



University "G. d'Annunzio"
Chieti-Pescara



SCUOLA SUPERIORE "G. d'Annunzio"

Ph.D. Course in

INNOVATIVE TECHNOLOGIES IN CLINICAL MEDICINE & DENTISTRY

CYCLE XXXVII

Identification of key biomarkers and therapeutic targets in human epithelial malignancies

Department of Neurosciences, Imaging and Clinical Sciences
SSD MEDS-04/A

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Tutor **Prof. Manuela Iezzi**

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Abstract

Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality worldwide, with high mortality rates driven by tumor recurrence and resistance to standard therapies such as 5-fluorouracil (5-FU). Increasing evidence suggests that CRC cells can exploit adaptive mechanisms to survive under metabolic stress. One such mechanism is autophagy, a catabolic process that recycles intracellular components and is increasingly recognized as a critical survival pathway under stress conditions. However, the molecular regulators driving these adaptive processes remain poorly understood. In this context, Trop-2, a transmembrane glycoprotein overexpressed in various human carcinomas, including CRC, has been associated with increased tumor aggressiveness and poor clinical outcomes. While Trop-2's role in tumor growth and metastasis is well-documented, its involvement in autophagy and metabolic stress adaptation has not been previously explored. In this study, we identify Trop-2 as a key modulator of cytoprotective autophagy in CRC. Using a combination of real-time impedance-based growth assays, confocal microscopy, immunoblotting, ultrastructural analysis and proteomics, we demonstrate that Trop-2 promotes CRC cell survival under nutrient-limiting conditions by enhancing autophagic flux. Trop-2 overexpression was associated with the upregulation of essential autophagy-related genes (ATGs), including ULK-1, Beclin-1, ATG4A, ATG5, and ATG7, as well as increased autophagolysosome formation and enhanced LC3 degradation. Additionally, our study reveals that TFEB activation is a downstream event of Trop-2 signaling, a novel finding not previously described. Mechanistically, Trop-2 triggers the nuclear translocation of TFEB, a master transcriptional regulator of autophagy and lysosomal biogenesis, potentially through calcium-dependent activation of calcineurin and sequestration of 14-3-3 ζ/δ at the membrane. Proteomic enrichment analysis further identified a molecular signature in Trop-2-expressing cells that resembles mTORC1 inhibition, supporting the hypothesis that Trop-2 enhances autophagy by repressing this key negative regulator. Electron microscopy confirmed that Trop-2 promotes autophagosome-lysosome fusion, facilitating efficient degradation and recycling of intracellular components under metabolic stress. We also investigated the functional relevance of Trop-2's proteolytic cleavage at the R87-T88 site, a cancer-specific event catalyzed by ADAM10. A cleavage-deficient Trop-2 mutant failed to induce autophagy and could not support CRC cell survival under nutrient-limiting conditions, highlighting the importance of Trop-2 activation through proteolysis for its pro-autophagic function. Moreover, inhibition of autophagy with Bafilomycin A1 (Baf A1) sensitized Trop-2-expressing cells to apoptotic death, underscoring the dependence on autophagy for survival and revealing a potential therapeutic vulnerability. Importantly, Trop-2-expressing cells exhibited significant resistance to 5-FU, a first-line chemotherapeutic agent for CRC. Co-treatment with Baf A1 restored drug sensitivity, suggesting that Trop-2-induced autophagy contributes to chemoresistance. These findings position Trop-2-mediated autophagy not only as a metabolic adaptation mechanism but also as a critical determinant of therapeutic resistance. In conclusion, our results identify Trop-2 as a central player in CRC's metabolic adaptation and chemoresistance through its regulation of autophagy. Trop-2 proteolytic activation acts as a molecular switch enabling CRC cells to survive nutrient stress and evade chemotherapy-induced apoptosis. These insights open new avenues for therapeutic intervention, including Trop-2-targeted strategies and combinatorial approaches with autophagy inhibitors to overcome resistance in CRC. Given Trop-2's widespread expression across tumor types, these findings may have broader implications for cancer therapy. Targeting Trop-2-mediated autophagy, particularly its cleavage-activated form, represents a promising strategy to overcome therapeutic resistance. This work also supports the stratification of CRC patients based on Trop-2 expression and cleavage status, laying the groundwork for the development of new therapeutic strategies combining autophagy inhibitors with standard chemotherapy or targeted anti-Trop-2 agents. Future studies will investigate the role of Trop-2 in autophagy regulation under additional tumor-relevant stressors, including hypoxia and immune infiltration, and assess its translational potential in advanced 3D models and preclinical CRC settings.

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INTRODUCTION

1. *Trop-2*

Trophoblast cell surface antigen 2 (Trop-2) is a type-I transmembrane glycoprotein and a calcium signal transducer, encoded by the *TROP2/TACSTD2* (tumor-associated calcium signal transducer 2) gene. This intronless gene is located on the short arm of human chromosome 1, at the 1p32 locus²) and belongs to the *TACSTD* gene family. The second member of this family, *TACSTD1* (also known as *EPCAM*), encodes the epithelial cell adhesion molecule (EpCAM). Trop-2 and EpCAM share 49% sequence identity and 67% similarity in their amino acid sequences, exhibiting a remarkable resemblance in the arrangement of hydrophilic and hydrophobic residues (1,2). Sequence analysis suggests that the *TACSTD* gene family evolved through exon shuffling. Notably, all nine exons of *TACSTD1* are present in the intronless *TACSTD2* gene, indicating that *TACSTD2* likely originated through retroposition from an mRNA intermediate (2).

1.1 *Protein structure of Trop-2*

Human Trop-2 is composed of 323 amino acids and its structure consists of an N-terminal extracellular domain (ECD, residues 27-274) connected to an intracytoplasmic C-terminal tail (ICD, residues 298–323) via a single-pass transmembrane portion (TM, residues 275–297) (3) (Fig. 1).

The ECD contains four N-glycosylation sites (4) and is structurally divided into two regions. The first half comprises a globular domain, stabilized by six disulfide bridges, which connect twelve interacting cysteines, defining two specific motifs: the GA733 type-I domain (residues 27-69) and a thyroglobulin type-IA motif (residues 70-148) (5). The second half of the ECD is a cysteine-free region that adopts a stem-like conformation, linking the globular moiety to the single hydrophobic transmembrane domain.

The ICD domain of Trop-2 comprises a short tail devoid of enzymatic domains, but it contains a HIKE motif (6), a regulatory site for protein-protein and protein-phospholipid interactions (7,8) and two serine residues, Ser303 and Ser322, which can be phosphorylated by protein kinase C α (PKC α) (9–11).

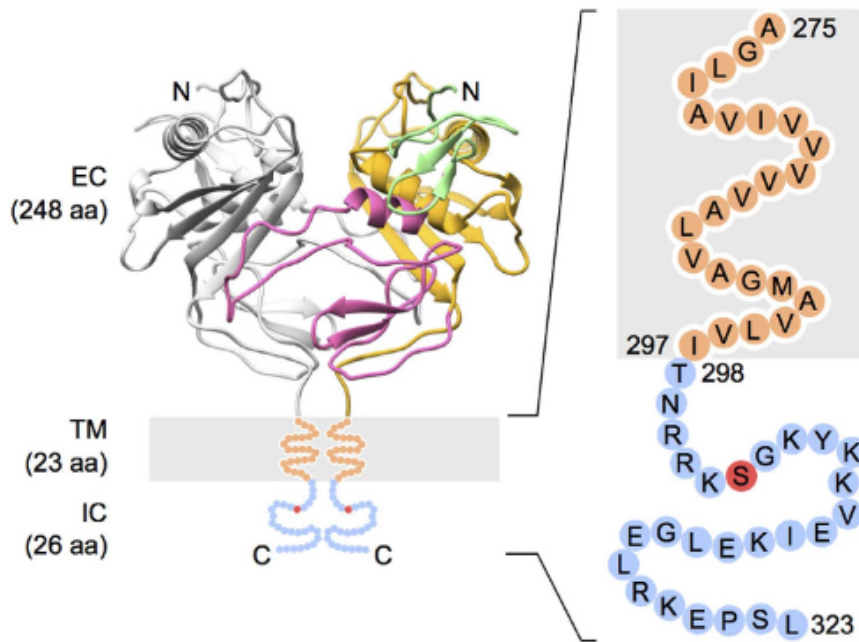


Fig. 1. Model of structural organization of Trop-2. This image is adapted from (10).

1.2 The Trop-2 signaling network

Trop-2 was initially identified as a surface antigen on invasive trophoblast cells (12). Next studies demonstrated that Trop-2 expression is upregulated in the majority of human carcinomas (13), including those of the lung (14), breast (15), pancreas (16), stomach (17), head and neck (18), colon-rectum (19), and ovary (20). Its expression levels correlate with cancer aggressiveness and poor prognosis. This thesis specifically focuses on colorectal cancer (CRC) (discussed in Chapter 3). In support of a model where Trop-2 acts as a key driver of tumour progression, normal colonic mucosa does not express Trop-2. A “switch-on” of Trop-2 expression is observed in dysplastic colon mucosa at the earliest stages of transformation. Trop-2 expression further increases during neoplastic transformation, highlighting its role in tumor development (13) (Fig. 2).

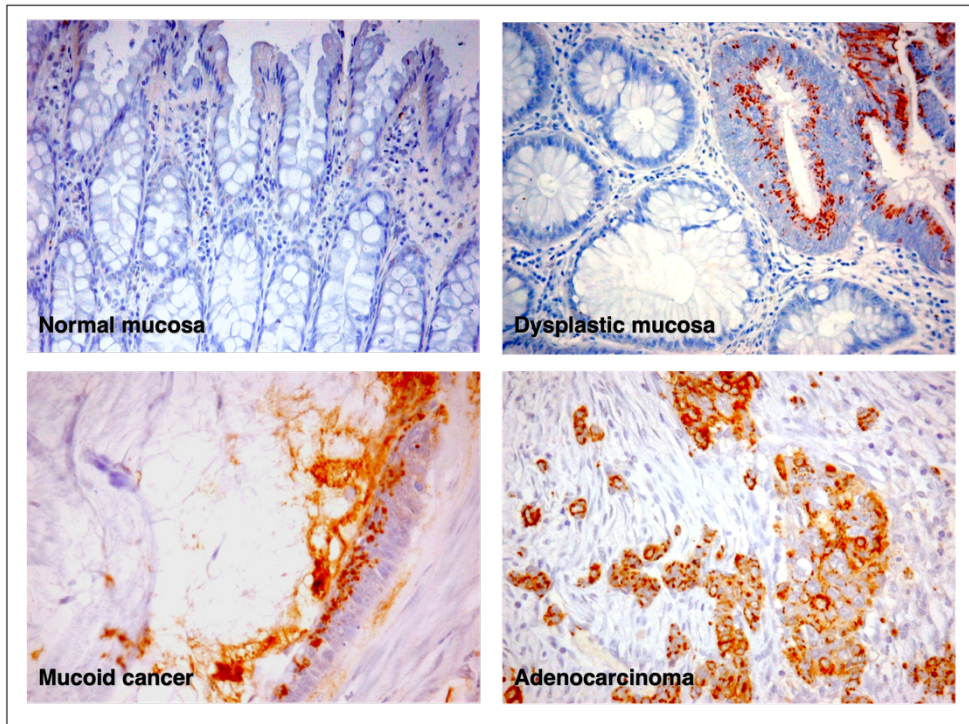


Fig. 2. Trop-2 protein expression in tumour progression. This image is adapted from (13).

Although cancer-specific mutations in the *TACSTD2* gene occur at very low frequency (0.2-0.3%), none have been identified as primary drivers of malignancy to date. Furthermore, the transcriptional network regulating Trop-2 expression in cancer remains to be fully elucidated.

Recent research, including studies from our group, has focused on identifying upstream regulators and downstream effectors of Trop-2 signaling. In line with these efforts, we have demonstrated that post-translational modifications play a crucial role in activating Trop-2 to promote cancer development and progression. Specifically, we discovered that Trop-2 undergoes cancer-specific, ADAM10-mediated proteolysis at the R87-T88 site in its ECD, and this event activates the molecule to promote tumor growth and metastasis (21). Trop-2 is subsequently further activated through regulated intramembrane proteolysis (RIP) by the ADAM17/TNF- α -converting enzyme (TACE) at the A187-V188 site in the ECD, followed by γ -secretase cleavage at the G285-V286 site within the transmembrane domain (22). Complete Trop-2 activation results in ECD shedding and nuclear translocation of the ICD, which promotes self-renewal, induces prostatic intraepithelial neoplasia (PIN), and

participates in β -catenin-dependent signaling. Notably, in human prostate cancer, nuclear β -catenin colocalizes with the nuclear ICD of Trop-2 and loss of β -catenin abolishes Trop-2-driven self-renewal and transformation (22).

This proteolytic cascade also disrupts cell-cell adhesion by triggering E-cadherin cleavage by ADAM10. This event leads to a functional, rather than transcriptional, epithelial-mesenchymal transition (EMT) (19). As a consequence, β -catenin, which is normally sequestered by E-cadherin, is released, thereby activating nuclear Trop-2 ICD/ β -catenin signaling (19).

Trop-2 has also been demonstrated to be involved in calcium (Ca^{2+}) signaling regulation upon crosslinking with antibodies, although the molecular mechanisms were not demonstrated (23). Recently, a research work from our group revealed that cancer-specific cleaved Trop-2 activates a dormant, ubiquitously expressed signaling super-complex composed of the Na^+/K^+ -ATPase ion pump, CD9, PKC α , and cofilin-1, which drives CRC growth and invasion (11) (Fig. 3). In this context, Trop-2 activation at the cell membrane leads to the release of intracellular Ca^{2+} , primarily from endoplasmic reticulum (ER) stores via Na^+/K^+ -ATPase (11). This facilitates the translocation of pre-activated, mTORC2-phosphorylated PKC α to the cell membrane, where it binds and phosphorylates the cytoplasmic tail of Trop-2, with CD9 acting as a crucial bridging factor(11). This interplay between Trop-2 and PKC α establishes a feed-forward activation loop in which Trop-2 transduces Ca^{2+} signals essential for PKC α activation and its own phosphorylation, thereby fully activating downstream effectors. These include the remodeling of the β -actin/ α -actinin/myosin II cytoskeleton via gelsolin, cofilin-1 and annexins A1/A6/A11, which in turn promotes malignant progression through cleavage of the β -actin-binding site of E-cadherin (19). This leads to downstream activation of key oncogenic pathways such as Akt (24), ERK, NFkB, and cyclin D1 (13,25,26).

To further support the functional relevance of these interactions, we demonstrated that deletion of the HIKE region, mutagenesis of the phosphorylation sites, or CRISPR-based inactivation of CD9 in CRC cells prevents Trop-2 from recruiting PKC α to the cell membrane (11). Consequently, this results in the inhibition of Trop-2-driven cell growth, highlighting PKC α as a key mediator of Trop-2's pro-tumorigenic effects.

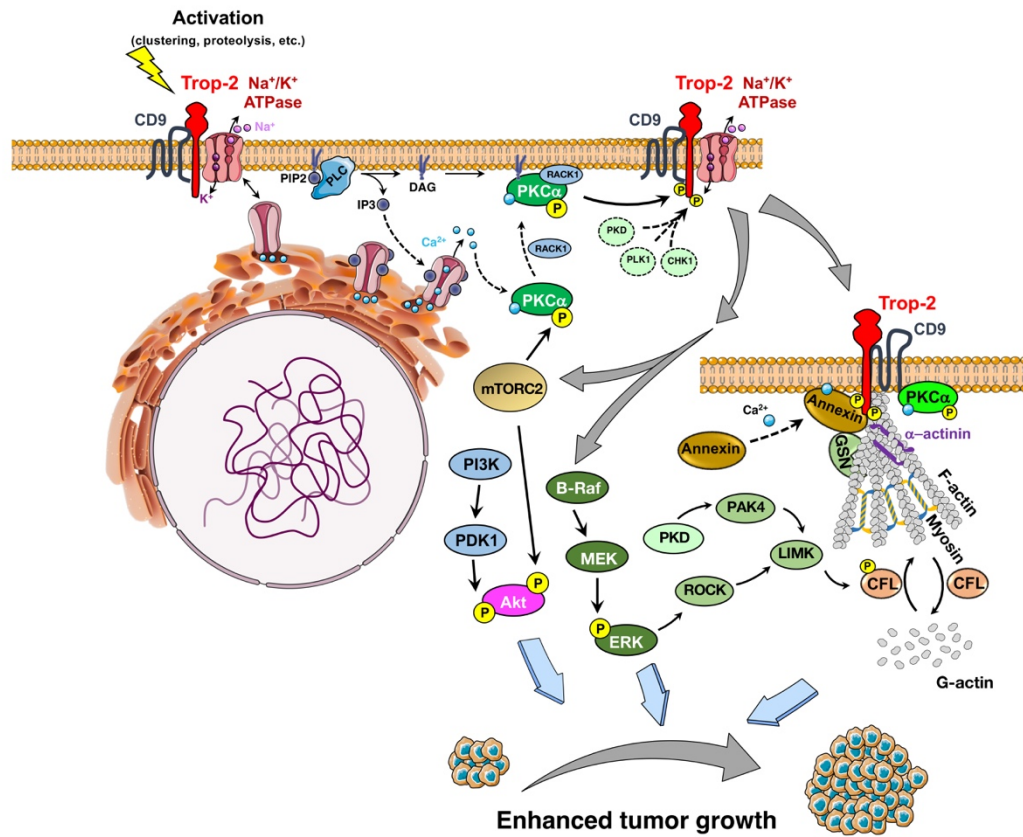


Fig. 3. Model of the major Trop-2-centered growth control signaling pathways. This image is adapted from (11).

The clinical relevance of these findings is underscored by prognostic analyses of various CRC case series. Overexpression of the components of the Trop-2–centered super-complex is shown to have a stronger prognostic impact than any individual parameter, correlating it with worse disease-free and overall survival in CRC patients (11).

At the molecular level, mTORC2 plays a pivotal role in the Trop-2/PKC α feed-forward activation loop, by phosphorylating and activating PKC α and Akt (11,27,28). Notably, proteomic profiling has identified Akt as a crucial hub within Trop-2-activated networks (24). Supporting this, treatment with Akt allosteric inhibitors selectively abolishes the growth of Trop-2-expressing tumors (24). Furthermore, mTORC2 upregulates NF κ B as a key Trop-2 downstream effector, which in turn activates the cyclin D1 and ERK pathways to drive tumor cell proliferation and metastatic dissemination (29).

Beyond protein-level regulation, Trop-2 signaling also intersects with oncogenic RNA processing events. Notably, trans-splicing between *CYCLIN D1* and *TROP2* generates the *CYCLIN D1-TROP2* mRNA chimera, with its expression regulated by both the fusion event and the altered stability of the spliced mRNA (26). This bicistronic chimera has been identified in human gastrointestinal, ovarian, and endometrial tumors, where it promotes oncogenic transformation through the synergistic action of *TROP2* and *CYCLIN D1*. The chimeric mRNA strongly enhances cancer cell proliferation *in vitro*, transforms naïve primary cells and induces aggressive tumor growth *in vivo*, particularly in the presence of activated RAS. Importantly, silencing this chimera results in inhibition of breast cancer growth, highlighting its potential as a therapeutic target (26).

In addition to these molecular mechanisms, Trop-2 is found to interact with several crucial signaling pathways. For example, it forms a complex with insulin-like growth factor 1 (IGF-1), thereby inhibiting IGF-1 receptor (IGF-1R) signaling (30). Given that IGF-1R activation can lead to MAPK pathway activation and PI3K/Akt signaling, this interaction places Trop-2 at a critical regulatory node. By suppressing IGF-1R signaling, Trop-2 downregulates the transcription factor activator protein 1 (AP-1) via the MAPK-ERK pathway (31).

Finally, Trop-2 plays an essential role in regulating cell adhesion, further contributing to its oncogenic potential. Recent findings have demonstrated that Trop-2 inhibits cell adhesion to fibronectin by enhancing $\beta 1$ integrin binding to the receptor for activated C kinase 1 (RACK1). This interaction subsequently activates c-Src and focal adhesion kinase (FAK), ultimately increasing invasiveness in prostate cancer cells (32).

1.3 Trop-2 in cancer therapy

Given its overexpression in various tumors relative to normal cells, Trop-2 represents a promising candidate for the development of target therapies, particularly for antibody-drug conjugate (ADC) directed against its extracellular region.

One example is Sacituzumab govitecan (SG), an ADC consisting of an anti-Trop-2 humanized monoclonal antibody conjugated to SN-38, the active metabolite of irinotecan, a topoisomerase I inhibitor. SG has been approved by the US Food and Drug Administration and the European Medicines Agency for the treatment of metastatic triple-negative breast cancer (33) and metastatic urothelial carcinomas (34). Upon binding Trop-2, SG undergoes

internalization, leading to intracellular SN-38 release, causing DNA double-strand breaks and apoptosis.

Another Trop-2-targeting ADC, Datopotamab deruxtecan (Dato-DXd), carries a potent DNA topoisomerase I inhibitor (DXd) via a tetrapeptide-based linker (35). Dato-DXd selectively binds Trop-2, undergoes internalization, and induces release of DXd, causing DNA damage and apoptosis in Trop-2-expressing tumor cells. It is currently being investigated in the phase I clinical trial TROPION-PanTumor01 for non-small-cell lung cancer demonstrating a 26 % objective response rate, a median progression-free survival of 6.9 months and an overall survival of 11.4 months (36).

Given Trop-2 expression in normal tissues, off-target effects remain a concern. While ADCs evolve toward more potent payloads, we have recently introduced a new anti-Trop-2 antibody, named 2G10, that has the unique feature of recognizing the cleaved Trop-2 molecule on cancer cells, avoiding unwanted binding to the uncleaved Trop-2 expressed on the surface of normal cells (37). *In vivo*, 2G10 was found to ablate growth of Trop-2-expressing breast, colon, prostate cancers, without any evidence of systemic toxicity. This paved the way for a paradigm shift in Trop-2-targeted therapy. However, understanding the Trop-2 biology, its activation mechanisms and the downstream cell signaling in tumor progression and chemoresistance may offer innovative strategies to selectively eliminate cancer cells and mitigate side toxicities.

