The role of FOXOs and autophagy in cancer and metastasis—Implications in therapeutic development

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Abstract
Autophagy is a highly conserved intracellular degradation process that plays a crucial role in cell survival and stress reactions as well as in cancer development and metastasis. Autophagy process involves several steps including sequestration, fusion of autophagosomes with lysosomes and degradation. Forkhead box O (FOXO) transcription factors regulate the expression of genes involved in cellular metabolic activity and signaling pathways of cancer growth and...
metastasis. Recent evidence suggests that FOXO proteins are also involved in autophagy regulation. The relationship among FOXOs, autophagy, and cancer has been drawing attention of many who work in the field. This study summarizes the role of FOXO proteins and autophagy in cancer growth and metastasis and analyzes their potential roles in cancer disease management.

**KEYWORDS**

autophagy, cancer, FOXO proteins, metastasis

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## 1 | INTRODUCTION

Cancer is generally characterized by a set of features that includes sustained chronic proliferation, insensitivity to growth suppressors, unlimited replicative potential, resistance to apoptosis, induction and sustained angiogenesis, tissue invasion, and metastatic formation.\(^1\) Metastases are accountable for ~90% of all cancer deaths.\(^2\) Their formation is quite complex requiring primary tumor cells to detach and enter the blood circulatory system to reach distant tissue sites, where they adapt to survive and thrive forming secondary tumors.\(^3\)\(^-\)\(^5\) Despite accumulating evidence for the occurrence of genetic and epigenetic alterations, mechanisms underlying cancer progression and metastatic formation are still poorly understood.\(^4\)

Forkhead box O (FOXO) transcription factors have been reported to influence the cellular responses to external energy changes, growth factors stimulation, and nutritional status. Evidence also suggest their involvement in several cellular processes and their dysfunction has been implicated in aging, diabetes, and cancer.\(^6\)\(^-\)\(^10\) In fact, FOXOs expression and transcriptional activity have been reported to be altered in many cancers. Due to their antiproliferative and proapoptotic functions, FOXOs are generally viewed as tumor suppressors.\(^11\) They have also been implicated in autophagy modulation in different cancer cells.\(^12\)\(^-\)\(^14\) Autophagy or macroautophagy is a biologically ancient and highly conserved process present in all cells.\(^15\) It involves the intracellular formation of specialized membranous vesicles, which engulf target proteins and organelles for transport to lysosomes for degradation.\(^16\) Many genes involved in autophagy have been identified and the basic process of autophagy is only partially understood.\(^17\)\(^,\)\(^18\) There is still a significant gap between our understanding of the signaling pathways that control autophagy and the impact of these pathways in cancer cell biology.\(^19\)\(^,\)\(^20\) Emerging evidence support a connection between FOXO proteins and autophagy in cancer suggesting their potential as leading targets for the development of new therapeutic approaches. This review summarizes the role of FOXO proteins and autophagy in cancer growth and metastasis and analyzes the most recent findings regarding the potential therapeutic opportunities related to FOXO’s-autophagy regulation.

## 2 | FOXO TRANSCRIPTION FACTORS

From FOXA to FOXS, forkhead genes encode proteins that contain a conserved DNA-binding domain (the forkhead box), often referred as a “winged-helix” due to its butterfly-like appearance.\(^21\)\(^-\)\(^23\) Mammalian FOXOs including FOXO1, FOXO3, FOXO4, and FOXO6 gene products are evolutionarily conserved and function as transcription factors by binding to target DNA through the forkhead domain leading to either inhibition or activation of downstream target genes.\(^24\) Accordingly, FOXO proteins participate in several crucial processes that range from cell proliferation to cell apoptosis including the regulation of autophagy, metabolism, inflammation, and differentiation (Table 1).
FOXOs transcriptional regulation is controlled by a variety of posttranslational modifications (PTMs), such as phosphorylation, ubiquitination, acetylation, methylation, glycosylation, and poly(ADP-ribosyl)ation (PARylation) that dictate FOXOs subcellular localization.

FOXOs regulation by insulin or insulin-like growth factor-1 occurs via the ubiquitous phosphatidylinositol 3-kinase-protein kinase B (PI3K-AKT) pathway. AKT phosphorylates nuclear FOXOs at three conserved serine/threonine residues (Thr32, Ser253, and Ser315 in FOXO3) leading to the binding of 14-3-3 proteins. With the exception of FOXO6, all FOXOs have these three evolutionarily conserved phosphorylation sites and binding of 14-3-3 promotes the translocation of FOXO proteins to the cytoplasm blocking their transcriptional activity.

FOXOs phosphorylation induced by other kinases such as serum/glucocorticoid regulated kinase 1, casein kinase 1 alpha 1 (CK1), dual-specificity tyrosine phosphorylation-regulated kinase 1A, extracellular signal-regulated kinase (ERK), and I kappa B kinase also inhibits FOXOs transcriptional activity. Conversely, FOXOs phosphorylation by upstream c-Jun N-terminal kinase (JNK), mammalian sterile 20-like kinase (MST1), and AMP-activated protein kinase (AMPK) promotes FOXOs transcriptional activity. Under oxidative stress conditions, JNK phosphorylates FOXO4 at Thr447 and Thr451 residues promoting its translocation from the cytoplasm to the nucleus.

