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A New Linkage between the Tumor Suppressor RKIP and Autophagy: Targeted Therapeutics

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Abstract

The complexities of molecular signaling in cancer cells have been hypothesized to mediate cross-network alterations of oncogenic processes such as uncontrolled cell growth, proliferation, acquisition of epithelial-to-mesenchymal transition (EMT) markers, and resistance to cytotoxic therapies. The two biochemically exclusive processes/proteins examined in the present review are the metastasis suppressor Raf-1 kinase inhibitory protein (RKIP) and the cell-intrinsic system of macroautophagy (hereafter referred to as autophagy). RKIP is poorly expressed in human cancer tissues, and low expression levels are correlated with high incidence of tumor growth, metastasis, poor treatment efficacy, and poor prognoses in cancer patients. By comparison, autophagy is a conserved cytoprotective degradation pathway that has been shown to influence the acquisition of resistance to hypoxia and nutrient depletion as well as the regulation of chemoimmuno-resistance and apoptotic evasion. Evidently, a broad library of cancer-relevant studies exists for RKIP and autophagy, although reports of the interactions between pathways involving RKIP and autophagy have been relatively sparse. To circumvent this limitation, the coordinate regulatory and effector mechanisms were examined for both RKIP and autophagy. Here, we propose three putative pathways that demonstrate the inherent pleiotropism and relevance of RKIP and the microtubule-associated protein 1 light chain 3 (MAP1LC3, LC3) on cell growth, proliferation, senescence, and EMT, among the hallmarks of cancer. Our findings suggest that signaling modules involving p53, signal transducer and activator of transcription 3 (STAT3), nuclear factor- κ B (NF- κ B), and Snail highlight the novel roles for RKIP in the control of autophagy and vice versa. The suggested potential crosstalk mechanisms are new areas of research in which to further study RKIP and autophagy in cancer models. These should lead to novel prognostic motifs and will provide alternative therapeutic strategies for the treatment of unresponsive aggressive cancer types.

Keywords

Autophagy; LC3; metastasis; NF- κ B; resistance; RKIP; survival; survival pathways; therapy

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I. INTRODUCTION

The progression from a nontumorigenic cell to a neo-plastic cell occurs through multiple steps of somatic mutations, epigenetic alterations, and metabolic re-arrangements that are perpetuated by aberrant signaling pathways.^{1–2} To date, many of the somatic and germline mutations involved in activating and inhibiting tumorigenesis have been compiled by the Cancer Gene Census (CGC) in the Catalogue of Somatic Mutations in Cancer (COSMIC) database.³ First published in 2004, COSMIC lists 719 curated entries of human gene mutations implicated in cancer development and predisposition. Beyond those well known to either exacerbate or attenuate tumorigenesis, several putative oncogenes and tumor suppressors continue to be discovered as a result of the obvious phenotypic impacts observed from RNAi screens and microarray analyses.^{4–9} Such studies highlight the utility of transcriptomic and proteomic screenings in further defining as yet unknown cellular abnormalities frequently seen in cancer cells. However, pinpointing of promising candidates for therapeutic targeting has been confounding due to the intricate circuitry that preserves tumor malignancy, resulting from the highly pleiotropic nature of such molecules.^{10–11}

The crosstalk that exists among diverse signaling pathways and biochemically distinct processes contributes to the extensive control over cellular division, differentiation, and survival in normal cells. At the transcriptional and post-transcriptional levels, tightly regulated signaling cascades mitigating conversion into neoplastic states are adversely affected by oncogenic events.^{12–13} Several genetic and epigenetic abnormalities present in these pathways have been identified as the primary drivers of oncogenesis and metastatic progression.^{14–18} Biologically promiscuous signaling proteins have been shown to cross-activate or cross-inhibit components of other pathways.^{19–22} Also, since many effectors of receptor tyrosine kinase (RTK) signaling pathways exist [including those of the Ras/Raf/mitogen-activated protein kinase kinase (MEK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways], it is unsurprising that a number of cross-pathway mediators can effect compensatory responses in the response to inhibitory molecules.²³ While these points of intersection create promising therapeutic targets, they also threaten to decrease drug responsiveness.

One hallmark of cancer development is the deregulation of the PI3K/Akt/mammalian target of rapamycin (mTOR) signaling axis, resulting in abnormal levels of glucose metabolism to support heightened anabolic requirements during tumor cell growth.^{2,24} In contribution to this cytoprotective function, the Akt/mTOR pathway, alongside the AMP-activated protein kinase (AMPK) pathway, also acts as canonical upstream regulators of autophagy by sensing ATP availability, growth factors, nutrient deprivation, and hypoxia.^{25,26} As a multistep cytoplasmic cargo-recycling program, autophagy is considered either a tumor-suppressive or a tumor-promoting mechanism depending on the stage of the cancer and on extrinsic factors, such as metabolic stress.²⁷ Interestingly, evidence of autophagy-dependent EMT induction and involvement of essential late-phase autophagy proteins [for example, p62/sequestosome 1 (SQSTM1)] in EMT regulation provide possible links to pathways controlling cancer cell motility and invasion.^{28,29}

RKIP is well recognized as a physiological regulator of the mitogen-activated protein kinase (MAPK) pathway.^{30,31} RKIP is also a critical regulator of the NF- κ B/Snail/Yin Yang 1 (YY1)/phosphatase and tensin homolog (PTEN)/RKIP circuit in cancer cells and becomes downregulated in a negative feedback loop by Snail and YY1, both of which modulate the expression of apoptosis-/metastasis-related gene products such as E-cadherin, Fas, and death receptor 5 (DR5).^{32–34} Similar interactions with other proto-oncogenes [i.e., Notch1 and melanoma differentiation-associated gene-9 (MDA-9)] reiterate the attenuation of EMT in cells expressing high levels of RKIP.^{35,36} Given RKIP's eponymous function in the Ras/Raf/MEK signaling axis and the characterization of various nodes of convergence between the MAPK pathway and the PI3K/Akt/mTOR pathway, it makes sense that RKIP should directly or indirectly influence autophagic activity in cancer cells.³⁷ Indeed, it was recently shown that RKIP acts as a gatekeeper of autophagy by binding to LC3, an essential protein in autophagosome formation, thus halting autophagy.³⁸

Extrapolating from the literature, this review summarizes the myriad roles that autophagy and RKIP play in tumorigenesis and the underlying molecular events. Given the multitude of autophagy-related (*ATG*) genes involved in regulating mammalian autophagy, we focus on the LC3 family of proteins due to its essential nature in autophagy and its value as a standard marker for autophagic activity.³⁹ The crosstalk between RKIP and LC3 has been the subject of very little research, particularly in cancer models. Nevertheless, the following survey of the classical regulatory axes for the respective molecules may reveal previously unseen connections between autophagy and RKIP signaling.