2. *The autophagic pathway*

Autophagy is an evolutionarily conserved catabolic process that plays a fundamental role in preserving cellular homeostasis by regulating the degradation and recycling of intracellular macromolecules and organelles via the lysosomal pathway in response to nutrient deprivation or cellular stress. The term “autophagy”, derived from the Greek for “self-eating”, was first introduced by Christian de Duve and has since become a cornerstone in the study of cellular dynamics. Significant advancements have been made in elucidating the molecular mechanisms underlying autophagy and its biological relevance across various contexts. A pivotal breakthrough in the field came from Yoshinori Ohsumi’s research, which, through a genetic screen in *Saccharomyces cerevisiae*, identified 15 autophagy-related genes (*ATGs*) essential for the transport of cellular components to the vacuole, the yeast equivalent of the lysosome (38). Ohsumi’s findings provided the foundation for understanding autophagic processes in eukaryotic cells and unveiled the molecular machinery responsible for this critical pathway. In recognition of his pioneering contributions, Ohsumi was awarded the Nobel Prize in Physiology or Medicine in 2016, highlighting the transformative impact of his work on autophagy research (39). Autophagy is classified into three distinct types based on how cellular cargo is sequestered: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy (40). Microautophagy involves the direct engulfment of cytoplasmic materials by the lysosomal membrane through invagination or protusion, followed by enzymatic degradation within the lysosome (41). CMA, on the other hand, is a highly selective process in which proteins containing a specific pentapeptide motif, related to the KFERQ sequence, are recognized by the heat shock cognate 71 kDa protein (Hsc70). These proteins are then transported across the lysosomal membrane via interaction with lysosome-associated membrane protein type 2A (LAMP-2A), leading to their degradation and recycling (42). Macroautophagy, the most extensively studied form of autophagy, is characterized by the formation of double-membrane vesicles known as autophagosomes, which encapsulate cytosolic material before fusing with lysosomes. Within these lysosomal compartments, the enclosed content is degraded by hydrolases, and the resulting breakdown products, such as amino acids, lipids, and sugars, are recycled to sustain cellular functions. This process is essential for energy balance, removal of damaged organelles, and cellular adaptation to stress conditions (43–45).

Due its central role in various physiological processes, including cellular maintenance, differentiation, immune regulation, and responses to nutrient deprivation, autophagy has been recognized as a key factor in both health and disease. Notably, aberrant autophagy has been related to numerous pathological conditions, such as cancer, neurodegenerative disorders, cardiovascular diseases, and metabolic syndromes. Consequently, elucidating the molecular mechanisms that control autophagy is essential for developing therapeutic strategies targeting this pathway. This thesis will specifically focus on macroautophagy, hereafter referred to simply as autophagy, and its implications in cancer development and progression.

2.1 Key stages of autophagy and core autophagic proteins

In mammalian cells, autophagy proceeds through five distinct stages: (a) initiation and (b) nucleation, during which autophagy-related proteins (ATGs) are recruited to a newly formed isolation membrane, giving rise to a cup-shaped structure known as the phagophore; (c) phagophore elongation, where the structure expands and encloses a portion of the cytoplasm; (d) fusion of autophagosomes, the fully enclosed phagophores, with lysosomes to form autophagolysosomes; and (e) degradation of the sequestered material within the autophagolysosomes. Each of these stages is tightly regulated by specific protein complexes that function in a hierarchical manner to orchestrate the autophagic process (46).

○ Induction and phagophore nucleation

The origin of the phagophore membrane has been the subject of extensive investigation, and multiple intracellular membrane compartments have been proposed as the source of the “initiation membrane”. However, accumulating experimental evidence suggests that the endoplasmic reticulum (ER) serve as the primary site for autophagosome biogenesis (47,48). The nucleation of the phagophore occurs at specialized ER subdomains known as omegasomes (49,50) (Fig. 4), which are characterized by the accumulation of phosphatidylinositol 3-phosphate (PI3P), a lipid that is typically absent from the ER (51). The growing phagophore forms in close association with the omegasome, establishing multiple membrane contact sites that facilitate lipid transfer from the ER. These lipids, synthesized in the ER, are transported through these contact points to support the expansion of the autophagic membrane.

The formation of omegasomes begins with the recruitment of the ULK1 (Unc-51-like kinase 1) complex, which consist of ULK1/2 Ser/Thr kinases, FIP200, ATG13, and ATG101 (52–54). As a key regulator of autophagy initiation and nucleation, the ULK1 complex activates downstream signaling by undergoing intermolecular autophosphorylation, which enhances ULK1 kinase activity. This activation is a critical step in initiating the autophagic process, as it regulates the recruitment of additional ATG proteins required for autophagosome formation. Furthermore, ULK1 phosphorylates key downstream regulators, such as ATG4B, ATG9, ATG14L, Beclin-1, AMBRA1, and SEC23B, which collectively contribute to the coordination of autophagosome biogenesis (54).

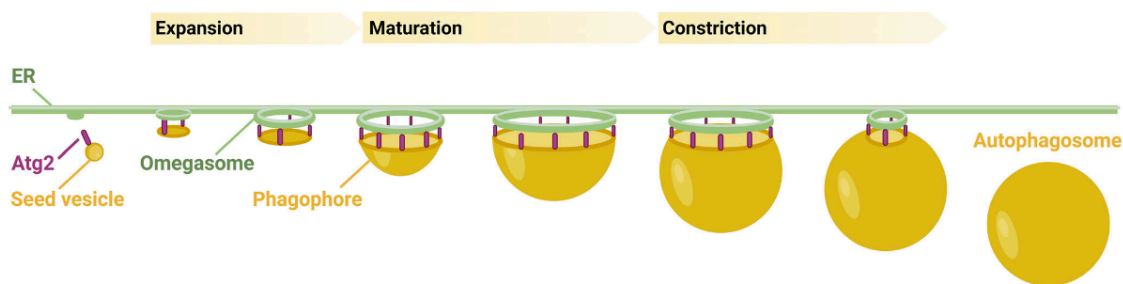


Fig. 4. Model of life cycle of the omegasomes. This image is adapted from (50).

○ *Phagophore elongation and autophagosome biogenesis*

The nucleation of the phagophore membrane is a highly complex and finely regulated process, with several aspects of its molecular mechanisms remaining yet to be fully elucidated (Fig. 5). Current models suggest that phagophore formation relies on the coordinated action of two key protein complexes: the ULK1 complex and the phosphatidylinositol 3-kinase complex I (PI3KC3-C1) (55,56). PI3KC3-C1 comprises VPS34, VPS15, Beclin-1, and ATG14L, with VPS34 catalyzing the conversion of phosphatidylinositol (PI) into PI3P (57,58). This lipid plays an essential role in both phagophore elongation and autophagosome maturation, serving as a signaling molecule that marks the site of autophagosome formation and recruits early autophagic effectors. Among these effectors, the WD-repeat proteins interacting with phosphoinositides (WIPI) family facilitates phagophore membrane expansion.

Phagophore elongation is closely associated with the recruitment of ATG9-containing vesicles (59), which originate from the Golgi apparatus and are actively transported to the

growing phagophore. ATG9A, the only transmembrane protein within the core autophagic machinery, is phosphorylated by ULK1 and plays a critical role in delivering lipids and proteins necessary for phagophore expansion (44). Once phosphorylated, ATG9A mediates the recruitment of LC3 (microtubule-associated protein 1 light chain 3) and WIPI1/2 to the phagophore, thereby promoting its enlargement. WIPI1/2, in particular, interact with PI3P and coordinate with other autophagic proteins to ensure the recruitment of additional factors required for autophagosome elongation. Among these, the WIPI1/2-ATG2 complex plays a fundamental role in maintaining the structural integrity of the autophagic membrane and supports the formation of LC3-positive autophagosomes. Acting as a membrane tether with lipid transfer capabilities, ATG2 collaborates with WIPI proteins to regulate the cycling of ATG9A vesicles, thus supplying membrane material essential for phagophore expansion (60). Moreover, recent studies have shown that recycling endosome GTPase RAB11A-positive membranes serve as a compartment from which the autophagosome originates (60). In particular, the interaction between WIPI2 and RAB11A is crucial for recruiting the autophagy machinery, including ATG16L1, thereby determining the sites of autophagosome formation (60).

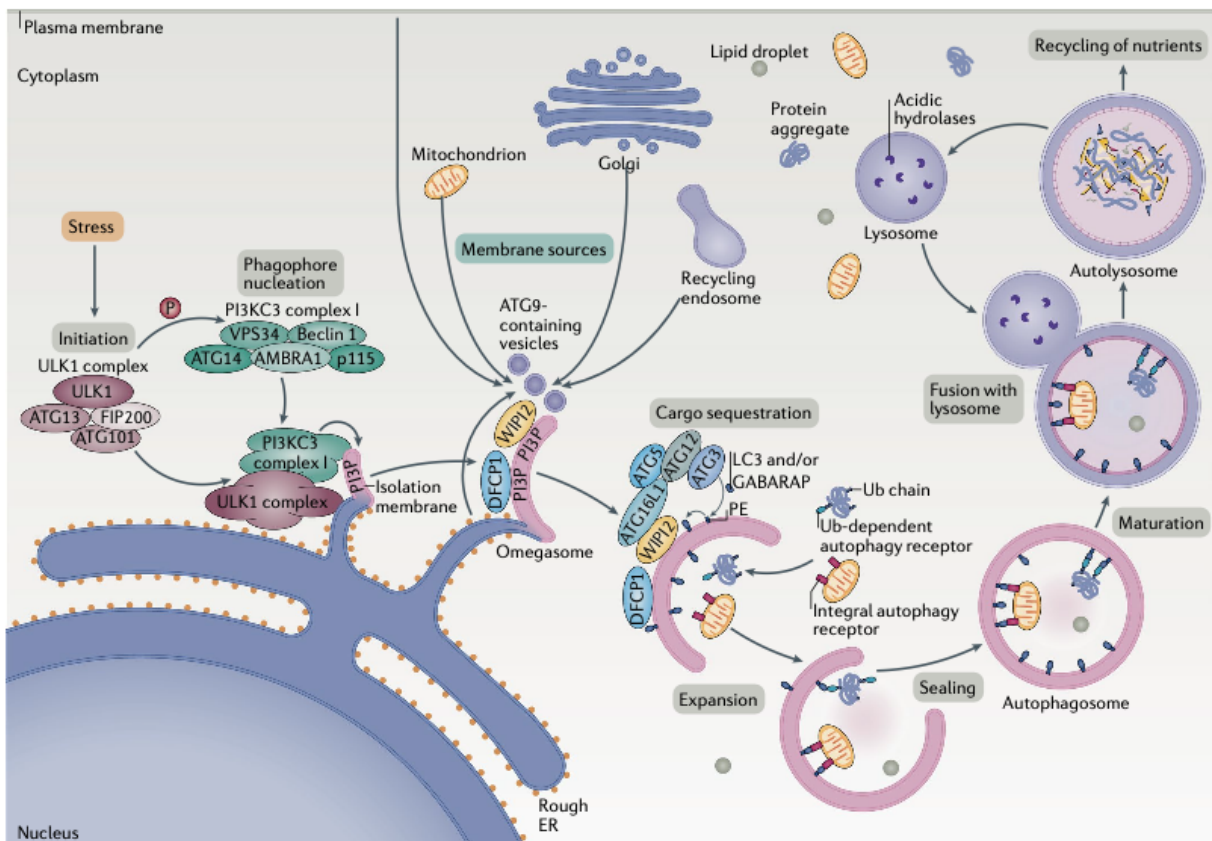


Fig. 5. Model of autophagy process. This image is adapted from (44).

Two ubiquitin-like conjugation systems are essential for autophagosome membrane elongation: the ATG12-ATG5-ATG16L1 complex and the LC3 conjugation system. The process begins with the activation of ATG12 by the E1 enzyme ATG7, followed by its conjugation to ATG5 through the action of the E2 enzyme ATG10. The resulting ATG12-ATG5 conjugate associates with ATG16L1, forming the ATG12-ATG5-ATG16L1 complex, that is subsequently recruited to the isolation membrane.

LC3 is initially synthesized as pro-LC3, an inactive precursor that undergoes C-terminal cleavage by ATG4B, exposing a glycine residue and generating LC3-I. Cytosolic LC3-I is then activated by the E1 enzyme ATG7, transferred to the E2 enzyme ATG3 and finally conjugated to phosphatidylethanolamine (PE) through the ATG12-ATG5-ATG16L1 complex, resulting in its conversion to LC3-II (61,62). This lipidated form of LC3 becomes an integral membrane component of autophagosomes, playing a pivotal role in their expansion, maturation and fusion with lysosomes. The lipidation of LC3 is, therefore, a critical step in the formation of functional autophagosomes capable of completing the autophagic process.

○ *Autophagosome maturation and lysosome fusion*

The final sealing of the double-membraned autophagosome is facilitated by the endosomal sorting complex required for transport (ESCRT), which is crucial for membrane scission. Following the closure of the autophagosome, various molecular factors are recruited to promote its fusion with the lysosome (63). The fusion of autophagosome with lysosome is a tightly regulated event, primarily mediated by SNARE proteins (64). Specifically, syntaxin 17 (STX17) and synaptosomal-associated protein 29 (SNAP29) are located on the autophagosome, while vesicle-associated membrane protein 8 (VAMP8) is present on the lysosome. These SNARE proteins interact to drive membrane fusion, ensuring the formation of the autophagolysosome (64). Additionally, the homotypic fusion and protein sorting (HOPS) complex plays a fundamental role in tethering the two membranes, further facilitating the fusion process. Upon fusion, the inner membrane of the autophagosome is degraded by lysosomal hydrolases, leading to the breakdown of its sequestered contents. The resulting macromolecules, including amino acids, lipids, and sugars, are released back into the cytoplasm and reused in various cellular processes. This metabolic recycling plays a key role in maintaining cellular homeostasis and supporting cellular adaptation under stress conditions.

2.2 Regulation and signaling of autophagy by mTORC1 and AMPK

A key regulator of autophagy is the mechanistic target of rapamycin (mTOR), a highly conserved serine/threonine kinase. mTOR is a core component of two functionally distinct complexes, mTORC1 and mTORC2, with mTORC1 playing a particularly significant role in the regulation of cell growth, metabolism and autophagy (65,66). mTORC1 functions as a negative regulator of autophagy, inhibiting the process when nutrients and growth factors are abundant. Amino acids availability is crucial for mTORC1 activation, and depletion of key amino acids, such as leucine and glutamine, inhibits mTOR activity and triggers the autophagic process (67–69).

mTORC1 and AMP-activated protein kinase (AMPK) tightly regulate the activity of the ULK1 complex. When nutrients are plentiful, the mTORC1 complex phosphorylates ULK1, preventing its association and activation by AMPK and leading to autophagy suppression. Conversely, during amino acid and glucose starvation, AMPK becomes active and inhibits mTORC1, leading to the dephosphorylation of Ser 757 (68). Hence, AMPK associates with, and directly phosphorylates, ULK1 on several sites and this modification activates ULK1 to trigger the autophagic process (68).

Beyond ULK1, mTORC1 and AMPK also regulate the activity of VPS34, a class III PI3K, essential for autophagosome formation. Under nutrient stress, AMPK stimulates autophagy by phosphorylating Beclin-1, which in turn activates the VPS34 complex. Conversely, mTORC1 exerts an inhibitory effect by phosphorylating ATG14L within the VPS34 complex, suppressing its lipid kinase activity and thereby blocking autophagy induction (70).

2.2.1 TFEB, master regulator of autophagy and lysosomal function

In addition to its direct effects on autophagy, mTORC1 also exerts a regulatory control at the transcriptional level by modulating the localization of the transcription factor EB (TFEB), a key regulator of lysosomal biogenesis and autophagy-related gene expression. TFEB belongs to the basic helix-loop-helix leucine-zipper family of transcription factors and governs the expression of genes within the Coordinated Lysosomal Expression and Regulation (CLEAR) network (71). By binding to the promoter regions of numerous ATGs, TFEB enhances autophagosome biogenesis and facilitates autophagosome-lysosome fusion (71,72). The

activity of TFEB is primarily controlled by nutrient availability and phosphorylation-dependent cytoplasm-nucleus shuttling (Fig. 6).

Under conditions of nutrient abundance, amino acids promote mTORC1 recruitment to the lysosomal membrane, where it directly phosphorylates TFEB at Ser142 and Ser211. These phosphorylation events result in retention of TFEB in the cytoplasm, preventing its translocation to the nucleus (71,73). Specifically, phosphorylation at Ser211 enables TFEB to bind the 14-3-3 chaperone proteins, which sequesters it in the cytoplasm, likely by masking its nuclear localization signal (NLS) (73,74).

When nutrients become limiting, mTORC1 is released from the lysosomal membrane and becomes inactive (75,76). Concurrently, the lysosomal Ca^{2+} channel mucolipin 1 (MCOLN1) facilitates the release of lysosomal Ca^{2+} , which activates the phosphatase calcineurin (77). Calcineurin subsequently dephosphorylates TFEB, allowing its rapid translocation to the nucleus. Once in the nucleus, TFEB upregulates genes within the CLEAR network, which encode lysosomal hydrolases, lysosomal v-ATPase pumps and autophagy regulators (77,78).

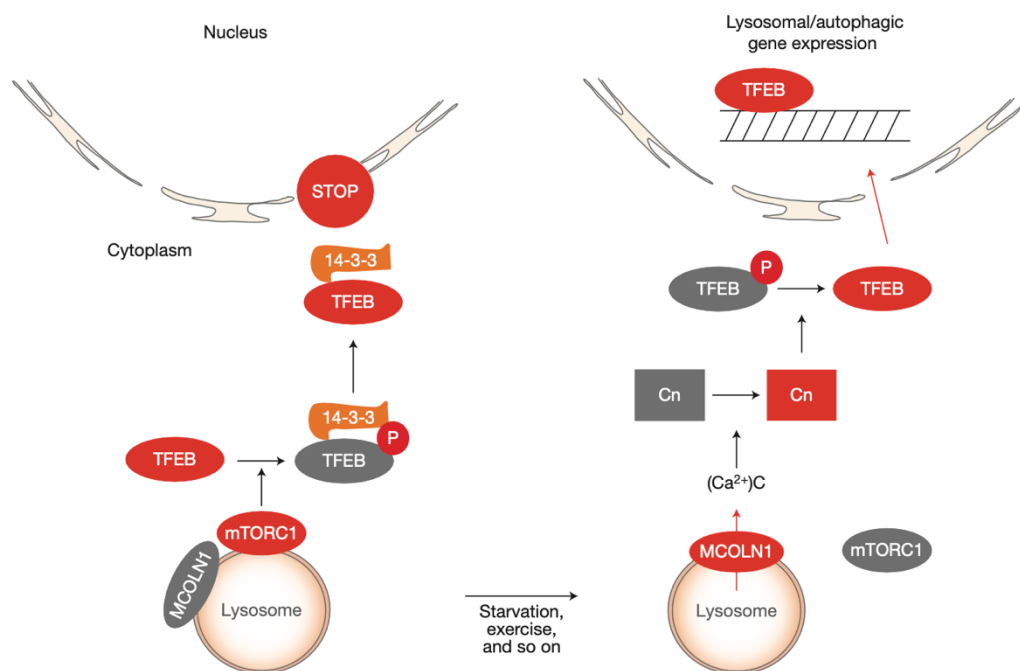


Fig. 6. Model of regulation of autophagy by TFEB. This image is adapted from (77).

2.3 Study of the autophagic process through targeted inhibition of key steps

Small molecules that inhibit autophagy are classified based on their targets, such as the ULK1 complex, Vps34, ATG4B, and the later stages of the process (79). The ULK1 complex plays a crucial role in initiating autophagy, and targeting ULK1/2 to block the early stages is an effective strategy. Compounds like MRT67307 and MRT68921 suppress ULK1/2 activity, inhibiting autophagy *in vitro* (80). Various Vps34 inhibitors have been developed, including 3-MA, wortmannin, LY294002, spautin-1, SAR405, autophinib, Vps34-IN1, and PIK-III. 3-MA inhibits Vps34 but also class I PI3K activity, complicating its interpretation in autophagy in autophagy regulation (81). Wortmannin specifically inhibits Vps34, offering a more reliable autophagy blockade, while LY294002 is more selective but still affects other kinases like mTOR (82,83). Newer selective Vps34 inhibitors, such as SAR405, autophinib, and Vps34-IN1, have emerged, with SAR405 enhancing the anti-proliferative effect of the mTOR inhibitor everolimus (84). Spautin-1 inhibits Beclin-1 through deubiquitinating enzyme inhibition, promoting Vps34 complex degradation and autophagy suppression (85). In addition to blocking autophagosome formation, inhibitors of later stages of autophagy are explored for cancer therapy. Lysosomotropic agents like chloroquine (CQ) and hydroxychloroquine (HCQ) raise lysosomal pH, inhibiting hydrolase activity. Lys05, a HCQ derivative, accumulates more in lysosomes, enhancing anticancer effects (86). DC661, a CQ dimer, induces autophagosome accumulation and lysosomal damage at lower concentrations than HCQ or Lys05, increasing cytotoxicity in melanoma cells. Palmitoyl-protein thioesterase 1 (PPT1) is crucial for lysosomal function, and its deficiency disrupts autophagy. V-ATPase inhibitors, such as Bafilomycin A1 (Baf A1), prevent lysosomal acidification and impair autophagosome-lysosome fusion, making them potent autophagy inhibitors (87).

2.4 Role of autophagy in cancer and progression

Autophagy plays a complex and dual role in tumorigenesis, acting as both a tumor suppressor in the early stages and a promoter of cancer progression in advanced stages (88–91) (Fig.7).

In the early stages of tumorigenesis, autophagy maintains cellular homeostasis by eliminating dysfunctional mitochondria (mitophagy) and oxidized protein aggregates (aggrephagy), preventing DNA damage, and limiting the accumulation of oncogenic proteins

such as SQSTM1/p62 (92,93). These processes help preserve genomic stability, reduce chronic inflammation, and protect cells from malignant transformation. Studies in mouse models have shown that loss or partial inactivation of essential autophagy genes, such as *Becn1* and *Atg5*, increases the risk of spontaneous tumor development, including lymphoma, lung cancer, and hepatocellular carcinoma (94,95).