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### Table 1

| Gene name | Human chromosomal location | Subcellular localization | Total amino acids | Expression pattern | Cellular Function | References |
|-----------|---------------------------|--------------------------|-------------------|-------------------|-----------------|------------|
| FOXO1     | 13                        | Nucleus/cytoplasm        | 655               | Ubiquitous with higher expression in adipose tissue | Proliferation (↑/↓) | 25-31      |
|           |                           |                          |                   |                   | Metabolism (↑)   |            |
|           |                           |                          |                   |                   | Apoptosis (↑)    |            |
|           |                           |                          |                   |                   | Inflammation (↑) |            |
|           |                           |                          |                   |                   | Autophagy (↑)    |            |
| FOXO3     | 6                         | Nucleus/cytoplasm        | 673               | Ubiquitous with higher expression in brain, heart, kidney and spleen | Proliferation (↑/↓) | 30-40      |
|           |                           |                          |                   |                   | Metabolism (↑)   |            |
|           |                           |                          |                   |                   | Apoptosis (↑)    |            |
|           |                           |                          |                   |                   | Inflammation (↑) |            |
|           |                           |                          |                   |                   | Differentiation (↑/↓) |            |
|           |                           |                          |                   |                   | Autophagy (↑/↓)  |            |
| FOXO4     | X                         | Nucleus/cytoplasm        | 505               | Ubiquitous with higher expression in skeletal muscle | Proliferation (↑) | 31,41-43   |
|           |                           |                          |                   |                   | Apoptosis (↑)    |            |
|           |                           |                          |                   |                   | Inflammation (↑) |            |
| FOXO6     | 1                         | Nucleus                  | 492               | Brain             | Proliferation (↑) | 31,44,45   |
|           |                           |                          |                   |                   | Metabolism (↑)   |            |

Abbreviation: FOXO, forkhead box O.
counteracting AKT signaling.\textsuperscript{60} Oxidative stress also promotes MST1-induced phosphorylation of FOXO3 at Ser207 leading to the disruption of 14-3-3 binding and to FOXOs nuclear relocalization.\textsuperscript{61} Similarly, under conditions of nutrient stress, AMPK was shown to phosphorylates FOXO3 at six residues (Thr179, Ser399, Ser413, Ser555, Ser588, and Ser626), triggering its transcriptional activity without interfering with its subcellular localization.\textsuperscript{62} FOXOs ubiquitination may involve mono- and polyubiquitination, both having different roles on their transcriptional activity regulation. Oxidative stress conditions induce the monoubiquitination of FOXO4 at K199 and K211 residues, triggering its relocalization into the cell nucleus and increasing its transcriptional activity.\textsuperscript{63} Accumulated FOXOs in the cytosol, as a result of AKT-mediated phosphorylation, are degraded by polyubiquitination through the ubiquitin-proteosome pathway.\textsuperscript{64} FOXO proteins function is also regulated by several acetylases and deacetylases.\textsuperscript{65} In response to oxidative stress, CREB-binding protein (CBP) and its paralog p300 (CBP/p300), acetylate FOXO proteins inhibiting their activity. CBP-induced acetylation of FOXO1 and FOXO4 leads to the reduction of their transcriptional activity.\textsuperscript{66,67} Silent information regulator 2 (SIRT2)-induced deacetylation of FOXO1 reverses the acetylation effect increasing FOXO1 transcriptional activity.\textsuperscript{67} FOXOs activity regulation may also result from the interplay of their PTMs. For example, acetylation decreases FOXO1 DNA-binding activity and consequently enhances its availability to AKT-mediated phosphorylation.\textsuperscript{68} Other PTMs such as methylation by protein arginine methyltransferase 1 (PRMT1), N- and O-linked glycosylation and PARylation by poly(ADP-ribose) polymerase 1 (PARP1), also regulate FOXOs transcriptional activity. Specifically, PRMT1 methylates FOXO1 inhibiting AKT-mediated phosphorylation and thereby preventing its nuclear translocation.\textsuperscript{47} Regarding FOXO1 glycosylation, in spite of not influencing its subcellular localization, it also enhances its transcriptional activity.\textsuperscript{48} Concerning PARP1-induced modifications, these have been shown to negatively regulate FOXOs transcriptional activity. Specifically, phosphorylation of FOXO3 by PARP1 prompts its nuclear export impairing its transcriptional activity.\textsuperscript{49,50}

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3 FOXOs IN CANCER AND METASTASIS

FOXO1, FOXO3, and FOXO4 were initially discovered because chromosomal translocations were detected in some forms of leukemia supporting their association with cancer development. While FOXO1 was found in alveolar rhabdomyosarcoma as a fusion protein involving DNA-binding domain of PAX3 or PAX7,\textsuperscript{69-71} FOXO3 and FOXO4 were discovered in acute myeloid leukemias (AMLs) as fusion proteins with mixed-lineage leukemia (MLL) gene.\textsuperscript{72,73} Nonetheless, the underlying mechanisms tying these genetic alterations with FOXOs role in carcinogenesis are still unclear.

