually dimerize before stimulation in living cells, and reported that the intensity of the signal actually increased when STAT3 dimers were phosphorylated, indicating a conformational change. Despite interesting, this can be misleading, as there is no solid information about the actual conformation of STAT3 dimers before and after stimulation. FRET requires the fluorophores to be at a very close proximity (100Å) to observe a FRET signal, and its experimental setup is not very user-friendly for high throughput screenings. On the other hand, our BiFC system would allow to detect protein-protein interactions occurring at higher distances, as it happens in macromolecular complexes, ad carry out high-throughput screenings. We believe both systems could provide complementary information about the behavior of STAT3 dimers.

The functionality of the Venus-BiFC system was tested in HEK293 and PC3 cells, transfected with the BiFC constructs (V1-STAT3 and V2-STAT3), separately or together. PC3 cells were chosen because they are STAT3-null, allowing us to study STAT3 in the absence of

endogenous interferences. In HEK293 cells, the localization of non-activated STAT3 dimers is cytoplasmic. This is congruent with the idea that non-activated STAT3 dimers are in a latent state in the cytoplasm "waiting" for stimuli to translocate to the nucleus. On the other hand, PC3 cells show fluorescence in both the cytoplasm and the nucleus, consistent with previous reports where latent STAT3 is described as shuttling permanently between nucleus and cytoplasm [3][73]. Upon stimulation with LIF or IL-6, only HEK cells showed STAT3 phosphorylation and nuclear translocation. This was very surprising, because PC3 cells express gp130, IL-6 and LIF receptors [27], so in theory they should respond to stimulation with either LIF or IL-6. We need to confirm the expression profile of our batch of PC3 cells regarding the key elements of the JAK/STAT pathway. However, these results are consistent with those from other authors who found that these cells do not respond to LIF or IL-6 in the context of proliferation and apoptosis assays [27, 51].

LIF and IL-6 induce STAT3 phosphorylation at the Tyr705 residue, essential for STAT3 activation [4, 22]. Our results indicate that this is correlated with STAT3 nuclear translocation, but not with the amount of dimers formed. This is consistent with the literature, confirming that STAT3 dimerizes prior to activation [20]. Cells were stimulated with cytokines after a period of serum starvation, in order to ensure that the effects observed were due to the cytokines and not to the presence of other contaminants existing in the serum. In the presence of serum, we were unable to see STAT3 nuclear translocation (Data not shown). In HEK293 cells stimulated with LIF, complete nuclear translocation occurs in around 45% of the cells (Figure 13). Cells that showed fluorescence in both the nucleus and cytoplasm were not included in this figure. It is noticeable that we and other authors observe phosphorylated STAT3 not only in the nucleus. In IL-6-activated PAECs and PASMCs cells, the majority of phosphorylated STAT3 is in the cytoplasm [74]. Therefore, depending on the cell type and context, a portion of activated STAT3 may have a cytoplasmic destination.

To further validate the BiFC system, we used Stattic, a specific inhibitor of STAT3 dimerization, phosphorylation and translocation to the nucleus. Cells were incubated with Stattic from transfection to study the effect of the inhibitor over STAT3 spontaneous dimerization. Flow cytometry results showed a decrease in the amount of fluorescent cells, with increasing concentrations of Stattic, confirming it´s inhibitory effect over STAT3 dimerization (**Erro! A origem da referência não foi encontrada.**). However, the same results were not verified when Stattic was introduced to the cells, after 19 h of transfection (Figure 17).

These results could be explained by the fact that Stattic was not powerful enough to reverse the dimerization of STAT3 once it is complete. In fact, in previous studies, the inhibitory effect of Stattic was tested by the binding of a phosphotyrosine-containing peptide to the STAT3 SH2 domain, not the full protein [69]. The binding of a small peptide to the SH2 domain of STAT3 is much simpler and possibly biased system than the actual binding between two SH2 domains of two full length proteins [73].

