

# The role of STAT3 in autophagy

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**Abbreviations:** ALK, anaplastic lymphoma receptor tyrosine kinase; ATF4, activating transcription factor 4; BNIP3, BCL2/adenovirus E1B 19kDa interacting protein 3; CNTF, ciliary neurotrophic factor; COX8, cytochrome c oxidase subunit VIII; ConA, concanavalin A; CTSB, cathepsin B; CTSL, cathepsin L; CuB, cucurbitacin B; CYCS, cytochrome c, somatic; EGF, epidermal growth factor; EIF2A, eukaryotic initiation factor 2A, 65kDa; EIF2AK2, eukaryotic translation initiation factor 2- $\alpha$  kinase 2; ER, endoplasmic reticulum; ETC, electron transport chain; FOXO1/3, forkhead box O1/3; HDAC3, histone deacetylase 3; HIF1A, hypoxia inducible factor 1,  $\alpha$  subunit (basic helix-loop-helix transcription factor); IL6, interleukin 6; IMM, inner mitochondrial membrane; KDR, kinase insert domain receptor; LMP, lysosomal membrane permeabilization; MAPK1, mitogen-activated protein kinase 1; MAP1LC3A, microtubule-associated protein 1 light chain 3  $\alpha$ ; miRNA, microRNA; mitoSTAT3, mitochondrial STAT3; MLS, mitochondrial localization sequence; MMP14, matrix metalloproteinase 14 (membrane-inserted); NDUFA13, NADH dehydrogenase (ubiquinone) 1  $\alpha$  subcomplex, 13; NES, nuclear export signal; NFkB1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; NLS, nuclear localization signal; PDGFRB, platelet-derived growth factor receptor,  $\beta$  polypeptide; PRKAA2, protein kinase, AMP-activated,  $\alpha$  2 catalytic subunit; PTPN2, protein tyrosine phosphatase, non-receptor type 2; PTPN6, protein tyrosine phosphatase, non-receptor type 6; PTPN11, protein tyrosine phosphatase, non-receptor type 11; ROS, reactive oxygen species; RTK, receptor tyrosine kinases; SH2, src homology 2; STAT3, signal transducer and activator of transcription 3 (acute-phase response factor); VHL, von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase; XPO1, exportin 1.

Autophagy is an evolutionarily conserved process in eukaryotes that eliminates harmful components and maintains cellular homeostasis in response to a series of extracellular insults. However, these insults may trigger the downstream signaling of another prominent stress responsive pathway, the STAT3 signaling pathway, which has been implicated in multiple aspects of the autophagic process. Recent reports further indicate that different subcellular localization patterns of STAT3 affect autophagy in various ways. For example, nuclear STAT3 fine-tunes autophagy via the transcriptional regulation of several autophagy-related genes such as *BCL2* family members, *BECN1*, *PIK3C3*, *CTSB*, *CTSL*, *PIK3R1*, *HIF1A*, *BNIP3*, and microRNAs with targets of autophagy modulators. Cytoplasmic STAT3 constitutively inhibits autophagy by sequestering EIF2AK2 as well as by interacting with other autophagy-related signaling molecules such as FOXO1 and FOXO3. Additionally, the mitochondrial translocation of STAT3 suppresses autophagy induced by oxidative stress and may effectively preserve mitochondria from being degraded

by mitophagy. Understanding the role of STAT3 signaling in the regulation of autophagy may provide insight into the classic autophagy model and also into cancer therapy, especially for the emerging targeted therapy, because a series of targeted agents execute antitumor activities via blocking STAT3 signaling, which inevitably affects the autophagy pathway. Here, we review several of the representative studies and the current understanding in this particular field.

## Introduction

Autophagy is an evolutionarily conserved process that sequesters nonessential intracellular components for lysosomal degradation in response to a variety of stress stimuli, including nutrient or growth factor deprivation, hypoxia, reactive oxygen species (ROS), DNA damage, protein aggregates, damaged organelles, and intracellular microorganisms.<sup>1</sup> Autophagy is generally categorized in 3 forms: macroautophagy, microautophagy, and chaperone-mediated autophagy. In this review, we focus on macroautophagy, which is generally referred to as ‘autophagy.’ During autophagy, nonessential cytoplasmic organelles or cytosolic components are sequestered by double-membrane vesicles named autophagosomes, which are delivered to and fuse with lysosomes for degradation and recycling, allowing cells to eliminate damaged or harmful components through catabolism and to maintain energy homeostasis.<sup>2</sup> Autophagy is widely involved in the pathogenesis of many diseases, especially cancer. Under physiological conditions, autophagy has been proposed to function as a tumor suppressive

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mechanism for the removal or mitigation of harmful stimuli, including oxidative stress, inflammation and genome instability, which may lead to cellular transformation. However, in established tumor cells, which are constantly exposed to mutations, radiation, chemotherapy or targeted agents, autophagy may act as an additional means for survival.<sup>3</sup> Although the exact role of autophagy is debatable in different tumor models or in different pathological conditions, certain tumors are proposed to be ‘autophagy addicted,’<sup>4</sup> and numerous crosstalk occurs between autophagy and other cancer-promoting pathways.<sup>5</sup>

STAT3 (signal transducer and activator of transcription 3 [acute-phase response factor]) is a latent transcription factor that mediates extracellular signals such as cytokines and growth factors through interaction with polypeptide receptors at the cell surface.<sup>6</sup> STAT3 protein becomes transcriptionally activated primarily by tyrosine phosphorylation. Activated STAT3 dimerizes, translocates to the nucleus, and binds to sequence-specific DNA elements for consequent transcription of target genes.<sup>7</sup> STAT3 is expressed at a basal level in cells but rapidly increases through self-activation once stimulated because the *STAT3* promoter contains a binding site for its own dimers.<sup>8,9</sup> STAT3 is constitutively activated or is required to maintain the transformed phenotype in a majority of malignant tumors such as breast cancer, head and neck cancer, brain tumors, lung cancer, ovarian cancer, prostate cancer, pancreatic cancer, renal carcinoma, thyroid cancer, melanoma, myeloma, hepatocellular carcinoma, lymphomas, and leukemia.<sup>10,11</sup> The constitutive activation of STAT3 on its own leads to fibroblast transformation, suggesting that STAT3 functions as an oncogene.<sup>12</sup> Additionally, numerous cancer cell lines undergo growth arrest or apoptosis when treated with antisense or dominant negative constructs against STAT3.<sup>10,12</sup> As the only embryonic lethal family member of the STAT family,<sup>13</sup> STAT3 is a prominent nuclear transcription factor that affects the expression of more than 1,000 gene products.<sup>9</sup> Recent literature has also endorsed the growing evidence of the role of STAT3 in the regulation of autophagy,<sup>2,4,14-16</sup> a well-known stress-induced survival response in eukaryotes. However, the