The increased metabolic demands associated with cancer progression causes tumor cells to reprogram their metabolism to grow and proliferate. Some of the metabolic alterations promoted by cancer cells affect both glucose and lipid metabolisms, which regulation has also been shown to be modulated by FOXOs transcriptional activity.\textsuperscript{74} To generate ATP, cancer cells resort to glycolysis, a mechanism promoted by the proto-oncogene Myc that is only used by healthy cells in hypoxic conditions.\textsuperscript{75,76} Several studies have reported that FOXOs antagonize Myc function, which is reported to be upregulated in many cancers, thus inhibiting glycolysis and, therefore, impairing glucose uptake.\textsuperscript{74} In fact, acetylated FOXOs were shown to promote the increase of Myc levels in glioblastoma and, upon energy stress conditions, FOXOs were shown to induce the upregulation of FOXO-induced long noncoding RNA 1 promoting the decrease of Myc protein levels in renal cancer cells.\textsuperscript{77,78} In contrast, FOXOs were shown to have a key role in the regulation of hepatic gluconeogenesis enhancing the glucose output via the activation of phosphoenolpyruvate carboxykinase 1 (PCK1) and glucose-6-phosphatase (G6Pase), thereby upregulating phosphoenolpyruvate carboxykinase 2 (PEPCK) levels.\textsuperscript{79,80} FOXO1 nuclear exclusion mediated by the tumor suppressor p53 was shown to impair the expression of both PCK1 and G6Pase and, therefore, to compromise gluconeogenesis in different cancer cell lines.\textsuperscript{81} Congruently, a different study reported that AKT loss mediated by the mammalian
target of rapamycin (mTOR) inhibition promoted FOXO1 nuclear retention and the upregulation of PEPCK levels, thus increasing the glucose output by cancer cells.\textsuperscript{82} Despite these evidence, FOXOs involvement in the regulation of gluconeogenesis in cancer cells is still unclear. Lipid metabolism rewiring by tumor cells is usually achieved by enhancing fatty acid synthesis, a process regulated by sterol regulatory element-binding proteins (SREBPs).\textsuperscript{83,84} Evidence suggest that FOXO1 is able to impair lipogenesis by reducing the transcriptional activity of SREBPs and thereby downregulating the expression of the enzyme fatty acid synthase.\textsuperscript{85} In contrast, FOXOs were shown to induce the expression of the enzyme adipose triacylglycerol lipase, consequently upregulating lipolysis in adipocytes.\textsuperscript{86} As the influence of FOXO proteins in cells metabolism has been extensively reported, more studies are still necessary to unravel their regulatory role in cancer cells metabolism.

3.1 | FOXOs as tumor suppressors

FOXOs are generally considered to suppress cancer. This view is sustained by the tumor-prone phenotype of FOXOs knockout mice. Studies from knockout mice with up to five deleted FOXO alleles resulted in a mild tumor phenotype.\textsuperscript{87} Nonetheless, the simultaneous deletion of all alleles lead to the development of thymic lymphomas and hemangiomas, indicating a redundant tumor suppressive function for these transcription factors in cancer.\textsuperscript{87} FOXOs suppressive role is further reinforced by studies reporting their inactivation in multiple human cancers by the PI3-AKT signaling pathway, which is commonly hyperactivated during cancer due to the occurrence of mutations in RAS, suppressor proteins PTEN or PI3K.\textsuperscript{88} FOXOs inactivation promotes the downregulation of genes implicated in cell cycle arrest, such as retinoblastoma-related protein p130 and cell cycle kinase inhibitor p27\textsuperscript{kip1}, and enhances cell survival decreasing the expression of the proapoptotic tumor necrosis factor-related apoptosis-inducing ligand.\textsuperscript{89} Downregulation of FOXO1 and increased levels of FOXO1 phosphorylation is reported to be correlated with poor prognosis in patients with soft tissue sarcoma,\textsuperscript{90} AML,\textsuperscript{91} prostate,\textsuperscript{92} and breast cancers.\textsuperscript{93} Likewise, the downregulation of FOXO3 correlated with poor survival in patients with neuroblastoma,\textsuperscript{94} breast,\textsuperscript{95-97} ovarian,\textsuperscript{96} and colorectal cancers.\textsuperscript{99} Similarly, the upregulation of FOXO3 phosphorylation levels were identified as adverse prognostic biomarkers in ovarian cancer\textsuperscript{100} and AML.\textsuperscript{101} FOXOs influence in metastasis formation and progression has also been reported. Specifically, the downregulation of FOXO3 was shown to be important for tumor metastasis progression in colorectal cancer\textsuperscript{99} and renal cell carcinoma.\textsuperscript{102} Moreover, FOXO3 nuclear localization was correlated with better prognosis and less frequent metastasis formation in estrogen receptor-positive breast cancer.\textsuperscript{97} Downregulation of FOXO4 in patients with prostate cancer was also correlated with earlier metastatic formation.\textsuperscript{103}