II. AUTOPHAGY

A. Autophagy Basics

In the decades following the discovery of autophagy, research on it has experienced great strides in both mechanistic and applied studies.^{40,41} It is well known that autophagy is ubiquitous in mammals as a lysosomal bulk degradation pathway that targets long-lived proteins, cellular organelles, and abnormal protein aggregates.^{25,42,43} This evolutionarily conserved process features the selective and nonselective sequestrations of potentially harmful and ambiguous cytoplasmic constituents. A highly regulated system of membrane rearrangement traffics the cargo to lysosomes, resulting in the cargo's content degradation by lysosomal hydrolases. Consistent with these functions, depending on the type of structures targeted for degradation, autophagy can be subclassified into pexophagy, mitophagy, xenophagy, reticulophagy, and so forth.⁴⁴ A core process in cellular development, autophagy has also been implicated in mouse models of proper neural and hematopoietic cell functions and development as well as cardiomyocyte and hepatocyte homeostasis.^{45,46} As it is an unalienable component of cell development, autophagy has been the subject of many studies aimed toward deducing its unquestionable alterations in the novel and established mechanisms of disease pathogenesis.^{47–50} The autophagy machinery is active in a basal state during periods of low cell stress in keeping with its role as a housekeeping program necessary for homeostasis and quality control.⁵¹ Functionally, the continuous turnover of macromolecules is vital to maintaining a steady pool of amino acids and fatty acids for synthesizing new proteins and membranes and feeding ATP production.⁵²

Autophagy is divided into three categories of biologically similar lysosome-dependent processes: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy. In CMA, proteins with a degenerate pentapeptide motif (KFERQ) are recognized and bound by the cytosolic chaperone protein Hsc70.⁵³ The cargo-Hsc70 complex translocates to the multimeric lysosome-associated membrane protein type 2A (LAMP2A) channel on the lysosomal membrane, where the targeted protein is unfolded and transported into the lumen for degradation.⁵⁴ In microautophagy, cytosolic substrates, the least studied of the three mechanisms, are engulfed by the vacuolar/lysosomal/endosomal membrane via direct invagination of the membrane for digestion.^{55,56} In comparison, the vast majority of the literature on autophagy is dedicated to elucidating the functions and roles of macroautophagy (hereafter referred to as autophagy) in cell development and disease pathogenesis. Autophagy is coordinated by 36 ATG proteins through five steps: initiation, vesicle nucleation, elongation, fusion, and degradation (Fig. 1).^{44,57–59}

Upon receiving proautophagic signals via nutrient deprivation or other environmental stress, the unc-51-like kinase (ULK) complex forms from the Atg13, Atg101, ULK1/2, and the family-interacting protein 200 (FIP200).⁶⁰ The major upstream regulator of this step, mTOR complex 1 (mTORC1), is inactivated in the absence of extracellular amino acids or insulin, allowing the ULK complex to initiate the nucleation step to form an Ω -like structure called the phagophore.^{61,62} The Class III PI3K complex [vacuolar protein sorting-associated protein 34 (VPS34) kinase complex]—composed of VPS34 (catalytic subunit, PIK3C3), VPS15 (PIK3R4), Beclin-1, Atg14 or UVRAG (UV radiation resistance-associated gene protein), and activating molecule in BECN1 regulated autophagy protein 1 (AMBRA1)—further mediates the formation of the double-membraned phagophore and constitutes another critical regulatory point.^{58,63,64} Elongation of the phagophore to form the autophagosome relies on two ubiquitin-like conjugation reactions.⁶⁵ In the first conjugation reaction, Atg12 covalently binds to Atg5 under the regulation of the E1-ubiquitin-activating enzyme Atg7 in conjunction with Atg10.⁶⁶ The Atg12-Atg5 complex then self-multimerizes with Atg16L1, creating the Atg12-Atg5-Atg16L1 complex (E3 ubiquitin ligase), which is needed as a scaffold for LC3 localization to the autophagosomal membrane and its subsequent lipidation.^{67,68} This relocation of the cytosolic form of LC3 (LC3-I) marks the initiation of the second ubiquitin-like reaction whereby Atg7 and Atg3, an E2 ubiquitin-conjugating enzyme, attaches phosphatidylethanolamine (PE) to LC3-I at the C-terminal Gly, forming the nonsoluble, membrane-associated LC3-II.⁶⁷

LC3-II is then incorporated into the growing autophagosome membrane. During this phase, the cargo ubiquitin-tagged for degradation is brought to LC3-II by the polyubiquitin-binding protein p62/SQSTM1. Other cargo receptors can mediate this interaction as well: toll-interacting protein (TOLLIP), nuclear dot protein 52 kDa (NDP52), optineurin (OPTN), and neighbor of BRCA1 gene 1 protein (NBR1).^{69,70} The capacity of cargo receptors to bind to LC3 at the LC3-interacting region (LIR), their propensity to aggregate, and their substrate-specificity are all important factors in mediating efficient selective autophagy.^{71,72} In addition, the autophagosome size and its expansion have been shown to be modulated not only by LC3 but also by the PI3K Class III products phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P₂] and ER-Golgi membrane trafficking.^{44,73,74} The now mature autophagosome enveloping the discarded substrates

fuses with lysosomes in a hemifusion mechanism facilitated by LC3-II and other fusion mediators such as VPS4/suppressor of K(+) transport growth defect 1 (SKD1), Rab 11, LAMP-2, intact microtubules, dynein, Rab7, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, and components of the homotypic fusion and protein-sorting (HOPS) complex.^{75,76} The final structure, called the autolysosome, degrades any cargo along with the inside membrane of the double-membraned autophagosome via the action of hydrolytic enzymes and low pH.⁷⁷ The resulting catabolites are then transported back into the cytoplasm for reuse.

Studies have established LC3 as an essential component of mid- to late-stage autophagy with implications for spatial and temporal regulation of autophagic flux. The following section explains how its structure relates to its function.

B. LC3: Families and Functions

First identified as a subunit of the neuronal micro-tubule-associated proteins (MAPs) in rats, LC3 is a soluble 17-kDa protein that is ubiquitously expressed in mammalian cells.^{78,79} Precursor LC3 proteins (proLC3s) are processed by Atg4 (cysteine protease) via cleavage of the C-terminal 22 amino acids to produce LC3-I with an exposed glycine residue.^{80,81} Interestingly, Atg4 can also recycle Atg8/LC3 by deconjugating it from PE after autophagosomal completion, rescuing it from degradation by the lysosome.⁸² This process has implications for the temporal regulation of LC3 by Atg4 by controlling its lipidation state both before and after autophagosome maturation. However, further work is prompted to study how Atg4 deconjugase activity is regulated in the context of ongoing autophagy.⁸³ As mammalian homologs of the yeast protein Apg8/Aut7p/Atg8, LC3 proteins are ubiquitin-like molecules that have been shown to dose-dependently determine the size of autophagosomes.^{84,85} Consistent with these findings, siRNA knockdown of GFP-LC3B in HeLa cells substantially decreased Atg16-positive puncta number and size, indicating reduced autophagosome biogenesis.⁸⁴ In agreement with LC3's function, its labeling and accumulation have been invaluable as biochemical tools for tracking autophagic flux.^{39,86,87} Studies have demonstrated the prognostic value of LC3 with other autophagy markers in specific cancer types, validated by immunostaining and assessment of autophagic flux in human cancer samples.^{88,89} GFP-LC3 is visualized in a punctate pattern localized to the cytoplasm during periods of increased autophagosome formation. This, in addition to monitoring the cargo for degradation and p62 accumulation, is currently the go-to method of elucidating the typical physiologic response in malignant cancer cells and the correlation of autophagy with various disease states.³⁹

Paralogs of the LC3 family of proteins— γ -aminobutyric acid receptor-associated protein (GABARAP), γ -aminobutyric acid receptor-associated protein-like (GABARAPL), and Golgi-associated ATPase enhancer of 16 kDa (GATE-16)—also function in liposome formation and maturation.⁹⁰ Weidberg and associates demonstrated that GABARAP/GATE-16 activity is important for membrane closure whereas LC3 activity is more involved in elongation of the autophagosome.⁸⁴ Similarly, Fujita et al. showed that catalytically inactive Atg4B (LC3 paralog-specific) mutants (Atg4B^{C74A}) hampered membrane closure but had little effect on membrane length.⁸¹ Thus, it was proposed that the GABARAP/

GATE-16 subfamily acts later and the LC3 family acts earlier during autophagosome biogenesis.⁸⁴ Targeting different LC3 paralogs may therefore be a viable strategy for slowing cancer cell proliferation or inducing Type II apoptosis by creating a blockade before the autophagosome-lysosome fusion event.⁹¹