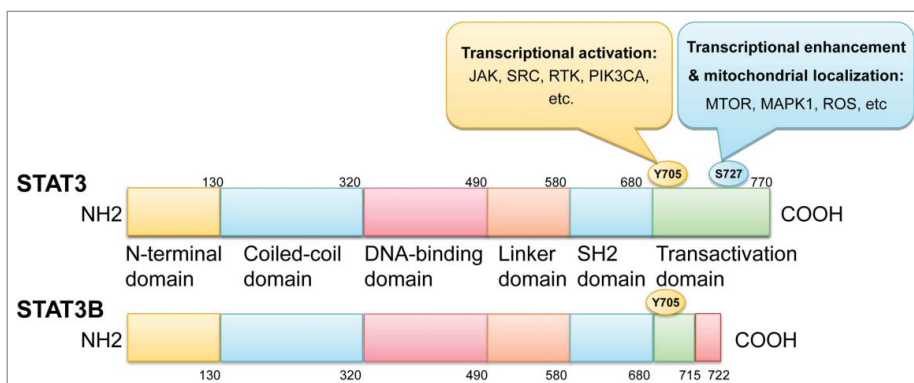
current data provide conflicting explanations of the mechanisms underlying this regulation, likely due to the different subcellular localizations of STAT3 protein. Differentially localized STAT3 is able to modulate autophagy both in a transcription-dependent and transcription-independent manner. We will now review the literature focusing on this novel understanding of STAT3 and its role in autophagy regulation.

## The structure and subcellular localization of STAT3

### Structure and functional domains of STAT3

STAT3 was first described as a transcriptional enhancer of acute phase genes that is activated by IL6 (interleukin 6).<sup>7</sup> The *STAT3* gene is located on chromosome 17q21 and encodes an 89-kDa protein. An alternative splicing transcript of the full-length *STAT3/STAT3α* encoding an 80-kDa protein exists and is known as *STAT3B/STAT3β*.<sup>17</sup> Furthermore, a 72-kDa C-terminal-truncated form of STAT3 produced by the limited proteolysis of STAT3 during granulocytic differentiation also exists and is known as *STAT3C/STAT3γ*,<sup>18,19</sup> and another 64-kDa truncated isoform known as *STAT3δ* that is expressed in the early stage of granulocytic differentiation has been described.<sup>20</sup> The STAT3 protein shares similar functional domains with other STAT family members, including an amino-terminal coiled-coil domain beginning at residue 130, which enables protein-protein interactions, a central DNA-binding domain between residues 320 and 490, a linker domain extending from residues 490 to 580 that affects DNA-binding stability, a classic Src homology 2 (SH2) domain between residues 580 and 680, a tyrosine residue at 705 and a carboxyl transactivation domain, which has a serine phosphorylation site at Ser727 and is absent in the alternative splicing variant *STAT3β*.<sup>6,21</sup> (Fig. 1). Phosphorylation of the conserved Tyr705 residue between the SH2 and carboxyl transactivation domain leads to the homo- or hetero-dimerization of 2 STAT molecules by reciprocal phosphotyrosine interactions between the SH2 domains of 2 monomers. This dimerization

alters the conformation of the STAT proteins to facilitate DNA binding.<sup>12</sup> Notably, in addition to STAT3 dimers, STAT1 and STAT3 heterodimers also exist and may possess transcriptional potential that differs from the potential of STAT1 or STAT3.<sup>9,22,23</sup> Additionally, the DNA-binding fold contains several β-sheets that are folded similarly to the sheets found in the DNA-binding domains of NFKB1 and TP53, which suggests that numerous crosstalk possibilities exist among these 3 important stress responsive pathways. For example, NFKB1 is activated through endoplasmic reticulum stress signals in starved cancer cells, resulting in the induction of IL6. Additionally, STAT3 is required for the induction of IL6 by NFKB1,



**Figure 1.** STAT3 structural domains and phosphorylation sites. The phosphorylation of Tyr705 by JAK, SRC, RTK, PIK3CA, and others activates the transcriptional ability of STAT3, whereas Ser727 phosphorylation in response to ROS or by MTOR, MAPK1, and others mediates the mitochondrial localization or enhances the transcriptional potential of STAT3.

and these proteins form identical nuclear complexes on proximal *IL6* promoters.<sup>24</sup> Moreover, the transcription factor TP53 piggybacks onto NFKB1-RELA and utilizes the κB motif at a cis-regulatory region to control *MIR21* expression. STAT3 is also necessary for the TP53-RELA complex to associate with this cis-element and for *MIR21* expression,<sup>25</sup> whereas *MIR21* targets *BCL2*.<sup>26</sup>

#### Tyrosine phosphorylation of STAT3: the canonical STAT3 pathway

STAT3 transcriptional activity is primarily activated by the phosphorylation of a single tyrosine residue, Tyr705. Tyrosine phosphorylation of STAT3 can be directly catalyzed by receptor tyrosine kinases (RTKs) such as EGFR, KDR, and MET or by nonreceptor tyrosine kinases such as JAKs.<sup>17,27</sup> The oncogene SRC also phosphorylates STAT3,<sup>28</sup> and SRC may be the intermediate that facilitates STAT3 phosphorylation by RTKs. Once activated, STAT3 transcriptionally modulates a range of targeted genes, several of which even convey contradictory responses. Specifically, STAT3 stimulates proliferation through the upregulation of the antiapoptotic gene *BCL2* in B cells, whereas STAT3 activation also leads to the downregulation of *MYC* and *MYB* and the induction of *JUNB* and *IRF1* in monocytic cells, contributing to their terminal differentiation and growth arrest.<sup>27</sup> Thus, STAT3-regulated genes (including both induced and repressed targets) have been reported in numerous cellular and organismal functions, including cell cycle progression, apoptosis, intermediate metabolism, inflammation, invasion, and angiogenesis.<sup>6,10,27,29</sup>