3.2 | FOXOs as tumor promoters

Despite being considered tumor suppressors by most studies, new evidence regarding FOXOs role in the promotion of cancer progression and in the resistance to cancer therapy in certain contexts has come to light. In fact, occurrences of FOXO1 single-nucleotide somatic mutations that promote its nuclear localization were correlated to a poor prognosis in large B-cell lymphomas.\textsuperscript{104} Likewise, increased levels of FOXO3 were associated with poor outcomes in hepatocellular carcinoma (HCC) and glioblastoma.\textsuperscript{105,106} Evidence also suggests the involvement of FOXO proteins in the regulation of leukemia-initiating cells (LIC) maintenance in chronic myelogenous leukemia (CML) and AML.\textsuperscript{107} Specifically, LICs were shown to present an increased nuclear localization of FOXO3 due to a decrease of AKT activation and these cells’ ability to cause CML was significantly reduced by FOXO3a deficiency.\textsuperscript{108} Likewise, active FOXOs were found in patients with AML and, either activation of AKT or FOXOs deletion, impaired leukemic cell growth and LIC function promoting the survival of an AML mice model.\textsuperscript{109} In line with these findings, high levels of FOXO3 correlated to a lower overall survival and recurrence free survival of
patients with AML. Similar roles for FOXO proteins in other cancer stem-like cells have also been reported. Despite the abovementioned evidence, linking FOXOs increased levels to reduced metastasis formation, several studies also suggest that FOXO proteins can facilitate metastasis. In fact, the nuclear localization of both FOXO3 and elevated β-catenin contents correlated with the formation of metastases and with a shorter survival in colorectal cancer. Likewise, FOXO3 overexpression correlated with lymph node metastasis formation and with a poor disease free survival of patients with triple-negative breast cancer. FOXOs’ facilitating role in metastasis has also been observed in breast cancer cell lines. Specifically, nuclear retention and activation of FOXO3, due to serum deprivation resultant from increased mass tumor, were shown to upregulate matrix metalloproteinase-9 (MMP-9) and MMP-13 levels which further promote invasive cellular migration. Likewise, FOXO1 was reported to directly regulate MMP-1 expression enhancing metastasis formation in breast cancer cells. FOXO3 knockdown was also reported to significantly suppress tumor formation and metastasis in pancreatic ductal adenocarcinoma. In summary, FOXOs present a paradoxical role in cancer and metastasis in which they are able to act as tumor suppressors, by inducing apoptosis and cell-cycle arrest but may also promote cancer progression (Figure 1).

4 | AUTOPHAGY

Autophagy is a highly conserved cellular process that involves the intracellular degradation and removal of damaged proteins and organelles supporting cellular renovation and homeostasis. This process may be induced by starvation or nuclear stress, initiating with the formation of autophagosomes that engulf the intracellular cargo and carry them to lysosomes for degradation and recycling. Autophagy initiation is controlled by the serine/
threonine kinase ULK1 complex, which is a direct target of mTOR pathway and AMP-activated kinase (AMPK). The mTOR pathway constitutes a major negative regulator of autophagy being activated downstream of PI3K-AKT signaling pathway which is frequently deregulated in cancer. Autophagy can also be initiated by AMPK activation, which inhibits mTOR. Once mTORC1 kinase activity is inhibited, autophagy initiates the formation of autophagosomes by activating ULK1 complex. This step is followed by the expansion of the autophagosomal membrane promoted by the activation of a second protein complex composed of vacuolar sorting protein 34 (VPS34), a class III PI3K, and beclin 1 (BECN1). Continued expansion of the autophagosomal membrane is regulated by the conjugation of essential autophagy genes Atg5 to Atg12 and of microtubule-associated protein 1 light chain 3 alpha (LC3) to phosphatylethanolamine (PE), forming LC3-PE conjugate (also known as LC3-II). The engulfment of the cytoplasmatic constituents then occurs both in a selectively, through the action of scaffolding proteins such as the adaptor protein p62/sequestosome 1, and nonselectively manner. This is followed by sealing of the two ends of the membrane to form a completed autophagosome which then fuses with lysosomes through a mechanism mediated by small GTPases such as RAB7 and SNARE-like proteins. The engulfment of the cytoplasmatic constituents then occurs both in a selectively, through the action of scaffolding proteins such as the adaptor protein p62/sequestosome 1, and nonselectively manner. This process is finalized by the degradation and recycling of autophagosome contents.

Multiple pieces of evidence suggest that autophagy is involved in tumor initiation and development, cancer progression, and metastasis. However, the precise role of autophagy in cancer is not clear due to the fact that it can either inhibit tumor cell initiation or promote tumor cell survival (Figure 2) depending on cancer type, stage of progression, and genetic context.

4.1 | Autophagy as tumor suppressor

The suppressive role of autophagy in cancer progression was initially suggested by studies showing that one allele of Atg6/BECN1 was depleted in 40% to 75% of human ovarian, breast and prostate cancers, that BECN1 upregulation inhibited the growth of human breast cancer cell lines and that BECN1 heterozygous mutant mice