C. Autophagy Regulation

The cell's ability to sense environmental stress through various mechanisms is intimately tied to autophagic control (Fig. 1).⁵⁸ The PI3K/Akt/mTOR axis exerts post-transcriptional control on constituents of two major autophagic control complexes: the ULK complex and the Class III PI3K complex.^{25,26} Insulin and amino acid excess activate mTOR through the G protein Rheb and the Rag proteins, respectively, leading to an increase in deactivating phosphorylation of ULK1 by mTOR at S757.^{25,92,93} Activation of upstream elements such as Akt causes S295 phosphorylation of Beclin-1, with this phosphorylated residue binding to the 14-3-3 protein/vimentin in a perinuclear pattern, sequestering it from the Class III PI3K complex and preventing autophagic induction.⁹⁴ Importantly, the mTOR pathway can cross-inhibit AMPK-ULK1 interaction, preventing AMPK from activating ULK1 during glucose abundance.⁹³ In contrast, in glucose starvation tuberous sclerosis complex 1 and 2 (TSC1/2) conduct proautophagic signals downstream of AMPK, deactivating Rheb and therefore mTOR.^{93,95}

Further regulation by the energetic status occurs through the B-cell leukemia/lymphoma-2 (Bcl-2) family of antiapoptotic proteins (Fig. 1). Pattingre and colleagues demonstrated that Bcl-2 and Bcl-extra large (Bcl-X_L) inhibit Class III PI3K complex aggregation by binding to Beclin-1 in a nutrient-dependent manner.⁹⁶ In addition, the target genes of hypoxia-inducible factor-1 (HIF-1)—Bcl-2/adenovirus E1B 19-kDa-interacting protein 3 (*BNIP3*, *BNIP3L*)—can displace Bcl-2/Bcl-X_L from Beclin-1 during hypoxia and normoxia due to a low but sufficient affinity for the antiautophagy regulators.⁹⁷ In an LC3-specific regulatory interaction, protein kinase A (PKA) can phosphorylate LC3 on S12, negatively affecting recruitment of LC3 to the autophagosome.⁹⁸ Phosphorylated LC3 decreased upon autophagy induction, indicating another potential regulatory checkpoint for LC3 alongside Atg4.⁹⁸

Current knowledge of transcriptional regulation of *ATG* genes has been published in extensive reviews.^{58,99,100} The forkhead box O (FoxO) family proteins are transcription factors described as up-regulating the expression of *ATG* genes centrally involved in autophagosome formation such as *LC3*, *Gabarp11*, and *ATG12* in rat neonatal cardiomyocytes.¹⁰¹ Activated by starvation, FoxO1 and FoxO3 increase autophagy gene expression following nuclear translocation, mitigating cell damage from metabolic and ischemic injury. Likewise, the E2F family proteins were characterized in upregulating LC3, Atg1, Atg5, and damage-regulated autophagy modulator (DRAM).¹⁰² Activation of an inducible E2F1 [ER (estrogen receptor)-E2F1] by 4-hydroxytamoxifen (OHT), a potent ER activator, and DNA damage led to its binding to consensus E2F-binding sites in the *LC3* and *ATG1* genes with a concurrent increase in LC3 puncta and a modest increase in autophagy. Opposing these proautophagy signals is Ume6, a zinc cluster protein that negatively regulates *ATG8* transcription by complexing with the corepressor Sin3 and the histone deacetylase Rpd3.^{103,104} As expected, Ume6-mediated repression of *ATG8* transcription is

reactive to nutritional cues and is deactivated by the proautophagic kinase regulator Rim15 upon glucose or nitrogen starvation.¹⁰⁴

Altogether, these mechanisms by no means encompass all regulatory pathways; rather, they underscore the necessity of a tightly linked web of rheostats that ensure nonaberrant autophagy activity during homeostatic states.

III. THE TWO FACES OF AUTOPHAGY

Current work on autophagy as a straightforward mechanism that underlies either tumorigenesis or tumor suppression has struggled with the confounding dichotomy of self-digestion in numerous contexts.²⁷ Indeed, many of the available anticancer agents induce autophagosome formation and autophagic flux, raising the necessity of investigating the mysterious interactions between autophagy and cytotoxic drug responses.¹⁰⁵ Data showing that dys-functional autophagy via *ATG5* or *ATG7* knockout accelerates neoplastic transformation in mouse livers or lungs delineate the links between autophagy and tumor suppression in the context of cancer initiation.^{106,107} In support of this hypothesis, researchers have discovered that allelic deletions of *BECN1*, the Beclin-1 gene, are often found in breast, prostate, and ovarian cancers with links to increased DNA damage and susceptibility to metabolic stress due to autophagic repression.^{108,109} Functional autophagy limits genomic instability and further accumulation of oncogenic mutations, consistent with the regulation of *ATG* genes by the DNA damage-inducible factors (i.e., E2F1, p53).^{102,110} This influence on replicative and metabolic stress further reinforces the hypothesis that autophagy is a tumor-suppressive mechanism, at least during early stages of tumor growth.

On the other hand, constitutive autophagy is often associated with tumor progression and poor survival outcomes in human cancers, including pancreatic cancer,¹¹¹ colorectal cancer (CRC),¹¹² gliomas,¹¹³ and esophageal cancer.¹¹⁴ Cancer cells are strongly adaptive in response to environmental insults, and thus the metabolic alterations characteristic to autophagy contribute to a phenomenon called “austerity,” or a tolerance to nutrient deprivation.^{43,112} Tumors that exhibit resistance to uneven perfusion and poor nutritive blood flow in the tumor environment, like pancreatic tumors, benefit from autophagy-mediated upkeep of metabolites, whereas reversal of austerity can be achieved via artificial repression of autophagy, leading to resensitization to starvation and subsequent cell death⁴³. Notably, inhibition of Akt in PTEN-null tumors sensitizes serum-starved cancer cells to lysosomotropic agents [e.g., bafilomycin A1, chloroquine (CQ), 3-methyladenine (3-MA)].¹¹⁵ Whereas Akt knockdown with serum starvation resulted in the accumulation of G₀/G₁-phase cells, the addition of autophagy inhibitors induced cell death dependent on the accumulation of abnormal acidic vesicular organelles (AVOs). In this case, autophagy is a double-edged sword that is detrimental to the cell when it is blocked, demonstrating the clinical significance of autophagy inhibitors in PTEN-null tumors.

Autophagy also has antiapoptotic properties as inhibition of autophagy by CQ facilitated p53-induced cell death in a *Myc*-induced model of lymphoma with tamoxifen (TAM)-inducible p53ER (p53ER^{TAM}) fusion constructs.¹¹⁶ Allelic knockout of *BECN1* and therefore autophagy can also increase cancer cell sensitivity to necrotic cell death under

starvation conditions via rampant metabolic stress.¹¹⁷ In addition, Ras-driven tumors are dependent on autophagy-mediated oxidative metabolism and mitochondrial maintenance for cell survival and growth.¹¹⁸ In fact, “autophagy addiction” was found in several human cancer cell lines, indicating the plausibility of applying autophagy inhibitors in tumors with constitutively high autophagic activity to mitigate tumor growth and survival.^{118,119} In this way, advances in identifying the crosstalk between autophagy and cell death pathways build upon current models of malignant cell circumvention of cell death.