#### Unphosphorylated STAT3

Although the presence of the phosphotyrosine dimer is important in target gene activation, unphosphorylated STAT3 may drive a range of different gene expression. Yang and coworkers employed a Y705F STAT3 mutant, which cannot be phosphorylated on residue 705, and showed that the levels of many mRNAs were strongly affected by Y705F STAT3, including several well-known oncoproteins such as *MRAS* and *MET*.<sup>9</sup> The exact mechanism underlying the transcriptional ability of unphosphorylated STAT3 is yet to be fully understood. However, one hypothesis suggests that the potential for protein-protein interactions is the primary mechanism, including the ability of STAT3 to interact with other transcription factors on target DNA and enhance transcription.<sup>9</sup>

#### Serine phosphorylation of STAT3

The literature suggests a modulatory role for Ser727 phosphorylation in STAT3 transcriptional activation, presumably through the enhanced recruitment of necessary transcriptional cofactors as occurs after the serine phosphorylation of STAT1.<sup>21,30,31</sup> The enzymes that catalyze the phosphorylation of STAT3 on S727 are numerous, including MTOR and MAPK1.<sup>5,31,32</sup> Studies have shown that STAT3 requires tyrosine and serine residues to be phosphorylated by independent protein kinase activities for the maximal activation of target gene transcription. Yokogami et al. showed that STAT3 Ser727 phosphorylation was inhibited by the MTOR inhibitor rapamycin during CNTF (ciliary neurotrophic factor) signaling and that the

maximal activation of STAT3 in CNTF-stimulated neuroblastoma cells depends on serine phosphorylation.<sup>30</sup>

#### Translocation of STAT3 to the nucleus and mitochondria

The STAT3 protein primarily dwells in the cytoplasm, and nuclear localization of STAT3 is transient, lasting no more than several hours.<sup>6,7,12,27</sup> Indeed, the activation of STAT3 is under tight control because the phosphorylated, active STAT3 dimers are regulated by nuclear and perinuclear tyrosine phosphatases such as PTPN6, PTPN11, and PTPN2.<sup>33</sup> According to most literature, the nuclear localization of STAT3 requires phosphorylation and dimerization.<sup>5,7,12,17,27,34</sup> However, Liu et al. reported that the nuclear import of STAT3 is mediated by a constitutively but not conditionally active nuclear localization signal (NLS) that requires the STAT3 coiled-coil domain to interact with a specific import carrier, importin-α3. This finding suggests that although phosphorylated dimers are required for STAT3 to bind to specific DNA target sites, nuclear import occurs constitutively and independently of tyrosine phosphorylation, and STAT3 proteins dynamically shuttle between cytoplasmic and nuclear compartments.<sup>35</sup> However, these observations contradict several primary reports that STAT3 protein merely conditionally translocates into the nucleus in response to IL6.<sup>7</sup> Thus, the conformational shift upon STAT3 dimerization may contribute to the access of the NLS. However, the diverse expression of nuclear transport proteins in various cell types may present different phenotypes. Reportedly, STAT3 nuclear export depends on nuclear export signal (NES) elements located within the DNA-binding domain, which can be recognized by XPO1 (exportin 1). The location of the NES sequences suggests that it is inaccessible to the nuclear receptor XPO1 when STAT3 is bound to DNA. Thus, only unbound STAT3 protein molecules can be actively exported from the nucleus after dephosphorylation.<sup>34,36</sup>

Apart from its well-described nuclear counterpart, recent evidence has also shown that a pool of mitochondrial STAT3 (mito-STAT3) exists that executes transcription-independent functions. Normally, the pool of mitoSTAT3 is approximately one-tenth of the amount of STAT3 in the cytosol, yet increasing accumulation of mitoSTAT3 appears in response to various stimuli such as ischemia.<sup>37,38</sup> The translocation of STAT3 into the mitochondria was reported to be regulated by NDUFA13, a component of the electron transport chain (ETC) complex I. Interestingly, NDUFA13 was found to colocalize and interact with Ser727-phosphorylated STAT3 in the mitochondria. As Wegrzyn and colleagues<sup>37</sup> found, the immunoprecipitates of mitochondrial extracts yielded from mouse liver captured by a monoclonal antibody specifically targeted to the components of the ETC complex I contained STAT3 and NDUFA13. Using highly purified rat heart mitochondria in vitro, Tammineni et al.<sup>39</sup> further discovered that STAT3 most likely resides in the inner mitochondrial membrane (IMM), facing toward the matrix. STAT3 import and translocation across the mitochondrial membrane requires membrane potential and, likely, energy. When STAT3 is imported alone, a small fraction of STAT3 associates with the IMM and matrix fractions. However, upon its co-import with NDUFA13, most of the STAT3 is recruited to the IMM. Thus,

NDUFA13 may act as a chaperone to facilitate STAT3 localization into the mitochondrial membranes, especially the inner membrane, by a common membrane receptor. NDUFA13 most likely alters the topology of mitochondrial STAT3 and promotes its integration into complex I. The C terminus of STAT3, especially Ser727, is required for the NDUFA13-dependent import and integration of STAT3 into complex I because mutating serine 727 to alanine reduces the import of STAT3 into mitochondria, even in the presence of NDUFA13.<sup>39</sup>

