STAT3 and metabolism: How many ways to use a single molecule?

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The transcription factor Signal Transducer and Activator of Transcription (STAT)3 has been considered as a potential anticancer target since its first description as an oncogene in 1999, recently leading to STAT3 inhibitors been brought to clinical trial for the treatment of solid tumors. However, the past 14 years of intense basic research have uncovered novel STAT3-mediated pathways that could affect the outcome of the designed therapies while at the same time help designing function-specific inhibitors. Particularly intriguing are the recent findings that suggest profound implications of STAT3 with the regulation of cellular metabolism in both canonical, that is transcriptional, and non-canonical ways. Here, after a short description of the main known features of STAT3 signaling and function, we review the recent literature on the role of STAT3 in regulating cellular metabolism and discuss the potential consequences on the therapeutic approaches currently under clinical experimentation.

Signal transducer and activator of transcription (STAT)3 was first described as a DNA-binding factor in acutely inflamed livers and in interleukin-6(IL-6)–stimulated hepatocytes, capable of selectively interacting with an enhancer element in the promoter of acute-phase genes.[1,2] This discovery was soon followed by the demonstration that STAT3 is not only the main transcription factor mediating the functions of all IL-6–family cytokines, but is also involved in the signaling of many other cytokines, growth factors and oncogenes.

STAT3 is activated by tyrosine phosphorylation (Y-P) at a single site next to its carboxy-terminal region (Y705) in response to cytokine stimulation.[3,4] Y-P is mediated by receptor-associated kinases belonging to the Janus kinase (JAK) family and required for the formation of functional dimers able to concentrate in the nucleus and to bind DNA. Targeted phosphorylation of STAT3 involves specific interactions between the STAT3 Src homology 2 (SH2) domain and four phospho-tyrosine sites on the glycoprotein 130 (gp130) cytoplasmic domain.[5] As for most cytokine-induced events, the activation of STAT3 must be tightly controlled by negative regulators, which mainly fall into three groups: phosphatases, suppressor of cytokine signaling (SOCS), and protein inhibitor of activated STAT (PIAS).[6–8] Cytosolic and membrane-bound phosphatases, like Src homology region 2 domain-containing phosphatase-1 (SHP1), Src homology region 2 domain-containing phosphatase-2 (SHP2), PTPRD,

PTPRT and protein tyrosine phosphatase 1B (PTP1B), counteract JAK-mediated phosphorylation events,[9,10] whereas nuclear phosphatases, such as T-cell PTP, terminate STAT3 signaling.[6] SOCS3 is the main STAT3 transcriptionally induced member of the SOCS family of cytoplasmic negative feedback inhibitors of JAKs activity.[11] Finally, PIAS3 prevents the binding of STAT3 to its target DNA.[12] Activation/inactivation of STAT proteins is a crucial step for their biological activities, and disruption of this control, leading to defective or constitutive STATs activation, always correlates with the development of pathological conditions.

STAT3 plays an important role in cell growth and survival, being the only member of the STATs family whose loss of function results in early embryonic lethality.[13] Some of its numerous physiological functions include the induction of acute-phase response genes in hepatoma cells and of tissue regeneration in the liver, stimulation of proliferation in B lymphocytes, activation of terminal differentiation and growth arrest in monocytes, lysosome-mediated apoptosis during mammary gland involution and maintenance of the pluripotency of embryonic stem cells.[14] This functional pleiotropy raises the interesting issue of how a single transcription factor can be involved in seemingly contradictory cell responses. The answer to this riddle may be found, at least in part, in the observation that distinct sets of target genes are induced by STAT3 in different cell types and under distinct conditions/stimulations.[14] This in turn may relate to cell-specific accessibility of genomic STAT3 binding sites,[15] cell- and signal-specific interactions with distinct co-factors and the STAT3 ability to activate transcription by favoring specific DNA binding of other factors, as in the case of IL-6–stimulated STAT3 activating the binding of Sp1 to the VEGF promoter in astrocytes.[16] Moreover, STAT3 can be subject to a range of post-transcriptional modifications in addition to Y-P that can be triggered by distinct signals and confer specific properties to the protein (see below).