![Figure 2](wileyonlinelibrary.com)
suffered from a high incidence of spontaneous tumor formation.\textsuperscript{134-138} BECN1, an important protein involved in the autophagosome formation step is regulated by UV radiation resistance-associated gene and Bax-interacting factor-1 whose impairment has been shown to promote tumor cell proliferation in several cancers.\textsuperscript{139-141} In addition, the deletion of vital autophagy genes Atg5 and Atg7 in mice induced the development of benign liver tumors.\textsuperscript{142} Possible mechanisms behind the autophagy tumor suppressive role have been already identified.\textsuperscript{132,143} The PI3K/AKT/mTOR signaling pathway has been shown to be commonly deregulated during cancer. Specifically, the constitutive activation of PI3K/AKT, which may be due to the occurrence to PI3K mutations, loss of PTEN or even overexpression of AKT, and subsequent stimulation of mTOR, inhibits the autophagic activity supporting its suppressive role in cancer progression.\textsuperscript{144-146} Failure in the control of damaged proteins and organelles due to autophagy inhibition leads to the accumulation of reactive oxygen species, increased DNA damage, and mitochondrial defects increasing the susceptibility for tumor development.\textsuperscript{143} Oxidative stress upregulation promotes the transcription of genes involved in the antioxidant defense by nuclear factor erythroid 2-related factor 2 (NRF2) which is activated by p62.\textsuperscript{143,147,148} In fact, defective autophagy was shown to increase p62 levels which promotes NRF2 activation to fight against oxidative stress and therefore enabling cell survival.\textsuperscript{143,149} Conversely, p62 ablation was shown to suppress tumorigenesis in mouse models with defective autophagy.\textsuperscript{147} Since p62 was also shown to regulate other oncogenic pathways involving nuclear factor-κB and mTOR as a signaling adaptor,\textsuperscript{150} it would be of great interest to ascertain its involvement in autophagy impairment and tumor promotion. Autophagy cancer suppressive function has also been associated to its aptitude to control early infiltration of regulatory T cells.\textsuperscript{151} In addition, in later stages of cancer, autophagy may have a protective role in tumor cells immunity, as its inhibition was shown to restrict cancer progression.\textsuperscript{151}

4.2 | Autophagy as tumor promoter

Despite being generally considered as a tumor suppressor in the initial stage of tumor development, in more-established cancers, autophagy promotes tumorigenesis since it tries to fulfill the increased biosynthetic and metabolic needs of tumor cells.\textsuperscript{135,152} Autophagy has been shown to be essential in the promotion of tumor cell survival undergoing hypoxia and that are under pressure from therapeutic interventions.\textsuperscript{135,153} In fact, hypoxic conditions promote hypoxia-inducible factor 1α-dependent and independent (via AMPK activation and mTOR inhibition) autophagy induction, thus enabling cell survival.\textsuperscript{154,155} Likewise, hypoxic activation of autophagy was shown to induce p62 downregulation which further promoted an increased Ras/ERK activity that contributed to cell survival and proliferation.\textsuperscript{156} Autophagy tumor promoter role was further established by studies performed in conditional autophagy gene FIP200 knockout mice showing that lack of FIP200 reduced the development of mammary tumors.\textsuperscript{157} Moreover, in cells transformed with oncogenic RAS, autophagy was shown to have an essential role in promoting tumor cells survival, growth, invasion, and metastasis.\textsuperscript{158-161} Therefore, by conferring tumor cells a survival advantage, autophagy may also be seen as a mechanism that facilitates cancer progression.

4.3 | Autophagy in cancer metastasis

As autophagy activation shares many of the environmental triggers known to promote metastatic formation, its activity was shown to be upregulated during metastasis. In fact, increased staining of the autophagy-associated protein LC3 was associated with lymph node metastasis and decreased survival rate in human breast cancer patient samples.\textsuperscript{162,163} Moreover, studies performed in melanoma metastasis samples revealed an increased expression of LC3 and BECN1.\textsuperscript{164} Increased LC3 expression also correlated with metastasis in HCC.\textsuperscript{165,166} As with primary cancer cells, autophagy has demonstrated both anti- and prometastatic effects.\textsuperscript{167,168} By supporting the survival of tumor cell under hypoxic and metabolic stress conditions, which results in the reduction of tumor cell necrosis and
subsequent infiltration of inflammatory cells, autophagy inhibits early -stage metastasis. Moreover, by directly mediating the secretion of immunomodulatory factors by the dying tumor cells, such as high-mobility group box 1 (HMGB1), autophagy is able to directly regulate the cancer-triggered inflammatory responses. In fact, released HMGB1 was reported to bind to dendritic cells Toll-like receptor 4 (TLR4) thereby triggering an antitumor immune response that restricts metastasis. In another study, AKT was actively phosphorylated by CXCL12 and several cytokines, and inhibition of the CXCR4/mTOR pathway induced autophagic cell death and inhibited metastasis of peritoneally disseminated gastric cancer cells. Loss of PTEN and subsequent activation of the AKT pathway in prostate cancer have also been pointed as an early prognostic marker for metastasis. The upregulation of PI3K/AKT/mTOR signaling pathway decreases the autophagic flux, thus promoting cancer progression. Accordingly, the inhibition of AKT pathway was shown to promote a decrease in metastasis incidence.

During advanced stages of metastasis progression, autophagy may promote metastasis by helping the tumor cells that detach from the extracellular matrix (ECM) to avoid anoikis thereby enhancing their survival and colonization in secondary sites. In spite of the precise mechanisms of autophagy protection towards anoikis remain unclear, recent evidence suggests that protein kinase R-like endoplasmic reticulum kinase promotes the survival of ECM-detached cells by inducing the autophagic activity and a proper antioxidant response. Autophagy inhibition in an animal model of HCC lung metastasis resulted in the decrease of metastasis formation by promoting anoikis resistance. Since tumor cells detachment is associated with a loss of extrinsic signaling that support proper nutrient and energy uptake, autophagy may work as a compensatory mechanism, as suggested by a study showing that autophagy is induced by either ECM detachment or β1 integrin inhibition. Thus, autophagy might postpone anoikis initiation by giving cells more time to connect with a suitable ECM. Disseminated cells that are unable to reconnect with ECM in the secondary sites may enter a dormant state promoted by autophagy. Understanding the contexts that dictate autophagy role in cancer progression and metastasis will be pivotal to the development of more effective therapies.