IV. RAF-1 KINASE INHIBITORY PROTEIN

A. General Properties

RAF-1 kinase inhibitory protein (RKIP) operates as a cell-intrinsic regulator of a number of well-studied cell survival and motility pathways, including the Raf/MEK/extracellular-signal-regulated kinase (ERK), NF- κ B, STAT3, and glycogen synthase kinase-3 β (GSK-3 β) pathways (Fig. 2).^{31,120–122} First purified from bovine brain as a 23-kDa protein, RKIP, or phosphatidylethanolamine binding protein 1 (PEBP1), is a member of the PEBP family.¹²³ It is encoded on the human 12q23 chromosomal region and shares homologs in rats, bovines, and plants.^{124,125} As its name implies, RKIP plays a major role in the MAPK pathway as a negative regulator of Raf-1 and downstream components, exerting an overall dampening effect on cell growth, proliferation, and metastatic potential. Due to its broad spectrum of downstream control targets, RKIP has been studied in diverse biochemical contexts in both pre-malignant and metastatic cancers. Proposed schemes of RKIP cross-signaling with antiapoptotic and prometastatic molecules have helped identify potential pathways that exacerbate human malignancies.^{126,127} Details on how RKIP influences its downstream targets and how the expression of the kinase inhibitor is regulated are summarized next.

B. Downstream Regulatory Targets

Yeung and associates first elucidated the role of RKIP in governing MAPK signaling using a yeast two-hybrid screen.³⁰ They found that RKIP binds Raf-1 in unstimulated cells as its primary target regardless of Raf-1 activation status, whereas mitogenic stimulation (e.g., epidermal growth factor, EGF) causes dissociation of Raf-1 from RKIP.³¹ Competitive inhibition of MEK phosphorylation and survival pathway signaling by RKIP was confirmed to increase prostate cancer cell sensitivity to drug-induced apoptosis.¹²⁰ Comparative inhibition of MEK and both activated and basal BRAF activity in human melanoma models suggests that not only Raf-1 but multiple MAPK-signaling nodes are involved suppressing survival responses.^{31,128} RKIP also regulates the spindle checkpoint by governing the activity of Aurora B kinase, another protein involved in spindle checkpoints and inhibition of cell cycle progression.¹²⁹ Raf-1 targeting by RKIP during early mitosis seems to be involved in regulating the mitotic index as Raf-1 siRNA knockdown increases mitotic index to control levels. Likewise, RKIP can inhibit STAT3 activity directly and indirectly. STAT3 is activated when bound to autophosphorylated c-Src (p-c-Src), and this interaction is disrupted when RKIP binds directly to STAT3 or inhibits c-Src autophosphorylation.¹³⁰ Investigators also found that RKIP can inhibit Raf-1, Janus kinase 1/2 (JAK1/2), and IL-6-mediated activation of STAT3.¹³⁰ In high-grade glioblastomas, the inverse association of

RKIP with STAT3 expression was determined to be prognostically significant, with the best overall survival rate seen in patients with tumors expressing high RKIP and low STAT3.¹³¹

In relation to NF- κ B, researchers showed that prostate and breast cancer cell lines resistant to chemotherapeutic-induced apoptosis become partially resensitized to cell death upon RKIP overexpression or NF- κ B super-repression.¹²⁰ Based on these observations, it was clear that RKIP is antagonistic to NF- κ B–related desensitization to apoptosis through an upstream inhibitory interaction. Mechanistically, RKIP has a high affinity for binding to the MAP kinase kinases (MAPKKK-) transforming growth factor beta-activated kinase 1 (TAK1, MAP3K7) and NF- κ B–inducing kinase (NIK) upon stimulation of the NF- κ B pathway by IL-1B or tumor necrosis factor α (TNF- α).¹³² The resulting complex inhibits inhibitor of κ B (I κ B) kinase (IKK) activation, leading to I κ B stabilization and unperturbed NF- κ B repression. A seemingly opposing inhibitory scheme has RKIP acting as a scaffold for TNF receptor–associated factor 6 (TRAF6) and the IKK complex, facilitating IKK activation and I κ B degradation, liberating NF- κ B, and promoting nuclear translocation of NF- κ B dimers.¹³³ Despite the apparent contrast, this contextual activation of NF- κ B results in the induction of A20 and *cyld* gene expressions, both encoding negative regulators of the NF- κ B axis.¹³³ The latter mechanism poses an intriguing concept of RKIP as a multifunctional pathway repressor that can enhance a negative autoregulatory loop as well as suppress signal transduction in a straightforward manner.

GSK-3 β is an important regulatory enzyme of energy metabolism and plays a prominent role as a tumor suppressor in WNT signaling and autophagy.^{134,135} Specifically, Snail, Slug, cyclin D1, and β -catenin are all substrates of GSK-3 β with contributions to the EMT phenotype.¹³⁶ RKIP stabilizes GSK-3 β via suppression of oxidative stress through an unclear mechanism and drives downregulation of the above mentioned GSK-3 β substrates. Suppression of reactive oxygen species (ROS) levels leads to inactivation of p38, an inhibitor of GSK-3 β , which deactivates GSK-3 β by phosphorylating residue T390.¹³⁶ *In vivo*, RKIP was observed to modulate GSK-3 β expression in a tissue-specific manner with a positive correlation in protein expression levels in human CRC samples.¹³⁶ RKIP-dependent Notch1 deactivation and cytoplasmic sequestration repress the expression of Notch1 target genes such as *CDH2*, *SNAI1*, and *VIM* in hypoxic conditions.³⁶ RKIP's capacity to counteract the prometastatic effect of hypoxia and pro-EMT protein expressions highlights its role as a metastasis suppressor.¹³⁷ Previous reports also described a potential for RKIP to suppress cancer cell stemness via regulation of stem cell factors Oct4, Sox2, KLF2, and Nanog.¹³⁸

C. RKIP Regulation

RKIP expression is intricately regulated by a network of cancer-related molecules, belying its participation in dysregulated loops that promote carcinogenesis (Fig. 2).¹³⁹ Low RKIP expression in metastatic clinical samples could be a consequence of negative feedback from abnormal signaling cascades and transcriptional/post-transcriptional repression by pro-EMT proteins. The polycomb protein enhancer of zeste homolog 2 (EZH2) is an epigenetic repressor (histone methyltransferase) of RKIP transcription that binds directly to the proximal E-box of the RKIP promoter and recruits suppressor of zeste 12 (Suz12) for

completion of the polycomb repressive complex 2 (PRC2) during epigenetic silencing.¹⁴⁰ Breast and prostate cancer cell lines exhibit a negative correlation between EZH2 and RKIP levels. Additional recruitment of Snail and histone deacetylases to the promoter region is required for efficient RKIP silencing.¹⁴⁰ Snail is a zinc finger transcriptional repressor that promotes EMT and downregulates metastatic suppressors; these processes are important in mass cell migration during embryogenesis.¹⁴¹ Studies confirmed that Snail interacts with the RKIP promoter at the proximal E-box and decreases RKIP expression.¹⁴² Bioinformatic analysis of cancer data sets revealed that Snail expression increases from normal to metastatic prostate cancers whereas RKIP expression does the opposite.¹⁴² In further defining the genetic landscape of the RKIP promoter, investigators found that the region -56 to +261 relative to the transcriptional start site contains cis-acting elements that recruit DNA-binding proteins Sp1, cAMP response element-binding protein (CREB), and p300.¹⁴³ Coordination of these transacting factors increases RKIP promoter activity, although the clinical significance of this interaction requires further study.