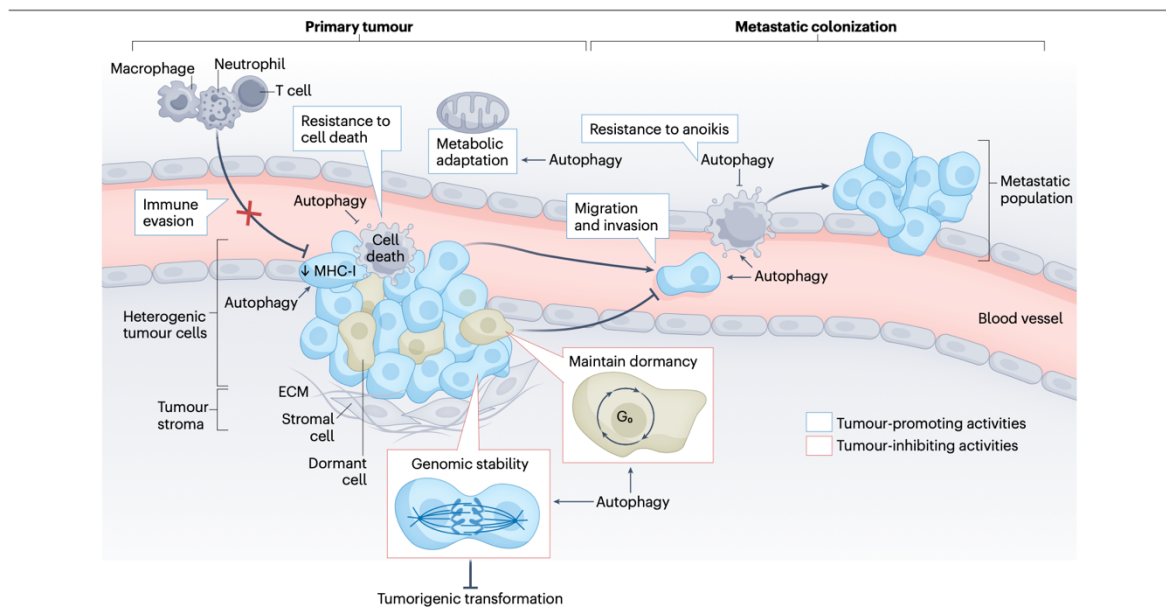


Fig. 7. Model of autophagy role in primary tumor and metastasis. This image is adapted from (95).

As tumors advance to later stages, autophagy shifts from a protective mechanism to a key survival strategy that supports the metabolic demands of cancer cells. By maintaining mitochondrial function, reducing oxidative stress, and preventing DNA damage, autophagy allows tumor cells to endure harsh conditions such as nutrient deprivation, hypoxia, metabolic stress, and chemotherapy (89).

Autophagy also plays a crucial role in metastasis (96). During early stage of metastasis, autophagy may act as a suppressor by preventing tumor necrosis and limiting inflammation. However, in advanced stages, it facilitates metastasis by enhancing cancer cell dissemination, supporting metastatic cell survival in circulation, and promoting colonization in secondary sites. Moreover, autophagy can induce dormancy in metastatic cells, allowing them to evade immune surveillance and resist therapy, ultimately contributing to cancer recurrence (95).

One of the major challenges in cancer treatment is the autophagy-mediated resistance to therapy. Autophagy is strongly induced in cancer cells in response to chemotherapy,

radiation therapy, and targeted treatments, acting as a cellular defense mechanism that helps tumor cells surviving therapeutic stress. This has been observed across multiple cancer types, where autophagy activation protects malignant cells from apoptosis and contributes to treatment failure. As a result, inhibition of autophagy has emerged as a potential strategy to enhance the efficacy of anticancer therapies. Given its significant role in tumor development, progression, and therapy resistance, targeting the autophagic machinery represents a promising avenue for the development of novel anticancer strategies (97). The modulation of autophagy, either through its inhibition in advanced cancers or its activation to restore tumor-suppressive functions, could provide new therapeutic opportunities to improve patient outcomes.

3. *Colorectal cancer (CRC)*

In 2022, CRC was the third most commonly diagnosed cancer worldwide, with 1.9 million new cases, as reported by the International Agency for Research on Cancer and the World Health Organization's GLOBOCAN database (98). It is preceded by lung cancer (2.5 million new cases) and breast cancer (2.3 million new cases) in terms of incidence. Furthermore, CRC is the second leading cause of cancer-related deaths globally. Generally, CRC develops at higher rate in the colon than in the rectum, with a typical ratio of 2:1 in industrialized countries, although this difference is less pronounced in non-industrialized regions (98).

While the majority of CRC cases are sporadic, with many patients having no family history, hereditary forms of CRC are associated with germline mutations in genes directly responsible for DNA repair or in those involved in the regulation of signaling pathways linked to DNA repair. Both sporadic and hereditary forms of CRC are genetically driven, arising from DNA alterations, including mutations. However, in addition to genetic mutations, epigenetic factors also contribute to the etiology of the sporadic forms of CRC. Four key genomic and epigenomic instabilities have been identified as underlying different CRC subtypes: chromosome instability (CIN), CpG island methylator phenotype (CIMP), microsatellite instability (MSI) and global DNA hypomethylation. CRC development is affected by various factors, including susceptibility alleles related to family history or chronic inflammation, the accumulation of new mutations, and the presence of pre-existing genetic mutations. Additionally, mutations in critical genes, such as *adenomatous polyposis coli* (APC), *Kirsten rat sarcoma* (KRAS), and *tumor protein 53* (TP53), are commonly involved in CRC initiation. Hereditary CRC represents a small proportion of all CRC cases and is associated with inherited mutations. It includes conditions such as familial adenomatous polyposis (FAP), MUTYH-associated polyposis (MAP), and Lynch syndrome (hereditary non-polyposis colorectal cancer) (99).

3.1 *Hereditary CRC*

3.1.1 *Lynch Syndrome (LS)*

LS accounts for 3% of CRC cases and is associated with a germline mutation in at least one of the four *mismatch repair* (MMR) genes: *MSH2*, *MSH6*, *MLH1*, *PMS2*, as well as rarer

mutations in the *EPCAM* gene (100,101), which lead to epigenetic inactivation of the *MSH2* gene (102). Individuals who inherit a mutation in one copy of one or more of the MMR genes have a high probability of acquiring a somatic mutation in the second copy, resulting in loss of function (103). Under normal conditions, the proteins encoded by the MMR genes recognize and correct base-pairing errors in the newly synthesized DNA strand. However, mutations in these genes result in the production of non-functional proteins that are unable to repair DNA damage during replication, leading to microsatellite instability (MSI) (104). MSI occurs both in LS and in sporadic forms of colon cancer, where MSI arises from hypermethylation of the *MLH1* gene promoter (105). The frequency of mutations in different MMR genes associated with LS varies. Mutations in *MSH2* and *MLH1* are found in approximately 90% of LS cases, while mutations in *PMS2* are rare, and those in *MSH6* account for about 10% of cases (106). The loss of function of these genes predisposes individuals to accumulate additional mutations, thereby increasing their risk of cancer. As a result, patients with LS are at an elevated risk of developing tumors in the small intestine, urethra, stomach, endometrium, ovaries, and even the skin. In terms of colon cancer, patients with LS develop tumors in the proximal colon in 70-85% of cases, with a higher incidence at a younger age compared to the general population (107). CRC associated with LS follow the classic adenoma-carcinoma sequence (discussed in paragraph 3.3) seen in other CRC tumors. However, adenomas in LS are non-polypoid (108) and tend to be poorly differentiated (109).

3.1.2 Familial Adenomatous Polyposis (FAP)

FAP is the second most common hereditary colorectal cancer syndrome, accounting for approximately 1% of all CRC cases. It is characterized by the development of hundreds of adenomatous polyps in the colon by the age of 35 in 95% of affected individuals (109). In about 10% of cases, FAP presents in an attenuated form, known as attenuated familial adenomatous polyposis (AFAP), where patients develop fewer than 100 adenomatous polyps (110). In addition to the clinical manifestations in the colon, some individuals with FAP may also exhibit extracolonic manifestations such as gastric polyps (30-88%), osteomas (65-80%), congenital hypertrophy of the retinal pigment epithelium (90%), thyroid carcinoma (12%), central nervous system tumors, skin lesions, and abnormalities in hepatobiliary system (111). FAP is an autosomal dominant disorder caused by frameshift and nonsense mutations in the *APC* tumor suppressor gene, located on the short arm of chromosome 5 (109). This gene encodes a protein

involved in cell adhesion and migration, microtubule assembly, chromosome segregation, and signal transduction (112). Under normal conditions, the protein encoded by *APC* binds to a complex consisting of GSK-3 β , CK1 α/ϵ , AXIN1, AXIN2, and β -catenin. This complex phosphorylates β -catenin, marking it for recognition and degradation by the ubiquitin-proteasome system. When a mutation occurs in the *APC* gene, a defective protein with reduced affinity for the complex is produced. As a result, the phosphorylation of β -catenin is impaired, leading to increased cytoplasmic levels of active β -catenin. This, in turn, activates the Wnt signaling pathway and facilitates the translocation of β -catenin into the nucleus, where it interacts with transcription factors to induce genes such as vascular endothelial growth factor (*VEGF*), *MYC*, and cyclin D1 (*CCND1*). Ultimately, this process activates signaling pathways that contribute to chromosomal instability.

3.1.3 MUTYH-Associated Polyposis (MAP)

MAP is a hereditary form of polyposis that differs from other syndromes due to its autosomal recessive inheritance pattern, resulting from mutations in the *MUTYH* gene located on the long arm of chromosome 1. The *MUTYH* gene encodes the MUTYH protein, a DNA glycosylase involved in the base excision repair (BER) system, which repairs oxidative DNA damage. Under normal conditions, the *MUTYH* protein recognizes oxidized guanine (8-oxoguanine), which incorrectly pairs with adenine, causing a transversion. Upon detecting this base-pairing error, *MUTYH* facilitates its repair by excising the incorrect base. This process allows DNA polymerase to restore the correct pairing between guanine and cytosine, and *OGG1* protein replaces the oxidized guanine with a normal base (113). Mutations that result in the loss of function of the *MUTYH* gene hinder this repair mechanism, leading to the accumulation of mutations, such as those in *K-ras* and *APC*, which contribute to carcinogenesis. Patients with MAP are at a significantly higher risk of developing CRC, with an incidence of 43-63% by age 60. This risk increases to 80-90% if patients do not undergo regular screening (114). Most individuals with MAP, like those with AFAP, present with fewer than 100 colonic polyps, and cancer primarily develops in the proximal colon. In addition to colonic lesions, patients may also present with extracolonic manifestations, including cancers of the breast, stomach, and duodenum.

3.2 *Sporadic CRC*

Sporadic CRC are the most prevalent, accounting for 90-95% of cases. These tumors typically arise from precancerous lesions known as adenomas, which can evolve into cancer over a period of 10 to 20 years due to the accumulation of somatic mutations in tumor suppressor genes and oncogenes. These mutations result in loss of function in tumor suppressor genes and gain of function in oncogenes. In sporadic tumors, approximately 138 driver genes are commonly implicated, with each individual tumor involving 2 to 8 of these genes (115). Sporadic CRC exhibit mutational heterogeneity, which allows for their classification into hypermutated and non-hypermutated forms (116). Non-hypermutated tumors primarily exhibit loss-of-function mutations in genes such as *TP53*, *APC*, *FBXW7*, *TCF7L2*, *SMAD4*, *PIK3CA*, and gain-of-function mutations in genes such as *KRAS* and *NRAS* (117). Hypermutated tumors, in contrast, are predominantly associated with mutations in mismatch repair genes, including *MSH3*, *MSH6*, *MLH3*, as well as hypermethylation of the *MLH1* gene. These alterations lead to MSI. However, there are certain genes, such as *APC*, whose mutations are involved in both types of tumors (115).

3.3 *CRC carcinogenesis*

The development of CRC from normal colonic mucosa follows the adenoma-carcinoma sequence, in which the accumulation of genetic mutations ultimately leads to the formation of cancer (118,119). In most cases, these mutations initiate the formation of polyps, which contain areas of aberrant crypts that eventually develop into an adenoma, typically less than 1 cm in size. Over time, the adenoma grows beyond 1 cm, becoming an advanced adenoma that ultimately transforms into carcinoma. This process typically takes about 10-15 years in sporadic cases, while in hereditary syndromes, cancer onset occurs earlier (120,121) (Fig. 8).

The adenoma-carcinoma sequence involves several mutations occurring at different stages. In the early stages, up to 80% of cases are characterized by a mutation in the *APC* gene, often accompanied by loss of heterozygosity through deletions or point mutations, predominantly in early adenomas. As such, *APC* mutation is considered a key event in the initiation phase of cancer. In about 18% of sporadic cases, hypermethylation of the *APC* gene promoter may occur, resulting in loss of gene function, which may also be associated with MSI. In addition to *APC* mutations, the *K-ras* oncogene is frequently mutated in the early stages, particularly in late adenomas, though it is less common in early adenomas. This suggests that

K-ras mutations play a significant role in promoting adenoma growth (122). The proto-oncogene *Ras* is involved in regulating the cell cycle, cell proliferation, apoptosis, and cell motility. Mutations in this gene lead to the formation of the *K-ras* oncogene, which promotes cell proliferation, induces inflammatory stimuli, angiogenesis, and suppression of apoptosis. Furthermore, mutations in *K-ras* predispose the cells to additional genetic alterations, contributing to cell proliferation and colonic epithelial hyperplasia, advancing the progression from an early adenoma to an intermediate adenoma during the promotion phase (123).

As the adenoma-carcinoma sequence progresses, additional mutations occur, including deletions in the *SMAD4* and *SMAD2* genes. These genes encode proteins that contribute to suppress the transforming growth factor beta (TGF- β) pathway, which inhibits cell growth and differentiation. Deletions in these genes disrupt the regulation of cell growth, contributing to the advancement of the intermediate adenoma toward carcinoma.

The mutation most commonly associated with the transition from adenoma to carcinoma is found in the *TP53* gene. Missense mutations in this gene result in the inactivation of the p53 protein, which normally causes cell proliferation arrest and activates genes for DNA repair mechanisms in response to DNA damage. The loss of p53 function prevents DNA repair, enabling mutations to be passed on to subsequent generations. The accumulation of these mutations further enhances the carcinoma's invasive properties, ultimately leading to metastasis (122,123). This sequence of events is commonly known as the Vogelstein progression model (124).

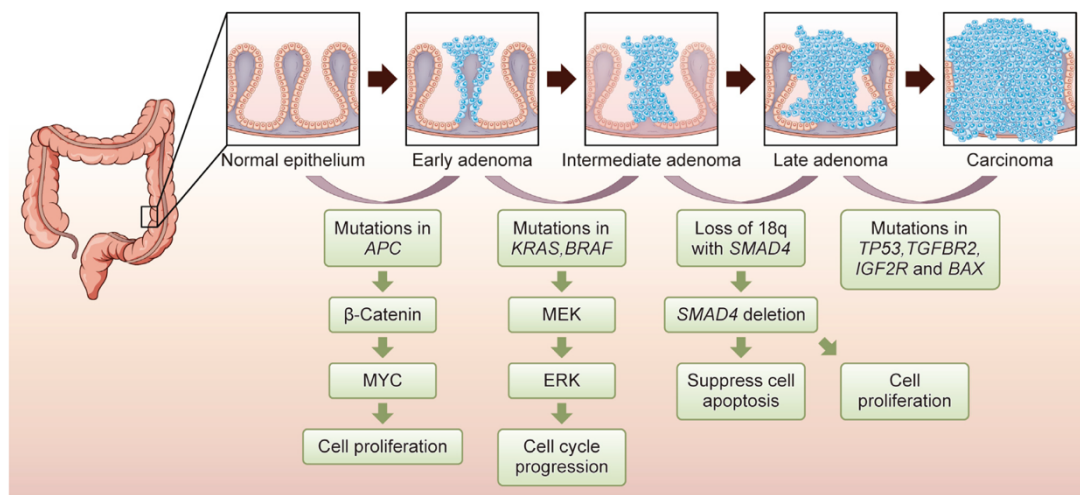


Fig. 8. Model of sequential progression of CRC and the corresponding cancer hallmarks. This image is adapted from (120).

More recently, in-depth genomic and transcriptomic analyses have revealed the molecular heterogeneity of CRC, leading to the definition of a molecular classification system consisting of four consensus molecular subtypes (CMS) (125,126):

- 1) CMS1 is characterized by MSI, hypermethylation, a hypermutation phenotype, and mutations in the *BRAF* gene. It also shows active immune cell infiltration in the tumour microenvironment, particularly CD8⁺ cytotoxic T lymphocytes, CD4⁺ T helper 1 cells and natural killer cells;
- 2) CMS2 exhibits epithelial characteristics, CIN, copy number alterations, and upregulation of Wnt/Myc signaling;
- 3) CMS3 shows metabolic dysregulation and is enriched in *Kras*-activating mutations;
- 4) CMS4 features strong activation of TGF- β and VEGFR pathways, stromal invasion, angiogenesis and mesenchymal characteristics.

The CMS system provides the most detailed gene-expression profile of CRC heterogeneity available so far. However, future advancements in disease classification, including the identification of intra-CMS subgroups and a more precise characterization of samples with mixed phenotypes, are expected (127). This system may offer insights into CRC prognosis and play a key role in drug development and its clinical use (128).

3.4 CRC treatments

At the time of diagnosis, approximately 80% of CRC patients present with the disease at an early stage (129). The first treatment typically involves the surgical resection of the tumor, with a 5-year survival rate of 99% for patients with stage I cancer, 80% for stage II, and 50% for stage III. However, despite surgery, recurrences may occur. For this reason, adjuvant chemotherapy is recommended, particularly for patients with stage III cancer. Despite this treatment, 40% of stage III patients still experience recurrences (129). Initially, adjuvant treatment involved the use of 5-fluorouracil (5-FU) or capecitabine, without the addition of oxaliplatin. It has been shown that adding oxaliplatin to fluoropyrimidines results in better therapeutic outcomes compared to using fluoropyrimidines alone. The combination of leucovorin (folinic acid), 5-FU, and oxaliplatin (FOLFOX) is considered the most effective chemotherapy regimen, particularly for patients with metastatic colon cancer, as well as for

stage II and III cancers with regional lymph node involvement (130). However, the addition of oxaliplatin is associated with increased post-treatment toxicity, including side effects such as neutropenia, vomiting, diarrhea, and peripheral neuropathy (131,132).

3.4.1 5-Fluorouracil (5-FU)

5-FU belongs to the class of antimetabolites and is characterized by the substitution of a hydrogen atom with a fluorine atom at the 5-carbon position of uracil. As mentioned earlier, 5-FU is one of the most widely used chemotherapeutics for adjuvant treatments in patients with stage III and II colon cancer following surgical resection. However, its use is not limited to colon cancer, as it is also used to treat malignant tumors of the skin, pancreas, stomach, breast, esophagus, and head and neck regions. Despite being considered one of the safest chemotherapeutics, patients treated with 5-FU may experience a variety of side effects, including diarrhea, fever, stomatitis, neuropathy, neutropenia, thrombocytopenia, and anemia. 5-FU is often administered in combination with leucovorin, which enhances its effect and reduces side effects, and with oxaliplatin to achieve a more effective treatment (133,134).

The precise mechanism of action of 5-FU remains not fully understood, but one known effect within tumor cells is the inhibition of the enzyme thymidylate synthase. After administration, 5-FU is taken up by the tumor cell and converted by uridine phosphorylase and uridine kinase into 5-fluorouridine monophosphate (FUMP), which is subsequently phosphorylated into fluorouridine diphosphate (FUDP). This can then be further phosphorylated into fluorouridine triphosphate (FUTP) or converted into fluorodeoxyuridine diphosphate (FdUDP) via a reaction catalyzed by the ribonucleotide reductase.

FdUDP is converted by thymidine phosphorylase and thymidine kinase into fluorodeoxyuridine monophosphate (FdUMP), which forms a ternary complex with 5-10 methylenetetrahydrofolate (CH₂-THF), inhibiting the action of thymidylate synthase by preventing it from binding to its substrate 2'-deoxyuridine-5'-monophosphate (dUMP) in a process that requires metabolites like folates, which are provided by the simultaneous administration of 5-FU with folinic acid.

Under normal conditions, thymidylate synthase catalyzes the conversion of dUMP into dTMP, ensuring thymidine synthesis. When 5-FU is administered, this enzyme is inhibited, and this leads to reduction of thymidine synthesis, accumulation of dUMP in the cell and

binding to the newly synthesized DNA strand, DNA strand breakage and consequent disruption of its synthesis (133,134).

Another potential mechanism of 5-FU is its incorporation, once converted to FUTP, into RNA molecules, especially tRNA, leading to post-transcriptional modifications. While this mechanism is still under investigation, it could result in the blockage of mRNA translation (135). The cytotoxic damage caused by 5-FU in tumor cells leads to an accumulation of cells in the S phase of the cell cycle, a block in the G2/M phase, and the arrest of cells in the G1-S phase (136).

3.4.2 5-FU chemoresistance and molecular mechanisms

5-FU resistance remains a major obstacle in the effective long-term management of metastatic CRC (137). One of the key mechanisms driving resistance is the alteration of the cellular pathways targeted by the drug, whose therapeutic efficacy is therefore compromised. Notably, changes in thymidylate synthase expression and the loss of *TP53* function have been shown to significantly impact CRC cell sensitivity to 5-FU treatment.

Increased thymidylate synthase expression has been strongly associated with a reduction in 5-FU sensitivity. Likewise, CRC cells with mutated or non-functional *TP53* exhibit diminished responses to 5-FU. In cells harboring *TP53* mutations, 5-FU typically induces mitochondria-mediated apoptosis by interacting with mitochondrial proteins, which, in turn, can enhance the cytotoxic effects of the drug. However, *TP53* mutations, which occur in approximately 50% of cancers, result in the loss of p53 functionality, further compromising the drug's therapeutic impact (138).

Autophagy has emerged as a crucial factor in promoting 5-FU resistance in CRC cells (139). Research has highlighted that the activation of the p38MAPK pathway plays a pivotal role in both autophagy and the cellular response to 5-FU. Inhibition of this pathway has been linked to a reduction of 5-FU-induced apoptosis, further promoting CRC cell survival. This resistance is primarily driven by an autophagic response, as blocking the p38MAPK pathway decreases p53-driven apoptosis without impacting p53-dependent autophagy. Therefore, the p38MAPK pathway is essential for balancing the opposing processes of apoptosis and autophagy, and its regulation is a key determinant in 5-FU resistance. Moreover, the induction of autophagy by 5-FU has been found to involve the p53-AMPK-mTOR signaling axis, by activating AMPK, p53 inhibits mTOR, triggering autophagy and contributing to 5-FU resistance (140).

