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Exploiting metabolic vulnerabilities caused by autophagy defects for cancer therapy

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For my parents

”In God we trust.
All others must bring data.”
W. E. Deming

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Lists of abbreviations

- 2DG = 2-deoxy-D-Glucose
- ATG = Autophagy-related gene
- ATP = Adenosine triphosphate
- CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats
- CNV = Copy number variation
- Ctrl = Control
- DCA = Dichloroacetate
- KO = Knockout
- mRNA = Messenger RNA
- NSCLC = Non-small cell lung cancer
- SCNA = Somatic copy number alterations
- WB = Western blot
- WT = Wild-type

Abstract

Autophagy is a catabolic process that recycles non-essential cellular components, making metabolites newly available for the cell and helping it to cope with starvation and other cellular stress conditions. For years autophagy has been drawing increasing interest because of the role it plays in normal cellular physiology and pathologies, including cancer. Indeed, during cancer progression tumor cells go through a series of changes that alter their metabolism and dramatically increase their need for nutrients. This kind of metabolic reprogramming makes them strongly dependent on the autophagy pathway to survive nutrient shortages or exposure to drugs that target cellular metabolism. Therefore, it would be an advantage to identify novel vulnerabilities created by autophagy deficiency in cancer and exploit such defects for metabolic drug therapy, opening an effective and selective therapeutic window.

In this study, we analyzed two types of defects in the autophagic pathway and tested whether and how they would impact the cell's ability to withstand treatment with metabolic drugs such as dichloroacetate and 2-deoxy-D-glucose. First, we studied a model of chemoresistance in which mTOR upregulation drives cancer resistance against the chemotherapeutic cisplatin, but at the same time suppresses autophagy and makes cells sensitive to metabolic inhibitors causing severe energetic stress. Second, we studied the effects of somatic copy number alterations on the autophagic pathway. Autophagy-related genes (ATGs) are rarely affected by point mutations in cancer. However, they are often affected by somatic copy number losses. It has been hypothesized that the accumulation of multiple non-homozygous ATG deletions would reduce the autophagic flux and cause a metabolic vulnerability, but formal proof was still missing. We used CRISPR-Cas9 to induce deletions of key ATGs and established cell lines carrying multiple non-homozygous ATG deletions. While complete knockout of one single ATG gene heavily sensitized cells to metabolic drugs, cells with accumulation of non-homozygous deletions of multiple ATGs remained mostly unaffected, demonstrating the resilience of the autophagy pathway against this type of alteration.

Zusammenfassung

Die Autophagie ist ein kataboler Prozess, bei dem nicht-essentielle Zellbestandteile recycelt werden, um die Zelle mit neuen Stoffwechselprodukten zu versorgen und ihr so das Überleben unter Nährstoffmangel und anderen Stressbedingungen zu ermöglichen. Seit Jahren gewinnt die Autophagie aufgrund ihrer Rolle in der normalen Zellphysiologie und -pathologie, einschließlich Krebs, zunehmend an Interesse. Tumorzellen durchlaufen während der Krebsentstehung eine Reihe von Veränderungen, die ihren Stoffwechsel beeinflussen und ihren Nährstoffbedarf drastisch erhöhen. Diese Art der metabolischen Umprogrammierung macht sie besonders abhängig von der Autophagie, um Nährstoffmangel oder die Einwirkung von metabolischen Medikamenten zu überleben. Daher wäre es von großem Nutzen, neuartige Angriffspunkte zu identifizieren, die durch Autophagiedefekte bei Krebszellen entstehen. Solche Defekte könnten für eine medikamentöse Stoffwechseltherapie genutzt werden und somit ein wirksames und selektives therapeutisches Fenster öffnen.

In dieser Studie haben wir zwei Arten von Defekten im Autophagie-Stoffwechselweg analysiert und getestet, ob und wie sie sich auf die Resistenz gegen Stoffwechselmedikamente wie Dichloracetat und 2-Desoxy-D-Glucose auswirken. Zunächst untersuchten wir ein Modell der Chemoresistenz, in welchem die Induktion von mTOR dazu führt, dass die Resistenz von Tumorzellen gegen das Chemotherapeutikum Cisplatin gefördert wird. Gleichzeitig wurde aber die Autophagie unterdrückt und die Zellen wurden empfindlicher gegenüber Stoffwechselhemmern, welche starken energetischen Stress verursachten. Weiterhin untersuchten wir die Auswirkungen von somatischen Kopiezahlvarianten auf die Autophagie. Autophagie-verwandte Gene (ATGs) sind bei Krebs selten von Punktmutationen betroffen, werden jedoch häufig durch somatische Kopiezahlverluste beeinträchtigt. Es wurde postuliert, dass die Anhäufung mehrerer nicht-homozygoter ATG-Deletionen den autophagischen Fluss reduzieren und eine metabolische Anfälligkeit verursachen könnte, aber ein formaler Beweis stand noch aus. Wir haben CRISPR-Cas9 verwendet, um Deletionen von Schlüssel-ATGs zu induzieren und Zelllinien zu etablieren, die mehrere nicht-homozygote ATG-Deletionen tragen. Während der vollständige *Knockout* eines einzelnen ATG-Gens die Zellen stark für

metabolische Medikamente sensibilisierte, blieben Zellen mit einer Anhäufung von nicht-homozygoten Deletionen mehrerer ATGs weitestgehend unbeeinflusst, was die Widerstandsfähigkeit des Autophagie-Stoffwechselweges gegenüber dieser Art von Veränderung zeigt.

1. Introduction

1.1. Autophagy

Autophagy, or autophagocytosis (from the Greek αὐτο-φάγος: “self-devouring” (“Henry George Liddell, Robert Scott, A Greek-English Lexicon,” n.d.)), is a physiological process that helps cells to cope with starvation and stressful conditions (Kobayashi 2015). The term was first coined by Christian de Duve in the 1960s after the discovery of the lysosome (Lu et al. 2022). First relevant discoveries of autophagy were obtained in the yeast *Saccharomyces cerevisiae*, and later the process was proven to be evolutionarily conserved in all eukaryotes (King 2012). Autophagy mediates the organized digestion of unnecessary and dysfunctional cellular components and the recycling of resulting nutrients for use in cellular homeostasis (Kobayashi 2015). The digestion is proteasome-independent, instead, it culminates with the degradation of the components inside lysosomes (Mizushima and Komatsu 2011). Three major types of autophagy have been described: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy (Parzych and Klionsky 2014). In microautophagy, cellular elements are taken directly into the lysosomes by endocytosis (W. Li, Li, and Bao 2012), In contrast, in CMA the degradation is very specific: the chaperone Hsc70 recognizes the 5-aminoacid sequence KFERQ in cytosolic proteins and interacts afterward with the CMA-receptors on the surface of lysosomes. The proteins get denatured and translocated into the lysosome lumen for degradation (Orenstein and Cuervo 2010; Bandyopadhyay et al. 2008). Lastly, macroautophagy is considered the “classical” autophagic form and is the best-characterized type. Macroautophagy (from now on simply referred to as “autophagy”) requires the formation of a double-membrane vesicle named “autophagosome”, which carries the elements for the degradation by fusing with the lysosome (Parzych and Klionsky 2014). Autophagy can sequester the cargo either in bulk or in a selective manner. In the second case, the process can be subdivided into further subcategories taking the name after the cargo that is degraded. For example, it is named mitophagy when the targets are mitochondria, xenophagy when targeting bacteria, aggrephagy for protein aggregates, and others (Mancias and Kimmelman 2016; Stolz, Ernst, and Dikic 2014). The selective targeting of the

elements to be degraded is mediated by several autophagy receptors that tether the cargo directly to the forming membrane of the autophagosomes. The targeting occurs either with or without the recognition of ubiquitin (Table 1, 2) (Kraft, Peter, and Hofmann 2010).

Table 1. Ubiquitin-dependent selective autophagy (Mancias and Kimmelman 2016) - modified.

Pathway	**Receptor(s)**	**Substrate**
Mitophagy	OPTN, NDP52, TAX1BP1, p62	Mitochondria
RNA granule disposal	NDP52, p62	RNA granules
Pexophagy	NBR1, p62	Peroxisome (PEX5)
Aggrephagy	p62, NBR1, OPTN TOLLIP, Cue5	Protein Aggregates
Xenophagy	p62, OPTN, NDP52, TAX1BP1	Bacteria
Proteaphagy	RPN10	Proteasomes
Midbody disposal	p62, NBR1	Midbody
Zymophagy	p62	Zymogen

Table 2. Ubiquitin-independent selective autophagy (Mancias and Kimmelman 2016) – modified.

Pathway	**Receptor(s)**	**Substrate**
ER-phagy	FAM134B, Atg40	Endoplasmic Reticulum
Ferritinophagy	NCOA4	Ferritin
Pexophagy	Atg30, Atg36	Peroxisomes
Mitophagy	NIX, BNIP3, FUNDC1, Atg32	Mitochondria
Aggrephagy	OPTN	Mutant HTT, SOD1
Virophagy	TRIM5α, SMURF1, p62	Viruses
Glycophagy	Stbd1	Glycogen
Nucleophagy	Atg39	Nuclear envelope
Lysophagy	Galectin-8/NDP52	Lysosomes

Xenophagy	Galectin-8/NDP52	Bacteria
Cvt targeting	Atg19, Atg34	Ape1, Ams1
Fatty acid synthase(FAS) disposal	FAS	FAS
Signalophagy	c-Cbl	Src
RHOA selective autophagy	SQSTM1	RHOA
Nuclear lamina autophagy	Lamin B1	Nuclear lamina/Nuclear
GATA4 selective autophagy	SQSTM1	GATA4

1.1.1. The autophagic pathway

The process of autophagy involves the cooperation of a complex protein apparatus, with the core machinery being encoded by autophagy-related genes (ATG) (Klionsky et al. 2003). Autophagy can be subdivided into 5 sequential phases: (1) induction, (2) nucleation, (3) elongation, (4) docking and fusion, (5) degradation and recycling (Chang and Zou 2020; X. Li, He, and Ma 2020).

1) Induction

Autophagy is induced by nutrient starvation, with shortage of amino acids being one of the most potent triggers (Mizushima 2007). Unsurprisingly, two main autophagy regulators are involved in nutrients and energy sensing: the mammalian target of rapamycin (mTOR) and the 5' adenosine monophosphate-activated protein kinase (AMPK) (Russell, Yuan, and Guan 2014). They both act by phosphorylating the same protein target, the Unc-51-like kinase 1 (ULK1), however with opposite effects: mTOR-mediated phosphorylation of Ser 757 asserts a negative effect on autophagy (Lamb, Yoshimori, and Tooze 2013), whereas AMPK-mediated phosphorylation of Ser 317 and Ser 777 has autophagy-activating effects (J. Kim et al. 2011). ULK1 can be placed at the apex of the autophagic pathway, forming the so-called pre-initiation complex together with ULK2, FIP200, ATG13, ATG17, and ATG101 (Chang and Zou 2020). ULK1 and its close homolog ULK2 are two similar forms of the same kinase, but ULK1 is critical for autophagy initiation. ULK2 can compensate for a ULK1 loss only in specific cell types (E.-J. Lee and Tournier 2011). The pre-initiation complex is

essential for the phosphorylation and activation of an important downstream element: the Beclin-1 complex (Russell et al. 2013).

2) Nucleation

Once activated, ULK1 phosphorylates both Beclin-1 and the class III PI3-kinase vacuolar protein sorting 34 (VPS34). Beclin-1 binds to VPS34 and p150. Together, they form a nucleation core for several other proteins with either an autophagy-promoting activity (ATG14, UVRAG, Bif1, and AMBRA-1) or with a downregulating role (Bcl2, BclxL, Rubicon) (Russell et al. 2013). While Beclin-1 acts as the main modulator of this complex, p150 anchors it to the surface of inner membranes, and the activated VPS34 produces phosphatidylinositol 3-phosphate (PI3P). PI3P accumulates on the surface of internal membranes such as the endoplasmic reticulum (ER) and serves as a docking element for effector proteins with PI3P-binding motifs. These proteins are required for the elongation and maturation of the nascent so-called isolation membrane (or phagophore) (Mannack and Lane 2015).

3) Elongation

The phagophore starts forming from zones on the membranes with locally high levels of PI3P, and a PI3P effector called double-FYVE-containing protein 1 (DFCP1) is recruited and deforms the membrane to form a structure called omegasome, due to the shape resembling an Ω (omega) letter of the greek alphabet (Axe et al. 2008). Another effector docking with PI3P on the surface of the omegasome is the WD-repeat-protein Interacting with the phosphoinositide (WIPI) class of proteins, important for the elongation of the membrane (Dooley et al. 2014). At this point, a major element of the pathway is required for the correct maturation of the autophagosome: the microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B, also known as LC3), a member of the ATG8 protein family and one of four proteins in the MAP1LC3 subfamily (MAP1LC3A, MAP1LC3C, MAP1LC3B2) (Shpilka et al. 2011, 8). It is considered a central player in the autophagic machinery (Tanida, Ueno, and Kominami 2008). To assert its action, LC3 must undergo a series of post-translational modifications. It requires the assembly of the E3 (ubiquitin-protein-ligase)-like ATG12/ATG5/ATG16 complex, in which the ATG12 unit is conjugated to the

ATG5 unit by the E1 (ubiquitin-activating-enzyme)-like enzyme ATG7, and the dimer is sequentially linked to ATG16 by the E2 (ubiquitin-conjugating enzyme)-like enzyme ATG10 (Chang and Zou 2020; Hanada et al. 2007). In parallel, newly synthesized pro-LC3 is proteolytically cleaved into LC3-I by ATG4, and subsequentially it gets covalently linked to a phosphatidylethanolamine (PE) molecule by the combined action of ATG7, ATG3, and the ATG12/ATG5/ATG16 complex (Chang and Zou 2020; Dooley et al. 2014; Hanada et al. 2007; Fujita, Itoh, et al. 2008; Noda, Fujita, and Yoshimori 2008). The lipidation of LC3 (LC3-II) allows its anchorage on the surface of the phagophore and it is essential for the correct maturation of the autophagosome (Fujita, Hayashi-Nishino, et al. 2008, 4). LC3-II also acts as a docking receptor for several adaptor proteins, including the p62/SQSTM1 (p62), a key element for ubiquitin-dependent selective autophagy (Pankiv et al. 2007).

4) Docking and fusion

After maturation, the loaded autophagosomes containing organelles and proteins fuse with the lysosomes. The fusion is aided by the action of several tethering factors, as well as SNARE complexes, which link the two vesicles together. The tethering factors can recognize either LC3, small GTPases, or several types of phosphoinositides present on the autophagosome membrane surface (Lőrincz and Juhász 2020; Nakamura and Yoshimori 2017). Autophagy can be efficiently blocked at this stage. Using compounds that prevent the fusion between autophagosomes and lysosomes such as chloroquine and bafilomycin is the most common experimental method of chemical autophagy inhibition (Mauthe et al. 2018).

5) Degradation and recycling

The vesicle originating from the fusion of the autophagosome with the lysosome is called autolysosome (Klionsky, Eskelinen, and Deretic 2014), and the lysosome's hydrolases and low pH allow the degradation of its content. Both LC3 and p62 present on the lumen surface get degraded together with the cargo, while LC3 on the external membrane is detached by ATG4 and is re-used in the process (Bjørkøy et al. 2009, 1; Y. Zhou et al. 2022). This step is fundamental for the recycling of the cargo and obtaining elementary components such as amino

acids, lipids, nucleosides, and carbohydrates that can be used in cellular metabolism to cope with nutrient shortage (Rabinowitz and White 2010) (Fig. 1).

Because both LC3-II and p62 get degraded with the autophagosomes at the end of the autophagic process, they are often used to assess autophagic activity. For example, the conversion rates of LC3-I into LC3-II (detected as two bands in western blotting) can give insight into the status of the autophagic flux: missed conversion of LC3-I into LC3-II can be interpreted as a sign of blocked autophagy at the early stages. Conversely, low levels of LC3-I but high LC3-II levels are a sign of a block in the autophagy pathway acting at the late stages. Accumulation of p62 is also considered a sign of blocked autophagy (Klionsky et al. 2021; Bjørkøy et al. 2009; Pankiv et al. 2007).

1.1.2. Roles of autophagy

Autophagy not only is involved in the recycling process of macromolecules, but it has been found increasingly often to play a role in different aspects of the cellular life cycle and human health: autophagy is linked to apoptosis, is involved in the response to pathogens, and can play a relevant role in human diseases such as neurodegenerative disorders, metabolic pathologies, and cancer (Khandia et al. 2019).

1.1.2.1. Autophagy and apoptosis

Although autophagy and apoptosis are considered two distinct processes, they are actually intertwined by a complex protein network. For example, the interaction of Beclin-1 with anti-apoptotic members of the Bcl-2 family may inhibit autophagy. In other cases, autophagy can stimulate or downregulate apoptosis by itself via the degradation of apoptosis-specific ligands (Fitzwalter and Thorburn 2015; Nezis et al. 2010; Thorburn et al. 2014; Delgado et al. 2014).

1.1.2.2. Autophagy against pathogens

Autophagy can counter infections both directly and indirectly. Cells can activate xenophagy as a mechanism to engulf pathogens such as bacteria, viruses, and parasites to directly digest them. Indirectly autophagy can stimulate an immune response by providing antigens derived from the pathogen degradation to activate inflammation (Sharma et al. 2018; Levine, Mizushima, and Virgin 2011).

1.1.2.3. Autophagy in neurodegenerative disorders

Multiple studies have highlighted the involvement of deregulated autophagy in several neurodegenerative pathologies. For example, its catabolic role is thought to prevent the harmful accumulation of β-amyloid which is the cause of Alzheimer's disease. Furthermore, dysregulated autophagy has also been observed in other conditions such as Parkinson's, and Huntington's diseases (Lim, Cho, and Kim 2016; Tooze and Schiavo 2008; Nixon 2013).

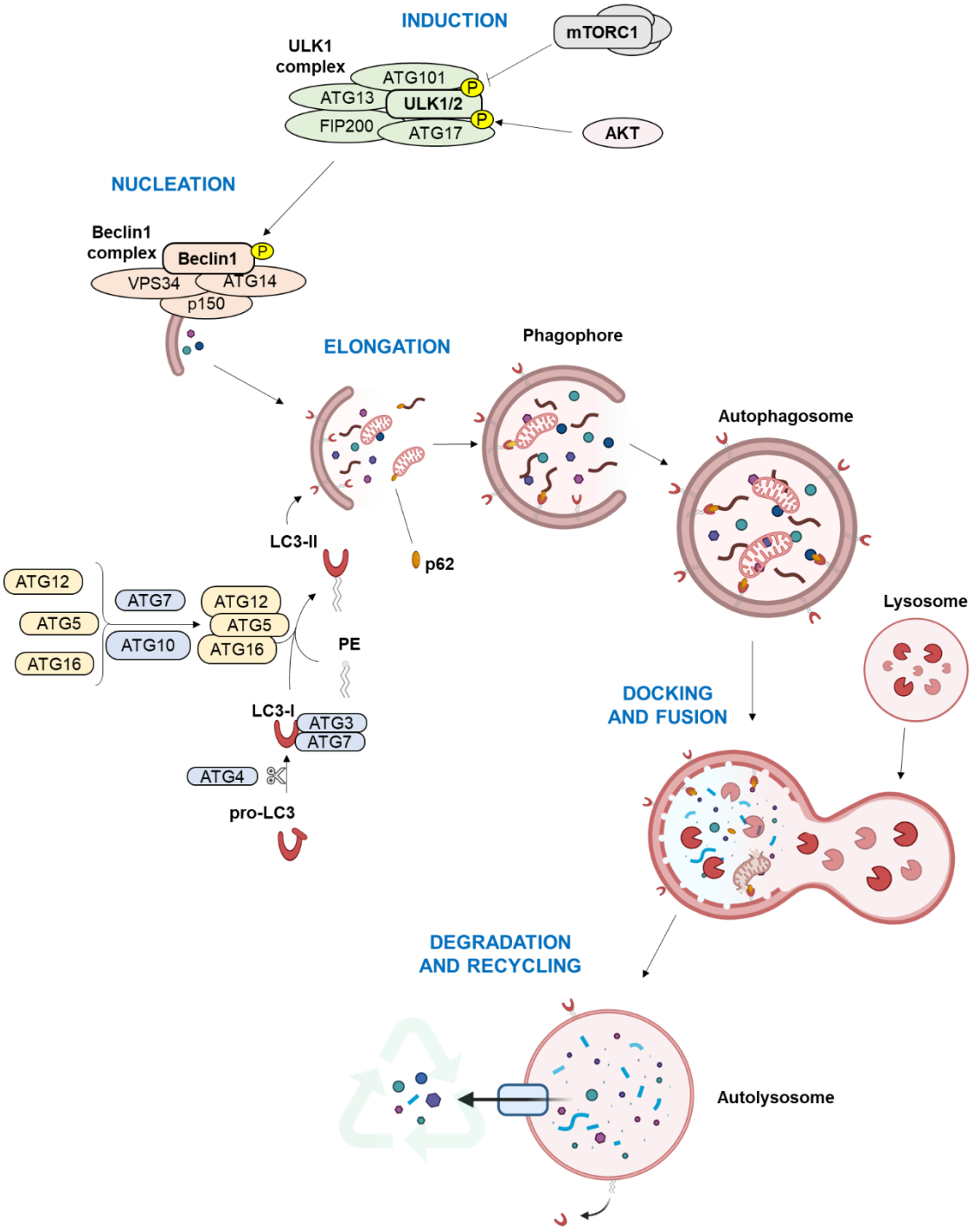


Fig. 1 The macroautophagy pathway
Schematic representation of the autophagic pathway and some of its key components. Figure created with BioRender.com

1.2. Autophagy in cancer

Autophagy elicits increasing interest because of its role in cancer. Due to its functions in maintaining cellular homeostasis and protein quality, autophagy has been proven to possess paradoxically a double nature: it exerts tumor-suppressive activity, preventing the malignant transformation of healthy cells, but it also a exerts cytoprotective effect in transformed cancer cells in already established tumors (Z. J. Yang et al. 2011; Chavez-Dominguez et al. 2020), making its role complex and context-dependent.

1.2.1. Autophagy in tumorigenesis

Several studies showed that the autophagic apparatus can exert a tumor suppressive function by promoting clearance of harmful misfolded protein aggregates, as well as by maintaining low reactive oxygen species (ROS) levels through scavenging of old or damaged mitochondria (mitophagy). This prevents chronic inflammation and harmful damage to the DNA that could lead to genomic instability and transformation (Z. J. Yang et al. 2011; Poillet-Perez et al. 2014; Kung et al. 2011).

On the contrary, defects in the autophagy machinery can contribute to cancer formation. Indeed, mice with deletion of genes such as *Atg5* and *Atg7* develop spontaneous hepatic adenomas due to the accumulation of damaged mitochondria and oxidative stress (Takamura et al. 2011). In addition, it has been reported that monoallelic deletion of the *BECN1* gene can promote tumorigenesis, although the mechanism is still not completely clear: *Becn1*$^{+/-}$ mice models develop tumors more frequently than their wild-type (WT) counterparts, and *BECN1* is found to be monoallelically deleted in 40-75% of cases of human sporadic breast, ovarian, and prostate cancer. For these reasons, *BECN1* is considered a haploinsufficient tumor suppressor (Qu et al. 2003; Yue et al. 2003; A. M. K. Choi, Ryter, and Levine 2013). Furthermore, aberrant accumulation of p62 is often observed in several types of cancer including prostate, liver, breast, and lung. Because p62 accumulation is considered a sign of autophagy inhibition, a block of autophagy is thought to be correlated with cancer progression and be involved in cancer initiation (X. Li, He, and Ma 2020). In conclusion, there is a consensus view that well-functioning

autophagy can prevent cancer formation. On the contrary, autophagy defects can contribute to tumorigenesis

1.2.2. Autophagy in tumor cell survival

Autophagy exerts a cytoprotective function in healthy cells and it can also promote the survival of cancer cells. One of the hallmarks of tumors is their high cell proliferation rates which translates into an elevated biomolecule synthesis. This is a metabolic and energetically demanding state which can lead to an energetic crisis. Considering that tumors may be poorly vascularized, their nutrient supply can be limited and some tumor regions can be hypoxic. The autophagy process can help tumor cells partially meet their metabolic needs by making building blocks newly available (Mathew and White 2011). When subjected to metabolic stress, autophagy-competent cancer cells induce an autophagic response that allows them to survive such conditions. On the contrary, autophagy-deficient cells are unable to withstand starvation and are highly susceptible to metabolic stress, both *in vitro* and *in vivo* (Lum et al. 2005; Degenhardt et al. 2006). Autophagy inhibition either by chemical or genetic means can induce cell death and decrease clonogenicity (White and DiPaola 2009; S. Yang et al. 2011; J. Y. Guo et al. 2011). In some cases, autophagy has been reported to contribute to cancer cell survival under chemo- and radiotherapy (Khandia et al. 2019; Eskelinen 2011). Furthermore, in already established tumors, functioning autophagy can contribute to the development of a more aggressive phenotype, while autophagy-deficient cells would generate more benign tumors as demonstrated in experiments with genetically modified mouse models (White, Mehnert, and Chan 2015).

