Role and regulation of autophagy in ALK positive cancers

Domenico Sorrentino

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THÈSE

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INTRODUCTION

1 Anaplastic Large Cell Lymphoma

Lymphoma is the general term for cancers that develop in the lymphatic system. Lymphoma originates in developing B-lymphocytes or T-lymphocytes, which have undergone a malignant change. There are two main categories of lymphomas. One kind is Hodgkin lymphoma (HL), which is marked by the presence of a specific type of cell called the Reed-Sternberg cell. These HL account for 20% of lymphomas. The other category is called non-Hodgkin lymphomas (NHL)[1]. It represents 80% of lymphomas and it includes the Anaplastic Large Cell Lymphoma type, which will be further developed in this manuscript.

1.1 Definition and Classification of ALCL

Anaplastic Large Cell Lymphoma, hereafter referred as ALCL, is an aggressive non-Hodgkin's lymphoma (NHL). Its identification and classification took many years [2-3]. Back in 1985, Dr Harald Stein, Dr Karl Lennert and colleagues identified a unique large cell lymphoma with anaplastic cytology (showing a poor cell differentiation) and strong expression of the antigen Ki-1, which was subsequently identified as an activation antigen (now designated CD30)[4]. This initial phenotypic description led to the misdiagnosis of ALCL as a neoplasm related to classic Hodgkin's lymphoma (cHL) or malignant histiocytis (MH), both characterized by CD30 expression. Subsequent immunophenotypic and T-cell receptor (TCR) gene rearrangement studies revealed its derivation from T cells and led to the recognition of ALCL as a unique subtype of peripheral T-cell lymphoma (PTCL) lacking expression of T-cell related surface proteins[4-5-6]. Based on these phenotypic evidences for a distinct disease, ALCL was included in the Kiel lymphoma classification [7].

Then, the t(2;5)(p23;q35) translocation was observed in a cell line established from a CD30 positive tumor[8-9-10]. Its molecular cloning by Dr Steve Morris and Dr Tom Look in 1994 [11] and the detection of the resulting fusion oncoprotein NPM-ALK (Nucleophosmin-Anaplastic Lymphoma Kinase) in a large subset of ALCL led to the recognition of ALK+ ALCL as a distinct clinical entity (and ALK- ALCL as a provisional entity) in the World Health Organization (WHO) 2008 classification[12]. Further studies over

the last decade led to the most recent 2016 revised WHO lymphoma classification[13], which now recognizes four different ALCL entities:

*the **systemic ALK-positive ALCL (ALK+ ALCL)**, which will be further developed in this manuscript;

*the **systemic ALK-negative ALCL (ALK- ALCL)**, which were lacking, until recently, of clear-cut criteria to distinguish them from other CD30-positive PTCLs (Peripheral T-Cell Lymphomas)[14]. Indeed, when compared to ALK+ ALCL, notable differences in epidemiology, in clinical outcomes, in gene expression profile and most of all, novel molecular findings specific for ALK-negative ALCL (mainly translocations involving IRF4/DUSP22 and TP63) now support their distinction as a clinical entity.

*the **primary cutaneous ALCL (pC-ALCL)**, which develop and stay localized in the skin. It is classically present as solitary, grouped or multifocal nodules on the upper half of the body that persist for at least 3 to 4 weeks. Initially reported cases of pC-ALCL were not noted to contain the anaplastic lymphoma kinase (ALK) gene translocation. However, a subset of pC-ALCL cases showed an ALK translocation, and its presence portends an increased likelihood of progression to systemic disease [15].

*the provisional entity **breast implant-associated ALCL (BI-ALCL)** [13]. This subgroup of ALCL is associated with the seroma forming around breast implants. The etiology and pathogenesis of BI-ALCL have been related to the immune reaction to silicone. Of note, BI-ALCL is negative for ALK expression[16].

2 ALK+ Anaplastic Large Cell Lymphoma

2.1 Origin

In early 1990s, a recurrent chromosomal translocation t(2;5) was described in systemic ALCL[10]. In 1994, the translocation was cloned by Dr Steve Morris and others at St Jude Children's Research Hospital in Memphis, Tennessee, and was found to involve a receptor tyrosine kinase called anaplastic lymphoma kinase (ALK) on chromosome 2p23 and nucleophosmin (NPM) on chromosome 5q35[17]. Because ALK is not normally expressed in lymphoid tissue, anti-ALK antibodies (which were developed for the first time by Dr Mason group in 1997) were used as a surrogate method for detecting the occurrence of the t(2;5) translocation and the resulting NPM-ALK fusion oncoprotein in lymphoma cells[18]. After widespread immunohistochemical analysis with anti-ALK antibodies, ALK+ ALCL was defined as a specific entity that typically affects children and young adults. Although ALCL cells are believed to correspond to mature $CD4^+$ T lymphocytes, recent studies suggest that the ALK gene translocation occurs in an immature thymic precursor cell with stem-like properties (figure 1)[19].

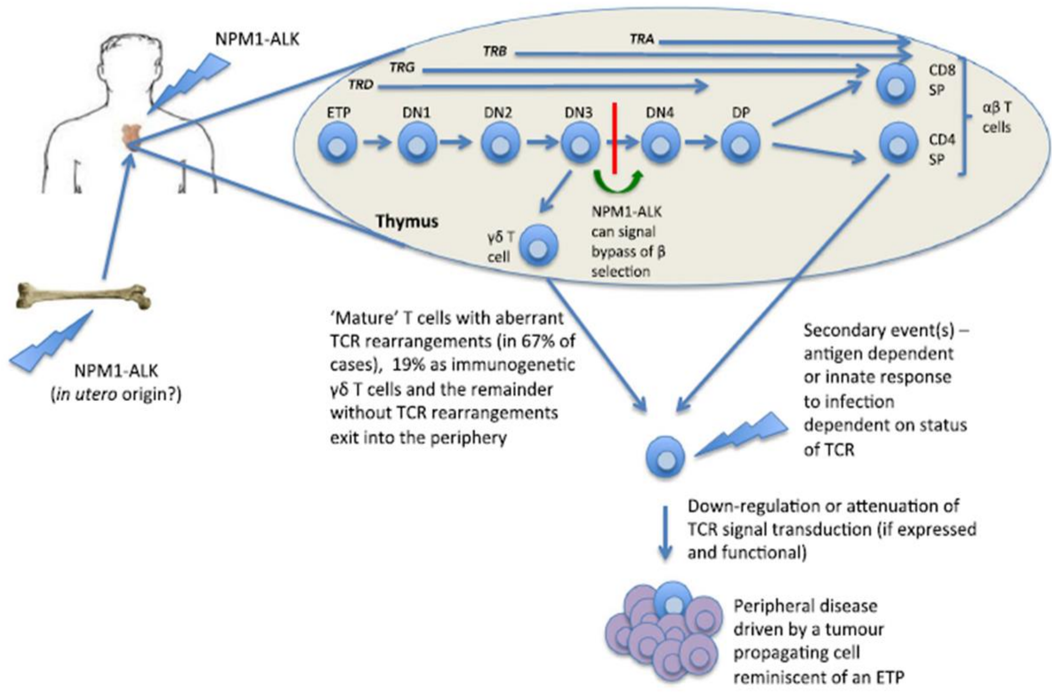


Figure 1. A thymic origin for ALCL. In this model, the t(2;5) or variant translocation occurs in haemopoietic stem cells or thymic progenitors whereby NPM1-ALK is permissive of cellular survival in the thymus despite aberrant TCR rearrangements. These 'primed' cells may go undetected until a secondary event(s) occurs that leads to clonal expansion and tumour development. This event may be induced as a consequence of an inflammatory response as evidenced by ALCL in the context of insect bites but might also be initiated in an innate manner *(Turner et al 2016).*

ETP: early thymic progenitor; DN: double negative thymocyte; DP: double positive thymocyte; SP:single positive

2.2 Clinical and diagnosis features

ALCL is primarily a pediatric tumor, accounting for 15% of all pediatric Non-Hodgkin Lymphoma (NHL) with an annual incidence ranging from 1.2 per million in children under 15 years, to approximately 2 per million in young adults between 25 and 34 years, translating to approximately 80 new pediatric cases diagnosed in Europe each year. Whilst the majority of pediatric cases are ALK-positive, about 50–60% of adult ALCL cases are ALK-negative[20]. ALK+ALCL show an aggressive behavior with rapidly progressive adenopathy and systemic symptoms such as fevers, night sweats, and weight loss.

At the time of diagnosis, most patients are in an advanced stage of disease (III–IV stage) according to the NHL classification (Table 1) with systemic symptoms (75%) and lymph node enlargement (90%), including mediastinal involvement (36%). Extranodal involvement is present in 40–68% of cases, including skin (26%), bone (14%), and soft tissues (15%), lung (12%), and liver (8%)[21]. Routine morphological examination of BM (bone marrow) and the use of immunohistochemical markers such as CD30 and ALK serve for patient diagnosis and stratification[22-23].

Table 1 - Statification of Non-Hodgkin Lymphoma. Approved by the Cancer.Net Editorial Board, 02/2018

Stratification	Characterization
Stage I	• The cancer is found in 1 lymph node region (stage I). • The cancer has invaded 1 extralymphatic organ or site (identified using the letter "E") but not any lymph node regions (stage IE).
Stage II	• The cancer is in 2 or more lymph node regions on the same side of the diaphragm (stage II). • The cancer involves 1 organ and its regional lymph nodes, with or without cancer in other lymph node regions on the same side of the diaphragm (stage IIE).
Stage III/IV	There is cancer in lymph node areas on both sides of the diaphragm (stage III), or the cancer has spread throughout the body beyond the lymph nodes (stage IV). Lymphoma most often spreads to the liver, bone marrow, or lungs.

2.3 Morphological characteristics

The characteristic ALK+ALCL neoplastic cells are large with abundant eosinophilic cytoplasm and prominent Golgi apparatus, stained as a clear perinuclear zone. Nuclei are large and show open chromatin with multiple nucleoli. These neoplastic cells with eccentric, horseshoe, or kidney-shaped nuclei have been referred to as "hallmark cells", because they are present in all morphological variants of this lymphoma[24]. According to the cytological and architectural features, five patterns have been

recognized in the WHO classification: the common pattern, the small cell pattern, the lymphohistiocytic pattern, the Hodgkin's-like pattern, and the composite pattern, as briefly detailed below.

*The ALK+ ALCL *common pattern* represents the most frequent morphological variant (60–70%). It consists predominantly of large pleomorphic cells with admixed "hallmark" cells: they have abundant clear or basophilic cytoplasm with large pleomorphic nuclei, finely dispersed nuclear chromatin, and multiple small nucleoli. Moreover these cells are strongly positive for CD30 and ALK staining (Figure 2)[25].

*The *small cell pattern* (5–10%) shows a predominant population of small to medium-sized neoplastic cells with clear cytoplasm and distinctive cell membranes exhibiting a "fried egg" appearance. The nucleus of the neoplastic cells can be horseshoe-shaped or round, and typical hallmark cells are always present and concentrated around the blood vessels, forming rosettes[26-27].

*The *lymphohistiocytic pattern* (10%) is characterized morphologically by the presence of small neoplastic cells admixed with abundant histiocytes. The histiocytes can predominate in this pattern, masking the neoplastic cells, making the diagnosis challenging without appropriate immunostaining[28].

*The *Hodgkin's like pattern* is present in only 3% of ALK+ ALCL. The morphological features include an architecture that resembles nodular sclerosis classical Hodgking Lymphoma (NScHL) with a prominent inflammatory background. ALK immunostaining is crucial in the differential diagnosis of this entity[29].

*In about 15% of the cases, more than one pattern can be seen in a lymph node biopsy, referred to as a "*composite pattern*"[30].

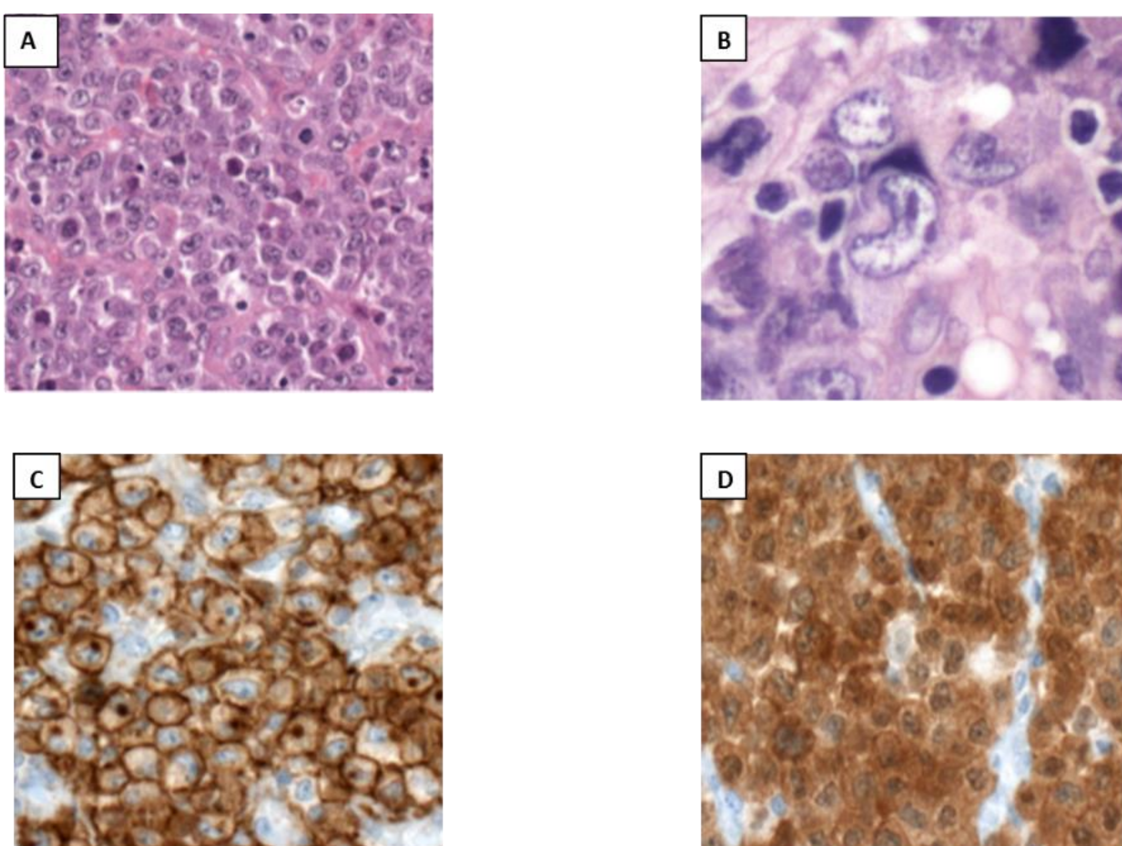


Figure 2. Morphological and immunohistochemical findings in ALK+ ALCL. (A) The large neoplastic cells are relatively monomorphic, showing abundant eosinophilic cytoplasm and pleomorphic nuclei and frequent apoptotic bodies (H&E stain, 400X); **(B)** A "hallmark cell", displaying an eccentric horseshoe-shaped nuclei with two nucleoli and prominent Golgi area (H&E stain, 630X); **(C)** The tumor cells are strongly and uniformly positive for CD30 with a membranous and Golgi zone pattern; **(D)** Strong nucleolar ALK staining in the neoplastic cells is observed *(Mojarro et al 2018)*.

2.4 Molecular characteristics of ALK

2.4.1 ALK structure

The ALK gene encodes a highly conserved receptor tyrosine kinase (RTK), which is a member of the insulin receptor superfamily, and is most closely related to leukocyte tyrosine kinase (LTK)[31-32.].The ALK receptor (Figure 3) is composed of an extracellular domain, a single-pass transmembrane region, and an intracellular kinase domain. The extracellular domain contains a glycine-rich region, two MAM segments (meprin, A5 protein, and receptor protein tyrosine phosphatase μ) and one LDLa domain (low density lipoprotein class A). The intracellular portion comprises a juxtamembrane segment, a protein kinase domain and a carboxyterminal tail[33-34-35].

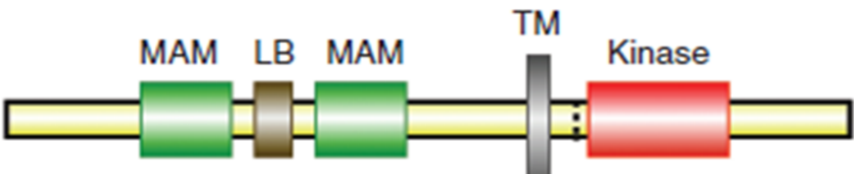


Figure 3. Structural organization of ALK. ALK is a receptor with a single transmembrane (TM) domain as well as 2 MAM domains and a putative ligand-binding (LB) domain in the extracellular region (*adapted from Hiroyuki Mano 2012).*

2.4.2 ALK Functional Role and ligands

The specific role of ALK in human development and physiology is still poorly understood but several studies on different animal models have partially clarified the ALK functions in development.

* In *Drosophila melanogaster*, ALK signaling is involved in the differentiation of mesenchymal cells, in the development of the visual system[36], the maturation of the neuromuscular junction and in the regulation of body size, learning and memory[37-38].

* In zebrafish, ALK contributes to neural crest nervous system embryogenesis. In this context, ALK is recognized by its ligand Jelly Belly (Jeb) leading to the activation of the downstream Ras-MAPK pathway[39].

* In mice, ALK expression patterns throughout the nervous system during embryogenesis suggest important roles in the central nervous system (CNS) development and function[33].

* Recently, human secreted small protein ALKAL1 which was previously reported as family-with-sequence-similarity150 (FAM150) has been shown to activate human ALK[40].

Finally, ALK receptor tyrosine kinase belongs to the functional family of so-called 'dependence receptors'. Such dependence receptors work with a dual signaling: in the presence of ligand or a situation of a mimicking ligand, the receptor exerts a pro-survival/anti-apoptotic effect on the cell; in contrast, in the absence of ligand and when the cell is submitted to environmental or genotoxic stress, the dependence receptor becomes pro-apoptotic [41].

2.4.3 Hypothetical ALK physiological activation

Although our knowledge of the mechanism of activation of mammalian ALK protein-tyrosine kinase is incomplete, Drs Lemmon and Schlessinger have described the mechanism of activation of several receptor protein-tyrosine kinases, providing us a hypothetic scheme for ALK activation. Upon ligand binding in the extracellular domain, the receptor protein-tyrosine kinase is activated by inducing receptor dimerization or oligomerization. A possible mechanism for ligand and dimer-induced activation of ALK involves the phosphorylation of one or more of the juxtamembrane tyrosine residues (Tyr 1078, 1092, 1096 and 1131), which in turn would be followed by consecutive phosphorylations until the active form of ALK is established[42]. Moreover, Tartari at al. have studied the molecular mechanism of NPM-ALK autoactivation by mutating three potential autophosphorylation sites (Tyr -> Phe) contained in the "Y*XXX*YY" motif of the ALK activation loop (Tyr-338, Tyr-342, and Tyr-343). Specifically, mutation of both the second and third tyrosine residues do not affect the kinase activity of NPM-ALK. In contrast, phosphorylation of the first tyrosine is necessary for the autoactivation of the NPM-ALK kinase domain[43].

2.5 ALK Gene Alterations in Cancers

The deregulation of tyrosine kinase (TK) activity is one of the major mechanisms of human carcinogenesis and can occur through several mechanisms such as chromosomal translocations, gene amplification or deregulation and point mutation. The abnormal TK activation leads to constitutive activation of several downstream signaling pathways, which contribute to the development of neoplastic phenotypes. ALK gene alterations (translocations, mutations and amplification) have been found in a large spectrum of cancers (figure 4). In ALK+ ALCL, ALK gene translocations are the oncogenic drivers[33].

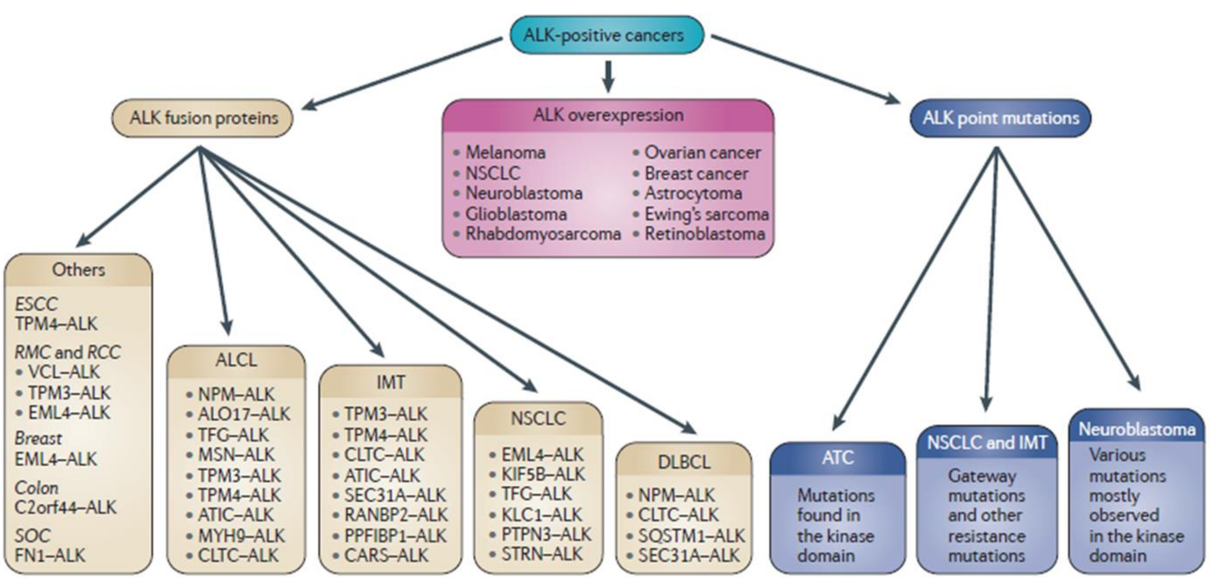


Figure 4. Schematic summary of the different categories of anaplastic lymphoma kinase (ALK)-positive cancers. ALK fusion proteins (shown in beige), in which the kinase domain of ALK is fused to the amino-terminal portion of various proteins, have been described in numerous cancers, such as anaplastic large cell lymphoma (ALCL), inflammatory myofibroblastic tumour (IMT), diffuse large B cell lymphoma (DLBCL), non-small-cell lung cancer (NSCLC), renal medulla carcinoma (RMC), renal cell carcinoma (RCC), breast cancer, colon carcinoma, serous ovarian carcinoma (SOC) and oesophageal squamous cell carcinoma (ESCC). ALK overexpression, although mechanistically not understood, has been reported in various cancer types and cell lines (pink). ALK mutations comprise a third category (blue) have been described in NSCLC, IMT and anaplastic thyroid cancer (ATC). ALK point mutations have been found mainly in neuroblastoma, as well as in NSCLC and ATC (*Hallberg and Palmer 2013*) .

2.5.1 Translocation NPM-ALK and others

Tyrosine kinase translocations are found in up to 3% of all human tumors [44]. Usually, translocations comprising transmembrane tyrosine kinase receptors take place between exons that encode the juxtamembrane region or the transmembrane domain. In both cases, these phenomena give rise to the elimination of the extracellular region and, consequently, the ligand-binding regulation, resulting in the constitutive and uncontrolled activation of the fusion typically through an obligatory dimerization dictated by the partner gene[45]. ALK breakpoints are almost invariably located between exons 19 and 20 of ALK. Each translocation creates a fusion protein in which the ALK TK-domain at the 3'-end is connected with distinct proteins portion of different partners at the 5'-end of the fusion, capable of providing constitutive dimerization[46]. ALK rearrangement was first described in 1994, in the anaplastic large cell lymphoma (ALCL) cell lines, with ALK being one of the fused partner in a recurrent chromosomal translocation t(2;5)(p23;q35) together with the nucleophosmin (NPM) gene located on chromosome 5[11]. This rearrangement produces a fusion gene resulting in the expression of an oncogenic fusion protein, NPM-ALK. NPM mediates the dimerization of the fusion protein, which leads to the constitutive activation of the ALK tyrosine kinase domain. This unrestrained kinase activity steams for the oncogenic potential of the fusion protein (figure 5)[31-32-47]. With the advent of next-generation sequencing (NSG)-based diagnostics, more than 20 different ALK fusion partners genes have been described in other type of cancers (i.e., colorectal cancer, breast cancer, esophageal cancer, ovarian cancer, renal cell cancer, diffuse large B-cell lymphoma and non-small-cell lung cancer) even though in low frequencies (Table 2)[48].

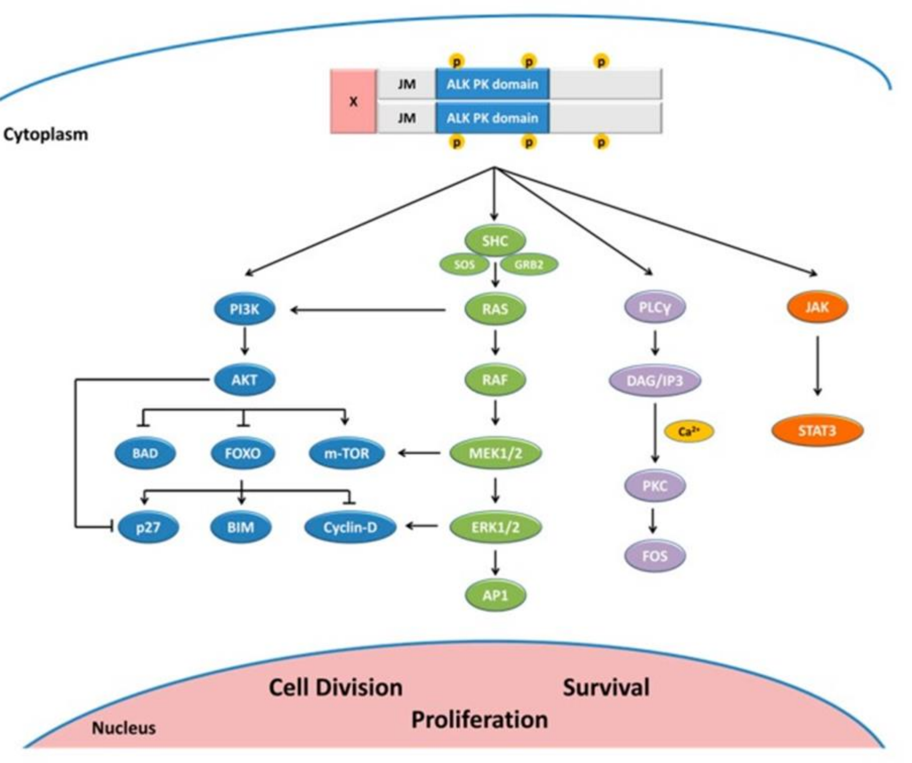


Figure 4 Anaplastic lymphoma kinase (ALK) mediates signaling via the PI3K/AKT, RAS/MAPK, phospholipase Cγ (PLCγ) and Janus kinase (JAK)-signal transducer and activator of transcription (STAT). (Adapted from *Sharma et al. Cancers, 2018).*

Cancer Type	ALK Fusion Partner (Chromosomal Localization)	Frequency %
ALCL	NPM1 (5q35.1) TPM3 (1q21.3) ATIC (2q35) TFG (3q12.2) TRAF1 (9q33.2) CLTC (17q23.1) RNF213 (17q25.3) TPM4 (19p13.1) MYH9 (22q12.3) MSN (Xq12) Aditional rare rearrangements	~55% (in adults)
Breast cancer	EML4 (2p21)	N.D.
Colorectal cancer	EML4 (2p21) WDCP (2p23.3)	<1%
DLBCL	RANBP2 (2q13) EML4 (2p21) SEC31A (4q21.22) SQSTM1 (5q35) NPM1 (5q35.1)	<1%
Esophageal cancer	TPM4 (19p13.1)	N.D.
IMT	TPM3 (1q21.3) RANBP2 (2q13) ATIC (2q35) SEC31A (4q21.22) CARS (11p15.4) PPFIBP1 (12p11) CLTC (17q23.1) TPM4 (19p13.1)	Up to 50%
NSCLC	EML4 (2p21) TPR (1q31.1) CRIM1 (2p22.2) STRN (2p22.1) TFG (3q12.2) HIP1 (7q11.23) PTPN3 (9q31) KIF5B (10p11.22) KLC1 (14q32.3) CLTC (17q23.1)	3-7%
Ovarian cancer	FN1 (2q35)	N.D.
RCC	VCL (10q22.2) TPM3 (1q21.2) EML4 (2p21) STRN (2p22.2)	<1%
RMC	VCL (10q22.2)	N.D.