In addition to these mechanisms, the overexpression of receptor-associated coactivator 3 (RAC3) in CRC cells under 5-FU treatment further exacerbates resistance. RAC3, a member of the SRC/p160 co-activator family, plays an essential role in tumor progression and survival. In CRC cells, RAC3 inhibits both apoptosis and autophagy, further promoting resistance. The activation of the p38MAPK and PI3K/Akt pathways in these cells leads to the inhibition of key apoptotic proteins, including caspase-8 and -9, and prevents the translocation of apoptosis-inducing factor 1 (AIF-1) from the mitochondria to the nucleus, thereby reinforcing the resistance mechanisms (141).

Thus, autophagy plays a central role in the development of 5-FU resistance in CRC. By modulating key signaling pathways such as p38MAPK and p53-AMPK-mTOR, CRC cells can evade apoptosis and continue to survive in the presence of chemotherapeutic treatments. These findings underscore the need for strategies targeting autophagy and related pathways to overcome 5-FU resistance and improve treatment outcomes for metastatic CRC patients.

RATIONALE

Colorectal cancer (CRC) remains one of the leading causes of cancer-related mortality worldwide, with tumor recurrence and chemoresistance representing significant challenges to effective treatment. Despite advancements in therapeutic strategies, CRC cells often develop adaptive mechanisms that allow them to survive under metabolic stress, underscoring the need to identify novel molecular targets that could enhance treatment efficacy and improve patient outcomes.

Trop-2, a transmembrane glycoprotein overexpressed in several human carcinomas, including CRC, has been widely associated with increased tumor aggressiveness and poor prognosis. While its role in tumor proliferation and metastasis has been extensively studied, its potential function in facilitating cellular adaptation to unfavorable conditions is much less known. Emerging evidence highlights autophagy as a crucial process in cancer progression, acting as a tumor-suppressive or a pro-survival mechanism depending on the context as well as on the stage of malignant progression. However, the involvement of Trop-2 in regulating autophagy has not yet been reported in the literature.

This study investigates the role of Trop-2 in promoting CRC cell survival under specific stress conditions through modulation of cytoprotective autophagy. In particular, we explore how Trop-2 affects the balance between cell survival and death under metabolic stress and whether this activity might be linked to signaling networks involved in the regulation of cell autophagy. Defining the mechanisms by which Trop-2 induces cell autophagy under metabolic stress is expected to have a highly relevant impact from a clinical point, as it may involve the big challenge of cancer chemoresistance. Therefore, we also examine the contribution of Trop-2 to CRC resistance to 5-fluorouracil, and the potential role of the autophagic process in mediating this function. By addressing these open questions, our study seeks to provide new insights into the function of Trop-2 in CRC metabolic adaptation, revealing potential vulnerabilities that could be leveraged for therapeutic intervention. A deeper understanding of how Trop-2 regulates autophagy and contributes to drug resistance could pave the way for innovative treatment strategies aimed at overcoming tumor resistance and enhancing clinical outcomes in CRC patients.

MATERIALS AND METHODS

1.1 Cell lines

KM12SM cell line was kindly provided by I. J. Fidler at the M. D. Anderson Cancer Center. KM12SM and KM12L4A cells were derived from the parental, non-metastatic line KM12C upon selection *in vivo*(142). Specifically, KM12C were injected into the spleen of nude mice, then tumor lesions were subsequently harvested from the liver and cultured *in vitro* to obtain the KM12L1 derivative(142). After three passages of *in vivo* selection (spleen injection → harvesting from liver metastasis), the KM12L4 cell line was derived. The KM12SM cell line was obtained from a rare liver metastasis that developed following the injection of KM12C cells into the cecum (142). HCT116 cells were obtained from Roche Diagnostics GmbH (Penzberg, Germany). SW48 cells were provided by Dr. A. Sacchetti (Erasmus MC, Rotterdam). HEK293T cells were a generous gift from Dr. A. Veronese (University ‘G. d’ Annunzio’, Chieti, Italy). Cells were incubated at 37°C in a humidified 5% CO₂ incubator and cultured in the following media: KM12SM in RPMI 1640 (10-040-CV, Corning) with 1% non-essential amino acids (25-025-CI, Corning) and 1% MEM vitamins (M6895, Merck); DU145 in RPMI 1640; HCT116 and HEK293T in Dulbecco’s Modified Eagle Medium low glucose (DMEM) (ECM0749, Euroclone) and SW48 in Dulbecco’s Modified Eagle Medium high glucose (DMEM) (ECM0102, Euroclone). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sial) and 1% penicillin/streptomycin (Sial). All cell lines were periodically checked for mycoplasma contamination by PCR.

1.2 Plasmids and cell transfections

The human *TROP2* cDNA (3) was amplified by PCR using the following primers:

F: 5’-GCGATTctcgagTCCGGTCCGCGTTCC-3’

R: 5’-GCGCCggtaccAAGCTCGGTTCCTTTC-3’

Wild-type *TROP2*, obtained by PCR, was subcloned into a pEYFP-N1 vector (Clontech) devoid of the EYFP coding sequence (pΔYFP), using XhoI/KpnI restriction sites.

The A87-A88 Trop-2 mutant was generated by site-specific mutagenesis of the Trop-2 proteolytic site using the Quickchange Site-directed mutagenesis kit (New England Biolabs), following the manufacturer's instructions, with the following primers:

F: 5'-AGCGCCCCCAAGAACGCCGCCGCGCTGGTGCGGCCG-3'

R: 5'-CGGCCGCACCAGCGCGGCGGCGTTCTTGGGGGCGCT-3'

DNA transfections were performed using Lipofectamine 3000 (L3000-001, Invitrogen) and OptiMem (31985070, Gibco) following the manufacturer's instructions. Stable transfectants were selected with 500 µg/mL Geneticin (G418) sulfate (sc-29065, Santa Cruz Biotechnology). Enrichment for stable transfectants was performed by FACS.

CMV-TFEB-GFP plasmid was kindly provided by Prof. A. Ballabio (Telethon Institute of Genetics and Medicine, Naples, Italy). The human TFEB-GFP was amplified by PCR using the following primers:

F: 5'-GCGACCGGTGCGCCACCATGGCGTCACGCATAGGGTTGCGC-3'-AgeI

R: 5'-CGCGAATTCTCACTTGTACAGCTCGTCCATGCC-3'-EcoRI

This PCR product was then inserted into the pULTRA-hot lentiviral vector (#24130, Addgene) devoid of the mCherry sequence (pULTRA-ΔmCh). To generate lentiviral particles to infect KM12SM cells, HEK293T packaging cells were co-transfected with 4 µg of pULTRA-ΔmCh-TFEB-GFP, and with the following packaging/helper plasmids: pMDL2g/pRRE (2µg) (a gift from D. Trono, #12251, Addgene), pMD2.G (1µg) (a gift from D. Trono, #12259, Addgene) and pRSV-Rev (1µg) (a gift from D. Trono, #12253, Addgene). Recombinant lentivirus-containing supernatant was harvested from HEK293T cells 48 hours after transfection, filtered through a 0.45 µm syringe filters, and used to infect the target cells in the presence of 8 µg/mL Polybrene (TR-1003, Merck). Enrichment for stable transfectants was performed by sorting with a FACS Aria III (Becton Dickinson).

EGFP-LC3 was a gift from K. Kirkegaard (#11546, Addgene) (143).

sgRNAs targeting the *TROP2/TACSTD2* gene were designed using the Zhang laboratory CRISPR design tools (<http://www.genome-engineering.org>). The sequences of the sgRNAs were as follows:

5'-ACACCGACGTCTGGTTGCAC-3' (strand antisense, designated as "T");

5'-GTCCTGCGCGGCCGTGTGGC-3' (strand antisense, designated as "D");

sgRNA sequences were cloned into the plasmid LentiCRISPRv2 (a gift from F. Zhang, #52961, Addgene). Then, lentiviruses were produced in HEK293T cells by co-transfection of

LentiCRISPRv2-sgRNA with the packaging plasmids pVSVg (a gift from R.A. Weinberg, #8454, Addgene) and psPAX2 (a gift from D. Trono, #12260, Addgene). Lentiviral particles were then utilized for infection of the target cells. Stable functional knock-out of the *TACSTD2* gene expression was obtained by selection of the infected cells with 3 ug/mL puromycin (ant-pr-1, Invivogen).

1.3 Flow cytometry

To assess membrane protein expression levels, cells were stained for flow cytometry and analyzed using a FACS Canto II (Becton Dickinson). Enrichment of transfectants expressing the target protein was performed by sorting with a FACS Aria III (Becton Dickinson).

1.4 Cell treatments

For experiments involving serum and amino acid starvation, cells were seeded (details provided below) and, after 6 days, they were rinsed twice with PBS and incubated in Earle's Balanced Salts (EBSS) (E3024, Merck) for 48 hours. For autophagy inhibition experiments, after 6 days from cell seeding, the culture medium was supplemented with 50 nM Baf A1 (tlrl-baf1, InvivoGen) for 48 hours. For treatments with 5-FU, 80 x 10³ KM12SM transfectants were seeded in E-plate L8 PET (see section 3.5 for more details), and after 48 hours, cells were treated with 6 µg/mL 5-Fluorouracil (5-FU) (F6627, Merck) and 50 nM Baf A1 in the culture medium for 72 hours. The culture medium was then replaced with fresh medium without drugs, and cell growth was monitored for up to 180 hours.

1.5 RTCA iCELLigence impedance assay

Proliferation assays were performed using the Real-Time Cell Analysis iCELLigence and xCELLigence system (Agilent). These systems are based on gold microelectrode biosensors embedded in each well of a microtiter plate to measure electrical impedance. Impedance is expressed as a Cell Index (CI) value, calculated as follows: (impedance at time n – impedance without cells) / nominal impedance value. The CI reflects the impedance caused by the interaction of adherent cells with the electrical current. Prior to cell seeding, the electric current

flows freely through the culture medium. After cell adhesion and growth, the current flow is impeded, providing a sensitive readout that is directly related to cell number, size, and viability. A total of 2×10^4 cells per well were seeded in electronic microtiter plates E-Plate L8 PET (300600870, Agilent), while 6×10^3 cells per well were seeded in electronic microtiter plates E-Plate 16 (5469830001, Agilent). The CI value was normalized to zero before starting proliferation measurements. CI values were recorded every 60 minutes in real-time following cell seeding. Baf A1 was added 144 hours after cell seeding. CI values were analyzed using the RTCA DATA Analysis software (Agilent).

1.6 Incucyte cell imaging for apoptosis assay

KM12SM cells were seeded in 96-well tissue culture plates at 4×10^3 cells per well in complete RPMI 1640 phenol red-free medium. After 6 days, Baf A1 was added to the medium to a final concentration of 50 nM. Immediately before treatment, cells were stained with Incucyte Caspase-3/7 Green Dye (final concentration 5 μ M; 4440, Sartorius) to detect apoptosis, following the manufacturer's instructions. Caspase-3/7 activation was monitored in real time using the IncuCyte S3 Live Cell Analysis System (Sartorius). The Caspase-3/7 assay utilizes a fluorogenic substrate, typically a peptide sequence as DEVD (Asp-Glu-Val-Asp), which is conjugated to a fluorescent dye. This peptide sequence serves as a preferred recognition motif for caspase-3 and caspase-7. Upon cleavage by these caspases, the fluorescent dye is released, generating a measurable fluorescent signal proportional to the active caspase-3 and -7 levels in the sample.

Using a 10X objective, four fields of view per well were collected every 4 hours for a total of 48 hours. Both phase contrast and green fluorescence (ex, 440/80 nm; em, 504/44 nm) were recorded for each well. Data were processed and analyzed using Incucyte S3 software. Apoptosis was quantified as the green object count per mm^2 .

1.7 Immunofluorescence confocal microscopy

1.2×10^5 cells per well were plated in 12-well tissue culture plates on poly-L-lysine-coated glass coverslips (0.1 mg/mL, diluted in PBS). For each time point, cells were fixed in 4% PFA

diluted in PBS for 20 minutes at RT, followed by quenching with 50 mM ammonium chloride (NH₄Cl) for 15 minutes at RT. Permeabilization and blocking were performed in PBS with 10% FBS and 0.1% saponin (SAE0073, Merck) for 20 minutes. Cells were then incubated with primary antibodies targeting the proteins of interest, diluted in PBS with 10% FBS and 0.1% saponin for 1 hour at RT. After three washes with PBS, cells were incubated with the secondary antibody conjugated to Alexa 488 and 633 for 45 minutes at RT. After an additional three washes with PBS, nuclei were counterstained with DAPI (D9542, Merck) for 5 minutes at RT. Coverslips were then mounted using Prolong Diamond Antifade (P36961, Invitrogen). Images were acquired with an LSM-800 confocal microscope (Zeiss) using a 63x, numerical aperture 1.4 oil immersion objective.

1.8 DAPI staining for nuclear fragmentation assessment

1.2 x 10⁵ cells per well were plated in 12-well tissue culture plates on poly-L-lysine-coated glass coverslips. After fixation and quenching of PFA (see above), nuclei were stained with DAPI in PBS for 5 minutes. Following staining, cells were washed with PBS to remove excess dye. Coverslips were mounted using Prolong Diamond Antifade (P36961, Invitrogen). Nuclear morphology was analyzed by confocal microscopy setting the excitation wavelength at 358 nm and the emission wavelength at 461 nm. At least 10 fields per sample were examined, and fragmented nuclei were identified by the presence of condensed, fragmented nuclear structures, as indicated by bright and irregular DAPI staining patterns.

1.9 Western blot

Cells were seeded in 100 mm tissue culture dishes at a density of 1 x 10⁶ cells per plate. After six days, cells were treated with 50 nM Baf A1 either in exhausted (spent) medium or in EBSS. After 48 hours of treatment, cells were washed with PBS, lysed by scraping in ice-cold RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1% NP-40, 0.05% SDS) supplemented with phosphatase (A32957, Thermo Fisher Scientific) and protease inhibitors (A32965, Thermo Fisher Scientific). After 10 min of incubation on ice, lysates were

centrifuged at 12,000 rpm for 10 minutes at 4°C. Supernatants were collected, and protein concentration was quantified using DC Protein Assay kit (Biorad). 50 µg of total protein lysates were incubated in 1x reducing Laemmli buffer at 99 °C for 5 minutes, then separated by SDS-PAGE on a gel containing 10% to 15% of a 37.5:1 acrylamide:bis-acrylamide solution (A3626, PanReac, Applichem) depending on the molecular weight of the protein of interest to analyze. A protein ladder (EPS026500, Euroclone) was used as a molecular weight standard. Proteins were transferred to a nitrocellulose membrane (GE10600001, Amersham Protan, Merck) by overnight electroblotting at 100 mA at 4 °C using 1x transfer buffer (25 mM Tris-base, 192 mM glycine and 20% methanol). Ponceau S staining (0.1% in 5% acetic acid) was used to verify the protein transfer and to confirm equal loading of the samples. After rinsing off the Ponceau staining solution with 1x TBS-T buffer (20 mM Tris-base, 13.6 mM NaCl, pH adjusted to 7.6 with HCl and 0,15% Tween-20), membranes were blocked for 1 hour with 5% skim milk or 3% bovine serum albumin (BSA) (A2153, Merck) in 1x TBS-T buffer. Following this, the membranes were incubated for 2 hours with the primary antibody (as listed in paragraph 1.12). Membranes were then washed three times with 1x TBS-T and incubated for 1 hour with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. After TBS-T washing, antibody binding was detected by chemiluminescence using ECL Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (34094, Thermo Fisher Scientific) with a Chemidoc Alliance Q9 (Uvitec Cambridge).

1.10 Subcellular fractionation

KM12SM transfectants were seeded in 100 mm tissue culture dishes at a density of 1.8×10^6 cells per plate. At each time point, to obtain nuclear and cytoplasmic fractions, cells were harvested at each time point with 1 mM EDTA, washed with PBS, and lysed in ice-cold cytoplasmic extraction buffer (10 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M Sucrose, 10% Glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na₃OV₄, 0.1 % Triton X-100 and 1x protease inhibitors). After 30 minutes of incubation on ice, the lysates were centrifuged at 3,300 rpm for 15 minutes at 4 °C. This first supernatant was kept as the cytoplasmic fraction. The pellet was then resuspended in nuclear extraction buffer (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT and 1x protease inhibitors) and incubated on ice for 30 minutes. After two cycles of probe

sonication (10 seconds each) on ice, the sample was centrifuged at 12,700 rpm for 15 minutes at 4 °C. This second supernatant was kept as the nuclear fraction.

1.11 Co-immunoprecipitation

KM12SM transfectants were lysed by scraping in TN buffer (50 mM Tris pH 7.5, 250 mM NaCl and 1 mM EDTA). After 15 minutes of incubation on a rotary shaker at 4°C, the cell lysates were sonicated for 5 minutes using a water-bath sonicator and centrifuged at 18,000 x g for 30 minutes at 4°C. Preclearing was then performed by incubating with Protein G-Sepharose 4 Fast Flow beads (17-0618-05, GE Healthcare) at 4 °C for 2 hours with head-over-head rotation. Binding to Trop-2-specific mAb-coupled Sepharose was carried out by overnight incubation with head-over-head rotation at 4 °C. After six washes with TN buffer, immunocomplexes were eluted twice with elution buffer (0.2 M glycine pH 2.3, and 0.5% Igepal CA-630) for 20 minutes at 37 °C. The eluate was neutralized using 1 M NH₄HCO₃, methanol and chloroform. After centrifugation at 18,000 x g for 10 minutes at 4 °C, the methanol/chloroform-precipitated proteins (pellet) were solubilized in 100 mM Tris pH 7.4 and 1% SDS.

1.12 Antibodies

The following antibodies were used in our experiments: anti-MAP LC3 α/β (Santa Cruz Biotechnology; Cat #398822); anti-ULK-1 (Santa Cruz Biotechnology; Cat #390904); anti-Becn-1 (Santa Cruz Biotechnology; Cat #48341); anti-ATG4A (Santa Cruz Biotechnology; Cat #517309); anti-ATG5 (Santa Cruz Biotechnology; Cat #133158); anti-ATG7 (Santa Cruz Biotechnology; Cat #376212); anti-LAMP2 (Santa Cruz Biotechnology; Cat #18822); anti-TFEB (Cell Signaling Technology; Cat #37785); HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich, Cat #401215); HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology; Cat #7074); anti-Trop-2(21); Alexa Fluor-488 Donkey Anti-Mouse IgG (Eugene; # A21202); Alexa Fluor-633 Donkey Anti-Mouse IgG (Eugene; #A21052);

1.13 Mass spectrometry

For shotgun label-free analysis (performed in collaboration with Prof. Maurizio Ronci, University “G.d’Annunzio” of Chieti-Pescara), samples from each culture conditions (controls in complete medium, cells incubated in EBSS and cells incubated in medium + Baf A1, as described above), were analyzed in technical triplicates by LC-MS/MS using a Proxeon EASY-nLCII (Thermo Fisher Scientific) chromatographic system coupled to a Maxis HD UHR-TOF (Bruker Daltonics GmbH, Bremen, Germany) mass spectrometer equipped with a nanoESI spray source. Peptides from trypsin digestion of the lysates were loaded on the EASY-Column C18 trapping column (2 cm L., 100 μ m I.D., 5 μ m ps, Thermo Fisher Scientific), and subsequently separated on an Acclaim PepMap100 C18 (75 μ m I.D., 25 cm L, 5 μ m ps, Thermo Fisher Scientific) nano scale chromatographic column. The flow rate was set to 300 nL/min and the gradient (mobile phase A: 0.1% formic acid in H₂O) was from 3 to 35% of mobile phase B (1% formic acid in acetonitrile) in 80 min followed by 35 to 45% in 10 min and from 45 to 90% in 11 min. The mass spectrometer was operated in positive ion polarity and Auto MS/MS mode (Data Dependent Acquisition - DDA), using N₂ as collision gas for CID fragmentation. Precursors in the range of 350 to 2,200 m/z (excluding 1,220.0–1,224.5 m/z) with a preferred charge state +2 to +5 (excluding mono-charged ions) and absolute intensity above 4,706 counts were selected for fragmentation in a maximum cycle time of 3 seconds. After acquiring one MS/MS spectrum, the precursors were actively excluded from selection for 30 seconds. Isolation width and collision energy for MS/MS fragmentation were set according to the mass and charge state of the precursor ions (from 3 to 9 Da and from 21 eV to 55 eV). In-source reference lock mass (1,221.9906 m/z) was acquired online throughout the runs. Each raw data file was converted to mzXML format and submitted to the LFQ processing. Raw mass spectrometry data were analyzed using the PEAKS® Studio 7.5 software using the “correct precursor only” option. Spectra were matched against the neXtProt database and false discovery rate (FDR) was set to 0.1% at the peptide-spectrum matches (PSM) level. The post-translational modification (PTM) profile was set as follows: fixed cysteine carbamidomethylation (Δ Mass: 57.02), variable methionine oxidation (Δ Mass: 15.99) and glutamine and asparagine deamidation (Δ Mass: 0.98). Non-specific cleavage was allowed to one end of the peptides, with a maximum of 2 missed cleavages and trypsin enzyme specificity. The highest error mass tolerances for precursors and fragments were set at 10 ppm and 0.05 Da, respectively. After processing every single raw data, the label free quantification (LFQ)

tool of PEAKS Studio was used to detect differentially expressed proteins. Parameters for LFQ were set as follows: mass error tolerance, 10.0 ppm; retention time shift tolerance, 2.0 min; FDR threshold, 0.5%. For quantitative analysis, the significance threshold at the protein level was set to ≥ 20 -10lgP with fold change ≥ 2.0 .

1.13.1 Pathway analysis

Gene Ontology and pathway analysis of differentially expressed proteins were performed with Ingenuity Pathway Analysis (IPA) (QIAGEN Redwood City, www.qiagen.com/ingenuity, version 122103623) and Omics Playground (www.bigomics.ch) (144). IPA core analysis provides not only genes functional annotation, canonical pathway and network discovery, but also estimates the status of upstream regulators and downstream effects associated with canonical pathways, diseases and functions. The upstream regulator analysis highlights the expected effects between transcriptional regulators and their target genes. The predicted activation or inhibition of each transcriptional regulator is inferred by the z score which in turn derives from the protein ratios in the dataset (z scores > 2.0 indicate that a molecule is activated, whereas z scores < -2.0 indicate the inhibition of target molecules). SwissProt accession numbers with corresponding ratios were imported into the software, and the analysis was performed selecting only direct relationships among genes and molecules in all species and confidence settings were set to high predicted or experimental observed. The resulting networks highlight the functional relationships among proteins based on known associations in the literature. IPA comparison analysis between the results of the different sections for every condition was also performed.