STAT3 as an Oncogene

In addition to cytokines, several growth factors and oncogenes can constitutively activate STAT3 through chronic tyrosine phosphorylation.[17] The activation leads to STAT3 dimerization, nuclear localization and transcriptional induction of specific target genes, in a variety of human tumors of both solid and non-solid origin.[18] The first direct evidence of STAT3 oncogenic properties derives from the generation and characterization of a constitutively activated STAT3 mutant (STAT3C).[19] The overexpression of STAT3C in 3T3 fibroblasts was able to induce cellular transformation, providing genetic evidence that aberrant STAT3 activity leads to a malignant phenotype.

In human cancers, persistent STAT3 activation mainly occurs either downstream of continuous stimulation[20] or as a consequence of the inactivation of negative regulators.[21] Cadherin-mediated cell–cell contacts can also lead to STAT3 activation,[22,23] as well as rarely occurring direct mutations in its coding region.[24] Interfering with STAT3 activity inhibits the growth of the vast majority of tumor cells displaying con-

stitutive phosphorylation of this factor.[25,26] These observations have prompted the development of a number of approaches to inhibit STAT3 transcriptional activity/tyrosine phosphorylation, with the goal of treating Y-P STAT3 positive cancers.

In addition to the canonical stimulation of its nuclear transcriptional activity elicited by Y-P, STAT3 can undergo several other post-translational modifications, most of which have been correlated with enhanced transactivating potential.

First, like most STATs, STAT3 can be phosphorylated on serine residue 727 (serine phosphorylation [S-P]) within the carboxy-terminal transcription activation domain (TAD), by several kinases such as mitogen-activated protein (MAP) kinases and mechanistic target of rapamycin (mTOR).[27,28] STAT3 S-P was reported to provide full transactivating properties to the Y-P protein, being required for optimal induction of a subset of target genes.[29] However, S-P can be detected on STAT3 in the absence of Y-P, and its levels correlate with the Gleason score in prostate cancer and with an estrogen receptor (ER) negative status and high tumor stage in breast cancer.[30,31] Although most of these reports correlate S-P with transcriptional activity, a novel, non-transcriptional role for STAT3 S-P has recently emerged (see below).

Second, STAT3 can be acetylated by the p300 co-activator in response to cytokines, favoring dimer stability and transcriptional activity.[32] Moreover, STAT3 acetylation mediates its interaction with DNA-methyltransferase 1 (DNMT1) and the hypermethylation of tumor suppressor gene promoters in cancer cells.[33]

Third, methylation was shown to regulate STAT3 activity both negatively and positively. Indeed, STAT3 bound to specific promoters can be negatively regulated *via* methylation on lysine 140 by the histone methyltransferase SET9.[34] On the other hand, trimethylation on lysine 180 by the enhancer of zeste homolog 2 (EZH2) component of the Polycomb complex 2 is required for STAT3 Y-P and transcriptional activity in glioblastoma and prostate cancer cells.[35]

In line with its contradictory functions in normal tissues, STAT3 was also associated with anti-oncogenic functions in thyroid tumors and in late stage adenomatous polyposis coli (APC)[Min] intestinal tumors,[36,37] underlining once more the complexity of its regulation and function.

Interestingly, unphosphorylated STAT3 has been shown to be able to dimerize, shuttle between nucleus and cytoplasm and trigger gene transcription.[38,39] Despite some indications about the specific set of induced genes, further studies are due to better elucidate both the transcriptional activation mechanisms and the target genes of non-phosphorylated but transcriptionally active forms of STAT3.