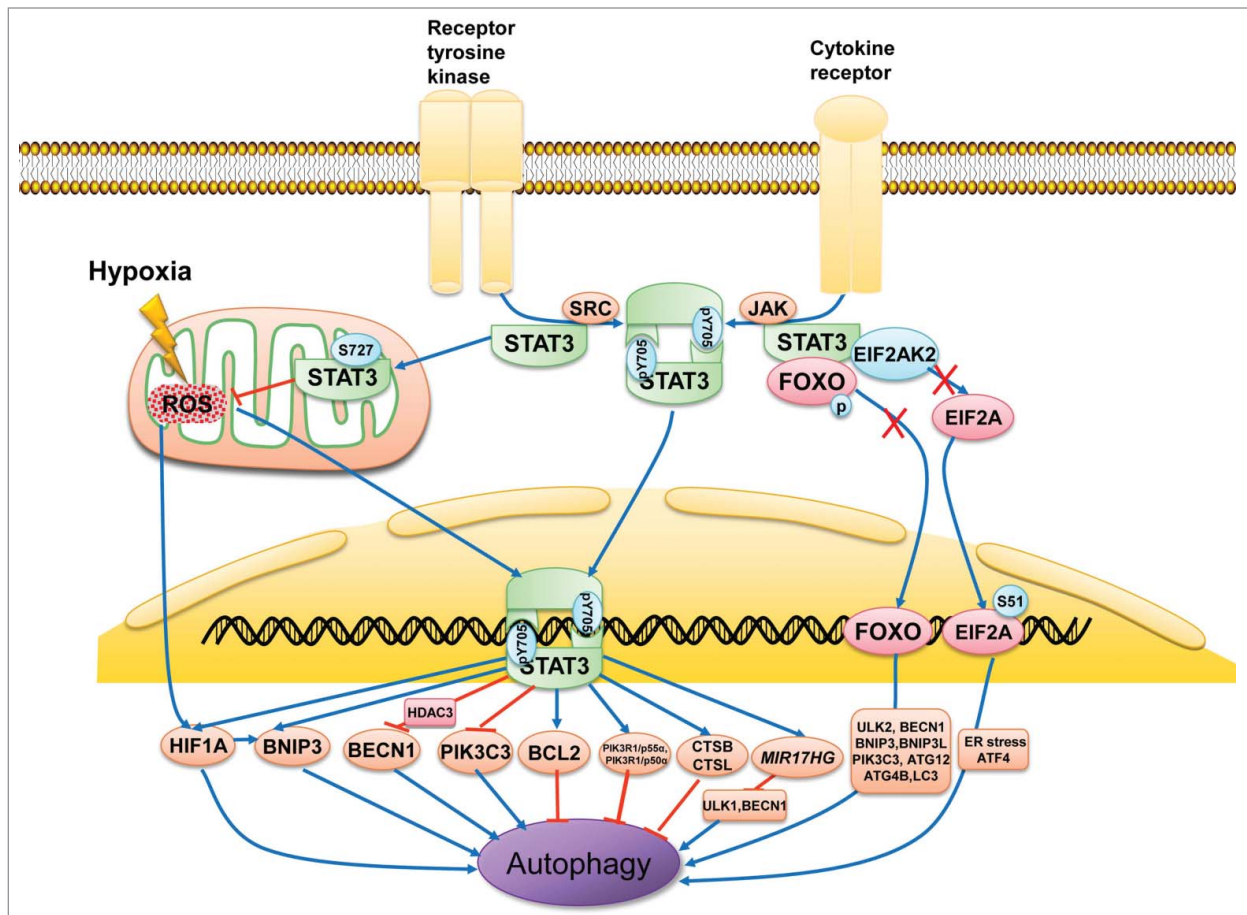
## STAT3 in autophagy

Autophagy is regulated by a network of intricate signaling pathways. The crosstalk between autophagy and other stress response pathways, including STAT3 signaling, may decide the survival or death of a cell.<sup>2,5</sup> Literature throughout the decade

has implicated STAT3 in several steps of autophagy, from the assembly of autophagosomes to their maturation. Furthermore, the role of STAT3 is further complicated by its cellular localization because differentially localized STAT3 regulates autophagy in distinct ways (Fig. 2). This regulation also works in concert with other autophagy-related signaling molecules.

### Regulation of autophagy by nuclear STAT3

STAT3 is the main transcriptional enhancer of several autophagy-related genes in the nucleus, and this activity contributes to a range of anti- vs. pro-autophagic functions of STAT3 in autophagy, which we will discuss separately below (Table 1). The anti-autophagic function primarily lies in its ability to disrupt the formation of the BECN1/PIK3C3 complex. One of its core components, BECN1, like other proapoptotic BH3-only proteins (such as BAX, BAD, BNIP3, and BCL2L11/BIM), contains a BH3 domain that may interact with the antiapoptotic BCL2 family



**Figure 2.** Subcellular localization of STAT3 in the regulation of autophagy. STAT3 monomers in the cytosol are phosphorylated by Src or JAK kinase on Tyr705 and subsequently form STAT3 dimers, which are then shuttled into the cell nucleus and bind with specific DNA elements to transcriptionally activate or suppress target genes such as *BCL2*, *BECN1*, *PIK3C3*, *CTSB*, *CTSL*, *PIK3R1/p55α*, *PIK3R1/p50α*, and *MIR17HG* as well as *HIF1A* and *BNIP3*, which either inhibit or stimulate autophagy depending on the different cellular context or stimulus. In contrast, unphosphorylated cytoplasmic STAT3 sequesters EIF2AK2, FOXO1, and FOXO3. EIF2AK2 promotes autophagy by phosphorylating EIF2A, an endoplasmic reticulum stress-related protein that upregulates ATF4. FOXO1 and FOXO3 also positively modulate autophagy by transcriptionally activating a series of autophagy-related genes such as *ULK2*, *BECN1*, *BNIP3*, *BNIP3L*, *PIK3C3*, *ATG12*, *ATG4B*, and *MAP1LC3A*. STAT3 monomers also translocate to the mitochondria and interact with complexes I and II of the ETC to repress ROS production. ROS induces autophagy by activating HIF1A, possibly by modulating the transcriptional ability of STAT3.