Omics Playground is a user-friendly and interactive self-service bioinformatics platform for the in-depth analysis, visualization and interpretation of transcriptomics and proteomics data. Omics Playground allows to identify patterns and groups with Clustering Analysis; identify key proteins across conditions with Differential Expression Analysis; visualize protein set activity and identify pathways with Protein Set Enrichment and Pathway Analysis; identify correlations between proteomics and transcriptomics datasets; identify compounds that produce proteomic signatures similar to the observed data; and understand functional relationships and interactions of proteins with protein network analysis.

1.14 Transmission Electron Microscopy (TEM) and immunogold analysis

1 x 10⁶ KM12SM transfectants were seeded in 100 mm tissue culture dishes and prepared for TEM. After 3 days and 6 days of culture without changing the medium, cells were rinsed with PBS, fixed with 2 % glutaraldehyde in 0.1 M sodium cacodylate (NaCaCO) buffer and stored at 4 °C. After 24 hours, cells were detached from the culture plate by scraping, and then centrifuged at 15,000 rpm for 15 minutes.

For embedding, cells were post-fixed in 2% OsO₄ in the same buffer for 2 hours and block-stained with saturated uranyl acetate. After dehydration, specimens were embedded in epoxy resin (Epon 812). Ultrathin sections (approximately 40 nm) were cut using a Leica Ultracut R microtome (Leica Microsystem) with a Diatome diamond knife (Diatome Ltd. CH-2501 Biel, Switzerland) and double-stained with uranyl acetate replacement and lead citrate. Sections were viewed and photographed in a 120 kV JEM-1400 Flash Transmission Electron Microscope (Jeol Ltd.), equipped with a CMOS Matataki and TEM center software (Jeol Ltd). Quantitative analysis was performed on random micrographs of KM12SM transfectants. The relative incidence of autolysosomes per area of the cell (50 µm²) was determined from electron micrographs randomly collected from transversal EM sections. The number of autolysosomes per unit area was counted and reported as number per 50 µm². For each cell line, approximately 60 representative cells were analyzed at a magnification of 8,900x. The average size of autolysosomes was measured by outlining each autolysosome using the Analy-SIS software (Olympus Soft Imaging Solutions). Only autolysosome with clearly defined borders were measured (sample size: approximately 180 autolysosome per cell line). The relative percentage (%) of the average cell area covered by autolysosomes was estimated as the ratio between the arithmetic total sum of the autolysosomes area within a cell to the total surface area of individual cell. TEM analysis was performed in collaboration with Prof. Simona Boncompagni and Dr. Giorgia Rastelli (University “G.d’Annunzio” of Chieti-Pescara). Immunogold labeling and analysis were performed in collaboration with Dr. Sophie Rome (CarMeN Laboratory, INSERM 1060/INRA 1397, Lyon 1 University, France).

1.15 Statistical Analysis

The data are expressed as mean $\pm$ standard deviation (SD). Statistical significance of the differences between the means was determined using ANOVA. Statistical analysis was performed using GraphPad Prism 9.0 software.

RESULTS

1.1 Trop-2 is a novel driver of CRC cell growth and long-term survival

We and other groups have previously demonstrated that Trop-2 expression is associated with biological aggressiveness and poor prognosis of human carcinomas, including cancers of pancreas, colon, prostate and breast (15,16,19). More recently, we showed that Trop-2 modulates key signalling networks to promote cell proliferation and growth, including those centered around Akt, PKC, E-Cadherin, CD9, Integrins, and mTORC (11,24).

Most assays designed to evaluate the role of Trop-2 in promoting cancer cell proliferation are typically conducted over a period of 48-96 hours. However, the long-term effects of Trop-2 on cancer cell survival under stress conditions have not been thoroughly investigated. To address whether Trop-2 confers a survival advantage under metabolic stress, we performed a Real-Time Cell Analysis (RTCA) assay, which allows dynamic long-term monitoring of cellular phenotypic changes in real time using impedance as a readout.

We focused on human CRC cells, one of the deadliest cancer types in Western countries, to evaluate the impact of Trop-2 on cell growth and long-term survival. Specifically, we monitored Trop-2-positive (“Trop-2”) and Trop-2-negative (“Vector”) CRC cell growth up to several days after they reached confluence without changing the culture medium (spent medium), thus mimicking severe nutrient consumption in culture. We performed RTCA assays in three CRC cell lines, and were able to identify the KM12SM cell line as the most responsive to the pro-growth and pro-survival effects of Trop-2. As shown in Fig.1A, in spent culture medium, KM12SM/Trop-2 cells survive for a significantly longer period of time compared to KM12SM/Vector cells. We observed that KM12SM/Trop-2 cells reach confluence after approximately 6 days after seeding, and following a brief plateau, continue to survive up to 10 days and over. In contrast, KM12SM/Vector cells are unable to survive beyond 6 days and undergo cell death.

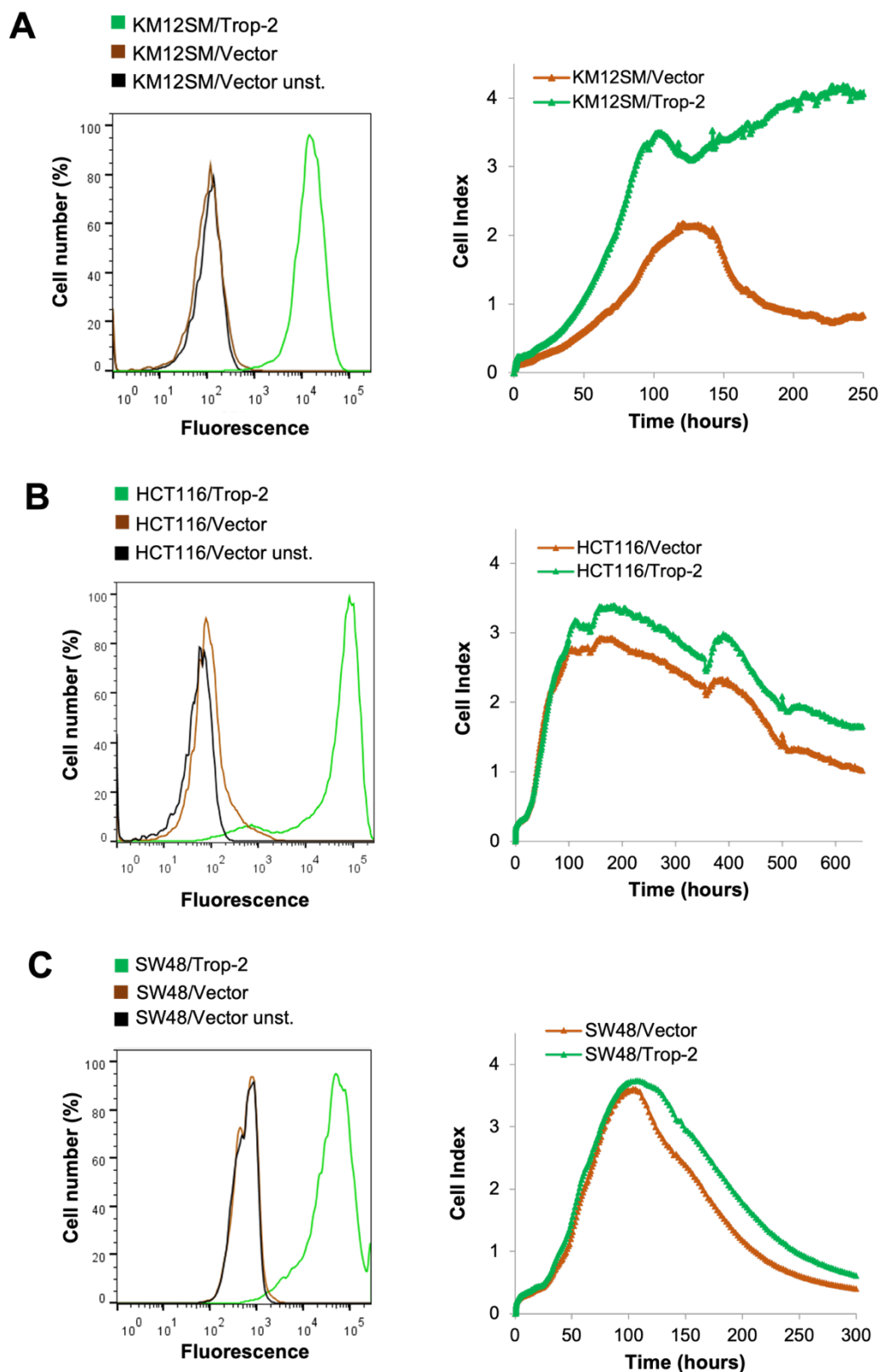


Fig. 1. Trop-2 is a key driver of CRC growth and long-term survival in spent medium. Impedance-based growth assays comparing Trop-2 (green) and Vector (brown) CRC cells. Left panels show flow cytometry analysis of Trop-2 expression, while right panels display real-time impedance measurements over time in spent medium, reflecting cell proliferation.

Conversely, in the CRC cell lines HCT116 and SW48, Trop-2 expression alone is found to be not sufficient to support cell proliferation and survival. Indeed, after exponential growth and upon reaching confluence, both Trop-2 and Vector cells undergo cell death (Fig. 1B-C).

1.2 Trop-2 promotes long-term survival in CRC by regulating autophagy

Under conditions of nutrient deprivation, stress, and hypoxia, cancer cells often depend on autophagy as a critical survival mechanism (89,95). Autophagy is a key catabolic process by which cells degrade and recycle damaged organelles and misfolded proteins, thus providing essential resources to maintain cellular homeostasis and promote survival.

To determine whether the Trop-2-dependent CRC cell survival occurs through the activation of autophagic pathways, we analyzed the expression of the most critical determinants of the autophagic cascade in KM12SM cells. Trop-2 was found to lead to the upregulation of key modulators of the various steps of the autophagic process (Fig. 2A). Specifically, Trop-2 appears to modulate autophagy initiation by promoting the activation of ULK-1 and Beclin-1. Moreover, Trop-2 induces elongation of the phagophore with consequent formation of autophagosomes by modulating the levels of ATG4A, ATG5 and ATG7 (Fig. 2A).

The autophagic flux inside the cell can be monitored by analyzing the rate of LC3-I to LC3-II conversion as well as the decrease of the LC3-I and LC3-II levels due to the completion of autophagic degradation of the substrates following the autophagosome-lysosome fusion (52,80). We observed enhanced consumption of LC3 in KM12SM/Trop-2 cells compared to the control cells, as highlighted by a significant reduction of both LC3-I and LC3-II. This is particularly evident in cells grown at high confluence (6 days after seeding), when nutrient availability becomes limited, and the Trop-2 cells begin to rely on autophagy for survival (Fig. 2B). In contrast, under the same culture conditions, in HCT116 cells, where we observed that the expression of Trop-2 does not promote cells survival (Fig. 1B) and in DU145 cells, which are autophagy-defective cells due to the absence of ATG5, the expression of LC3 remained unchanged regardless of the presence or absence of Trop-2 (Fig. 2B).

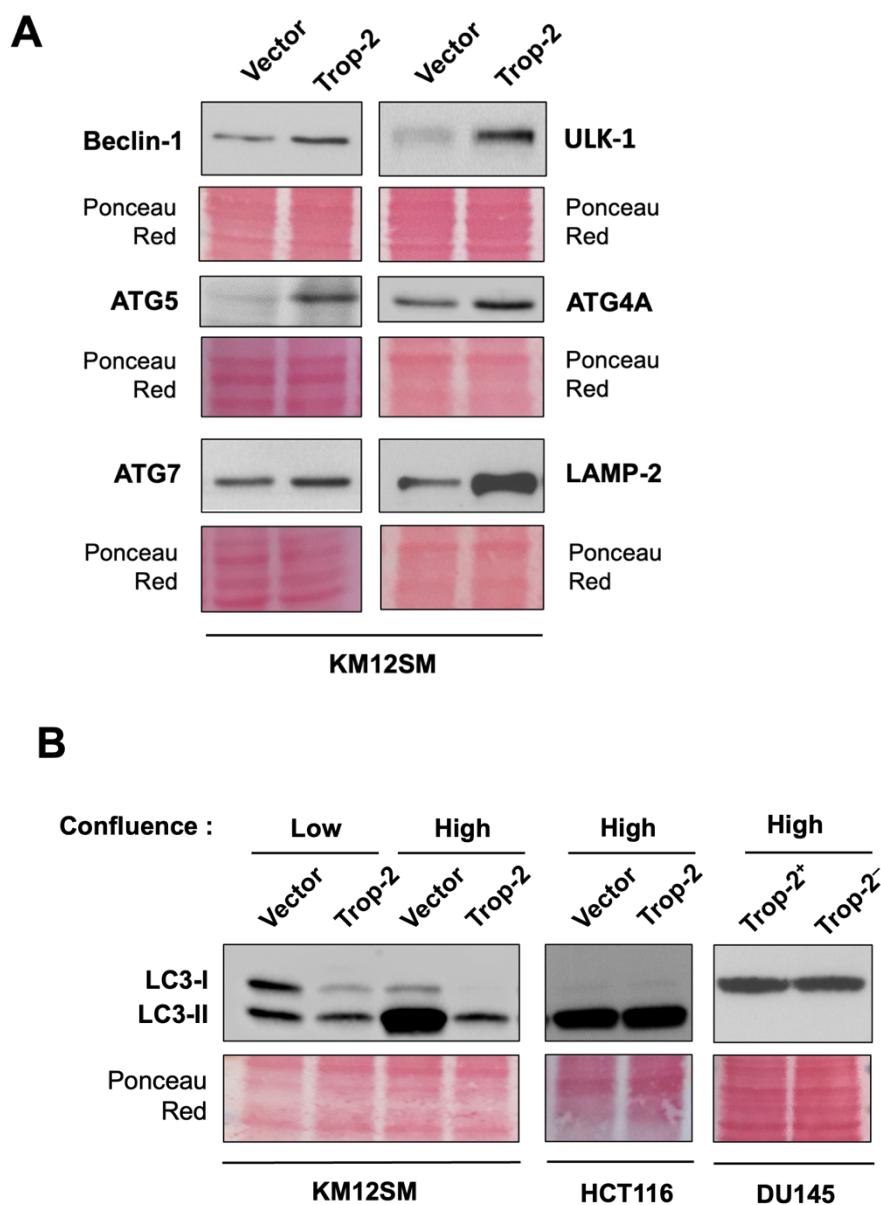


Fig. 2. Trop-2 modulates CRC cell autophagy. (A) Western blot analysis showing the expression levels of key autophagy-related proteins in KM12SM/Vector and KM12SM/Trop-2 CRC cells. Trop-2 upregulates critical modulators involved in different stages of the autophagic process. (B) Western blot analysis of LC3 expression in KM12SM and HCT116 CRC cells and DU145 prostate cells (autophagy-defective) cultured in spent medium under low (short culture duration) and high (long culture duration) confluence conditions. Ponceau S staining serves as a protein loading control.

To further validate our hypothesis, we examined LC3 expression in both Trop-2 and Vector KM12SM cells under conditions of autophagy induction using EBSS as well as the autophagy inhibitor Baf A1. Under nutrient-deprivation culture conditions, in KM12SM/Trop-2 cells stably expressing LC3-GFP, we observed significantly fewer LC3-GFP puncta compared to Vector cells (Fig. 3A, top), suggesting that Trop-2 promotes enhanced degradation of LC3 and faster autophagic flux. When autophagy is inhibited by treatment with Baf A1, the number of LC3-GFP puncta increases significantly (from 7 to 40 in Vector cells, and from 3 to 41 in Trop-2 cells), but the effect of Trop-2 is neutralized as the number of LC3-GFP puncta becomes comparable in Vector and Trop-2 cells (Fig. 3A, bottom). All of these findings are confirmed when we induce autophagy by treating the cells with EBSS, which leads to reduction of LC3-I and LC3-II in both KM12SM/Vector and KM12SM/Trop-2 cells (Fig. 3B). Also, when we inhibit autophagy by treatment with Baf A1, both LC3-I and LC3-II forms accumulate in the cells independent of Trop-2 expression (Fig. 3B). Altogether, these findings indicate that Trop-2 is a key inducer of the autophagic process in CRC cells.

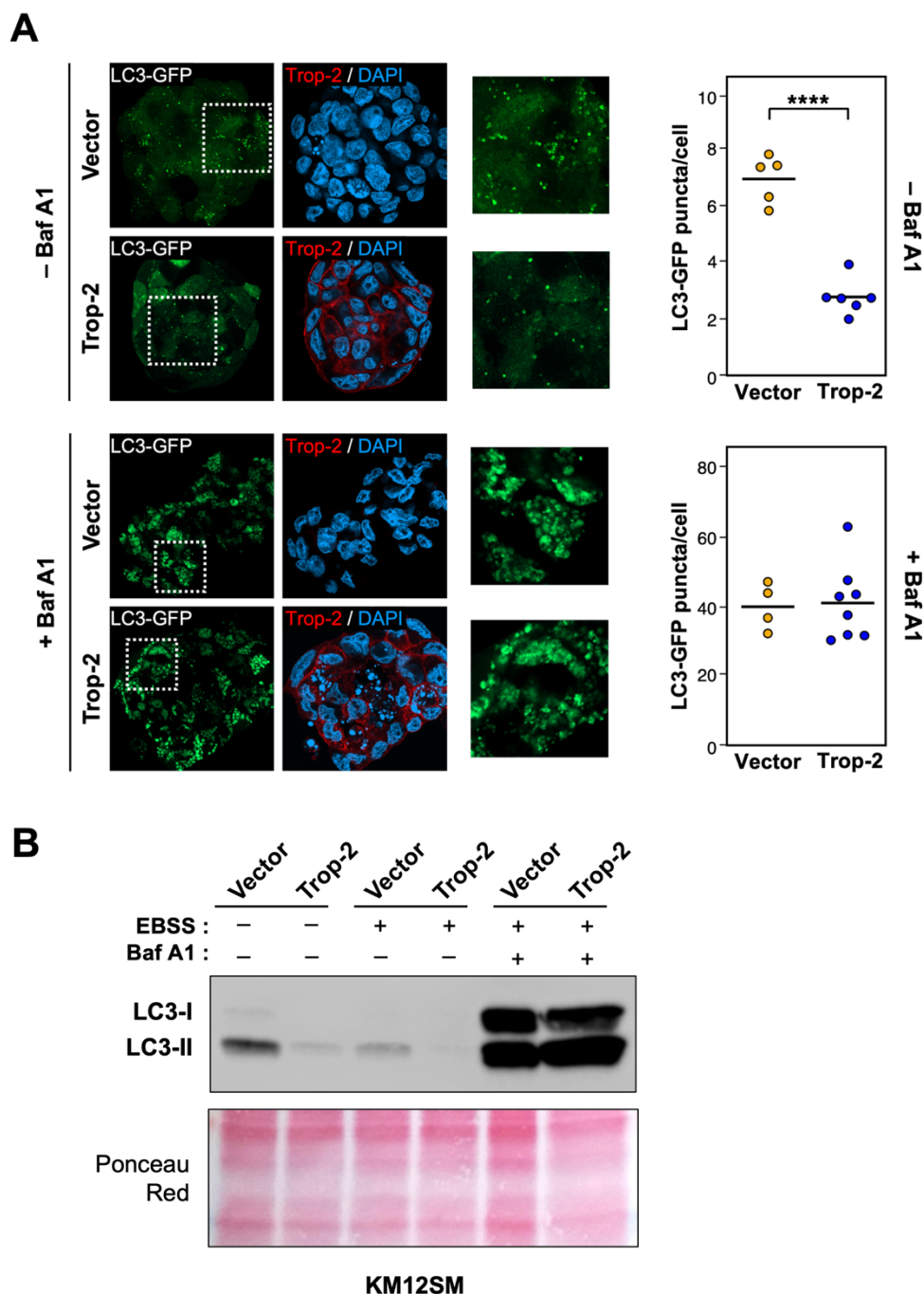


Fig. 3. Trop-2 promotes LC3 lipidation in CRC cells. (A) Fluorescence confocal microscopy representative images (*left*) and the relative quantification (*right*) of Vector and Trop-2 KM12SM cells expressing LC3-GFP in spent medium vs Baf A1 treatment. Nuclei were counterstained with DAPI. In nutrient deprivation conditions Trop-2 led to a more prominent degradation of LC3. (B) Western blot analysis of LC3 expression in KM12SM cells in spent medium, with autophagy induction (EBSS treatment) and inhibition (Baf A1 treatment). Ponceau S staining serves as a protein loading control.

1.3 Proteomic analysis of Trop-2 role in regulation of autophagy

To understand the molecular mechanisms underlying the induction of cytoprotective/pro-survival autophagy by Trop-2, we performed proteomic analysis of KM12SM/Trop-2 and KM12SM/Vector cells, comparing conditions of low-nutrient availability, autophagy induction (EBSS) and autophagy inhibition (Baf A1). We identified several proteins that were dysregulated in untreated, overconfluent Trop-2 vs Vector cells kept under nutrient-limited conditions in spent medium (comparison 1). In particular, 326 proteins were significantly overexpressed and 336 proteins were significantly inhibited. We also computed the differentially expressed proteins detected in two further conditions: EBSS vs untreated KM12SM/Vector cells (comparison 2: 264 upregulated proteins and 236 downregulated proteins) and KM12SM/Trop-2 treated with Baf A1 vs untreated (comparison 3: 372 upregulated proteins and 365 downregulated proteins). Among the top-ten targets across the three comparisons, we found that three upregulated proteins (ACP3, MSMB and KLK3) and one downregulated protein (SCD) in comparison 1 were correspondingly altered in the comparison 2 (autophagy induced in Vector cells by EBSS) and were changed in the opposite direction in the comparison 3 (autophagy inhibited in Trop-2 cells by Baf A1) (Fig. 4). We also extended the analysis to the whole dataset of altered proteins in the three conditions, and found that 19 proteins (CD44, SEMG1, JPT1, NDUFAF3, MSMB, AZGP1, TUBA1C, PHLDA1, CNTNAP2, SEMG2, GLA, SLC12A2, ACP3, REEP4, PIP, CDC42BPB, IL18, ALB, KLK3) were upregulated in comparisons 1 and 2 and downregulated in comparison 3, while 17 proteins (NRP1, TMX3, GNE, TPBG, RAB27B, NDUFB8, ABCC3, SCD, DGAT1, H1-0, NT5E, YIPF5, SPATS2L, TFRC, KRT6A, SLC2A1, NUP210) were downregulated in comparisons 1 and 2 and upregulated in comparison 3. These findings suggest the molecular mechanisms underlying the Trop-2-driven induction of cytoprotective autophagy under cellular stress such as limited nutrient availability, frequently reported to occur during cancer progression.