1.3. Autophagy in anticancer strategies

Because of the evident crucial role that autophagy is playing in cancer cells, it is gaining increasing interest in cancer research. Autophagy modulation is seen as a promising strategy of combinational therapies as a way to increase the efficiency of the primary drug or to overcome resistance mechanisms (Chang and Zou 2020; Aveic et al. 2018). Several compounds have been identified that can modulate autophagy at different stages of the pathway (Table 3), with mTOR inhibitors (e.g. rapamycin) being the most common autophagy inducers, and the autophagosome-lysosome fusion inhibitors (e.g. chloroquine, bafilomycin) being the most common autophagy blockers.

Another possible autophagy-centered anticancer strategy is to exploit the metabolic vulnerability shown in some tumors with intrinsic impaired autophagy: by employing metabolic inhibitors it would be possible, in principle, to create therapies with greater selectivity, or to overcome drug resistance phenomena by means of combinational treatments.

Table 3. Ubiquitin-independent Selective Autophagy (Hale et al. 2013).

Autophagy inducer	Action
MG132	Inhibit 26S proteasome
Bortezomib	Inhibit 26S proteasome
Tunicamycin	Induce ER stress by inhibiting N-acetylglucosamine phosphotransferase
Thapsigargin	Induce ER stress via ATP2A1 inhibition Prevent autophagosome/lysosomal fusion by inhibiting RAB7 recruitment
Fluspirilene	Stabilize ATG12–ATG5 by preventing Ca2+-mediated CAPN1 activation
Resveratrol	Sirtuin-mediated deacetylation of cytoplasmic proteins
Spermidine	Sirtuin-mediated deacetylation of cytoplasmic proteins
Rapamycin	Inhibit MTOR
Lithium chloride	Increase PtdIns3P levels by inhibition of IMPAD1
L-690,330	Increase PtdIns3P levels by inhibition of IMPAD1
Carbamazepine	Increase PtdIns3P levels by inhibition of IMPAD1
Xestospongin B	Antagonize ITPR1 (and ITPR1-mediated BCL2–BECN1 interaction)
Xestospongin C	Inhibit ITPR1 and ER Ca2+ receptors

Autophagy inhibitor	Action
3-methyladenine	Inhibit PtdIns3K and PtdIns3K (PIK3CB stably, and PIK3C3 transiently)
Wortmannin	Inhibit PtdIns3K and PtdIns3K (PIK3CB and PIK3C3)
Bafilomycin A1	Inhibit vacuolar ATPase
Spautin-1	Inhibit USP10 and USP13 deubiquitinase activity
Chloroquine, hydroxychloroquine	Alkalize lysosome environment

1.3.1 Exploiting metabolic vulnerabilities caused by autophagy defects

Cancer cells are characterized by high proliferation rates, and consequently, they are subjected to high metabolic demand to sustain the elevated biomass production and energetic needs. These changes have since long been considered one of the hallmarks of cancer (Martínez-Reyes and Chandel 2021; Hanahan and Weinberg 2011). Already at the beginning of the 20th century, the physiologist Otto Warburg noticed in malignant cells a shift from normal aerobic respiration in favor of glycolysis even in the abundance of oxygen: a phenotype that today is called the “Warburg effect” (Jang, Kim, and Lee 2013; Warburg 1956). Considerable interest is placed in the study of these phenomena, and multiple compounds targeting metabolic alterations are continuously under development (Schmidt et al. 2021). Examples of such compounds are 2-deoxy-D-glucose (2DG) and dichloroacetate (DCA) (Goel 2021; Al-Azawi et al. 2021) (Fig. 2). 2DG is introduced in the cell through glucose transporters and undergoes phosphorylation by hexokinase, however, it cannot be further processed and competitively inhibits glycolysis at the very beginning (Aft, Zhang, and Gius 2002). DCA’s mechanism of action consists, instead, in the inhibition of pyruvate dehydrogenase kinase (PDK), the negative regulator of the pyruvate dehydrogenase (PDH) complex, whose activity is to convert pyruvate into acetyl-CoA. The final effect is an increased shift from aerobic glycolysis and lactate production to the TCA cycle (Tataranni and Piccoli 2019). For these reasons, both molecules are considered anti-Warburg compounds, and both have been tested

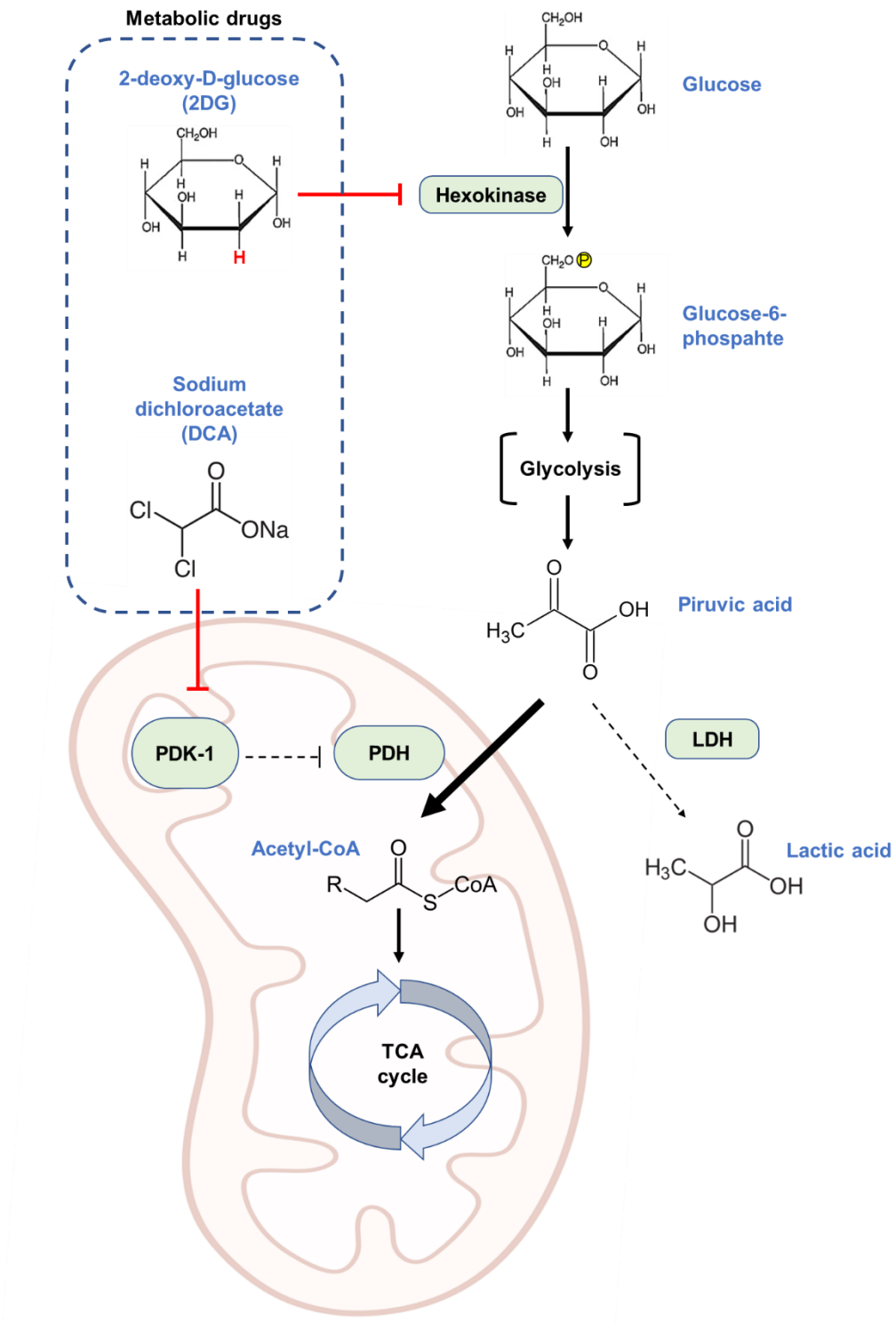


Fig. 2 Modes of action of 2-deoxy-D-glucose and sodium dichloroacetate Schematic representation of the 2 modes of action of 2-deoxy-D-glucose (2DG) and sodium dichloroacetate (DCA) acting at different stages in the glycolysis pathway. Figure created with BioRender.com

as anti-cancer drugs. However, their toxicity and lack of efficacy evidence prevent their use in the clinic (Garon et al. 2014; Landau 1958; Vander Heiden and G 2011; Mohanti et al. 1996). Nonetheless, they are useful tools that can be

employed in preclinical research to dissect metabolic vulnerabilities and discover new therapeutic windows for cancer treatment. In this work, we made use of both DCA and 2DG since they were found highly cytotoxic against cells with autophagy defects (Gremke et al. 2020). We used them to explore metabolic druggability in two case studies of cancer cells with autophagy defects.

1.3.1.1. mTOR upregulation

A common feature found in cancer is the upregulation of the mTOR axis. mTOR is a serine/threonine kinase, a downstream effector of the receptor tyrosine kinases (RTKs) pathway, and is a major regulator of cellular growth, proliferation, survival, and also protein synthesis and autophagy (J. Kim et al. 2011; Xu, Liu, and Wei 2014; Hay and Sonenberg 2004). mTOR is the core subunit of two distinct complexes: mTOR complex 1 and 2 (mTORC1 and mTORC2).

mTORC1 is composed of mTOR, its regulatory-associated protein Raptor, the components mLST8 and PRAS40, and the inhibitory subunit DEPTOR. mTORC1 is mainly involved in nutrient sensing and promotion of protein synthesis through phosphorylation of effector targets such as the P70-S6 Kinase 1 (p70S6K) which subsequently phosphorylates and activates the ribosomal protein S6 (rpS6), and 4EBP1. Besides, mTORC1 can also inhibit autophagy by phosphorylation of ULK1 (Bond 2016; Sabatini 2022; Peterson and Schreiber 1998). Activation of the mTORC1 complex takes place via signal originating from the RTKs and is transduced either through the MAPK/ERK kinase cascade or the PI3K/AKT pathway. Both pathways influence two proximal mTORC1 regulators: Rheb and the TSC1/TSC2 complex. Rheb (Ras homolog enriched in the brain) is a GTP-binding protein and is a mTORC1 activator when bound to GTP, but inactive when bound to GDP. The TSC1/TSC2 (tuberous sclerosis complex subunit 1 and 2) complex interacts with Rheb and stimulates Rheb hydrolysis of GTP to GDP, inactivating the enzyme (Zou et al. 2011). TSC1/2 ultimate effect is therefore a downregulation of the mTOR activity (Huang and Manning 2009). Opposite to the MAPK/ERK and PI3K/AKT pathways, the AMP-activated protein kinase (AMPK) can negatively regulate mTORC1. When nutrients are scarce and the ADP/ATP ratio is increased, AMPK is active and phosphorylates TSC2, enabling its GTPase activity (Hardie 2007).

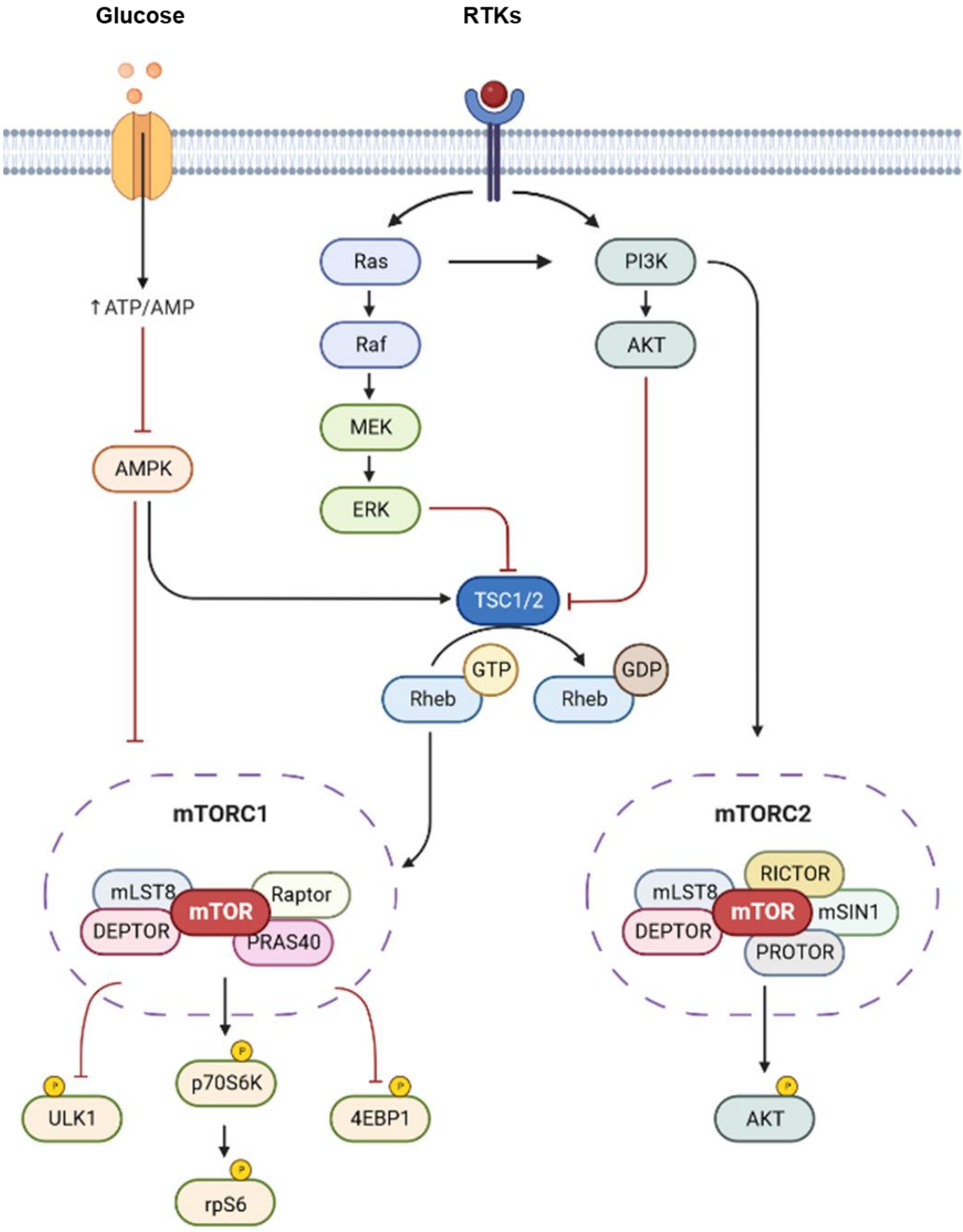


Fig. 3 mTOR signaling
Schematic representation of the mTOR pathway with upstream regulators. Some of the known downstream targets of mTORC1 and mTORC2 are shown that were used as markers of the complexes' activity in this study. Figure created with BioRender.com

The second complex, mTOR Complex 2 (mTORC2), comprises instead the rapamycin-insensitive companion of mTOR (RICTOR), MLST8, PROTOR, and the mSIN1 component. mTORC2 has a role in the regulation of the actin cytoskeleton and is also able to activate the AKT kinase by phosphorylation on Ser 473, which, in turn, is an important controller of survival and cellular metabolism. It is activated by growth factors through the PI3K axis (Saxton and Sabatini 2017; X. Chen et al. 2018) (Fig. 3).

Besides promoting cancer cell survival by acting downstream of important oncogenic drivers such as the Ras-driven MAPK and PI3K/AKT pathways, high mTOR activity can cause chemotherapeutic resistance phenomena in several types of cancer like breast, melanoma, and lung cancer (Ilagan and Manning 2016; B.-H. Jiang and Liu 2008). Our group highlighted in previous work how upregulation of mTOR activity can cause acquired resistance to cisplatin (CDDP), a frontline DNA-crosslinking chemotherapeutic agent used against a variety of tumors that induces double-strand breaks in DNA (Wanzel et al. 2016). Cisplatin resistance is often caused by enhanced DNA repair capabilities induced by mTOR, which can regulate the Fanconi anemia pathway, essential for the repair of DNA interstrand crosslinks (F. Guo et al. 2013; Shen et al. 2013). Indeed, FANCD2, a central player of this pathway, was found to be upregulated in chemoresistant cells in an mTOR-dependent manner, and it was accompanied by high levels of specific markers for mTOR activity such as 4EBP1 and p70S6K phosphorylation (Wanzel et al. 2016).

mTOR inhibition is therefore considered an appealing target in cancer therapy, and an entire class of drugs called rapalogs (rapamycin and its analogs) was generated with that aim (Hua et al. 2019). However, this first generation of mTOR inhibitors proved to be effective as monotherapy only in a few rare cancer entities and showed to possess mostly cytostatic properties. The possible reason for the limited activity of the first-generation inhibitors is their selective blocking of the mTORC1 complex only (Y.-J. Zhang, Duan, and Zheng 2011; Easton and Houghton 2006). Therefore, the second generation of drugs, mTORC1/mTORC2 dual inhibitors were developed. Although they proved to be more potent than rapalogs, and despise the initial promising results, they raised concerns about possible long-term toxicity due to the broader effects (Zaytseva et al. 2012). Another important issue about existing inhibitors is their limited selectivity towards

cancer cells. For these reasons, the discovery of new druggable vulnerabilities and new therapeutic approaches against chemoresistant cancer are in high demand.

Such an opportunity could be provided by the reprogrammed metabolism caused by mTOR itself. Elevated mTOR signaling causes higher proliferation and consequently higher rates of macromolecule synthesis. However, at the same time, it causes an early inhibition of the autophagic pathway by phosphorylation of ULK1 at Ser 757 (Magaway, Kim, and Jacinto 2019). This, together with the enhanced nutrient requirements, can create a metabolic vulnerability that can be targeted by metabolic inhibitors such as 2DG and DCA (Gremke et al. 2020).

1.3.1.2. Somatic copy number alterations

Based on previous findings, it is known that a block of autophagy, for example by chemical inhibition or due to high mTOR activity, can increase tumor cells' response to metabolic drugs (Gremke et al. 2020). However, these are not the only cases that can generate a vulnerability. In theory, an autophagy block can be induced by genetic alterations. In cancer, autophagy genes are rarely hit by missense mutations that provoke their loss of function (Lebovitz et al. 2015). However, a haploinsufficiency network-based analysis shows that the autophagic pathway is frequently subjected to somatic copy number alterations (SCNAs) (Joe Ryan Delaney et al. 2017), a type of mutations that involves structural variations of a large portion of the genome, such as allelic deletions or duplications (Harbers et al. 2021). It is also reported, that SCNAs affecting the autophagic pathway comprise more often deletions rather than gains, and that these are usually not homozygous losses, but instead monoallelic deletions that can involve multiple ATG genes simultaneously (Joe Ryan Delaney et al. 2017). Notably, a considerable fraction of cancer types were found to carry SCNAs of key autophagy genes such as *BECN1, MAP1LC3B, and ATG10* (Qu et al. 2003; Joe Ryan Delaney et al. 2017). Since monoallelic deletions can reduce the transcription levels of a gene, it has been speculated that multiple monoallelic deletions of core autophagic genes might lead to the choking of the autophagy flux, in turn reducing the ability of cancer cells to cope with nutrient deprivation and stress conditions (Joe Ryan Delaney et al. 2017). This could, in principle, create an actionable vulnerability to metabolic drugs to be exploited in cancer

metabolic therapy. In such cases, the SCNAs could be used as a biomarker when evaluating therapeutic options for patients. However, any proof of concept that the accumulation of multiple monoallelic deletions would make cells susceptible to metabolic drugs was still to be provided.

1.4. CRISPR-Cas9 genome editing

In order to generate and study the allelic deletions described in the previous chapter, and to generate knockouts of autophagy genes, we made use of the CRISPR-Cas9 system. CRISPR (clustered regularly interspaced short palindromic repeats) are DNA sequences found in the procaryotic genome that, together with a Cas (CRISPR-associated protein) endonuclease, work as a defense mechanism against bacteriophage infections. These sequences are made of genomic fragments of viruses that infected bacteria in the past and became part of their own genome. When they are transcribed, the resulting mRNA can couple with a Cas, which uses CRISPR as a guide to target complementary sequences. This allows prokaryotes to cleave eventual DNA from viral infections, working as a kind of bacterial immune system (Barrangou 2015; Barrangou et al. 2007). In 2012, Emmanuelle Charpentier and Jennifer Doudna demonstrated how the CRISPR-Cas system could be used as a potent and relatively easy tool to perform gene editing (Jinek et al. 2012), which granted them the Nobel prize in chemistry in 2020 (“The Nobel Prize in Chemistry 2020” n.d.). CRISPR-Cas systems from different organisms have been identified and used for genome editing, with the CRISPR-Cas9 from *Streptococcus pyogenes* being the most widely known and used. To exert its action, the system would require three major components: the Cas9 endonuclease, and two RNA molecules (crRNA and tracrRNA) which are required to correctly direct the Cas9 to the target site. However, the current system developed by Charpentier and Doudna consists of only Cas9 and one chimeric single-guide RNA (sgRNA) (Barrangou 2015; Jinek et al. 2012). The sgRNA is composed of a constant “scaffold” portion which is recognized by the Cas9, and a variable target-specific sequence of usually 20 nucleotides (nt) at the 5’ end of the sgRNA that is complementary to the target. To perform the cut, the Cas requires an endonuclease-specific motif: the protospacer adjacent motif (PAM) on the target sequence. For Cas9, the PAM

sequence corresponds to 5′-NGG-3′ and the cutting site is present 3 base pairs (bp) upstream of it, between the sgRNA variable sequence's 17th and 18th nucleotides (Barrangou 2015; Jinek et al. 2012). Once the complex sgRNA-Cas9 is assembled and reached the target sequence, a blunt double-strand break (DSB) takes place on both strands, and the DNA undergoes a repair process. Homology-directed repair (HDR) and nonhomologous end-joining (NHEJ) are the two primary repair mechanisms that the cell at this point can adopt. HDR is an accurate, error-free method of repairing DNA that needs a homologous template sequence from either another chromatid, a donor homology plasmid, or a single-stranded oligodeoxynucleotide (ssODN). This pathway can be exploited in genome editing because it allows the insertion of virtually any desired sequence in the cell's genome. The sequence of interest must be flanked by homology arms that are complementary to the parts in direct proximity upstream and downstream of the Cas9 cut site. The NHEJ requires no homologous template and repairs together the cut ends of a DSB. This mechanism happens at a faster rate than HDR, but at the same time is error-prone. This often results in INDEL mutations that cause the effective knocking out (KO) of the gene when the DSB is located on an exon. Indeed, INDEL can provoke frameshift mutations, nonsense-mediated mRNA decay, etc (Sansbury, Hewes, and Kmiec 2019; H. Yang et al. 2020) (Fig. 4). It is also possible to use CRISPR-Cas9 to induce large deletions, spanning the entire length of one gene if desired, by employing two sgRNAs targeting respectively an upstream (5´) and a downstream (3´) target sequences. The DNA portion within the two target sequences is eliminated, and the two cut sites are joined together via NHEJ (Pulido-Quetglas et al. 2017).

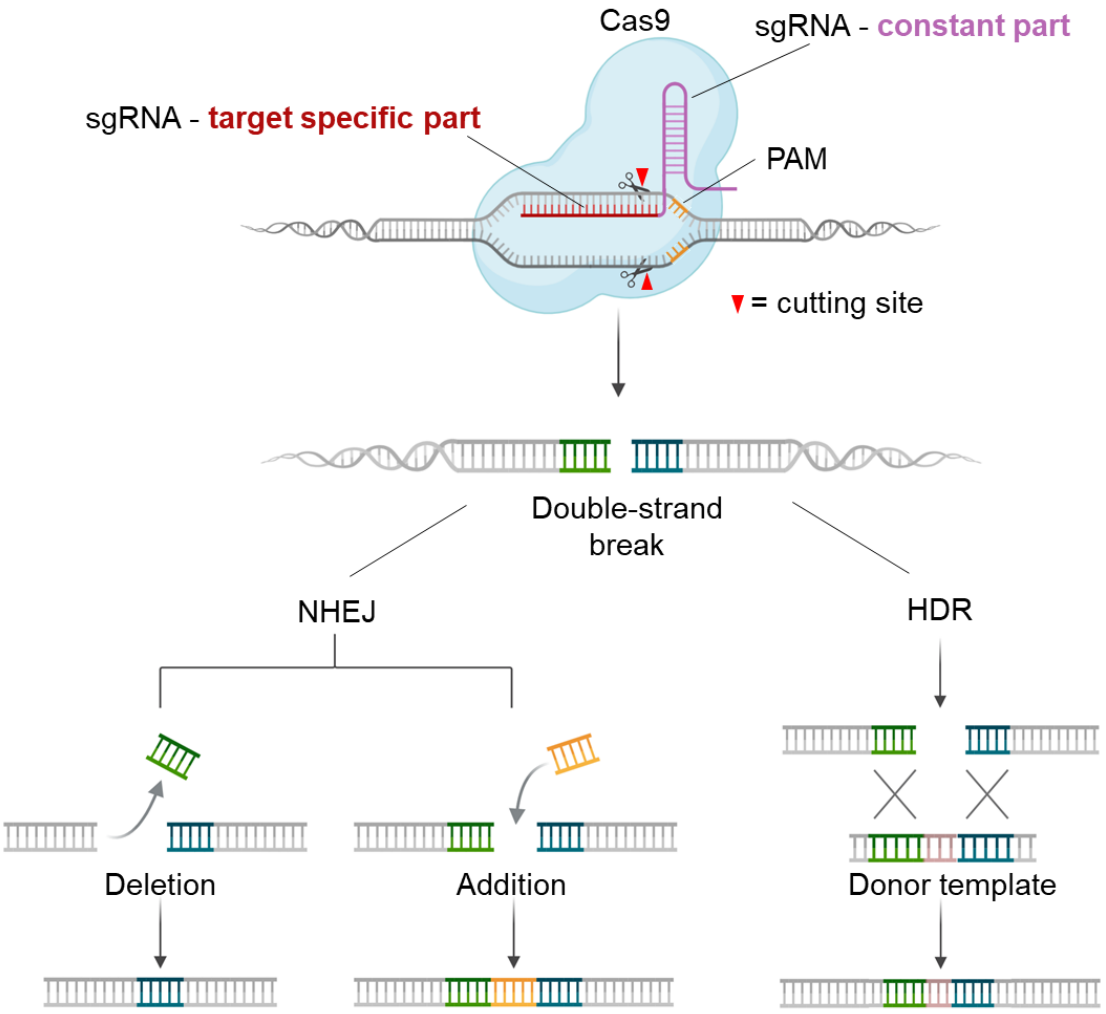


Fig. 4 The CRISPR-Cas9 system
Schematic representation of the CRISPR-Cas9 system components and mode of action, with following mechanisms of DNA repair. Figure created with BioRender.com

1.5. Aim of the study

Cancer cells undergo metabolic reprogramming that dramatically increases their nutrient requirements. This makes them more susceptible to energy depletion and metabolic stress, and more dependent on the autophagic pathway. Indeed, autophagy permits cells to momentarily cope with energy stress and protects cells from compounds targeting cancer metabolism by recycling unnecessary cellular components. However, in some tumor cells, the autophagic pathway could be either inhibited by altered signaling or impaired by mutations that reduce autophagic protein levels. Such sort of autophagy deficiencies are vulnerabilities that can be exploited by the use of metabolic inhibitors such as DCA and 2DG. Our aim was to provide experimental support to two models of metabolic vulnerability caused by autophagy defects that could be exploited as new therapeutic windows for cancer treatment:

1) Validate a model in which mTOR upregulation causes resistance to DNA-damaging chemotherapeutics such as cisplatin and, at the same time, induces hypersensitivity to metabolic drugs due to autophagy inhibition.
   - By assessing the effects of mTOR chemical inhibition in chemoresistant cells.
   - Evaluating the effects of artificial mTOR upregulation by creating cell lines expressing constitutively active mTOR mutants or acting on upstream regulators.
   - Evaluating the effects caused by autophagy inhibition by creating autophagy-deficient cell lines with the use of CRISPR-Cas9-induced mutagenesis.
   - Understanding the causes of cell death caused by metabolic inhibitors.