**Table 1.** Nuclear STAT3-regulated genes in autophagy

Target genes	Anti/pro-autophagy	Mechanisms	Reference
<i>BCL2</i> family members	anti-	STAT3 transcriptionally activates <i>BCL2</i> and <i>MCL1</i> expression, which leads to autophagy inhibition	41,42
<i>BECN1</i>	anti-	STAT3 directly binds to the promoter region of <i>BECN1</i> and represses its transcription by recruiting HDAC3	43
<i>PIK3C3</i>	anti-	STAT3 downregulates <i>PIK3C3</i> , which is an essential component of the BECN1-PIK3C3 complex	44
<i>PIK3R1/p55<math>\alpha</math></i> , <i>PIK3R1/p50<math>\alpha</math></i>	anti-	STAT3 upregulates the expression of <i>PIK3R1/p55<math>\alpha</math></i> and <i>PIK3R1/p50<math>\alpha</math></i> , which compete with <i>PIK3R1/p85<math>\alpha</math></i> to inhibit autophagy.	46
<i>CTSB</i> , <i>CTSL</i>	possibly anti-	STAT3 upregulates <i>CTSB</i> and <i>CTSL</i> , which enhances cell death by LMP; LMP impairs the autophagy flux.	45
<i>HIF1A</i>	pro- in hypoxia anti- in normoxia.	STAT3 transcriptionally activates and stabilizes HIF1A, which induces autophagy in hypoxia and represses autophagy in normoxia.	47,48,50
<i>BNIP3</i>	pro-	STAT3 phosphorylation upregulates <i>BNIP3</i> expression; BNIP3 is a pro-autophagic BH3-only protein.	51

members (such as *BCL2*, *BCL2L1*, and *MCL1*). In this regard, the antiapoptotic family members are important negative regulators of *BECN1*, whereas members of the proapoptotic BH3-only proteins displace *BECN1* to retain its proautophagic activity.<sup>2</sup>

Nuclear STAT3 has the ability to transcriptionally activate *BCL2*, *BCL2L1*, and *MCL1*.<sup>11,12,40</sup> Upon activation, nuclear STAT3 upregulates *BCL2* expression and consequently leads to autophagy inhibition, and the dominant negative inhibition of STAT3 typically induces the upregulation of autophagy. Feng et al.<sup>41</sup> showed that the biguanide metformin downregulates STAT3 activity and *BCL2* expression and induces autophagy both in vitro and in vivo. A similar report by Tai and colleagues<sup>42</sup> showed that sorafenib, a small molecular inhibitor of KDR and PDGFRB, activates autophagy in a dose- and time-dependent manner in multiple HCC cell lines through the downregulation of phospho-STAT3 (pSTAT3) and the subsequent reduction of *MCL1*. Conversely, the ectopic expression of *MCL1* reverses the effect of sorafenib on autophagy.

The findings of Miao et al.<sup>43</sup> indicate that *BECN1* may also be a direct transcriptional target of STAT3 because IL6 treatment significantly promotes STAT3 phosphorylation while reducing *BECN1* mRNA and protein levels in AGS and NCI-H1650 cells. A significant reduction of *BECN1* occurs in cells transfected with constitutively activated STAT3, and a dominant-interfering STAT3 mutant (STAT3Y705F) increases *BECN1* mRNA and protein levels in AGS and NCI-H1650 cells.<sup>43</sup> Further studies revealed that STAT3 directly binds to the promoter region of *BECN1* and represses its transcription through the recruitment of HDAC3 (histone deacetylase 3).<sup>43</sup>

In addition, *PIK3C3* is also regulated by STAT3 tyrosine phosphorylation. Yamada et al.<sup>44</sup> found that increased STAT3Y705 phosphorylation is associated with the downregulation of *PIK3C3*. The expression of the STAT3Y705F mutant markedly increases autophagic flow in the WT tibialis anterior muscle, along with a marked induction of *PIK3C3* protein expression, suggesting the possible indirect regulation of *PIK3C3* protein levels by nuclear STAT3.

One crucial stage of autophagy is the degradation of autophagosome, which involves fusion with lysosome to form

autolysosome and degradation of the inner membrane together with its luminal contents. Thus functional lysosomes are indispensable for the progression of autophagic flux. STAT3 plays an important role in lysosomal-mediated programmed cell death during post-lactational regression (involution) of the mammary gland.<sup>45</sup> A recent study found that autophagy is strikingly induced at 24 h of involution, whereas STAT3 inhibits this pro-survival autophagy that occurs concomitantly with lysosomal-mediated programmed cell death. STAT3 suppresses autophagy in mammary gland cells by directly upregulating the *PIK3R1/p55 $\alpha$*  and *PIK3R1/p50 $\alpha$*  (phosphoinositide-3-kinase, regulatory subunit 1 [ $\alpha$ ], p55 $\alpha$  and p50 $\alpha$  isoforms), which function as inhibitors of *PIK3R1/p85 $\alpha$* -mediated autophagy.<sup>46</sup> In addition, STAT3 transcriptionally enhances the expression of *CTSB* (cathepsin B) and *CTSL* (cathepsin L), possibly through the downregulation of *Serpina3g*, a known target of NF $\kappa$ B1 in memory T cells.<sup>45</sup> STAT3 can inhibit the transcriptional activity of NF $\kappa$ B1 by binding to NF $\kappa$ B1 and RELA, subsequently downregulating its target genes, including *Serpina3g*. Thus STAT3-mediated upregulation of *CTSB* and *CTSL* enhances cell death by lysosomal membrane permeabilization (LMP) during mammary gland involution,<sup>45</sup> whereas LMP impairs autophagy because the autophagy flux requires functional lysosomes.<sup>46</sup>

As described above, nuclear STAT3 executes anti-autophagic functions by upregulating negative regulators of autophagy such as *BCL2*, *BCL2L1*, *MCL1*, *PIK3R1/p55 $\alpha$* , and *PIK3R1/p50 $\alpha$* , or by downregulating essential autophagy genes such as *BECN1* and *PIK3C3*. However, STAT3 also executes its pro-autophagic function by modulating the hypoxic expression of HIF1A (hypoxia inducible factor 1,  $\alpha$  subunit [basic helix-loop-helix transcription factor]) and *BNIP3*.

The regulation of HIF1A by STAT3 is 2-fold. On the one hand, STAT3 transcriptionally upregulates *HIF1A*;<sup>47</sup> on the other hand, STAT3 interacts with the C-terminal domain of HIF1A and stabilizes the protein from ubiquitination mediated by VHL (von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase).<sup>48</sup> Interestingly, HIF1A may also modulate autophagy in a context-dependent manner. HIF is a heterodimer of a constitutive  $\beta$  subunit and an oxygen-regulated  $\alpha$  subunit

that is stabilized and expressed when oxygen concentrations decline below a threshold of 5%. Upon moderate hypoxia (1%-3%), HIF activates the transcription of *BNIP3* and *BNIP3L*, genes encoding 2 BH3-only proteins that disrupt the inhibitory interaction between BECN1 and BCL2 in favor of autophagy induction.<sup>2,49</sup> On the other hand, under normoxic conditions, the report by Yuan et al.<sup>50</sup> demonstrates that the stable overexpression of HIF1A reverses the induction of autophagy by the JAK2-STAT3 inhibitor cucurbitacin I in U251 cells.