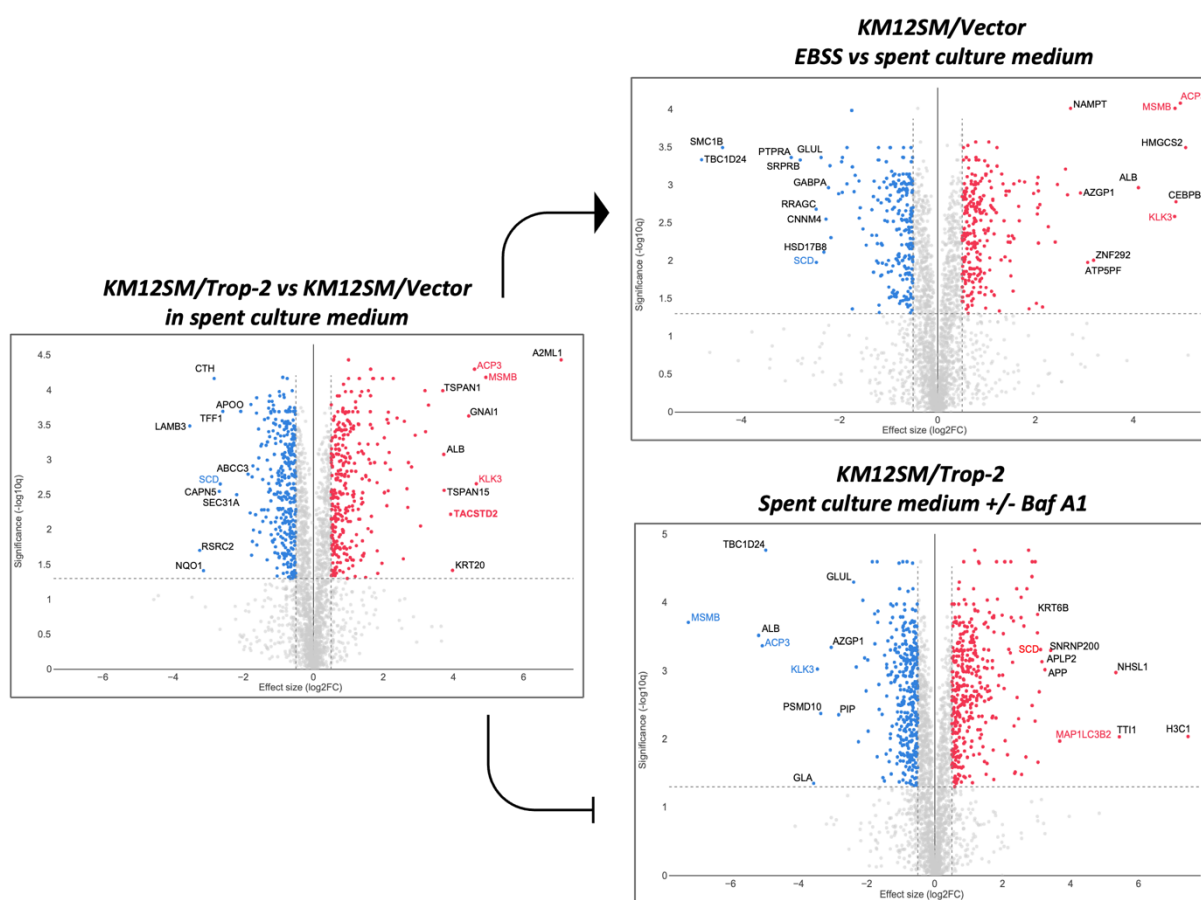


Fig. 4. Proteomic analysis of KM12SM cells under different autophagy-modulating conditions. Volcano plots showing differentially expressed proteins in KM12SM/Trop-2 and KM12SM/Vector cells under low-nutrient availability and autophagy-modulating conditions. (Left) Proteins dysregulated in Trop-2 vs Vector cells cultured in nutrient-limited spent medium. (Top right) Comparison of EBSS-treated vs untreated KM12SM/Vector cells (autophagy induction). (Bottom right) Comparison of Baf A1-treated vs untreated KM12SM/Vector cells (autophagy inhibition). Significantly upregulated proteins are highlighted in red, while downregulated proteins are shown in blue.

We went on to perform signaling pathway analysis in KM12SM/Trop-2 vs KM12SM/Vector cells cultivated to over confluence in spent medium. This revealed that Trop-2 drives autophagic survival of CRC cells through inhibition of the mTORC1 complex, a known negative regulator of autophagy (Fig. 5). Furthermore, key contributions to the overall effect of Trop-2 expression in CRC cells survival under stress are provided by inhibition of apoptosis and p53 signaling (Fig. 5).

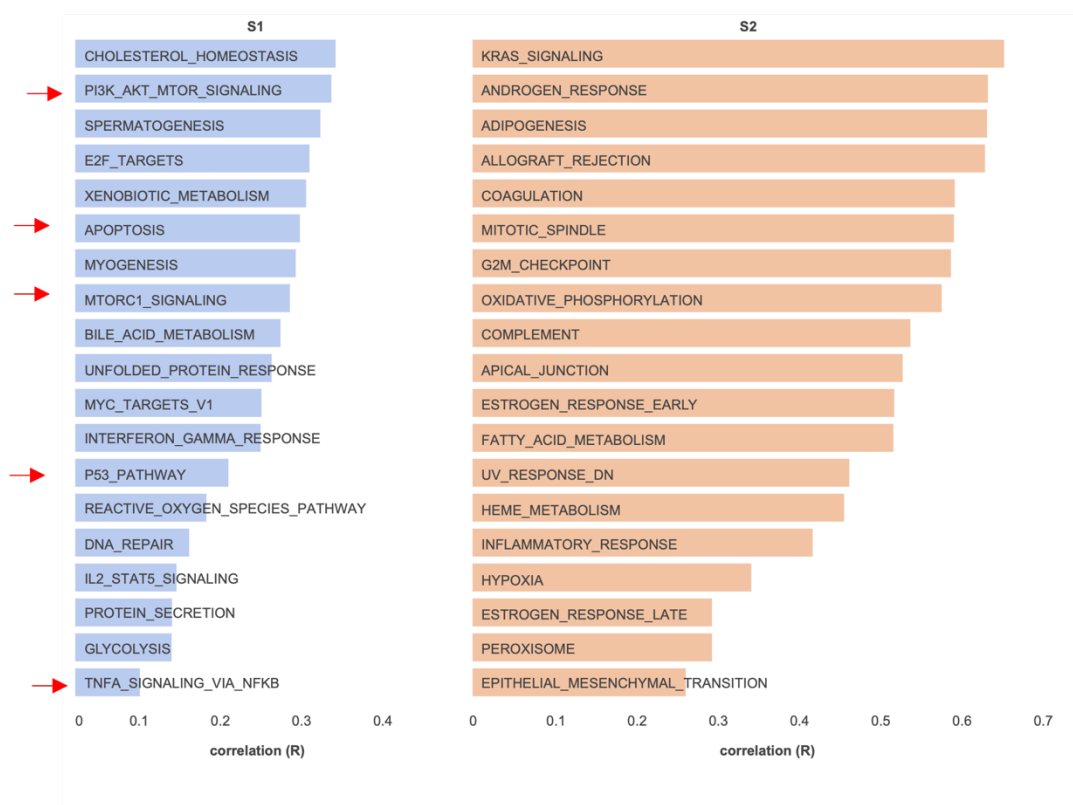


Fig. 5. *Trop-2* promotes CRC survival by inhibiting *mTORC1*. Signaling pathway analysis in KM12SM/*Trop-2* vs KM12SM/Vector cells cultured in spent medium. Downregulated pathways are shown in blue, while upregulated pathways are in red.

Drug enrichment analysis was carried out, and led to the identification of various drug signatures matching the proteomic signature of the comparison 1. In this “Drug Connectivity Map” panel we identified: (a) positive correlations with several known mTOR inhibitors, such as OSI-027, WYE-354, WYE-125132, NVP-BEZ235, deforolimus and torin-1 (Fig. 6, top); (b) negative correlations with known inhibitors of cancer growth, such as the topoisomerase inhibitors irinotecan, camptothecin, doxorubicin, SN-38, amsacrine, teniposide (Fig. 6, bottom). These data further support a role for *Trop-2* as a key inducer of CRC cell autophagy. In summary, all experimental data presented above confirm the hypothesis that CRC cells with high expression of *Trop-2* can efficiently adapt to environmental stress, such as nutrient-limiting conditions, by activating cytoprotective autophagy.

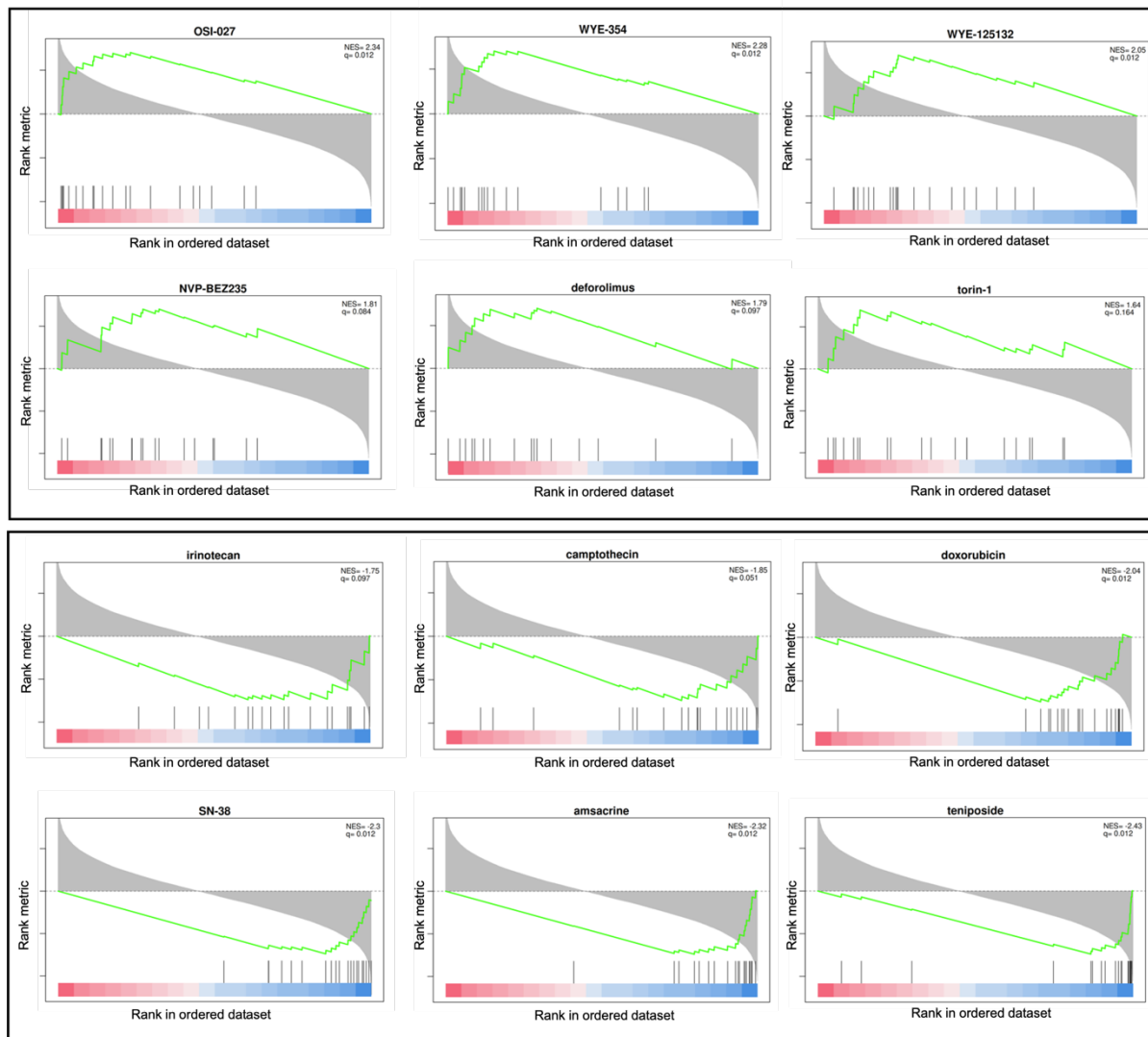


Fig. 6. Drug Connectivity Map with positive correlation with mTOR inhibitors (*top*) and negative correlation with topoisomerase inhibitors (*bottom*).

1.4 Trop-2 regulates the size of autophagolysosomes

We decided to investigate the ultrastructural organization of KM12SM/Vector and KM12SM/Trop-2 cells grown to over confluence in spent medium using transmission electron microscopy (TEM). As shown in Fig. 7, both groups showed numerous organelles resembling autophagolysosomes (indicated by empty arrows in panels A-B and E-F). At higher magnification, these structures contained vacuoles (multivesicular internal compartments; Fig. 7D and G) as well as membrane layers (multilammellar internal structures; Fig. 7C and H).

Systematic quantification of the autophagolysosome-like structures revealed that Trop-2 cells exhibited a statistically greater abundance of these organelles, which were also larger in size and occupied a larger area of the cell compared to Vector cells (Fig. 7 I, L and M).

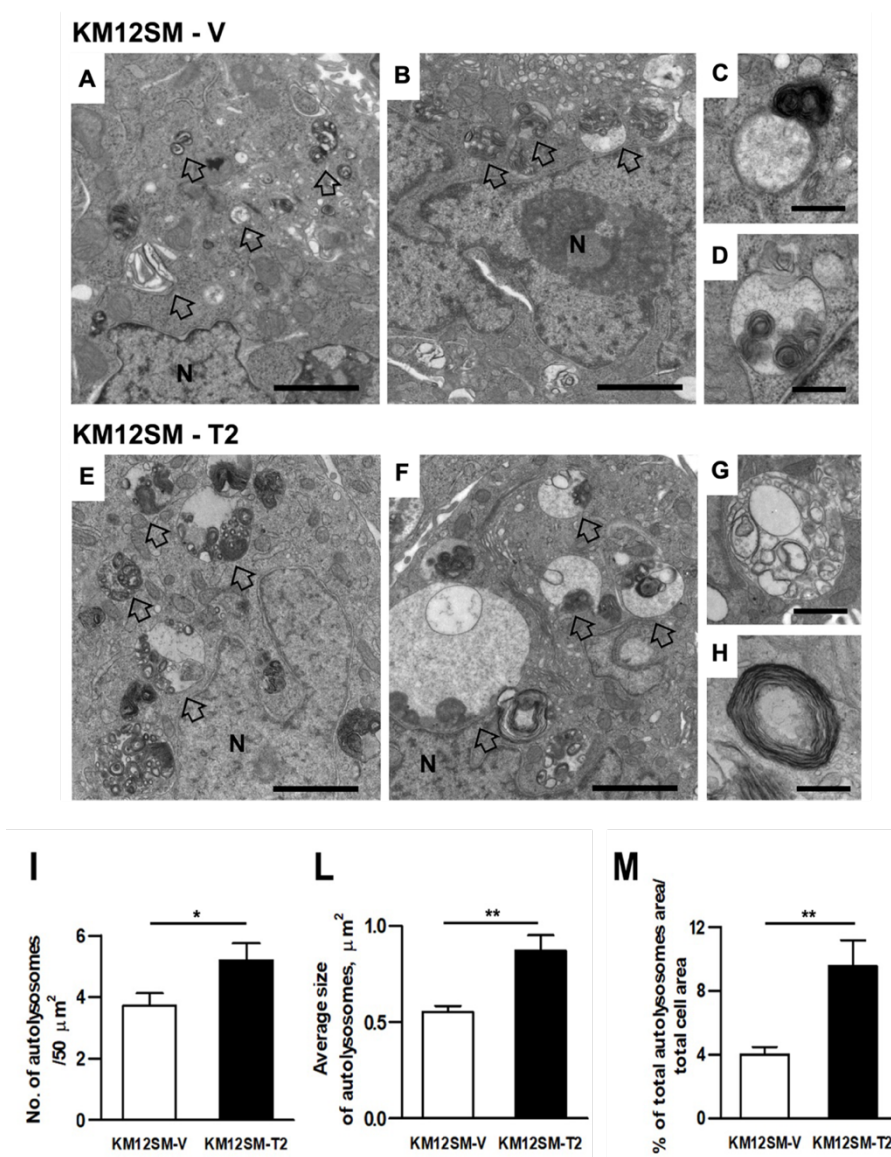


Fig. 7. Trop-2 induces fusion of autophagosomes with lysosomes. Representative TEM images of KM12SM/Vector (**A-D**) and KM12SM/Trop-2 (**E-H**) CRC cells in spent medium. Scale bars: A-B and E-F, 2 μm ; C-D and G-H, 0.5 μm . (**I**) Histograms of the number of autolysosomes per 50 μm^2 of sectional cell area; (**L**) Histograms of the average size of autolysosomes (μm^2); (**M**) Histograms of the average percentage of total cell area occupied by autolysosomes; * $p < 0.05$; ** $p < 0.001$. Data are shown as mean $\pm$ SEM.

To confirm the hypothesis that these intracellular organelles were autophagolysosomes, we carried out immunofluorescence and confocal microscopy analysis of the localization

pattern of the lysosomal membrane protein LAMP-2. LAMP-2-positive structures were found to be significantly larger in Trop-2 cells relative to controls, thus supporting our hypothesis (Fig. 8A). Furthermore, immunogold TEM co-labelling of LC3 and LAMP-2 was performed to enable precise characterization of the autophagolysosomes. Both proteins were found colocalized on their membrane as well as in the lumen (Fig. 8B), thus confirming their identity. These results indicate that, Trop-2 induces the fusion of autophagosomes with lysosomes, leading to the formation of larger autophagolysosomes, thereby stimulating the autophagic flux.

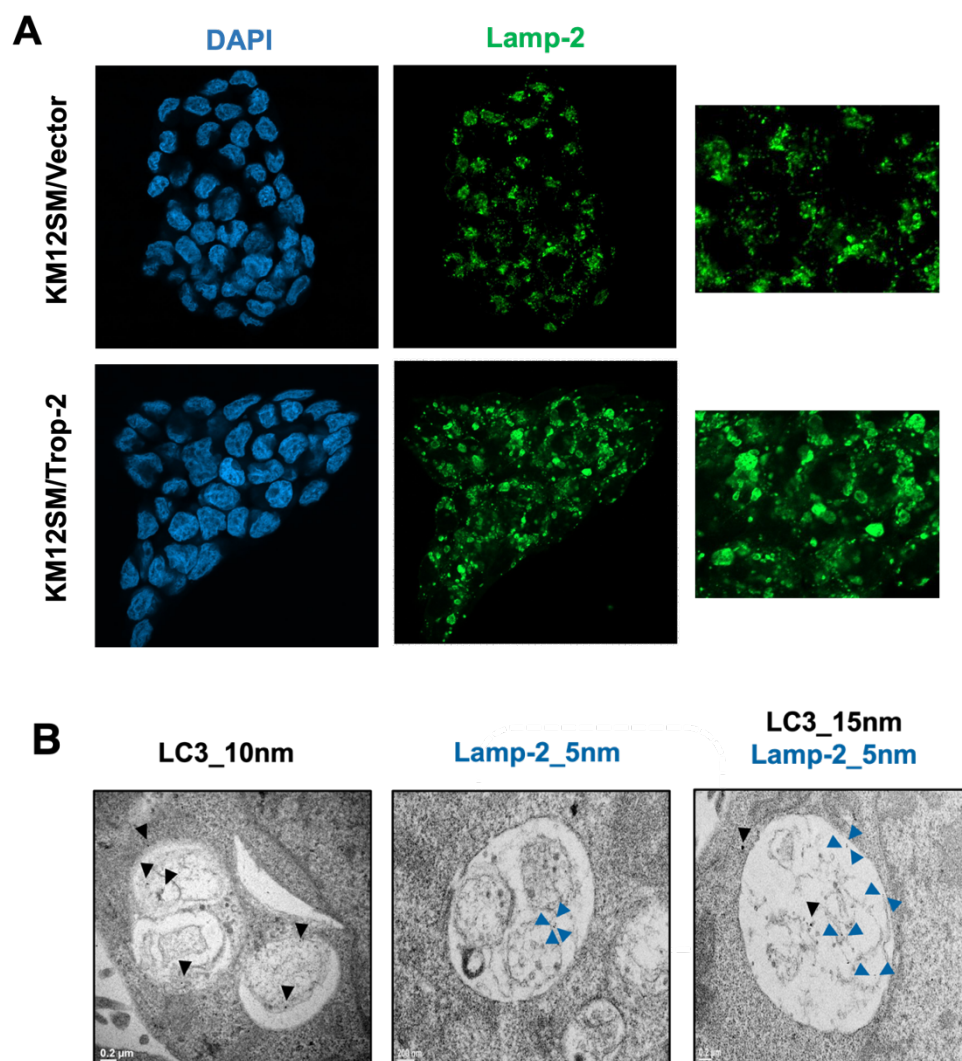


Fig. 8. Trop-2 CRC cells have large autophagolysosomes. (A) Fluorescence confocal microscopy representative images of DAPI (blue) and LAMP-2 (green) expression in KM12SM/ Vector (*top*) and KM12SM/Trop-2 (*bottom*) cells. (B) Immunogold TEM labelling to detect LC3 (*left*), LAMP-2 (*middle*) and LC3/LAMP-2 (*right*) at the surface and inside autophagolysosomes in KM12SM/Trop-2 cells. Black arrowheads, LC3, 10 or 15nm gold particles; blue arrowheads, LAMP-2, 5nm gold particles. Scale bars, 200 nm.

1.5 Analysis of Trop-2 role in cell death induction upon inhibition of autophagy

Baf A1 is a potent inhibitor of vacuolar-type H⁺ ATPase, which prevents the fusion between autophagosomes and lysosomes, thus effectively blocking the autophagic flux. Baf A1 is widely used *in vitro* to study the mechanisms of cellular autophagy (79).