Another transcription factor, BTB and CNC homology 1 (BACH1), negatively regulates RKIP by participating in a double-negative feedback loop with it.¹⁴⁴ BACH1 binds to the -373-bp region of the RKIP promoter and represses RKIP expression in triple-negative breast cancer (TNBC) cells; EZH2 binding contributes to the degree of repression, but is not necessary for BACH1 repressive activity. Even so, BACH1 binding is required for EZH2 recruitment. Conversely, RKIP induces BACH1 loss by activating let-7 in an antimetastatic signaling cascade.¹⁴⁵ Together, RKIP and BACH1 form a “bistable circuit” in which protein levels are inverse to each other in any one stable state, although the exact mechanisms of perturbations that shift the balance have not been pursued in depth.¹⁴⁴

Post-transcriptionally, RKIP mRNA is targeted by miR-27a and miR-224.^{146,147} miR-27a decreases RKIP protein expression in lung adenocarcinoma cells while increasing cisplatin resistance and EMT gene expression.¹⁴⁶ Furthermore, high expression of miR-27a is inversely correlated with RKIP in clinical samples of cisplatin-insensitive advanced lung adenocarcinoma. Similarly, miR-224 reverses RKIP-dependent decreases in cell viability and S-phase fraction and increases in apoptotic gastric cancer cells.¹⁴⁷

Importantly, phosphorylation of RKIP by protein kinase C (PKC) at S153 triggers a switch in the binding site affinity for Raf-1 to G protein-coupled receptor kinase 2 (GRK-2).¹⁴⁸ GRK-2 negatively regulates the G protein-coupled receptor (GPCR) cascade in a negative feedback mechanism by phosphorylating the GPCR, initiating its subsequent internalization and degradation. Phosphorylated RKIP (p-RKIP) dimerizes and attenuates GRK-2 inhibition of GPCR signaling at the expense of enhanced MAPK signaling, thereby generating a positive feedforward loop between GPCR and RKIP.¹⁴⁹ Interestingly, GRK-2 also exerts negative control over the MAPK-activating GPCRs while GRK-2 inhibitors enhance the cardioprotective effect of MAPK signaling and cell proliferation.¹⁵⁰ Exogenous expression of RKIP in GRK-2-inhibited cells induces higher levels of apoptosis, consistent with its previously demonstrated antagonism toward the MAPK axis. Altogether, these studies highlight the importance of detecting the phosphorylation state of RKIP in clinical samples while preventing false associations between specific cancers and RKIP expression.

D. RKIP Clinical Significance

RKIP has been reported to control cell cycle kinetics and metastatic progression through differential regulation of a variety of pathways and effectors involved in modulating cell proliferation, motility, and apoptosis.^{34,151} Clinical analyses of aggressive and high-grade cancers such as gastric,^{152,153} breast,¹⁵⁴ prostate,¹⁵⁵ urinary bladder,¹⁵⁶ endometrial,¹⁵⁷ glioma,^{131,158} melanoma,¹⁵⁹ nasopharyngeal,¹⁶⁰ lung adenocarcinoma,¹⁴ and clear cell renal cell carcinoma¹⁶¹ point to absent and lowered RKIP expression as indicating poor prognoses and metastatic potential. Oncogenic KRAS is inversely correlated with RKIP expression in pancreatic ductal adenocarcinoma cells through the MAPK-ERK pathway–dependent upstream regulation of RKIP.¹⁶² Loss of RKIP in this manner turns on the expression of EMT factors and the acquisition of chemo-immuno-resistance. Prostate tumor and melanoma cells commonly exhibit lower levels of RKIP in metastatic cells versus primary tumor cells.^{125,128,163} Primary prostate tumors that express high RKIP have a lower 7-year prostate-specific antigen (PSA) recurrence rate than do tumors with comparatively low RKIP. RKIP expression is predictably low in lymph node involvement versus in primary tumors, reinforcing its prognostic significance in the listed cancers.¹⁵²

The participation of RKIP in prometastatic networks and cascades as a negative regulator is well documented (Fig. 2). For example, RKIP mediates overall restriction of cell motility by downregulating matrix metalloproteinases (MMPs), which degrade components of the extracellular matrix during EMT.^{164,165} RKIP also reduces mRNA and protein expressions of cellular adhesion molecules—vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1)—which facilitate cell migration and metastasis.¹⁶⁶ The NF- κ B/Snail/YY1/RKIP/PTEN circuit dictates EMT-related gene expression and chemo-immuno-resistance in tumor cells.¹⁶⁷ RKIP induction in this dysregulated loop resensitizes tumor cells to chemotherapeutic and immunotherapeutic drugs with concomitant downregulation of the EMT-promoting factors Snail, YY1, and NF- κ B.³³ In breast cancers, the RKIP/Myc/LIN28/let-7/high-mobility group A2 (HMGA2)/BACH1 module governs expression of the bone metastasis genes *MMPI*, osteopontin (*OPN*), and chemokine (C-X-C motif) receptor 4 (*CXCR4*).¹⁶⁸ This study suggests that a combined approach to experimentally validating novel metastasis signatures involving RKIP from bioinformatic analyses of gathered clinical data can help identify signatures that predict metastatic potential.^{168,169}

V. RKIP-LC3 CROSSTALK

A. Emergence of an RKIP-LC3 Connection

In the previous sections, we focused solely on the independent functions and regulatory mechanisms of autophagy and RKIP and existing crosstalk with prominent cancer pathways. However, the links between RKIP and autophagy are anything but obvious in the literature. It is possible to extrapolate crosstalk interactions based on relationships between upstream and downstream pathways, but any such extrapolation is limited by the dearth of experimental data on both RKIP and autophagy.^{37,170–172} To date, only three studies have been published on the role of RKIP in autophagy and vice versa, or the closely related

process of adipogenesis; only one of those three studies established a direct link between RKIP and LC3.^{38,173,174}

Neuronal death is a common occurrence in Parkinson's disease (PD), and overactivation of the ERK/MAPK pathway has been implicated in stimulating cell cycle progression and neuronal loss. Because RKIP is the physiological inhibitor of Raf kinase, Wen et al. sought to show how autophagy promotes loss of RKIP in PD models. By treating primary cortical neurons with etoposide to induce DNA damage or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to induce neural toxicity, the researchers showed that these insults activate cyclin-dependent kinase 5 (CDK5), which in turn phosphorylates RKIP at T42.¹⁷³ T42 phosphorylation has the same effect on the switching of Raf-1 affinity to GRK-2 affinity as S153 phosphorylation of RKIP, although p-(T42)-RKIP has the added effect of preparing RKIP for degradation by CMA. In addition, a conserved motif (KLYEQ) is needed for targeting of Hsc70 to p-RKIP and the ensuing translocation to the lysosome. Upon CMA-mediated depletion of RKIP, uninhibited signal transduction through Raf-1 to the ERK/MAPK axis promotes S-phase reentry and proliferation. It is unclear where macroautophagy fits in this model and further study is needed to uncover how the loss of RKIP influences the previously described cytoprotective role of autophagy in neurodegenerative diseases.⁴⁷

Preadipocytes undergo a process during adipogenesis similar to that during autophagosome maturation. Lipid droplet production and adipogenic transcription factor expression increase following overexpression of LC3 in 3T3-L1 preadipocytes.¹⁷⁴ Interestingly, p-(S153)-RKIP increases in a fashion parallel to ERK1 activation with adipogenic progression. Suppression of RKIP inhibitory activity also corresponds to intracellular accumulation of triacylglycerides in dexamethasone, 1-methyl-3-isobutylxanthine, and insulin (MDI-) induced mitotic expansion of growth-arrested preadipocytes. The evidence strongly suggests a probable regulatory loop between LC3 and RKIP that becomes active after autophagic initiation and is dependent on either the upstream regulation of both molecules or an as yet unidentified feedback mechanism.