In the case of BNIP3 regulation by STAT3, research performed by Pratt and Annabi<sup>51</sup> showed that concanavalin A (ConA) induces autophagy, evidenced by the formation of acidic vacuoles and the induction of BNIP3 expression. However, the genetic or pharmaceutical inhibition of STAT3 abrogates ConA-induced BNIP3 expression and subsequent autophagy, indicating that STAT3 phosphorylation potentially upregulates autophagy via increased BNIP3 expression. Notably, no other autophagosome biomarkers have been examined except for the acidic vacuolar organelles. Thus, further study is required to validate that the acidic vacuolar organelles induced by ConA are autophagosomes and, if so, that this accumulation of autophagosomes represents the induction of autophagy rather than a failure to clear autophagosomes.

Furthermore, STAT3 has been reported to regulate the expression of a series of microRNAs (miRNAs) that target autophagy-related genes, which may be another possible mechanism for nuclear STAT3 regulation of autophagy. For example, STAT3 upregulates *MIR17HG* (miR-17-92 cluster host gene [non-protein coding]) through a highly conserved STAT3 binding site in the promoter region,<sup>52</sup> and members of the *MIR17HG* cluster have been reported to target the autophagy-related genes *ULK1*, *BECN1*, and *BCL2L11*.<sup>53-55</sup> Several miRNAs that have been transcriptionally modulated by STAT3 and have been reported to target autophagy pathways are summarized in **Table 2**.<sup>26,52-64</sup> However, the role of miRNAs in the regulation of STAT3-mediated autophagy has not been well established, and this need to be further investigated.

### Regulation of autophagy by cytoplasmic STAT3

Nuclear STAT3 regulates autophagy in a multifaceted manner. For example, STAT3 inhibits autophagy by transcriptionally activating *BCL2* or stimulates autophagy by upregulating and stabilizing HIF1A under hypoxia. However, as previously mentioned, to effectively transduce extracellular signals, a large fraction of STAT3 protein resides in the cytoplasm, where the main autophagy process occurs. A frequently cited report by the Kroemer group<sup>14</sup> unraveled a novel mechanism to explain how

cytoplasmic STAT3 inhibits autophagy by inhibiting EIF2AK2 (eukaryotic translation initiation factor 2- $\alpha$  kinase 2) activity. In a library screen for autophagy inducers, a series of pharmacological inhibitors of STAT3 were identified as potent activators of autophagy. Small interfering RNA (siRNA)-mediated knock-down of STAT3 also showed that decreasing the total STAT3 level induces autophagy. Further investigation revealed that cytoplasmic STAT3 regulates autophagy in a more direct manner. The SH2 domain of cytoplasmic STAT3 exhibits a conformational fold that resembles the C terminus of the EIF2AK2 substrate EIF2A (eukaryotic initiation factor 2A, 65kDa), and interacts with the catalytic domain of EIF2AK2, thus inhibiting EIF2AK2 enzymatic activity and preventing it from phosphorylating EIF2A, a known autophagy activator. Wild-type STAT3 and nonphosphorylatable STAT3<sup>Y705F</sup> transfected into U2OS cells inhibit baseline as well as starvation-induced EIF2A phosphorylation, further confirming this hypothesis.<sup>14</sup> The phosphorylation of EIF2A mediates general translational arrest and promotes the selective transactivation of stress response genes. Cells carrying a nonphosphorylatable EIF2A (S51A) mutant fail to induce autophagy in starvation conditions, suggesting that EIF2A phosphorylation of serine 51 plays a major role in autophagy regulation.<sup>65</sup> Currently, how EIF2A phosphorylation contributes to autophagy initiation has not been thoroughly described. One speculation is that EIF2A phosphorylation may affect the endoplasmic reticulum in a manner that promotes the physical formation of the phagophore. Another possibility is that EIF2A phosphorylation stimulates autophagy via its effects on the transactivation of autophagy genes. EIF2A phosphorylation stimulates the selective translation of the ATF4 transcription factor while suspending general translation, which subsequently stimulates MAP1LC3A expression.<sup>66</sup> This revelation suggests that cytoplasmic STAT3 functions as a negative regulator of autophagy in a transcription-independent manner.

In addition to its direct effect on EIF2AK2 activity, cytoplasmic STAT3 also interacts with the autophagy-related proteins FOXO1 and FOXO3. Following its dephosphorylation, FOXO3 translocates into the nucleus and upregulates multiple autophagy-related genes such as *ULK2*, *BECN1*, *PIK3C3*, *BNIP3*, *BNIP3L*, *ATG12*, *ATG4B*, and *MAP1LC3A*.<sup>67</sup> Oh et al.<sup>68</sup> showed that active FOXO1 and FOXO3 reside exclusively in the nucleus of naive T cells, whereas inactive phosphorylated FOXO1 and FOXO3 are most abundant in activated T cells, sequestered in their cytoplasm in association with unphosphorylated STAT3. When the JAK-STAT3 pathway is activated by IL6 or IL10, FOXO1 and FOXO3 rapidly relocalize into the nucleus in response to STAT3 phosphorylation, and the accumulation of FOXO1 and FOXO3 in the nuclei coincides with the increased expression of target genes. STAT3 inhibitors abrogate the cytokine-induced translocation of FOXO1 and FOXO3 into the nucleus.

As depicted above, cytoplasmic STAT3 sequesters EIF2AK2, FOXO1, and FOXO3 under normal conditions, suggesting an inhibitory role. However, upon immediate STAT3 activation, EIF2AK2, FOXO1, and FOXO3 are released from cytoplasmic anchorage and mediate the proautophagic response through the

**Table 2.** STAT3 regulated microRNAs that target autophagy pathway

microRNA	Anti/pro-autophagy	Target gene	Reference
MIR17HG	pro-	ULK1, BECN1, BCL2L11	52-55
MIR34A	anti-	BCL2	56,57
Mir30c	pro-	Atg5	58,59
MIR155	anti-	MTOR pathway genes	60,61
MIR21	pro-	BCL2	26,62
MIR204-5p	anti-	MAP1LC3A	63,64

phosphorylation of EIF2A, FOXO1, and FOXO3 through translocation to the nucleus and upregulation of autophagy-related gene products. Thus, a proautophagic signaling pathway may also lie within STAT3 activation.