We treated KM12SM cells with Baf A1 and assessed DNA fragmentation by confocal microscopy. We observed that KM12SM/Vector cells were unable to survive in spent medium upon reaching overconfluence (-Baf A1), as indicated by extensive DNA fragmentation in untreated cells (Fig. 9A, left). In contrast, KM12SM/Trop-2 cells easily overcome this stress and survive with no evident signs of death, as highlighted by the presence of intact nuclei in the vast majority of the examined fields of view (Fig. 9A, right). However, treatment with Baf A1 led to a significant reduction in the survival of KM12SM/Trop-2 cells, which showed extensive DNA fragmentation (Fig. 9A, right).

To elucidate the molecular mechanisms underlying the induction of cell death in KM12SM/Trop-2 cells upon autophagy inhibition, we performed a signaling pathway analysis using Ingenuity Pathway Analysis (IPA). This revealed that Baf A1 treatment induced apoptosis in KM12SM/Trop-2, while this apoptotic response was unaffected or rather even inhibited in KM12SM/Vector cells (Fig. 9B), possibly because of the high levels of apoptotic death already detectable in the untreated Vector cells. These findings support our hypothesis that Trop-2 promotes cell survival via autophagy induction, and that inhibition of autophagy strongly determines induction of apoptosis specifically in Trop-2 cells.

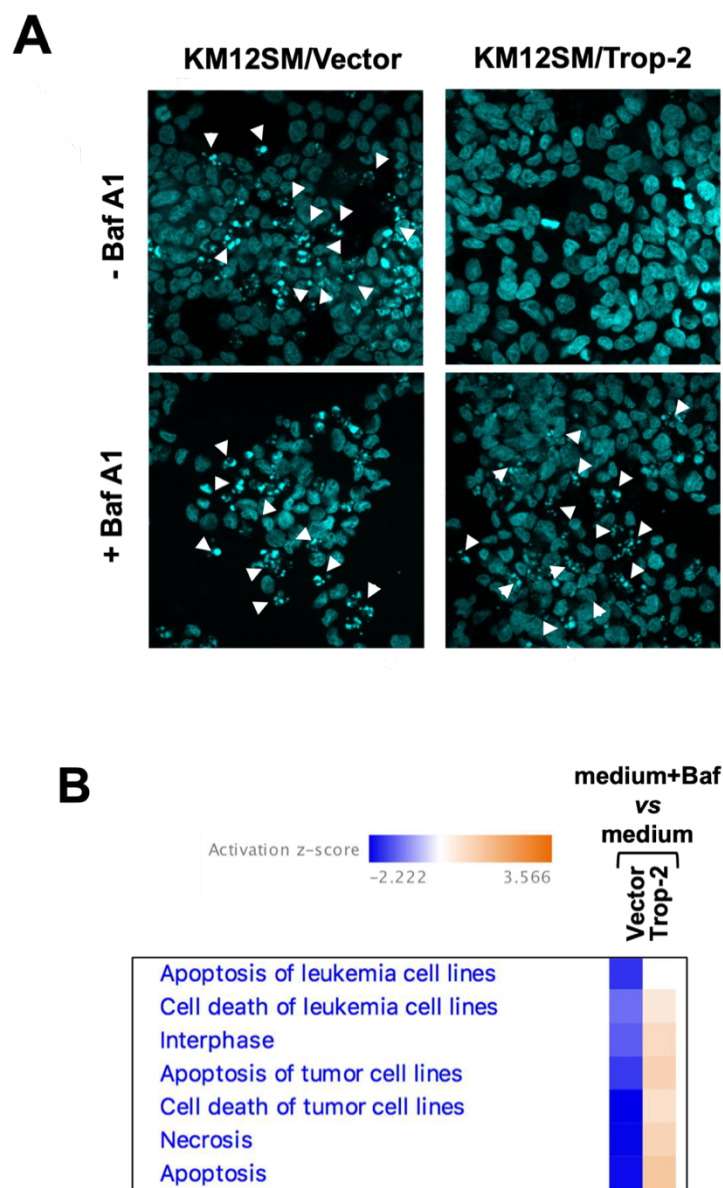


Fig. 9. Inhibition of autophagy induce cell death in Trop-2 CRC cells. (A) Fluorescence confocal microscopy representative images of DAPI of KM12SM/Vector and KM12SM/Trop-2 in spent medium (top panel) and after Baf A1 treatment. (B) Proteomic analysis of Trop-2 and Vector cells in presence vs absence of Baf A1. Apoptotic pathways in KM12SM/Trop-2 were significantly induced compared to controls upon autophagic inhibition.

1.6 Proteolytic cleavage of Trop-2 is critical to modulate CRC cell autophagy

Cancer specific mutations of the *TACSTD2* gene, which encodes Trop-2, have been observed at a very low frequency and no one of them has been demonstrated to act as a driver of

malignancy so far. We have recently demonstrated that Trop-2 undergoes a cancer-specific, ADAM10-mediated post-translational cleavage at the R87-T88 site (21). Additionally, we have shown that this proteolytic cleavage is critical to prime Trop-2 to CRC cell growth stimulation (21). Based on these findings, we aimed to investigate whether cleavage at the R87-T88 site acts as an activator switch for Trop-2 in promoting CRC cell survival.

To test this hypothesis, we performed Western blot analysis of LC3 expression in KM12SM cells expressing either wild-type Trop-2 or a variant Trop-2 harboring the R87A-T88A mutation (Trop-2^{R87A-T88A}). We found that the cleavage-impaired Trop-2 mutant failed to stimulate autophagy, similar to Vector cells, in which we observed higher expression of both LC3 forms compared to autophagic Trop-2 cells, where both LC3-I and LC3-II were expressed at significantly lower levels (Fig. 10B).

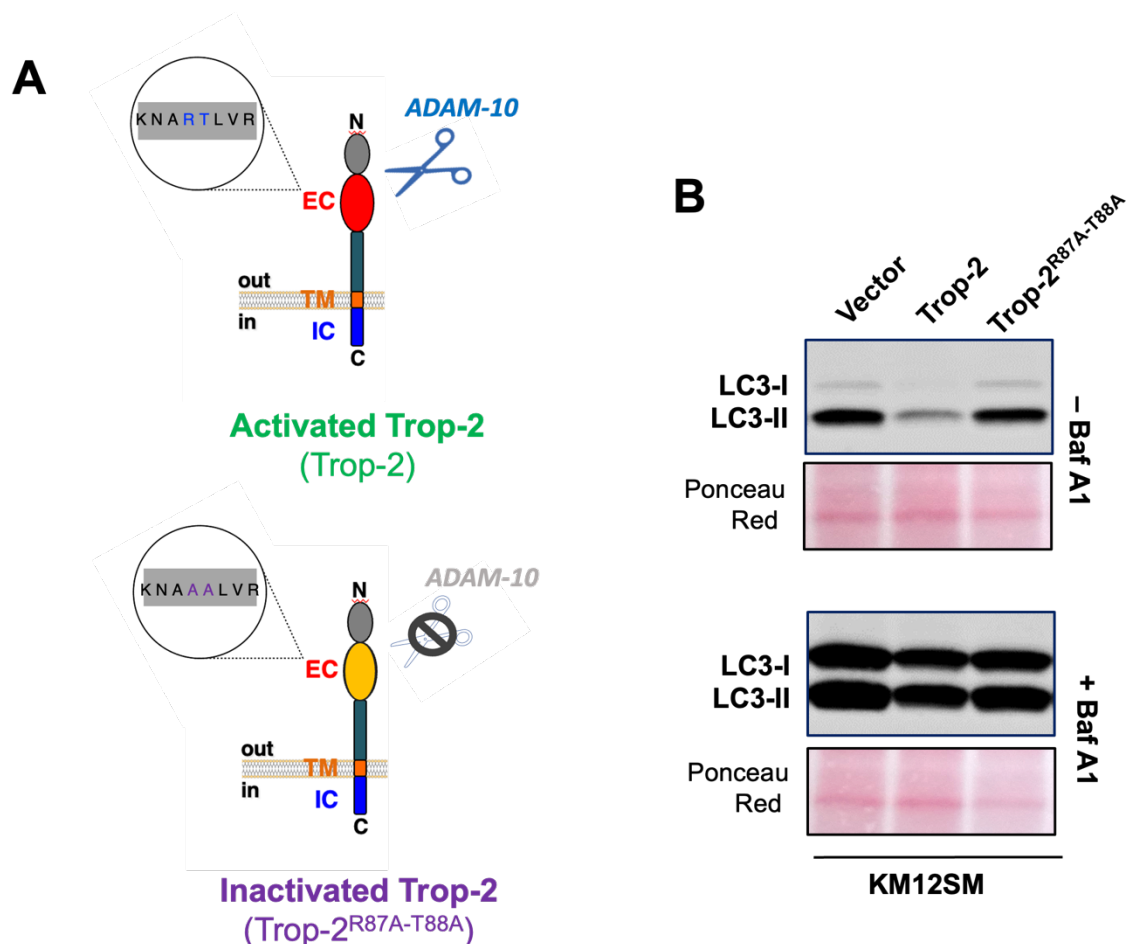


Fig. 10. Proteolytic cleavage of Trop-2 is necessary to modulate the autophagic flux. Western blot analysis of expression levels of LC3 in CRC cells expressing either wild type or mutant Trop-2, carrying a R87A-T88A mutation at the cleavage site. Treatment with Baf A1 induced accumulation of LC3 independently on the expression of Trop-2. Ponceau S staining serves as a protein loading control.

Systematic immunofluorescence/confocal microscopy analysis Lamp-2 revealed that Trop-2^{R87A-T88A} cells have significantly smaller autophagic organelles compared to Trop-2 cells (Fig. 11, left), as well as significantly less autophagolysosomes (Fig. 11, right). This indicates that the proteolytic cleavage of Trop-2 is essential for inducing fusion between autophagosomes and lysosomes (Fig. 11, left).

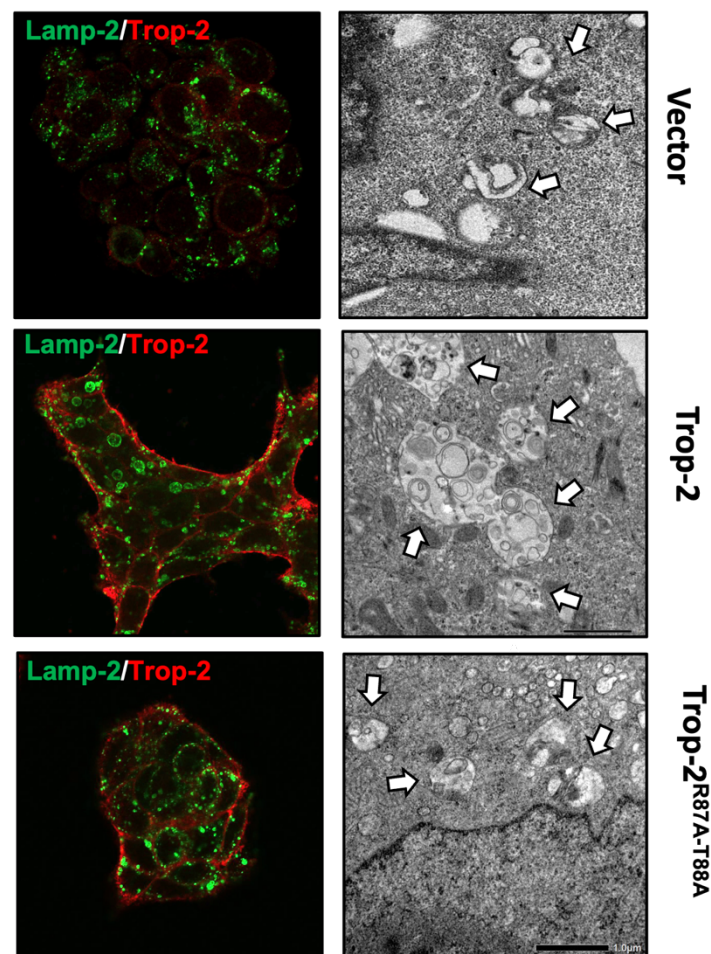


Fig. 11. Proteolytic cleavage of Trop-2 is crucial for late stages of autophagy. (left) Fluorescence confocal microscopy representative images of LAMP-2 (green) and Trop-2 (red) expression in Vector (top), wt Trop-2 (middle) and Trop-2^{R87A-T88A} (bottom) KM12SM transfectants. (Right) TEM representative images of Vector (top), wt Trop-2 (middle) and Trop-2^{R87A-T88A} (bottom) KM12SM transfectants.

These findings led us to investigate whether the Trop-2 cleavage is essential for the long-term survival of CRC cells under stress conditions. To this end, we performed an RTCA

analysis over a 10-day period without medium changing on KM12SM cells expressing either wild-type Trop-2 or the Trop-2^{R87A-T88A} mutant. The mutation at the cleavage site resulted in the inhibition of the “Trop-2 window” for cell growth stimulation and survival in spent medium (Fig.12, left). Both Trop-2^{R87A-T88A} cells and Vector cells failed to survive upon reaching confluence and began to die, as highlighted by the percentage of spontaneous cell death compared to Trop-2 cells, in which the cells continue to proliferate. Exposure of Trop-2 cells to Baf A1, after 6 days in culture, impaired cell survival and led to faster cell death than Vector and Trop-2^{R87A-T88A} cells, as shown by the calculated percentage of cell death due to Baf A1 treatment vs spontaneous death (Fig. 12, right).

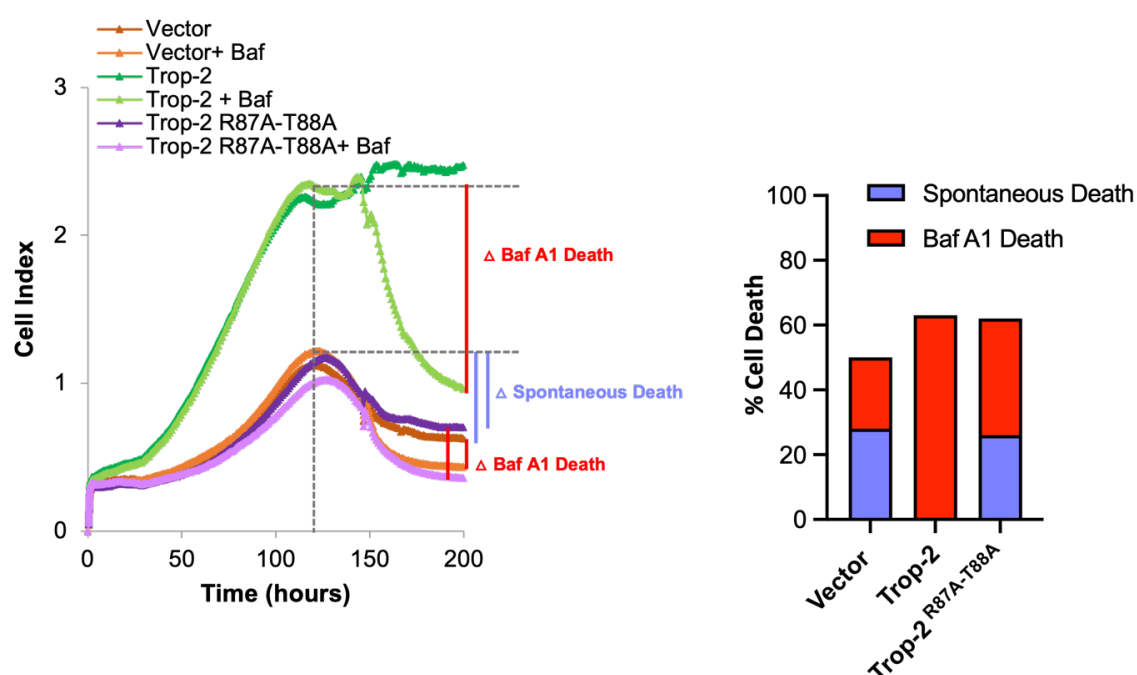


Fig. 12. Impedance RTCA assay of KM12SM transfectants (Vector, wt Trop-2 and Trop-2^{R87A-T88A}) cultured for 10 days without changing the medium. Cells were treated with Baf A1 at maximum confluence, when nutrients become limiting. Cell death was quantified as the percentage of Baf-A1-induced death relative to spontaneous cell death observed in the untreated control.

To define the molecular mechanisms of Trop-2-triggered growth vs death upon autophagy inhibition, we performed a Caspase 3-7 activity assay using the Incucyte S3 Live Cell Analysis System. KM12SM cells expressing Vector, Trop-2 and Trop-2^{R87A-T88A} were treated with Baf A1, after 6 days in culture without medium changes. Caspase 3-7 activity was carried out, and allowed us to demonstrate that the Baf A1-dependent inhibition of autophagy in Trop-2 cells leads to a significantly higher number of fluorescent, apoptotic cells compared to the Vector

and Trop-2^{R87A-T88A} cells (Fig. 13). These findings suggest that the proteolytic cleavage of Trop-2 is essential for modulating to autophagic flux in stress condition, and that Trop-2 plays a critical role in CRC cell resistance to apoptotic death through cytoprotective autophagy.

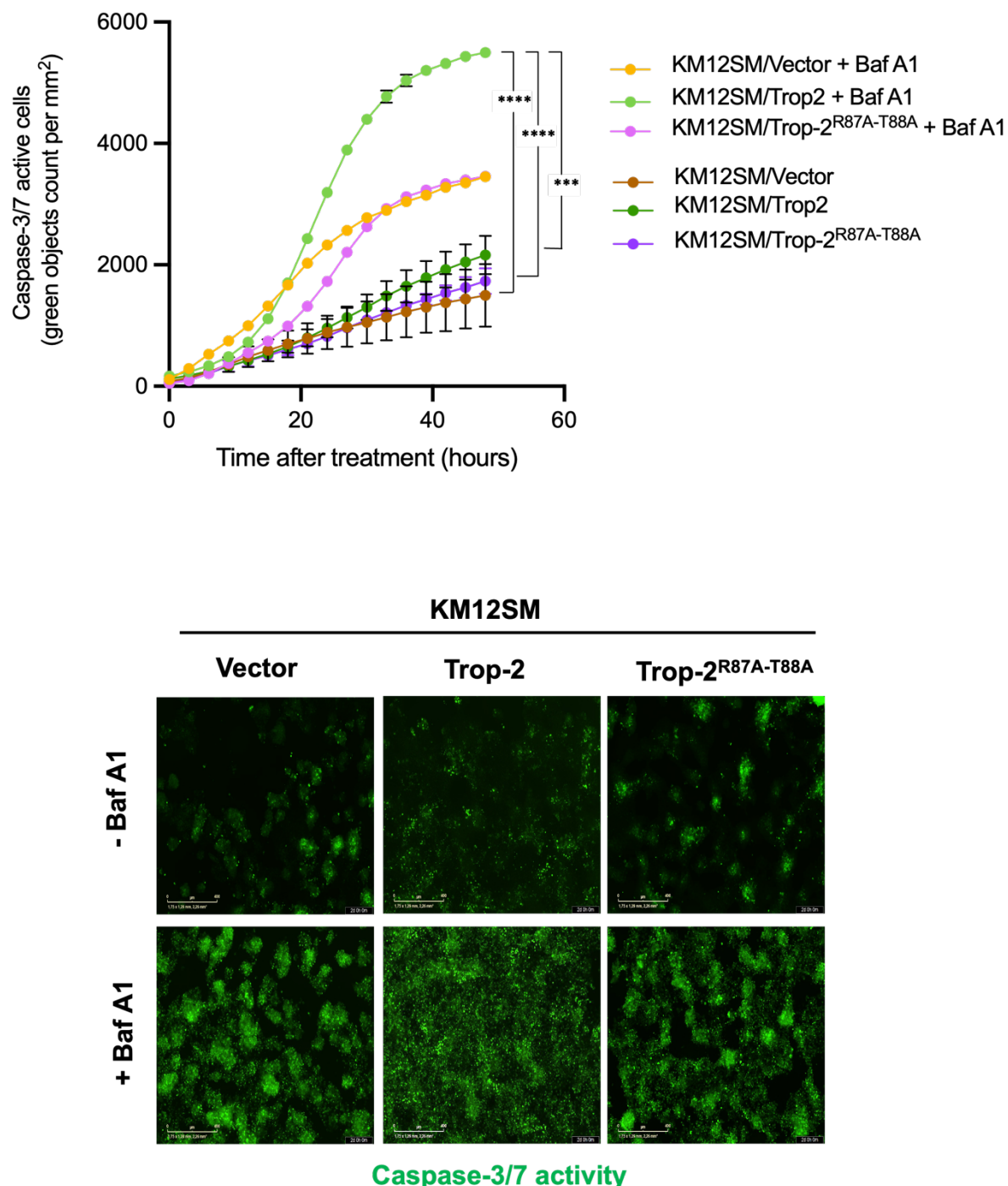


Fig. 13. Trop-2 protects CRC cells from apoptosis by activating cytoprotective autophagy under stress. (Top) Quantification of Caspase 3-7 fluorescence in KM12SM/Vector, KM12SM/Trop-2 and KM12SM/Trop-2^{R87A-T88A} cells treated with Baf A1 after 6 days in culture without medium changes, using Incucyte Live cell Imaging. The y-axis represents the green object count per mm². Data are expressed as mean $\pm$ standard deviation. **** p < 0.0001. (Bottom) Representative images of Caspase 3-7 activation in KM12SM cells treated with vehicle or Baf A1.

1.7 Trop-2 overexpression is associated with nuclear translocation of TFEB and activation of autophagy

To characterize the molecular determinants of the Trop-2-driven induction of cytoprotective autophagy we performed an immunoprecipitation of Trop-2 from KM12SM grown at overconfluence in spent medium, followed by a mass spectrometry analysis for identification of its potential interactors. The data revealed an interaction of Trop-2 with the 14-3-3 ζ/δ isoform (YWHAZ) (Fig. 14A). The discovery that 14-3-3 ζ/δ is a novel Trop-2 interactor is potentially of direct relevance for this study, as 14-3-3 ζ/δ plays a key role in the nuclear-cytoplasm shuttling of the Transcription Factor EB (TFEB), a major regulator of cell autophagy (77).