Several lines of evidence support a connection between FOXO proteins and autophagy. FOXOs have been shown to regulate autophagy by both transcriptional-dependent and transcriptional-independent mechanisms. While located in the nucleus, FOXOs are able to promote the transcription of several autophagy genes involved in the different steps of the autophagic process, but when translocated to the cytosol, FOXOs also regulate autophagy by interacting directly with cytosolic autophagy proteins. During muscle atrophy, FOXO3 was reported to upregulate several autophagy-associated genes, like LC3, BNIP3, Gabarapl1, VPS34, ULK2, and Atg12. FOXO1 deacetylation was shown to occur in mouse cardiomyocytes with the assistance of NAD-dependent deacetylase SIRT1 (sirtuins) which induces the expression of RAB7A, GTP-binding protein. This protein aids in the fusion of lysosomes and mature autophagic vesicles. Study of PI3K-AKT-FOXO pathway revealed that FOXOs upregulation of glutamine synthetase results in mTOR inhibition and consequently promotes autophagy. This is indicative that autophagy induction by FOXOs-mediated increase of glutamine metabolism might allow cellular survival during starvation by protecting cells from damage accumulation. FOXOs induction of Sestrin3 and RICTOR was also reported to enhance the autophagic flux by inhibiting mTOR1. In addition, studies performed in mouse embryonic fibroblast and embryonic kidney (HEK293T) cell lines showed that FOXO3 coordinately regulates FOXO1 to induce transcription-dependent autophagy. FOXO proteins were also shown to control the autophagic flux by epigenetic mechanisms and more recently autophagy was reported as being able to degrade FOXO proteins (reviewed in detail by Cheng).
The exact role of FOXOs regulation of autophagy in tumor biology is still unclear. The hyperactivation of PI3K/AKT signaling pathway in cancers promotes FOXOs phosphorylation thereby promoting their nuclear exclusion. Under stress conditions, cytosolic FOXO1 was shown to induce autophagy in human colon cancer cells by interacting with Atg7. In this study, FOXO1-mediated autophagy induction resulted in an increase of cell apoptosis, which is associated with FOXO1 antineoplastic effect. Epigenetic modulation of FOXO proteins by histone deacetylase inhibition was reported to induce autophagy and apoptosis through FOXO1-mTOR signaling and FOXO1 transactivation of autophagy genes in human cancer cells. Conversely, FOXO3 was shown to inhibit the cytosolic accumulation and expression of FOXO1 in multiple cancer cell lines, resulting in autophagy suppression. FOXO3 was also reported to trigger autophagy in neuroblastoma cells by upregulating the decidual protein induced by progesterone (C10ORF10/DEPP) expression. In this study, FOXO3-induced autophagy partly protected neuroblastoma cells from apoptosis, and pharmacological inhibition of autophagy enhanced chemotherapeutic intervention outcomes. Some studies have also been reporting that autophagy deregulation in cancer may promote FOXO proteins turnover mediated by AKT induced phosphorylation. More recently, FOXO3 was shown to be degraded by basal autophagy by acting as its cargo. In fact, autophagy inhibition in cancer cells was shown to upregulate FOXO3 activity consequently increasing the proapoptotic protein p53 upregulated modulator of apoptosis (PUMA) levels conferring the cells a higher sensitivity to an apoptosis inducer. This can explain how autophagy inhibition can increase apoptosis upon the administration of anticancer drugs.

6 | CANCER THERAPY

6.1 | Targeting FOXOs for cancer therapy

Cancer therapies based in restoring the expression and activation of FOXOs may be effective in tumor suppression. In fact, FOXOs are involved in the mediation of cancer cell apoptosis promoted by the use of chemotherapeutic drugs such as 5-fluorouracil, paclitaxel, and resveratrol, and inhibitors of BCR-ABL, PI3K, or AKT. Nonetheless, the effectiveness of the treatments is often vulnerable to the development of multidrug resistance (MDR) as demonstrated by studies showing that FOXO1 and FOXO3 are key regulators of the MDR1 in adriamycin-resistant breast cancer cells and in doxorubicin chemotherapy, respectively. Therapeutic strategies designed to target FOXO proteins may also be jeopardized by feedback mechanisms that promote cell survival. Upon treatment with inhibitors of PI3K or AKT, cancer cells have the ability to readjust to the loss of oncogenic signaling by FOXOs-mediated reactivation of PI3K-AKT pathway. In fact, the activation of FOXO3 by doxorubicin promoted K562 CML cell survival by upregulating the expression of PI3K catalytic subunit p110α and thereby activating PI3K/AKT pathway. Also, the inhibition of the PI3K-AKT pathway in renal cell carcinoma increased FOXOs activity, which enhanced the expression of TORC2 component RICTOR promoting AKT activation. Altogether, these findings reinforce the paradoxical function of FOXOs in cancer.