Role of STAT3 in Cellular Metabolism

The STAT3-dependent gene expression signature in cancer is heterogeneous, reflecting the involvement of this factor in multiple steps of the oncogenic program. However, the addiction of tumors of different origins and at different stages to
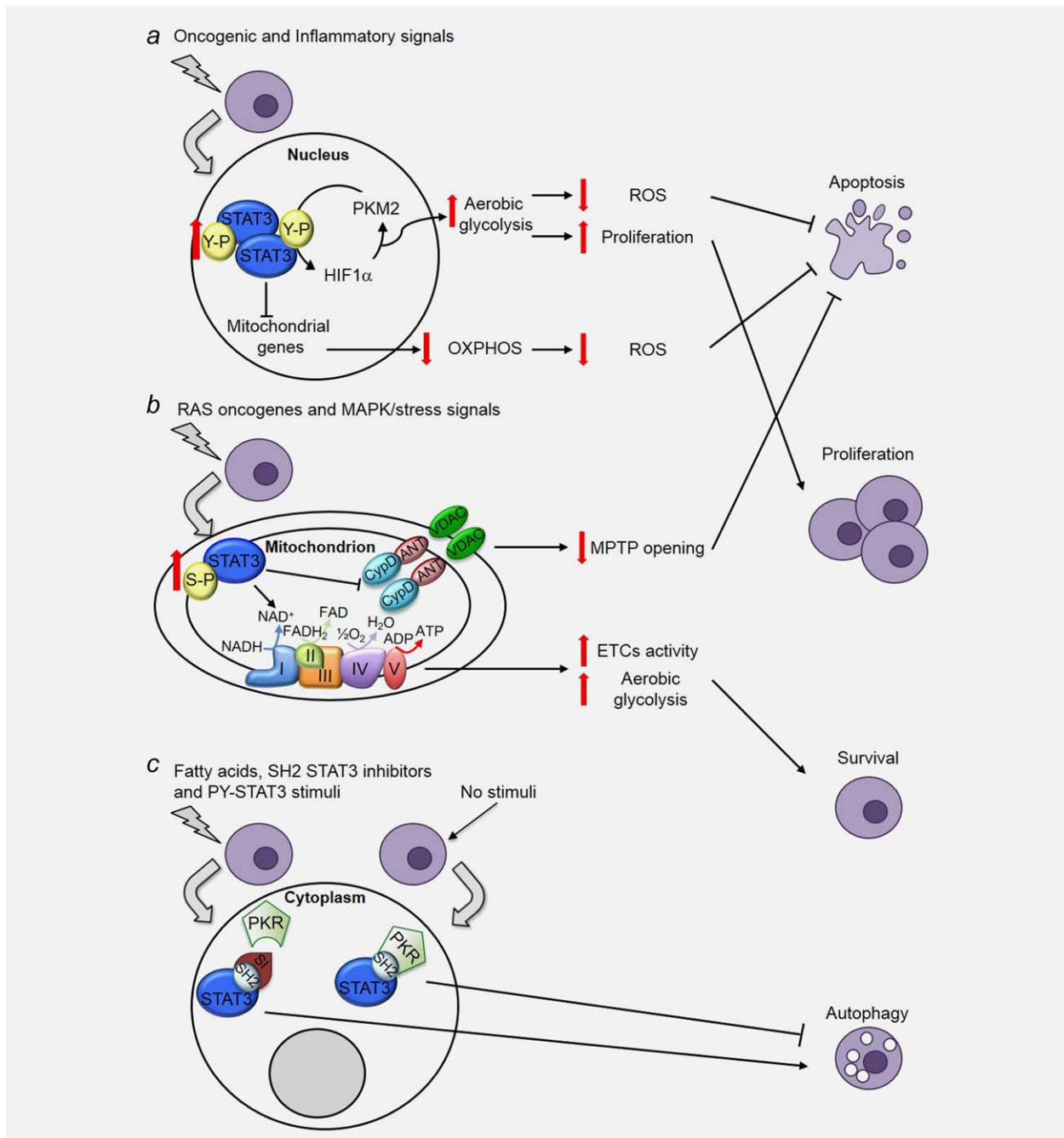
Figure 1. Multiple metabolic roles of STAT3 according to stimulus, post-transcriptional modification and subcellular localization. In both normal and tumor cells STAT3 can be found in the mitochondrial matrix (mSTAT3), in the nucleus (nSTAT3) and in the cytoplasm (cSTAT3), depending on specific post-translational modifications (*i.e.*, Y-P, S-P or none) that are triggered by different upstream stimuli. (*a*) Many pro-oncogenic signals including inflammatory cytokines, growth factors and oncogenes mainly induce P-Y nSTAT3, which mediates the upregulation of the oxygen sensor Hif-1α thus leading to increased glycolysis and metabolic advantages for proliferating cells. Moreover, STAT3 can be chronically activated by the HIF-1-induced PKM2, thus initiating a positive feedback loop to support cell proliferation and protection from apoptosis and senescence. On the other hand, nSTAT3 decreases mitochondrial activity in an HIF-1α-independent manner by downregulating a significant number of mRNAs encoding for protein components of the ETC complexes, thus leading to reduce mitochondrial respiration, ROS production and apoptosis. (*b*) P-S mSTAT3, induced among others by RAS oncogenic signals and the activation of MAP kinases, can improve cell survival by enhancing both oxidative phosphorylation and aerobic glycolysis, and protects cells from apoptosis by inhibiting the opening of the MPTP *via* its interactions with the pore component Cyclophylin D (CypD). (*c*) Non-phosphorylated cSTAT3 has been recently shown to inhibit autophagy by interacting *via* its SH2 domain with the cytoplasmic PKR. STAT3 inhibitors (SI) interfering with the SH2 domain can disrupt this interaction, leading to enhanced autophagy. Adenine nucleotide translocator (ANT); Voltage-dependent anion channel (VDAC); Oxidative phosphorylation (OXPHOS); Nicotinamide adenine dinucleotide (NAD$^+$/NADH); Flavin adenin dinucleotide (FAD$^+$/FADH); Adenosine triphosphate (ATP); Adenosine diphosphate (ADP). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