The Noh group was the first to map a sequence-specific LIR (WXXL) motif in PEBP1 (RKIP).^{38,72} As PE is a substrate for RKIP, the researchers tested whether the LIR is required for RKIP to bind to LC3. In substituting the tryptophan (W) and leucine (L) residues for alanine (A), they discovered that RKIP loses its capacity for binding LC3, suggesting the LIR is vital for efficient binding. It was additionally discovered that RKIP binds only the unconjugated LC3-I because it is localized to cytoplasmic GFP-LC3 in normal conditions and not to GFP-LC3 puncta (LC3-II) during starvation. RKIP-LC3 complexing reduces autophagic flux under starvation conditions by sequestration of LC3-I prior to lipidation, essentially blocking LC3 incorporation into the autophagosome. Phosphorylation of RKIP at S153 results in dissociation from LC3-I, relieving the blockade on autophagosome formation. Also, RKIP expression is necessary for Akt/mTORC1 activation in starved cells, giving rise to increased inhibitory phosphorylation of ULK1. Neither MAPK3/ERK1 nor MAPK1/ERK2 changes in activation, indicating that RKIP modulates autophagy independent of the MAPK-ERK pathway. Altogether, the evidence

suggests a novel regulatory mechanism for LC3 processing by RKIP contingent on two conditions: an unaltered LIR motif and the RKIP phosphorylation state.

Through these findings, it is rational to hypothesize multiple mechanisms of crosstalk between RKIP and LC3 and, by extension, autophagy. In the following sections, we explore the literature to construct potential regulatory schemes based in large part on well-represented molecular nodes: p53, STAT3, Snail, and IKK.

B. A p53-Mediated Senescence Intersection

p53 is a prominent tumor suppressor and master regulator of DNA damage, apoptotic responses, senescence, and metabolism.¹⁷⁵ Monoallelic loss of *tp53* can greatly increase the incidence of double-stranded DNA (dsDNA) breaks and overall genomic instability, whereas genotoxic agents can stimulate a DNA damage response in cells with intact *tp53* loci. Examination of the literature revealed conflicting functions of RKIP and autophagy in modulating senescence within overlapping contexts of nutrient starvation and DNA damage (Fig. 3). RKIP transcription is inducible in a p53-dependent manner.¹⁷⁶ DNA damage by Adriamycin (Adr) promotes activation of p53 phosphorylation at S46, as does oncogenic Ras activation and insulin-like growth factor 1 (IGF-1) stimulation. Phosphorylation of p53 is required for binding to putative p53 consensus binding sites in the RKIP promoter region. Upregulation of RKIP expression is sufficient to suppress the ERK-MAPK cell proliferation pathway while driving the acquisition of senescence characteristics in human cancer cell lines.¹⁷⁶ Meanwhile, treatment of mouse embryonic fibroblasts (MEFs) with etoposide (Etop) revealed a DNA damage response pathway in which p53 activates AMPK and its downstream effectors TSC1/2 in conjunction with PTEN upregulation.¹⁷⁷ Like classical induction of autophagy by nutrient starvation, glucose starvation transiently activates p53 via S15 phosphorylation, suggesting that p53 is involved in sensing cellular stress upstream of autophagy.^{177,178} Furthermore, DNA damage-inducible mTOR inhibition elicits a proautophagic downstream response complete with increased levels of LC3-II and concurrent vacuole formation.¹⁷⁷

In contrast to the positive regulation of senescence by RKIP, autophagy inhibits cell senescence when activated in serum-starved cells in a p53-dependent manner (Fig. 3).¹⁷⁸ In this model, autophagy is important for evading starvation-induced cell death and rescuing cells from senescence via a cytoprotective mechanism. It is interesting that p53 can also moderate autophagic activity by post-transcriptionally downregulating LC3 during prolonged nutrient starvation.¹⁷⁹ In an apparent conflict with the cell survival role of autophagy in starvation, p53 prevents abnormal accumulation of autophagosomes and stalling of autophagy in constitutively starved cells, thereby preventing a type of autophagy-related apoptosis. Nevertheless, autophagy is critical in maintaining genomic stability. A recent study provided evidence that autophagy functions in a tumor-suppressive capacity by limiting DNA damage and protecting *tp53* integrity.¹¹⁰ Total inhibition of autophagy in heterozygous mutant p53 zebrafish embryos accelerates loss of *tp53* heterozygosity through rampant DNA damage, leading to increased tumor formation. Therefore, RKIP and LC3 may act in opposite ways to coordinate a dynamic regulation of cell senescence. Such a delicate balance may be important in chemotherapeutic treatment of poorly perfused tumors

exhibiting constitutively high basal autophagy. How the cell manages the unidentified set of stimuli that threaten to tip the balance for or against senescence remains to be seen.

C. RKIP Regulation of *ATG* Genes via STAT3

STAT3 is a transcription factor that controls the expression of a series of cell-cycle regulators, angiogenic factors, inflammatory signaling molecules, and antiapoptosis genes.¹⁸⁰ We discussed how RKIP negatively regulates STAT3 activity in a previous section. Recently, a review by You et al. outlined the multifaceted function of STAT3 in upregulating antiautophagy genes and downregulating proautophagy genes.¹⁸¹ Nuclear STAT3 (phosphorylated/active dimer) transcriptionally activates Bcl-2 and represses the expression of *BECN1* and *PIK3C3*, genes encoding Beclin-1 and VPS34, respectively (Fig. 4).^{182–184} The loss of crucial autophagy-regulating proteins precludes efficient autophagy activation.

Cytoplasmic STAT3 was found to impede the *ATG* gene, including MAP1LC3A, transcriptional activation by eukaryotic translation initiation factor 2- α (eIF2 α), and FoxO1/FoxO3a.^{185–187} The SH2 domain of cytoplasmic, unphosphorylated STAT3 emulates the C terminus of eIF2 α and inhibits the eIF2 α activator eIF2 α kinase 2 (EIF2AK2) by binding to the EIF2AK2 catalytic domain.¹⁸⁷ Normally, EIF2AK2 activation of eIF2 α phosphorylation causes translational arrest and autophagic activation. Furthermore, eIF2 α can induce selective translation of activating transcription factor 4 (ATF4), which then upregulates LC3B and thus autophagy.¹⁸⁵ Unphosphorylated STAT3 resides in the cytoplasm and can isolate FoxO1/FoxO3a from the nucleus, preventing *LC3* transcriptional activation.¹⁸⁶ Since high RKIP inhibits STAT3 phosphorylation, the latter interaction may constitute yet another mechanism by which RKIP antagonizes *LC3*-dependent autophagy. However, it would be important to delineate the regions on STAT3 that bind RKIP and whether RKIP disrupts only the activation of STAT3 phosphorylation or if it displaces FoxO1/FoxO3a or EIF2AK2 from STAT3 monomers, effectively nullifying *LC3* repression. For this it is necessary to study the context of STAT3 activation in cancer cells with high autophagic activity.

D. Regulation of EMT by a Context-Dependent Loop

Snail and NF- κ B have been studied together as inducers of EMT and metastasis. The extensively studied NF- κ B/Snail/YY1/PTEN/RKIP dysregulated loop in turn shows RKIP to be the central negative regulator of NF- κ B and Snail.^{32,188} In short, RKIP suppresses IKK activation, leading to I κ B stabilization on NF- κ B, which prevents activation of the latter (Fig. 5A). Abundant evidence on EMT regulation suggests that NF- κ B promotes Snail transcriptional activation and post-translational stabilization.^{189,190} Snail then represses RKIP activity, closing the loop on the RKIP branch of the scheme. As for autophagy, recent reports described a process in which autophagy attenuates endothelial-to-mesenchymal transition (EndMT) in human cardiac microvascular endothelial cells via Snail degradation.¹⁹¹ The autophagy cargo receptor p62 is crucial for recruiting Snail to LC3 to initiate Snail degradation under hypoxic conditions. Since EndMT is physiologically similar to EMT (epithelial-to-mesenchymal transition), we can conjecture that autophagy also affects Snail levels in malignant cancer cells.¹⁹² Per Noh et al., unphosphorylated RKIP (u-RKIP) binds directly to LC3-I and hinders its lipidation to LC3-II under normal conditions.³⁸ An insufficient pool of LC3-II precludes autophagosome formation in classical autophagy,

relieving Snail of its inhibition by autophagy during homeostasis. Altogether, the evidence points to RKIP as the primary regulator of this schematic loop under basal conditions when autophagy is further inactivated by Akt/mTOR.