   The resulting effects were compared with those observed in the chemoresistant cells.

2) Provide evidence on whether non-homozygous deletions affecting multiple autophagy genes simultaneously make cells vulnerable to metabolic drug treatment.
    - By creating cell lines with progressive allelic deletions of either one or multiple ATGs, and creating cell lines with complete KOs using CRISPR-Cas9.
    - Comparing the response of these cell lines to metabolic inhibitors.

2. Materials and Methods

2.1. Materials:

2.1.1. Bacterial strains

Bacterial strains	Source
DH10B ElectroMAX	LifeTechnologies

2.1.2. Plasmids

Name	Insert	Source
pcDNA3-FLAG-MTOR-L1460P	FLAG-MTOR-L1460P	Addgene #69006
pcDNA3-FLAG-MTOR-S2215Y	FLAG-MTOR-S2215Y	Addgene #69013
pcDNA3-FLAG-MTOR-I2500F	FLAG-MTOR-I2500F	Addgene #69014
PB-EF1α-MCS-IRES-Neo PiggyBac cDNA Cloning and Expression Vector	FLAG-MTOR (L1460P/S2215Y/I2500F)	System Biosciences
pSpCas9(BB)-2A-Puro vector pX459 V2.0	sgRNAs	Addgene #62988
pCMV-HAhyPBase	-	Thermo Fisher
FLAG pLJM1 Rheb1	FLAG pLJM1 Rheb1	Addgene #19312
pQCXI Puro DsRed-LC3-GFP	DsRed-LC3-GFP	Addgene #31182

2.1.3. Cell lines

Cell line	Source
NCI-H460	American Tissue Collection Center
PC-9	American Tissue Collection Center

2.1.4. Oligonucleotides

PCR primers

#	Name	Sequence
1	LC3_del_FW	CAGACCTCAGTGCCTCGGTCGA
2	LC3_del_RV	AGAGAACGCCGCAGATCCAGGT
3	BECN1_del_FW	TCCACGGCCTCAGGGATGGAAG
4	BECN1_del_RV	GTGCACCCCTGGGCAGTTTTCA
5	ATG10_del_FW	TACAGCACCTAGAGTCCCGT
6	ATG10_del_RV	GGAAGCAGGGAGAAAAAATCCTC
7	ATG7ex2_FW	AGTTGTGTTTCAAGGTAGCCTG

8	ATG7ex2_RV	CCGTGAGGATAACAGAAGATGATG
9	BECN1_int_FW	TTCACCACATTGGCCGGACTGC
10	BECN1_int_RV	AGGGCGGATGTCACCAAGCTCT
11	BECN1 ex4 FW	AGGGCATTCTGTCCTCTGCCCC
12	BECN1 ex4 RV	GCCATGCTGGTCTTCCACAGGG
13	LC3_int_FW	TGGAGGAAGGACTGGGTCTC
14	LC3_int_RV	GGCTGTCTGGTGATTCCTGTAA
15	ATG10_int_FW	ACTCCCTTTTCCTTGCCTCATAG
16	ATG10_int_RV	CACGCTGAAGTCTTGATACCCT

Single-guide RNA (sgRNA)

#	**Name**	**Sequence**
1	ATG7	AGAAATAATGGCGGCAGCTA
2	LC3sg1	GGGAAGCACCGTGTTCATCG
3	LC3sg5	AGTTGTGACCTGCTACACAT
4	BECN1sg1	TCCCTGTAACAACCCGTACG
5	BECN1sg5	GATCACATCACATGGTGACC
6	BECN1sgExon4A	ATTGAAACTCCTCGCCAGGA
7	ATG10sg3	TCCATCCGTAAGTTTTCAAG
8	ATG10sg4	CAAGGAGCTCCTGTAGACTG
9	ATG14sg1	TGAAGGCCTTCTCAAAAC
10	ATG14sg2	AGCTTTACAGTCGAGCACAA,
11	ATG14sg3	AGAAAAAGGAGA AGATTCAG
12	ATG14sg4	CTCGATTGGAAAAATGACAG,
13	ATG14sg5	CCAATCG AGGAAGTAAAGAC
14	FIP200sg1	CTGGTTAGGCACTCCAACAG
15	FIP200sg2	AG GAGAGAGCACCAGTTCAG
16	FIP200sg3	AACCTCATTTCCCAAGTCAG
17	FIP200sg4	GATACCGCAGATGCTGAAAG
18	FIP200sg5	TCAAGATAGACCTAATGATG

Small interfering RNA (siRNA)

#	**Name**	**Sequence**
1	TSC1/2-si1	CGACACGGCUGA UAACUGA
		GCAUUAAUCUCUUACCAUA
2	TSC1/2-si2	CGGC UGAUGUUGUUAAAUA
		GGAUUACCCUUCCAACGAA

2.1.5. Enzymes

Name	Source
NotI	New England Biolabs
EcoRI	New England Biolabs
BbsI	New England Biolabs
GoTaq® G2 DNA Polymerase	Promega
Q5® Hot Start High-Fidelity DNA Polymerase	New England Biolabs
T4 DNA Ligase	LifeTechnologies

2.1.6. Antibodies

Primary antibodies

Antigen	Clone	Dilution	Source
FANCD2	sc-20022	1:500	Santa Cruz
FLAG-tag	F1804	1:1000	Sigma-Aldrich
phospho-ULK1 (Ser757)	#14202	1:1000	Cell Signaling
ULK1	R600 #4773	1:1000	Cell Signaling
p62/SQSTM1	P0067	1:1000	Sigma-Aldrich
LC3B (LC3-I/II)	ab48394	1:1000	Abcam
p70S6Kinase	H9 sc-8418	1:500	Santa Cruz
phospho-4E-BP1 (Thr37/46)	236B4 #2855	1:1000	Cell Signaling
4E-BP1	R-113, sc-6936	1:200	Santa Cruz
phospho-S6 Ribosomal protein (Ser240/244)	#2215	1:1000	Cell Signaling
phospho-AKT (Ser473)	D9E #4060	1:1000	Cell Signaling
AKT	#9272	1:1000	Cell Signaling
Tuberin/ TSC2	D93F12 #4308		Cell Signaling
phospho-AMPKα (Thr172)	#2535	1:1000	Cell Signaling
AMPKα	23A3 #2603	1:1000	Cell Signaling
phospho-Acetyl-CoA carboxylase (Ser79)	#3661	1:1000	Cell Signaling
Acetyl-CoA carboxylase	C83B10 #3676	1:1000	Cell Signaling
ATG7	D12B11 #8558	1:1000	Cell Signaling
FIP200	D10D11 #12436	1:1000	Cell Signaling
β-Actin	AC-15, ab6276	1:10.000	Abcam
ATG14	#5504	1:1000	Cell Signaling
Phospho-Histone H2A.X (Ser139)	#2577	1:1000	Cell Signaling
BECN1	E8, sc-48341	1:1000	Santa Cruz
ATG10	EPR4804, ab124711	1:1000	Abcam

Secondary antibodies

Antigen	Dilution	Source
sheep anti-mouse IgG-HRP	1:5000	GE Healthcare
goat anti-mouse IgG-HRP	1:5000	Thermo Fisher Scientific
donkey anti-rabbit IgG-HRP	1:5000	GE Healthcare
Alexa Fluor 488-linked anti-rabbit-IgG	1:200	Life Technologies

2.1.7. Chemicals

Standard drug concentrations used in all the experiments, unless stated differently in the figures.

Name	Concentration	Source
Cisplatin (CDDP)	1 µg/ml	Sigma-Aldrich
Dichloroacetate (DCA)	40 mM	Sigma-Aldrich
2-deoxy-D-glucose	10 mM	Sigma-Aldrich
AZD8055	250 nM	Selleckchem
Rapamycin	250 nM	Selleckchem
Everolimus	250 nM	Absource Diagnostic
Puromycin	1 µg/ml	Thermo Fisher Scientific
Geneticin	400 µg/ml	Thermo Fisher Scientific

2.2. Methods

2.2.1. Cells culture

H460 cells were cultured in complete RPMI1640 medium with 10% fetal bovine serum (FBS) supplement and 1% penicillin (10.000 U/ml)/streptomycin (10 mg/ml) at 37 °C in a 5% CO_2 humidified incubator. Passaging of cells was performed twice per week by washing once with PBS and trypsinization for 5 min, followed by resuspension in medium and plating. Cell count was performed by using a Beckman Culter Z-series counter. They were periodically tested to ensure negativity from mycoplasma contaminations.

Reagents:

• RPMI high glucose cell culture medium, with L-glutamine (#21875034, Life Technologies)

• FBS (Fetal Bovine Serum) (#F0804, Sigma-Aldrich)

• Pen/Strep (Penicillin 100 µg /ml, Streptomycin 100 µg/ml) (#15140-122, Life Technologies)

• PBS (phosphate-buffered saline) without $Ca2^+$ and $Mg2^+$ (#14190 Life Technologies)

• Trypsin-EDTA solution (#T4174, Sigma-Aldrich)

For all experiments (unless stated otherwise in the corresponding figure), the used drug concentrations are reported in chapter 2.1.7 “Chemicals”.

2.2.2. Generation of drug-resistant cell lines

Repeated exposure of $H460^{WT}$ cells to increasing concentrations of CDDP (5 nM-2.56 M) led to the production of CDDP-resistant $H460^{Res}$ cells. Resistant cell lines were maintained in culture with 1 µg/ml CDDP. Cells were cultured without CDDP for one week before an experiment.

2.2.3. Cells transfection

To transfect cells with CRISPR-Cas9 expressing plasmids, cells were seeded in 6 well plates 24h before transfection to obtain about 70% confluency. The next

day, the cell medium was switched to a serum/antibiotic-free medium, and Lipofectamine 2000 (#11668027, LifeTechnologies) was used as transfection agent. 8 µl Lipofectamine and 2,5 µg plasmid DNA were mixed in 400 µl Opti-MEM serum-free medium following the manufacturer´s protocol. The lipoplexes were added drop-wise to the plate wells. The cell medium was changed again to complete RPMI 4-6h after transfection. After 48 h transfected cells were re-seeded into 10 cm dishes with complete medium with the addition of 1µg/ml puromycin for three days to perform antibiotic selection. Afterward, the supernatant in the dishes was changed to complete RPMI medium.

2.2.4. Golden Gate cloning

The CRISPR-Cas9 system requires the expression of both the Cas9 and the sgRNA components. To this end, for our experiments, we transfected cells with the PX459 vector containing the sequences for both the Cas9 and the scaffold portion of the sgRNA. However, sgRNAs must be cloned into the plasmids through Golden gate assembly: a cloning method based on the use of type IIS restriction enzymes that allows the directional and highly efficient assembly of DNA fragments into the cloning site. After their design, the single-strand(ss) sgRNA oligonucleotide pairs were obtained and a reannealing reaction was set in place:

Reannealing buffer:
- 10 µl 1 M (pH = 7.5)
- 10 µl 5 M NaCl
- 980 µl H2O

Reannealing reaction:
- 16 µl reannealing buffer
- 4 µl 10 µM sense + antisense oligonucleotide

Thermocycler program:
95ºC 5 min > gradual cooling 1°C / 30 seconds > down to 21ºC

Afterward, the reannealed oligos (ds-sgRNA) were used for the cloning reaction:

- 2.5 µl ds-sgRNA
- 1 µl pX459 vector (1µg)
- 1 µl BbsI enzyme (10U)
- 0.5 µl DNA ligase (30 U)
- 2 µl NEB 2.1 buffer
- 1 µl 10 mM ATP
- 1 µl 100 mM DTT
- H2O up to 20 µl in total

Thermocycler program:

37°C - 2 min
20°C - 5 min
} 50x
65°C - 25 min
4°C ∞

2.2.5. Bacterial electroporation and culture for plasmid expansion

Plasmids from cloning reactions were subsequently electroporated in bacteria (DH10B ElectroMAX). Frozen bacteria aliquots were thawed on ice and 1 µl of the cloning product was added. The bacteria mixture was then pipetted into ice-cold electroporation cuvettes followed by electric pulsation in a micropulser (BioRad). Electroporated bacteria were then incubated at 37°C with 500 µl of LB medium in shaking conditions to allow the expression of antibiotic resistance. A suitable antibiotic was added to 100 µl of bacteria solution after 30 minutes, which was then plated on LB agar plates and cultured overnight at 37°C. After 24h, we picked the colonies and used them for plasmid isolation.

Reagents:

- LB medium: 5 g/l NaCl, 5 g/l yeast extract, 10 g/l Bactotryptone
- LB agar plates: 1.5% agar-agar in LB medium supplemented with either Ampicillin (100 µg/ml) or Kanamycin (50 µg/ml) antibiotics

2.2.6. Plasmid isolation (Mini- and Midi-preps)

Mini-prep

To rapidly assess whether the picked bacterial colony contains a plasmid with the correct cloning insertion, small-scale plasmid isolation (mini-prep) was performed for screening with either restriction digestion or Sanger sequencing. Colonies were picked with a sterile micropipette tip and dropped in tubes containing 4ml of LB medium supplemented with the suitable antibiotic (Ampicillin 100µg/ml or Kanamycin 50µg/ml). Bacteria solutions were left growing overnight at 37°C in shaking conditions. The following day 2 ml of the bacterial culture was poured into 2 ml tubes and centrifuged to obtain a pellet. The pellet was resuspended in 300µl of P1 buffer and subsequently, 300µl of P2 buffer was added for cell lysing (5 minutes on ice). Afterward, the reaction was neutralized by the addition of 300µl P3 buffer. The mixture was then centrifuged (10 minutes at 13.000g) and the supernatant containing the plasmid DNA was taken. DNA precipitation was obtained by the addition of 700µl isopropanol and pelleted by centrifugation (30 minutes at 13.000g). The pellet was then washed with ethanol 70% and let dry at 40°C until the complete evaporation of the liquid. DNA pellet was then resuspended in 40-50µl of water and used for screening.

Reagents:

- P1 buffer: 50 mM Tris HCl pH 7.5, 10 mM EDTA pH 8.0, 100 µg/ml RNAse-A
- P2 buffer: 200 mM NaOH, 1% SDS
- P3 buffer: 3 M potassium acetate pH 5.5

Midi-prep

To obtain larger quantities of plasmid that would be further used for transfections in cancer cell lines, medium-scale preparations (Midi-preps) were performed. Bacterial suspension from Mini-preps was inoculated in 100ml of LB medium supplemented with the suitable antibiotic and let grow overnight in a shaker at 37°C. The following day, DNA extraction and purification were performed using the Nucleobond Xtra Midi Kit (Macherey-Nagel #740410.100) following the manufacturer´s protocol.

2.2.7. Western blotting

To detect protein levels, western blotting was performed.

Cells were seeded one day prior to treatment on 10 cm dishes and treated for 48h with the standard drug concentration unless stated differently in the experiment figure. After treatment, cells were washed once in PBS and kept on ice. Afterward, they were harvested by scraping them in 1ml of NP-40 Lysis Buffer supplemented with protease and phosphatase inhibitors. Samples were subjected to 3 cycles of freeze and thawing in liquid nitrogen and warm bath to ensure complete cell lysing, and centrifuged at 13.000g at 4°C for 20 min to remove membranes and debris before transferring the supernatant into a new tube. Following the manufacturer's instructions, the Bradford Bio-Rad Protein Assay was used to test the protein concentrations of each sample to ensure that the same amount of protein was loaded into each sample. By adding LDS sample buffer and a reducing agent to protein lysates, followed by heating for five minutes at 95°C, protein lysates were denatured.

Protein preparations were loaded on precast gels with gel density chosen based on the molecular weight of the proteins of interest. PageRuler Prestained Protein Ladder was used as molecular weight marker. Gels were submerged in SDS MOPS running buffer and subjected to one initial voltage of 90V to allow proteins to align in the stacking gel for 15 minutes, and afterward were run at 120V until the marker would reach the gel bottom. Proteins were then blotted from gels onto PVDF membranes using NuPAGE Transfer Buffer and 15% methanol at constant 100V and 450mA for 90 minutes.

After blotting, membranes were blocked in a TBST solution containing 5% nonfat dry milk and incubated overnight at 4°C in the blocking solution with the addition of the desired antibody. The next day, membranes were washed three times with TBST and incubated at room temperature with blocking solution and HRP-conjugated secondary antibody for 2h. Afterward, they were further washed in TBST, and protein detection was performed with HRP ECL Substrate Sirius kit by Gel Doc XR System (Biorad).

Reagents:

- NP-40 lysis buffer 2% (pH 8.0)

- Protease inhibitor cocktail complete ULTRA tablets EASYpack (#04693116001, Roche)
- Phosphatase inhibitor PhosSTOP (#4906837001, Roche)
- Bradford Bio-Rad Protein Assay (#5000006, Bio-Rad)
- Loading LDS sample buffer (#NP0008, Life Technologies)
- Reducing agent (#NP0009, Life Technologies)
-SDS-Page precast gels NuPAGE Novex Bis-Tris Mini and Midi Gels (#WG1402BOX, Life Technologies)
- Running buffer NuPAGE MOPS SDS Running Buffer (#NP0001, Life Technologies)
- Protein ladder PageRuler Prestained Protein Ladder (#26616, Thermo Scientific)
- Transfer buffer NuPAGE® Transfer Buffer (#NP00061, (Life Technologies)
- PVDF membranes (Immobilon-P PVDF Membrane, # IPVH00010)
- TBST 15 mM NaCl, 5 mM Tris HCl pH 7.5, 0.1% Tween 20
- Nonfat dry milk Skim Milk Powder (Sigma #70166)
- HRP substrate WesternBright ECL Substrate Sirius (Biozym #541021)

2.2.8. Genomic DNA (gDNA) extraction

Using the manufacturer's instructions, genomic DNA was extracted and purified with the QIAamp DNA Blood Mini Kit (#51104, Qiagen). DNA yield and quality were analyzed by use of the Nanodrop ND-1000 spectrophotometer.

2.2.9. PCR

For genotyping the modifications performed on the cell lines by CRISPR-Cas9, PCR was performed to amplify the target DNA sequence.

For each reaction:
- gDNA template 150 ng
- Forward + Reverse primers (10 µM) 2 µl
- PCR buffer 4 µl
- dNTPs 0.4 µl
- DMSO 1 µl
- GoTaq polymerase (M3005, Promega) 0.2 µl

- H2O up to 20 µl

Thermocycler program:

95 °C - 2 min

95 °C - 0:30 min
56 °C - 1min/kb min
72 °C - 0:30 min
} x35

72 °C - 5 min

4 °C - ∞

For Sanger Sequencing, PCR amplicons were sent to and analyzed by LGC Genomics GmbH (Berlin, Germany).

2.2.10. Cloning of paired gRNAs into one single vector

Generation of CRISPR-Cas9 vectors expressing two sgRNAs was performed as described in the paper “Rapid and efficient one-step generation of paired gRNA CRISPR-Cas9 libraries” (Vidigal and Ventura 2015).

2.2.11. Clonogenic growth assays

To observe the effects on clonogenic growth provoked by drugs, colony formation assays were performed. Cells were seeded in 6-cm dishes one day before the exposure to the drugs and treated for 10 days before fixation with 70% ethanol unless confluency was reached before. Because continuous exposure to mTOR inhibitors causes cytostatic effects, they were given 72h before co-treating with other compounds and removed with the first medium change. Staining was obtained by use of a crystal violet solution for 30 minutes, afterward, the dishes were washed with water and let completely dry. Images of the dishes were acquired with a scanner. Subsequent quantification was obtained by de-staining the dishes for 10 minutes on a shaker using a 20% acetic acid solution. The resulting solution was diluted 1:20 in water and its optical density was measured in triplicates using a Cytation 3 (Biotek) plate reader at 590 nm. Yields were then normalized to the untreated control samples to obtain relative clonogenic values for each sample. Data were analyzed using Microsoft Excel and Graphpad prism 8.0.

Reagents:

- Crystal violet 1:20 in 20% ethanol (#HT90132, Sigma-Aldrich)

2.2.12. Real-Time Live Cell Imaging

Effects on cell proliferation were assessed through live cell imaging using the IncuCyte S3 Live-Cell Analysis System (Sartorius), equipped with a phase-contrast microscope at 10X magnification. For each condition, we seeded cells on 96-well plates in triplicates, 1.000 cells for each well. From the time of treatment, pictures of each well were taken every 4h up to 7 days. Analysis of the confluence was performed with the IncuCyte S3-2018A software, while photo editing and the addition of scale bars were done with the ImageJ software. Area under the curve (AUC) values were calculated with GraphPad Prism 8.0 and were normalized to the untreated control.

2.2.13. Flow cytometry

To assess the effects on apoptosis caused by drug treatment, we measured the sub-G1 population of cells after propidium iodide staining through flow cytometry. Cells were seeded one day prior to treatment on 10-cm dishes. Cells were treated using the standard drug concentration for 4 days unless stated differently in the experiment figure. After treatment, the supernatant was collected and cells were washed, trypsinized, and finally resuspended in the previously collected supernatant. Afterward, cells were centrifuged to obtain one pellet that was resuspended in 5 ml PBS, and subsequently, the cell suspension was fixed by adding 10 ml glacial ethanol (90%) during agitation and stored at -21°C overnight. Cells were stained the following day using 10 µg/ml propidium iodide plus 100 µg/ml RNase A. Data were analyzed using the FlowJo Software (BD Bioscience) after the Accuri C6 flow cytometer (BD Bioscience) was used to measure the sub-G1 population.

2.2.14. Agarose gel electrophoresis

The separation of DNA fragments from either PCR or plasmid restriction was performed through agarose gel electrophoresis, with gel percentage (from 0,8% up to 2%) variable according to the size of the band to isolate. Agarose was added to TAE buffer and made to boil until complete dissolution. Prior gel casting, Safe-

Red™ was added to allow DNA visualization. After loading on the gel, samples were run at a constant voltage. Band visualization was achieved using UV light at the Gel iX Imagersystem (Intas).

- Agarose NEEO Ultra-Quality Carl Roth, #2267
- TAE buffer 40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8.0
- RedSafe DNA stain (#21141, Chembio)
- Loading buffer DNA IV Applichem , #A3481,0010
- GeneRuler DNA Ladder Mix Fermentas, #SM0332

2.2.15. DNA band extraction and purification

Purification of DNA bands cut from the gels was done using the Wizard® SV Gel and PCR cleanup system (Promega #A9282) following the manufacturer protocol.

2.2.16. Fluorescence microscopy

For visualizing autophagosomes with the DsRed-LC3-GFP reporter, cells were seeded on an eight-well chamber slide (Sarstedt) the day previous to treatment with the indicated drugs. Afterward, cells were washed with PBS, fixed in a 3.7% solution of Paraformaldehyde (Sigma-Aldrich) in PBS (adjusted at pH 7,4) for 20' at RT, and again washed. Finally, the chamber was taken off the slide, and cells were treated with a drop of 200 nM DAPI (Molecular Probes) for 45 minutes at room temperature before being mounted on the coverslip. Pictures were obtained with the use of a Leica DM4 B Fluorescence microscope, X63 magnification objective with oil immersion. Merged images of all three channels (DsRed, DAPI, GFP) were obtained and analyzed with the Aperio Software (Leica).