Table2. ALK fusion partners in different cancer types. Abbreviations (alphabetic order): ALK, anaplastic lymphoma kinase; ALCL, anaplastic large-cell lymphoma; ATIC, 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase/IMP Cyclohydrolase; CARS, cysteinyl-tRNA synthetase; CLTC, clatherin heavy chain; CRIM1, cysteine rich transmembrane BMP regulator 1; DLBCL, diffuse large B-cell lymphoma; EML4, echinoderm microtubule-associated protein-like 4; FN1, fibronectin 1; HIP1, huntingtin interacting protein 1; IMT, inflammatory myofibroblastic tumor; KIF5B, kinesin family member 5B; KLC1, kinesin light chain 1; MSN, moesin; MYH9, myosin heavy chain 9; N.D., not described; NPM1, nucleophosmin; NSCLC, non-small-cell lung cancer; PPFIBP1, PPFIA binding protein 1; PTPN3, protein tyrosine phosphatase, non-receptor type 3; RANBP2, RAN binding protein 2; RCC, renal cell carcinoma; RMC, renal medullary carcinoma; RNF213, ring finger protein 213; SEC31A, SEC31 Homolog A; SQSTM1, sequestosome 1; STRN, Striatin; TFG, TRK-fused gene; TPM3, tropomyosin 3; TPM4, tropomyosin 4; TPR, translocated promoter region, nuclear basket protein; TRAF1, TNF receptor associated factor 1; VCL, vinculin; WDCP, WD repeat and coiled coil containing (*Sharma et al 2018).*

2.5.2 ALK Mutations

Activating point mutations and small deletions in ALK have been described in neuroblastoma, thyroid cancer and NSCLC (non-small-cell lung cancer). For instance, about 10% of sporadic neuroblastoma cases harbor somatic nonsynonymous mutations within ALK, including K1062M, F1174L/C/I, F1245C/V/L, and R1275Q amino acid substitutions. Importantly, these mutations do not confer equal transforming ability. Knockdown experiments revealed that the growth of neuroblastoma cell lines was

dependent to a greater extent on the F1174L mutant than on R1275Q. The mutant ALK proteins thus contribute substantially to the transformation process in neuroblastoma, but the extent to which they do so varies among the mutation types. These mutations also differentially affect the sensitivity of neuroblastoma to ALK inhibitors, which may not be surprising given that point mutations within the kinase domain of ALK affect its 3-dimensional structure and thereby influence inhibitor binding[49].

2.5.3 ALK Amplification

ALK gene amplification have been described in Melanoma, NSCLC, Neuroblastoma, Glioblastoma and Rhabdomyosarcoma[33]. Indeed, rare cases of ALK gene amplification in neuroblastoma have been reported. While its clinical relevance is yet to be clarified, *ALK* amplification frequently co-occurs with amplification of *MYCN* amplification, a known growth driver for this disorder, suggesting that *ALK* also contributes to carcinogenesis[50-51]. Recently, Dr van Gaal and colleagues have discovered frequent copy number gain of *ALK* in rhabdomyosarcoma accompanied with an increased level of ALK protein[52]. Interestingly, contrary to neuroblastoma, rhabdomyosarcoma with *ALK* amplification do not carry *MYCN* amplification. Such *ALK* anomaly is likely to be connected to carcinogenesis because *ALK* gain was associated with poor survival and the occurrence of metastases[49].

3 NPM-ALK Signaling

The NPM-ALK protein is expressed as a homodimer, which becomes autophosphorylated through reciprocal ALK tyrosine kinase activity and, therefore, it is strongly and persistently activated. NPM-ALK mimics physiological pro-growth signals and activates multiple intracellular signal transduction pathways, which chronic activation leads, *in fine*, to persistent expression of genes that are involved in the promotion of cell proliferation and the protection from apoptotic cell death [53]. The most studied pathways, which are deregulated by NPM-ALK, are the PLCϒ, the PI3K-AKT, the MAPK/ERK, the mTOR and the JAK-STAT pathways (Figure 6)[54].

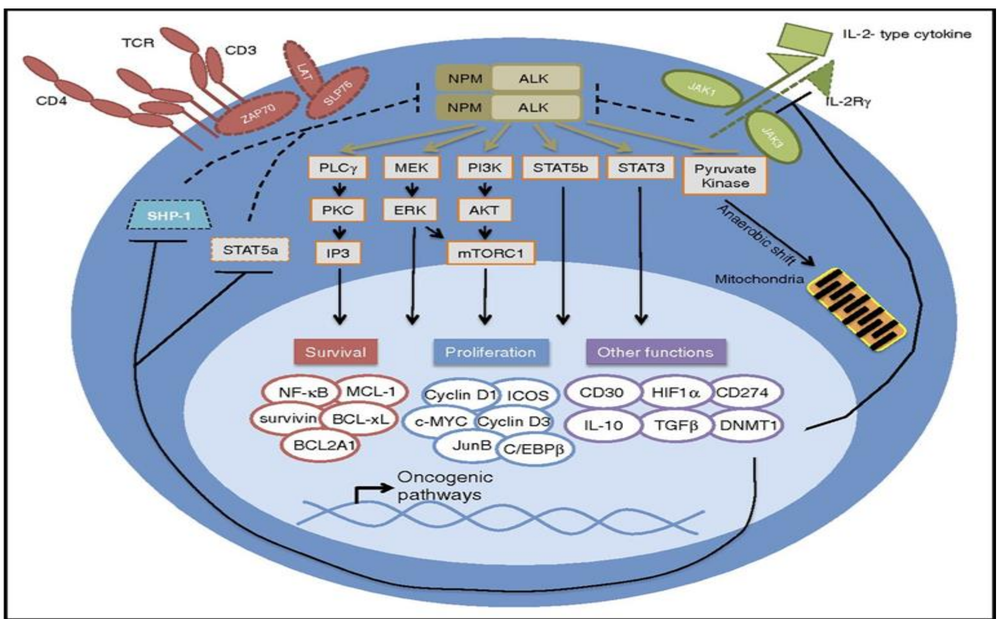


Figure 6. Aberrant NPM-ALK kinase activity. NPM-ALK hijacks key cell signaling pathways physiologically activated by TCR and IL-2 signaling pathways. Transcriptional targets directly downstream of these signaling pathways include key transcription factors and other cell cycle and apoptosis regulators that normally promote growth and survival but lead to oncogenesis in ALK+ ALCL. Phosphoproteomic analysis has revealed that in addition to modulating important signaling pathways, NPM-ALK alters key metabolic pathways through phosphorylation, such as that of the rate-limiting enzyme pyruvate kinase, whose phosphorylation inactivates the enzyme promoting a shift from aerobic to anaerobic glycolysis *(Wasik et al 2016).*

3.1 NPM-ALK and PLCϒ pathway

NPM-ALK has been found to be associated with the signal transducer PLC-ϒ. PLC-ϒ activation downstream of NPM-ALK leads to the generation of diacylglycerol and inositol triphosphate (IP3), which in turn activate protein kinase C. PLC-ϒ activity seems to be important for DNA synthesis and for the delivery of a mitogenic signal[55] (figure 6).

3.2 NPM-ALK and PI3K-AKT pathway

The phosphoinositide 3-kinase (PI3K) signaling pathway is one of the most frequently altered pathways in human cancer and has a critical role in driving tumor initiation and progression. Although PI3K and its lipid product phosphatidylinositol-3,4,5-trisphosphate (PIP3) have been shown to activate multiple downstream signaling proteins, the vast majority of studies have focused on the protein kinase AKT as the dominant effector of PI3K signaling[53]. Pr Wasik et al have shown that the PI3K-Akt pathway is constitutively activated in NPM-ALK-transformed murine hematopoietic cell lines and in lymphoma tissues from ALK-positive ALCL patients. In addition, they showed that both PI3K and Akt are essential for the growth factor independence and lymphomagenic activity of NPM/ALK-transfected cells (figure 6)[56].

3.3 NPM-ALK and MAPK/ERK pathway

The MEK/ERK pathway is physiologically activated by growth factors, serum and phorbol esters and, to a lesser degree, cytokines and osmotic stress. The activated MEK/ERK signaling pathway impacts multiple cell functions, including proliferation, survival, migration, division and differentiation, and thus, is frequently deregulated in cancers. The key mitogen-activated protein kinase (MAPK) signaling pathway results in activation of the extracellular-regulated kinase (ERK) 1 and 2 complexes. The other, upstream proteins of the pathway are three related MAPK kinase kinases (MAPKKK) A-Raf, B-Raf, c-Raf and the MAPK kinases (MAPKK) MEK1 and MEK2, which directly activate ERK1 and ERK2 by

phosphorylation. In turn, the activated ERK1/2 complex activates numerous substrates in all cellular compartments, including various membrane proteins, cytoskeletal proteins, and nuclear substrates such as NF-AT, c-Fos, c-Myc and STAT3 in physiological condition[57]. Pr Wasik *et al*. have shown that NPM/ALK induces activation of MEK1/2 and ERK1/2. This activation is c-Raf independent as shown by functional inhibition (using two c-Raf small molecule inhibitors: RI and ZM336372) and by the depletion of c-Raf (using small interference RNA). However, ERK1/2 is stringently dependent on the MEK1/2 activity. Regarding the cell function, it has been shown that the inhibition of the MEK/ERK pathway adversely affects the proliferation and survival of the ALK+ ALCL cells. These findings identified MEK/ERK as an important and druggable signaling pathway in the ALK-induced malignant cell (figure 6) [58].

3.4 NPM-ALK and mTOR pathway

Mammalian target of rapamycin (mTOR) is an ubiquitously expressed serine/threonine kinase which affects a number of key cell functions including protein synthesis and cell proliferation[59-60]. mTOR is associated with either protein called raptor or another named rictor. The exact mechanisms of mTOR activation are still under investigation but at least two distinct signals have been described for its activation. One is provided by the cell membrane receptors for growth factors, such as insulin; the second is generated by nutrients[61-62]. Dr Marzec et al demonstrated that NPM/ALK activates the mTOR pathway through the MEK/ERK and, to a much lesser degree, the PI3K/Akt pathways. The mTOR inhibitor rapamycin profoundly suppresses proliferation and enhances the apoptotic rate of ALK+ALCL cells (figure 6)[63-64].

3.5 NPM-ALK and JAK-STAT pathway

STAT3 and STAT5 proteins are oncogenic downstream mediators of the JAK–STAT pathway. Nuclear phosphorylated STAT3 and STAT5 regulate cell-type-specific transcription profiles *via* binding to promoter elements and show more complex functions involving interaction with various transcriptional coactivators or corepressors and chromatin remodeling proteins. Dr Chiarle et al. confirmed that NPM-

ALK efficiently activates STAT3 *in vivo* and *in vitro*. The deregulated STAT3 signaling promotes NPM-ALK cancer cell proliferation and survival in association with other cancer pathways[65].

STAT5 is composed by STAT5a and STAT5b, which are encoded by two related but distinct genes. The specific roles of STAT5a and STAT5b in the malignant cell transformation are still poorly characterized. However, the activation of STAT5b through ALK was observed in NPM-ALK-transfected BaF3 cells, suggesting that NPM-ALK might activate different STATs depending on the cell origin and/or their stage of differentiation. Moreover, STAT5a and STAT5b play opposite roles in ALK+ALCL cells. Whereas STAT5b is persistently activated by NPM-ALK and contributes to oncogenesis by promoting cell growth and survival, STAT5a, which acts as a potent tumor suppressor, notably by downregulation NPM-ALK expression, is epigenetically silenced (figure 6)[53].

3.6 Other functional roles of NPM-ALK activation

Although the role of NPM-ALK in cell proliferation and survival is well established, as described above, it has become evident in the last decade that several other pro-oncogenic mechanisms are upregulated through modulation of gene expression, primarily *via* STAT3, or by changing the functional status of proteins through phosphorylation. Below, we will highlight some of these other and recently described functions of NPM-ALK, which all contribute to tumorigenesis (figure 7):

- Evasion of anti-tumor immune response

NPM-ALK acts through STAT3 to induce expression of transforming growth factor beta (TGF-b) and IL-10 as well as the cell surface receptor PD-L1 (CD274, B7-H1) on the tumor cells, which thus create an immune evasion prone tumor microenvironment[66-67]. Furthermore, ALK+ ALCL cells do not express the immunomodulatory molecule TNFa (tumor necrosis factor alpha), because of the methylation of its gene promoter (figure 7).

-Tolerance to hypoxia and induction of angiogenesis.

STAT3 also induces expression of HIF1α (Hypoxia Inducible Factors Alpha) through direct binding to the HIF1α promoter allowing ALK+ALCL cells to adapt to hypoxic conditions that typically emerge in fast-growing tumors[68]. HIF1α contributes to tumor angiogenesis by inducing expression of VEGF (Vascular

endothelial growth factor)[68-69]. Moreover, our group previously showed that ALK collaborates with HIF1α to increase VEGF expression by down-regulating miR-16 which allows the VEGF mRNA degradation[70] (figure 7).

-Silencing of tumor suppressor genes

STAT3, which is activated by NPM-ALK, has a deep inhibitory effect on gene expression by recruiting the epigenetic gene-silencing complex that contains DNA methyltransferases (DNMTs) 1, 3a, and 3b and histone deacetylase 1[71-72]. The ALK dependent epigenetic gene silencing system has been shown for SHP-1[71], STAT5a[73], and IL2RgY[72]. In fact, protein products of these genes act in ALK+ ALCL cells as tumor suppressors by interfering either with phosphorylation and/or expression of NPM-ALK (figure 7).

-DNA repair

NPM-ALK has been found to affect activity of selected mismatch repair (MMR) proteins[74]. NPM-ALK binds the MMR protein MSH2 and consequently impairs MSH2:MSH6 heterodimerization and function. The MMR function is restored by disrupting NPM-ALK binding to MSH2[75]. NPM-ALK shows its inhibitory effect by phosphorylating MSH2 at tyrosine 238 (Y238). Therefore, the introduction of the Y238F mutant leads to marked restoration of the MMR function[76]. These findings indicate that NPM-ALK can induce genome-wide destabilization by interfering with DNA damage repair processes (figure 7).

-Tissue invasiveness and tumor spread through induction of a stem cell–like program.

NPM-ALK affects cell proliferation, invasiveness, and metastatic spread through the reversion to a more undifferentiated phenotype by the expression of embryonic genes such as SOX2[77]. SOX2 is a known STAT3 target in embryonic and neural stem cells[78]. SOX2 is expressed by ALK+ALCL cell lines and primary tumors, and its expression is enriched in a subpopulation of cells with stem cell–like properties on the basis of side population analysis[79]. These SOX2 positive ALK+ALCL cells exhibit better invasiveness and tumorigenesis[80] (figure 7).

-NPM-ALK promotes activation of cell metabolism

Pyruvate kinase PKM2 is a NPM-ALK target; its inhibition by NPM-ALK–mediated tyrosine phosphorylation results in a shift from oxidative phosphorylation to anaerobic glycolysis. This metabolism shift enhanced cell proliferation *in vitro* and tumorigenesis *in vivo*. A small molecule

activator of PKM2 suppressed cell growth, suggesting that this specific kinase or that the tumor metabolism could represent a new promising therapeutic target in ALK+ALCL[81] (figure 7).

-Oncogenic ALK blocks the tumour suppressive functions of the TGF-β pathway

TGF-β signalling plays critical roles in tumorigenesis by regulating cell proliferation, apoptosis, angiogenesis, immune surveillance and metastasis[82-83]. TGF-β binds to its dual-specificity kinase receptor complex, consisting of type II and type I receptors, which phosphorylates SMAD2 (Mothers against decapentaplegic homolog 2) and/or SMAD3[84]. Phosphorylated SMAD2 and/or SMAD3 then forms an oligomeric complex with SMAD4. The SMAD complex is transported into the nucleus, where SMADs bind to promoters of target genes such as CDKN2B (Cyclin-dependent kinase inhibitor 2 B), CDKN1A and CDKN1C to confer cell cycle arrest[85]. Given its essential role in TGF-β signal transduction, the activity of the SMAD4 tumour suppressor protein must be tightly regulated. Oncogenic ALK was found to directly phosphorylate SMAD4 on Tyr 95 in various cancer cells and tissues, including lymphoma, lung tumours and neuroblastoma. This phosphorylation disables the DNA-binding activity of SMAD4, and consequently disarms TGF-β tumour suppressing responses[86].

-Modification of the (actin) cytoskeleton via small G proteins

Many studies pointed out that Rho family GTPases could have a critical role in the biology of T-cell lymphoma. In ALCL, the Rho family GTPases Cdc42 and Rac1 are activated by the ALK oncogenic activity. Ambrogio et al. have shown that NPM-ALK regulates the shape of ALCL cells and F-actin filament assembly in a pattern similar to T-cell receptor–stimulated cells. In particular, NPM-ALK forms a complex with the guanine exchange factor VAV1, enhancing its activation through phosphorylation. Subsequently, VAV1 increases Cdc42 activity, and in turn, Cdc42 regulates the shape and migration of ALCL cells[87].

Similarly, Colomba et al. have shown that Rac1 GTPase, a known cytoskeletal regulator, is activated by NPM-ALK and that Vav3 is one of the exchange factors involved in Rac1 activation[88,89].

Moreover, Chiarle et al. have shown that either Cdc42 or Rac1 deletion impaired lymphoma development, modified lymphoma morphology, actin filament distribution, and migration properties of lymphoma cells *in vivo*[90].

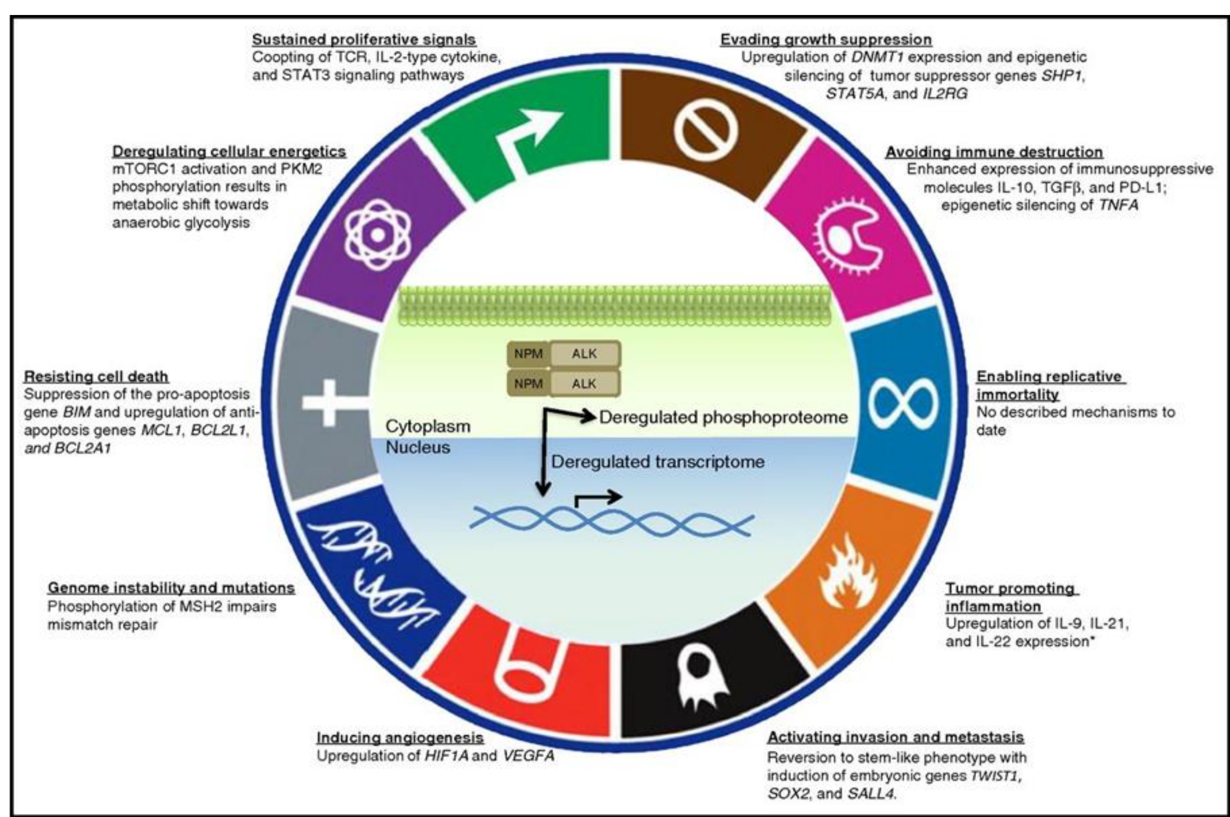


Figure 7. NPM-ALK achieves most of the "Hallmarks of Cancer." NPM-ALK is the protein product of a chromosomal translocation. Through activation of T-cell signaling pathways and dysregulation of the phosphoproteome, NPM-ALK drives the transformation of T cells into ALK1 ALCL by multiple oncogenic mechanisms *(Wasik et al 2016).*

4 Current Treatment in ALCL

ALK+ ALCL is associated with favorable prognosis, whereas ALK- ALCL shows heterogeneous clinical, phenotypical, and genetic features, often associated with unfavorable prognosis[91]. In this chapter, we will focus our attention on the current and experimental ALK+ ALCL treatments.

4.1 First line treatment

Prior to the classification of ALK+ ALCL as a distinct Non Hodgkin Lymphoma (NHL) entity in 1989 (see above), most patients were enrolled in both B- and T-cell NHL trials and thus were treated with the chemotherapeutic regimen used for these diseases. In Europe, the leading NHL-Berlin-Frankfurt-Munster (NHL-BFM) working group used a multi-agent chemotherapy[92]. The drugs included and their modes of action are listed below.

- **Cyclophosphamide** is an alkylating agent which binds to DNA and forms cross-links, resulting in DNA damages.

- **Ifosfamide** is similar in chemical structure to cyclophosphamide. This drug is also an alkylating agent.

- **Etoposide** inhibits DNA synthesis by forming a complex with topoisomerase II and DNA. This complex induces breaks in double stranded DNA and prevents repair by topoisomerase II binding. Accumulated breaks in DNA prevent entry into the mitotic phase of cell division, and lead to cell death.

- **Methotrexate** is an anti-neoplastic anti-metabolite with immunosuppressant properties. It is an inhibitor of tetrahydrofolate dehydrogenase and prevents the formation of tetrahydrofolate, necessary for synthesis of thymidylate, an essential component of DNA.

- **Cytarabine** (or ARA-C) is an anti-metabolite anti-neoplastic agent inhibiting the synthesis of DNA. It also has immunosuppressive properties.

- Doxorubicin hydrochloride is an antineoplastic agent of the anthracycline class. Doxorubicin has antimitotic and cytotoxic activity. It forms complexes with DNA by intercalation between base pairs, and it inhibits topoisomerase II activity by stabilizing the DNA-topoisomerase II complex.

- Vincristine is part of a group of drugs called vinca alkaloids. This drug binds to the microtubular proteins of the mitotic spindle, leading to crystallization of the microtubule and mitotic arrest or cell death. Vincristine has some immunosuppressant effect.

- Prednisone is a glucocorticoid similar to cortisol used for its anti-inflammatory, immunosuppressive, anti-neoplastic, and vasoconstrictive effects.

Through these regimens and trials were not primarily aimed for ALCL, a retrospective analysis revealed:

- 81% Overall Survival (OS). This criteria is the percentage of people who are still alive after a certain period of time following the diagnosis or the beginning of the therapeutic treatment. The overall survival rate is often stated as a five-year survival rate, which thus corresponds to the percentage of people who are alive five years after their diagnosis or the start of treatment.

- 83% Event Free Survival (EFS). This criteria represents the percentage of people who show a particular group of defined and adverse events (i.e. bone pain) or disease progression after a defined treatment duration.

In clinical trials, measuring OS and EFS is indicative on how well a new treatment works.

These results motivated the European Inter-group for Childhood NHL (EICNHL) to launch the first international ALCL-specific clinical trial, in 1999. This trial remains to date the largest clinical trial dedicated to pediatric ALCL. It enrolled 352 children over 7 years in 11 European countries and Japan, regardless of ALK status. It was based on the previous NHL-BFM protocol and was specifically designed to compare the efficiency of two doses of methotrexate (MTX1 or MTX3) administered either via intrathecal injection (injection into the spinal canal or into the subarachnoid space) or intravenously, respectively, and to assess if vinblastine (VBL), which binds the microtubular proteins of the mitotic spindle leading to mitotic arrest or to cell death, could be a valuable addition to the protocol. The conclusions were that MTX3 was less toxic than MTX1 and that VBL addition did not improve the 2-year

EFS of 74%. The trial reported also a 2-year OS of 92%, which is excellent and defined ALCL99 frontline regimen as gold standard for paediatric ALCL [93-94-95]. Of importance, other clinical trials in Europe and North America (listed in Table 3) used different chemotherapeutic backbones. The American Children's Oncology Group (COG) notably designed trials based on Adriamycin (Doxorubicin), prednisone (an anti-inflammatory drug of the glucocorticosteroid class) and vincristine (APO) chemotherapy backbone, which were as efficient as European regimen since a similar EFS of 76% at 5 years was observed[96]. However, since the European ALCL99 presented less long term cardiac toxicity than the American APO backbone, the ALCL99 regimen is now internationally accepted[20].

Trial Acromym	Other	Cyc	Ifo	Doxo	Eto	MTX (I/T)	MTX (IV)	ARA-C (IV)	ARA-C (I/T)	VCR	VND	VBL	EFS (YEAR)	OS (YEAR)	Country
NHL-BFM90-(K2 arm)		■	■	■	■	■	■	■	■				73% (5)	N/A	Germany
NHL-BFM90-(K3 arm)		■	■	■	■	■	■	■	■	■			76% (5)	N/A	Germany
NHL-BFM95(R1/2)		■	■	■	■	■	■	■	■	■			N/A	N/A	Germany
NHL-BFM95(R3/4)		■			■	■	■		■		■		N/A	N/A	Germany
EICNHL-ALCL99(MTX1-arm)		■	■	■	■	■			■				74% (2)	90% (2)	Europe
EICNHL-ALCL99(MTX3-arm)		■	■	■	■		■		■				75% (2)	95% (2)	Europe
EICNHL-ALCL99-VBL		■	■	■	■	■	■		■			■	70% (2)	94% (2)	Europe
POG9315(APO arm)				■		■				■			71% (5)	88% (4)	Netherlands
POG9315(IDM-HiDAC arm)				■		■	■	■		■			71% (4)	88% (4)	Netherlands
CCG-5941		■		■	■	■	■	■	■	■			68% (5)	80% (5)	Netherlands
LNH-92	+ Daun	■			■	■	■	■	■	■			69% (5)	74% (5)	Netherlands
ANHL0131(APO arm)				■		■				■			74% (3)	84% (3)	Italy
ANHL0131(APV arm)				■		■				■		■	79% (3)	86% (3)	Italy

Table 3. Treatment strategies for childhood ALCL. ARA-C = cytarabine; Cyc = cyclophosphamide; Daun = daunorobicin; Doxo = doxorubicin; Eto = etoposide; EFS=Event Free Survival OS= Overall Survival IDM-HiDAC = intermediate dose MTX high-dose Cytarabine; Ifo = ifosfamide; I/T = intrathecal; IV= Intravenous; MTX = methotrexate; TT = topotecan; VBL = vinblastine; VCR = vincristine; VND =Vindesine *(adapted from Prokoph et al 2018)*

There are no prospective randomized trials for adult ALK+ ALCL. Most published first line regimens include usually CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone) in adult. A retrospective analysis of 78 ALCL found that overall survival (OS) of adult ALK+ ALCL (53/78) was far superior to ALK-negative ALCL (71% vs. 15%). In conclusion, the majority of adult patients are cured with standard first line approaches, with a long-term survival of at least 70%, depending on a number of disease and patient-related factors[97].