However, the roles change under physiological stress (Fig. 5B). Autophagy is activated by a multitude of injuries, and GPCRs constitute one mechanism of nutrient sensing and upstream autophagic activation.¹⁹³ GPCR activation can also trigger the phosphorylation of RKIP at S153, which we now know disrupts the RKIP-LC3-I interaction.^{38,148} Therefore, stress-induced activation of GPCR reactivates autophagy via LC3-I to LC3-II conversion, which then promotes Snail degradation. Starvation-stimulated IKK can also increase LC3 expression and processing.¹⁹⁴ Comb and colleagues showed that knockdown of IKK catalytic subunits IKK α /IKK β significantly decreases LC3 mRNA and protein levels. Furthermore, loss of IKK catalytic function corresponds to an inability to convert LC3-I to LC3-II. The effect of IKK inhibition on inactivating autophagy was recapitulated in another study.¹⁹⁵ Interestingly, both studies concluded that autophagic control by IKK is independent of NF- κ B. As an important point of clarification, IKK is only minimally involved in regulating autophagy under basal conditions, whereas a much more prominent upregulation of autophagy machinery is seen in starved cells.^{194,196} As NF- κ B is a stress-reactive factor, its positive regulation of Snail plausibly may also be present in the starved condition loop. However, since autophagy controls Snail degradation, it may have a role as a downstream regulatory mechanism of Snail-mediated EMT. Again, the proposed regulatory scheme is context-dependent: normal/basal versus starvation (Fig. 5A and B). Further study is required to experimentally validate the proposed scheme in cancer cells.

VI. CONCLUDING REMARKS

Mendoza et al. consolidated evidence demonstrating an intricate web of crosstalk between the Ras-ERK and PI3K-mTOR pathways.³⁷ Although each pathway regulates mutually exclusive sets of downstream effector proteins, many nodes of overlap pose significant challenges to therapeutic modalities and their clinical efficacies. The same can be said about the two pathways' related proteins/processes, RKIP and autophagy. RKIP and autophagy have garnered substantial attention from groups aiming to unravel the complex interrelationships of each with cancer development. Our survey of the genetic and molecular bases of RKIP and autophagy provides the necessary foundation for establishing previously unseen linkages. Cancer research on autophagy has itself been in flux with attempts to resolve the ambiguity of cellular self-digestion on tumorigenesis, although solid evidence points to a direct influence on cell death resistance and evasion. Due to ample literature on the role of RKIP in modulating EMT and reversing chemo-immuno-resistance in tumor cells, it is logical to juxtapose available RKIP and autophagy data. However, how RKIP may alter autophagy and vice versa has been the subject of very limited studies. Likewise, the lack of bioinformatics or proteomics analyses in histological studies provides little information on the possible correlations between RKIP expressions and the widely used autophagy marker LC3.

We proposed three crosstalk schemes between RKIP and LC3 rooted in a compilation of novel regulatory and effector mechanisms. The mechanisms were extrapolated from

interactions with pro-/antitumorigenic molecules such as p53, STAT3, NF- κ B, and Snail, which hint at the possible functions of an RKIP/LC3 cancer pathway. Through the three pathways, we rationalized, RKIP-LC3 crosstalk can affect tumor cell senescence, EMT/motility, and cellular growth. RKIP unambiguously opposes EMT gene products and halts cell growth while promoting the senescence phenotype. RKIP additionally reverses chemo-immuno-resistance in cells that have acquired or exhibit resistance to common therapeutic agents. In a similar fashion, autophagy can protect against metastatic progression through degradation of the EMT-inducer Snail, albeit in a manner dependent on activation by physiological stress. The activation of autophagy in instances whereby oncogenic molecules such as STAT3 are in a deactivated state suggests that autophagy may be a functional component of decreased tumorigenicity. Nevertheless, it is entirely possible that autophagy may also play a tumor-supportive role in the prototypical functions of cellular health maintenance and catabolite abundance. In fact, a number of ongoing clinical studies are evaluating the antitumoral effects of autophagy inhibition using CQ or hydrochloroquine (HCQ) in several cancer subtypes such as lung cancer and multiple myeloma. Our findings suggest that the suppression of senescence by autophagy is a consequence of mitigating damage from oxidative stress and genotoxic agents. However, the same function can adversely affect therapeutic options by lowering cell sensitivity. The exploitation of autophagy by the cancer cell can, in theory, override antitumorigenic factors and increase oncogenic potential. Another possibility is that autophagy is crucial for cellular maintenance before oncogenesis as a preventative mechanism in accordance with previous evidence. For example, RKIP may act in conjunction with autophagy to decrease cell cycle reentry and reestablish genome stability, respectively, during periods of oncogenic stress to inhibit mutagenic activation of protooncogenes. Our findings suggest that the RKIP/autophagy axis can serve as diagnostic marker for human malignancies in predicting metastatic potential and loss of drug susceptibility. In addition, the RKIP/autophagy axis emphasizes its relevance as a target for tumor suppression, metastasis inhibition, and chemo-immuno-resistance reversal.

VII. FUTURE DIRECTIONS

Without direct validation of the crosstalk mechanisms proposed here our extrapolations remain tenuous. The critical next step, then, is to investigate data sets for the correlative expression between RKIP and LC3 transcripts and protein levels in cancer tissue biopsies. Examination of expression profiles in relation to the aggressiveness of the cancer and the stage of treatment will help determine effects on treatment efficacy. *In vivo* studies on phenotypic alterations from RKIP knockdown and overexpression of key autophagy inducers may confirm the findings presented in this review. If both processes are in fact mutually antagonistic to each other, one expects an external stimulus (chemotherapeutics, immunotherapeutics, proinflammatory signaling molecules, growth factor stimulation) to shift the equilibrium toward either tumor latency or tumor expansion. Likewise, examining these profiles in preneoplastic cells may confirm cooperative tumor suppression by both RKIP and autophagy. Thus, it will be important to characterize the temporal and conditional regulations of RKIP-autophagy crosstalk. Further expansion of the known subset of proteins targeted for degradation by autophagy may identify regulatory mechanisms for either tumor

suppressors or onco-proteins. Such studies should improve upon current cancer therapeutics by providing novel targets in pathways that mediate cancer development in early stages and treatment resistance in later stages.

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ABBREVIATIONS:

AMPK	AMP-activated protein kinase
ATF4	activating transcription factor 4
ATG	autophagy-related
BACH1	BTB and CNC homology 1
Bcl-2	B-cell leukemia/lymphoma-2
Bcl-X_L	B-cell leukemia/lymphoma-extra large
CQ	chloroquine
CMA	chaperone-mediated autophagy
eIF2α	eukaryotic translation initiation factor 2- α
EIF2AK2	eIF2 α kinase 2
EMT	epithelial-to-mesenchymal transition
ERK	extracellular-signal-regulated kinase
EZH2	enhancer of zeste homolog 2
FoxO1/3	forkhead box O 1/3
GABARAP	γ -aminobutyric acid receptor-associated protein
GABARAPL	γ -aminobutyric acid receptor-associated protein-like
GATE-16	Golgi-associated ATPase enhancer of 16 kDa
GPCR	G protein-coupled receptor
GRK-2	G protein-coupled receptor kinase 2
GSK-3β	glycogen synthase kinase-3 β
HMG A2	high-mobility group A2

IκB	inhibitor of κ B
IKK	I κ B kinase
LC3	microtubule-associated 1 light chain 3
LIR	LC3-interacting region
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
NF-κB	nuclear factor- κ B
PE	phosphatidylethanolamine
PI3K	phosphatidylinositol 3-kinase
PTEN	phosphatase and tensin homolog
RKIP	Raf-1 kinase inhibitory protein
STAT3	signal transducer and activator of transcription 3
TSC1/2	tuberous sclerosis complex 1/2
ULK1/2	unc-51-like kinase 1/2
VPS34	vacuolar protein sorting-associated protein 34
YY1	Yin Yang 1

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FIG. 1:

Autophagy stages and regulatory checkpoints. Functional autophagy relies on a complex system of membrane rearrangements moderated by dozens of ATG proteins with nonredundant functions categorized into five discrete steps. The proteins bound by light red represent the major negative regulatory molecules and complexes mentioned in this review.