2.2.17. RNAi

To generate transient gene knockdown, cells were seeded to reach about 70% confluency in 6-well plates, and the next day they were transfected with siRNA. Lipofectamine RNAiMAX was used as a transfection agent (Invitrogen) and used to transfect the desired siRNA according to the manufacturer's protocol.

2.2.18. Statistics

To manage all data, generate graphs, and calculate statistics, Microsoft Excel and GraphPad Prism were used. The corresponding statistical method of analysis and statistical significance were described in the text or figure legends.

3. Results

3.1. mTOR upregulation

3.1.1. Chemoresistance to cisplatin and vulnerability to metabolic inhibitors are linked to mTOR activity

To investigate the interplay between mTOR activity and sensitivity to either chemotherapeutic agents or metabolic drugs in chemotherapy-resistant cells, the non-small cell lung cancer (NSCLC) NCI-H460 (abbr. H460) cell line was chosen. Chemoresistant cells ($H460^{Res}$) were generated in a previous study: the parental line ($H460^{WT}$) underwent a dose-escalation treatment with cisplatin (CDDP, a common frontline chemotherapeutic used in NSCLC) until cells became CDDP-resistant. Cisplatin-resistant $H460^{Res}$ cells demonstrated cross-resistance to a variety of cross-linking agents such as oxaliplatin, carmustine, and mitomycin C (Wanzel et al. 2016).

First, we tested the sensitivity of $H460^{Res}$ to different metabolic drugs and CDDP in a clonogenic assay. As expected, the clonogenicity of CDDP-resistant cells was not affected by CCDP. However, when exposed to the metabolic inhibitors DCA and 2DG, the proliferation of $H460^{Res}$ cells was strongly reduced, in stark contrast to parental cells (Fig. 5 a).

It is known that mTOR upregulation can drive resistance to CDDP, for instance by increasing levels of the FANCD2 protein, a central component of the Fanconi anemia pathway essential for the repair of DNA damage induced by crosslinking agents (F. Guo et al. 2013; Shen et al. 2013; H. Kim and D'Andrea 2012). We compared, therefore, mTOR activity in resistant and parental cells by analyzing the phosphorylation levels of several known mTOR targets together with FANCD2. Furthermore, to confirm the contribution of mTOR to the resistant phenotype, we blocked mTOR activity by using the dual mTORC1-2 inhibitor AZD8055 (Chresta et al. 2010). Cells were exposed to a wide concentration range to find the optimal treatment conditions. We observed that, in basal conditions, $H460^{WT}$ cells exhibit moderate mTORC1 and mTORC2 activity by examining the phosphorylation levels of mTORC1 ($pULK1^{S757}$, $pp70S6K^{T389}$,

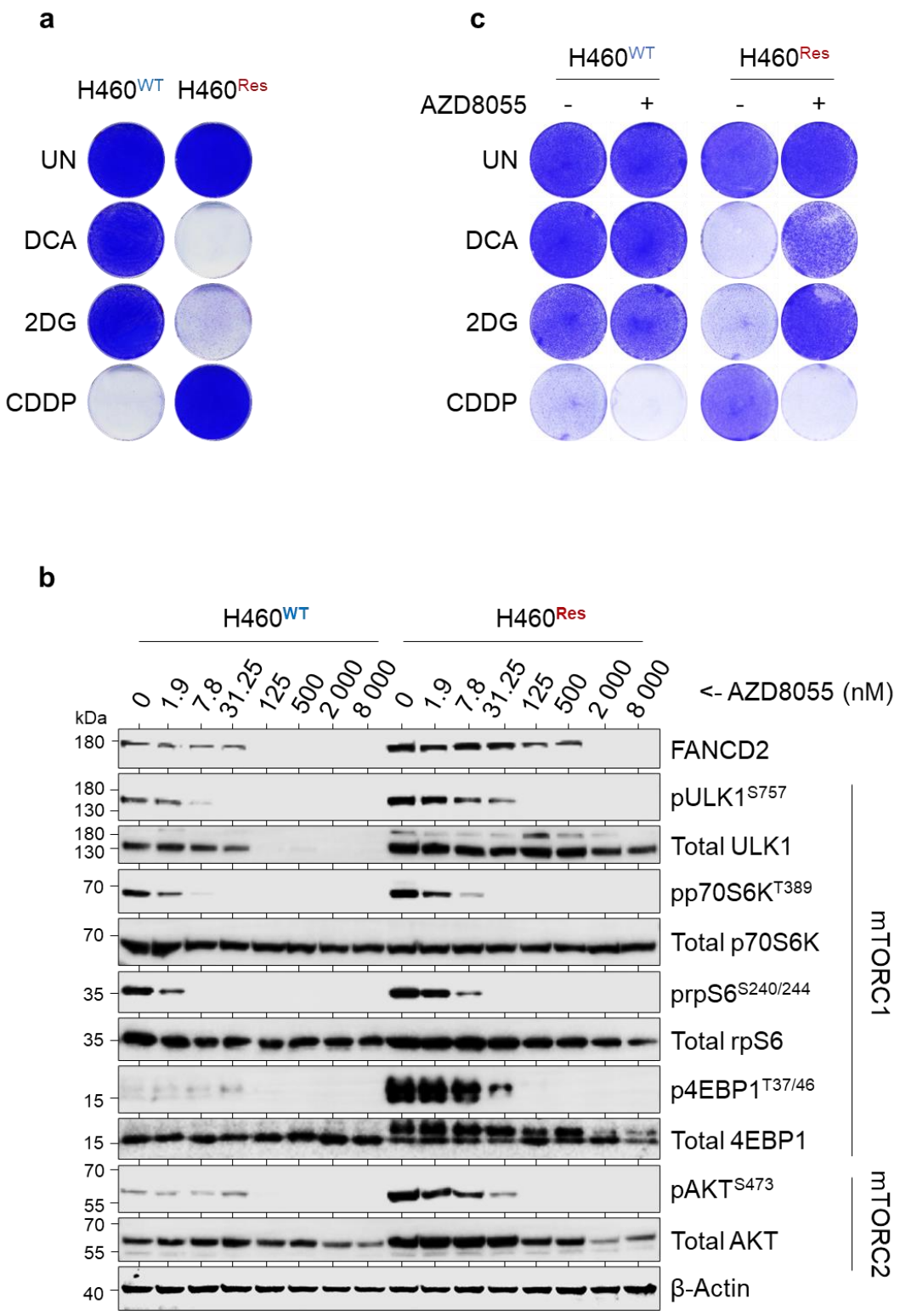

a
H460WT
H460Res
UN
DCA
2DG
CDDP
c
H460WT
H460Res
AZD8055
-
+
-
+
UN
DCA
2DG
CDDP
b
H460WT
H460Res
0
1.9
7.8
31.25
125
500
2 000
8 000
<- AZD8055 (nM)
kDa
180
FANCD2
180
130
pULK1S757
180
130
Total ULK1
70
pp70S6KT389
70
Total p70S6K
35
prpS6S240/244
35
Total rpS6
15
p4EBP1T37/46
15
Total 4EBP1
70
55
pAKTS473
70
55
Total AKT
40
β-Actin
mTORC1
mTORC2

Fig. 5 Chemoresistant cells are vulnerable to metabolic drugs and show elevated mTOR activity

a) Colony formation assay. Effects on clonogenic growth caused by the indicated drugs in parental ($H460^{WT}$) and chemoresistant ($H460^{Res}$) cells. DCA= 40mM; 2DG= 10mM; CDDP= 1µg/ml.
b) Western blot. Detection of FANCD2 levels and phosphorylation rates of mTORC1 and mTORC2 targets in $H460^{WT}$ and $H460^{Res}$ cells. Cells were treated for 3 days with AZD8055. β-Actin was used as loading control.
c) Colony formation assay. Effects on clonogenic growth caused by the indicated drugs in $H460^{WT}$ and $H460^{Res}$ cells.

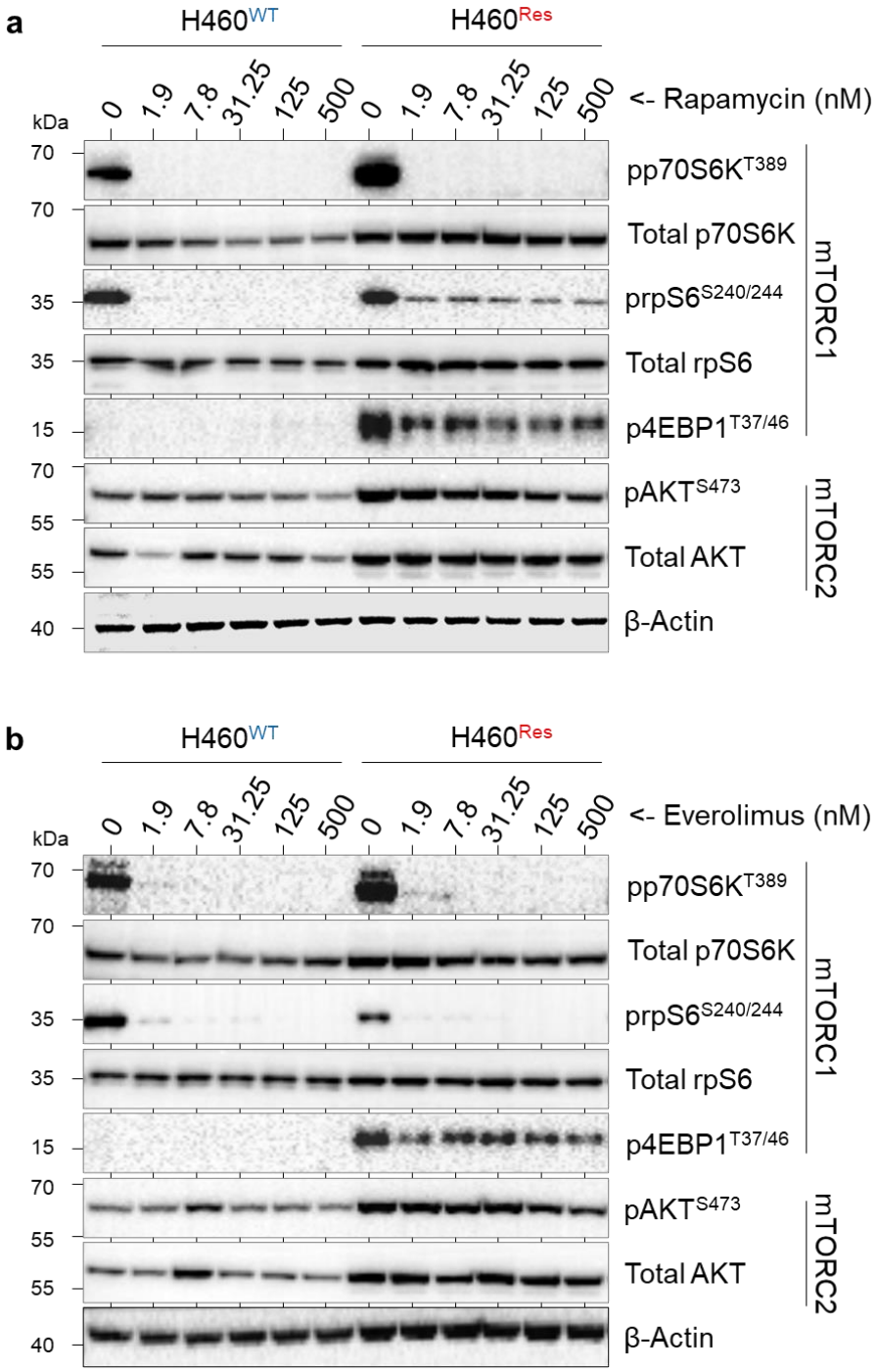
$4EBP1^{T37/46}$, $rpS6^{S240/244}$), and mTORC2 ($pAKT^{S473}$) targets respectively, together with moderate expression of the protein FANCD2. The addition of AZD8055 with increasing concentration reduced FANCD2 levels and the phosphorylation rate of all the mTORC1/2 targets in a dose-dependent manner but did not reduce their total protein amount, with exception of total ULK1.

In comparison to $H460^{WT}$, $H460^{Res}$ cells showed markedly elevated mTORC1 and mTORC2 activity, as seen by the higher phosphorylation rates of their corresponding targets, while having total protein levels similar to WT cells (with exception of higher total ULK1 and total AKT). More importantly, the chemoresistant cells exhibited higher FANCD2 expression. Remarkably, $H460^{Res}$ cells were less sensitive to mTOR inhibition by AZ8055 compared to parental cells, since higher inhibitor concentrations were required to “switch off” mTOR’s markers phosphorylation and to decrease FANCD2 levels compared to $H460^{WT}$ (Fig. 5 b). Interestingly, some proteins showed a decrease also in the total protein level after AZD8055 treatment. These proteins were ULK1, AKT, and FANCD2, the same we found with higher amounts in the basal conditions. Collectively, these results provide strong evidence of the upregulation of the mTOR pathway in chemoresistant cells. Based on the results shown in Fig. 5C we chose 250 nM as the AZD8055 concentration to use in further experiments because at 250 nM the mTORC1/2 activity was fully blocked in $H460^{Res}$ cells and FANCD2 was diminished to levels similar to basal in $H460^{WT}$ cells.

We tested then the effects of AZD8055 in combination with the beforementioned metabolic drugs in a colony formation assay. Strikingly, exposure of $H460^{Res}$ to AZD8055 reverted the phenotype to the parental state: cells became resensitized

to CDDP and lost the vulnerability to DCA and 2DG. In contrast, the clonogenic growth of $H460^{WT}$ cells was mostly unaffected (Fig. 5 c). These results provided the first evidence of the involvement of mTOR signaling in conferring simultaneously resistance against CDDP and sensitivity to DCA/2DG.

In addition, to dissect the roles of the two mTOR complexes (mTORC1 and mTORC2) in conferring the sensitivity to metabolic inhibitors, we treated cells with either rapamycin or everolimus. While AZD8055 inhibits both mTORC1 and 2, rapamycin and its derivative everolimus are first-generation mTOR inhibitors affecting only the mTORC1 complex (Y.-J. Zhang, Duan, and Zheng 2011). We treated the $H460^{WT}$ and $H460^{Res}$ cells with an increasing concentration of both rapamycin and everolimus and assayed the mTOR activity using western blot. Also in this case, the basal phosphorylation levels of all the mTOR targets (both mTORC1 and 2) were greater in the resistant cells. Exposure to the lowest drug concentration tested induced inhibition of the mTORC1 complex as seen from the reduced phosphorylation levels of $p70S6K^{T389}$, $4E\text{-}BP1^{T37/46}$, and $RPS6^{S240/244}$, but not of the mTORC2 target AKT^{S473} (Fig. 6 a, b). In parallel, we examined the effects of rapamycin and everolimus on the sensitivity to either DCA or 2DG in a clonogenic assay. None of the drugs had any relevant impact on the clonogenicity of $H460^{WT}$ cells, nor did any of their combinations. However, while $H460^{Res}$ strongly reduced growth under the single treatment with DCA and 2DG, the combination with either rapamycin or everolimus rescued clonogenic growth back to levels comparable to the WT-like phenotype (Fig. 6 c). This suggests that mTORC1 activity is essential to confer the sensitivity to DCA and 2DG.


a
H460WT
H460Res
0
1.9
7.8
31.25
125
500
<- Rapamycin (nM)
kDa
70
pp70S6KT389
Total p70S6K
35
prpS6S240/244
Total rpS6
15
p4EBP1T37/46
mTORC1
pAKTS473
55
Total AKT
40
β-Actin
mTORC2
b
<- Everolimus (nM)

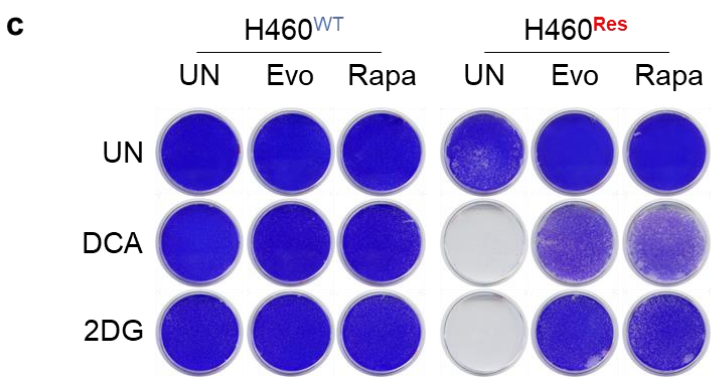

c
H460WT
H460Res
UN
Evo
Rapa
DCA
2DG

Fig. 6 mTORC1 activity is enough to confer hypersensitivity to metabolic drugs

a) Western blot. Detection of mTORC1 and mTORC2 targets and corresponding phosphorylation rates in parental and chemoresistant cells. Cells were treated for 3 days with increasing concentration of rapamycin. β-Actin was used as loading control.
b) Western blot. Detection of mTORC1 and mTORC2 targets and relative phosphorylation rates in parental and chemoresistant cells. Cells were treated for 3 days with increasing concentration of everolimus. β-Actin was used as loading control.
c) Colony formation assay. Effects on clonogenic growth caused by the indicated drugs in parental ($H460^{WT}$) and chemoresistant (460^{Res}) cells. DCA= 40mM; 2DG= 10mM; CDDP= 1μg/ml.

3.1.2. The role of mTOR in metabolic drug sensitivity

To gather more evidence of whether mTOR upregulation is responsible for metabolic drug sensitivity, we enhanced the mTOR signaling either by creating cell lines stably expressing mTOR hyperactive mutants or by acting on upstream mTOR regulators.

3.1.2.1. Creation of mTOR hyperactive mutants

In a previous study, a comprehensive survey of cancer-associated mTOR mutations revealed an array of several hyperactivating variants (Grabiner et al. 2014). From this pool, we chose three mTOR mutations among those exhibiting the highest mTORC1 and mTORC2 activity rates: L1460P, S2215Y, and I2500F (Fig. 7). These mutations are reported to not affect the assembly of the mTOR complex, but the L1460P mutation is located on the FAT domain of mTOR, which reduces the binding affinity to the DEPTOR endogenous inhibitory subunit. S2215Y and I2500F are instead situated in the catalytic kinase domain (Fig. 7). They all have the overall effect of increasing the mTORC1 phosphorylation

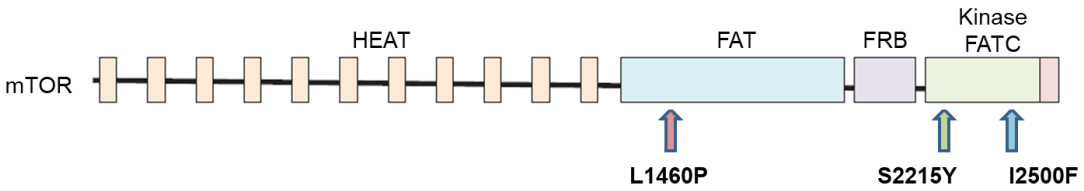


Fig. 7 mTORC1 and mTORC2 activating mutations
Schematic location of the selected mutations in the mTOR protein. (Modified figure from Grabiner et. al. 2014.)

activity, with the exception of I2500F, which is characterized also by elevated mTORC2 activity. Donor plasmids containing mTOR cDNA sequences with the L1460P, I2500F, and S2215Y mutations and N-terminal FLAG-tag were obtained from Addgene (#69006, #69014, #69013). The cDNA sequences were subcloned into the PiggyBac transposon expression vector PB-EF1α-MCS-IRES-Neo (System Biosciences #PB533A-2). The PiggyBac is equipped with two inverted terminal repeats (ITRs) flanking the expression cassette. ITRs are recognized by a transposase that stably integrates the construct into the genome at TTAA sites ("PB-EF1α-MCS-IRES-Neo PiggyBac CDNA Cloning and Expression Vector" n.d.) (Fig. 8). For the cloning, the NotI restriction enzyme was used to excise the coding sequences from their donor plasmid backbones followed by agarose gel purification (0,8% agarose). The PiggyBac recipient plasmid was linearized at the multiple cloning site (MCS) using NotI, and to prevent re-circularization it was successively dephosphorylated with alkaline phosphatase. At last, mTOR cDNA sequence fragments and the linearized PiggyBac were mixed and ligated using T4 DNA ligase (Life Technologies, #EL0014). To screen whether the insertion happened successfully and with the mTOR insert in the right orientation, a

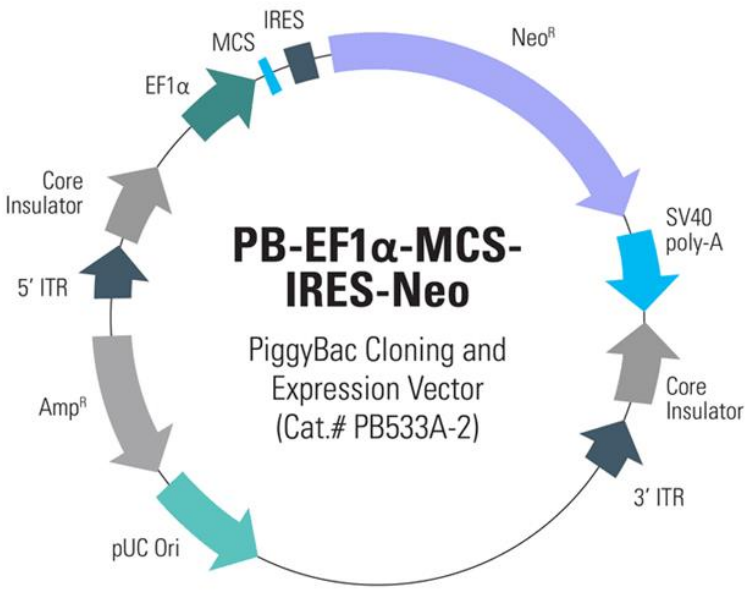


Fig. 8 The PiggyBac plasmid
Structure of the PiggyBac PB-EF1α-MCS-IRES-Neo expression system. Image from https://www.systembio.com/pb-ef1-mcs-ires-neo-piggybac-cdna-cloning-and-expression-vector.

restriction preliminary screening was performed using the EcoRI enzyme (New England Biolabs #R0101). Insertion with the right orientation would originate three bands of 10,5 kbp, 3,1 kbp, and 1 kbp. Incorrect insertion would show different band sizes: 10,1 kbp, 3,5 kbp, and 1 kbp (Fig. 9 a,b). The positive plasmids were further validated by Sanger sequencing to confirm the correct orientation and mutation (Fig. 9 c).

Expression vectors encoding mTOR variants were used to transfect $H460^{WT}$ cells in combination with the plasmid expressing the Super PiggyBac transposase, required for the stable transgene integration in the genome. After transfection, cells underwent selection with Geneticin, and single-cell clones were generated. Finally, we identified the clones expressing the highest levels of FLAG-mTOR using a western blot (Fig 10). We picked clone #1 for L1460P, clone #2 for S2215Y, and clone #2 for I2500F for further examination.

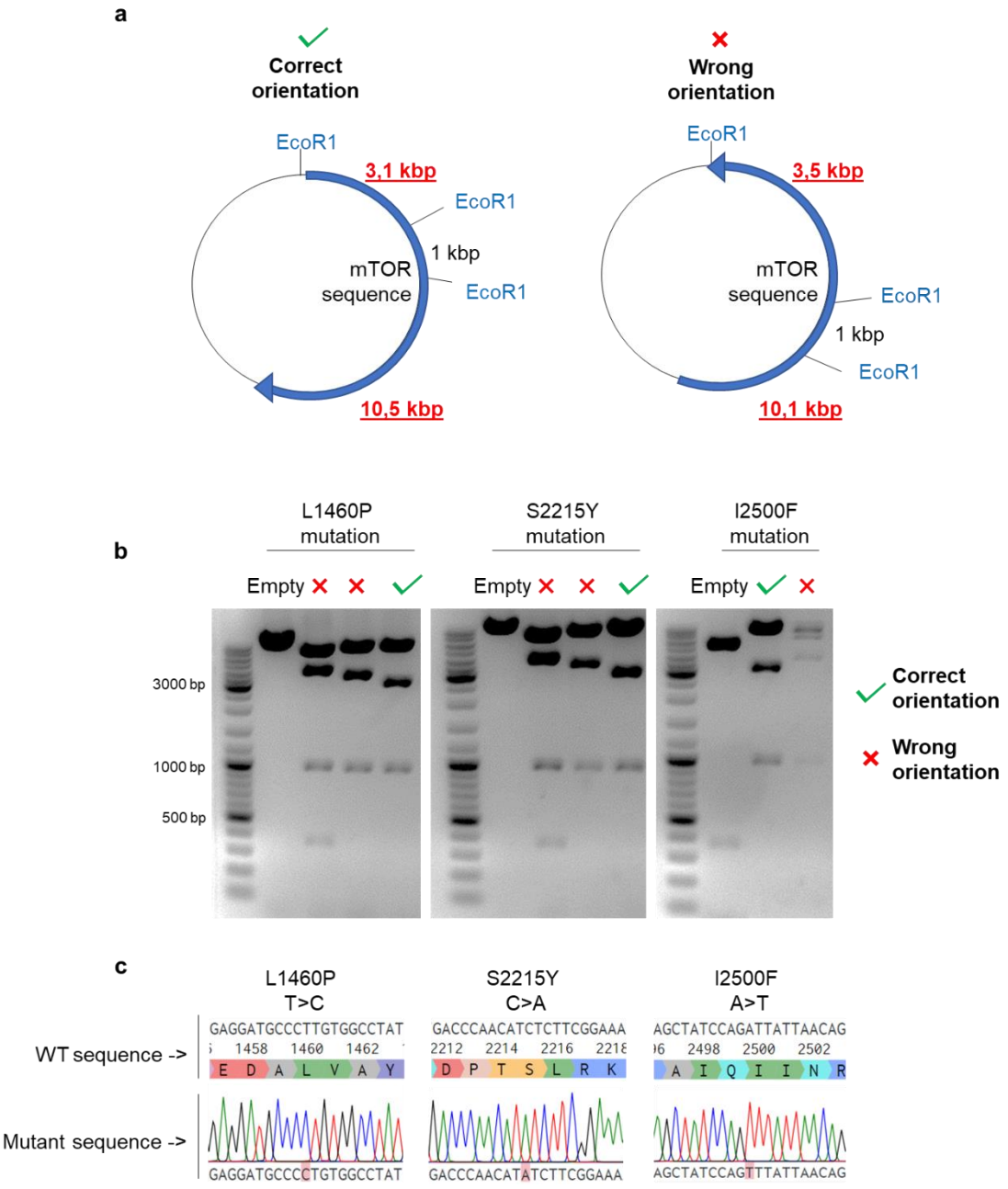


Fig. 9 mTOR cDNA subcloning

a) Schematic of the plasmid screening with the restriction enzyme EcoRI.
b) Agarose gel electrophoresis showing the plasmid fragments originating from EcoRI digestion. Empty= empty control vector
c) Sanger sequencing of the plasmid DNA for each mTOR mutation.