4.2 Treatment of relapsed or refractory ALK+ALCL

Following ALK+ ALCL diagnosis and chemotherapy according to the ALCL99 defined standard regimen, it was observed that some patients never regressed and are thus defined as refractory to the treatment or regressed but subsequently relapsed. The refractory patients account for 10-15% of the cases, the relapsed patients account for 20 to 40% of the cases. Of note, the causes for disease progression and relapse are still not known, and the concerned patients suffer from a bad prognosis.

There is no clear consensus for the treatment of refractory and relapsed ALK+ ALCL. Two main strategies, Hematopoietic Stem Cell Transplantation (HSCT) and chemotherapy, have been tested:

*in line with the standard of care for children with other forms of NHL, ALK+ ALCL relapsed patients have been treated by HSCT, either allogeneic (stem cells from a genetically similar donor) or autologous (stem cells are first removed, stored, and later given back to the same person). Several retrospective European and Japanese studies suggest a trend towards better patient's outcome when treated with allogeneic versus autologous SCT [3]. This was further confirmed recently by the EICNHL-ALCL-RELAPSE trial [20].

*Other important studies showed that chemotherapy was still effective in relapsed patients [98]. In particular single-agent chemotherapy, using Vinblastine at low dose within a long-term regimen (24 months), was found to achieve high remission rates for both relapsed and refractory patients [99]. Thus, ALK+ ALCL is a peculiar disease with relapses still being chemosensitive.

4.3 Biomarkers and Prognostic factors

Some clinical factors, such as visceral or mediastinal disease and bone marrow involvement, initially showed promise as predictors of treatment failure[100-23-101]. Unfortunately, these prognostic features were identified in retrospective studies, and have not been demonstrated to be effective in a prospective randomised clinical trial. Potential biomarkers, such as circulating tumor cells in bone marrow or peripheral blood, may have prognostic value[102]. Tumor cells in peripheral blood, or minimal disseminated disease (MDD), has gained traction in recent years as a biomarker for ALCL. Indeed,

studies have shown significant differences in progression-free survival when retrospectively stratified using a combination of MDD and anti-ALK autoantibody titers, the latter being another promising biomarker for ALCL[103]. Anti-ALK autoantibodies can be identified and quantified in a majority of patients, and may, on their own, help to predict risk of relapse[104-105]. Other potential biomarkers for relapse risk are micro-RNAs (miRNA) detected in exosomes within the peripheral blood of patients. In particular, miR-103a-3p and miR-223-3p, when detected in exosomes, are predictive of relapse, potentially because they are thought to increase the invasiveness of ALCL cells (figure 8)[3].

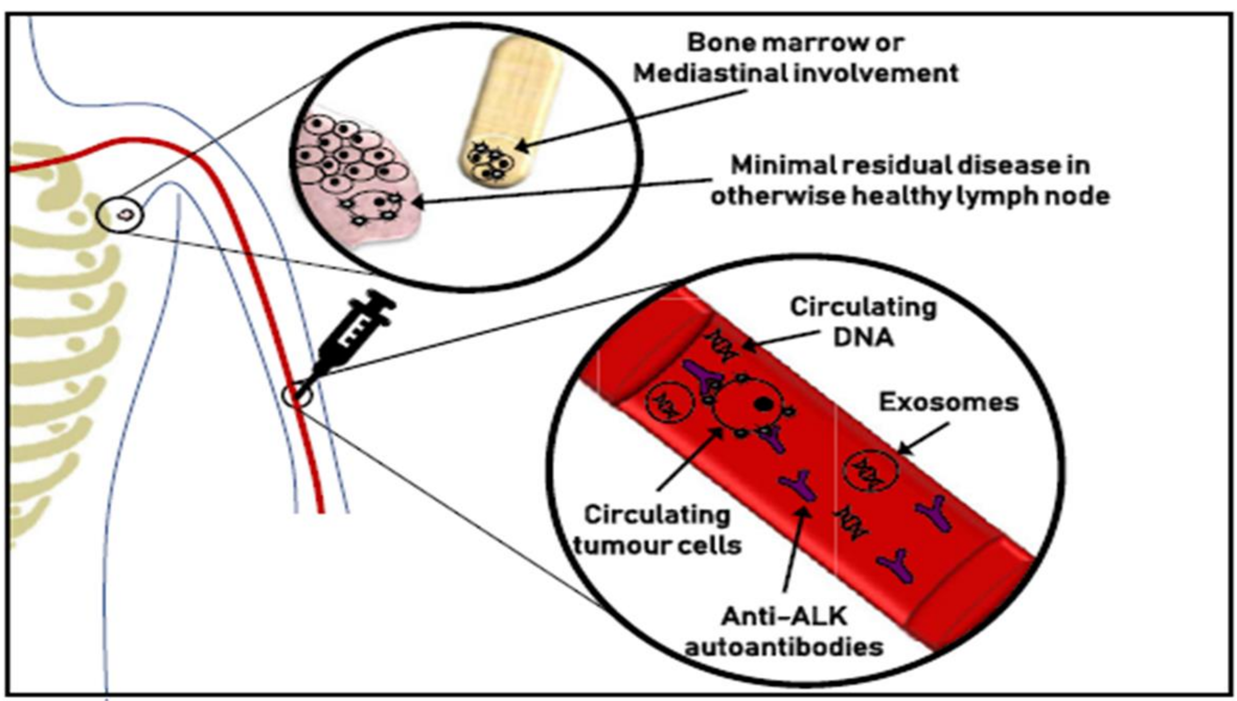


Figure 8. Graphic representation of the main biomarkers in ALCL. The most obvious prognostic biomarkers are of a clinical nature, including bone-marrow or mediastinal involvement. Minimal residual disease is also a predictive biomarker. More recent liquid biopsy-based biomarkers show promise, though have yet to be validated in prospective clinical trials, these include exosomes (and miRNA profiles), circulating tumour cells (minimal disseminated disease), circulating DNA and anti-anaplastic lymphoma kinase (ALK) autoantibodies. A combination of these may yet prove to be sufficient to predict ALCL prognosis or relapse *(Larose et al 2019).*

4.4. Knowledges from basic sciences: effective translation to the clinics

Two mean caveats have emerged from the retrospective analysis of the clinical trials data:

***10 to 15%** of the ALK+ ALCL diagnosed patients do not survive[106-20].

***24 to 40%** of the survivors suffer from the late effects of the intensive therapy and/or transplantation (sterility, secondary malignancies) required to cure them. These side effects are particularly important in the pediatric population[107].

Therefore, there is still a clear need for the development of new therapies, which implies a strong fundamental research on ALCL. In this paragraph, we will focus on the knowledges from basic science which have been successfully translated in clinics.

4.4.1 Brentuximab Vedotin

Therapies targeting CD30 have been developed and assessed in ALCL patients[108]. After an initial phase where several anti-CD30 antibodies (i.e. the human Ig G1k antibody MDX-060[109], the human antibody 5F11[110], the chimeric antibody SGN-30[111] and the immunotoxin ki-4dgA [112]) showed considerable *in vitro* activity, clinical studies from patients with CD30-positive lymphomas (i.e. Hodgkin lymphoma and ALCL) demonstrated only modest activity. However, another compound, Brentuximab vedotin (BV or SGN-35), seemed to be more promising. This agent is a conjugate constituted by the antitubulin agent monomethyl auristatin E and a CD30-specific monoclonal antibody that has shown excellent activity both in Hodgkin lymphoma and ALCL[113]. It thus received FDA approval as a frontline single-agent therapy in ALCL only for use in chemo-resistant, relapsed adult ALCL patients. A paediatric-specific phase I/II trial for relapsed or refractory ALCL patients was launched in 2012 (NCT01492088). Unfortunately, results from this study were disappointing as only 53% of ALCL patients achieved an overall response, and all patients on the study experienced adverse events[114]. Therefore, other strategies targeting CD30 are still under study (see paragraph 4.6.4).

4.4.2 Imatinib

Several studies have linked NPM-ALK expression with the induction of the activator protein 1 (AP-1) transcription factors JUNB and JUN [115-116]. Moreover, it has been shown that JUN and JUNB promote lymphoma development and tumor dissemination through transcriptional regulation of platelet-derived growth factor receptor-b (PDGFRB) in NPM-ALK mouse model. Thus, inhibitors of PDGFR such

as Imatinib (inhibitor of BCR–ABL kinase, receptor tyrosine kinase KIT and PDGFRβ), may provide a viable therapeutic approach (figure 9). Indeed, a dramatic increase in overall survival was observed in Imatinib treated CD4-NPM–ALK transgenic mice. In addition, treatment of a refractory, late-stage NPM–ALK+ ALCL patient with Imatinib led to complete and sustained remission[117-118]. These findings suggest that targeting PDGFRβ is a promising alternative therapeutic option for ALCL, which is currently being explored in a clinical trial (EudraCT Nr.: 2013-003505-26).

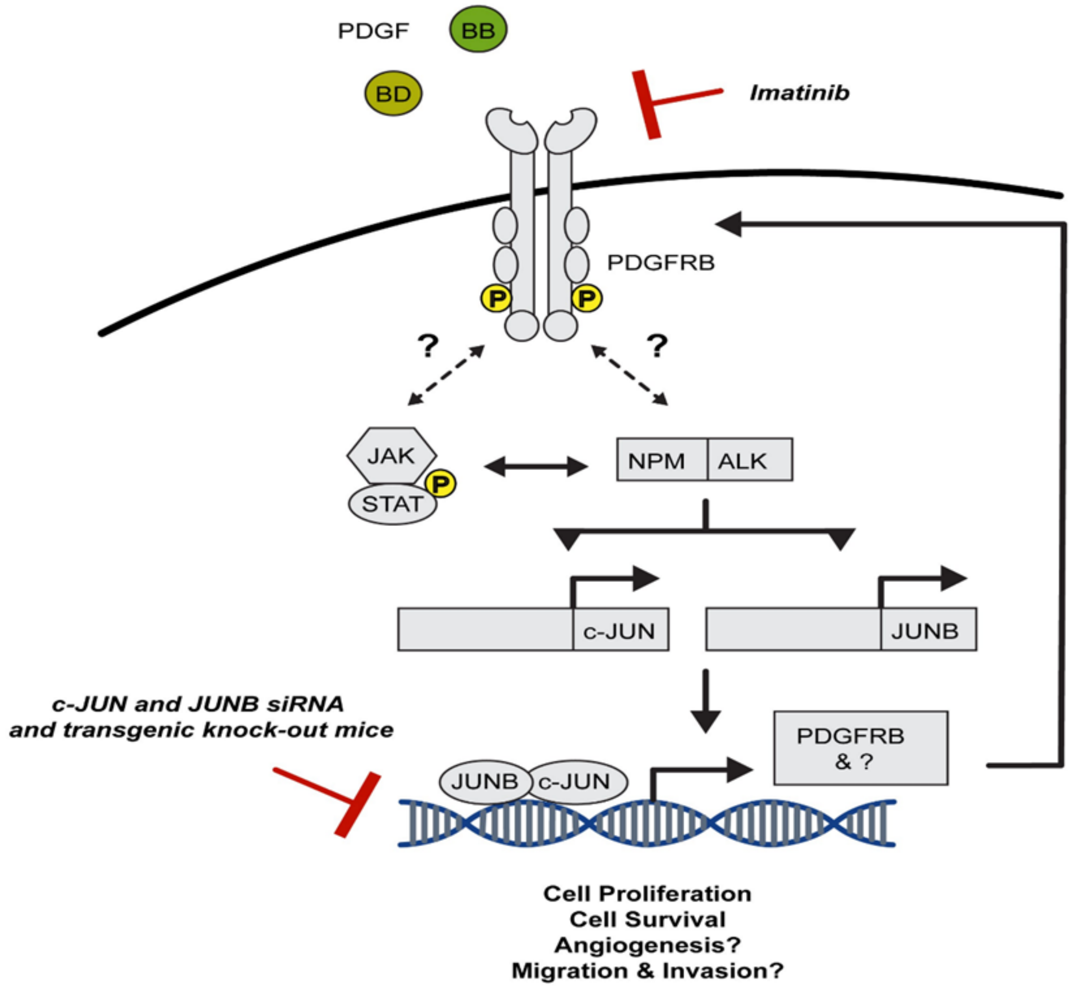


Figure 9. Blockade of the PDGFRB/STAT/NPM-ALK/JUN signalling cascade in ALK+Anaplastic Large Cell Lymphoma (ALCL). PDGFRB phosphorylates the JAK/STAT signalling cascade, which is known to interact with the oncogenic NPM–ALK fusion. The downstream effector targets of NPM–ALK, c-JUN and JUNB, directly bind to the PDGFBRB promoter resulting in an increase in PDGF expression, overall propagating a malignant signalling cascade. Treatment with Imatinib, a tyrosine kinase inhibitor, results in a decrease in tumour burden and increased survival of c-JUN and JUNB transgenic knock-out mice harbouring the constitutively active NPM–ALK fusion in CD4+ T cells (*Alonso et al 2018*).

4.4.3 ALK Tyrosine kinase inhibitors class

** Crizotinib: A First-Generation ALK Inhibitor*

ALK is arguably the ideal target for ALK-positive disease due to:

- The tumour addiction to its expression. Indeed, the inhibition of NPM-ALK alone in cells and murine models leads to tumour regression[33].

- The absence of its expression in healthy tissues. [119-120]

PF-02341066 (Crizotinib), is an orally bioavailable small molecule inhibitor produced by Pfizer that caused complete regression of NPM-ALK xenografts at pharmacologically relevant doses. Crizotinib was originally discovered as a c-Met kinase inhibitor[121]. Ironically, the drug was found to have off-target effects on other kinases including ALK. The crystal structure of crizotinib bound to ALK revealed a similar binding mode as for c-Met, involving a conserved hydrogen bond (Hb) with hinge region residues (Figure 10)[122]. Following a number of successful *in vitro* studies showing the efficacy of crizotinib in ALK inhibition[123-121], the molecule entered clinical trials, first for ALK+ NSCLC, and then for other ALK-dependant malignancies including IMT, NB and ALCL.

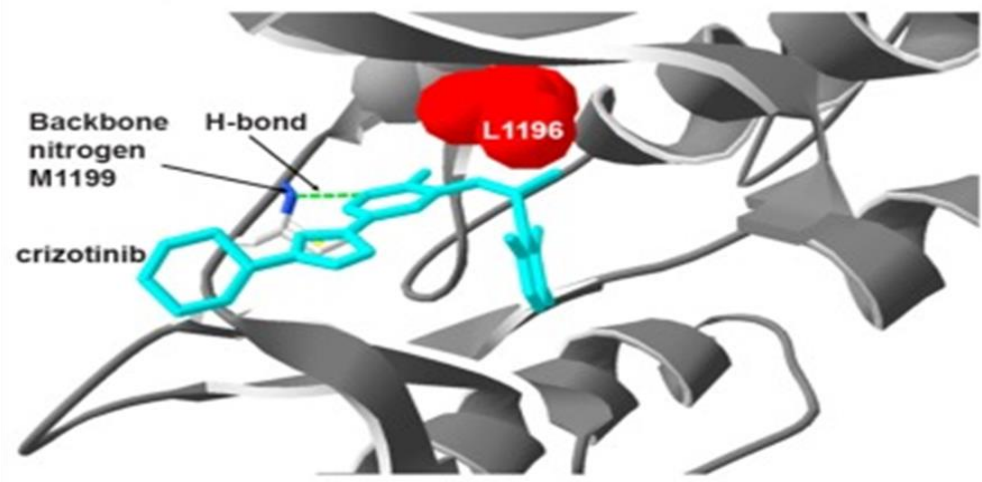


Figure 10. Crystal structure of ALK in complex with crizotinib (PDB: 2XP2). Close view of crizotinib bound in the active site of wild-type ALK. The gatekeeper residue L1196 is shown as red surface. Crizotinib is shown as cyan sticks. The green dashed line indicates the hydrogen bonding to the backbone nitrogen of M1199 (indicated in sticks). Secondary structures are represented with grey ribbon *(Sharma et al 2018).*

*Concerning ALK+ NSCLC, an early phase I study (PROFILE 1001) showed a sustained response in locally advanced or metastatic NSCLC patients carrying the EML4-ALK fusion gene. Subsequently, crizotinib was evaluated in a phase II study (PROFILE 1005) which positive results supported the clinical benefits of using crizotinib in ALK-positive NSCLC that had progressed on previous chemotherapy regimens. Two phase III studies, PROFILE 1007 and PROFILE 1014, provided further proofs in favor of the use of crizotinib over standard second-line chemotherapy and over first-line chemotherapy, respectively in advanced ALK-positive NSCLC[48].

*Concerning Inflammatory myfibroblastic Tumor (IMT), the clinical trial NCT00585195 showed a sustained partial response to crizotinib in a patient with ALK-translocated IMT, as compared with no observed activity in another patient without the ALK translocation. These results support the dependence of ALK-rearranged tumors on ALK-mediated signaling and suggest a therapeutic strategy for genomically identified patients with the aggressive form of this soft-tissue tumor[124].

*Concerning Neuroblastoma (NB), early results from the clinical trial (NCT00939770), evaluating the pharmacokinetic profile of crizotinib were disappointing; of eleven patients with known activating ALK mutations, only one had a complete response and two had stable disease. This is consistent with the differential sensitivity of ALK mutants to crizotinib [125].

*Concerning ALCL, results are promising, particularly for refractory adult ALCL patients, for whom the odds of survival have improved significantly, from 30% to 73% [126]. Another study called PROFILE 1013 (NCT01121588) has evaluated the safety and antitumor activity of single-agent, oral crizotinib among 18 young adult patients with advanced ALCL. Results of PROFILE 1013 showed notable and durable antitumor activity of crizotinib as monotherapy for patients with advanced, relapsed or refractory ALK-positive ALCL. Moreover, this study suggests that crizotinib may also offer a potential long-term treatment option, with an OS at 2 years similar to that reported with first-line CHOP chemotherapy in patients with ALK-positive ALCL. Recent data in a paediatric setting have also shown encouraging outcomes, with response rates for refractory and relapse ALCL patients of between 80% and 90% depending on dosage[127]. Finally in Japan, the trial UMIN000028075 is investigating the efficacy and safety of crizotinib as monotherapy for children with recurrent or refractory ALK-positive ALCL. Results are expected in 2022.

** Resistance against Crizotinib*

Despite the remarkable responses that have been observed in patients with ALK rearrangements, resistance to crizotinib eventually develops and rather quickly, making durable response unachievable, particularly in NSCLC. Resistance to crizotinib was also reported in NB[125], in IMT[128] and ALCL[129]. One of the resistance acquired mechanisms to crizotinib is the selection of point mutations within the drug target that alter drug sensitivity. These point mutations are reported in Table 4.

Table 4. Mutational profile of ALK that induce Crizotinib resistance (adapted from Sharma *et al 2018)*.

TKI	Sensitive Mutants	Resistant Mutants	Disease	Evidence (In Vitro/ In Vivo/Clinical)
Crizotinib	L1198F	I1151Tins	NSCLC	Clinical
		L1152R	NSCLC	Clinical
		C1156Y	NSCLC	Clinical
		I1171T/N	NSCLC	Clinical
		F1174L	IMT	Clinical
		L1196M	NSCLC	Clinical
		L1196Q	NSCLC	Clinical
		L1198P	EML4-ALK BaF3 cells	In vitro
		G1202R	NSCLC	Clinical
		D1203N	NSCLC	Clinical
		S1206Y	NSCLC	Clinical
		G1269A	NSCLC, IMT	Clinical

The L1196M mutation: The first case of resistance against crizotinib was reported in an EML4-ALK-positive NSCLC patient. The tumor resumed growth after an initial partial response over a period of 5 months. Deep sequencing analysis of the patient sample revealed a L1196M mutation[130]. The L1196 residue is a conserved gatekeeper residue located close to the ATP pocket and crizotinib binding site. In this secondary mutation, a smaller residue (leucine) is replaced by a larger residue (methionine). In contrast to small leucine residue, the large methionine did block the access of the inhibitor to the adjacent hydrophobic pocket. This methionine substitution, in addition, has been reported to increase the enzyme activity by strengthening the hydrophobic R-spine which then promotes the formation of the active protein conformation[131]. L1196M mutant EML4-ALK protein was found to have higher phosphorylation levels. These results show that the L1196M substitution confers drug resistance by increasing the protein kinase activity (figure 11)[132]. Together with L1196M mutation in NSCLC patients, it has been found a C1156Y substitution at a relatively high frequency. C1156Y mutation creates a displacement of crizotinib along with some conformational changes in the binding site of the drug that

eventually decreases crizotinib affinity and leads to drug resistance[129]. In figure 12 are showed all the keys residues associated with Crizotinib resistance.

In ALK+ALCL patients, the mutations Q1064R, I1171N and M1328I have been described to confer resistance to Crizotinib. All these mutations were not present in samples obtained before crizotinib treatment. Since these residues do not form direct contacts with crizotinib, they probably interact with different structures within the catalytic domain [126].

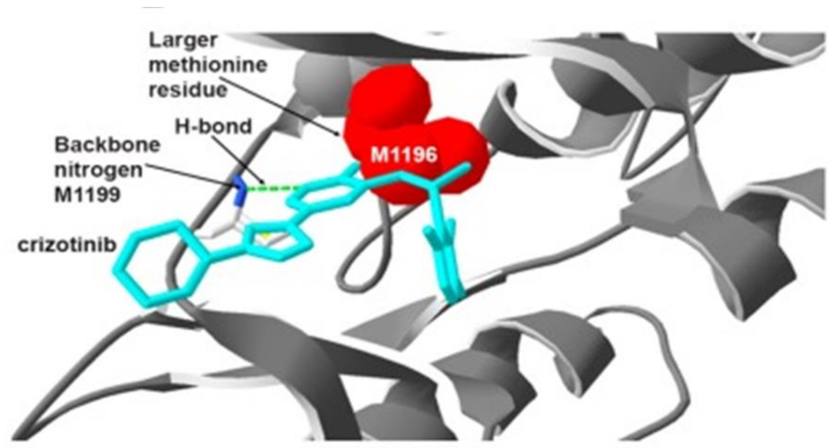


Figure 11. Crystal structure of M1196L mutated ALK in complex with crizotinib The L1196M mutation show steric clash with crizotinib which confer resistance (*Sharma et al 2018).*

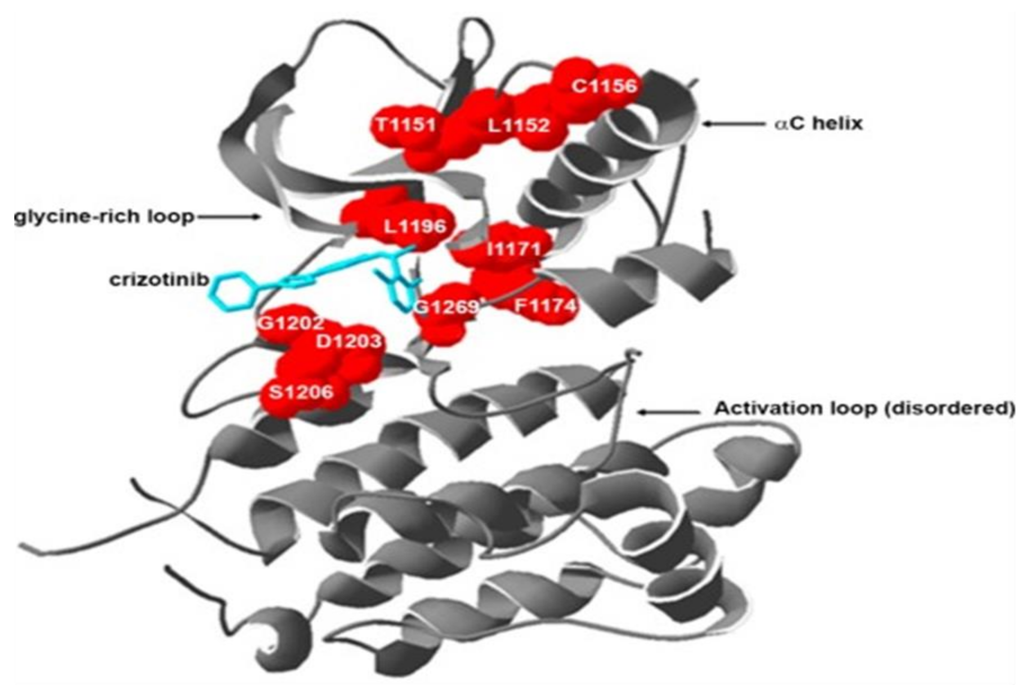


Figure 12. Overall architecture of ALK bound to crizotinib. Key residues associated with resistance to crizotinib are shown as red surface and labeled. Some important regulatory regions of the kinase are indicated by arrows *(Sharma et al 2018).*

4.4.5 Second and Third Generation ALK Inhibitors

Interestingly, crizotinib-resistant tumors were often found to still continue to be ALK-dependent for their growth. Indeed, around 30% of the cases of crizotinib resistance are due to the occurrence of secondary mutations in the ALK TK domain. Therefore, more potent, selective and structurally different next-generation ALK inhibitors have been developed or are in the pipeline to overcome crizotinib resistance. Although they are not functionally or structurally related to crizotinib (except lorlatinib) they are usually referred as second-generation and third-generation inhibitors as they were all developed to tackle crizotinib-resistance mutants (Table 5)[48]. So far, they have been tested in ALK+ NSCLC trials (as discussed below), but it is likely that their use will be extended in a near future to the treatment of ALK+ ALCL.

Table 5. ALK inhibitors, indication and active ALCL trials

Name	Company	Generation	FDA approval (country)	Active ALCL clinical trials	Phase	Notes
Crizotinib (Xalkori)	Pfizer	First	Locally advanced and metastatic NSCLC (US)	NCT00939770	1/2	Pediatric ALCL, CNS tumors (monotherapy)
				NCT02487316	4	Adult ALCL (with or without chemotherapy)
				NCT01606878	1	Pediatric ALCL (combination with chemotherapy)
				NCT02584634	1	Adult NSCLC (combination avelumab with crizotinib or lorlatinib)
Ceritinib (LDK378, Zykadia)	Novartis	Second	Metastatic crizotinib-resistant NSCLC (US)			
Alectinib (CH5424802, AF802)	Roche/Chugai	Second	Crizotinib-resistant NSCLC (US); ALK+ NSCLC (Japan)	NCT01588028	1	ALK+ NSCLC
ASP3026	Astellas	Second		NCT01284192	1	Adult refractory solid tumor or B-cell lymphoma
Brigatinib (AP26113)	Ariad	Second		NCT01449461	1/2	Adult NSCLC, ALCL, DLCL, IMFT
				NCT02094573	2	Adult NSCLC
Lorlatinib (PF-06463922)	Pfizer	Third		NCT01970865	1/2	Adult ALK+ and ROS1+ NSCLC
				NCT02584634	1	Adult NSCLC, avelumab (with crizotinib or lorlatinib)
				NCT02569554	1	Adult, healthy, PPI, and food effect

CNS, central nervous system; DLCL, diffuse large cell lymphoma; IMFT, inflammatory myofibroblasic tumor; PPI, proton pump inhibitor; US, United States *(Wasik et al 2016)*.

Ceritinib (LDK378; Zykadia; Novartis)

Ceritinib is an ATP-competitive, selective oral ALK inhibitor that was found to be 20 fold more potent than crizotinib in enzymatic assays. Ceritinib inhibited *in vitro* and *in vivo* the growth of ALK-positive cells carrying crizotinib-resistant mutations, L1196M, G1269A, I1171T, and S1206Y but failed to inhibit the growth of G1202R and F1174V/C mutants [133]. In 2016, ceritinib received an accelerated approval from FDA for the treatment of ALK-positive metastatic NSCLC patients with disease progression or intolerance to crizotinib[134]. Indeed, several trials (ASCEND-1, ASCEND-2and ASCEND-3) revealed the higher efficacy of ceritinib in ALK-rearranged NSCLC [135,136].