6.2 | Targeting autophagy for cancer therapy

Treatments aiming at targeting autophagy in tumor cells have been focusing on either its induction and/or inhibition. While the induction of cytoprotective autophagy in premalignant lesions may prevent cancer progression, in more established cancers autophagy inhibition may prevent tumors from growing and spreading. Autophagy-inducing drugs include mTOR1 inhibitors like rapamycin and rapamycin derivatives temsirolimus (CCI-779) and everolimus (RAD001), which have been used in the treatment of different cancers. Other anticancer and autophagy stimulatory compounds have also been identified, including metformin that upregulates AMPK; resveratrol that activates SIRT1; and perifosine that inhibits AKT and mTOR. Despite the
induction of cytoprotective autophagy in early-stage tumors may suppress cancer progression, its potentiation by chemotherapy and radiation therapy has been identified as a resistance mechanism in several cancers. Together with the fact that tumor cells from advanced cancers benefit from autophagic activity to survive the augmented metabolic demands, current anticancer therapies have been mainly focused on autophagy inhibition combined with different types of anticancer approaches. The rationale behind the employment of combination therapies is to render cells the sensitivity to the treatments. Different autophagy inhibitors actuate on the different phases of the autophagic process. Inhibitors that actuate at the initiation stage, by blocking autophagosome formation through class III PI3K targeting, include wortmannin, 3-methyladenine, and LY294002. Other inhibitors such as chloroquine (CQ), hydroxychloroquine (HCQ), monensin, and Bafilomycin A1, the latter being a specific inhibitor of vacuolar-ATPase, inhibit the autophagosome-lysosome fusion. By being able to cross the lysosomal membrane, these drugs inhibit autophagy by preventing lysosomes acidification and blocking the fusion of autophagosomes with lysosomes. Curiously, CQ antitumor actions and ability to sensitize cancer cells to chemotherapy have also been described to occur through autophagy-independent mechanisms. At the moment, CQ and HCQ are the only clinically available inhibitors and they have been evaluated alone or in combination with other anticancer therapies in different cancers (Table 2). Past clinical trials demonstrated that CQ and HCQ are safe autophagy inhibitors and despite presenting diverging clinical outcomes its administration still holds encouraging therapeutic benefits. More importantly, current research suggests the need to design new delivery approaches able to improve the drugs penetration into cancer cells, and to develop more potent and specific autophagy inhibitors. In this sense, new promising autophagy inhibitors have been developed envisioning their application in forthcoming clinical trials and include ULK1, VPS34, ATG4B, palmitoyl-protein thioesterase 1, and phosphoinositide kinase-type finger-containing kinase (PIKFYVE) inhibitors (Table 3) (reviewed in detail by Amaravadi et al). At the moment, an ongoing phase 1 clinical trial is testing the safety and pharmacokinetics of the PIKFYVE inhibitor apilimod in patients with non-Hodgkin’s lymphoma (NCT02594384). The discovery of more reliable biomarkers to screen the tumors before treatment may also result in better therapeutic outcomes.

6.3 Therapeutic opportunities related to FOXOs-autophagy regulation

Evidence regarding FOXOs-autophagy regulation in cancer may allow the emergence of trustworthy biomarkers and novel therapeutic strategies aiming to halt cancer progression. In fact, the subcellular localization of FOXO3 upon PI3K inhibition was reported to function as a biomarker to predict the outcome of therapies combining PI3K and autophagy inhibitors in cervical cancer cells with PIK3CA mutations. As the use of PI3K inhibitors is often accompanied by the development of drug resistance, combined treatment with HCQ was shown to enhance PI3K inhibitor efficacy in cells that exhibited FOXO3 nuclear translocation. In a different study, FOXO1 turnover was shown to be promoted by ERK-induced interaction of FOXO1 with X-box-binding protein 1 (XBP1). XBP1 was found to be inversely proportional to FOXO1 and autophagy as shown by XBP1 upregulation and FOXO1 downregulation in human colon cancer samples. Therefore, ERK inhibition may impair cancer progression by inducing FOXO1 expression and autophagy. However, in RAS- or BRAF-mutant cancers, autophagy induction by MAPK inhibition was recently associated with the development of treatment resistance. Therefore, the combination of MAPK inhibitors with autophagy inhibitors may offer promising results. treatment, a natural isothiocyanate, was shown to inhibit PI3K/AKT and mitogen-activated protein kinase/ERK signaling pathways thereby promoting FOXOs transcriptional activity in pancreatic cancer cells. In another study, benzyl isothiocyanate treatment promoted FOXO1 acetylation thus inducing the upregulation of autophagy and inhibiting the growth of human breast-cancer cells. Interferon regulatory factor-4 binding protein, whose expression has been highly correlated with the malignancy and invasiveness of human breast cancer cells, was shown to have a suppressive effect on these cells autophagic activity that relied on the activation of mTOR2/AKT/FOXO3
| Cancer                                      | Inhibitor | Clinical trial phase | Combination treatment          | Biomarkers                                               | Reference |
|--------------------------------------------|-----------|----------------------|--------------------------------|----------------------------------------------------------|-----------|
| Breast cancer, NSCLC, and brain metastases | CQ        | Phase 2              | Radiation                      | Not analyzed                                             | 224       |
| Benign (solid) tumor                       | HCQ       | Phase 1              | Vorinostat                     | EM analysis of PBMCs for AVs                            | 225       |
|                                            |           |                      |                                | IHC detection of LC3II                                   |           |
| Glioblastoma                               | CQ        | Phase 3              | Radiation and TMZ              | Not analyzed                                             | 226       |
| Benign tumor and melanoma                  | HCQ       | Phase 1              | Temsirolimus                   | EM analysis of PBMCs for AVs                            | 227       |
| Relapsed glioblastoma                      | CQ        | Five patients case series | Radiation                      | Not analyzed                                             | 228       |
| Benign tumor                               | HCQ       | Phase 1              | Docetaxel, rapamycin with metronomic Cyproterone | Not analyzed                                             | 229       |
| Pancreatic tumor                           | HCQ       | Phase 2              | None                           | Analysis of LC3II in peripheral lymphocyte from metastatic pancreatic cancer patient | 230       |
| Advanced pancreatic cancer                 | HCQ       | Phase 2              | Gemcitabine and nab-paclitaxel | Not analyzed                                             | 223       |
| Renal cell carcinoma                       | HCQ       | Phase 1/phase 2      | Everolimus                     | Not analyzed                                             | 231       |