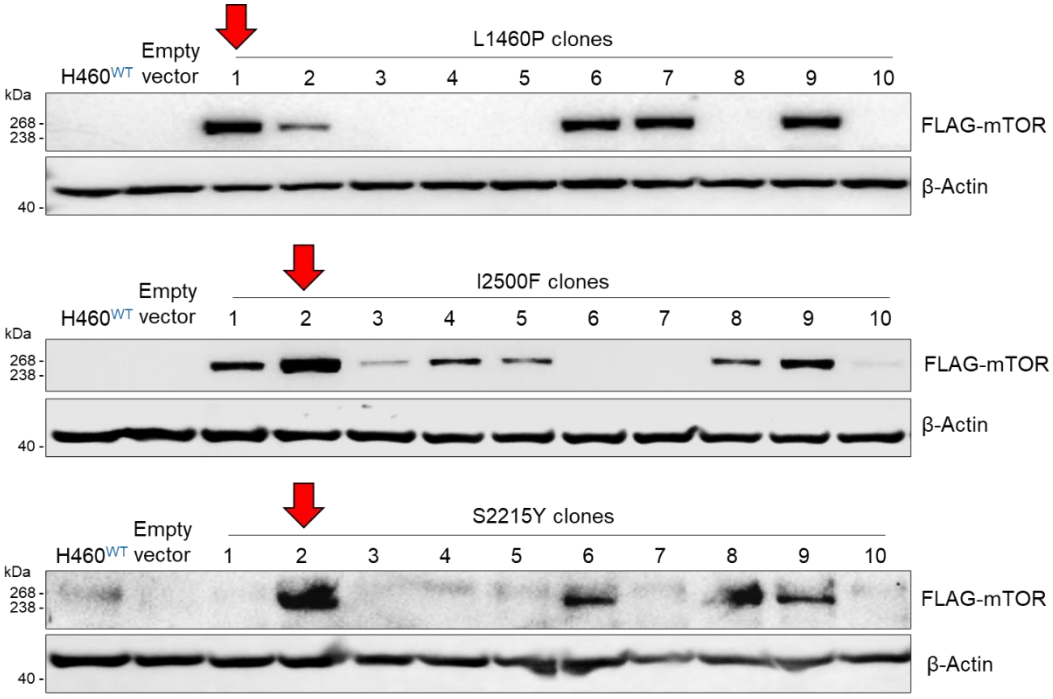


Fig. 10 mutant mTOR clones
Western blot analysis showing the FLAG-mTOR levels in single-cell clones for each mutation type. Clones were obtained after Geneticin selection. The red arrows indicate the clones picked for further analysis.

3.1.2.2. Cells expressing hyperactive mTOR mutants show higher sensitivity to metabolic drugs

Next, we analyzed the phenotype of the established mutant cell lines. Western blotting revealed higher phosphorylation levels of both of the mTOR targets ULK1 and 4EBP1 in three mutant-expressing cell lines compared to parental cells ($H460^{WT}$) and mock control, indicating higher mTOR activity. However, none of the clones did match the activity exhibited by the chemoresistant cells ($H460^{Res}$), given by the more marked levels of pULK1 and p4EBP1 (Fig.11 a). Subsequently, we evaluated whether the higher mTOR activity would translate into sensitivity to metabolic drugs. With this aim, we tested the clonogenicity of cells expressing hyperactive mTOR variants in a colony formation assay and confirmed that all three mutant clones were indeed more sensitive to both DCA and 2DG compared to the WT controls, and even exhibited modest resistance to CDDP (Fig. 11 b). Similar results were also obtained by evaluating the percentage of apoptotic cells assessed through Propidium iodide (PI) staining followed by flow cytometry analysis. After 5 days of treatment, about 20% of the cell population in each

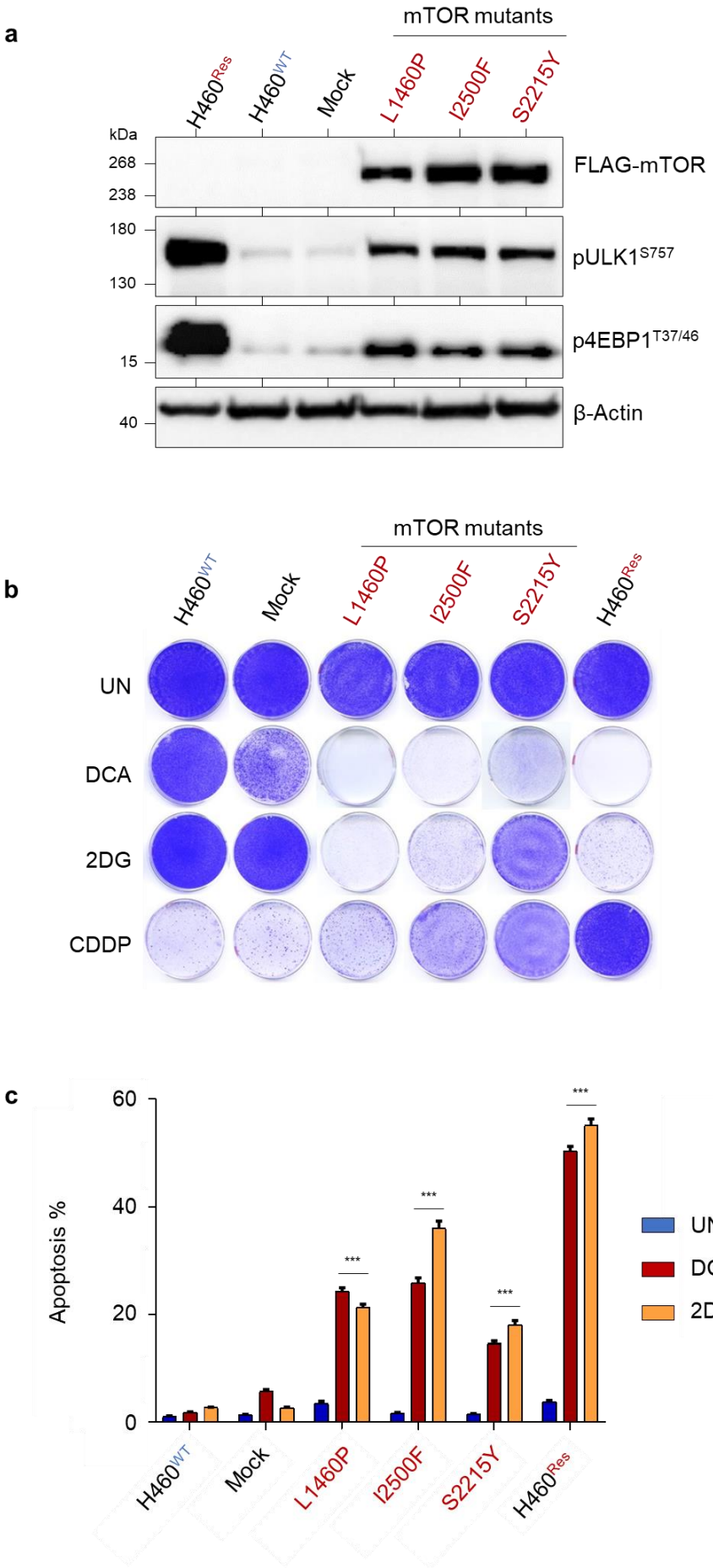

a
mTOR mutants
H460Res
H460WT
Mock
L1460P
I2500F
S2215Y
kDa
268
238
180
130
15
40
FLAG-mTOR
pULK1S757
p4EBP1T37/46
β-Actin
b
mTOR mutants
H460WT
Mock
L1460P
I2500F
S2215Y
H460Res
UN
DCA
2DG
CDDP
c
Apoptosis %
60
40
20
0
***
UN
DCA
2DG
H460WT
Mock
L1460P
I2500F
S2215Y
H460Res

Fig. 11 Clones expressing mutant hyperactive mTOR are sensitive to metabolic drugs

a) Western blot analysis. Phosphorylation levels of mTOR activity markers in mTOR mutant clones.
b) Colony formation assay. Effects on clonogenic growth caused by the indicated drugs in mTOR mutant clones. DCA= 40mM; 2DG= 10mM; CDDP= 1µg/ml.
c) Percentage of apoptotic cells (sub-G1) determined by flow cytometry analysis. Cells were fixed and PI-stained after 5 days of treatment with the indicated drugs. Bars represent mean ± SD, n = 3. Significance through two-way ANOVA with Dunnet multiple comparisons test. ***p<0.001 compared to the WT parental ctrl. DCA= 40mM; 2DG= 10mM.

mTOR mutant line was found in a sub-G1 state when treated with DCA. Similarly, about 20% of the mTOR mutant cells treated with 2DG were apoptotic, and up to 40% in the case of I2500F. On the contrary, the parental controls were unaffected. Thus, clones expressing hyperactive mTOR mutants proved to be significantly more susceptible to DCA and 2DG treatment, although they did not reach the sensitivity of the $H460^{Res}$ cells, which were found about 50% apoptotic (Fig. 11 c). These results confirm that increased mTOR signaling is not only required but sufficient to make cells more susceptible to metabolic perturbations caused by DCA and 2DG.

3.1.2.3. mTOR upregulation by acting on upstream regulators

The conclusions from the mTOR hyperactive mutants were further supported by another experiment using a different adenocarcinoma cell line, the PC-9 cells. To achieve higher endogenous mTOR signaling, we acted upon two of the upstream mTOR regulators: Rheb1 and the TSC1/TSC2 complex. PC-9 cells with stable overexpression of Rheb1 ($PC\text{-}9^{Rheb}$) were previously generated by infection with a FLAG-Rheb1 lentiviral vector (FLAG pLJM1 Rheb1; Addgene #19312). Starting from these cells, we continued by knocking down the TSC1/TSC2 complex through RNA interference trying to obtain an even stronger mTOR activation. $PC\text{-}9^{Rheb}$ cells were transfected with two different anti-TSC1/TSC2 siRNA mixes (siRNA 1 and 2), and we observed their phenotype. By performing a western blot (Fig. 12 a), we could detect the correct expression of FLAG-Rheb1, together with the successful knockdown of both the TSC1 and TSC2 subunits. In all the $PC\text{-}9^{Rheb}$ samples we could observe an increase in the phosphorylation levels of

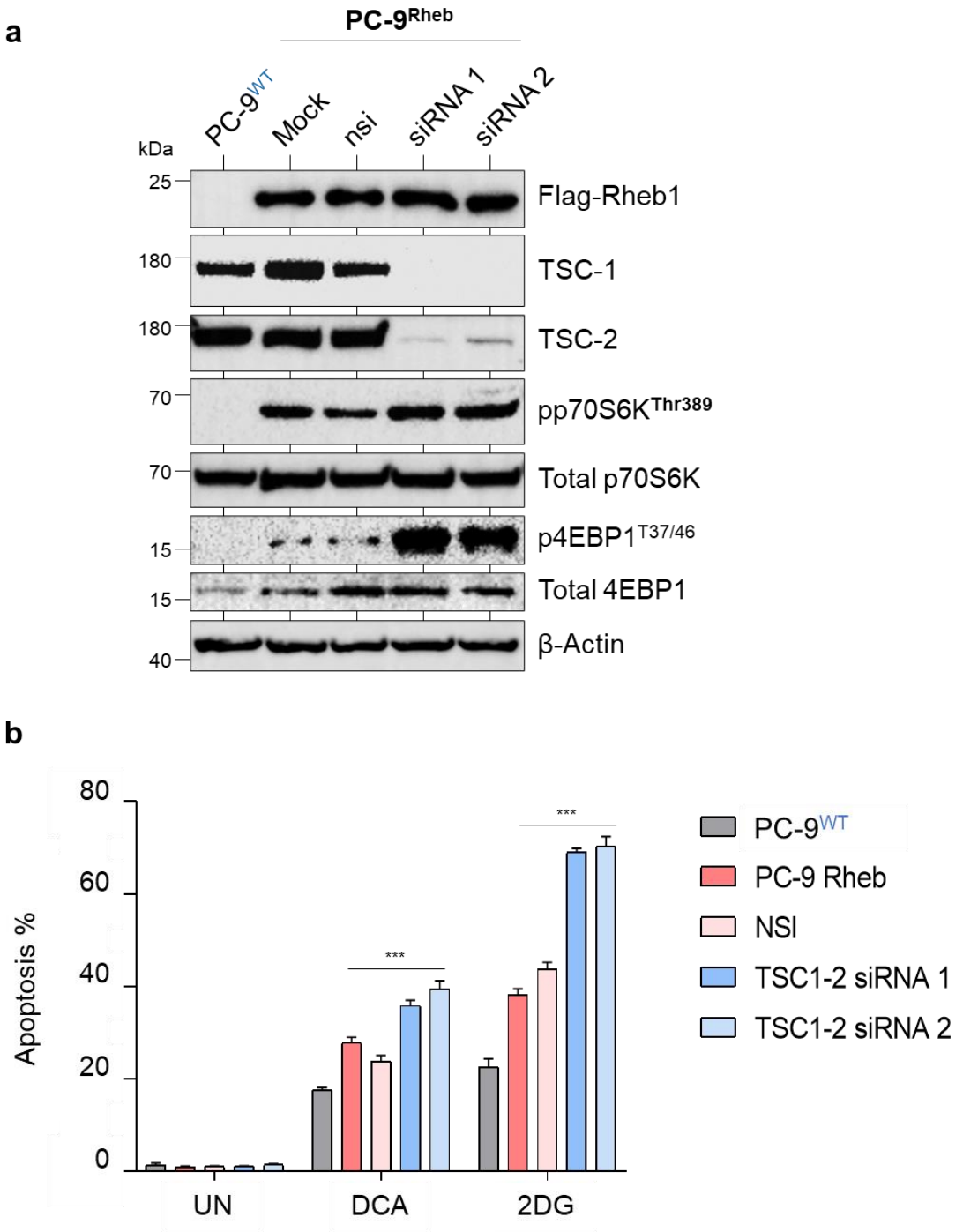


Fig. 12 Artificial activation of endogenous mTOR increase sensitivity to metabolic drugs

a) Western blot analysis. Phosphorylation levels of mTOR activity markers in PC-9 cells overexpressing Rheb1, and transfected with two siRNA mixes against the TSC1/TSC2 complex.

b) Percentage of apoptotic cells (sub-G1) determined by flow cytometry analysis. Cells were fixed and PI-stained after 5 days of treatment with the indicated drugs. Bars represent mean ± SD, n = 3. Significance through two-way ANOVA with Dunnet multiple comparisons test. ***p<0.001 compared to the WT parental ctrl. DCA= 40mM; 2DG= 10mM.

p70S6K and 4EBP1, and depletion of the TSC1/TSC2 inhibitory complex caused mTOR activity to rise even further. More importantly, we tested their sensitivity to metabolic drugs by analyzing their relative sub-G1 population through flow cytometry and saw that their response rate to DCA and 2DG was proportional to their mTOR activity. While WT cells showed about 20% apoptotic cells after treatment with DCA, they reached almost 30% when overexpressing Rheb1, and further up to 40% when combined with TSC1/2 knockdown. Even greater effects were observed with 2DG: while the drug caused about 20% of WT cells to become apoptotic, the percentage doubled in PC-9^{Rheb} cells and reached a significant 80% when combined with TSC1/2 knockdown (Fig. 12 b).

These findings, obtained by artificially increasing mTOR signaling by acting on proximal upstream regulators, further supported our hypothesis: mTOR upregulation is sufficient to create a metabolic vulnerability that can be targeted by drugs such as DCA and 2DG.

3.1.3. Metabolic vulnerability in chemoresistant cells is caused by inhibition of autophagy

After observing that chemoresistant cells do show higher mTOR signaling, and after establishing that a higher mTOR signaling is enough to induce sensitivity to metabolic drugs, we focused on determining the reasons for such sensitivity. mTOR is a master regulator of the cell's metabolism and it controls processes that involve proliferation and protein synthesis, energy sensing, and acts as a main inhibitor for autophagy. Autophagy, on the other hand, is essential for cells when lacking nutrients or when under metabolic stress, since it provides valuable substrates to overcome such conditions. On this basis, we developed our hypothesis that the metabolic vulnerability observed in chemoresistant cells is caused by an mTOR-dependent inhibition of autophagy, which in turn makes cells unable to cope with the metabolic stress induced by drugs such as DCA and 2DG.

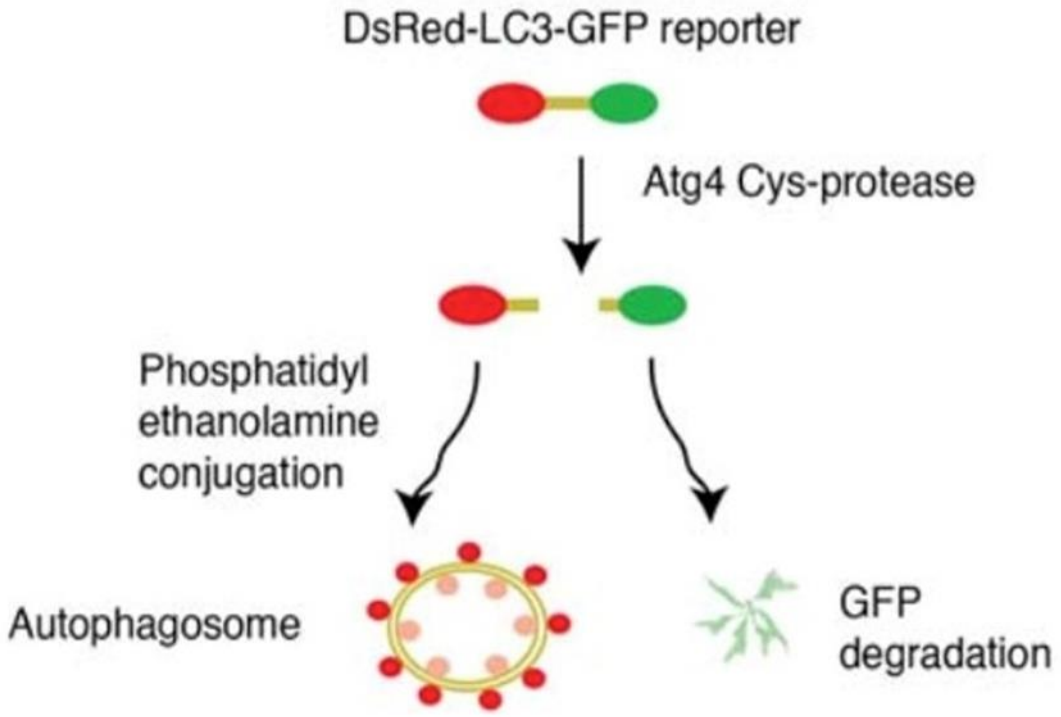


Fig. 13 The DsRed-LC3-GFP reporter
Schematic structure and functioning of the DsRed-LC3-GFP dual color reporter. Image from https://www.addgene.org/31182/

3.1.3.1. Chemoresistant cells exhibit a block of autophagy and signs of energy stress

To prove this hypothesis, members of our team Dr. Wanzel and Dr. Gremke evaluated whether autophagy is indeed blocked in chemoresistant cells. With this aim, both $H460^{WT}$ and $H460^{Res}$ cells were infected with a retrovirus containing the DsRed-LC3-GFP expression vector (pQCXI Puro DsRed-LC3-GFP, Addgene plasmid #31182). This reporter construct has LC3 linked at its N-terminus to a DsRed fluorophore, and the C-terminus to EGFP. This construct is designed to quantify the autophagic activity in the cells and can be used to mark the LC3-autophagosome/puncta in the cells, an established method to assess autophagy (Sheen et al. 2011). In baseline conditions, the fluorescence generated by the dual color DsRed-LC3-GFP reporter is homogeneously diffused through the cytosol. However, an increase in autophagy rates causes LC3 cleavage by ATG4, including the LC3 portion of the reporter. This causes the two fluorophores to separate, and while the GFP part is degraded, the DsRed accumulates with LC3

on the surface of autophagosomes, originating distinctive puncta that are possible to observe through fluorescence microscopy (Fig. 13). It is also possible to observe a general shift in the total fluorescence towards red, which can be measured by flow cytometry. $H460^{WT}$ and $H460^{Res}$ cells expressing the reporter were obtained and treated with both DCA and 2DG, and observed under the fluorescence microscope. In $H460^{WT}$ cells it was possible to observe the formation of numerous red puncta representing the autophagosomes after the treatment with both 2DG and DCA, indicating the induction of a significant autophagic response. However, in $H460^{Res}$ cells, no red puncta were visible, suggesting an autophagy block at an early stage of the pathway, with no LC3 processing (Fig. 14 a). This is in line with the action of mTOR, which inhibits autophagy by phosphorylating ULK1, the apical switch of the whole autophagic cascade.

In parallel, we wanted to know whether there were signs of energy stress in chemoresistant cells when exposed to metabolic drugs. To this end, Dr, Wanzel performed a WB and examined the phosphorylation levels of two known energy-stress markers: AMPK and ACC. The 5' AMP-activated protein kinase (AMPK) is an enzyme that plays a role in energy homeostasis, able to sense a depletion in ATP levels and influence pathways connected to glucose and fatty acids uptake and oxidation. When the intracellular ATP/AMP ratio decreases in favor of AMP, AMPK undergoes activating phosphorylation at threonine-172 (Hawley et al. 1996). One of the targets of AMPK is the acetyl-CoA carboxylase (ACC), one enzyme whose primary function is to provide substrate for the synthesis of fatty acids. When nutrients are scarce, its activity gets blocked through inhibitory phosphorylation on multiple sites including serine-79 (L. Tong 2005).