Brigatinib (AP26113; Ariad)

Brigatinib, another orally available potent next-generation-ALK/ROS1/EGFR inhibitor, had displayed activity against the tyrosine kinases as well as some of their mutant forms in cellular and pre-clinical models. Preclinical data showed that Brigatinib has pan-ALK inhibitory profile (i.e., blocks all crizotinib-resistant mutants) in cellular models at clinically achievable levels, although it still suffers a significant loss of activity against the G1202R mutant[137]. On 28 April, 2017, the FDA granted an accelerated

approval to brigatinib for the treatment of ALK+ metastatic NSCLC patients. A phase III trial, ALTA-1L (NCT02737501) is ongoing to compare the efficacy and safety of brigatinib with those of crizotinib as a first-line treatment in patients with ALK+ metastatic NSCLC[138].

Lorlatinib (PF-06463922; Pfizer)

Lorlatinib is a third generation TKI developed by Pfizer through cyclization and further modification of their first-generation compound, crizotinib, with the aim to improve brain penetration and inhibition of drug-resistant ALK mutants. Indeed, lorlatinib inhibited wilt-type and mutant ALK, including the highly resistant G1202R mutant, at a sub-nanomolar concentration in cell-line models. Thus, lorlatinib appears as an effective therapeutic approach for patients with ALK-driven NSCLC who have become resistant to the currently available TKIs, including second-generation ALK TKIs[139]. Moreover, a phase III study comparing lorlatinib with crizotinib as monotherapy in terms of prolonging progression-free survival and overall survival in the treatment of naïve advanced ALK-positive NSCLC patients is currently ongoing (NCT03052608). Even though lorlatinib is a potent inhibitor, the L1198F resistant mutation was reported in one ALK+ NSCLC patient after receiving lorlatinib treatment for 8 months. The patient had been treated with two prior TKIs, crizotinib and ceritinib, and became refractory to both of them. Surprisingly, the L1198F lorlatinib resistant tumor regained sensitivity to crizotinib [140].

Resistance to Second and third- generation ALK TKIs

Even though the second generation of ALK inhibitors is proven to be more potent and highly selective with tolerable adverse events, the biggest setback still stays in the form of acquired resistance against them. For example, while ceritinib was able to overcome some of the secondary ALK resistance mutations that arise after crizotinib treatment, G1202R, F1174C/V mutations were reported to be selected by ceritinib. Structural analysis revealed that G1202R substitution causes a significant loss in ceritinib binding due to steric hindrance [133]. On the other hand, alectinib was shown to be effective against crizotinib or ceritinib resistant mutations, but leads to the acquisition of I1171T and V1180L resistant mutations *in vitro* and in patients. Interestingly, these two mutations could be overcomed with ceritinib treatment which supports the idea of using two different inhibitors/combinatorial

therapy. Again, the G1202R emerged as a highly intractable mutant[141]. The current therapeutic paradigm for patients with ALK-positive malignancies is to treat with sequential ALK targeted therapies, often moving from first- to second- to third-generation ALK inhibitors. Resistance against the third-generation ALK-TKI lorlatinib, which is currently the later line of therapy, has been reported in different double compound mutations found via mutagenesis screening and clinical samples[142-143-144-145] .

In order to implement these combination therapies in ALK-positive cancers, it is crucial to identify possible additional tumor cells vulnerabilities, which would help in developing effective combined therapeutic strategies for ALK+ cancer patients.

4.5 Combined therapies

4.5.1 ALCL99 + targeted therapies (BV or TKI) as frontline treatment

*In USA, the Children Oncology Group (COG) trial ANHL12P1 (NCT01979536) is currently testing the addition of BV (Brentuximab Vedotin) or crizotinib to ALCL99 chemotherapy for newly diagnosed patients with ALCL. Patients have been randomized to receive either BV (18 mg/m2 once per cycle) or crizotinib (165 mg/m2 BID (bi-dayly) for 21 days each cycle) along with the ALCL99 backbone. The trial opened in november 2013 in 135 institutions and has enrolled 123 patients as of 1st october 2018. Data should be available in march 2021.

*In Europe, the EICNHL (European Inter-Group for Childhood Non-Hodgkin Lymphoma) is planning also to trial an ALK inhibitor in combination with the ALCL99 chemotherapeutic backbone as frontline treatment in a phase I safety study. Unfortunately, so far, no ALK inhibitor has been selected or agreed for use in this study, although crizotinib is the obvious candidate due to its longer history of use in adults, proven safety, and efficacy in ALK-positive NSCLC. Indeed, crizotinib and combination chemotherapy have already been tested for treating younger patients with relapsed or refractory Solid Tumors or Anaplastic Large Cell Lymphoma (NCT01606878). Moreover, a trial for adults with ALK-positive ALCL is underway (NCT02419287)[20].The other potential candidate is ceritinib, although its use

is associated with significant gastrointestinal toxicities, which may limit its application in a paediatric population. However, ceritinib has shown long-lasting responses in three ALK-positive adult ALCL relapsed patients. In this study, the high remission rate, long duration of remission, and acceptable tolerability of treatment support the use of ceritinib in the treatment of patients with ALK-positive ALCL[146].

4.6 Knowledges from basic sciences: recent findings and possible evolution of the therapy

Numbers of potential therapeutic targets have been identified in research laboratories but most have yet to make headway at the bedside. For instance, the signalling pathways which are deregulated in ALCL have been extensively studied and documented, providing multiple potential targets for therapy [147-148-56-149-150-151-152-153-154]. Moreover, different immunological strategies to treat ALK+ALCL have been designed [155]. In this paragraph will be discussed some of these new approaches.

4.6.1 Targeting STAT-3

STAT3 is a critical mediator of NPM-ALK–induced tumorigenesis[149]; indeed, STAT3 upregulation seems to be a frequent feature of ALCL regardless of ALK expression[156-157]. STAT3 is phosphorylated in normal cells by members of the JAK family in response to cytokine-cytokine receptor interaction. The phosphorylated form of STAT3 translocates into the nucleus and acts as a transcriptional activator. Several lines of evidence support its role in ALCL. First, STAT3 is required for ALK-mediated tumorigenesis in lymphoma[149]. Second, a strong correlation exists between ALK-dependent and IL-2-dependent transcriptional changes in ALK+ ALCL, the latest ones being known to be dependent on STAT3[158]. Third, convergent mutations of JAK1 and/or STAT3 itself in ALK-negative ALCL also result in STAT3 activation[156]. Therefore, STAT3 is an attractive drug target. However, the currently available STAT3 inhibitors have serious limitations because they are poor tissue-penetrating oligonucleotides or small molecules with rather low specificity[159].

4.6.2 Targeting immune evasion

The immune therapy of cancer has gained a huge interest over the last decade. ALK+ ALCL cells have been shown previously to evade the anti-tumour immune response by downregulating CD48 (lymphocyte activation marker), thereby promoting immune evasion. This process is reversed by inhibiting ALK [160]. Moreover, immune evasion is facilitated by NPM-ALK-induced expression of Programmed Cell-Death Ligand 1 (PDL-1, also termed CD274) on the surface of ALCL cells, which has been confirmed in patient tumours [161]. Mechanistically, the activation of the transcription factors STAT3, IRF4 and BATF3, downstream of NPM-ALK, was found recently to induce the expression of PD-L1 [162]. Finally, there are case reports of adolescent or young adult patients with relapsed ALCL responding to Nivolumab (which is a fully human IgG4 antibody targeting PDL-1) [163-164]. For this reason Nivolumab was put into clinical trials for both adult and pediatric relapsed and refractory ALK+ ALCL (NCT03703050, trial 'Nivo-ALCL').

4.6.3 Vaccine Therapy

Many evidences support that ALK has unique biological characteristics that are attractive for a tumor antigen. First, ALK is not expressed in obviously detectable levels by non-tumoral cells with the exception of specific regions of the central nervous system and the testis, both immunologically privileged sites. Second, tumors are known to be addicted to the ALK oncogene. Third, circulating antibodies against NPM-ALK and EML4-ALK proteins were found in ALK-positive ACLC and NSCLC, respectively[48].

*Anti-ALK Vaccine for ALK lymphoma:

As a proof of principle for the efficiency of an ALK-targeted vaccine, Dr Chiarle et al. showed in a ALK+ lymphoma mouse model the potential of a vaccination therapy with truncated ALK DNA, as well as the benefit of this approach when combined with chemotherapy. Altogether, this preclinical study and the existence of a "boostable" autologous response against ALK in humans support the design of an ALK epitope-directed vaccination study in patients in remission after chemotherapy. The most suitable patients would be those with a pre-existing immune response. Patients with a very weak immune

response against ALK usually relapse very early, within three months after therapy, which makes them less suitable for a vaccination approach. It is expected that this approach could either prevent or delay relapses.[155]

*Anti-ALK Vaccine for ALK lung cancer:

In the continuity of the work performed in ALK+ lymphoma, the same group has shown that ALK vaccination induced a strong and specific immune response either prophylactically or therapeutically against EML4-ALK+ lung tumors in preclinical models. This response was associated with an increase of ALK specific cytotoxic T-cells[165]. This result suggests that an ALK-directed vaccine therapy could have clinical efficacy in humans and that combination of such an anti-ALK vaccine therapy with checkpoint inhibitors should also be tested.

4.6.4 CAR-T Cell against CD30

Another recent CD30 targeting therapy uses T-cells engineered to express CD30 Chimeric Antigen Receptors (CAR-T cells) [3]. CAR-T cells are CD8+ T cells engineered to express a chimeric T cell receptor allowing the recognition of CD30 on the targeted tumor cells. CAR-T cells have received regulatory designation by the FDA for relapsed or refractory myeloma, and three clinical trials are currently ongoing in CD30-positive relapsed lymphomas in adults (NCT02259556, NCT01316146, NCT02274584). Preliminary results in patients have shown CAR-T cells to be reasonably safe and remissions of variable lengths were achieved. However, the authors note a large number of adverse events and the safety profile has yet to be tested in paediatric patients [166].

Targeting alternative pathways to improve ALK+ALCL treatment

Autophagy pathway has been shown to be induced upon therapies in different kinds of ALK-associated cancers. The development of drugs, which specifically inhibit or activate the autophagic process, and the search for the “right” therapeutic combinations, which could promote the appropriate autophagic response (i.e., cytotoxicity) are expanding research fields. To benefit from these advances, it is important to first investigate, when possible, the autophagic status in a patient’s tumor. The search for autophagy gene abnormalities (amplification, deletion, mutations) in ALK-associated cancers would

also bring useful information on the status of autophagy in primary and/or relapsed ALK-associated tumors. The development of biomarkers of the autophagic status in bodily fluids of cancer patients could also help at diagnosis, and could potentially orient the therapeutic strategy. In the next chapter will be extensively explored the autophagy pathway and its therapeutic modulation.

5 Autophagy

Autophagy is a primarily degradative pathway present in all eukaryotic cells. It has many functions, the most important are:

- Recycling cytoplasm contents to generate macromolecular building blocks and energy under stress conditions.

- Removing damaged organelles and proteins to maintain cellular homeostasis.

- Taking part to various aspects of immunity including the elimination of invasive microbes and its participation in antigen presentation[167].

5.1 Historical landmarks of autophagy

The term "autophagy" (from the Greek for *self-eating*) was coined by Pr Christian de Duve (Nobel prize in Physiology or Medicine 1974) at the CIBA Foundation Symposium on Lysosomes in 1963. The definition of this term arose from different influential works which begun with its own observations. Indeed, he found a latency of acid phosphatase activity during cell fractionation of rat liver homogenate. Further biochemical studies revealed a novel organelle enwrapping an acid phosphatase and also various kinds of hydrolytic enzymes with optimal activity at acidic pH. He named this unique organelle the "lysosome", the name is due to its role as an organelle for lytic function[168].

Soon afterwards, Dr A. Novikoff et al. observed, by electron microscopy (EM), an isolated lysosome-enriched fraction which proved this single membrane vesicles as a unique morphological entity[169]. Additional works using Electron Microscopy (EM) showed lysosomes containing cytoplasm or organelles such as mitochondria and endoplasmic reticulum (ER)[170-171]. Finally, Drs Arstila and Trump skillfully showed that a double membrane bound structure containing a portion of cytoplasm and organelles without hydrolytic enzymes, known as the autophagosome, is formed at first. This structure is subsequently observed as a single membrane structure, referred to as the autophagolysosome,

showing various stages of organelle degradation by lysosomal enzymes[172]. Based upon these observations, Pr de Duve defined this mode of delivery of cytoplasmic materials to the lysosomes for degradation as “autophagy”[173]. Although autophagy was initially revealed in mammalian systems, the molecular understanding of this degradation machinery was largely expanded and facilitated through genetic studies in yeast carried out in the Prs Ohsumi and Thumm laboratories [174-175].

Subsequently, a series of studies uncovered the connections between autophagy and pathophysiological conditions, such as pathogen infection[176] and neurodegeneration [177], and established its dual role in cell growth and death[178-179]. Nowadays, it has been described three types of autophagy: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA), the latter only occurring in mammalian cells. Both micro- and macroautophagy can be selective or nonselective in regards to the substrates they degrade. In this chapter, we will present very briefly the microautophagy and CMA, and we will discuss more in detail the macroautophagy pathway.

5.2 Microautophagy

Microautophagy is a form of autophagy by which cytoplasmic contents enter into the lysosome via direct membrane invagination (figure 13A)[180]. This process has been observed in yeast[181], plants[182], Drosophila Melanogaster and mammals[183]. Microautophagy in yeast, which is commonly known as “endosomal microautophagy”, has been involved in the degradation of portion of the nucleus[184], damaged mitochondria[185], lipid droplets[186] and peroxisomes[181]. Due to the limited number of tools available for the study of microautophagy, it is known relatively little about this process regulation and possible roles in human health, but a molecular signature of the process has begun to emerge.

5.3 Chaperone-mediated autophagy

Chaperone mediated autophagy (CMA) refers to the direct delivery of cytosolic proteins, marked for degradation, to the lysosome (figure 13B). The main CMA feature is that membrane invaginations are not required, because the cytosolic proteins are delivered to the lysosomal lumen through a protein-translocation complex at the lysosomal membrane[187]. As this process only degrades soluble proteins

containing KFERQ-like motif[188] but not organelles, lipids and nucleic acids[183], it is a protein selective degradation process, involving the recognition of the KFERQ motif by the heat shock 70kDa protein 8 (HSPA8/HSC70). The translocation of chaperone-bound autophagy substrates across the lysosomal membrane is led by a specific splicing isoform of LAMP2 (Lysosome-associated membrane protein 2), namely, LAMP2A(figure)[189]. The CMA functions involve: *(i)* the regulation of transcription by degradation of several transcription factors; *(ii)* the control of cell cycle progression through degradation of cell cycle arrest proteins; *(iii)* the regulation of neuronal survival by degrading inactive forms of the transcription factor MEF2D (myocyte enhancer factor D); *(iv)* the T cell activation by the ability to timely degrade the negative regulators of T cell activation Itch and RCAN1; *(v)* the immune response through the presentation of antigens in macrophages[190]. How CMA may impact cancer initiation and progression is poorly understood. However, it is clear that CMA is required for optimal cell growth and tumorigenesis[191].

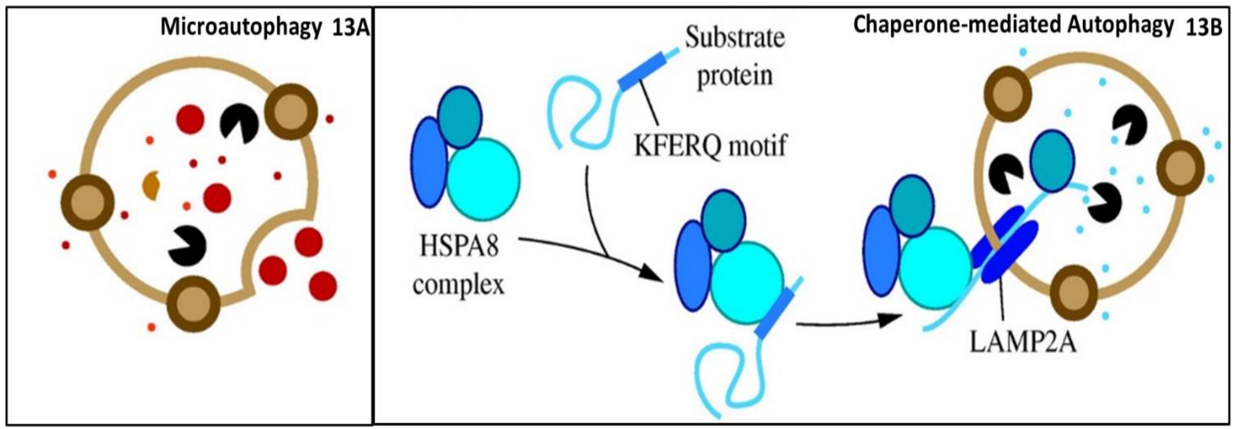


Figure 13. Microautophagy and Chaperone-mediated autophagy process. 13A. Microautophagy involves the direct uptake of cargo through invagination of the lysosomal membrane. **12B.** Chaperone-mediated autophagy transports individual unfolded proteins directly across the lysosomel membrane *(Adapted from Klionsky et al. ANTIOXIDANTS & REDOX SIGNALING 2014).*

5.4 Macroautophagy pathway: Morphological basis and progression

The marcroautophagy pathway (herein referred as autophagy) has an unique morphological feature that distinguish it from other intracellular vesicle-mediated pathways: the sequestering vesicle, called autophagosome, is formed *de novo* rather than from a preexisting membrane[192] (figure 14). This peculiar characteristic has been shown first in yeast; indeed, autophagy induction leads to the formation of autophagosomes at a single perivacuolar site called phagophore assembly site (PAS)[193].

For the mammalian system, initial studies showed that endoplastic reticulum (ER) and omegasomes served as initiation sites[194-195]. However, it was recently demonstrated that the autophagosome formation begins at multiple sites all over the cytoplasm rather than at single PAS[196] and that the source of membranes for the phagophore expansion might derive from the plasma membrane[197], ER[194], Golgi complex[198], and mitochondria[199]. Thus, this question is still not completely answered.

During the elongation phase, the phagophore bends to generate a spherical autophagosome containing a cytoplasmic cargo[180]. The size of the autophagosome varies based on organism and cargo type. For example, the diameter of autophagosomes ranges from 0.4 to 0.9 µm in yeast, and 0.5 to 1.5 µm in mammals[200-201-202]. Once the autophagosome is completely formed and sealed, it delivers its cargo through fusion to the lysosome in mammals, or to the functionally related vacuole in yeast and plants. The product of fusion between an autophagosome and a lysosome in mammalian cells is referred to as an autolysosome[203]. The acidic lumen and the hydrolases of the lysosome allow the degradation of the autophagosome inner membrane and then its cargo. Finally, cargo components are exported back into the cytoplasm, through lysosomal permeases, to be used in biosynthetic processes or generate energy[204]. To sum it up: the autophagy process is divided into morphological and mechanical distinct steps, including the: Induction, Nucleation, Elongation and closure of the autophagosome, Autophagosome-Lysosome fusion, Breakdown of the cargo followed by release of the degradation products back into the cytosol.

All these steps will be developed in details in the following paragraphs.

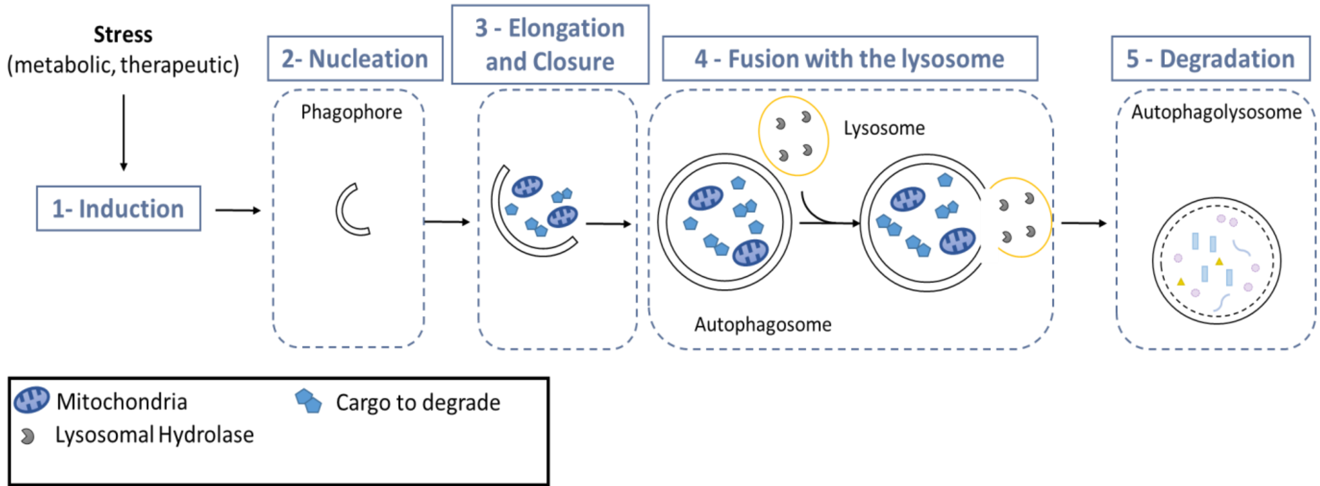


Figure 14. The macroautophagy process. Macroautophagy takes place in five main steps: (1) Induction occurs after a metabolic or therapeutic stress (2) During the nucleation step, the formation of the phagophore (or isolation membrane) is initiated. (3) During the Elongation and closure step, the phagophore bends to generate a spherical autophagosome. (4) Once formed, the autophagosome containing a cytosolic cargo will fuse with the lysosome, which triggers (5) the degradation of its content and the release of primary components in the cytosol for recycling. The lysosome can then be regenerated so that the process can start again. *(Giuriato et al. Cancers, 2017).*

5.5 Autophagy: the core machinery

The autophagy machinery has been mainly described by Pr Yoshinori Ohsumi, for which he was awarded the Nobel Prize in physiology or medicine in 2016. The interest of the scientific community towards this process has rapidly been expanding so that in 2010, more than 45 papers a week were published on the subject[205].

Induction: The ULK1 complex

Under normal conditions, the autophagy basal level is very low; indeed, an efficient mechanism to induce autophagy is necessary for cells to respond to stress conditions. One of the most important and well-studied proteins which inhibits autophagy in a nutrient-rich condition is the serine/threonine protein kinase TOR (Target of Rapamycin) specifically, TOR complex 1 (TORC1)[206-207-208].

For example in yeast, upon nutrient-rich conditions, Tor negatively regulates another serine/threonine kinase named Atg1. It has been shown that upon Tor inhibition by starvation or rapamycin treatment, the kinase activity of Atg1 is activated and promotes the formation of Atg1-Atg13-Atg17 scaffold and the recruitment of multiple Atg proteins to the PAS (phagophore assembly site) to initiate autophagosome formation[209-210-208-211-212]. In more details, the hyperphosphorylation of Atg13 by

TORC1, upon nutrient-rich conditions, prevents the association of Atg1 with Atg13 which is bound to Atg17, Atg31 and Atg29. In this scenario, the autophagy is inhibited by blocking the induction step. The inactivation of TORC1 by starvation allows the association of Atg1 with Atg13, Atg17, Atg31 and Atg29 complex, activating the process (figure 15A)[213-214-215].

Differently than yeast, mammalian ULK (ULK1 or ULK2, the homologs of yeast Atg1) make a stable complex with mammalian Atg13, focal adhesion kinase family interacting protein of 200 kDa (FIP200) which is a putative counterpart of yeast Atg17 and Atg101 (an Atg13-binding protein). The autophagy process is negatively regulated upon nutrient-rich conditions when TORC1 associates with the ULK complex, phosphorylating ULK1 or ULK2 and hyperphosphorylating Atg13. When TORC1 is inactivated by starvation, it dissociates from the ULK complex, preventing these inhibitory phosphorylations of Atg13 and ULK1/2; moreover, ULK1/2 phosphorylates Atg13, autophosphorylates and hyperphosphorylates FIP200 at other activation sites (figure 15B). Thus, the ULK complex is indispensable for proteins recruitment and autophagy induction[216-217]. The ser/thr kinase ULK1 will be extensively discussed in the following chapter.

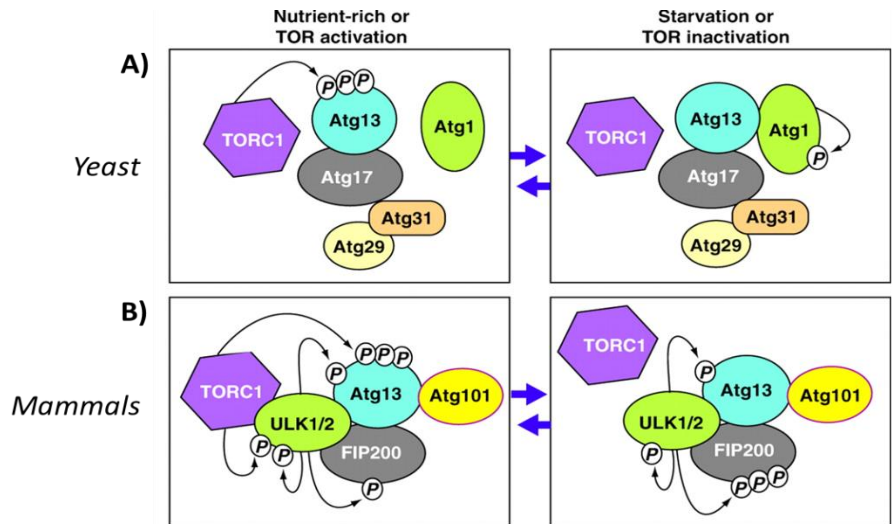


Figure 15. Regulation of ATG1 complex by TORC1 upon Starvation or TOR inactivation in yeast and mammals. A) In yeast, under nutrient-rich condition, TORC1 hyperphosphorylates Atg13 and block the association of Atg1 with Atg13-Atg17-Atg31-Atg29 complex. Upon Starvation condition, TORC1 is unable to phosphorylates Atg13 and this allows the association of Atg1 to the complex and its autophosphorylation. **B)** In mammals, upon nutrien-rich condition, TORC1 regulates negatively the ULK1/2-Atg13-FIP200-Atg101 complex by phosphorylatating ULK1/2 and hyperphosphorylating Atg13. Upon starvation condition, TORC1 dissociates from the complex and allows the hyperphosphorylation of FIP200 by ULK1/2 *(Adapted from Klionsky et al. Journal of cell science. 2011).*

Nucleation: the ATG14 complex

The next complex recruited to the PAS is the ATG14-containing class III phosphatidylinositol 3-kinase (PtdIns3K) complex[196]. This complex generates PtdIns3P, which is essential for nucleation in both yeast and mammals. The proteins present in the complex are: PIK3C3/VPS34, PIK3R4/p150 (Vps15 in yeast), and BECN1 (Vps30/Atg6 in yeast) (Figure 16). Regulation of the PtdIns3K complex occurs largely through proteins that interact with BECN1, which is essential for autophagy. One of them is the antiapoptotic protein BCL2 which binds BECN1 and prevents its interaction with PIK3C3. Thus, the BCL2-BECN1 association inhibits the nucleation step[218-219-220]. Moreover, it has been shown that Bcl-2 originating from the ER rather than mitochodria is responsible for negatively regulating autophagy[221].