In some of the cells studied we realized the presence of aggregates, that increased in amount when cells were treated with Stattic or stimulated with LIF (Figure 20). Despite the fact that this phenomenon cannot yet be clarified, we would like to put forward some possible explanations. First, the non-physiologically high expression of STAT3 in these cells could lead to the formation of aggregates. Second, the N-terminal region of STAT3 is also important to the formation of unphosphorylated dimers [73], and the Venus halves located there could lead to conformational changes that disturb the protein folding. Third, the reassembly of Venus halves could lead itself to the formation of dimers prone to aggregation. Finally, the introduction of Venus halves could also be leading to conformational changes that inhibit the association of STAT3 dimers with modulators and chaperones, leading to its accumulation in the cytoplasm [75].

With the optimization of the BiFC system completed, we decided to test a group of dinuclear complexes synthetized by Dr. Rita Delgado´s group (ITQB-NOVA, Portugal), designed to bind to phosphorylated substrates. In a recent published paper, the parent molecule of these dinuclear complexes was able to decrease STAT3 activity [70]. All the dinuclear complexes have the same parent molecule, but with the addition of Cu2+ (complexes number 2, 4, 6 and 8) or Zn2+ (complexes number 1, 3, 5 and 7). Only drug 2 seemed to have a tendency to decrease the percentage of fluorescent cells. However, incubation was for short periods of 4 hours after transfection so, as already mentioned for Stattic, these drugs may not be powerful enough to reverse dimerization, once it is completed. Since these dinuclear complexes were synthetized to bind to phosphorylated substrates, we treated the cells with the dinuclear complexes in the presence of LIF, but the results were also negative (Figure 23). As already mentioned, STAT3 dimers form spontaneously. If the dinuclear complexes bind to the phosphorylated residue of STAT3, dimerization of STAT3 could still occur through the interaction of other STAT3 domain, such as the N-terminus, or even in complex with the dinuclear complexes. To better understand this, it could be interesting to have more information about the conformation of unphosphorylated and phosphorylated STAT3 dimers. The

conformation of the dimers can protect the pY residue and impede the dinuclear complexes to reach it. Despite these results, we would like to continue our experiments by increasing the time of incubation with the dinuclear complexes up to 24 h or treating the cells from the time of transfection. To investigate the effect of the dinuclear complexes on STAT3 nuclear translocation, microscopy images were quantified for the percentage of cells with nuclear translocation (Figure 25). In a surprising way, drugs number 1, 2, 5 and 7 completely inhibited nuclear translocation.

Regarding the phosphorylation state of STAT3, we can confirm that Drug 1, 5 and 7 inhibit STAT3 phosphorylation at Tyr 705 residue. All these drugs have in common the fact that Zn2+ was added to the parent molecule in substitution of Cu2+ (Figure 27). We do not have a current explanation for this fact, but work is being done to better understand these results. We apologize for not delivering the structure of these complexes, but they are not yet published, and their publication in this thesis would jeopardize our chances for publication in international journals. Dinuclear complex number 2 inhibited nuclear translocation without inhibiting STAT3 phosphorylation. However, this could be explained by the fact that this drug has shown to be toxic in LDH assays (Figure 26) and by microscopic observations. Since the cells seemed damaged or dying upon incubation with drug number 2, the apparent inhibition of nuclear translocation could have been an artifact. It would be necessary to find a non-toxic concentration for this drug, to elucidate if the compound is really active against STAT3.

As can be seen, LDH assays and microscopy observations gave the opposite results to MTT assays regarding drug number 2. The reduction of MTT in the MTT assay depends on the metabolic activity of the cells due to the NADPH flux, and it is used to evaluate a decrease in metabolic activity due to early cell damage. LDH assays, on the other hand, determine LDH released from the cell due to membrane damage that occurs in later stages in the cell death process [76]. If the results with drug number 2 had a biological basis, it could be possible that MTT indicated cell damage when LDH assay would not, but not the other way around. Since microscopy and LDH results are consistent, we can only conclude that compound number 2 interferes with the MTT assay, but we are not sure how this happens.