When treating $H460^{Res}$ cells with DCA/2DG it was possible to observe clear signs of energy depletion manifested with a marked increase in the phosphorylation levels of AMPK and ACC, but not in $H460^{WT}$ (Fig. 14 b). In addition, autophagic markers were detected. As expected, $H460^{Res}$ cells exhibited higher inhibitory-phosphorylation of ULK1 (S757), and these levels did not decrease after treatment with DCA/2DG, but surprisingly they increased even further. p62 and LC3 levels were also detected to estimate autophagic activity. In chemoresistant, but not in WT cells, we could see a massive accumulation of the cargo protein p62. Consistent with the notion that p62 gets degraded together with the

autophagosomes, its accumulation suggests a block in the autophagic process. The detection of LC3 also suggests a block in resistant cells: DCA/2DG provokes in H460^{WT} not only an increase in total LC3 levels but more importantly a conversion from LC3-I to LC3-II. This is what normally is observed when

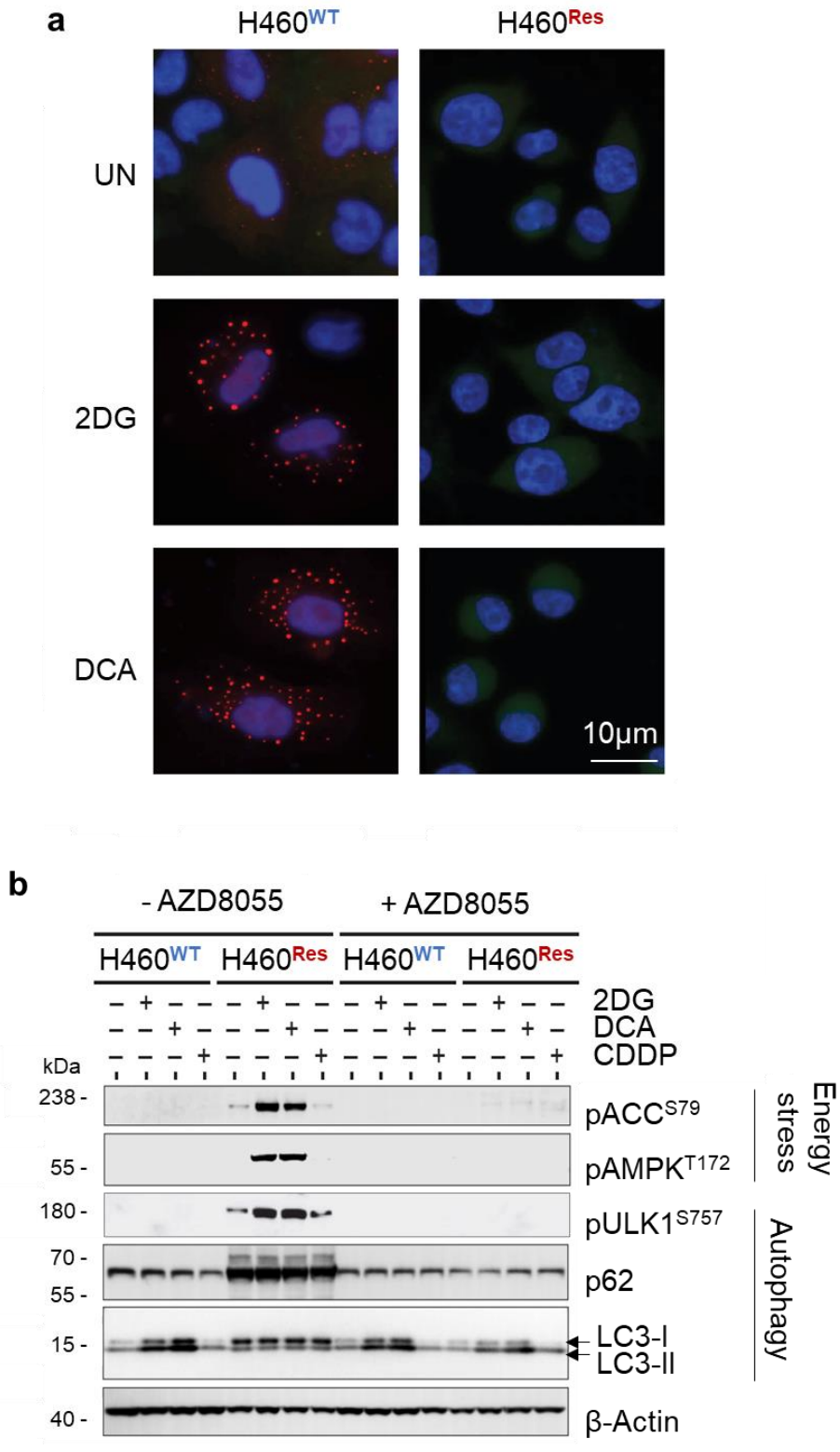


Fig. 14 Chemoresistant cells show an autophagy block, and signs of energy stress

a) Fluorescence microscopy pictures. Representative images of $H460^{WT}$ and $H460^{Res}$ cells expressing the DsRed-LC3-GFP reporter and treated for 48h with the indicated drugs. DCA= 40mM; 2DG= 10mM. Data courtesy of Dr. Gremke
b) Western blot analysis. $H460^{WT}$ and $H460^{Res}$ cells were treated with the indicated drugs for 48h. Phosphorylated forms of ACC and AMPK were taken as markers for energy stress, while ULK1, p62 and LC3 were used as autophagy makers. DCA= 40mM; 2DG= 10mM; CDDP= 1µg/ml. Data courtesy of Dr. Wanzel.

autophagy is ongoing. However, in $H460^{Res}$ LC3 accumulates in its unprocessed form (LC3-I) and the conversion to LC3-II is only minimal. Noteworthy, all these results were specific only to drugs having effects on metabolism (DCA/2DG) and not CDDP. Finally, the additional combination with the mTOR inhibitor AZD8055 reverted entirely the $H460^{Res}$ response, corroborating the hypothesis that the metabolic stress is mTOR-dependent.

In conclusion from these results, we can observe in $H460^{Res}$ a block of autophagy, and we can deduce that the block is happening at an early stage of the pathway, caused by mTOR-dependent inhibition of ULK1. Furthermore, we could observe significant energy stress levels caused by DCA and 2DG that were selective only to cells with blocked autophagy.

3.1.3.2. Suppression of autophagy causes vulnerability to DCA/2DG

After confirming that mTOR causes indeed a block of autophagy in chemoresistant cells and that these cells show signs of energy stress when exposed to DCA/2DG, we investigated whether autophagy inhibition is sufficient to cause sensitivity to metabolic drugs, giving a possible explanation for the vulnerability. To this end, the CRISPR-Cas9 system was used to target *ATG7*'s exon 2 in H460 cells to induce disrupting indel mutations and obtained single-cell clones containing either homo- ($ATG7^{-/-}$) or heterozygous ($ATG7^{+/-}$) mutations. ATG7 is one of the ATG enzymes required for the correct processing of LC3, an essential component for the progression of the autophagic pathway (Xiong 2015). We proceeded, therefore, to treat these clones with either DCA or 2DG, and performed a western blot. Complete disruption of *ATG7* exhibited signs of

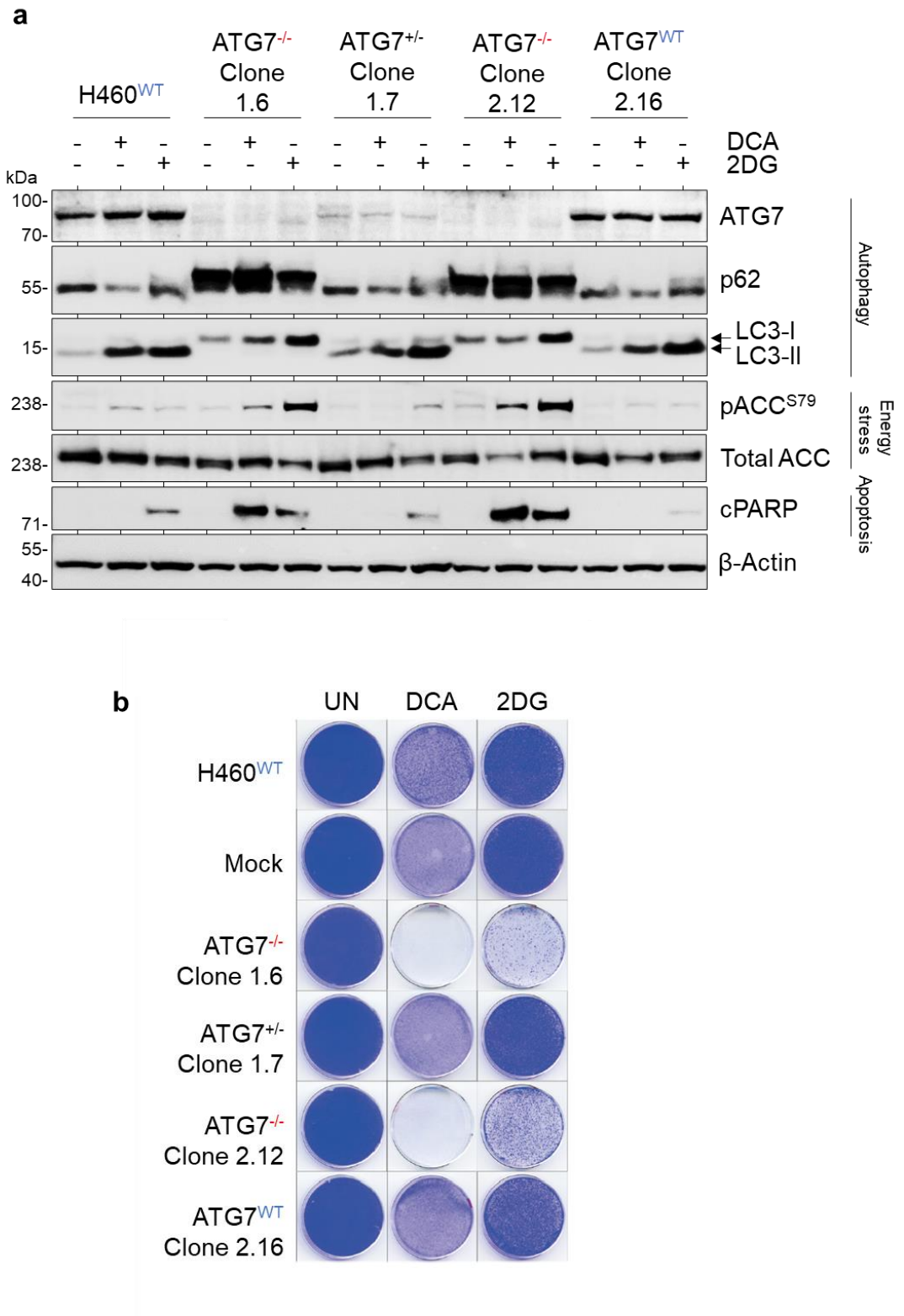


Fig. 15 Autophagy inhibition sensitizes cells to metabolic drugs

a) Western blot analysis. Cells with the indicated ATG7 status were treated with the indicated drugs for 48h.

b) Colony formation assay. Effects on clonogenic growth caused by the indicated drugs in clones with different ATG7 status. DCA= 40mM; 2DG= 10mM. Data courtesy of Dr. Wanzel.

autophagic inhibition as shown by the p62 and LC3 markers: clones lacking *ATG7* displayed a massive accumulation of p62, and while DCA/2DG treatment did lead to an accumulation of LC3 in all cells, the knockouts could not convert LC3-I into LC3-II as expected. Of note, the *ATG7*$^{+/-}$ clone, although possessing reduced levels of *ATG7*, could maintain its ability to convert LC3, and showed no obvious signs of p62 accumulation. This suggests that cells holding at least one allelic copy of *ATG7* are still autophagy-competent. In addition, we could observe signs of DCA/2DG-induced energy stress and apoptosis by means of increased pACC and cleaved PARP (cPARP) levels respectively. However, these signs were visible preferentially in the two *ATG7*$^{-/-}$ clones, but not in the WT controls, nor in the heterozygous *ATG7*$^{+/-}$, which maintained a WT-like phenotype (Fig. 15 a). Clonogenicity growth was also significantly affected. In a colony formation assay performed by Dr. Wanzel, we could see how the *ATG7*$^{-/-}$ clones demonstrate reduced proliferation when treated with metabolic drugs, unlike the other autophagy-competent cell lines. Also in this case *ATG7*$^{+/-}$ displayed WT-like behavior (Fig. 15 b).

To exclude the possibility that the observed effects were *ATG7*-specific, but rather a consequence of a general inhibition of autophagy, we targeted other key autophagic elements. We designed sgRNAs against exon 5 of *ATG14* and exon 7 of *FIP200*, performing a knockout with the CRISPR-Cas9 system. *FIP200* is found at the beginning of the pathway, belonging to the ULK1 complex regulating the initiation, while *ATG14* belongs to the *BECN1* complex that is required for the phagophore elongation. A western blot could confirm the successful knockout of both genes, and we could observe the expression status of LC3 and p62. *ATG14* and *FIP200* are not involved in the processing of LC3, therefore, unlike in the *ATG7*$^{-/-}$ control, the conversion from LC3-I to LC3-II is still possible. Nonetheless, in *ATG14* and *FIP200* KOs, we can notice a reduction in LC3-I's conversion rates when compared to the WT controls, in line with the effects that we would observe in case of an early block of the pathway. More importantly, we could also detect a strong accumulation of p62, suggesting that although LC3 conversion was still in place, the overall autophagic flux was nonetheless blocked (Fig. 16 a). A colony formation assay demonstrated also that these KO cells were equally sensitive to DCA/2DG as the *ATG7*$^{-/-}$ or even the H460^{Res} lines. Of note, the autophagy inhibition caused by the complete disruption of *ATG7*, *FIP200*, and *ATG14* did

not alter the response to CDDP (Fig. 16 b). These results associate autophagy inhibition with sensitivity to DCA/2DG, demonstrating that faulty autophagy is sufficient to cause a metabolic vulnerability that could be exploited in cancer treatment.

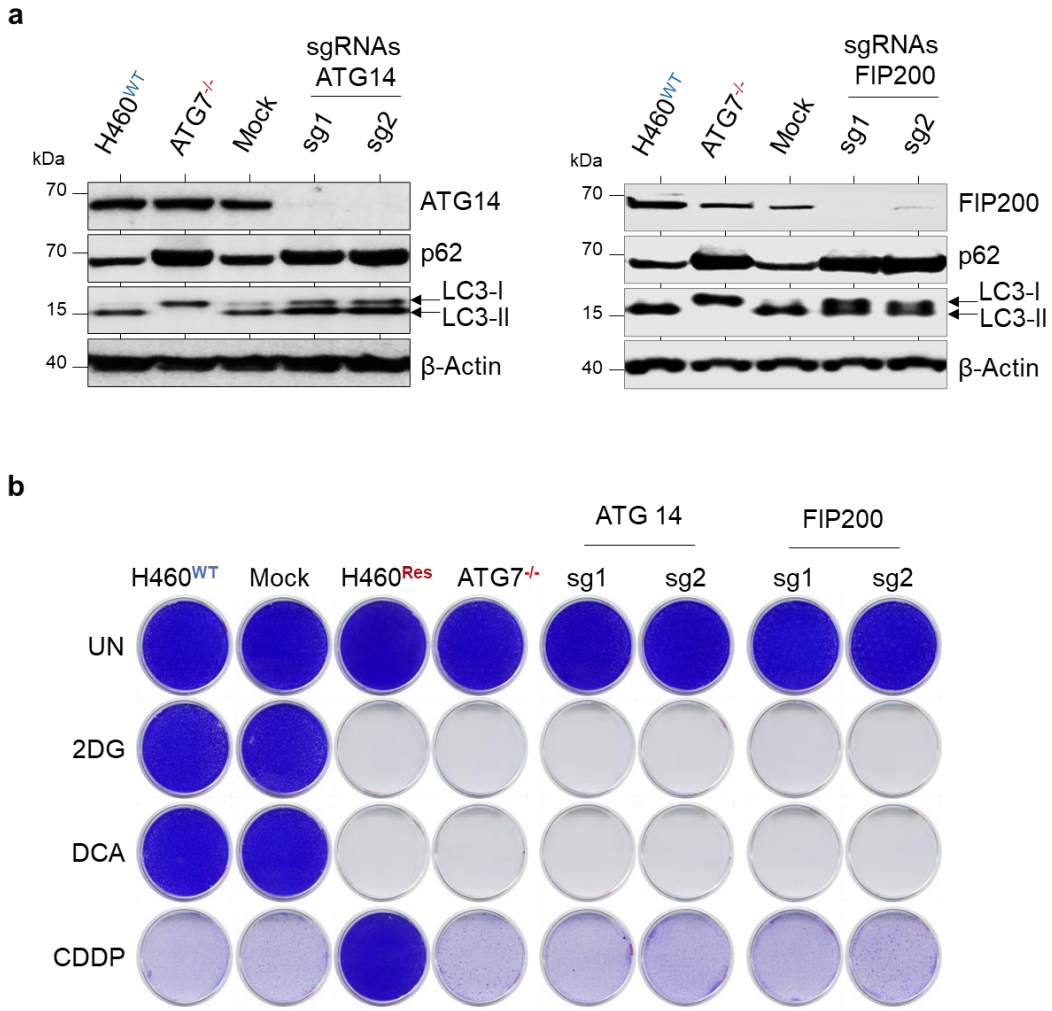


Fig. 16 Autophagy inhibition sensitizes cells to metabolic drugs

a) Westen blot analysis. Pool of cells transfected with CRISPR-Cas9 and sgRNA against ATG14 and FIP200. P62 and LC3 were used as markers to assess autophagic activity.

b) Colony formation assay. Effects on clonogenic growth caused by the indicated drugs in cells with deleted ATG14 and FIP200. DCA= 40mM; 2DG= 10mM; CDDP= 1µg/ml.

3.1.4. Other mechanisms beyond autophagy might contribute to the hypersensitivity to metabolic drugs

We demonstrated how mTOR upregulation mediates autophagy inhibition and how blocks in autophagy are sufficient to generate a metabolic vulnerability. Nonetheless, during our experiments, we repeatedly observed one detail when using the DCA/2DG drugs: $H460^{Res}$ cells' response was always much stronger and faster than the autophagy-KO lines. This suggested that chemoresistant cells possess higher susceptibility to metabolic inhibitors, possibly due to other mechanisms in addition to energy stress that contributes to their death. To test this hypothesis, we first compared the apoptosis rate displayed by $H460^{Res}$ cells and two autophagy-deficient cell lines (*ATG7*-KO and *ATG10*-KO, generation described later in paragraph 3.2.1) treated with half the usual DCA/2DG drug concentration employed in our tests in a FACS timecourse experiment. This way, we hoped to have a wider timeframe to observe the differential sensitivity to the drugs. Indeed, the $H460^{Res}$ sub-G1 population was always higher than both the $ATG7^{-/-}$ and $ATG10^{-/-}$ lines when treated with either DCA or 2DG, even showing double the percentage of apoptotic cells after 4 days of treatment (Fig. 17). This suggests that there might be other factors that contribute to $H460^{Res}$ death in addition to the sole energetic shortage.

Cancer cells possess an altered metabolism that is required to maintain their elevated proliferation rates. It is also well-known the pro-growth role that mTOR exerts in the cell, stimulating proliferation and biomass synthesis namely proteins, lipids, and, importantly, nucleotides. These last are indispensable building components not only of the DNA but also of RNA, the fundamental constituent in ribosomes (Xu, Liu, and Wei 2014; Ben-Sahra et al. 2014; Valvezan et al. 2017). Therefore, it is reasonable to hypothesize that cancer cells with upregulated mTOR signaling require particularly higher nucleic acid provision. Many tumor cells rely on the non-oxidative arm of the pentose phosphate pathway to synthesize ribose 5-phosphate that can be used for *de novo* nucleotide synthesis (X. Tong, Zhao, and Thompson 2009). Because such a pathway requires glucose 6-phosphate from glycolysis, metabolic drugs could decrease this substrate availability, therefore interfering with the nucleotide synthesis. This, together with

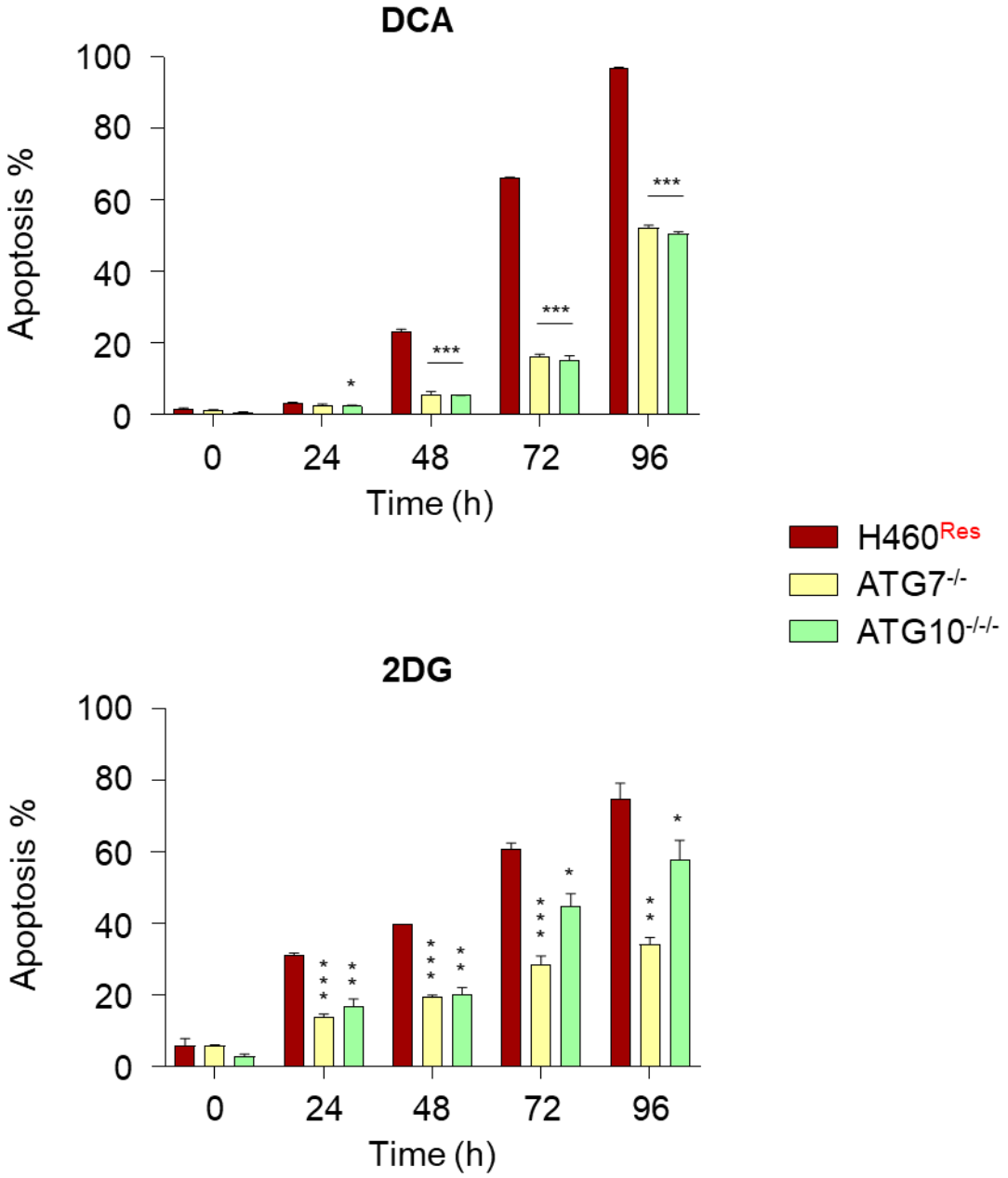


Fig. 17 Chemoresistant cells die at a higher rate than autophagy-KO cells
Percentage of apoptotic cells (sub-G1) determined by flow cytometry analysis. Cells were fixed and PI-stained every 24h interval of treatment with the indicated drugs. Bars represent mean ± SD, n = 3. Significance through two-way ANOVA with Dunnet multiple comparisons test. ***p<0.001, **p<0.01, *p<0.05 compared to $H460^{Res}$ ctrl. DCA= 40mM; 2DG= 10mM,

impaired substrate recycling caused by autophagy inhibition might lead to DNA replication stress and, ultimately, DNA damage (Valvezan et al. 2017; J. Y. Guo et al. 2016; Austin et al. 2012).

To test this theory, firstly we controlled if there were any signs of DNA damage in chemoresistant cells when treated with DCA/2DG. We made use of the

phosphorylated form of histone H2AX (S139), known as γH2AX. γH2AX is a widely known and used marker of DNA damage. Specifically, it is an early response to DNA double-strand breaks (DSB) thought to loosen the chromatin structure in proximity to the lesion and to function as a docking station for repairing factors (Mah, El-Osta, and Karagiannis 2010). Western blotting analysis revealed that phosphorylation of histone H2AX was dramatically increased in $H460^{Res}$ after treatment with either DCA or 2DG, but not in WT cells (Fig. 18 a). Discrete clusters of such histone modifications, the so-called DNA damage foci, can be detected through microscopy imaging after immunofluorescent staining (Rothkamm et al. 2015). We performed, therefore, an immunofluorescence assay and observed whether there was the formation of such clusters. CDDP was used as a positive control as it is known to cause DNA crosslinking and induce an increase of γH2AX (Olive and Banáth 2009). As expected, foci were detected in $H460^{WT}$ cells by treatment with CDDP, but not in $H460^{Res}$ thanks to their higher DNA-repairing capacity. On the contrary, treatment with DCA/2DG did not cause DNA damage in WT cells. However, in $H460^{Res}$ despite the greater DNA-repair capabilities, there was a clear formation of γH2AX foci (Fig. 18 b). These results confirmed the presence of DNA damage caused by metabolic drugs in $H460^{Res}$ by means of increased γH2AX levels and foci formation.