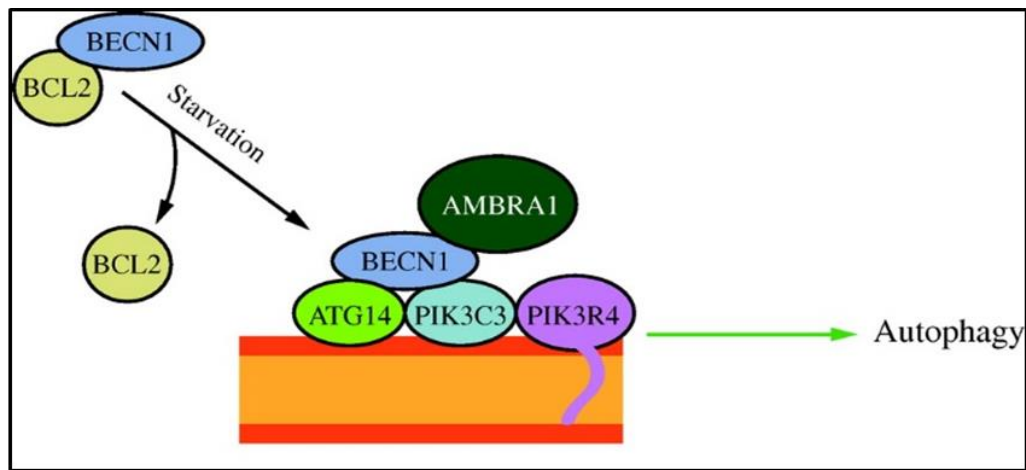


Figure 16. Activity of The ATG14 complex (ATG14-BECN1-PIK3C3-PIK3R4) is required for autophagy. The ATG14 complex can be positively regulated by AMBRA1 and negatively regulated by BCL2 binding to BECN1and preventing association with the complex *(Adapted from Klionsky et al, ANTIOXIDANTS & REDOX SIGNALING, 2014)*

Elongation and closure of the autophagosome: the Ubl conjugation systems

In the elongation and closure step, two conjugation systems involving ubiquitin-like (UBL) proteins[222] have been identified. The main characteristic of the first conjugation system is the formation of the Atg12–Atg5-Atg16 complex. In both yeast and mammals, this complex is generated by the covalent bound between Atg12 and Atg5 due to the E1 activating enzyme Atg7 and the E2 conjugating enzyme Atg10. After the Atg12–Atg5 conjugation, Atg16L1 binds to Atg5 in a non-covalent way and dimerizes

to form a larger complex. Mammalian orthologs of this system, ATG5, ATG12 and ATG16L1, have been identified, and function as in yeast [180](figure 17).

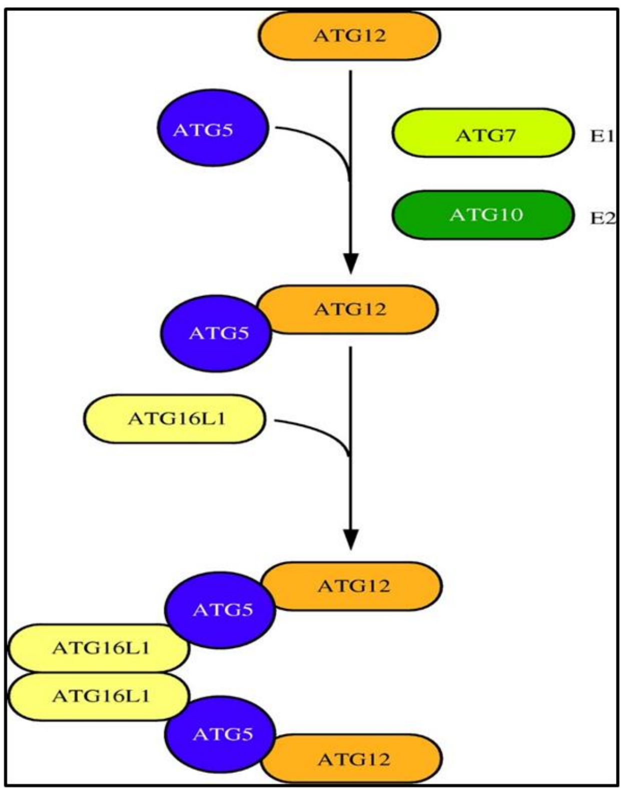


Figure 17. ATG12–ATG5-ATG16L1 conjugation complex. The ubiquitin-like protein ATG12 is irreversibly conjugated to ATG5 in an ATG7- and ATG10-dependent manner. ATG7 and ATG10 function as E1 activating and E2 conjugating enzymes, respectively. The ATG12–ATG5 conjugate binds ATG16L1 through ATG5. ATG16L1 dimerizes and allows association with the phagophore, promoting membrane expansion.(*Klionsky et al, ANTIOXIDANTS & REDOX SIGNALING, 2014).*

The second UBL system is called the Atg8 system and it is also involved in phagophore expansion. In yeast, this conjugation pathway starts with the cysteine protease Atg4 which processes Atg8 to allow the exposition of a glycine residue at C terminus[223]. Then, the E1-like enzyme Atg7 activates the processed Atg8 and transfers it to the E2-like enzyme Atg3[224]. Finally, the C-terminal glycine of Atg8 is covalently conjugated to the lipid phosphatidylethanolamine (PE).The Atg12–Atg5 conjugate, which may act as an E3 ligase, facilitates this final step[225]. Atg8–PE is membrane-associated, but can be released from membranes as a result of a second Atg4-mediated cleavage[223]. The mechanism of regulation of the second Atg4-dependent processing event, referred to as deconjugation, is not known;

however, this appears to be an important step in autophagy because defects in cleavage result in partial autophagic dysfunction[226].

Mammalian homologs of the Atg8 system function similarly to their yeast counterparts (Figure 18)[222]. Different from yeast, which has only one Atg4, mammalian cells have four isoforms of ATG4. Moreover, mammalian cells have multiple Atg8 homologs including LC3A (Microtubule-associated proteins 1A/1B light chain 3A), LC3B, LC3C, gamma-aminobutyric acid type A receptor associated protein (GABARAP), GABARAP-like 1, 2 and 3 (GABARAPL1, GABARAPL2, and GABARAPL3, respectively)[227]. Among the Atg8-like proteins in mammals, LC3 has been the best characterized. The ATG4-processed form of LC3 is referred as LC3-I and the PE-conjugated form is called LC3-II. Lipidation of LC3 in mammalian cells is accelerated under conditions of nutrient starvation or other types of stress. Whereas both subfamilies can localize with autophagosomes, it has been proposed that they function at different steps in phagophore elongation and completion, with the LC3 subfamily acting before the GABARAP subfamily[222](Figure18).

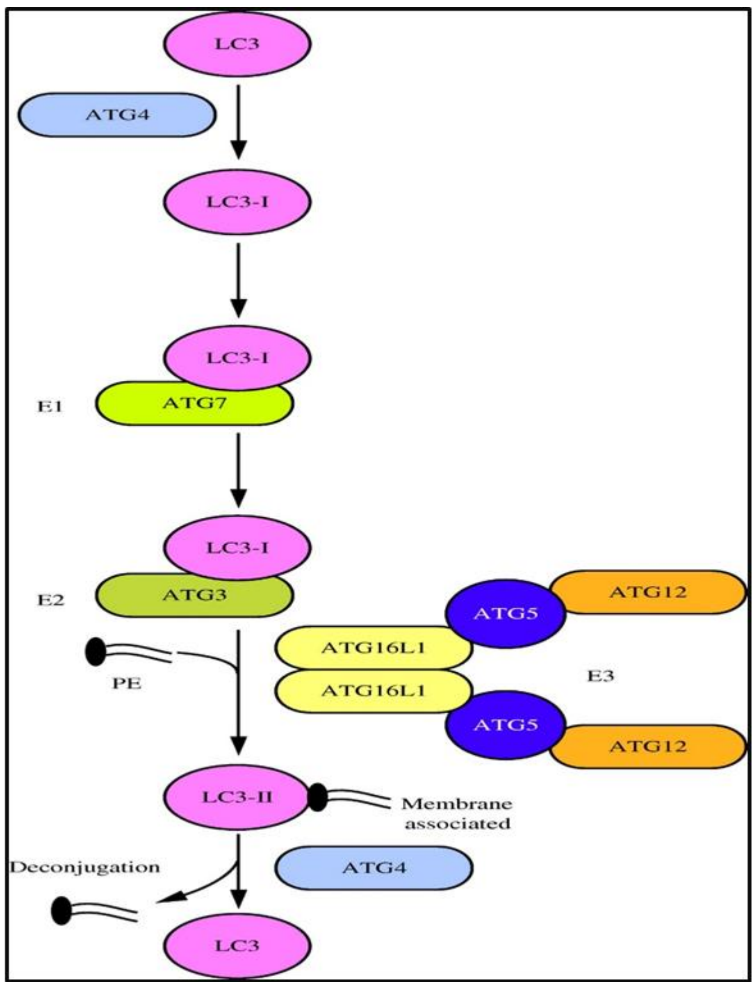


Figure 18. The LC3 conjugation system. LC3 is processed by ATG4 to reveal a C-terminal glycine (LC3-I). ATG7, an E1-like enzyme, activates LC3-I and transfers it to the E2-like enzyme ATG3. The ATG12–ATG5-ATG16L1 complex may participate as an E3 ligase in the conjugation of PE to LC3-I to create LC3-II, which can associate with the phagophore. LC3-II can subsequently be cleaved by ATG4 to release LC3(deconjugation). PE, phosphatidylethanolamine. (*Klionsky et al, ANTIOXIDANTS & REDOX SIGNALING, 2014).*

Vesicle fusion and autophagosome breakdown

In yeast, the machinery consists of the Rab family GTPase Ypt7 (the homolog of Rab7), the NSF homolog Sec18, the SNARE proteins Vam3, Vam7, Vti1, and Ykt6, the class C Vps/HOPS complex proteins, and two other proteins, Ccz1 and Mon1[193]. In 2012, it has been identified another SNARE, syntaxin 17, which localizes to completed autophagosomes and is required for fusion with the endosome/lysosome[228]. After fusion, degradation of the inner vesicle is dependent on a series of lysosomal hydrolases, including proteinases A and B (encoded by PEP4 and PRB1, respectively) and the lipase Atg15[229]. The resulting small molecules from the degradation, particularly amino acids, are transported back to the cytosol for protein synthesis and maintenance of cellular functions under starvation conditions. The identification of Atg22, together with other vacuolar permeases (such as Avt3 and Avt4) as vacuolar amino acid effluxers during yeast autophagy, has helped in the understanding of the mechanisms of nutrient recycling; these permeases represent the last step in the degradation and recycling process[230].

In mammalian cells, once the autophagosome is mature, its movement to reach the lysosome has been shown to be dependent on microtubules[231]. Then, the fusion event requires the lysosomal membrane protein LAMP-2 and the small GTPase Rab7, although the mechanism is still not well characterized[232-233]. After fusion, the cargo degradation is ensured by the cathepsin B, D and L lysosomal enzymes [234].

5.6 Transcriptional and Epigenetic Regulation of Autophagy

FoxO3

Autophagy genes are regulated at the transcriptional level in response to stress. For instance, under starvation conditions, transcription of the autophagosome marker Atg8/LC3 is rapidly upregulated in yeast and mammals[235]. FoxO3 (Forkhead box transcription factor class O) was the first transcription factor described to induce autophagy in the Drosophila larval fat body[236]. In mammalian cells, autophagy transcription via FoxO3 was demonstrated in protein degradation studies during muscle atrophy [237]. Moreover, it has been shown that FoxO3 induces the transcription of multiple autophagy genes, including *Gabarapl1, LC3B, atg12, atg4B,ulk2, vps34, beclin 1, Bnip3,* and *Bnip3l*. Of note, FoxO3

directly binds to the promoters of *LC3B, Gabarapl1, atg12, Bnip3l,* and *Bnip3* to activate gene transcription. Constitutively active FoxO3 is sufficient to induce autophagosome formation in adult mouse skeletal muscle, which promotes lysosomal proteolysis and leads to muscle wasting[235].

STAT3

STAT3 (signal transducer and activator of transcription 3) is a latent transcription factor that mediates extracellular signals such as cytokines and growth factors through interaction with polypeptide receptors at the cell surface[238].

- Nuclear STAT3 is the major transcriptional enhancer of several autophagy- related genes in the nucleus. Moreover, its activity contributes to a range of anti- vs. pro-autophagic functions (Table 6). For example, STAT3 might inhibits autophagy by activating BCL2. On the contrary, STAT3 can stimulates autophagy by upregulating and stabilizing HIF1A (Hypoxia Inducible Factor 1 Subunit Alpha) under hypoxia[239].

Table 6. Nuclear STAT3-regulated genes in autophagy *(adapted from You et al. Autophagy, 2015)*

Target genes	Anti/pro-autophagy	Mechanisms
BCL2 family members	anti-	STAT3 transcriptionally activates *BCL2* and *MCL1* expression, which leads to autophagy inhibition
BECN1	anti-	STAT3 directly binds to the promoter region of *BECN1* and represses its transcription by recruiting HDAC3
PIK3C3	anti-	STAT3 downregulates *PIK3C3*, which is an essential component of the BECN1-PIK3C3 complex
PIK3R1/p55α, PIK3R1/p50α	anti-	STAT3 upregulates the expression of PIK3R1/p55α and PIK3R1/p50α, which compete with PIK3R1/p85α to inhibit autophagy.
CTSB, CTSL	possibly anti-	STAT3 upregulates *CTSB* and *CTSL*, which enhances cell death by LMP; LMP impairs the autophagy flux.
HIF1A	pro- in hypoxia anti- in normoxia.	STAT3 transcriptionally activates and stabilizes HIF1A, which induces autophagy in hypoxia and represses autophagy in normoxia.
BNIP3	pro-	STAT3 phosphorylation upregulates *BNIP3* expression; BNIP3 is a pro-autophagic BH3-only protein.

- A large fraction of STAT3 protein is found in the cytoplasm, where the main autophagy steps occurs. A novel function of cytoplasmic STAT3 was described by Pr Kroemer group. They found a novel mechanism to explain how cytoplasmic STAT3 inhibits autophagy by inhibiting EIF2AK2 (eukaryotic translation initiation factor 2-a kinase 2) activity. Further investigations have shown that cytoplasmic STAT3 regulates autophagy in direct manner. It was found that the SH2 domain of cytoplasmic STAT3

exhibits a conformational fold that match with the C terminus of the EIF2AK2 substrate EIF2A (eukaryotic initiation factor 2A, 65kDa), and interacts with the catalytic domain of EIF2AK2, thus inhibiting EIF2AK2 enzymatic activity and preventing it from phosphorylating EIF2A, a known autophagy activator[240].

Chromosome modifications

In the literature, there are many studies that highlight the important role of epigenetic in regulating autophagy in different pathological conditions [235]. For example, alteration of the acetylation status through chemical or genetic inhibition of histone deacetylases (HDACs) lead to autophagy induction[241]. Specific transcriptional effects on ATG genes by HDAC suppression have also been reported. It has been shown that, in patients with chronic obstructive pulmonary disease caused by cigarette smoking, the inhibition of HDAC activity increases the binding of Egr-1 (early growth response-1) and E2F transcription factors to the LC3B promoter region, and activates LC3B expression[242]. Future studies to define the specific functions of different histone deacetylases on autophagy genes are much awaited.

5.7 Classical Measure of Autophagy

Guidelines to monitor autophagy are published and are frequently updated[243]. In this paragraph, we will highlight a few common methods that are used to monitor autophagy.

5.7.1. Transmission Electron Microscopy (TEM)

TEM was the first technique that was used to detect autophagy. It is the only autophagy monitoring technique enabling the visualization of autophagic structures and their position within the cell. Although TEM remains the traditional method that is used in the field, it requires high technical expertise to analyze the obtained pictures and to identify organelles and autophagosomes in particular.

It is also very time consuming, and a special care has to be taken to guarantee proper handling of the samples.

5.7.2 Turnover of PE-Conjugated ATG8 Proteins

MAP1LC3 (microtubule associated protein 1 light chain 3), best known as LC3, belongs to the ATG8 family of proteins. This family is divided into two subgroups, i.e., the LC3 and the GABARAP (gamma-aminobutyric acid receptor-associated protein) proteins. Four LC3 isoforms (LC3A, B, B2 and C) and three GABARAP isoforms (GABARAP, GABARAPL1 and GABARAPL2/GATE16 (Golgi-associated ATPase enhancer of 16 kDa)) have been identified in mammals. The maturation of these ATG8 proteins involves the cleavage of their precursor by a cysteine protease (ATG4), and their subsequent lipidation (addition of a phosphatidylethanolamine molecule (PE)). The non-lipidated forms are referred to LC3-I or GABARAP-I, and the lipidated forms (which are the ones that are associated with the autophagosomal membranes) are referred to LC3-II or GABARAP-II. In the literature, the induction of autophagic flux is traditionally evaluated by observing the difference by western blot in the amount of LC3B-II in both the presence and absence of lysosomal inhibitors, such as bafilomycin A1 and chloroquine. If autophagy is induced, the amount of LC3B-II will be higher in the presence of the inhibitor than in its absence. The same reasoning applies for GABARAP-I and GABARAP-II. It should be noted that when using this technique, the researchers are limited by the specificity of the antibodies that are commercially available. For instance, cross-reactions between LC3A and LC3B, as well as between GABARAP and GABARAPL1, are known and can lead to result misinterpretation. Regarding GABARAP as a marker for autophagy measurement, it is important to point out that the PE-conjugated GABARAP forms are usually undetectable in mammalian cells without autophagy induction. In addition, autophagy was found to be LC3 independent in certain cell types. In those cases, GABARAP is absolutely required to fulfill the autophagy process[244]. Because of these particularities, it has been proposed that this subfamily of protein might be more sensitive than the LC3 family to monitor autophagy induction. However, for now, LC3B remains the most common autophagy marker used in the literature.

5.7.3 Fluorescent LC3B Probes

Techniques using fluorescence probes to monitor autophagy are also frequently used. As an example, autophagy induction can be monitored by the ectopic expression of GFP (green fluorescent protein)-LC3 in cells. During autophagy induction, the cytoplasmic GFP-LC3 protein will relocate to the autophagosomes, inducing the formation of fluorescent puncta, which can be visualized and quantified by fluorescent microscopy. This technique is more sensitive than the monitoring of LC3B-II by western blotting, but it often requires some technical optimization rounds. The autophagic flux can also be evaluated with an RFP (red fluorescent protein)-GFP-LC3 tandem construct[245]. While autophagosomes bearing this fusion protein will appear as yellow dots (i.e., both RFP and GFP positive), the autophagolysosomes will appear in red (RFP), as the GFP fluorescence is quenched upon lysosomal acidification ($pH < 5$). The autophagic flux induction can thus be quantified by the loss of the GFP fluorescence. This characteristic can also be measured by using a flow cytometer, and allows for the ratiometric quantification of autophagy induction[246]. The critical point for an accurate quantification of autophagosomes and autophagolysosomes, and for a correct assessment of the autophagic flux with these fluorescent probes, is the sensitivity of the green fluorescent protein to acidic pH. Thus, after the initial development of the RFP-GFP tagged LC3B construct[245] , other groups have generated refined versions of this probe, replacing GFP with mWasabi[247] or pHluorin[248], which appeared to be more suitable to precisely monitor autophagic structures. Recently, a new autophagic flux probe, i.e., GFP-LC3-RFP-LC3DG, has been developed by the group of Pr Mizushima[249-250]. It is based on the equimolar release of GFP-LC3 and RFP-LC3ΔG upon cleavage by endogenous ATG4 proteases. The RFP-LC3DG fusion protein does not contain the C-terminal glycine residue mandatory for lipidation, it will thus stay free in the cytosol and serves as an internal control. On the contrary, GFP-LC3 can relocate to the autophagosomes, and after its fusion with lysosomes, the fluorescence signal will ultimately be lost. The measured GFP:RFP signal ratio inversely correlates with autophagy flux activation. This system appears to be more sensitive than the classical RFP-GFP-LC3B probe because it avoids the late degradation of RFP within autophagolysosomes.

6 The incredible ULK1

As evoked in the previous chapter, autophagy is a highly regulated process; indeed, it has been described 35 autophagy-related (ATG) genes which modulate the pathway[251]. One of the most studied ATG protein is UNC-51-like kinase 1 (ULK1), also known as the ortholog of Atg1. ULK1 was the first gene cloned in yeast and the encoded protein has the same autophagy-initiating function in mammalians[252]. As mentioned in paragraph 3.5, ULK complex is required to initiate the autophagic process. It is composed of ULK1 and three other members: mATG13, FIP200, and ATG101. Moreover, several studies have shown that ULK1 has different types of post-translational modifications[253-254] which regulate the process. In this paragraph, we will discuss the structure, interactions, regulation and functions of ULK1.

6.1 ULK1 structure

ULK1 is a cytoplasmic kinase whose open reading frame is composed of 1050 (Homo sapiens) or 1051 (Mus musculus) amino acids with a molecular weight of 112.6 or 113 kDa, respectively. It is a highly conserved protein, showing an overall similarity of 29% with its yeast homolog Atg1. ULK1 structure consists of an N-terminal kinase domain (KD) (residues 16−278) and a C-terminal domain (CTD) (residues 833−1050) containing two tandem microtubule-interacting and transport (MIT) domains. Moreover, the region connecting the KD and MIT domains, for approximately 500 amino acids, is less conserved and is named proline/serine-rich (PS) region [255-256] (Figure 19). The ULK1 KD crystal structure has revealed a typical fold, which is similar to the KD of the most extensively characterized protein kinase A (PKA). The KD consists of two globular folds (helical C-lobe and N-lobe), which are composed of a α-helix named αC and a five-stranded β-sheet. Of note, adenosine triphosphate (ATP) is usually bound to the cleft that is formed between the two lobes and also covered by a lid that is named the P-loop or Gly-rich loop[257].

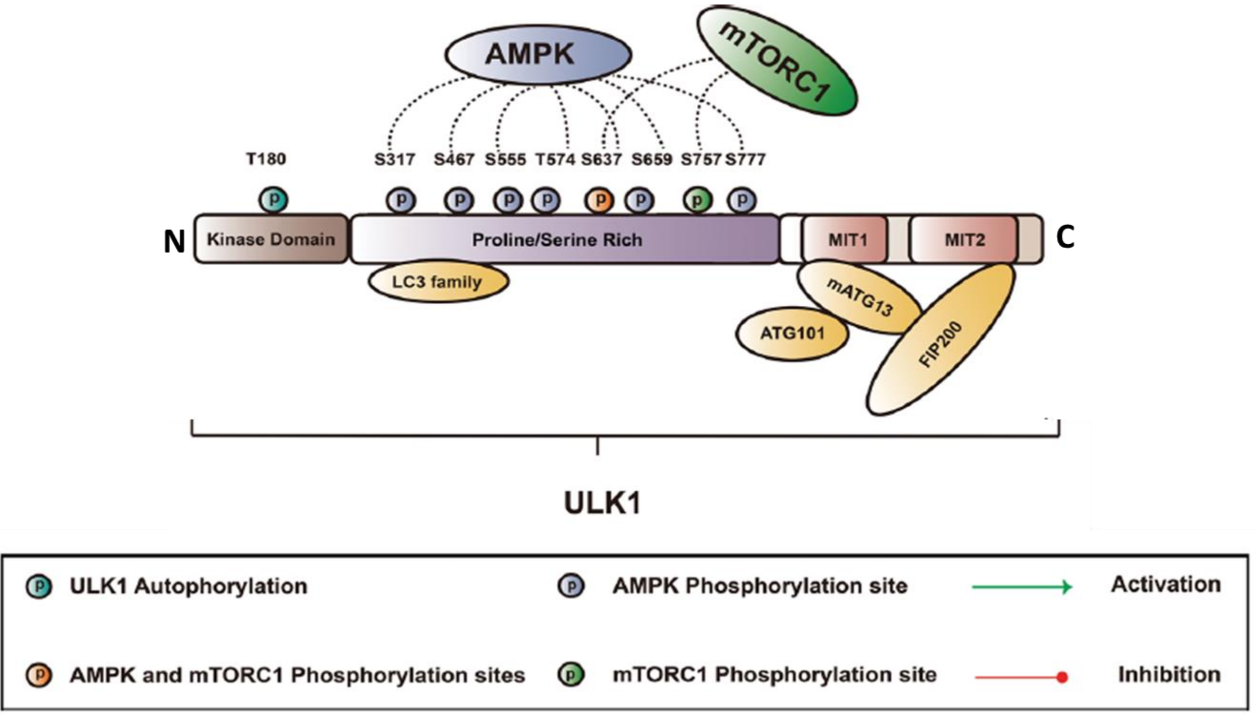


Figure 19. Structure and activation of ULK1. Domain structure of ULK1 is composed of the respective domains, including kinase domain (KD), proline/serine-rich (PS) region, and C-terminal domain (CTD) containing MIT1/2. The upstream kinases AMPK and mTORC1 are play crucial roles in regulating ULK1 by phosphorylation (amino acid sites are based upon sequence in mouse) *Adapted from Zhang et al, Journal of medicinal Chemistry, 2018.*

6.2 ULK1 interactions

The ULK complex contains ULK1, mATG13, FIP200, and ATG101, which may interact with each other to activate the downstream signaling pathways.

FIP200 is the first protein found to interact with ULK1 in mammalian cells. This interaction is essential for autophagosome formation[258]. The second protein which is associated with ULK1 and FIP200 is mATG13. FIP200 and mATG13 bindings site are mapped into ULK1 C-terminal regions and the two proteins may together regulate ULK1 kinase activity. Specifically, either of them can exert its function in a compensatory mechanism, and the maximal activity of ULK1 can be reached by cooperation of mATG13 and FIP200[259]. Finally, ATG101 was the last protein to be identified in the ULK1 complex where it binds on ATG13. Although the function of ATG101 remains unclear, it is one of the essential

components of the ULK complex in mammals. Interestingly, there is not any homolog or functional equivalent of ATG101 in yeast[260].

6.3 ULK1 complex regulation

ULK1 activity can be controlled by different post-translational modifications, among which phosphorylations have been the most studied. These phosphorylations are ensured essentially by two crucial stress sensor proteins: AMPK (AMP-activated protein kinase) and mTORC1. As mentioned before, mTORC1 is a negative autophagy regulator and can exerts its functions by phosphorylating ULK1 at Ser637 (Mus musculus)/Ser638 (Homo sapiens) and Ser757 (Mus musculus)/Ser758 (Homo sapiens) or directly phosphorylate mATG13 at Ser258 to prevent ULK1 activation[261-262].

In addition, AMPK can reduce TORC1-mediated inhibition by phosphorylating Raptor (member of mTORC1 complex) at Ser722 and Ser792. It can also directly activate ULK1 by phosphorylation at Ser317, Ser467, Thr574, Ser555, Ser637 (Mus musculus)/Ser638 (Homo sapiens), and Ser777[263-264] (Figure 19). It has been shown recently that ULK1 can be phosphorylated by type I interferon receptor (IFNR) at Ser757, which is a phosphorylation site of mTORC1 to inhibit ULK1; thereby, ULK1 can be activated after the engagement of type I IFNR[265]. Of note, ULK1 autophosphorylation at Thr180 promotes its ubiquitylation and degradation by kelchlike protein 20 (KLHL20), which governs the degradation of the Beclin1 complex in prolonged starvation[266].

It has been described other types of post-transcriptional modifications, such as ubiquitylation and acetylation. An example is the Activating molecule in BECN1-regulated autophagy protein 1 (AMBRA) which may interact with the E3-ligase TRAF6 to support the ubiquitylation of ULK1[267]. All the post-translational modifications of the ULK are listed in table 7.

Table 7.Post-translational modifications of the ULK

Substrate	Modification	Modifying enzyme
ULK1	Phosphorylation at Ser637, Ser757(*Mus musculus*)/Ser758(*Homo sapiens*)	mTOR
	Phosphorylation at Ser317, Ser467, Thr575, Ser637(*Mus musculus*)/Ser638(*Homo sapiens*), Ser555, Ser777	AMPK
	Dephosphorylation at Ser637	PPM1D
	Phosphorylation at Ser757	IFNR
	Autophosphorylation at Thr180	ULK1
	Poly-Ub (Lys63-linked)	TRAF6
	Poly-Ub (multiple linkages)	KLHL20
	Poly-Ub at Lys925 and Lys933	NEDD4L

(Zhang et al, Journal of medicinal Chemistry, 2018)

6.4 Modulation of ULK1 expression levels

Negative and positive regulation of ULK1 can be viewed as a regulatory mechanism that protects cells against excessive autophagy, which could compromise cell viability. It has been found that, after the first few hours of starvation, ULK1 protein levels are downregulated by the E3 ligase NEDD4L; in more detail, NEDD4L ubiquitinates ULK1 by both K27- and K29-linked ubiquitin chains and thus mediates its proteasomal degradation. At same time, ULK1 mRNA is actively transcribed and, after that, translated upon MTOR-dependent reactivation of translation[268]. According to this model, MTORC1 activity can drive alternating periods of mutually exclusive autophagy and protein synthesis (figure 20)[269]. Finally, a recent study has shown that ULK1 is targeted by the E3 ligase CUL3/CULLIN-3 for proteasomal degradation at the end of the autophagy response[270].