The inhibitory effect of some of the dinuclear complexes over STAT3 phosphorylation raised an interesting question. If the complexes are designed to bind to phosphorylated residues, then it does not make much sense that they would inhibit STAT3 phosphorylation. However, we find three possible explanations for this. The hypothesis that the parental dinuclear complex bind to the phosphotyrosine residue of STAT3 was based on NMR and X-ray studies [70]. They

showed that the dinuclear complex bind to a small phosphopeptide. However, these dinuclear complexes have high affinity to phosphate groups in general, and they could have captured the soluble phosphate molecules before they are used for STAT3 phosphorylation. In our protocol, the dinuclear complexes were added to the cells before stimulation with LIF. It could be the case that the dinuclear complexes sequestered the phosphate before it could have had the change to phosphorylate STAT3, for example, when JAKs are trans phosphorylating the receptor. The second explanation could be that the dinuclear complexes are actually strong enough to sequester the phosphate from STAT3. The third hypothesis is that they can bind to the phosphorylated residue and make STAT3 undetectable with a P-STAT3 antibody. It would be very interesting to continue investigating these hypotheses. We could reverse our protocol, and add LIF prior to treating the cells with the dinuclear complexes. If we still found a decrease in the amount of P-STAT3(Y705) it could be confirming the second hypothesis. If we found no decrease in the amount of P-STAT3(Y705), it would support the first hypothesis. In this case, the dinuclear complexes could still be able to bind to the phosphotyrosine of STAT3 and remain bonded as part of a complex.

Phosphorylation events play an important role in the JAK/STAT3 pathway. We wanted to determine whether phosphorylation pathways were also relevant for spontaneous STAT3 dimerization of latent STAT3. We tested 63 inhibitors, and our preliminary results indicated that 3 of them (D8, G6 and G10) were able to reduce the percentage of fluorescent cells by more than 50%. However, two of them presented very high levels of toxicity, remaining only G10 as a potential candidate (Figure 29). G10 produced a slight decrease in STAT3 expression, but the signal of GAPDH was also somewhat decreased, leaving our results inconclusive. The actual name of G10 is DCC-2036 and it is a kinase inhibitor that targets the kinases of the Src-bcr-Abl family. In the rest of the library, there is only one more compound that targets Src-bcr-Abl (tested by my labmate Joana Ferreira) and it also decreased the dimerization of STAT3 (Data not shown). For these reasons, we are currently carrying out additional experiments to confirm or reflect these results, and elucidate if the Src-bcr-Abl family is able to regulate the dimerization of latent STAT3.

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Conclusion

Abundant evidence suggests that STAT3 is crucial for many physiological processes but also for the development of many diseases. However, there is still lack of effective therapeutics leading to its inhibition. Since STAT3 does not have its own enzymatic activity it is not easy to target it directly. Furthermore, it still remains to be clarified the molecules that associate with STAT3 and the pathways that can activate it. The Venus-STAT3 BiFC system presented in this thesis could help understand these aspects.

The main goal of this work was to optimize the Venus-STAT3 BIFC system. We accomplished this goal by demonstrating that: 1) Stattic inhibited the formation of dimers; 2) LIF induced STAT3 phosphorylation without increasing the amount of dimers; and 3) LIF promoted the translocation of STAT3 dimers to the nucleus. We also found evidence that STAT3 dimerizes prior to activation, which is congruent with recent literature.

The secondary goals of this work were: 1) to test the effect of novel STAT3 inhibitors on STAT3 dimerization; and 2) to analyze the possible role of phosphorylation pathways on the spontaneous dimerization of non-activated STAT3. We tested a range of dinuclear macrocyclic complexes synthetized by Dr. Rita Delgado (ITQB-NOVA, Lisbon). We found that dinuclear complexes of Zn2+ were able to inhibit STAT3 phosphorylation and translocation to the nucleus. Further work is currently being done to confirm these results and find possible explanations for them. We also used the Venus-BiFC system to perform a molecular screening using part of a kinase inhibitors library. We found one compound from this library that inhibited STAT3 dimerization, and further experiments are being performed to confirm these results and/or find other candidate compounds of the same library. This could help clarify if other molecules outside the canonical JAK/STAT3 pathway can regulate STAT3 spontaneous dimerization.

With this work, we were able to accomplish our main and secondary goals. We optimized a BiFC system that showed to be a user-friendly method with the potential to study a large range of protein-protein interactions. We also opened other opportunities of study, and current work is being done in continuation of this thesis.

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