(I) Initiation: Initiation of autophagy begins with the formation of the ULK complex. Abundant nutrients and amino acids sustain Akt/mTORC1 inhibition of ULK1, whereas nutrient starvation de-represses ULK1 and transmits proautophagic stimuli through AMPK and TSC1/2. (II) Nucleation: Completion of the ULK complex facilitates aggregation of the Class III PI3K complex components along with nucleation of the membranes destined to become the double-membraned autophagosome. During this step, active Akt can phosphorylate Beclin-1 to sequester it to the 14-3-3 proteins' proximal to intermediate filaments. This, in addition to binding of Bcl-2/Bcl-X_L to Beclin-1, disrupts complex completion and prevents autophagy from progressing further. (III) Elongation: Past the blockade, conjugation of Atg12 with Atg5 and Atg16L1 occurs in a ubiquitin-like reaction catalyzed by Atg7; the Atg12-Atg5-Atg16L1 complex along with Atg3 and Atg7 facilitates LC3 lipidation and membrane localization. The phagophore elongates with the inclusion of lipidated LC3 (LC3-II) on the outer membrane. Posttranscriptional regulation of Atg4-processed LC3-I occurs by Atg4B cleavage of PE from LC3-II, freeing LC3-I for another round of autophagy, or it occurs by PKA-mediated deactivating phosphorylation of LC3-I. (IV) Fusion: As the autophagosome matures, the lysosome is recruited and fuses to the autophagosome. (V) Degradation: The vesicular contents are degraded by the lysosomal enzymes. (VI) Transcription regulation: *ATG* gene expression is upregulated by the FoxO proteins and E2F1 downstream of nutrient-sensing networks while Ume6 represses *LC3* transcription through Sin3 and Rpd3. Rim15

can suppress Ume6 during glucose starvation when it is relieved of the inhibitory influence of PKA.

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FIG. 2:

Downstream effectors and upstream regulatory pathways of RKIP. RKIP is an important regulator in a complex web of interactions. While it predominantly acts as a metastasis suppressor in the EMT pathways (Notch1, let-7, NF- κ B), it also affects and is affected by pathways controlling apoptosis and cellular proliferation. (I) RKIP is the only physiological inhibitor of the Raf-1/MEK/ERK axis that binds directly to Raf-1, BRAF, or MEK (not shown) to prevent signal transduction to ERK and beyond. RKIP curtails MAPK-mediated cell proliferation through several mechanisms; for instance, it prevents ERK from repressing Aurora B kinase activity, leading to an active spindle checkpoint blockade of cell cycle reentry. (II) it inhibits the EMT-inducer Notch1 and its pro-EMT downstream targets N-cadherin, vimentin, and Snail. (III) Alternatively, it stabilizes GSK-3 β and subsequently upregulates pro-EMT molecules such as Snail, Slug, cyclin-D1, and β -catenin. (IV) Among those described, the RKIP/Myc/LIN28/let-7/HMGA2/BACH1 axis (minimally shown) is systematically identified as a pathway controlling expression of a bone metastasis signature (BMS) consisting of OPN, CXCR4, and MMP1. RKIP activates let-7, which in turn inhibits HMGA2 and BACH1 from activating the BMS candidates just mentioned. (V) Phosphorylation of RKIP by PKC increases the former's affinity for GRK-2, a negative regulator of GPCR signaling. Once bound to RKIP, GRK-2 is isolated from GPCRs, allowing enhanced GPCR signaling. (VI) Due to its ubiquity in human tissues, RKIP has been shown to downregulate the adhesion molecules ICAM-1 and VCAM-1 as well as MMPs responsible for mesenchymal transformation in diverse cell types. (VII) Transcriptional repression of RKIP occurs through binding of EZH2 as part of the PRC2 to the RKIP promoter. Post-transcriptional regulation of RKIP mRNA is governed by miR-27a and miR-224. (VIII) Metastatic tumor cells often exhibit a dysregulated NF- κ B/Snail/YY1/

PTEN/RKIP loop that drives metastatic transition. In the nonmalignant example of the loop, RKIP suppresses NF- κ B activity through inhibition of IKK. Without RKIP, NF- κ B is free to upregulate oncoproteins YY1 and Snail. Snail then downregulates total RKIP levels or inhibits PTEN and enhances PI3K/Akt pathway activity. (IX) RKIP inhibits STAT3 activation both directly by binding to STAT3 monomers and indirectly by preventing c-Src autophosphorylation.

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FIG. 3:

Effects of RKIP and autophagy on p53-mediated senescence. This schematic outlines the addition of RKIP and autophagy to a cell senescence response induced by DNA damage and cellular stress. In the presence of DNA-damaging agents (solid lines), p53 responds by activating TSC1/2, thus also activating AMPK and inhibiting mTOR. The resultant proautophagic signal upregulates LC3, and therefore, autophagy. Here, autophagy acts as a cytoprotective mechanism by reversing senescence and mitigating DNA damage. p53 also moderates LC3 levels during extended starvation to prevent cell death from aberrant vesicle accumulation. RKIP is activated by phosphorylated p53 triggered by Adr-mediated genomic instability. In contrast, RKIP promotes senescence under cellular stress. The opposite interactions obtain during normal conditions (dashed lines).

FIG. 4:

Modulation of *ATG* gene expression by RKIP and STAT3. Overexpression of RKIP can inhibit STAT3 activation and subsequent nuclear translocation. Loss of RKIP thus upregulates *BCL2* and downregulates *BECN1* and *PIK3C3* in a STAT3-dependent manner. When STAT3 is retained in the cytoplasm, its monomeric form isolates FoxO1/FoxO3a from the nucleus and represses *LC3* transcriptional activation. Cytoplasmic STAT3 also displaces eIF2a from the catalytic domain of EIF2AK2, thereby reactivating translation and suppressing ATF4. In the absence of upstream STAT3 control, ATF4 binds to the *LC3* promoter and induces autophagy.

FIG. 5:

The condition-dependent RKIP/LC3/Snail/NF- κ B loop. Either RKIP or autophagy (bold in the respective figures) influences EMT via Snail or NF- κ B in a specific intracellular context. (A) Under basal conditions, RKIP inhibits NF- κ B-mediated Snail expression. Low Snail expression alleviates RKIP repression and creates a positive loop in favor of high RKIP levels. However, the pool of unphosphorylated RKIP also abrogates LC3 conversion and therefore autophagy. (B) When the cell encounters a physiologic stress, stress-sensing receptors such as GPCRs are activated. Active GPCR signaling can increase RKIP phosphorylation and its dissociation from LC3-I. Starvation-activated GPCRs also generate proautophagic signals, further enhancing LC3 conversion rates. Additional evidence indicates that starvation-induced IKK activity can upregulate LC3 levels and conversion to the lipidated form. Consequently, cargo receptors target Snail for degradation through active autophagy. When RKIP is phosphorylated, autophagy plays a more prominent role in suppressing EMT.