In the next two paragraphs, we will describe how ULK1 modulates upstream and downstream autophagic pathways.

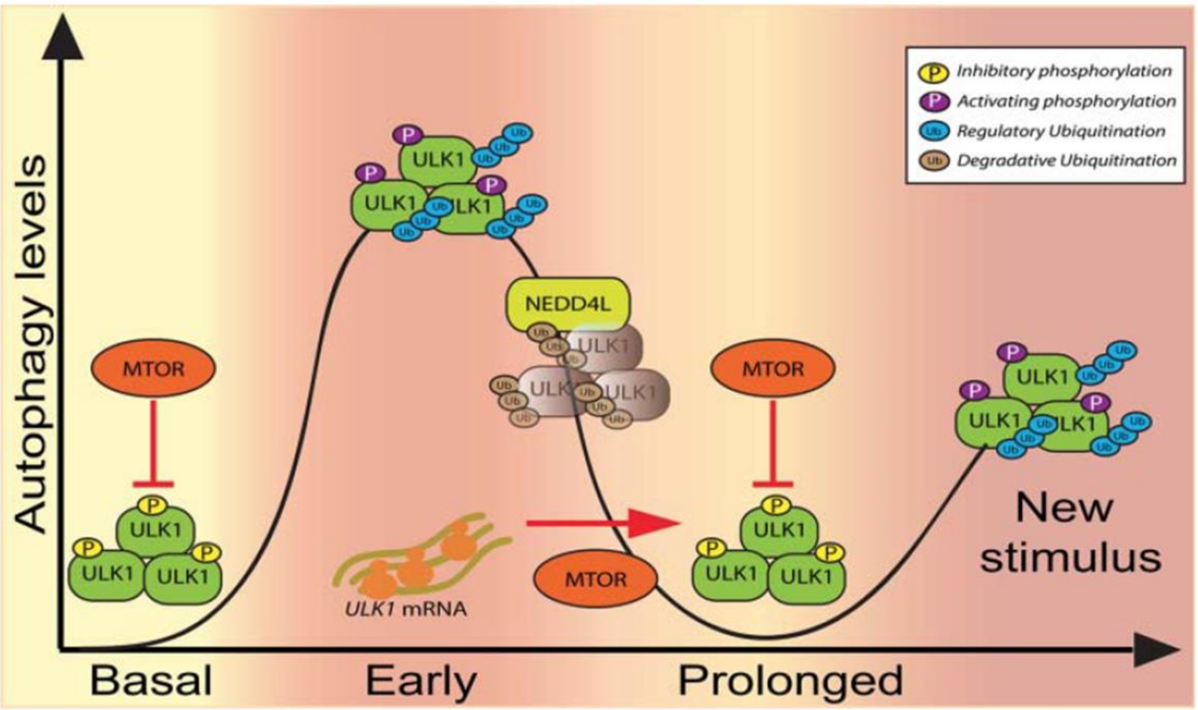


Figure 20. Autophagy oscillates through ULK1-MTOR crosstalk. In basal conditions, the ULK1 complex is maintained in an inhibited state by MTOR. During the early phase of autophagy induction, ULK1 is activated by both phosphorylation and regulatory ubiquitination; in enduring starvation, NEDD4L promotes ULK1 degradation by the proteasome, while ULK1 mRNA is actively transcribed. During prolonged starvation, MTOR is reactivated and ULK1 mRNA is translated and then inhibited again by MTOR, preventing excessive autophagy. When a new stimulus occurs, the system is thus ready for a new round of autophagy. This regulation permits an oscillatory autophagy pattern *(Cecconi et al.Autophagy,2017).*

6.5 ULK1 regulates upstream mTORC1 and AMPK kinases

In rich-nutrients condition, AMPK is inactive and mTORC1 is linked to the ULK complex through interactions with Raptor and ULK1. Under starvation condition, ULK1 phosphorylates Raptor at Ser855, Ser859, Ser792 and Ser683, thus, promoting mTORC1 inhibition by hindrance of substrate binding to Raptor[271]. In conditions where ATP/AMP ratio decreases, AMPK is active and inhibits mTORC1 either by phosphorylation or by promoting mTORC1 and ULK1 disassembling[263-254]. Later on, to stop the

autophagy process, AMPK might be negatively regulated by ULK1-mediated phosphorylation, thereby generating a negative feedback loop[272].

6.6 ULK1 and Beclin1

Beclin1 (mammalian ortholog of yeast Atg6) is a downstream target of ULK1 and can be found in complex with vacuolar protein sorting 34 (VPS34), VPS15 and ATG14L. In this scenario, ATG14L can recruit ULK1 to Beclin1, promoting Beclin1 phosphorylation. Under amino acid starvation or mTORC1 inhibition, ULK1 can phosphorylate Beclin1 on Ser14 (Mus musculus)/Ser15 (Homo sapiens), increasing the activity of the Beclin1[273]. Moreover, ULK1 can also phosphorylate ATG14L at Ser29 in a mTOR-dependent manner, thus resulting in an increased ATG14L- VPS34 lipid kinase activity for autophagy modulation [274]. Finally, Beclin1 can also be phosphorylated at Ser30 by ULK1 to activate ATG14L-VPS34 complex[275]. Thus, ULK1 is a key protein of the autophagic signaling network, able to control not only downstream BECN1, but also to retro-control upstream AMPK and mTOR.

6.7 ULK1 and Cancer

ULK1 has been found downregulated in different type of cancer tissues[276]. Moreover, reduced expression of ULK1 and autophagy pathway is associated with tumor progression, suggesting its expression as a new prognostic factor in breast cancer, particularly in triple negative breast cancer[277]. On the contrary, upregulation of ULK1 was also found in other types of cancer tissues. Thus, blocking ULK1 activity could be a promising therapeutic strategy. For example, AMPK-ULK1 pathway is shown to be involved with the bromodomain and extra-terminal domain (BET) inhibitor JQ1 in leukemia stem cells (LSCs) resistant to the BET inhibitors. This study suggest that prosurvival autophagy is a potential mechanism in resistance of acute myeloid leukemia (AML) and LSCs to JQ1[278].

6.8 ULK1 inhibitors and activators

Nowadays, a number of small molecules have been identified to modulate ULK1/ULK1-mediated autophagic pathways, which could represent a benefit for autophagy-related therapies. ULK1 compounds can be further divided into two types, inhibitors or activators. Here some examples:

ULK1 Inhibitors:

- ✓ SBI-0206965 shows an high selectivity to ULK1 (IC50 = 108 nM) and ULK2 (IC50 = 711 nM). SBI-0206965 can suppress ULK1-mediated phosphorylation of VPS34 to regulate autophagy. Moreover, SBI-0206965 can synergize with the mTOR inhibitors to kill tumor cells, suggesting their combined use in clinic[279].
- ✓ MRT67307 potently inhibited both ULK1 (IC50 = 45 nM) and ULK2 (IC50 = 38 nM) and its analog MRT68921 showed an increased affinity for ULK1 (IC50 =2.9 nM) and ULK2 (IC50 =1.1 nM). Unfortunately, MRT67307 and MRT68921 also showed nonspecificity toward ULK1. In addition, MRT68921 can inhibit autophagosome formation in an ULK1-dependent manner[280].
- ✓ WP1130 is a deubiquitinases inhibitor which leads to the increase of ULK1 ubiquitination and subsequent degradation[281].

ULK1 Activators

- ✓ LYN-1604 was the first ULK1 activator. It showed an high affinity for ULK1 with an EC50 of 18.94 nM. Moreover, LYN-1604 showed a good antiproliferative activity against breast cancer. It was able to induce cancer cell death, associated with autophagy activation[282].
- ✓ Temozolomide can induce autophagy via the ATM-AMPK-ULK1 axis for the treatment of O6-methylguanine DNA methyltransferase-negative gliomas[283].
- ✓ Tetrandrine was shown to decrease human oral cancer SAS cells viability via induction of autophagic cell death[284].
- ✓ Baicalein showed an autophagic cell death induction by the activation of AMPK-ULK1 pathway, as well as by the inhibition of mTORC1 in breast carcinoma[285].

7 Roles and Therapeutic modulation of Autophagy in Cancer

Since the introduction of the term autophagy by Pr Christian de Duve in 1963, enormous advancements in understanding this pathway and its modulation to improve clinical outcomes have been achieved. Moreover, the recent Pr Yoshinori Ohsumi Nobel Prize for Physiology or Medicine, has highlighted the importance of autophagy in health and diseases. In the context of cancers, many studies have shown that autophagy prevents cancer development. Indeed, in premalignant lesions, an high autophagy activity has been shown to prevent cancer development[286]. Inversely, once cancer is established, autophagy supports tumor cell survival and growth[287-288]. In the therapy settings, both inhibition or activation of autophagy have been proposed as therapeutic strategies[289-290]. Based on an abundant literature, it is now well established that autophagy is a key process in cancer prevention, development and response to therapy. In this paragraph, we will discuss only the role of autophagy in cancer therapy, with an emphasis for ALK+ ALCL.

7.1 The dual role of Autophagy in Cancer Therapy

Autophagy activation following cancer therapies has been associated mainly with either cancer cell survival or cancer cell death[291-292]. A small number of studies also report a cytostatic and a non-protective function for autophagy, but these two responses have been less studied thus far[293-294] (table 8). The cellular and molecular mechanisms underlying the two main and opposite outcomes in the tumor cells' fate (survival or death) following autophagy activation are not clearly understood yet. So far, it has been proposed that the mechanisms explaining the cytoprotective function of autophagy following therapy mainly rely on the impairment of the apoptotic cell death pathway. This can involve *(i)* the clearance of drug-induced cytotoxic reactive oxygen species (ROS)[295]; and, *(ii)* the degradation of pro-apoptotic proteins[296]. Additionally and importantly, cytoprotective autophagy has been shown also to *(iii)* protect cancer stem cells through the induction of tumor dormancy[297-298]. Conversely, the

cytotoxic function of autophagy has been described to mainly rely on the promotion of diverse cell death mechanisms. This can involve *(i)* the priming of tumor cells to apoptotic cell death, *(ii)* the induction of apoptosis through the activation of key apoptosis protein[299-300]; *(iii)* the degradation of negative regulator of apoptosis[301-302]; (*iv)* the use of elongating autophagic membranes as a scaffold for the assembly of the apoptosis protein complex (called apoptosome)[300]; *(v)* the induction of necroptosis[303]; and, *(vi)* the induction of immunogenic cell death[304-305]. Notably, autophagy on its own can also lead to cell death through an excessive and lethal self-digestion[306].

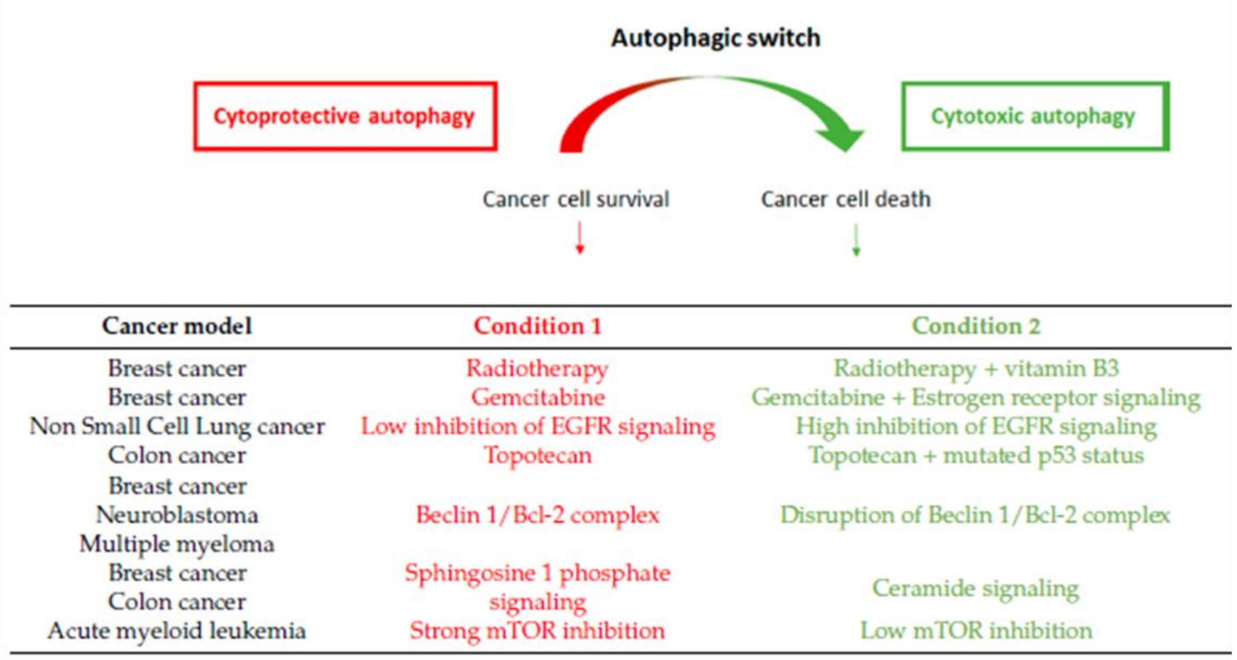
Table 8. Characteristics of the four functional forms of autophagy

Forms of autophagy	Characteristics
Cytoprotective	a. May confer resistance to therapy b. Increased sensitivity to therapy when blocked c. Increased apoptosis when blocked d. Possibly involved in normal tissue homeostasis
Cytotoxic	a. Promotes cell death when induced b. Cell death may be associated with subsequent apoptosis c. Reduced sensitivity to therapy when blocked d. Unlikely to mediate actions of conventional therapeutic modalities
Cytostatic	a. Mediates growth inhibition b. Results in reduced clonogenic survival c. Potentially associated with senescence d. Involved in tumor growth delay/ dormancy?
Nonprotective	a. Does not differ in intensity from other forms b. Inhibition does not influence sensitivity to therapy c. Relevance related to efforts to enhance response to therapy through autophagy inhibition

Gewirtz,Cancer Research,2014

7.2 Switching from a Cytoprotective to a Cytotoxic autophagy in Cancers

Adding complexity in identifying the function of autophagy following cancer therapy, a growing amount of studies are pointing to the possibility for a singular cancer cell to undergo a shift from cytoprotective to cytotoxic autophagy. This switch is beginning to be understood at a molecular level, and usually relies on an additional signaling partner (or pathway), which modifies the autophagy magnitude within the treated cells. Examples of autophagic switches are reported in Table 9.



Cancer model	Condition 1	Condition 2
Breast cancer	Radiotherapy	Radiotherapy + vitamin B3
Breast cancer	Gemcitabine	Gemcitabine + Estrogen receptor signaling
Non Small Cell Lung cancer	Low inhibition of EGFR signaling	High inhibition of EGFR signaling
Colon cancer	Topotecan	Topotecan + mutated p53 status
Breast cancer Neuroblastoma Multiple myeloma	Beclin 1/Bcl-2 complex	Disruption of Beclin 1/Bcl-2 complex
Breast cancer Colon cancer	Sphingosine 1 phosphate signaling	Ceramide signaling
Acute myeloid leukemia	Strong mTOR inhibition	Low mTOR inhibition

Table 9. Autophagic switch from a cytoprotective to a cytotoxic function. Cytoprotective autophagy prevents the cancer cell death and is induced by some particular conditions in various cancer models. All the conditions leading to cytoprotective autophagy are colored in red. When an autophagic switch occurs, cytoprotective autophagy turns into cytotoxic autophagy, and helps to kill the cancer cells. All the conditions leading to the induction of cytotoxic autophagy are colored in green *(Giuriato et al,Cancers,2017).*

A series of studies carried out in breast tumor cells demonstrated the switch from cytoprotective autophagy in cells that were submitted to radiation alone, to cytotoxic autophagy in cells submitted to radiosensitization combined to vitamin D3 [307-308]. In the same line, the recent work of Sheng et al. showed that the estrogen receptor (ER) status in breast cancer cells influenced the gemcitabine

efficacy: ER expression promoted cytotoxic autophagy through the enforced activation of the ER-ERK-p62/SQSTM1 pathway, whereas ER negative cells underwent cytoprotective autophagy [309].

Similarly, a growing number of studies highlight that although the inhibition of EGFR signaling in non-small cell lung cancer cells induced cytoprotective autophagy at first, its further activation (through the addition of rapamycin[310] or through longer hypoxia exposure [311]), led to a switch towards autophagic cell death[312].

The control of autophagy intensity by the anti-apoptotic Bcl2 family of proteins has also been abundantly studied. The literature in this field identifies a “Beclin1/Bcl2 rheostat”, acting in the control of cell survival and death decisions. The Bcl-2 and Bcl-xL proteins inhibit autophagy by binding to Beclin1 [313-314]. The disruption of these interactions increases the level of free Beclin1, which can subsequently strongly activate the autophagy process. Thus, the combination of the molecular depletion of Bcl-2 (through siRNA) with chemotherapy in breast cancer cells [315], with nutrient starvation in neuroblastoma [316], and importantly, with crizotinib in NPM-ALK+ ALCL was shown to potentiate autophagy and to promote massive tumor cell death[317]. This last study, performed in our laboratory, will be further developed in the next paragraph.

Finally, as a last example to illustrate the importance of this “Beclin1/Bcl2 rheostat”, Lamy et al. reported in multiple myeloma that caspase-10 inactivation (using Q-AEVD-OPH or shRNA targeting caspase-10) led to the stabilization of the BCLAF1/Bcl-2 complex and the unleashed activation of Beclin1, which is responsible for the autophagy process over-activation, culminating in autophagy-mediated cell death [318].

The orientation towards an autophagy-mediated cell survival or cell death was shown to be controlled also by sphingolipid rheostat as well[319], i.e., by the balance between ceramide and sphingosine-1-phosphate levels [320]. Indeed, Scarlatti et al. reported that ceramide-induced autophagy triggered autophagic cell death[321], and Lavieu et al. demonstrated that sphingosine-1-phosphate induced survival autophagy[322].

Finally, different doses of a therapeutic compound could also trigger autophagy, but with opposite outcomes in a same cell line. In this context, Willems et al. reported that Acute Myeloid Leukemia (AML) cells treated with either low or high doses of the mTORC1 catalytic inhibitor (AZD8055) undergo

autophagy with cytotoxic or cytoprotective functions, respectively[323]. Consequently, the authors suggest that combining chemotherapy (which induces cytoprotective autophagy) with low-dose AZD8055 or conversely, combining high-dose AZD8055 with autophagy inhibitors, may represent new strategies for improving AML treatment.

7.3 The role of Autophagy and possible modulation in ALK+ALCL

ALK aberrant oncogenic activity results either from ALK gene amplification, mutations, or chromosomal rearrangements. A growing spectrum of cancers has been associated to the ALK oncogene, which has boosted the research towards ALK tyrosine kinase inhibition. The table 10 summarizes the role of autophagy in different ALK+related cancers in response to therapy. In this paragraph, we will focus our attention on ALK+ALCL.

Table 10. Studies reporting autophagy induction following therapy in Anaplastic Lymphoma Kinase (ALK)-associated cancers.

Cancer Type	*ALK* Gene Aberration	Treatment	Method(s) Used to Monitor Autophagy	Role of Autophagy	Signaling Pathway Involved
ALK+ALCL	Translocation (mainly NPM-ALK)	Crizotinib	Electron microscopy Western Blotting Immunohistochemistry Autophagy array (qPCR) Acridine Orange	Cytoprotective	Akt-mTOR suggested
ALK+NSCLC	Translocation (mainly EML4-ALK)	Crizotinib	Western blotting Electron microscopy	Cytoprotective	Akt-mTOR
NB	Mutations Amplification	Entrectinib AZD3463	Western blotting	Cytoprotective Cytotoxic role suggested	Not studied PI3K/Akt/mTOR
ARMS	Gain in copy number	Crizotinib	Acridine Orange Western blotting	Cytotoxic role suggested	PI3K/Akt/mTOR suggested
GBM	No aberration reported	THC	Immunohistochemistry Western blotting	Cytotoxic	Midkine through Akt/mTORC1

(Giuriato et al,Cancers,2017).

Our team showed that autophagy was induced in ALK-positive ALCL cell lines following pharmacological (Crizotinib) or molecular inactivation (through ALK-targeted siRNA) of NPM-ALK . This was assessed by a combination of complementary approaches: increased acidic vesicular organelles (AVOs) formation,

increased number of degradative autophagic vacuoles (as detected by electron microscopy), and increased LC3-II dot staining. Moreover, autophagic flux activation was demonstrated by the classical LC3 turnover assay using either Chloroquine or siRNA targeting ATG7 as inhibitors of the autophagy degradation process. We further demonstrated, both in vitro (by performing viability, apoptosis and clonogenic assays) and in vivo (by measuring xenografted tumor growth), that the combination of autophagy and ALK inhibitions led to the potentiation of the targeted therapy, thus highlighting the cytoprotective function of autophagy in these settings[324]. We have pursued this work by investigating whether we could induce an autophagic switch from cytoprotection to cytotoxicity, in Crizotinib-treated cells. In a recent work of our team, we have shown that BCL-2 is involved in the regulation of this switch [325], as previously reported in other models.

In my thesis work, we identified a microRNA (miR7-5p) which overexpression potentiated the Crizotinib-induced autophagic flux and decreased the cell viability. Thus, our data indicate another possibility to balance from cytoprotective autophagy occurring upon crizotinib single treatment, to autophagy associated with cell death, in crizotinib treated and miR-7-5p overexpressing ALK-positive ALCL cells.

In the next chapter, we will discuss the role of microRNAs in regulating autophagy and cancer.

8 Role of miRNAs in autophagy regulation and cancer

MicroRNAs (miRNAs) are small non-coding RNAs involved in the regulation of gene expression by controlling stability and translation of RNAs messenger (mRNAs) of coding-protein genes. This mechanism controls and modulates different cellular pathways including cell growth, apoptosis, migration, differentiation and autophagy[326-327-328]. Therefore, miRNAs dysregulation (e.g up- or downregulation) is often associated with human diseases including different cancer types. Moreover, the differential expression between normal tissues and tumors allows the use of microRNAs as potent and high predictable cancer markers[329-330]. In the last decade, a growing number of articles were highlighting the role of miRNAs in autophagy regulation and cancers[331].

In this chapter, we will present first the microRNAs machinery, then the regulation of autophagy by miRNAs and we will highlight the role of miR-7-5p, specifically, in cancers.

8.1 MicroRNAs machinery

miRNAs have been found in a wide range of living organisms, suggesting an evolutionary conserved function in the control of gene expression [332]. MicroRNAs are single-stranded, non-coding RNAs composed of 17-25 nucleotides (nt) in length[333]. *In-silico* predictions shows that around 60% of all human genes have potential miRNAs binding sites, suggesting their regulation by this mechanism[334]. At genome level, miRNA genes and clusters can be found in both intergenic and intronic regions. Cellular levels of intronic miRNAs depend on the expression of the host protein-coding gene[335]. Generally, pri-miRNAs are 60–70 nt length RNA transcripts which are usually transcribed from miRNA genes in an RNA polymerase II (pol II)-dependent manner[336]; even though, some miRNAs may depend on RNA polymerase III (pol III)[337]. As protein-coding mRNAs, pri-miRNAs possess a 5'cap and a 3'poly-A tail, they can also go through splicing[336]and different RNAse-dependent reactions are necessary for processing intermediary RNA oligonucleotides and producing mature miRNAs. After transcription in the nucleus, pre-miRNAs are processed by a core ribonuclease complex, including Drosha and its regulatory subunit

DGCR8 (DiGeorge syndrome critical region 8) to generate hairpin-structured pre-miRNAs of 60–70 nt. After cleavage, pre-miRNAs are recognized by exportin-5 and carried out from the nucleus to the cytoplasm where DICER protein generates ~21–22 nt long miRNA duplexes by cleaving the harpin structures. Subsequently, these duplex are loaded into RNA-induced silencing complex (RISC) where Argonaute (AGO) guide single-stranded mature miRNAs to their target mRNAs. The degree of complementarity between mature miRNA sequences and miRNA response elements (MRE) on mRNA sequences is ~8 nt in the core region of the miRNA. The base pairing with the guide miRNA causes an endonuclease-dependent cleavage of the target mRNA (Figure 21)[338-339].

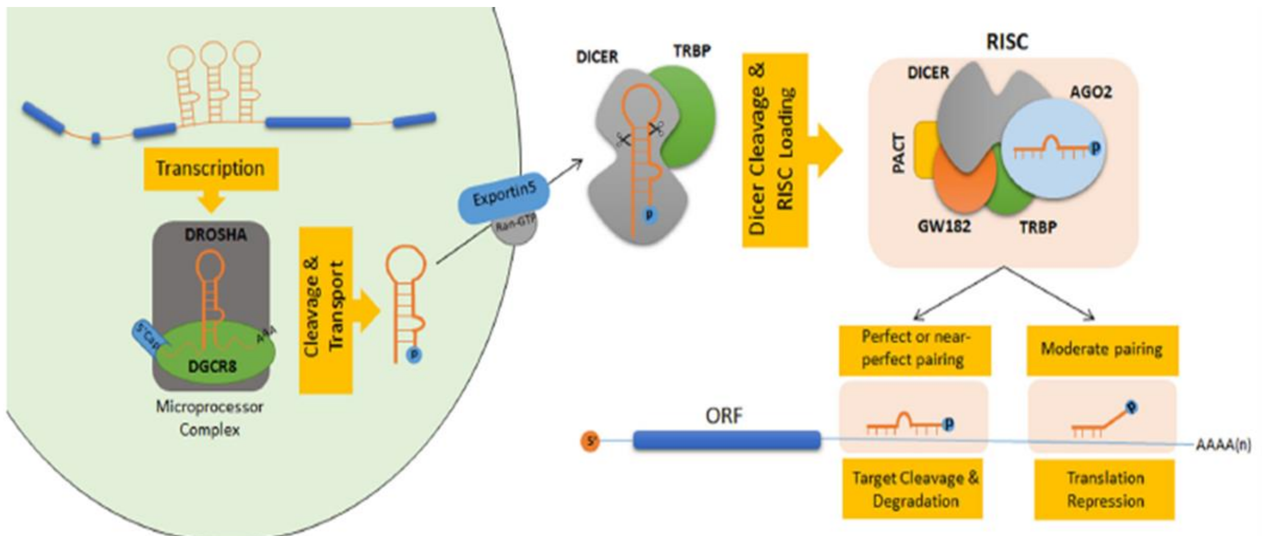


Figure 21. Schematic model of microRNA (miRNA) biogenesis and maturation in human. Nuclear cleavage events performed by protein complexes showing ribonuclease III activity lead to the processing of pri-miRNAs into small hairpin-shaped pre-miRNAs. The core ribonuclease complex (the microprocessor complex) consists of a heterotetramer of Drosha and DGCR8 (DiGeorge syndrome critical region gene 8 or Pasha) proteins. During this reaction, flanking ssRNA–dsRNA junctions in pri-miRNAs are recognized by DGCR8 which guides Drosha to specific cleavage sites around 11 bp away from the stem–ssRNA junctions. Pre-miRNAs that are released after Drosha cleavage exhibit characteristic features of RNase III endonuclease products having 5′ phosphate groups and 2 nt overhangs at their 3′ sequences. After cleavage by Drosha, 3′ overhangs are recognized by exportin-5 (XPO5) complexes in the nucleus . Pre-miRNAs are then transferred to the cytosol by canonical Ran-GTP-dependent transport mechanisms. In the cytosol, another RNase III-type endonuclease, called the Dicer, cleaves pre-miRNAs near their terminal loops, and leads to their conversion to double-stranded 20–22 nt miRNA duplexes.
Terminal loop of pre-miRNAs are recognized through the N-terminal helicase domain of Dicer. Its PAZ domain interacts with 2 nt 3′ overhangs at the termini of pre-miRNAs and directs them to its catalytic RNase III domain for cleavage. RNA-induced silencing complex (RISC) captures the cleavage product through its Argonaute (AGO) protein component. ATP-dependent chaperone activity of Hsc70/Hsp90 proteins is important for small RNA duplex loading onto Ago proteins. Following passenger-strand degradation or ejection, AGO proteins remain in complex with a single-strand guide miRNA. In humans, among the four AGO proteins (AGO1–4), only the AGO2 protein has the ability to slice target mRNAs *(Gozuacik et al,Frontiers in Oncology,2017).*

8.2 Regulation of autophagy by miRNAs

A summary of all miRNAs known to be involved in autophagy regulation is extensively described in this review[331]. Below, we will highlight just a few examples of miRNAs involved in the different steps of the autophagy machinery.