STAT3-activity suggests that a core function related to cellular survival might exist.

Several observations have suggested important correlations between STAT3 and cellular metabolism, a process that is aberrantly regulated in cancer cells. We have shown that constitutively active STAT3 promotes a glycolysis-like state through the transcriptional induction of the hypoxia-responsive factor hypoxia responsive factor 1 alpha (HIF-1α) and the downregulation of mitochondrial activity (Fig. 1a).[40] STAT3 inhibition in a tumor xenograft model triggers decreased glucose intake prior to tumor growth arrest, suggesting that indeed this metabolic function strongly contributes to STAT3 pro-oncogenic activity. Further implications with the regulation of cell metabolism were suggested by the observation that STAT3 can be chronically activated via Y-P by the HIF-1-induced pyruvate kinase M2 isoform (PKM2), initiating a positive feedback loop that supports cell proliferation and survival (Fig. 1a).[41,42] The switch toward aerobic glycolysis is thought to be required for rapid proliferation of cancer cells and in addition can drive their plasticity to adapt and survive in environments of limiting oxygen concentrations, thus partially explaining the addiction for STAT3 shown by many biologically different tumors.[40,43]

Additionally, STAT3 transcriptional signaling is positively regulated by the nutrient-sensing mTOR pathway.[44,45] mTOR has been recently shown to potentiate STAT3-mediated transcription and tumor angiogenesis by inducing its phosphorylation on serine, which is negatively regulated by p53-induced GADD45A.[46]