Abbreviations: AVs, autophagic vesicles; CQ, chloroquine; EM, electron microscopy; HCQ, hydroxychloroquine; IHC, immunohistochemistry; LC3, microtubule-associated protein 1 light chain 3 alpha; NSCLC, non–small cell lung cancer; PBMC, peripheral blood mononuclear cell.
pathway. The development and induced innate immunity of natural killer (NK) cells were also shown to be dependent of FOXOs-mediated autophagy. The efficacy of anticancer immunotherapies resorting to NK could therefore be enhanced by upregulating FOXOs transcriptional activity and autophagy. The recent discovery that autophagy modulates FOXO3 turnover is another example of how the synergy between them may pave the way to the development of more effective therapies, as the inhibition of autophagy caused cancer cells to become more sensitive to anticancer drugs due to the upregulation of FOXO3 activity and consequent increase of PUMA levels.

### 7 | CONCLUSION AND FUTURE DIRECTIONS

Emerging evidence has shown that FOXO proteins and autophagy are promising therapeutic targets for cancer treatments. However, despite their great potential, it is still necessary to fully comprehend their role in cancer progression. Identifying the contexts where FOXOs support or suppress cancer progression is paramount for the development of more effective therapeutic approaches. The supportive role for FOXO proteins in cancer progression complicates treatment strategies aiming the reactivation of FOXOs activity. Therefore, alternative strategies may include a combination of FOXO protein inhibitors and drugs able to target PI3K to avoid the acquisition of drug resistance. Improvement of drug specificity and development of suitable methods to measure FOXOs activity before and after treatment also pose as valuable tools to enhance therapeutic efficacy.

Autophagy studies addressing whether it should be induced or inhibited, and which patients would benefit from autophagy manipulation will also result in better therapeutic outcomes. Current clinical trials involving autophagy modulation were designed to evaluate autophagy inhibition alone or in combination with other treatments. Nonetheless, determining which is the better therapy combination and which tumors are more susceptible

### TABLE 3  Novel autophagy inhibitors for cancer therapy

| Inhibitor       | Targets                                      | Inhibition point                                      |
|-----------------|---------------------------------------------|-------------------------------------------------------|
| ULK101          | ULK1                                        | Initiation of autophagic vesicle nucleation            |
| SBI-0206965     |                                             |                                                       |
| SAR405          | VPS34                                       | Initiation of autophagic vesicle nucleation            |
| Compound 13     |                                             |                                                       |
| SB02024         |                                             |                                                       |
| Autophinib      |                                             |                                                       |
| S130            | Atg4B                                       | Autophagosome formation and/or autophagosome fusion with the lysosome |
| FMK-9a          |                                             |                                                       |
| NSC185058       |                                             |                                                       |
| LV-320          |                                             |                                                       |
| Lys05           | Palmitoyl-protein thiosterase 1              | Autophagosome fusion with the lysosome                  |
| DQ661           |                                             |                                                       |
| DC661           |                                             |                                                       |
| Apilimod        | PIKFYVE                                     | Lysosomal degradation                                 |

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to autophagy modulation is still a challenge. The development of more reliable biomarkers to measure the autophagic flux in vivo will facilitate the patients that could benefit from treatments envisioning autophagy inhibition.

Efforts have been made to understand the mechanisms underlying FOXOs-mediated autophagy in cancer and its therapeutic potential. A great body of evidence suggest that FOXOs are key autophagy inducers promoting both health and illness depending on the different contexts. In fact, autophagy regulation by FOXO proteins in tumor cells is different from its regulation in healthy conditions and their established paradoxical role in carcinogenesis further complicates the understanding of the mechanisms underlying the regulation of this axis during cancer. The modulation of FOXOs transcriptional activity through its upstream regulators, such as PI3K or ERK, could offer a potential approach to regulate the autophagic activity of tumor cells. Nonetheless, the establishment of potential treatment resistance mechanisms would also need to be addressed. The recent discovery that autophagy inhibition upregulates FOXO3 proteins rendering cells a higher responsiveness to chemotherapy opens new doors to searching of new approaches. Many questions still need to be addressed to fully comprehend how to benefit from FOXO-autophagy regulation during cancer.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

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