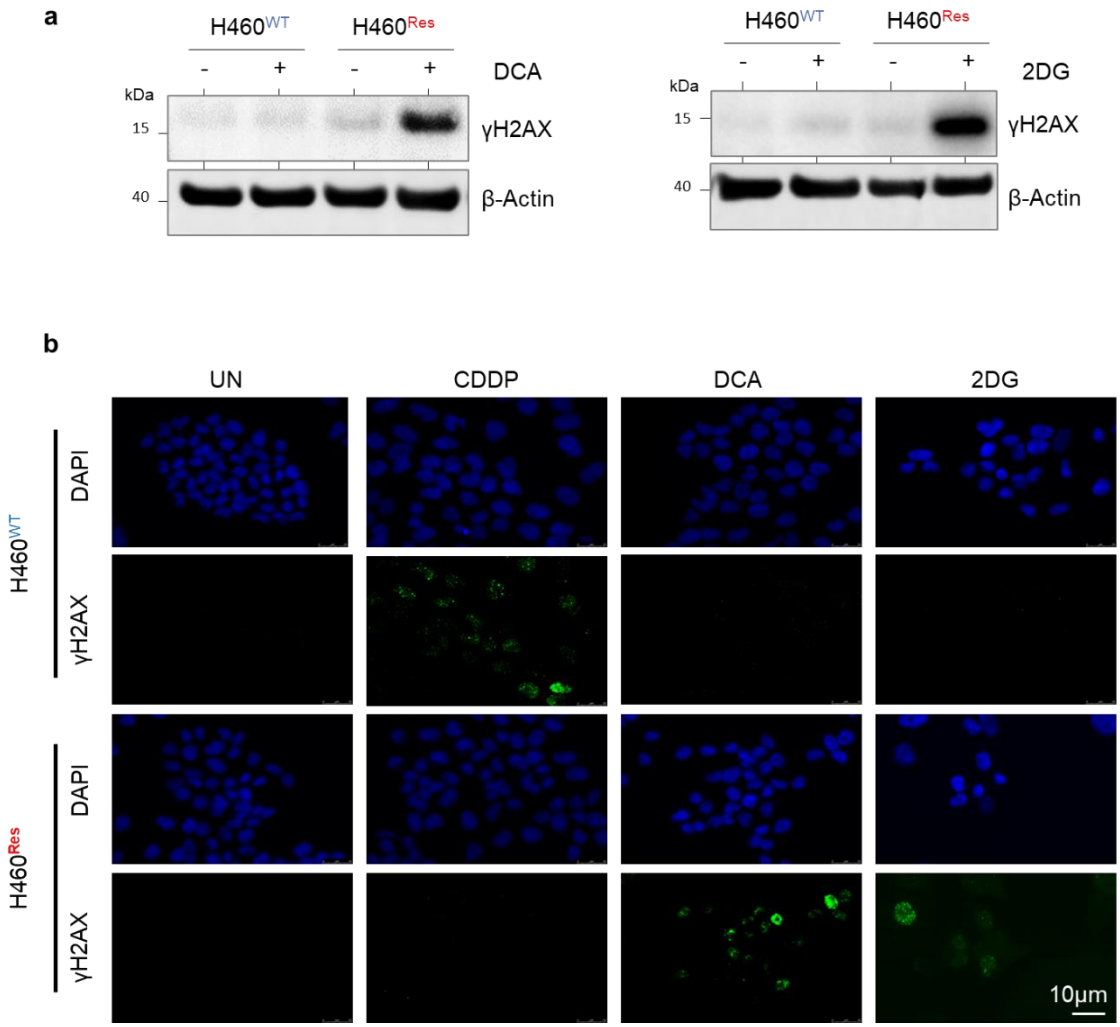


Fig. 18 Chemoresistant cells display DNA damage upon treatment with DCA/2DG

a) Western blot analysis. $H460^{WT}$ and $H460^{Res}$ cells were treated with either DCA or 2DG, and γH2AX was detected as a marker of DNA ds breaks. DCA= 40mM; 2DG= 10mM; CDDP= 1μg/ml.

b) Immunofluorescence microscopy. Representative images from $H460^{WT}$ and $H460^{Res}$ cells treated with either CDDP, DCA or 2DG. γH2AX foci were detected as marker of DNA ds breaks. DCA= 40mM; 2DG= 10mM; CDDP= 1μg/ml.

3.2. SCNAs affecting autophagic genes

We showed how altered regulatory pathways or the complete disruption of single autophagy genes is enough to stop the autophagic process and to make cancer cells hypersensitive to metabolic drugs. Nonetheless, it was our interest to gain deeper insight into whether other types of autophagy defects could be exploited against cancer. Although in cancer autophagy genes are rarely hit by disrupting mutations such as indels or single-nucleotide variants, a haploinsufficiency networks study has reported that they are highly prone to somatic copy number alterations (SCNAs) (Joe Ryan Delaney et al. 2017). Such alterations include allelic deletions or duplications. However, in the vast majority of cases, they are reported to be monoallelic deletions. Such monoallelic deletions can affect multiple ATG genes at the same time, with *BECN1, MAP1LC3B* (LC3), and *ATG10* being some of the most affected. Because allelic deletions can have repercussions on mRNA levels of the hit genes (Read 2017), it has been speculated that concurrent monoallelic deletions of core autophagic genes might lead to the choking of the autophagy flux. This in turn would reduce the ability of cancer cells to cope with the effects of autophagy-stressing drugs (Joe Ryan Delaney et al. 2017). Such a vulnerability could be exploited by the use of metabolic drugs, and the SCNAs used as a biomarker when evaluating therapeutic options for patients. However, formal proof of concept for such hypothesis was yet to be provided. To this end, we used the CRISPR-Cas9 gene-editing tool to target the key autophagy genes *BECN1, LC3*, and *ATG10*. We created an isogenic cell line model harboring an accumulation of heterozygous deletions on these genes simultaneously. Subsequently, we evaluated the effects of these alterations in response to treatment with metabolic inhibitors.

3.2.1. Generation of the allelic deletions and clone screening

For the generation of the ATG-deletions, we chose the H460 cell line, based on prior findings on the use of metabolic drugs. The H460 cell line is autophagy-competent and increased autophagic activity is observed after treatment with the drugs DCA and 2DG. Additionally, it was observed that the KO of *ATG7* provokes a block of the autophagic flux and induces an energy stress response, together with increased apoptosis (Fig. 14) (Gremke et al. 2020). This suggests that these cells are dependent on autophagy to maintain their metabolic equilibrium

whenever this is altered by inhibition of aerobic glycolysis. Furthermore, they possess a hypotriploid karyotype, with 3 copies of the *BECN1* and *ATG10* genes, and 2 copies of *LC3* and *ATG7* (J. Liu et al. 2004; Jeong et al. 2018). Because of these features, the H460 is an excellent model for studying how multiple copy number losses of autophagy genes affect the response to pressure caused by metabolic inhibitors.

The deletion of one or more gene copies was achieved by using the CRISPR-Cas9 technology, designing sgRNA-couples targeting intron sequences at the very beginning (5') and end (3') of the gene of interest (Fig. 19). We targeted

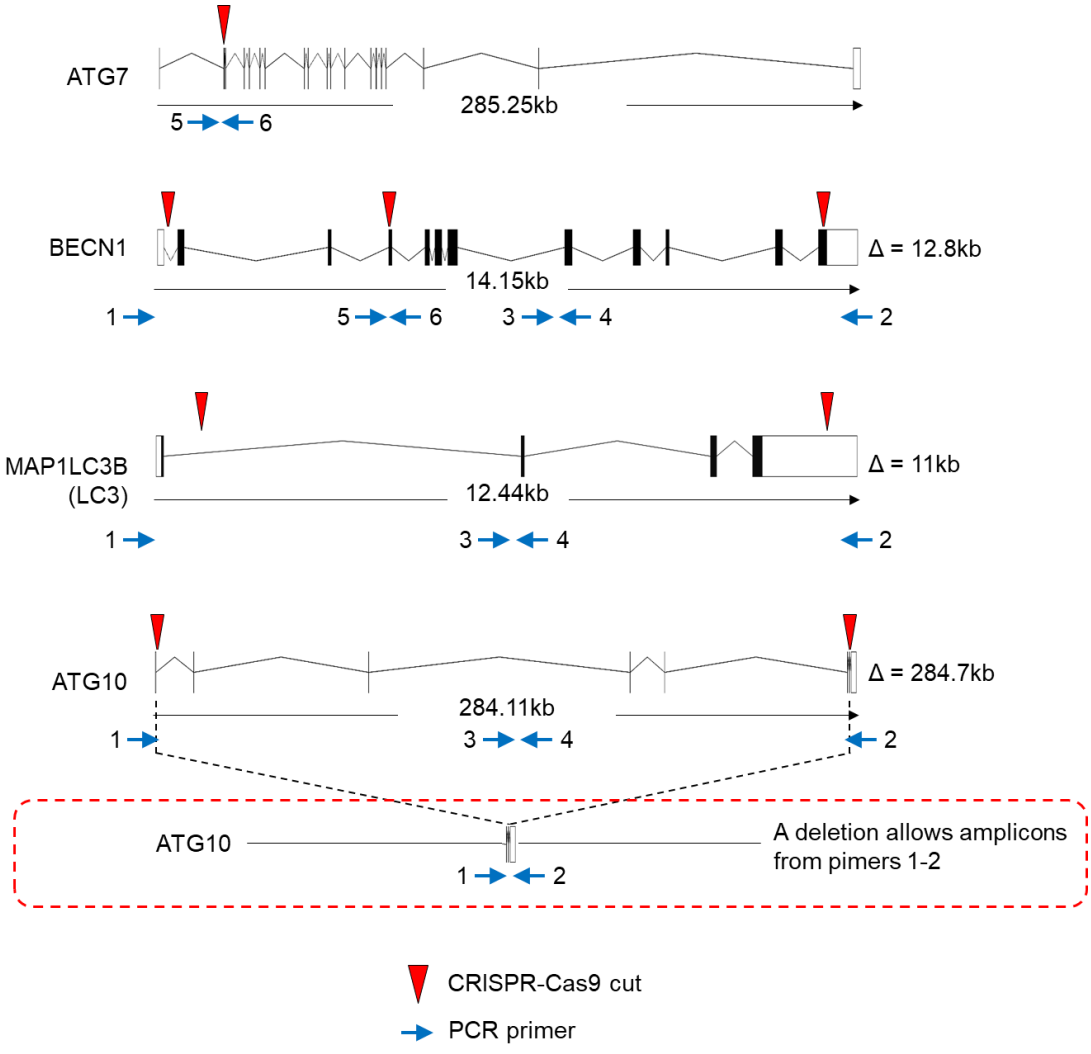


Fig. 19 Design of sgRNA and PCR primers
Schematic representation of the ATG7, BECN1, MAP1LC3B, and ATG10 gene loci. In red triangles are indicated the CRISPR-Cas9 nucleases cutting sites. Δ = deletion size. In blue arrows the primers used for PCR analysis: primers 1-2 originate one amplicon only in the presence of an allelic deletion; primers 3-4 amplify one internal fragment in non-deleted alleles; primers 5-6 amplify cutting sites on exons.

introns because we were interested to obtain large deletions rather than disrupting indels in exons. After assembling the plasmids containing sgRNA-Cas9, H460 cells were transfected, and after antibiotic selection, single-cell clones were generated and screened through PCR. For the screening, PCR primers (pair 1-2, see Fig. 19) were designed to amplify regions spanning the entire length of the genes (>10kbp), including both the Cas9 cutting sites. Because of the great distance between primer 1 and 2, no amplicon can originate using standard PCR settings, usually able to amplify sequences up to 1kbp in size. However, a PCR product can be obtained in presence of a deletion. PCR screening using primer 1-2 could discern clones with at least one allelic loss, but a second primer couple (3-4) internal to the gene was used to understand whether such clones had at least one remaining allele (Fig. 20 a). For *BECN1* and *ATG10*, possessing three allelic copies, Sanger sequencing was performed to discriminate between a +/+/- and +/-/- status. PCR products from 1-2 primers spanning the fusion point were sequenced, and the detection of two different fusion sequences indicated a clone with two allelic deletions (+/-/-) (Fig. 20 b). For *BECN1* it was not possible to obtain a $BECN1^{-/-/-}$ clone by inducing large deletions of all three alleles, therefore, $BECN1^{+/-/-}$ cells were re-transfected with Cas-9-sgRNA targeting exon 4. Re-cloning followed by screening-sequencing of the exon 4 (primers 5-6) allowed us to identify two clones with frameshift mutations ($BECN1^{fs/-/-}$) which were complete knockouts (KO) (Fig. 20 c). Using the previously described strategy, we could isolate $BECN1^{+/+/-}$, $BECN1^{+/-/-}$ (named “B”), $BECN1^{fs/-/-}$ ($BECN1^{KO}$), $ATG10^{+/+/-}$, $ATG10^{+/-/-}$ (named “A”), $ATG10^{-/-/-}$ ($ATG10^{KO}$), $LC3^{+/-}$ (named “L”), and $LC3^{-/-}$ ($LC3^{KO}$) clones from H460 cells.

Finally, the generation of cell lines with the accumulation of multiple allelic deletions was obtained in the following way: starting from the $LC3^{+/-}$ cells, they were transfected using the beforementioned sgRNA pairs against *BECN1* first, screened, and $BECN1^{+/-/-}$ cells were selected. Clones with the $LC3^{+/-}$; $BECN1^{+/-/-}$ status were named “LB”. Afterward, starting from LB cells, the same process was repeated using sgRNA pairs against *ATG10*. The obtained clones with the $LC3^{+/-}$; $BECN1^{+/-/-}$; $ATG10^{+/-/-}$ genotype were named “LBA” (Fig. 21 a, b). $ATG7^{+/-}$ and $ATG7^{-/-}$ cell lines were previously created and used as control references (Gremke et al. 2020).

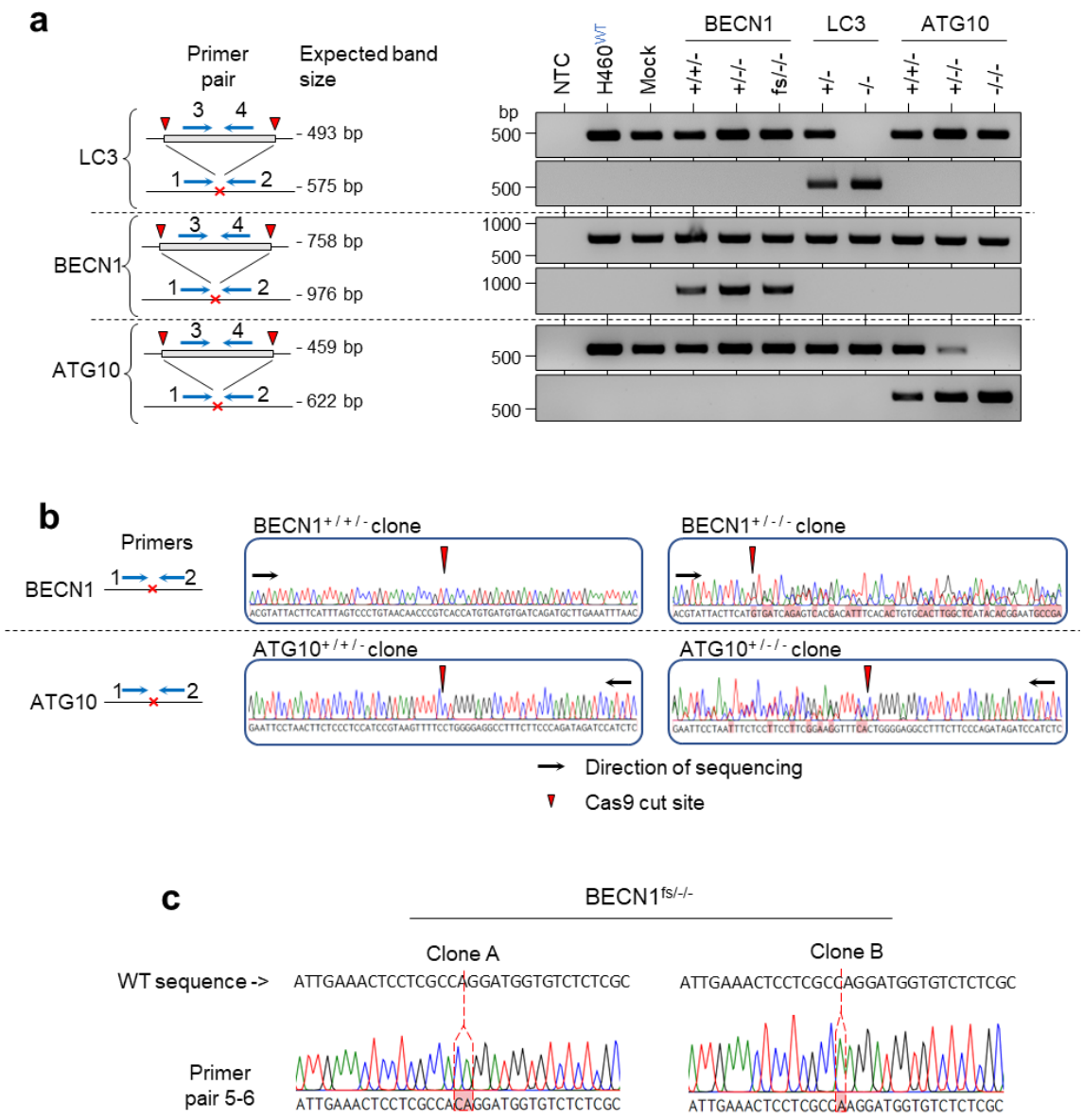


Fig. 20 Clones genotyping

a) Clones genotype was validated through PCR. For each gene (LC3, BECN1 and ATG10), primers 1-2 were used to detect the presence of at least one deleted allele, and primers 3-4 to detect any non-deleted allele remaining. NTC= No Template control; Mock= control clone from cells transfected with non-targeting nuclease. PCR kindly performed by Dr. Gremke.

b) Sanger sequencing of PCR amplicons from primers 1-2 in respectively BECN1$^{+/+/-}$, BECN1$^{+/-/-}$, ATG10$^{+/+/-}$, and ATG10$^{+/-/-}$ clones. Chromatograms from the deleted gene's fusion point validated the loss of either one or two alleles.

c) Sanger sequencing of PCR amplicon from primers 5-6 in two BECN1$^{+/-/-}$-exon-targeted clones. Sequencing validated the presence of frameshift mutations (BECN1$^{fs/-/-}$) in the remaining BECN1 allele.

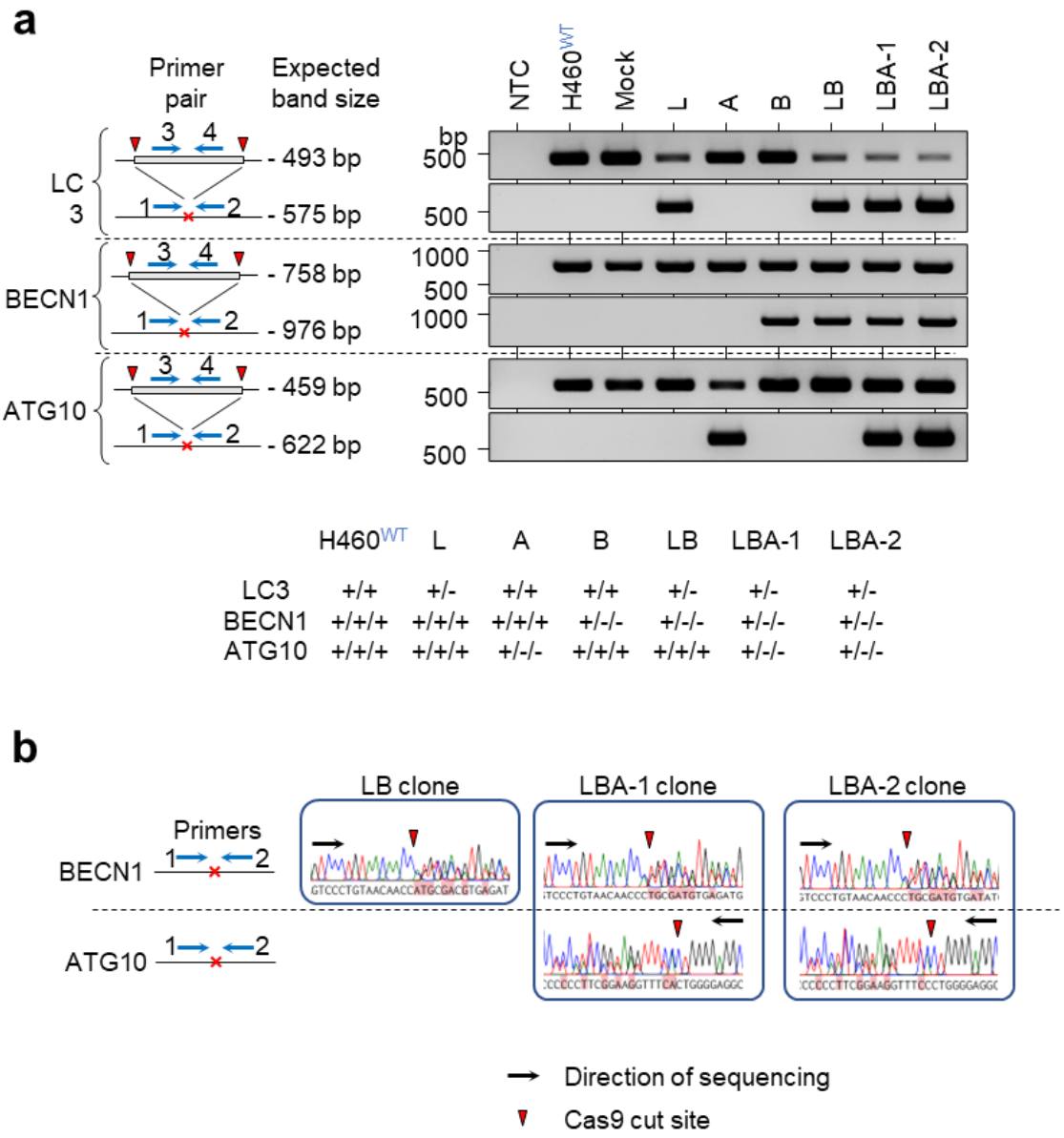


Fig. 21 Clones genotyping

a) Clones genotype was validated through PCR. For each gene (LC3, BECN1 and ATG10), primers 1-2 were used to detect the presence of at least one deleted allele, and primers 3-4 to detect any non-deleted allele remaining. NTC= No Template control; Mock= control clone from cells transfected with non-targeting nuclease. Under the electrophoresis picture, a short nomenclature for the clones with different ATG KOs. PCR kindly performed by Dr. Gremke.

b) Sanger sequencing of PCR amplicon from primers 1-2 in respectively LB, LBA-1 and LBA-2 clones. Chromatograms from the deleted gene's fusion point validated the loss of either one or two alleles.

3.2.2. Knockout of *LC3*, *BECN1*, or *ATG10* sensitizes cells to aerobic glycolysis' inhibitors

Our first interest was to assess whether the complete KO of either *BECN1, LC3*, or *ATG10* would have the same impact observed with the KO of other key autophagy genes such as *ATG7, ATG14*, or *FIP200*. In a western blot kindly performed by Dr. Wanzel, we could see the absence of the targeted proteins in the correspondent knockout clones. In addition, we could observe a missed conversion of LC3-I into LC3-II in the $ATG10^{KO}$ clone, similar to what we could see in $ATG7^{KO}$ since both enzymes are involved in the processing of LC3. As expected, in *BECN1* knockouts the LC3 conversion was possible, but we could detect an accumulation of LC3-II. We could also see that the autophagy marker p62 accumulated to varying extents in all of the knockout clones (Fig. 22 a). All of these findings confirmed the successful knockout and, most importantly, they suggested a probable block, or at least considerable reduction, of the autophagic activity in these lines. Therefore, we continued and checked whether such a block would translate into susceptibility to metabolic inhibitors. We assessed the knockouts' clonogenic growth when exposed to anti-Warburg drugs in a colony formation assay, and we saw that they suffered significantly from the treatment with both DCA and 2DG to an extent comparable to the autophagy-deficient $ATG7^{KO}$ control (Fig. 22 b). Furthermore, we monitored their growth through live-cell imaging. While none of the KOs displayed any dramatic decrease in proliferation in untreated conditions, their proliferation was severely slowed down when exposed to metabolic drugs, as evaluated by the area under the curve (AUC) analysis. While in the WT control, the proliferation decreased only moderately (-20-30%) under treatment with DCA/2DG, all KOs exhibited a substantial reduction (-70-90%) (Fig. 22 c, d, e). Therefore, we could confirm that the targeted genes *BECN1, LC3*, and *ATG10* are important in maintaining the metabolic equilibrium similar to *ATG7*, *ATG14*, or *FIP200* since their complete deletion provokes sensitivity to glycolysis inhibitors.