### Regulation of autophagy by mitochondrial STAT3

Complementing the roles of nuclear and cytoplasmic STAT3, the aforementioned translocation of STAT3 to the mitochondria may play a critical role in autophagy regulation. Recent discoveries unveiled a novel role for STAT3 translocated to the mitochondria in regulating the activity of the electron transport chain, which represents a major source of ROS production.<sup>38</sup> Wegrzyn et al.<sup>37</sup> reconstituted STAT3 into STAT3<sup>-/-</sup> B cells with a retrovirus expressing STAT3. Oxidative phosphorylation in mitochondria significantly decreased in the STAT3<sup>-/-</sup> pro-B cells (40% decrease of complex I activity and 85% decrease of complex II activity in STAT3<sup>-/-</sup> cells compared with WT pro-B cells); however, the expression of STAT3 restored the activities of complexes I and II. To confirm that the defects were due to a lack of mitoSTAT3, a STAT3 construct containing the COX8 (cytochrome c oxidase subunit VIII) mitochondrial localization sequence (MLS) fused to the N terminus of STAT3 (MLS-STAT3) was generated. The MLS places the protein of interest in the inner mitochondrial membrane. Mutations in either Tyr705 or the DNA-binding domain of MLS-STAT3 restore the activity of complexes I and II. Interestingly, the phosphomimicking mutant MLS-STAT3<sup>Y705F,S727D</sup> reconstitutes the activities of complexes I and II, whereas the dominant negative mutant MLS-STAT3<sup>Y705F,S727A</sup> is ineffective, suggesting that serine but not tyrosine phosphorylation is important for the activity of STAT3 in the mitochondria and that STAT3 most likely exerts its actions as a monomer. Mitochondria from the hearts of *stat3<sup>fllox/flloxCre</sup>* (*stat3<sup>-/-</sup>*) and *Stat3<sup>fllox/fllox</sup>* (wild-type) mice were assayed for complex I and II activities.<sup>37</sup> ETC assays confirmed that *stat3<sup>-/-</sup>* heart mitochondria have defects in complexes I and II but have normal complex III activity. Using transgenic mice generated with cardiomyocyte-restricted expression of STAT3 that was targeted to the mitochondria with a MLS and containing mutations in the DNA-binding domain (MLS-STAT3E), Szczepanek et al.<sup>69</sup> discovered that ischemia increases the release of H<sub>2</sub>O<sub>2</sub> in WT mitochondria respiring on glutamate and malate, whereas no such effect is observed in mitochondria expressing MLS-STAT3E. Additionally, the maximal capacity of complex I to produce H<sub>2</sub>O<sub>2</sub> after ischemia, established by inhibiting complex I with rotenone and supplying glutamate and malate as a substrate, is decreased in mitochondria expressing MLS-STAT3E compared with WT mitochondria. Thus, at this point, STAT3 is considered to interact with complexes I and II of the ETC to modulate their activities through protein-protein interactions or post-translational modifications, and the loss of STAT3 results in a significant increase in ROS.<sup>70</sup> Several studies have implicated mitochondrial ROS in the induction of autophagy, especially the selective autophagic degradation of mitochondria, also known as mitophagy.<sup>71</sup> Thus, the mitochondrial translocation of STAT3 inevitably suppresses autophagy induced by oxidative stress and effectively preserves mitochondria from

mitophagy. This phenomenon supports the prosurvival features of STAT3 because MLS-STAT3 expression inhibits CYCS (cytochrome c, somatic) release by preventing the opening of the mitochondrial permeability transition pore during ischemia.<sup>69</sup>

### Implication of STAT3-regulated autophagy in cancer therapeutics

The last decade marked a revolutionary improvement for targeted treatments and chemotherapy agents directed against numerous oncogenic pathways, especially for agents targeting receptor tyrosine kinases. Autophagy is well known for its prosurvival phenotype in numerous diseases, including cancer. Additionally, autophagy-related chemoresistance and targeted therapy resistance have been extensively studied in recent years.<sup>3,72,73</sup> Currently, several agents targeting the JAK-STAT pathway have been under investigation in clinical trials for cancer treatment, and the same attention must be paid to the resistances provoked by the upregulation of autophagy.

Tyrosine kinases are the primary oncogenic pathways in numerous malignancies. Many targeted therapies focus their efforts on blocking these kinases and their corresponding pathways. However, these interferences inevitably trigger the activation of bypassing mechanisms, including autophagy. A wide range of RTKs phosphorylate STAT3 directly or via SRC kinase, and several studies have proven that blocking kinases such as KDR, PDGFRB, and ALK results in enhanced autophagy by subsequent inhibition of the STAT3 pathway (Table 3).<sup>14,41,42,50,74-83</sup> Currently, several JAK-STAT inhibitors are being examined in clinical trials for cancer therapy. For example, in a phase II clinical trial to treat metastatic pancreatic cancer, ruxolitinib together with capecitabine showed a benefit for overall survival and progression-free survival compared with a placebo plus capecitabine used in second-line therapy, promoting the application of JAK-STAT inhibitors in cancer therapy.<sup>84</sup> However, similar to other targeted therapies, acquired resistance causes problems for the further application of such agents, among which is the secondary mutation JAK2<sup>V617F</sup> in JAK kinase.<sup>85</sup> A number of other factors also need to be considered, including the upregulation of autophagy. In the research conducted by Yuan et al.<sup>86</sup> human malignant glioma U251 and A172 cells were treated with a STAT3 inhibitor, WP1066, or a short hairpin RNA plasmid targeting *STAT3* to suppress the activation of STAT3 signaling. STAT3 inhibition enhances the radiosensitivity of glioma cells; however, WP1066 or sh*STAT3* treatment and ionizing radiation exposure induce the lipidation of MAP1LC3A and increase BECN1 expression. Furthermore, the combination of ionizing radiation exposure and STAT3 inhibition trigger a pronounced increase of autophagy flux. The inhibition of autophagy by 3-methyladenine or *ATG5* siRNA strengthens the radiosensitizing effects of STAT3 inhibition, and the apoptosis of more cells is induced by the cotargeting of autophagy and STAT3 signaling, indicating a prosurvival role of STAT3 inhibition-induced autophagy. Our recent work also indicated that the autophagy inhibitor static induces cytoprotective autophagy in