Phosphorylation on Serine 727 (S727) has emerged as a key regulator of metabolic processes. Indeed, STAT3 was found to localize to mitochondria, where its S-P enhances coupled Complex I and II activity and reduces reactive oxygen species (ROS) production (Fig. 1b).[47,48] This function appears to be essential for cellular survival under certain stress conditions, such as heart ischemia. In this scenario, cardiac expression of a mitochondrially targeted STAT3 protects cells by preserving Complex I activity, reducing ROS production and caspase-3 activation.[49] Moreover, STAT3 interaction with Cyclofilin D in the mitochondrial matrix of the heart left ventricle inhibits the opening of the mitochondrial permeability transition pore (MPTP), thus opposing calcium-induced apoptosis and necrosis (Fig. 1b).[50] In agreement with a positive role during survival, mitochondrial STAT3 could also support tumorigenesis, as RAS-mediated transformation relies on mitochondrial STAT3 to increase both aerobic glycolysis and the activity of the electron transport chain (ETC) (Fig. 1b).[47] Interestingly, a constitutively activated form of SHP2 that triggers enhanced mitochondrial metabolism, oxidative stress and cellular senescence, might do so via decreased phosphorylation of mitochondrial STAT3 on both tyrosine and serine.[51] Finally, mitochondrially targeted expression of a S727A STAT3 mutant inhibits tumor growth and metastatic ability of the breast cancer cell line

4T1, correlating with reduction of Complex I activity under hypoxia and enhanced production of ROS.[52]

Therefore, STAT3 appears to function as a hub to integrate different pro-survival and growth signals at the level of the energy and respiratory metabolism, via both mitochondrial and nuclear activities (Figs. 1a and 1b).[53] However, retinoic-interferon-induced mortality 19 gene (GRIM19)-mediated transport of STAT3 into mitochondria has been implicated in tumor necrosis factor (TNF)-induced necroptosis, highlighting once more the multifaceted functions of this factor.[54] Moreover, mitochondrial STAT3 has also been shown to act independently of survival pathways to promote specific cellular responses such as regulating nerve growth factor (NGF)-dependent neurite outgrowth or inducing insulin resistance in response to high aminoacid levels in the plasma.[55,56] However, how STAT3 would exert its activities within the mitochondrion is still an open question, since its very low abundance in this organelle challenges the proposed mechanism based on the interaction with specific electron transport complexes of the mitochondrial inner membrane or with cyclin D (CycD) in the mitochondrial matrix.[57]

Interestingly, STAT3 was recently implicated in the regulation of the autophagic pathway, another important metabolic process, since chemical or genetic STAT3 inhibition was shown to accelerate the autophagic flux due to an inhibitory interaction of the STAT3 SH2 domain with the cytoplasmic protein kinase R (PKR)/eukaryotic translation initiation factor 2-alpha knase 2 (EIF2AK2) (Fig. 1c).[58] Interfering with either STAT3 cytoplasmic localization, through constitutive or acute Y-P activation, or with its SH2 domain by means of specific drugs such as STATTIC or S3I, may increase the pool of free PKR and basal autophagy via PKR-mediated eukaryotic translation initiation factor 2, subunit 1 (EIF2S1) phosphorylation (Fig. 1c).[59] Several fatty acids, including palmitate, were shown to trigger autophagy via a pathway that involves disruption of the STAT3-PKR complex.[59] However, it is puzzling that also inhibitors that indirectly prevent STAT3 phosphorylation, such as the JAK inhibitor WP1066, are able to increase basal autophagy in the absence of constitutively active STAT3 Y-P.

Strikingly, these novel and often opposing STAT3 metabolic functions depend on specific phosphorylation events, which in turn regulate both protein activity and localization in the nucleus, the cytoplasm or the mitochondria. Thus, the balance between these different forms must be of primary importance to dictate functional outcomes. This balance will be highly variable under different physiological and pathological conditions, making the dissection of the single STAT3 activities and of the expected outcome of drug treatments even more challenging.

Anti-STAT3 Therapeutic Strategies: Where, When and How?