Induction

mTOR complex and other proteins of its pathway were shown to be targets of different miRNAs. In particular, five different components of the mTOR pathway (p70S6K, eIF4E, Mknk1, Mknk2, and Mapkap1) are direct targets of miR-7. On this line, Fang Y et al. showed miR-7-5p as a key regulator of the PI3K/Akt pathway and of mTOR, p70S6K and PIK3CD in hepatocellular carcinoma (HCC) cells[340]. Moreover, miR-199a and miR-101 could directly target mTOR in different cell types[341-342]. Components of the ULK1/2 complex are also direct targets of miRNAs. For example, miR-25 is showed to be a novel regulator of autophagy and cell death through its direct effects on ULK1 expression[343].

Nucleation

miR-30a was one of the first miRNAs to be involved in autophagy regulation. Zhu et al. showed that autophagy was inhibited by direct interaction between miR-30a and BECN1 in MCF-7 cells[344]. Furthermore, combined treatment of Imatinib and miR-30a increased drug sensitivity in chronic myeloid leukemia cells through regulation of ATG5 and BECN1[345]. Regulators of the BECN1/-VPS34 complex are also modulated by miRNAs. For example, AMBRA1 was identified as a miR-23a target in dermal human fibroblast and by using a miR-23a-specific antagomirs, it was possible to show an increased autophagy[346].

Elongation

Different studies showed that miR-181a, miR-30a, miR-374a, and miR-224-3p could target *ATG5* mRNA. Moreover, miR-30d, miR-630, and miR-200b target *ATG12* and miR-519A could affect levels of both *ATG16* and *ATG10*. Finally, a study showed an indirect correlation between miR-204 and LC3 levels. Upregulation of miR-204 levels triggers an increase in LC3 protein levels in adult rat models[331].

Autophagosome maturation and Lysosome fusion

RAB proteins (RAB1B, RAB22A, RAB14), which regulate endocytic pathways, have been shown to be targeted by miR-502, miR-373, and miR-451[347-348]. Moreover, UVRAG (regulator of endosomal trafficking and autophagosome maturation) was shown to be a target of miR-374, miR-630, miR-125, and miR-351, which have an inhibitory effect on autophagy[349-350]***.***

8.3 miR-7-5p in cancer

Recent studies have shown that miR-7-5p plays an important role in cancer. In this paragraph, we will describe the regulation and the role of miR-7-5p.

8.3.1 Genomic organization and regulation of miR-7-5p in cancer

At genomic level, miR-7-5p (miR-7) is located in three different loci in the human genome and its regulation is poorly understood[351].

One locus is placed within an intron of the ubiquitously expressed hnRNP-K (Heterogeneous nuclear ribonucleoprotein K), while the other two loci are intergenic. Of note, the hnRNPK locus is responsible for the most miR-7 expression and a forced expression of the transcription factor HOXD10 (Homeobox D10) which directly interacts with the miR-7 chromatin, increases miR-7 level showing a tumor-suppressive phenotype.

In another study has been shown that knockdown of the ubiquitin-specific peptidase Usp18 increases miR-7 level followed by downstream repression of EGFR expression. Mechanistically, miR-7 acts downstream of Usp18 to regulate EGFR mRNA translation via the 3'-UTR. Nevertheless, the direct knockdown of EGFR, showed an high increase of miR-7 level and the cause of this effect is due to the direct association of c-myc with a putative miR-7 promoter suggesting an interesting feedback loop with EGFR[352].

Finally, Circular RNAs (circRNA) represent a novel class of widespread and diverse endogenous RNAs that regulate gene expression in mammals. ciRS-7 (also termed CDR1as), is a circular miR-7 inhibitor, which harbors more than 70 conventional miR-7 binding sites. Since miR-7 modulates the expression

of several oncogenes, disclosing the regulation of miR-7 activity will likely advance the understanding of various cancer etiologies[352].

8.3.2 Tumor suppressive and oncogenic role of miR-7

Nowadays, several studies have shown the implication of miR-7 in different pathways and diseases. For example, miR-7 has been proposed to have a role in Parkinson disease being a direct regulator of alpha-synuclein[353]. Moreover, miR-7 has been suggested as possible therapeutic target in diabetes since its putative cause of low beta-cell renewal[354]. In the oncology field, miR-7 directly targets and downregulates central oncogenic factors in cancer-associated signaling pathways, including EGF receptor (EGFR), IRS-1, IRS-2[355], Pak1[356], Raf1[357], Ack1 [358], and PIK3CD [359], indicating a clear tumor-suppressive role for miR-7. This role is further corroborated by the fact that miR-7 is the most reduced miRNA in cancer stem-like cells [360]. Moreover, miR-7 was among the most downregulated miRNAs in colorectal cancer [361] . Another example of miR-7 involvement in cancer has been reported in Tazawa et al study. They generated an oncolytic adenovirus that upregulated miR-7 expression and induced autophagy cell death of human tumor cells through the activation of the transcription factor E2F1/miR-7/EGFR pathway, suggesting a potential new method of inducing tumor cell death by controlling miR-7 levels[362].

Many evidences are supporting a tumor-suppressive role for miR-7, but opposite effects have also been reported. An example is the association of miR-7 overexpression with the poor prognosis of lung carcinomas[363].

The Table 11 below highlights the known targets of miR-7 in different tumor types.

Table 11. The targets of miR-7 in different tumor types

Tumor type	Target site(s)	Role of target site
Breast cancer	FAK, PAK1, EGFR, KLF4, HoxB3, SET8	Suppressor
Lung cancer	PA28γ, BCL-2, EGFR, SLC7A5, HuR	Suppressor
Hepatocellular carcinoma	CCNE1, PI3KCD, mTOR, p70S6 K, GSK-3B	Suppressor
Glioma	EGFR, C-KIT, FAK, ERK, ciRS-7	Suppressor
Colorectal cancer	XRCC2, PAX6, YY1	Suppressor
Gastric cancer	EGFR, IGF1R	Suppressor
Acute myelocytic leukemia	TET2	Suppressor
Urothelium carcinoma	FGFR3	Suppressor
Cervical cancer	XIAP	Suppressor
Renal carcinoma	None reported	Oncogene
Melanoma	IRS-2	Suppressor
Ovarian cancer	EGFR, ERK	Suppressor
Oral cancer	RECK	Oncogene
Neuroblastoma	4-HPR, EGCG	Suppressor
Pancreatic carcinoma	MAPK	suppressor
Pleural mesothelioma	None reported	Suppressor
Schwannoma tumors	EGFR, ACK1, PAK1	Suppressor
Tongue squamous carcinoma	IGF1R	Suppressor

FAK focal adhesion kinase, *PAK1* p21 activated kinase 1, *EGFR* epidermal growth factor receptor, *KLF4* Krüppel like factor 4, *HoxB3* homeobox B3, *SET8* SET domaincontaining (lysine methyltransferase) 8, *PA28γ* proteasome activator 28 subunit γ, *HuR* human antigen R, *PI3KCD* phosphoinositide 3-kinase catalytic subunit delta, *mTOR* mammalian target of rapamycin, *p70S6 K* ribosomal protein S6 kinase, *GSK-3β* glycogen synthase kinase 3, *ciRS-7* circular RNA sponge for miR-7, *XRCC2* X-ray repair complementing defective repair in Chinese hamster cells 2, *PAX6* paired box 6, *YY1* Yin Yang 1, *IGF1R* insulin-like growth factor 1 receptor, *TET2* tet methylcytosine dioxygenase 2, *FGFR3* fibroblast growth factor receptor 3, *XIAP* X-linked inhibitor of apoptosis protein, *IRS-2* insulin receptor substrate 2, *RECK* reversion-inducing cysteine-rich protein with Kazal motifs, *4-HPR* *N*-(4-hydroxyphenyl) retinamide, *EGCG* (-)-epigallocatechin-3-gallate, *MAPK* mitogen-activated protein kinase, *ACK1* associated cdc42 kinase 1 (adapted from Zhao *et al. Cancer Cell Int ,2015)*

AIM OF THE THESIS

9 Aim of the thesis

1) In NPM-ALK+ ALCL cell lines, it has been demonstrated that autophagy was induced upon NPM-ALK inactivation, and was endowed with cytoprotective functions. Indeed, autophagy inhibition combined to crizotinib treatment, increased the cytokilling effect of the TKI[364]. Deregulation of miRNA expression levels have been extensively described in cancers, including in NPM-ALK+ ALCL[365], and have also been shown to modulate the responses to therapies[366-367]. As autophagy manipulation is known to impact responses to cancer therapy, a strong interest in the identification of miRNAs involved in autophagy regulation occurred during the past years[331]. In addition, several studies have shown in different cancers type, the possibility to switch from cytoprotective to cytotoxic autophagy. In line with our previous studies, we pursued our work on the identification of possible microRNAs and their targets that could be therapeutically modulated, in addition to crizotinib treatment to drive autophagy towards cytotoxic functions and the outcome of tumor cells death.

2) It has been demonstrated that several fusion oncogenes, notably PML-RAR in Acute Promyelocytic Leukemia (APL)[368], BCR-ABL in Chronic Myelogenous Leukemia (CML)[369], and FLT3-ITD in Acute Myeloid Leukemia (AML)[370] can be degraded through autophagy induced by specific treatment, i.e., arsenic trioxide or all-trans retinoic acid in APL; arsenic trioxide in CML and an inhibitor of the proteasome known to induce autophagy, the bortezomib, in AML. Since these leukemic cells are known to be addicted to their leading oncogene, autophagy activation could be therapeutically exploited to force oncogene degradation and subsequently to lead to tumor regression. Following this line, the second part of my thesis was to try to purify the autophagosomal fraction from ALK+ALCL cell line and check for the presence of NPM-ALK.

RESULTS

RESULTS

Part I : Review « Targeting Autophagy in ALK-Associated Cancers »

1.1 Introduction

A Special Issue entitled "Targeting ALK in Cancer" had been launched in the journal "Cancers" in 2017. The purpose was to extensively review the pathobiology of ALK in human cancers. Indeed, as stated in chapter 2-5 of this manuscript, ALK gene alterations have been found in several cancer types. Being member of the European Research Initiative group on ALK-related malignancies (ERIA) consortium, we have been contacted for a contribution in this Special Issue. We thought it could be interesting to review what was known in the literature regarding autophagy in response to therapy in these various ALK-dependent cancers.

1.2 Autophagy responses upon therapy in ALK+ cancers

Our review is divided in two parts. First, we have presented the autophagy process, a few classical methods to measure the autophagy flux and its functions in cancer therapy. In the second part, we have discussed the reported roles of autophagy in the treatment of ALK-associated cancers. The table 1 *(from the review)* below summarizes these studies. Our review has been accepted for publication in december 2017. Moreover, we have been asked to prepare the cover of this Special Issue, which highlights the growing interest of the scientific community in this field. The cover and the review are included below.

cancers-09-00161 (2).pdf

Part II: Article 1 « miR-7-5p overexpression potentiates crizotinib-induced cytokilling and autophagic flux by targeting RAF1 in NPM-ALK positive lymphoma cells »

2.1 Introduction

In 2015 our laboratory demostrated that autophagy was induced upon NPM-ALK inactivation in NPM-ALK+ALCL cell lines and that was endowed with cytoprotective functions. Indeed, autophagy inhibition combined to crizotinib treatment, increased the cytokilling effect of the TKI[364]. Moreover, in 2019, our team has shown that crizotinib-mediated inactivation of ALK caused an increase in BCL2 levels that restrained the cytotoxic effects of the drug. BCL2 downregulation in combination with crizotinib treatment potentiated loss of cell viability through both an increase in autophagic flux and cell death[317]. Following the same line, we wanted to investigate the possibility to increase the cytotoxic effect of Crizotinib through autophagy modulation. Since deregulation of miRNA expression levels have been extensively described in cancers, including NPM-ALK+ ALCL, we tried to identify possible microRNAs and their targets that could be therapeutically modulated, in addition to crizotinib treatment to drive autophagy towards cytotoxic functions.

2.2 Results of the study

2.2.1 Interest in miR-7-5p as a regulator of the autophagy flux in crizotinib-treated ALK+ ALCL

In an attempt to identify miRNAs involved in the survival response upon targeted therapy in ALK+ALCL, we treated Karpas-299 cells with crizotinib (500nM) for 24h and analyzed the miRNA expression profile using a commercially available microarray. We found predominantly downregulated rather than upregulated miRNAs (Figure 1A, Article 1). We decided to focus our attention on miR-7-5p for three main reasons: (i) it was the most significantly downregulated miRNA in our assay (Table 1, Article 1); (ii) it was known in the literature to harbor predominantly tumor suppressive functions. Thus, we reasoned that its downregulation in crizotinib-treated cells could participate in survival responses upon therapy and failure in the efficacy of the treatment to kill tumor cells; (iii) it was reported to impact on the

autophagy process in other cancer. We thus pursue our work by confirming these three points in our model and we found indeed (i) the downregulation (by RT-qPCR) of miR7-5p in the NPM-ALK positive Karpas-299 and SU-DHL-1 cells, submitted either to crizotinib treatment or to ALK knockdown (using siRNA targeting ALK) (Figure 1B and 1C, Article 1); (ii) that miR-7-5p overexpression (using mimics transfection) resulted in a further decrease in cell viability in comparison with single treatment with crizotinib (Figure 2A and 2B, Article 1); (iii) that miR-7-5p overexpression (using mimics transfection) resulted in an increased basal and crizotinib-induced autophagy flux (Figure 2C, Article 1), as measured using clonal RFP-GFP-LC3 expressing Karpas-299 cells.

2.2.2 Identification of RAF1 as a target of miR-7-5p

In order to find miR-7-5p target(s) which could account for the effect described above, we first looked at experimentally validated miR-7-5p targets and then focused our attention on the ones which had been shown to have a role in the autophagy process.

Using a biotinylated miRNA pulldown assay, we found a selective binding of the RAF1 mRNA to the biotinylated microRNA miR-7-5p (figure 3A, Article 1). To support the results obtained, we validated that miR-7-5p overexpression in Karpas-299 cells resulted in both RAF1 mRNA and protein level decreases (figure 3C, 3D, Article1).

2.2.3 miR7-5p mimics or RAF1 inhibition impacts on cell viability and autophagy flux

After having shown that (i) miR-7-5p was significantly downregulated in Karpas-299 and SU-DHL-1 cells upon NPM-ALK inactivation, (ii) the effect of miR-7-5p overexpression improve crizotinib-induced cytotoxic effects and (iii) increased autophagy flux, we focused our attention on its target RAF1. Specifically, we investigated whether the pharmacological inhibition of RAF1 could reproduce the results obtained by overexpression of miR-7-5p on cell viability and on the autophagic flux. Treating Karpas-299 with Vemurafenib (ATP-competitive oral inhibitor of RAF1 and BRAF) and Crizotinib, we observed a significant decrease in cell viability compared with the single drug treatment (figure 4A, Article 1). Following the same experimental procedure, we also observed a potentiated autophagic flux

when RFP-EGFP-LC3 Karpas-299 cells were treated with both compounds (figure 4B, Article 1). Similarly, a slight but significant potentiation of autophagic flux and cell death was observed upon RAF1 molecular downregulation using siRNA targeting RAF1(figure 4D,supplemental figure 4, Article 1).

2.2.4 RAF1 controls the autophagy machinery through ULK1

Having found that RAF1 inhibition has an impact on autophagic flux in NPM-ALK+ ALCL cell lines, we hypothesized that this effect could due to its kinase activity on the autophagy machinery. We focused our interest on ULK1 since its regulation through serine phosphorylation, notably by two major kinases controlling i.e. mTOR and AMPK, had been largely documented.

We first investigated if the pharmacological inhibition of RAF1 (using vemurafenib) could impact the phosphorylation of ULK1 at its phosphoSer757 site (inhibitory phosphorylation).

We found a significant decrease of the phosphoSer757 ULK1 signal in Karpas-299 cells submitted to the combined NPM-ALK and RAF1 inactivation (using crizotinib and vemurafenib, respectively) compared with the single drug treatment (figure 5A, Article 1). We obtained similar results when Karpas-299 cells were transfected either with siRNA targeting RAF1, or with miR-7-5p, and treated with crizotinib (Supplemental figure 5B, Article 1). Moreover, we retained important to highlight that the function of RAF1 in regulating ULK1 phosphorylation occurred independently of MEK/ERK signaling cascade. In accordance, we found that the combination of Mirdametinib (MEK inhibitor) and Crizotinib didn't affect the phosphorylation of Ser757 ULK1 (figure 5B, Article 1) and the autophagic flux (figure 5C, Article 1). Finally, we investigated whether RAF1 could directly phosphorylate ULK1 at Ser757 inhibitory residue by performing an in vitro kinase assay. We found that the recombinant RAF1 enzyme phosphorylated the ULK1 protein as a substrate on its Ser757 residue (figure 6, Article 1).

These results are the core of a paper submitted to the journal "Cancers", as shown below.

Cancers 2020, 110220 Sorrentino D et al final.pdf

Supplemental Figures 110220 Sorrentino et al.pdf

Supplemental figure legend 110220 Sorrentino et al.pdf

Part III: Revision of the article; ongoing work

Our study has been found interesting by two reviewers which are experts in the field. There comments have been useful and their major requests will improve our paper. Below are described additional results that will be included in the revised version, and new experiments which are planned for the next 3 months.

3.1 Assessment of autophagy flux potentiation upon combined ALK and RAF1 inhibitions

We have generated in our laboratory Karpas-299 clonal cells, which stably express the RFP-GFP-LC3 tandem probe. These cells allow an easy, quantitative and rapid measurement of the autophagy flux by flow cytometry. We have used them extensively throughout our paper. To confirm our results, we plan to analyze LC3 dots contents upon the various treatments using immunofluorescence. Indeed, this technique is well known in our team; it is a very classical one, highly recognized in the autophagy community, and it is more sensitive than LC3 western-blot. We are currently setting up the conditions for LC3 immunofluorescence stainings in Karpas-299 cells. Then, we will plan further use of this technique to measure the autophagy flux in cells submitted or not to ALK and RAF1 single or combined inactivation.

3.2 Decreased ULK1 phosphorylation upon combined ALK and RAF1 inhibitions

Our data showed that ALK inhibition (using crizotinib) combined to RAF1 pharmacological inactivation (using vemurafenib) or molecular knockdown (using siRAF1 or miR-7-5p mimics) impaired the phosphorylation of ULK1 on the serine 757 residue. To further confirm this result, we have generated Karpas-299 cells genetically invalidated for RAF1 using the CRISPR/Cas9 system. We used to delete Raf1 the lentiCRISPR vector (plasmid #52961) established by the Zhang lab. The plasmid encodes the humanized Streptococcus pyogenes species of Cas9 (hSpCas9) nuclease that specifically recognizes and cleaves the DNA sequence directly adjacent to the PAM sequence 5'-NGG-3'. The design of the target

gRNA sequences against RAF1 was performed using the platform: http://crispr.mit.edu/. The cloning of the gRNA sequences against RAF1 was performed as recommended by the Zhang Lab GeCKO website http://www.genome-engineering.org/gecko/. These cells, when submitting to crizotinib treatment, harbored a clear decrease in the PhosphoSer757-ULK1 signal, as shown below (figure 22).

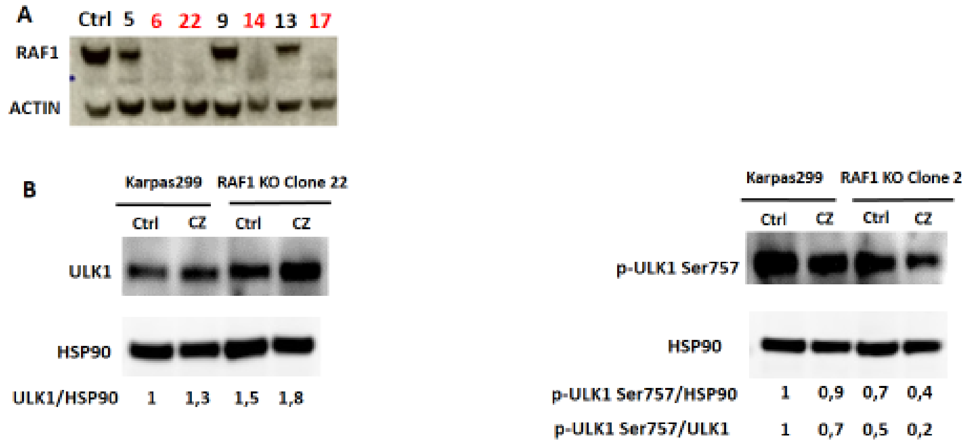


Figure 22. Effect of combined ALK inhibition and RAF knockout on ULK1 Serine 757 phosphorylation. (A) Whole cell lysates were loaded on a same gel and probed for RAF1 and ACTIN contents by western blot. In red are highlighted the RAF1 knockout positive clones. **(B)** Wilde type Karpas-299 cells and RAF1 knockout(KO) cells were treated or not (Ctrl) overnight (16h) with crizotinib (CZ) 250nM. Whole cell lysates were loaded twice on a same gel and probed for total ULK1 and phospho-Ser757 ULK1 contents by western blot. Densitometry analysis of the phospho-ULK1 (Serine757) levels was calculated relative to the control samples (normalized over the hsp90 signals) and relative to the total ULK1 signals.

3.3 Demonstration *in vivo* of the efficiency of the combined ALK and RAF1 inhibition for the treatment of ALK+ ALCL.

We have shown *in vitro* that ALK inactivation (using crizotinib) combined to miR-7-5p overexpression or vemurafenib treatment strongly impaired cell viability (figure 2A-B and 4A, Article 1). To further validate these results and support their translation to clinics, we plan to perform in vivo experiments, using immunodeficient mice harboring ALK+ ALCL xenografted tumors. Our experimental design will be to subcutaneously inject 4 million of Karpas-299 cells in both flanks of 16 mice. Once tumors will be palpable, the mice will be divided into 4 groups (of 4 mice each): (i) the first “control” group will correspond to untreated mice, to leave the tumor grow over time; (ii) the second “Crizo” group will correspond to mice treated with crizotinib only; (iii) the third “VEM” group will correspond to mice

treated with vemurafenib only; (iv) the fourth "Combo" group will correspond to mice treated with the combination crizotinib + vemurafenib. Of note, a pilot experiment will be performed to determine the doses of vemurafenib and crizotinib to be used.

The effects of the different treatments on tumor growth will be assessed by measuring tumors every 2 days using calipers, followed by tumor volume calculation. Around 28 days post-injection, mice will be humanely sacrificed and tumors harvested for subsequent immunohistological analysis of classical autophagy markers (LC3).

Part IV: Purification of autophagosomes. Ongoing work

4.1 Interest in isolating autophagosomes

It has been shown in several studies that key oncogenes proteins are degraded by autophagy pathway[371] . Thus, autophagy induction could represent an interesting therapeutic strategy to force cells to degrade oncoproteins. In this context, we want to study whether NPM-ALK is located into autophagosomes cargo.

Another reason that focus our interest in purifying autophagosomes, is their documented role as cellular signalling platforms that allow efficient spatial coordination of oncogenic pathways[372] . Indeed, whether or not NPM-ALK could use autophagosomal membranes as scaffold to mediate its oncogenic potential has never been studied so far.

Finally, it has been shown that an enhanced autophagy could mediate the degradation of anti-apoptosis[373] or anti-necroptosis proteins[374], thereby promoting cell death. Thus, it would be interesting to analyze if such proteins could be recovered in ALK+ ALCL isolated autophagosome fraction, in conditions where we found that enhanced autophagy resulted in increased cell death.

4.2 Methods to isolate autophagosomes

-Extrusion in the culture medium

This protocol is based on the fact that a cell with an high autophagosomes content is able to extrude them in the culture medium. This phenomenom, called “exophagy”, is not well described. Nevertheless, such purification of autophagosome was reported in the literature and we adapted the protocol to our ALK positive ALCL cell line[375].

Karpas 299 cells were treated with:

-Chloroquine (well-known blocker of Autophagosome-Lysosome fusion)

-Crizotinib (Autophagy inducer)

-Bortezomib (Proteasome inhibitor)

The goal of the treatment is to increase the autophagosomes production by Crizotinib, block the NPM-ALK degradation by proteasome (Bortezomib)[376] and keep intact the autophagosomal fraction (AF) by Chloroquine.

The cell suspension was pre-cleared by centrifugation at 1600 rpm for 10 min, and the extruded autophagosomes in the supernatant were then recovered by centrifugation at 12,000 rpm for 30min, washed with PBS and pelleted again by a second 30 min centrifugation at 12,000 rpm. The AF purity was then assessed by western-blot.

-Cavitation/ultracentifugation method

This protocol aims at purifying the autophagosomes, present within cells, after N2 pressure disruption of the plasma membranes. It has been developed by the group of Dr A.M. Cuervo and we adapted it to our cell model, as described below.

$400X10^6$ Karpas-299 cells were treated with Chloroquine (20µM for 16h) to accumulate autophagosomes. After treatment, cells were collected and resuspended in cold sucrose (0.25M), put into a cavitation chamber and disrupted by Nitrogen cavitation (35psi for 1min). Cell disruption by nitrogen decompression is a rapid and effective way to homogenize cells and to release intact organelles. The pellet was recovered after centrifugation (12000g, 12min) and resuspended in 1ml of cold sucrose (0.25M). The organelles suspension was then loaded at the bottom of an ultracentrifugation tube and then was added Nycodenz at decreasing concentrations (26%-24%-20%,-15%) in order to build different interphases. The tube is ultracentrifuged at 104500g for 3h. Each interphase is collected and analyzed by Western Blot.

4.3 Preliminary results in ALK+ ALCL

4.3.1 Extrusion method

Our preliminary results indicate that an autophagosome rich fraction (AF) (as assessed by LC3-II and p62 enrichment) could be recovered from the tumor cell culture medium (figure 23). Interestingly, NPM-ALK could be detected in this fraction. However, we were concerned by the purity of this autophagosome preparation, since it has also been detected Actin which is a cytosol marker.

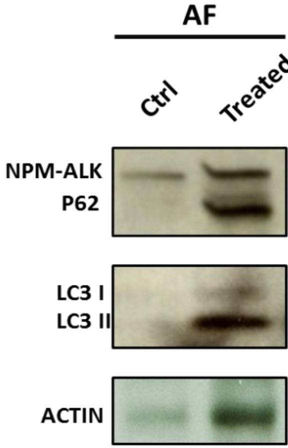


Figure 23. Autophagomal fraction (AF) from Karpas 299. Cells were treated or not (Ctrl) with Crizotinib (500nM), Bortezomib (10nM) and Chloroquine (10uM) for 16h. The recovered AF were analyzed by Western Blot.

4.3.2 Cavitation/ultracentifugation method

As shown in figure 24 A, each Nycodenz interphases should correspond to a specific organelles. In this preliminary result, we focus our attention to delineate the purity and the presence of NPM-ALK in the interphases corresponding to the autophagosomes and autophagolysosomes fractions. For this reason, we decided to detect:

-LC3II (Autophagosome/AutophagoLysosome marker)

-PCNA (Nucleus marker)

-HSP90 and Actin (Cytosol marker)

In figure 24B, we show that all the interphases, except the interphase 26-50% (Mitocondria fraction) are not contaminated by cytosol and nucleus contents. We suppose that the ultracentrifugation step was not efficient enough for the contents separation in the lower part of the tube.