**Table 3.** Targeted and chemical agents that induced autophagy via the STAT3 pathway

Agent	Target gene	Tumor type	Mechanism	References
Sorafenib	<i>KDR, PDGFRB, RAF</i>	Prostate cancer, Glioma, hepatocellular carcinoma	Downregulation of pSTAT3 and <i>MCL1</i> expression	42,74,75
Crizotinib	<i>ALK</i> fusion gene	anaplastic large cell lymphoma	Downregulation of pSTAT3 and <i>BCL2</i> family	76
Stattic	<i>STAT3</i>	Osteosarcoma	Blockage of the interaction between cytoplasmic STAT3 and <i>EIF2AK2</i>	14
Cucurbitacin I	<i>JAK2</i>	Glioblastoma	Downregulation of pSTAT3 and <i>HIF1A</i>	50
Metformin	<i>PRKAA2?</i>	esophageal squamous cell carcinoma	Downregulation of pSTAT3 and <i>BCL2</i>	41
Cucurbitacin B (CuB)	?	melanoma	Downregulation of pSTAT3 and <i>BCL2</i>	77
Nexrutine	<i>STAT3?</i>	pancreatic cancer	Modulation of STAT3 and decrease of ROS generation	78
Obatoclox	<i>PTPN6</i>	hepatocellular carcinoma	Downregulation of pSTAT3 and <i>MCL1</i>	79,80
Niclosamide	?	myeloma	Inhibition of pSTAT3 and upregulation of autophagy	81
Arsenic trioxide	<i>STAT3?</i>	Glioblastoma	Inhibition of pSTAT3 and upregulation of autophagy	82
Concanavalin A	<i>MMP14</i>	Glioblastoma	Upregulation of pSTAT3 and <i>BNIP3</i>	83

hepatic tumor cells and xenografts, whereas cotargeting of the autophagy pathway sensitizes the antitumor effect of STAT3 inhibition (unpublished data).

### Issues and Perspectives

The regulation of autophagy by STAT3 primarily features prosurvival characteristics regardless of its subcellular localization. The translocation of STAT3 affects both the early-phase autophagic flux and the prolonged autophagic response. As a well-recognized stress responsive nuclear factor, STAT3 responds to a panel of autophagy inducers, including hypoxia, ROS, growth factor deprivation, and a number of targeting agents. For example, during growth factor deprivation, the absence of RTK or SRC phosphorylation represses the tyrosine phosphorylation of STAT3 and its target gene *BCL2*; however, the expression of *BECN1* and *PIK3C3* are elevated. All of these effects accommodate the formation of the *BECN1-PIK3C3* complex, initiating autophagy. As a consequence, the amount of total STAT3 may also be reduced because STAT3 transcription is self-activated, rendering the pro-autophagic proteins *EIF2AK2*, *FOXO1*, and *FOXO3* free to phosphorylate *EIF2A* or to upregulate autophagy-related genes in promoting cytoprotective autophagy. In the case of hypoxia, the low oxygen concentration results in the hypoxic expression of *HIF1A*, which leads to STAT3 phosphorylation. In turn, pSTAT3 contributes to *HIF1A* transcriptional activation and stabilization, followed by the transcription of the BH3-only proteins *BNIP3* and *BNIP3L*. Meanwhile, ROS production is increased in mitochondria, which mediates the mitochondrial translocation of STAT3. mitoSTAT3 stabilizes and protects complexes I and II of the ETC from hypoxic insult, consequently reducing the ROS level and preserving the mitochondria from ROS-induced mitophagy as well as preventing the release of *CYCS* due to mitochondrial outer membrane permeabilization, thereby avoiding mitochondrial-dependent apoptosis. However, many questions remain that require further study. For example, as a prominent nuclear factor, STAT3 reportedly regulates the expression of more than 1,000 gene products. As described in this paper, not all of these products modulate

autophagy in the same direction. Although most of this modulation occurs in a context-dependent manner, the mechanism underlying how specific stimuli drive STAT3 signaling to accommodate or restrain autophagy has not been fully explained. The regulation of autophagy by cytoplasmic STAT3 involves multiple protein-protein interactions, but the different affinities of autophagy-related molecules to cytoplasmic STAT3 remain unclear, and whether these proteins comprise a large interactome or bind to different partners in response to different signals remains puzzling. Notably, a number of studies have contributed to the revelation of the role of STAT3 in autophagy. However, several of the recent studies have not distinguished between the transcriptional and nontranscriptional functions of STAT3 signaling in autophagy. Thus, future studies may shed greater insights into the role of STAT3 if they address these differences.

Further study of the role of STAT3 in autophagy may provide us with an in-depth understanding of its physiology as well as with a novel strategy for cancer therapeutics. Although the existing data do not bode well for an eminent outcome for the clinical application of autophagy inhibition in cancer treatment, the cotargeting of the autophagic pathway may prove beneficial in specific types of cancer. Presently, most of the STAT3-targeted agents are in early trials, with only a few reports of autophagy-related resistance. However, in an attempt to raise the efficacy of these agents, due consideration is needed to anticipate the resistance caused by the upregulation of cellular autophagy. To date, autophagy inhibition has been shown to be an efficacious addition to several cytotoxic agents and targeted therapeutics in multiple preclinical models.<sup>3</sup> Currently, combination treatments using targeted agents and autophagy inhibitors are in trials for clinical application. The latest reports have shown that the autophagy inhibitor hydroxychloroquine is tolerable and potentially effective in combination with the *MTOR*-targeted agent temsirolimus<sup>87</sup> and the proteasome inhibitor bortezomib<sup>88</sup> in phase I trials and may be a valid complement to STAT3-targeted therapy. Nevertheless, the systemic inhibition of autophagy introduces another problem involving the impairment of the toxicity tolerance of noncancerous cells. Autophagy in normal cells represents a defense mechanism, and reports have demonstrated that the activation of autophagy protects hepatocytes against chemical-induced hepatotoxicity.<sup>89</sup>



Thus, the rational selection of an autophagy-targeted subpopulation or the development of a more targeted delivery method remain as challenges for further studies.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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