Many STAT3 inhibitors have been tested in pre-clinical models, but only two have been assessed in active clinical trials

(others are recruiting), and both inhibitors are based on interfering with STAT3 transcriptional activity. The first drug is a decoy oligonucleotide recently assessed in phase 0 trials for the treatment of head and neck cancers.[60] The decoy was designed to scavenge STAT3 from chromatin by competitive binding to the STAT3 DNA-binding domain, and was proven to be effective in both cell lines and animal models.[60] The second drug (OPB-31121) is a small molecule currently in Phase 1 for advanced solid tumors, which acts by downregulating the expression of the upstream kinase JAK2 and of the signaling receptor gp130, thus indirectly decreasing the rate of STAT3 Y-P.[61]

We have shown that chronic STAT3 transcriptional activity is able to act as a first hit in the process of malignant transformation.[62] Continuously Y-P STAT3 is a hallmark of chronic inflammation, featuring elevated systemic and local levels of the main STAT3 activator IL-6.[63] Indeed, both IL-6 and STAT3 are considered central players in the onset of inflammation-related cancer.[63] Interestingly, circulating IL-6 levels are known to increase with age, and IL-6 production represents an important feature of the senescence-associated secretory phenotype.[64,65] This suggests that aging may indirectly trigger chronic STAT3 tyrosine-activation, decreased pool of cytoplasmic and mitochondrial STAT3, reduced autophagy and mitochondrial respiratory activity, and increased ROS production. Since aging is the single factor with the highest impact on cancer initiation, interfering with STAT3 transcriptional activity may be useful as a prophylactic therapy to reduce the rate of cellular transformation. Systemic prophylactic STAT3 blockade by specific inhibitors would presumably be too toxic, due to continued inhibition of its physiological functions. However, effective results might be achieved by chronic anti-inflammatory treatments or by using non-toxic inhibitors, such as several phytochemicals, which are considered to act more like "network inhibitors" by down-modulating multiple pro-tumorigenic and pro-inflammatory factors simultaneously.[66]

In light of what discussed above though, STAT3 transcriptional inhibition may eventually increase the pool of monomeric protein in cancer cells. We have shown that chemically interfering with the constitutively activated form of STAT3 leads to a "counter-switch" to oxidative phosphorylation.[40] On one side, this effect should initially slow down proliferation and compromise the adaptation to a hypoxic environment. On the other side, the reduced nuclear localization of STAT3 will increase its availability for mitochondrial localization, which might eventually lead to enhanced adenosine triphosphate (ATP) production and glycolysis rate, as previously described.[48] Moreover, constitutively activated STAT3 might indirectly favor autophagy by increasing the pool of active PKR in the cytoplasm. Conversely, many STAT3 inhibitors that interfere with STAT3 Y-P, increasing the pool of cytoplasmic STAT3 able to interact with PKR, might in turn inhibit its activity and impair autophagy. In contrast, STAT3 inhibitors that block its PKR-interacting SH2 domain should inhibit its transcriptional activities and at the same time enhance autophagy by releasing potentially active PKR. As autophagy can have both beneficial and pathogenic effects in cancer,[67] the end results of such treatments may vary according to the tumor type and stage. Therefore, the contrasting functions of STAT3 in tumorigenesis may very well relate to different metabolic needs of specific tumors.

Despite the concept of STAT3 as an oncogene gaining strength during the last years, the new twists discussed here suggest that its metabolic functions, both transcription-dependent and -independent, need to be kept in strong consideration for the development of new therapeutic strategies. First, combinatorial therapies based on inhibiting STAT3 transcriptional activity and interfering with cell metabolism may be either redundant or synergistic, depending on the target; second, therapies based on inhibiting STAT3 transcriptional activity might have different and opposite outcomes depending on the oncogenic signals activated and on the molecular approach used; third, since cancer cells might rely on STAT3 also independently from its dimerization, its tyrosine phosphorylation might not be a suitable diagnostic for STAT3-dependent cancer or even a suitable therapeutic target. These considerations underline the need to further elucidate the roles of the different STAT3 forms (Y-P *vs.* S-P, nuclear *vs.* cytoplasmic or mitochondrial, dimeric *vs.* monomeric) and their relative distribution in different kinds of tumors and downstream of distinct oncogenic pathways. In turn, this new knowledge will help defining specific effective inhibition strategies as well as establishing guidelines to assess treatment outcomes.

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