The presence of LC3II is relegated only to the Autophagosome and Autophagolysosome fractions (interphases 15-20%/20-24% respectively), which indicates a good purity. Moreover, NPM-ALK is found in those two fractions. We are concerned by the presence of LC3II in the mitocondria fraction indicating that the majority of the Autophagosome and Autophagolysosome fraction did not migrate correctly.

We retained that cavitation/ultracentrifugation method allowed us to exclude cytosol contents from the autophagosome and autophagolysosome fractions, but we still need to exclude the presence of others organelles from the fractions of interest. To solve this problem, we are going to use specific markers (e.g. COXIV for Mitocondria and LAMP2 for Lysosomes) to assess that the detected signal of NPM-ALK is relegated uniquely to Autophagosomes contents.

These preliminary results are encouraging but further work is needed to optimize the purification of autophagosomes form NPM-ALK positive ALCL cells.

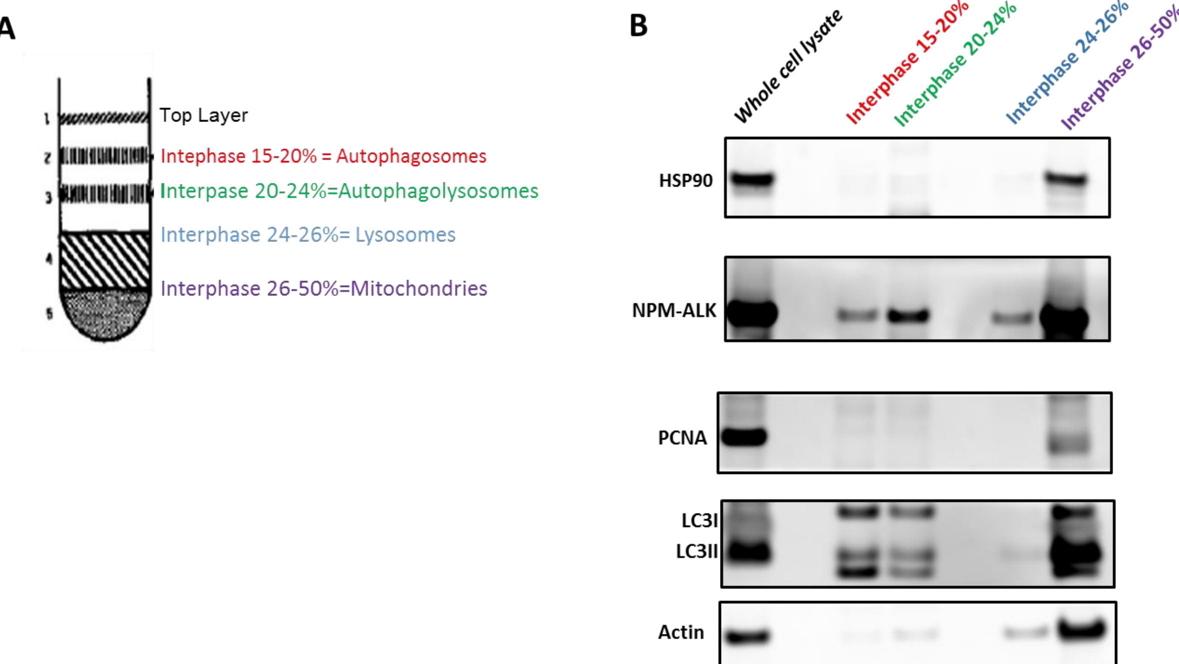


Figure 24. Detection of NPM-ALK in the Autophagosome and Autophagolysosome fraction. **(A)** Schematic representation of each Nycodenz interphases which correspond to different organelles fractions **(B)** Karpas-299 cells were treated with Chloroquine (20µM for 16h). The cells were submitted to cavitation/ultracentrifugation method and the different interphases were loaded on the same gel and probed for HSP90,NPM-ALK,PCNA,LC3,ACTIN.

DISCUSSION AND PERSPECTIVES

Discussion and perspectives

The role of autophagy in cancer therapy has abundantly been studied over the past decade. It is now well established that autophagy can lead, depending on the cancer and on the therapy, to either tumor cell survival or demise. This duality, although fascinating, makes it difficult to decide whether autophagy inhibitors or, inversely, autophagy activators should be used to improve a given anti-cancer therapy. Adding complexity, but also creating new therapeutic opportunities, switches from cytoprotective to cytotoxic autophagy have been reported.

My thesis work was conducted in this field. Indeed, one year before my arrival in Toulouse, my host team had demonstrated the induction of a cytoprotective autophagy in ALK+ Anaplastic Large Cell Lymphoma following crizotinib targeted therapy. My project was to identify new therapeutic ways to shift survival autophagy towards lethal autophagy. The aim was to identify and propose a new therapeutic strategy, based on the manipulation of autophagy, to improve the cytotoxic effects of crizotinib, and ultimately, to prevent tumor relapse. As a starting point, we performed a microRNA expression profile analysis in ALK+ ALCL cells submitted or not to crizotinib.

During my PhD, I have shown that (i) the microRNA 7-5p was downregulated with the highest reproducibility in crizotinib-treated ALK+ ALCL cells; (ii) the ectopic expression of miR-7-5p (using mimics) increased both the crizotinib-induced loss of cell viability and the crizotinib-induced autophagy flux; (iii) one of the target of miR-7-5p was RAF1; (iv) the molecular or pharmacological inhibition of RAF1 recapitulated the effects of miR7-5p mimics; (v) the combined ALK and RAF1 inhibition potentiated autophagy and increased cell death; (vi) RAF1 could phosphorylate ULK1 on inhibitory site to restrain autophagy. A representative model highlighting the main molecular characteristics that underlie the roles of autophagy in ALK+ALCL therapy (ALK and RAF1 inhibition) are illustrated in figure 25.

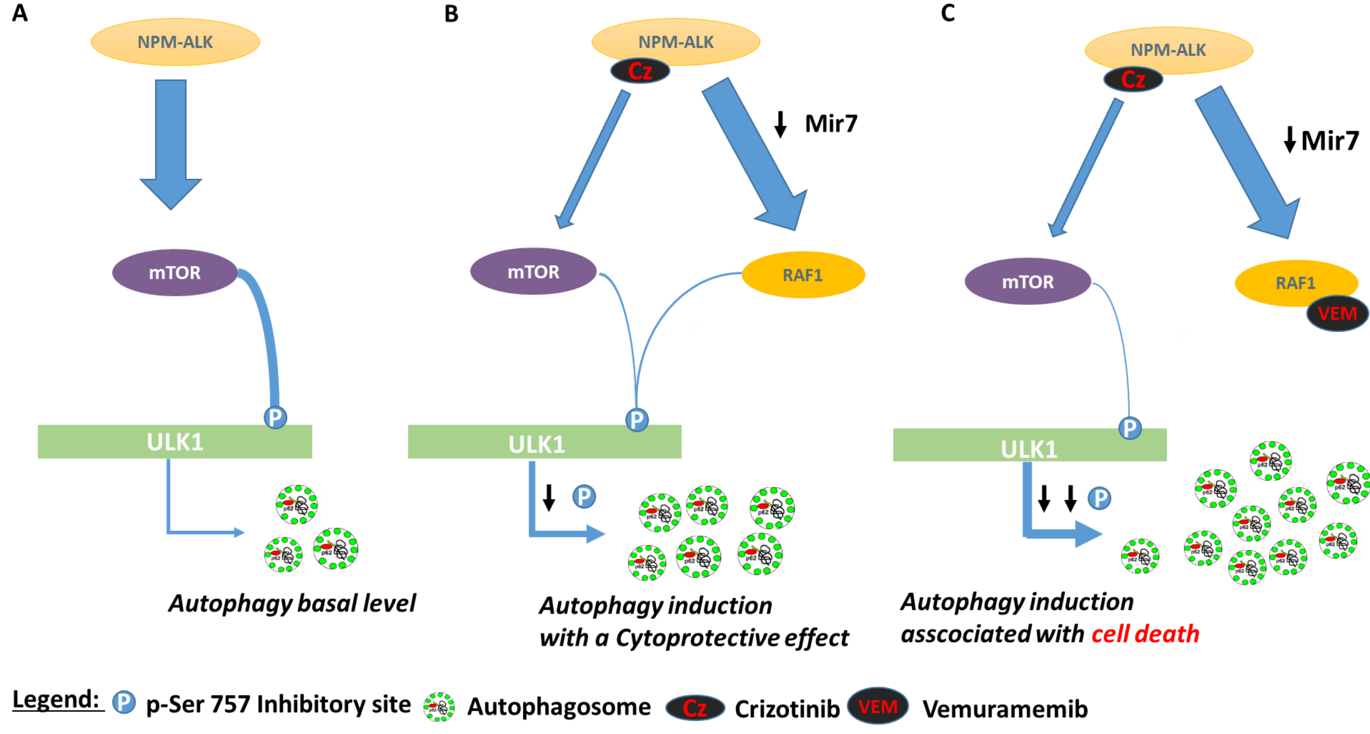


Figure 25. Representative model of the molecular mechanism underlying the autophagy pathway upon ALK and RAF1 inhibition. **(A)** NPM-ALK increases mTOR activity and also involves the phosphorylation of ULK1 at p-Serine 757 residue. **(B)** The inactivation of NPM-ALK by Crizotinib reduces the phosphorylation of ULK1 at p-Serine 757 and increase the RAF1 protein level (through Mir7 downregulation) which phosphorylate ULK1 at same residue. The autophagy flux is increased and shows a cytoprotective effect. **(C)** ALK and RAF1 inhibition decreases drastically the ULK1 phosphorylation at p-Serine 757 which further increases the autophagic flux and is associated with cell death.

Autophagy potentiation and tumor cell death

*The connection between autophagy and cell death is complicated, as denoted by the terms "Autophagic Cell Death (ACD)" or "Autophagy associated with cell death", which have been extensively debated [306]. According to the recommendations of the "Nomenclature Committee on Cell Death (NCCD)", true ACD corresponds to situations where cell death is mediated exclusively by autophagy and can be suppressed by disruption of the autophagy process [377]. For purists, this included only cases where a too excessive cellular self-digestion is no more compatible with cell survival. Therefore, for the other cases where autophagy was reported to support and to allow the occurrence of other cell death pathways, such as apoptosis or necroptosis, and even if autophagy inhibition in these settings

prevented the occurrence of these subsequent cell death modalities, it does not correspond to true ACD but rather to "Autophagy associated with cell death" [378,379]. Finally, this last term of "Autophagy associated with cell death" also includes cases of cell death, where autophagy markers and autophagy flux are increased, but where autophagy inhibition does not suppress the death outcome; in other words, those are cases where autophagy accompanies the death process, but is not a direct or even indirect executor of the death outcome.

*In ALK positive Anaplastic Large Cell Lymphoma, two studies from our laboratory report the possibility to shift crizotinib-induced cytoprotective autophagy to autophagy associated with cell death:

(i) the recently published work of Dr Avédis Torossian, under the supervision of Pr Estelle Espinos, described that crizotinib treatment resulted in the upregulation of BCL2 mRNA and protein levels. The knockdown of BCL2 (through the use of siRNA or miR34a mimics to target BCL2), concomitantly to crizotinib treatment, resulted in an increased loss in cell viability, associated with enhanced autophagy and apoptosis [325]. In these settings, the blockade of autophagy (through the use of siRNA targeting ULK1) resulted in a partial rescue in the loss of cell viability. Thus, according to the definition of the NCCD explained above, autophagy appeared to be associated with the lymphoma cells demise.

(ii) my thesis work identified miR7-5p and its target RAF1 as regulators of the intensity of the autophagy flux. As we found that RAF was able to phosphorylate ULK1 at inhibitory site, we proposed that it could restrain the crizotinib-induced autophagy flux. Therefore, RAF1 inactivation unleashed the autophagy response, which was associated with increased cell death, including apoptosis. Furthermore, as autophagy inhibition in these settings was not able to rescue from the loss in cell viability (personal data not presented in the manuscript), it suggested that the enhanced autophagy flux was likely to support other cell death modalities, but then became dispensable for the execution of cell death.

*In a mechanistic point of view, the explanations in the literature for the balance from cytoprotective autophagy to lethal autophagy are including:

(i) *the extend and duration of autophagy, indicating that a critical threshold of autophagy may account for its protective or death functions.* In ALK positive Anaplastic Large Cell Lymphoma, previous work of the team demonstrated that enhanced or sustained autophagy elicited by single crizotinib treatment was still endowed with cytoprotective functions (Dr Julie Frentzel, unpublished data). Thus, we do not

believe in the notion that an autophagy threshold accounts for the cell survival or death fate. Interestingly, the combination of crizotinib with another therapeutic treatment (Bcl2 downregulation or RAF1 inhibition) potentiates autophagy and leads to cell death. Thus, these results rather suggest that ALK inactivation, concomitant with the inhibition of other molecular determinants, now engages autophagy towards cell death outcome.

(ii) *the selective removal of autophagy substrates, which promotes cell death.* Whether or not enhanced autophagy, through the excessive degradation of key survival factors, may account for cell death in our model has not been investigated yet. In this context, the hypothesis of NPM-ALK degradation through excessive autophagy should be studied. Indeed, previous reports did show the relocation and degradation of fusion oncogenes upon anti-cancer treatment [368,369]. Another interesting substrate, which autophagic degradation was reported to lead to tumor cell death, is the reactive oxygen species (ROS) scavenger catalase [374]Indeed, such autophagy-mediated ROS accumulation accounts for membrane lipid oxidation, loss of membrane integrity and subsequent cell demise. Interestingly, ALK positive ALCL cells were found to produce high level of ROS by a pathway involving lipoxygenases (LOX) [380]. Thus, it is tempting to speculate that excessive autophagy, by degrading ROS catalase could further increase the ROS content in ALK positive ALCL cells until reaching toxic levels responsible for subsequent cell death. It would be interesting to pursue investigations to confirm this hypothesis, notably to determine if ROS catalase could be detected in autophagosomes purified from NPM-ALK positive ALCL cells. Finally, the same reasoning could apply for the excessive autophagy mediated degradation of anti-apoptosis proteins, such as Fap-1, an inhibitor of Fas-mediated apoptosis, as described in BJAB lymphoma cells [373]. Thus, whether or not Fap-1 or others anti-apoptosis proteins or molecules could be detected in autophagosomes and degraded, through excessive autophagy, in ALK positive ALCL cells would be interesting to investigate.

(iii) the use of autophagosome membranes as a support for other cell death modalities, such as apoptosis or necroptosis. Indeed, Young et al. demonstrated in MEF cells treated with sphingosine kinase inhibitor (SKI) that ATG5 and ATG16L positive autophagosomal membranes were indispensable for the formation of an intracellular death-inducing signaling complex (iDISC) containing FADD and caspase-8 apoptosis related proteins [381]. In the same line, Basit et al. demonstrated that the recruitment of FADD, RIPK1 and RIPK3 proteins, which constitute the necrosome core, on autophagosomal

membranes allowed Obatoclax (a BCL2 inhibitor) mediated cell death in rhabdomyosarcoma cells [382]. In this framework, it is clear that further investigations should be conducted In ALK positive ALCL to determine whether apoptosis and/or necroptosis could occur, secondary to autophagy in crizotinib-treated cells, to ensure lymphoma cells full eradication as observed in combined therapy.

All the events underlying the autophagy switch from cytoprotection to cell death are presented in the table below.

Cancer Type	Events underlying the autophagy switch from cytoprotective to cell death	Mechanisms	References
L929/A cell line(Fibroblast)	**Degradation of ROS scavenger catalase**	zVAD -> autophagy -> degradation of catalase -> ROS accumulation -> non-apoptotic death	*Yu et al* PNAS 2005
B-cell lymphoma (BJAB cell line)	**Degradation of anti-apoptosis factors**	Autophagic degradation of the phosphatase Fap-1 -> Fas apoptosis	*Gump et al* Nat cell Bio 2014
Acute myeloid leukemia (AML)	**Autophagy membranes as scaffold for apoptosis (iDISC formation)**	Autophagosomal membrane serves as a platform for caspase-8 activation -> apoptotic cell death	*Young et al* BJC 2012
Rhabdomyosarcoma (RMS)	**Autophagy membranes as scaffold for necroptosis (RIPK1/3)**	Obatoclax promotes the assembly of the necrosome on autophagosomal membranes	*Fulda et al* Cell death 2015
Anaplastic Large Cell Lymphoma (NPM-ALK+ ALCL)	**Excessive autophagy**	Crizotinib+BCL2 downregulation -> autophagy and apoptosis -> cell death	*Torossian et al* Hematologica 2019
Acute promyelocytic leukemia (APL)	**Degradation of the fusion oncogene PML/RARA by Autophagy**	Retinoic acid-> autophagy-> PML/RARA degradation	*Simonsen et al* Blood 2010
Acute myeloid leukemia (AML)	**Degradation of the FLT3-ITD oncogene by Autophagy**	Bortezomib ->autophagy->FLT3-ITD degradation	*Larrue et al* Blood 2016
Chronic myelogenous leukemia (BCR-ABL)	**Degradation of the fusion oncogene BCR-ABL by Autophagy**	Arsenic trioxide -> autophagy->BCR-ABL degradation	*Goussetis et al* Blood 2012

Prospects for therapeutic use in patients

*Our work highlighted a new promising combined therapy to improve the treatment of ALK positive ALCL patients. It is based on the combination of the crizotinib drug with a treatment aiming at downregulate RAF1 activity. We found miR-7-5p mimics, siRNA targeting RAF1 and vemurafenib as equivalent strategies to enhance crizotinib-induced autophagy flux and cytokilling effects. This work shed light on a previously undescribed role for RAF1 in ALK positive ALCL, consisting in negatively regulating the autophagy flux through phosphorylation of the ULK1 serine 757 inhibitory residue. Furthermore, it showed for the first time the benefit, *in vitro*, of combining crizotinib to vemurafenib,

a drug which present the advantage to be already used in clinics for the treatment of RAF-associated cancers. We found that vemurafenib enhanced both basal and crizotinib-induced autophagy flux. This result is in accordance with the literature showing that vemurafenib single treatment in BRAF mutated thyroid cancers elicited a cytoprotective autophagy response [383] but that vemurafenib and obatoclax co-treatment, by inhibiting BCL2 and potentially increasing autophagy, improved cytokilling in vemurafenib refractory thyroid cancers [384]. Finally, our results should further stimulate investigations aiming at the delivery of miR7-5p mimics for tumor therapy. Based on its known tumor suppressive functions, the development of such miR7-5p replacement therapy already started. Three methods have been described so far to allow miR7-5p delivery *in vivo*; one involved the encapsulation of miR7-5p in cationic liposomes [385]; another described the use of RGD (Arg-Gly-Asp) nanoparticules [386]; and recently, a nanoparticle-mediated co-delivery of a chemotherapeutic drug (paclitaxel) and miR-7-5p was proven feasible [387]. Despite encouraging, the main challenge to date remains to translate these findings, obtained in mouse models for cancers, to clinical use for patients.

*Our work may support the targeting of autophagy in ALK positive ALCL stem cells, to prevent tumor relapse. This assumption came from results obtained by Dr Luca Mologni group, showing that ALK inactivation (using lorlatinib) combined with mTOR inhibition (using temsirolimus) was more effective than each single treatment in inducing cell cycle arrest and apoptosis [64]. Furthermore, the team provided evidence that this co-treatment strongly delayed tumor relapse *in vivo*, after cessation of the therapy. This result leads us to suggest that ALK positive tumor cells, endowed with stem cells properties, might be more sensitive to the co-treatment conditions. As autophagy has been involved both in tumor dormancy [388] and in the acquisition of stem cell features upon therapy [298], we formulate the hypothesis that enhancing autophagy, notably by inhibiting mTOR (the most potent inhibitor of the autophagy process), might lead to the death of this ALK positive ALCL stem-like cell population, thereby preventing or delaying tumor relapse. It would be interesting to confirm this hypothesis either by using ALK positive side population cells which do express a signature of genes associated with 'stemness' and pluripotency, as described by the group of Dr Suzannne Turner [389] or by using ALK positive cells engineered to harbor stem-like features through their responsiveness to the Sox2 transcription factor, as described by the group of Pr Raymond Lai [390].

CONCLUSION

Conclusion

NPM-ALK+ ALCL treatment still needs improvement, to prevent refractory/relapse cases following standard chemo- or NPM-ALK targeted-therapies. Our study provides evidence, for the first time, that dual inhibition of NPM-ALK and RAF1 (using pharmacological (vemurafenib) or molecular approaches (siRNA or miRNA (miR-7-5p mimics) targeting RAF1)) may be superior than single NPM-ALK targeted therapy (crizotinib) in killing tumor cells. We found that this combined therapy triggers massive autophagy, notably through the relieved inhibition of the ULK1 protein, and ultimately leads to lymphoma cell death. Thus, our work stresses the importance of autophagy in the responses to anti-cancer drugs and highlights a new therapeutic approach for NPM-ALK+ ALCL.

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Abstract : Anaplastic Lymphoma Kinase positive Anaplastic Large Cell Lymphomas (ALK+ ALCL) are an aggressive pediatric disease. They are characterized by chromosomal translocations involving the *ALK* gene with various translocation partner genes. NPM-ALK is the most prominent fusion protein observed. It results from the t(2;5)(p23;q35) chromosomal translocation and leads to the constitutive activation of the tyrosine kinase domain, which is driving lymphomagenesis through the activation of multiple survival/proliferation pathways. Therapeutic options comprise chemotherapy, which is efficient in about 70% of the patients, and targeted therapies, such as crizotinib (an ALK tyrosine kinase inhibitor) used in refractory/relapsed cases. Efforts converged also towards the development of combined therapies to improve treatment. In this context, we studied whether autophagy could be modulated to improve crizotinib therapy. Autophagy is a vesicular recycling pathway, known to be associated either with cell survival or cell death depending on cancers and therapies. The Unc-51-like kinase-1 (ULK1) protein plays a critical role in the autophagy initiation stage and is regulated mainly through AMPK- or mTOR- mediated serine/threonine phosphorylations on key residues. In NPM-ALK+ ALCL cell lines, previous work of my host team had demonstrated that autophagy was induced upon NPM-ALK inactivation, and was endowed with cytoprotective functions. During my PhD, I first reviewed the cytoprotective, or inversely, cytotoxic roles of autophagy upon therapy in various other ALK-dependent cancers. Then, my main thesis project was to identify microRNAs (miRNAs) and their potential targets that could be therapeutically modulated, in addition to crizotinib treatment, to drive autophagy towards cytotoxic functions and the outcome of tumor cells death. Indeed, deregulation of miRNA expression levels have been extensively described in cancers, including in NPM-ALK+ ALCL, and have also been shown to modulate the autophagy responses upon therapies. My work led to the demonstration that miRNA-7-5p, known primarily to harbor tumor suppressive functions in diverse cancer types, and RAF1, one of its targets, play essential roles in NPM-ALK+ ALCL, by controlling autophagy flux and tumor cell fate. RAF1 is a serine/threonine kinase, best known to connect RAS to the MEK/ERK pathway. However, the mechanism by which RAF1 inhibition, specifically, could induce autophagy had not been described so far. Our work points out for the first time the possible phosphorylation of ULK1 on its serine757 inhibitory residue by RAF1 (and not MEK or ERK), which opens up a new therapeutic avenue to modulate the autophagy flux in ALK+ ALCL. In a second part of my project, and to better understand the potential link between RAF1 and ULK1, I gave several attempts to purify autophagosomes from NPM-ALK+ ALCL cells. While our results suggest that NPM-ALK could be detected in an autophagosome-enriched fraction, further investigations will determine whether RAF1 and ULK1 could co-localized at autophagosomal membranes. Altogether, our results strengthens that autophagy lays at a center place for NPM-ALK+ ALCL tumor cells fate upon crizotinib treatment, and stressed that NPM-ALK and RAF1 combined inactivation, by increasing autophagy flux, might be beneficial for ALK+ ALCL patients.

Résumé : Le lymphome anaplasique à grandes cellules ALK positif (LAGC ALK+) est un cancer pédiatrique très agressif. Il se caractérise par des translocations chromosomiques impliquant toujours le gène ALK et différents autres gènes, partenaires de translocation. NPM-ALK (Nucleophosmine- Anaplastic Lymphoma Kinase) est la protéine de fusion à activité tyrosine kinase la plus fréquemment observée. Elle résulte de la translocation chromosomique t(2;5)(p23 ;q35) et l'activation constitutive de son domaine catalytique permet le développement du lymphome, par l'activation de nombreuses voies de signalisation de survie et de prolifération. Les thérapies actuelles sont la chimiothérapie, qui est efficace dans 70% des cas, ou une thérapie ciblant l'oncogène ALK (notamment l'inhibiteur tyrosine kinase Crizotinib), qui est utilisée chez les patients réfractaires ou en rechute. Les efforts de la communauté scientifique convergent également au développement de thérapies combinées, pour améliorer le traitement de ces lymphomes. Dans ce contexte, nous étudions si l'autophagie peut être modulée pour améliorer la réponse au crizotinib. L'autophagie est un processus vésiculaire d'autodigestion, connu pour être associé soit à la survie, soit à la mort cellulaire, selon le type de cancer et de thérapie. La protéine ULK1 (Unc-51-like kinase-1) joue un rôle majeur dans l'initiation de ce processus et est régulée principalement par des phosphorylations sur des résidus sérine ou thréonine, qui sont assurées par les kinases mTOR ou AMPK. Dans les lignées de LAGC ALK+, des travaux antérieurs de l'équipe ont montré qu'une autophagie cytoprotectrice était induite lors de l'inactivation de l'oncogène NPM-ALK. Durant ma thèse, j'ai tout d'abord répertorié le rôle cytoprotecteur ou, à l'inverse, cytotoxique de l'autophagie dans différents cancers dépendants de l'oncogène ALK, et soumis à différentes thérapies. Mon projet de thèse principal a ensuite été d'identifier des microARNs ainsi que leurs cibles potentielles, dont la manipulation thérapeutique pouvait, en association avec le crizotinib, conduire à une autophagie associée à la mort des cellules tumorales. En effet, des dérégulations du niveau d'expression des microARNs ont été abondamment décrites dans différents cancers, incluant les LAGC ALK+, et leurs capacités à moduler la réponse autophagique sous thérapie a également été démontrée. Mon travail de thèse a permis de démontrer que le microARN-7-5p, connu pour ses propriétés anti-tumorales, et RAF1, une de ses cibles, jouaient un rôle crucial dans les LAGC ALK+, en contrôlant le flux autophagique et le devenir cellulaire. RAF1 est une sérine /thréonine kinase, essentiellement connue pour connecter RAS à la voie de prolifération MEK/ERK. Cependant, le mécanisme par lequel l'inhibition spécifique de RAF1 peut influer sur l'autophagie n'a encore jamais été décrit. Nos travaux montrent pour la première fois la capacité pour RAF1 (et non MEK ou ERK) de phosphoryler ULK1 sur le résidu inhibiteur sérine 757, ce qui offre une nouvelle voie thérapeutique pour moduler le flux autophagique dans les LAGC ALK+. Dans une seconde partie de ma thèse, et afin de mieux comprendre ce lien potentiel entre RAF1 et ULK1, nous nous sommes attachés, selon deux protocoles différents, à purifier les autophagosomes à partir d'une lignée cellulaire de LAGC ALK+. Nos résultats suggèrent la présence de NPM-ALK dans nos fractions enrichies en autophagosomes, et ces travaux seront poursuivis pour détecter RAF1 et ULK1 dans ces mêmes fractions. Ainsi, nos résultats démontrent

l'importance de l'autophagie dans la réponse des LAGC ALK+ au crizotinib et mettent au jour que la combinaison thérapeutique consistant à inhiber NPM-ALK et RAF1, en potentialisant le flux autophagique, pourrait être bénéfique pour les patients atteints de ce lymphome.