To directly assess the nuclear-cytoplasmic shuttling kinetics of TFEB in response to nutrient availability, we performed a time-course fractionation in KM12SM cells cultured for 4 to 7 days without changing the medium. We observed that in Trop-2 cells, nutrient exhaustion (from the culture day 5 on) triggered the translocation of TFEB to the nucleus, followed by activation of autophagy (Fig. 14B). Imaging of Vector and Trop-2 cells revealed that after 6 days, TFEB shuttled from the cytoplasm to the nucleus only in the Trop-2 group (Fig. 14C), indicating that TFEB undergoes active nuclear import and that Trop-2 triggers this event. The interaction between Trop-2 and 14-3-3 ζ/δ suggests that Trop-2 could retain this protein in the membrane, preventing its binding to phosphorylated TFEB, which is consequently free to translocate to the nucleus. Starvation has been reported to induce an intracellular increase of Ca^{2+} released from the lysosomes, and this primes the phosphatase calcineurin to dephosphorylate TFEB at Ser 211 and Ser 142 and induce its nuclear translocation (77). Our formerly published data that Trop-2 overexpression results in a strong increase of the intracellular Ca^{2+} levels suggest that Trop-2 can induce a calcineurin-dependent dephosphorylation of TFEB, leading to a massive translocation of this protein to the nucleus to activate pro-autophagy genes, among them *BECN1* and *ATG5*. This appears to be the case, as both Beclin-1 and ATG5 are significantly up-regulated in Trop-2 cells compared with the Vector counterpart.

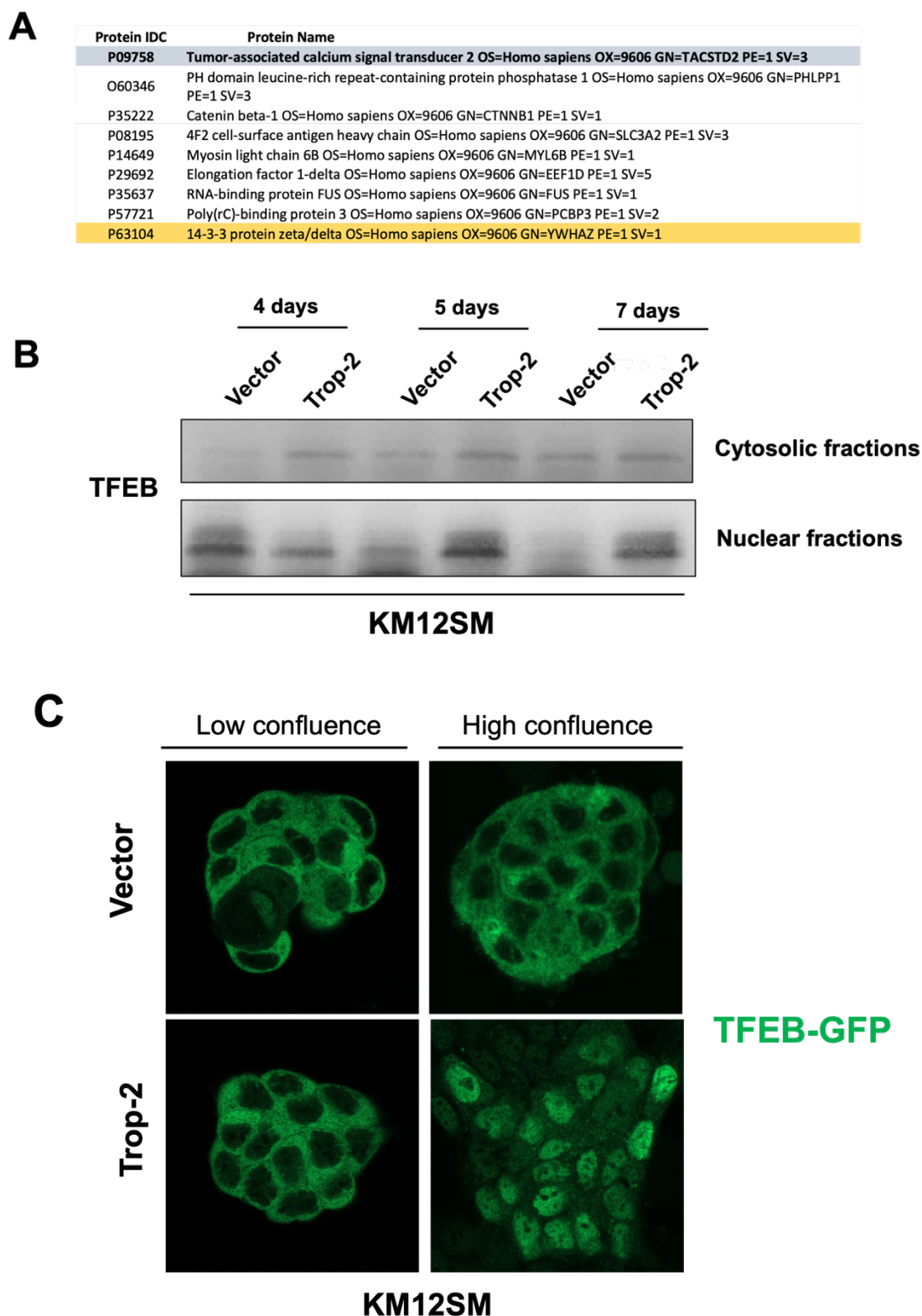


Fig. 14. TFEB, master regulator of Trop-2-driven autophagy. (A) Immunoprecipitation of KM12SM/Trop-2 cultured in spent medium. (B) Western blot analysis of TFEB expression in KM12SM/Vector and KM12SM/Trop-2 cells after nuclear/cytosolic fractionation. (C) Representative images of KM12SM Vector and Trop-2 cells overexpressing TFEB cultured at low and high confluence without medium changes.

1.8 Inhibition of autophagy overcomes Trop-2 resistance to 5-FU

It has been frequently reported that induction of cytoprotective autophagy can be directly correlated to the acquisition of chemo- and radioresistance by metastatic tumor cells (137). In CRC, 5-fluorouracil (5-FU) represents the primary first-line chemotherapeutic agent for adjuvant or neoadjuvant treatment (132). However, its clinical effectiveness has been significantly limited by the development of intrinsic or acquired chemoresistance in the majority of patients with metastatic CRC (132,134).

We asked whether Trop-2 could act as an inducer of CRC cell chemoresistance through induction of autophagy. First, we treated KM12SM/Vector and KM12SM/Trop-2 cells with increasing concentrations of 5-FU, in order to evaluate potentially different responses to treatment due to a specific effect of Trop-2. As shown in Fig. 15A, we observed a significantly lower mortality of Trop-2 cells compared to Vector cells throughout a wide range of 5-FU concentrations (12-48 $\mu\text{g/mL}$). A dose-response curve was generated, and the IC₅₀ values for 5-FU were determined. Specifically, the IC₅₀ value for Vector cells was 11.42 $\mu\text{g/mL}$, whereas for Trop-2 cells, the IC₅₀ increased to 34.32 $\mu\text{g/mL}$, suggesting that the presence of Trop-2 confers enhanced resistance to 5-FU treatment (Fig. 15B).

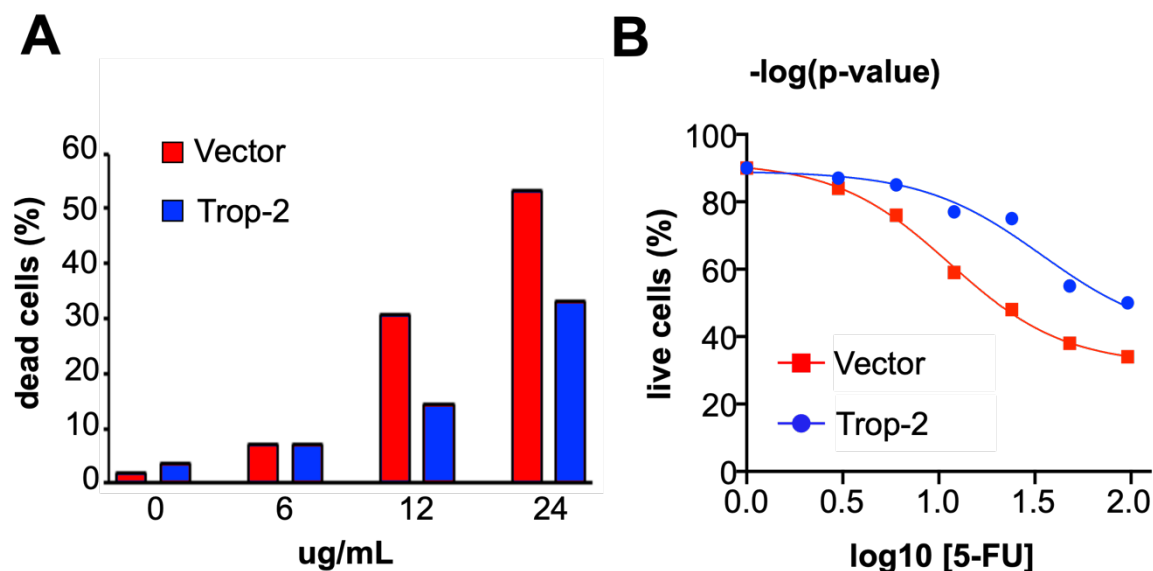


Fig. 15. (A) Histogram of mortality in KM12SM/Trop-2 and KM12SM/Vector cells following treatment with different concentrations of 5-FU for 72 hours. Both cell types were exposed to the same drug concentrations (6 $\mu\text{g/mL}$, 12 $\mu\text{g/mL}$, 24 $\mu\text{g/mL}$). The x-axis indicates both cell types (Vector and Trop-2) exposed to the different concentrations of 5-FU. The y-axis shows the mortality values as

percentages. **(B)** Dose-response curve of 5-FU treatment at the concentrations indicated in the graph in (A).

Second, we examined whether targeting autophagy in KM12SM/Trop-2 cells could represent a potential strategy to overcome resistance to 5-FU. We performed a RTCA analysis of KM12SM treated with 5-FU in combination with Baf A1. Compared with Vector cells, which showed extreme sensitivity to 5-FU treatment, either in absence or in presence of Baf A1, Trop-2 cells were found to easily rescue the stress of the treatment of 5-FU after washing out the drug only in the case where this agent was not provided to the cells in combination with Baf A1. Indeed, when Baf A1 was added to 5-FU, Trop-2 cells underwent massive cell death and were unable to recover after washing out the chemotherapeutics (Fig 16).

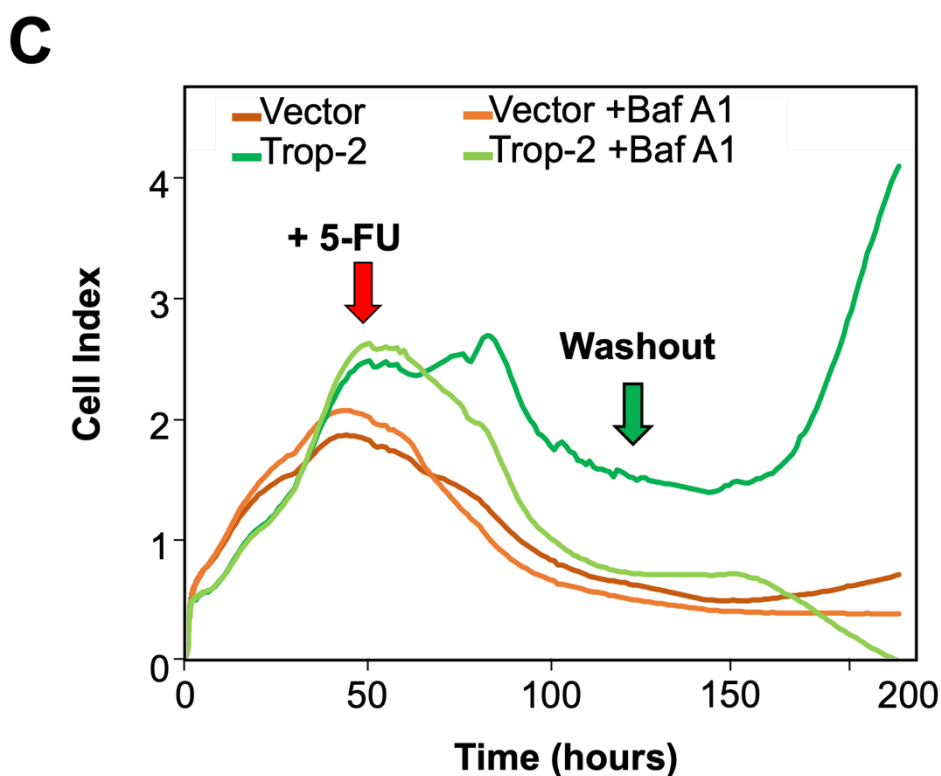


Fig. 16. Inhibition of autophagy in Trop-2 CRC cells overcomes resistance to 5-FU. KM12SM/Vector and KM12SM/Trop-2 were treated with 5-FU with or without Baf A1, and monitored over time using the RTCA system.

DISCUSSION

Colorectal cancer (CRC) remains one of the leading causes of cancer-related mortality worldwide, with tumor recurrence and chemoresistance representing major obstacles to effective treatment. Despite advancements in therapeutic strategies, CRC cells frequently develop adaptive mechanisms that enable them to survive under metabolic stress. Identifying these mechanisms is therefore essential for the development of novel therapeutic approaches aimed at improving treatment efficacy and patient outcomes.

In this study, we showed that Trop-2 is a key regulator of CRC cell growth and long-term survival under metabolic stress conditions of nutrient exhaustion, through induction of cytoprotective autophagy. Trop-2 has been widely recognized as an oncogenic driver in various carcinomas, with numerous studies linking its overexpression to tumor aggressiveness, metastasis and poor prognosis. However, its role in cellular metabolism and stress adaptation had not been investigated prior to this study. Our findings significantly expand current knowledge by demonstrating for the first time that Trop-2 sustains CRC cell survival through autophagy induction.

Our real time impedance-based assays revealed that Trop-2-positive KM12SM CRC cells exhibit a significant survival advantage compared to Trop-2-negative counterparts, under nutrient-limiting conditions. This suggests that Trop-2 functions as a metabolic regulator, enhancing cellular resilience under adverse environmental conditions. However, Trop-2 expression was insufficient to overcome naturally occurring cell death in overconfluence/nutrient exhaustion conditions in the HCT116 and SW48 CRC cell lines, indicating that its pro-survival effects may be context-dependent and rely on yet unknown molecular determinants, such as hypoxia.

Given the well-established role of autophagy as a metabolic adaptation mechanism (80,89), we hypothesized that Trop-2 promotes CRC survival by modulating the autophagic pathway. Consistent with this hypothesis, Trop-2 overexpression was found to lead to strong upregulation of essential autophagy-related genes (ATG), including ULK-1, Beclin-1, ATG4A, ATG5, and ATG7, which are critical for the initiation and elongation of the phagophore. Furthermore, LC3 lipidation analysis revealed reduction of both LC3-I and LC3-II forms in Trop-2-positive cells. This difference is no longer observed upon treatment with the

autophagy inhibitor Bafilomycin A1 (Baf A1), suggesting an accelerated degradation of LC3 due to an increased autophagic activity of Trop-2-positive cells under high cell density and nutrient consumption conditions. These findings reinforce the idea that Trop-2 is specifically activated in metabolic stress environments to sustain cell viability through autophagy.

Our study also identifies TFEB activation as a downstream event of Trop-2 signaling, a novel aspect not previously described. Our data indicate that Trop-2 stimulates TFEB nuclear translocation, leading to the upregulation of ATG genes. Phosphorylated TFEB interacts with 14-3-3 ζ/δ , and this binding contributes to sequester TFEB in the cytoplasm and prevent its nuclear translocation, which occurs following dephosphorylation of TFEB by calcineurin and dissociation of the TFEB/14-3-3 ζ/δ complex (71,77). Our proteomics analysis of Trop-2 immunoprecipitates revealed a binding between Trop-2 and 14-3-3 ζ/δ , suggesting that 14-3-3 ζ/δ might be confined at the cell membrane, leading to TFEB translocation into the nucleus. In further support to this hypothesis, our recent findings that Trop-2 drives an intracellular Ca^{2+} increase in cancer cells may indicate a Trop-2-dependent activation of calcineurin which in turn dephosphorylates and activates TFEB.

To further elucidate the molecular mechanisms underlying Trop-2-driven autophagy, we performed proteomic analyses in nutrient consumption conditions. Specifically, drug enrichment analysis performed on the proteomic data revealed that the molecular signature of Trop-2-expressing CRC cells is comparable to the signature obtained using a treatment of the cells with mTOR inhibitors. This suggests that Trop-2 can promote CRC survival by inhibiting the mTORC1 signaling complex, which is a well-known negative regulator of autophagy in nutrient-rich conditions, and whose inhibition in stress conditions is considered as a hallmark of autophagy-dependent survival (65,70). Additionally, Trop-2-driven autophagy was associated with the downregulation of apoptotic and p53 signaling, allowing CRC cells to evade cell death while sustaining metabolic balance.

Furthermore, Trop-2 interacts with insulin-like growth factor 1 (IGF-1), thereby inhibiting IGF-1 receptor (IGF-1R) signaling (30,31). Given that IGF-1R inhibition and suppression of mTORC1 are known to stimulate autophagy (31), our findings suggest that Trop-2 may enhance autophagic activity through IGF-1R modulation.

Our ultrastructural assays showed that Trop-2 induces fusion of autophagosomes with lysosomes, thereby accelerating degradation and recycling of the intracellular resources. This was demonstrated by the increased number and size of autophagolysosomes observed in Trop-

2-positive CRC cells compared to controls. These findings suggest that Trop-2 can be actively involved not only in autophagy initiation but also in the final stages of autophagic degradation, thereby enhancing CRC metabolic adaptation.

Trop-2 undergoes cancer-specific proteolysis by ADAM10 at the R87-T88 site (21); hence, we investigated whether this proteolytic cleavage is actually required for its pro-autophagic function. Our results indicate that a cleavage-deficient Trop-2 mutant (Trop-2 R87A2^{R87A-T88A}) fails to induce autophagy, leading to impaired CRC cell survival under nutrient-limiting conditions. These observations indicate that proteolytic activation of Trop-2 serves as a molecular switch, enabling CRC cells to adapt to metabolic stress through autophagy induction.

Further supporting this hypothesis, our results demonstrate that Trop-2 cleavage is essential for sustaining autophagic flux and preventing apoptosis under stress conditions. CRC cells expressing the cleavage-deficient Trop-2 mutant exhibited impaired autophagosome maturation and autophagolysosome formation, highlighting the critical role of Trop-2 cleavage in facilitating lysosomal fusion. Moreover, the survival advantage conferred by Trop-2 under nutrient consumption was abolished in the cleavage-deficient mutant, reinforcing the notion that this post-translational modification is necessary for Trop-2-driven metabolic adaptation.

Importantly, we demonstrated that inhibition of autophagy with Baf A1 triggers apoptotic death specifically in Trop-2-positive cells. In contrast, control cells and cleavage-deficient mutant cells, which inherently exhibited lower autophagic activity, showed minimal apoptotic response to Baf A1, suggesting that Trop-2-expressing cells rely on autophagy to evade cell death. This indicates that Trop-2 not only promotes autophagy as a survival mechanism but also renders CRC cells highly susceptible to apoptosis when autophagy is inhibited. Collectively, these findings highlight the crucial role of Trop-2 proteolytic cleavage in regulating CRC cell fate under metabolic stress.

A major challenge in CRC treatment is the emergence of resistance to first-line agents such as 5-fluorouracil (5-FU). As cytoprotective autophagy plays a key role in drug resistance (137), we hypothesized that Trop-2-induced autophagy might result in enhanced CRC resistance to 5-FU. Consistent with this hypothesis, Trop-2-expressing CRC cells exhibited significantly lower mortality across a range of 5-FU concentrations. Notably, co-treatment with Baf A1 effectively re-sensitized Trop-2-expressing cells to 5-FU, leading to massive cell death

upon drug withdrawal. These findings strongly suggest that Trop-2-driven autophagy acts as a survival mechanism that protects CRC cells from the cytotoxic effects of chemotherapy.

Overall, the experimental results obtained in this study strongly suggest that targeting Trop-2-mediated autophagy may indeed represent a promising strategy to overcome drug resistance in CRC. Future clinical exploitation of our findings could involve (1) new CRC patient stratification criteria based on Trop-2 expression, in order to identify potential responders/non-responders to treatment with 5-FU, and elaborate novel targeted treatment options, and (2) development of highly innovative therapeutic strategies, involving for example the use anti-autophagic compounds encapsulated into vehicles delivered to Trop-2-expressing CRC tumors based on the capability to selectively recognize the cancer-specific cleaved Trop-2 form, or the development of next generation antibody-drug conjugates armed with anti-autophagic payloads.

CONCLUSIONS

This study identifies Trop-2 as a critical regulator of CRC survival and chemoresistance, which modulates them through the induction of autophagy. We demonstrate that Trop-2 stimulates autophagic flux by promoting autophagolysosome formation and downregulating mTORC1 signaling under stress conditions of limiting nutrient availability. Notably, we reveal that Trop-2 proteolytic cleavage is a key activation step for its pro-autophagic function, suggesting that post-translational modifications of Trop-2 serve as molecular switches governing CRC survival strategies. Known stressors such as hypoxia and the tumor microenvironment (i.e. extracellular matrix stiffness, effect of immune cells infiltrating the cancerous mass etc.) will be investigated in the near future to discriminate tumor-intrinsic from tumor-extrinsic mechanisms driven by Trop-2. Moreover, we will also run further studies to identify specific autophagic pathways that may be preferentially activated in specific environmental conditions or in CRC cells harbouring specific CMSs.

Although our study provides a solid molecular basis for understanding the role of Trop-2 in CRC survival and chemoresistance, further investigations are necessary to translate these findings into clinical applications. Specifically, advanced *in vitro* models of cancer growth and progression (including spheroids, organoids/tumoroids, and organ-on-a-chip systems) and preclinical mouse models (such as patient-derived xenografts, genetically modified organisms of malignant progression, and carcinogen-induced models of cancer growth) will be essential to validate the therapeutic potential of targeting Trop-2-driven autophagy.

Our data confirm that Trop-2 enables CRC cells to survive nutrient stress by triggering a cytoprotective autophagy program, a mechanism that also underlies enhanced resistance to 5-FU chemotherapy. These *in vitro* findings have significant implications for CRC therapy, suggesting new strategies for CRC patient stratification based on Trop-2 expression levels and its activatory cleavage. Furthermore, future studies exploring combinatorial treatments involving autophagy inhibitors and standard chemotherapeutics, such as 5-FU, or agents targeting Trop-2, could provide clinical validation for novel treatment schemes aimed at improving clinical outcome for CRC patients. Finally, given that Trop-2 is overexpressed in various human carcinomas, it will be important to extend this research to explore whether Trop-2-driven autophagy plays a similar role in tumor progression and therapeutic resistance across other cancer types. Addressing these metabolic weaknesses could enhance the efficacy of new

therapeutic strategies, thereby broadening the potential clinical impact of targeting Trop-2 in oncology.

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