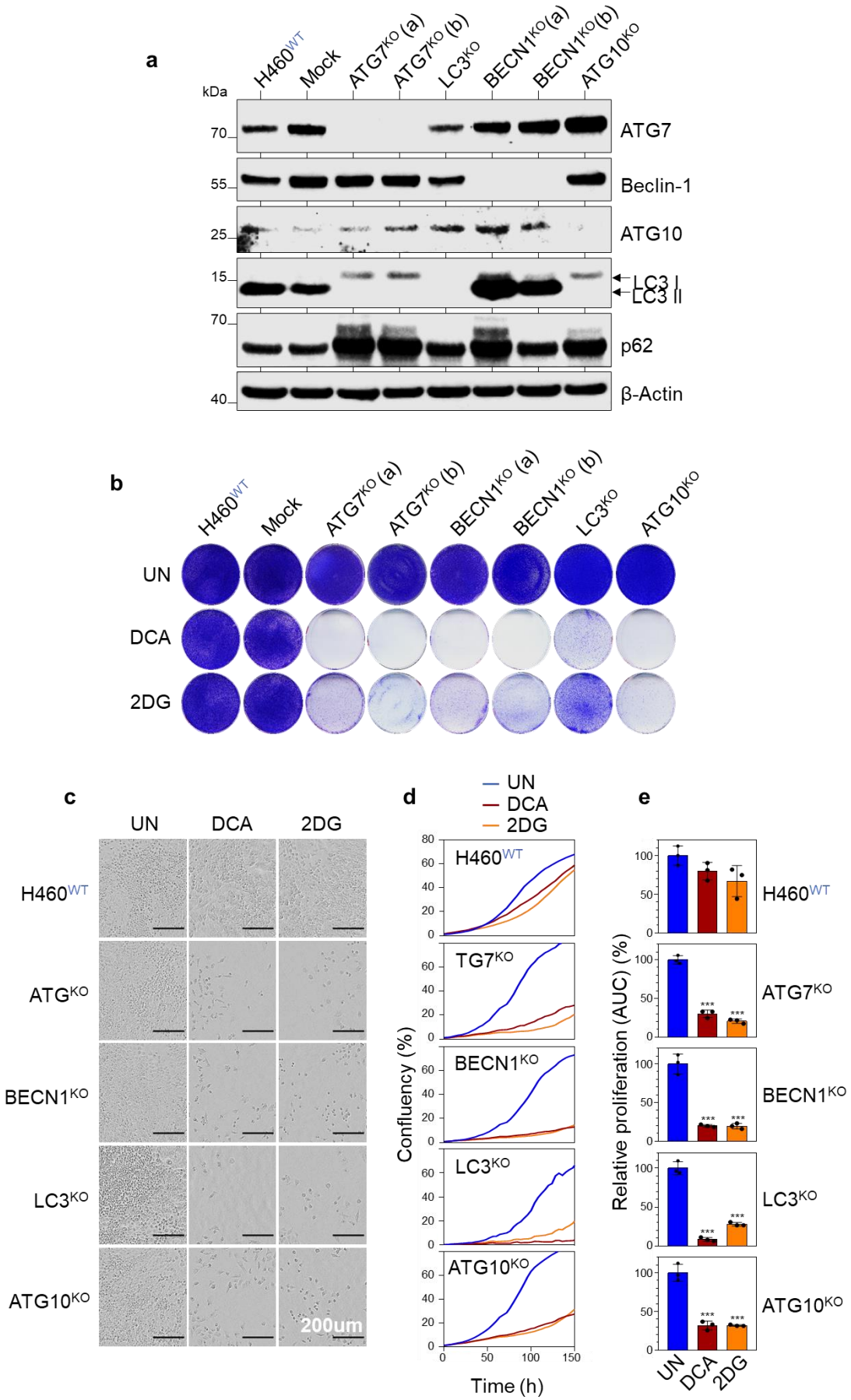

a
H460WT
Mock
ATG7KO (a)
ATG7KO (b)
LC3KO
BECN1KO(a)
BECN1KO(b)
ATG10KO
kDa
70
55
25
15
70
40
ATG7
Beclin-1
ATG10
LC3 I
LC3 II
p62
β-Actin
b
H460WT
Mock
ATG7KO (a)
ATG7KO (b)
BECN1KO (a)
BECN1KO (b)
LC3KO
ATG10KO
UN
DCA
2DG
c
UN
DCA
2DG
H460WT
ATGKO
BECN1KO
LC3KO
ATG10KO
200um
d
UN
DCA
2DG
H460WT
TG7KO
BECN1KO
LC3KO
ATG10KO
Confluency (%)
Time (h)
e
Relative proliferation (AUC) (%)
H460WT
ATG7KO
BECN1KO
LC3KO
ATG10KO
UN
DCA
2DG

Fig. 22 KO of BECN1, LC3 or ATG10 sensitizes cells to DCA/2DG

a) Western blot analysis. The indicated autophagy markers were detected in the knockout clones. p62 and LC3 were used as markers to assess autophagic activity. Kindly performed by Dr. Wanzel.
b) Colony formation assay. Effects on clonogenic growth caused by the indicated drugs in cells with the indicated gene KO.
c) Live cell imaging. Representative images of cell proliferation taken at day = 7. Scale bars = 200 µm.
d) Proliferation curves from live-cell imaging, reported as cell % confluency over time.
e) Proliferation reported as area under the curve (AUC) values from (d) normalized to the untreated. Bars represent mean ± SD, n = 3. Significance through one-way ANOVA with Dunnet multiple comparisons test. ***p<0.001 compared to WT ctrl.

DCA= 40mM; 2DG= 10mM.

3.2.3. Cells with heterozygous deletion of one single autophagy gene retain their ability to proliferate under metabolic drug treatment

After assessing the phenotype of complete KOs, we evaluated whether the partial loss of allele copies affecting one single autophagy gene would have the same impact. Therefore, we compared the CRISPR-Cas9-engineered *BECN1*$^{+/+/-}$, *BECN1*$^{+/-/-}$, *LC3*$^{+/-}$, *ATG10*$^{+/+/-}$ and *ATG10*$^{+/-/-}$ clones with their correspondent KO and WT counterpart. We analyzed in a western blot kindly performed by Dr. Wanzel if the protein levels expressed in such clones were directly proportional to their relative allele number. Interestingly, the heterozygously-deleted genes showed only a slight decrease in the correspondent protein level, and we observed their absence only in KO cells (Fig. 23 a). This suggests that even in the case of only one allele copy remaining, this is sufficient to generate enough protein to maintain levels similar to the wild-type. Consistently with these notions, in *BECN1*$^{+/+/-}$, *BECN1*$^{+/-/-}$, *LC3*$^{+/-}$, *ATG10*$^{+/+/-}$ and *ATG10*$^{+/-/-}$ clones the LC3 marker was normally processed, in the same way the WT cells do. Furthermore, both LC3 and p62 showed little to no signs of accumulation. These results are the opposite of what we observed in complete KO clones and indicate that autophagy is still possible. We studied, then, the response of these cells to metabolic inhibitors. In a colony formation assay, we could observe that these clones maintained their clonogenic potential when exposed to DCA and 2DG, in

direct contraposition to the complete KOs (Fig. 23 b). In addition, with live-cell imaging, we could record their proliferation. All cell lines had comparable proliferation rates in untreated conditions. However, while KOs growth rates were significantly reduced when treated with DCA/2DG, clones with one remaining allele of either *ATG7, BECN1, LC3*, or *ATG10* showed no statistically significant proliferative reduction compared to WT cells (Fig. 24 a, b). All these results prove that even one single allelic copy of these genes is enough to keep WT-like protein levels, and in turn, maintain the autophagic process and withstand the metabolic stress caused by the glycolysis inhibitors DCA and 2DG.

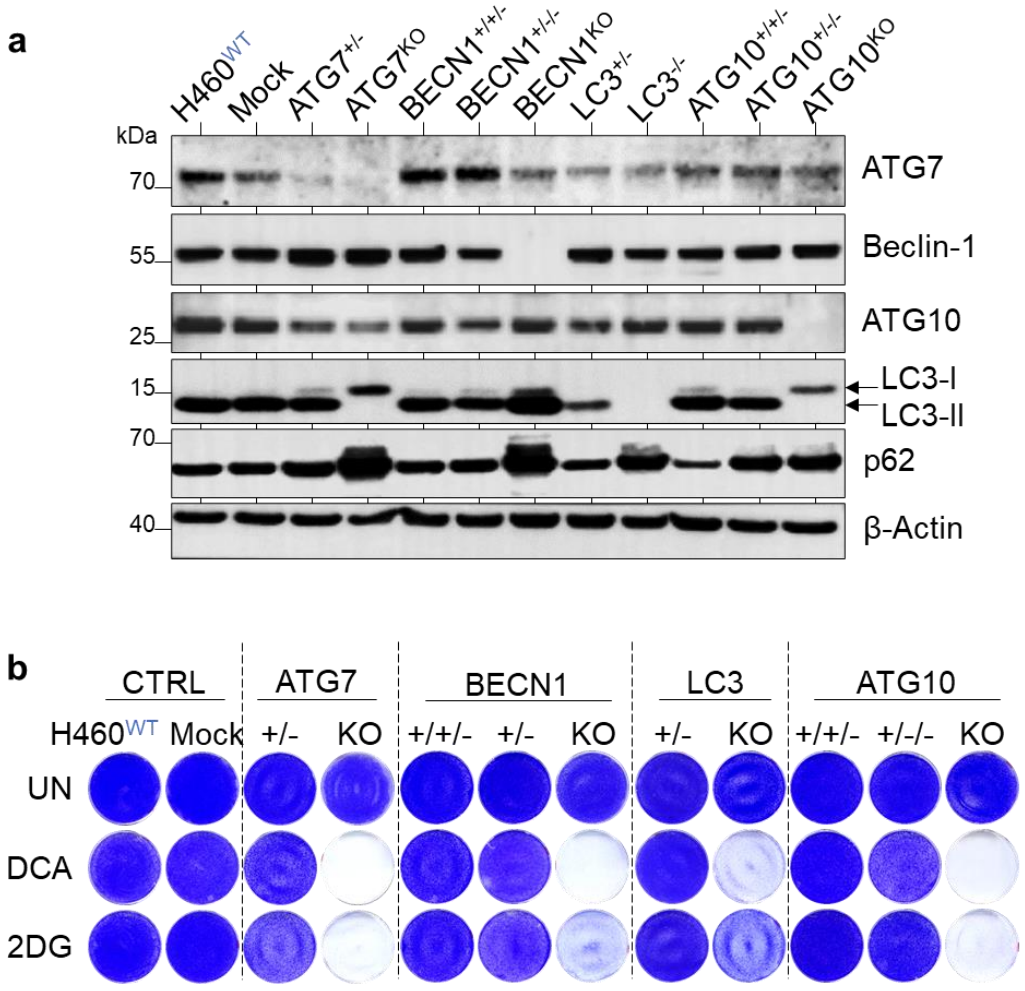


Fig. 23 Clones with non-homozygous deletion of single autophagy genes uphold wild-type-like proliferative growth when exposed to metabolic inhibitors

a) Western blot analysis. Autophagic proteins were detected in the indicated clones. p62 and LC3 were used as markers to assess autophagic activity. Kindly performed by Dr. Wanzel.

b) Colony formation assay. Effects on clonogenic growth caused by metabolic drugs in cells with non-homozygous deletion of one single gene. DCA= 40mM; 2DG= 10mM.

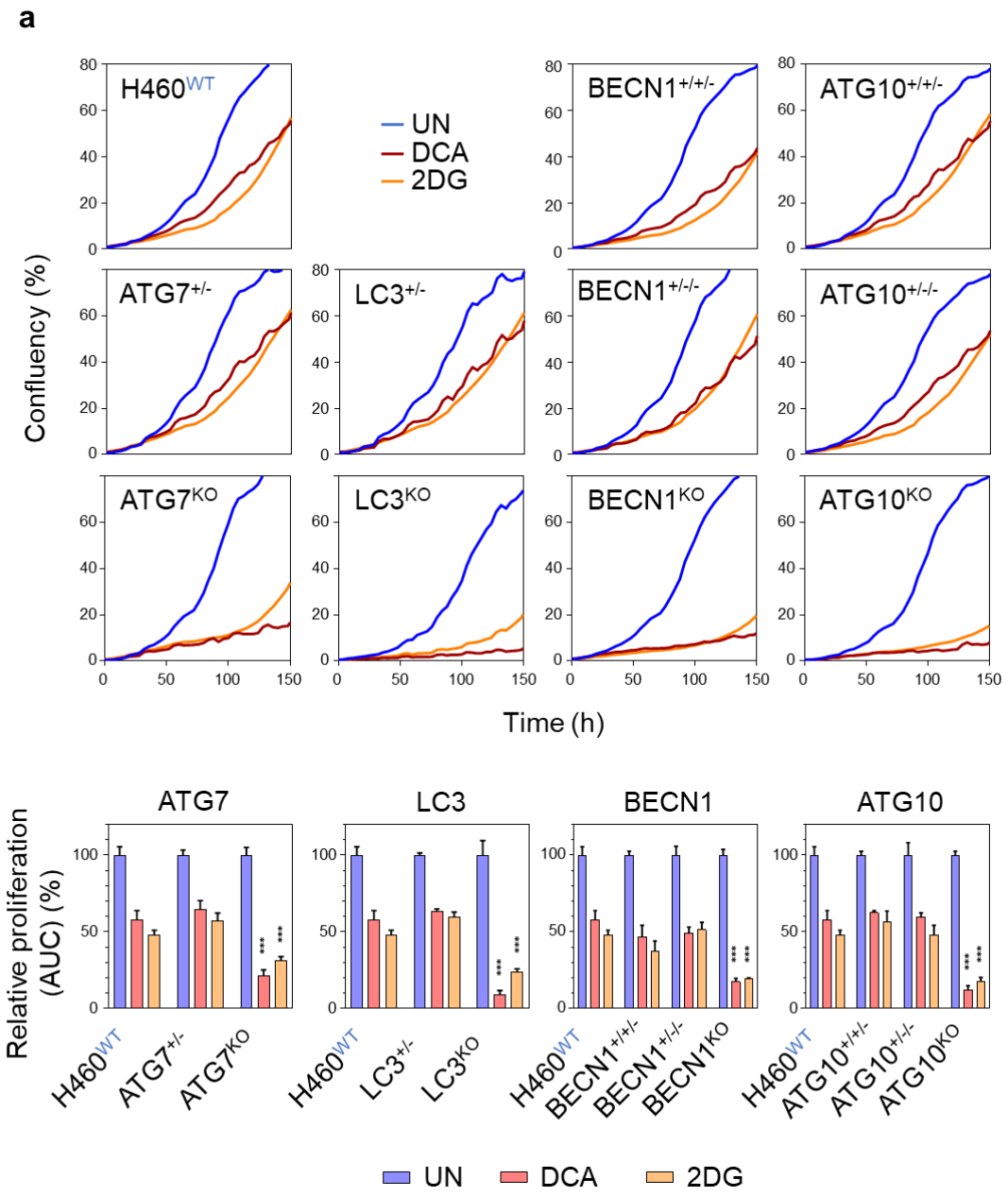


Fig. 24 Clones with non-homozygous deletion of single autophagy genes uphold wild-type-like proliferative growth when exposed to metabolic inhibitors

a) Proliferation curves from live-cell imaging, reported as cell % confluency over time. DCA= 40mM; 2DG= 10mM.

b) Proliferation reported as area under the curve (AUC) values from (a) normalized to the untreated. Mean ± SD. n =3 with two-way ANOVA analysis with Dunnett's multiple comparisons test for statistical significance. ***p<0.001 compared to WT ctrl. DCA= 40mM; 2DG= 10mM.

3.2.4. Cells with accumulation of heterozygous deletions on multiple autophagy genes, retain their ability to proliferate under metabolic drug treatment

We demonstrated that the knockout of a single autophagic gene is enough to induce a metabolic vulnerability. On the contrary, the presence of even one remaining allele does not influence cells' sensitivity to metabolic inhibitors. At this point, we proceeded to analyze whether the accumulation of non-homozygous deletions affecting multiple genes simultaneously could cooperate to produce an autophagy defect that could cause a druggable metabolic vulnerability. To this end, we utilized the LB (*LC3*$^{+/-}$*; BECN1*$^{+/-/-}$) and LBA-1/2 (*LC3*$^{+/-}$*; BECN1*$^{+/-/-}$*; ATG10*$^{+/-/-}$) clones described previously in chapter 3.2.1. (Fig 19). Once more, in a WB kindly performed by Dr. Wanzel, we evaluated if the targeting of these genes had an impact on their protein expression levels. In the LB clone, LC3 and Beclin-1 levels were modestly reduced. In the same way, also the LBA-1 and LBA-2 clones showed a modest reduction of all three proteins (LC3, Beclin-1, and ATG10). Nonetheless, in LB and LBA-1/2 cells, LC3-I could be normally processed into LC3-II, and p62 did not accumulate as seen in the *ATG7*^{KO} control (Fig. 25 a). This indicates that the autophagic process is not only still possible, but it is also not severely affected by these mutations in untreated conditions. We proceeded then to assess their response to metabolic inhibitors. We performed a clonogenic assay in which LB cells did not show any sign of sensitivity to metabolic drug treatment. Although it was possible to see a slight reduction of colony formation in the LBA-1/2 lines after treatment with DCA/2DG, the effects were vastly inferior to what we could observe in the complete *ATG7*^{KO} control (Fig. 25 b). The same results were observed using live cell imaging (performed by Dr. Wanzel, analyzed by Polo P.): AUC analysis showed that the non-homozygous deletion clones (including LB and LBA1-2) exhibited only a -10/30% further reduction to proliferation when compared to the WT control after treatment with DCA/2DG. This was way inferior to the -50% of the KO control (Fig. 26 a, b).

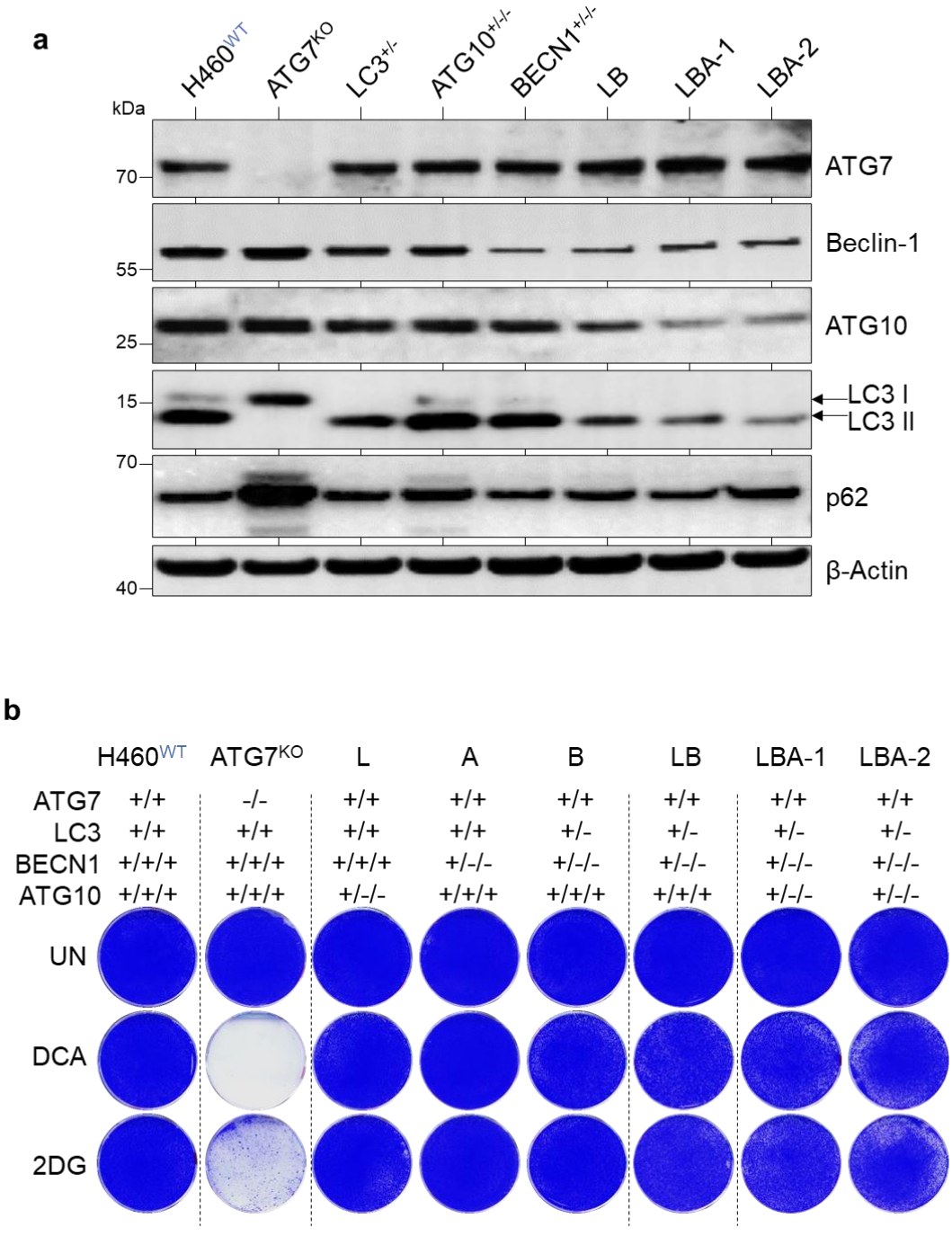


Fig. 25 Clones with accumulation of heterozygous deletions on multiple autophagy genes do not show hypersensitivity to metabolic inhibitors

a) Western blot analysis. Autophagic proteins were detected in the indicated clones. p62 and LC3 were used as markers to assess autophagic activity. Kindly performed by Dr. Wanzel.

b) Colony formation assay. Effects on clonogenic growth caused by metabolic drugs in cells with accumulation of heterozygous deletion on multiple autophagy genes. DCA= 40mM; 2DG= 10mM.

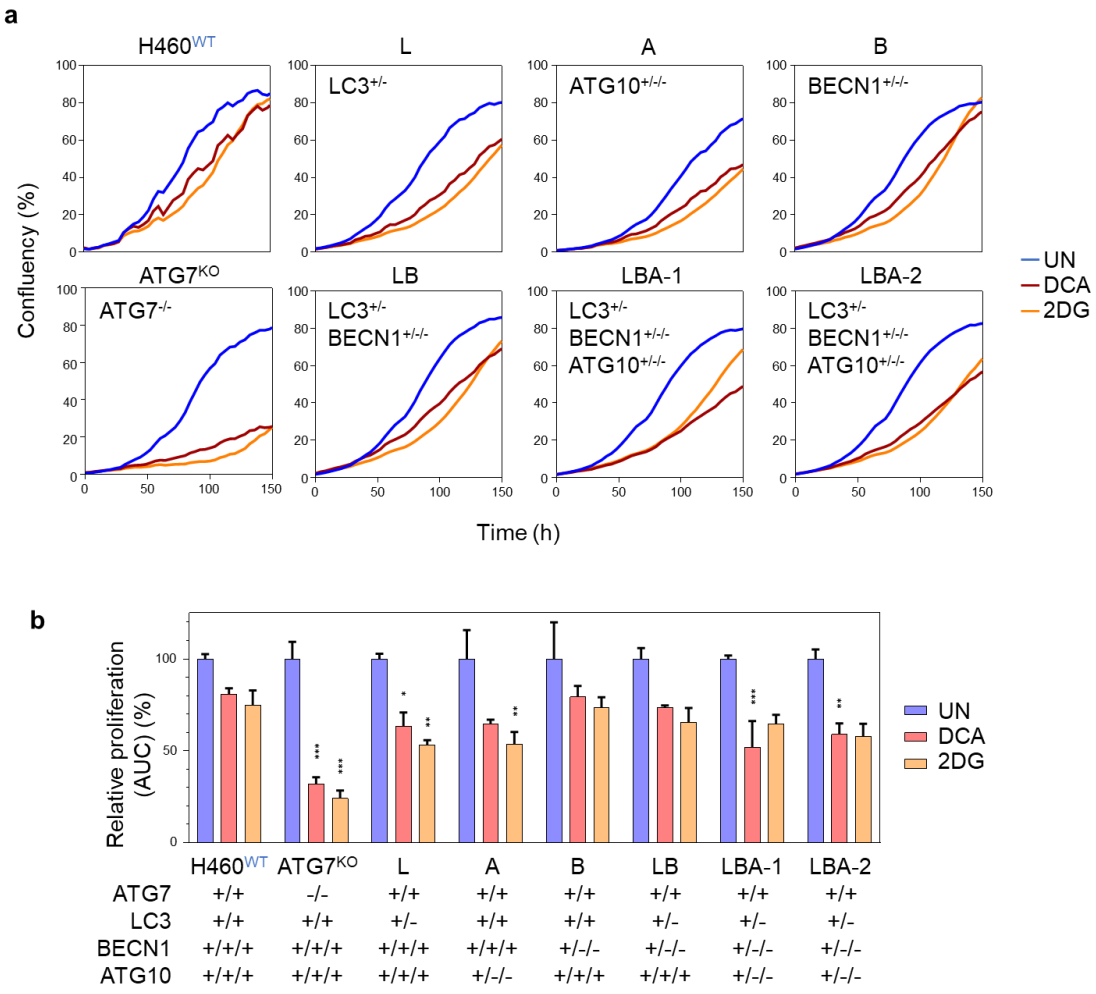


Fig. 26 Clones with accumulation of heterozygous deletions on multiple autophagy genes do not show hypersensitivity to metabolic inhibitors

a) Proliferation curves from live-cell imaging, reported as cell % confluency over time. Kindly performed by Dr. Wanzel, analysys by Polo P. DCA= 40mM; 2DG= 10mM.

b) Proliferation reported as area under the curve (AUC) values from (c) normalized to the untreated. Mean ± SD. n =3 with two-way ANOVA analysis with Dunnett's multiple comparisons test for statistical significance. ***p<0.001 compared to WT ctrl. DCA= 40mM; 2DG= 10mM.

To detect even minor changes in sensitivity that were not immediately evident with the experimental settings used up to this point, we also modified the colony formation assay setup to obtain a quantitative output. We seeded cells with a much lower clonal density, and after drug treatment, we manually counted the number of formed colonies. We could observe that DCA induced no statistically significant reduction in the number of forming colonies neither in the LB nor in the LBA-1/2 cells when compared to the WT control. On the other hand, 2DG caused a significant decrease in clonogenicity in the LB and LBA-1 clones (-60% compared to -20% of the WT), but not in the LBA-2 clone. Of note, the effect did not increase with more deletions. In addition, the reduction was only modest when compared to the $ATG7^{KO}$ control cells, which exhibited a near-to-zero number of remaining colonies after treatment with both DCA and 2DG (Fig. 27 a, b).

Finally, to verify whether some differential sensitivity could be observed at higher drug dosages, we extended the concentration range of the metabolic inhibitors and observed their effects on cells' clonogenicity once again. By increasing the concentration of DCA from 40 mM to 50 mM we obtained a dose-dependent response in all lines and we observed a somewhat relatively higher sensitivity in the multiple heterozygous lines compared to the parental WT and monoallelic-deletion control cells. At 60 mM DCA, the concentration revealed to be too elevated, even for the WT. Similar results were obtained also by increasing 2DG concentration from 20 mM to 30 mM and 40 mM. At 30-40 mM 2DG, the LB and LBA lines showed slightly more sensitivity than the WT and the $ATG7^{+/-}$ cells, but interestingly, not much more than the monoallelic $BECN1^{+/-/-}$ control line. In any case, none of the cell lines, and none of the drug concentrations tested exhibited a degree of sensitivity comparable to the homozygous KO $ATG7^{KO}$ (Fig. 28 a, b). Altogether the data show how the presence of at least one allelic copy of the targeted genes (*LC3, BECN1, ATG10*) is enough for cells to withstand the metabolic stress induced by the drugs DCA/2DG, even when non-homozygous deletions affect multiple genes simultaneously.

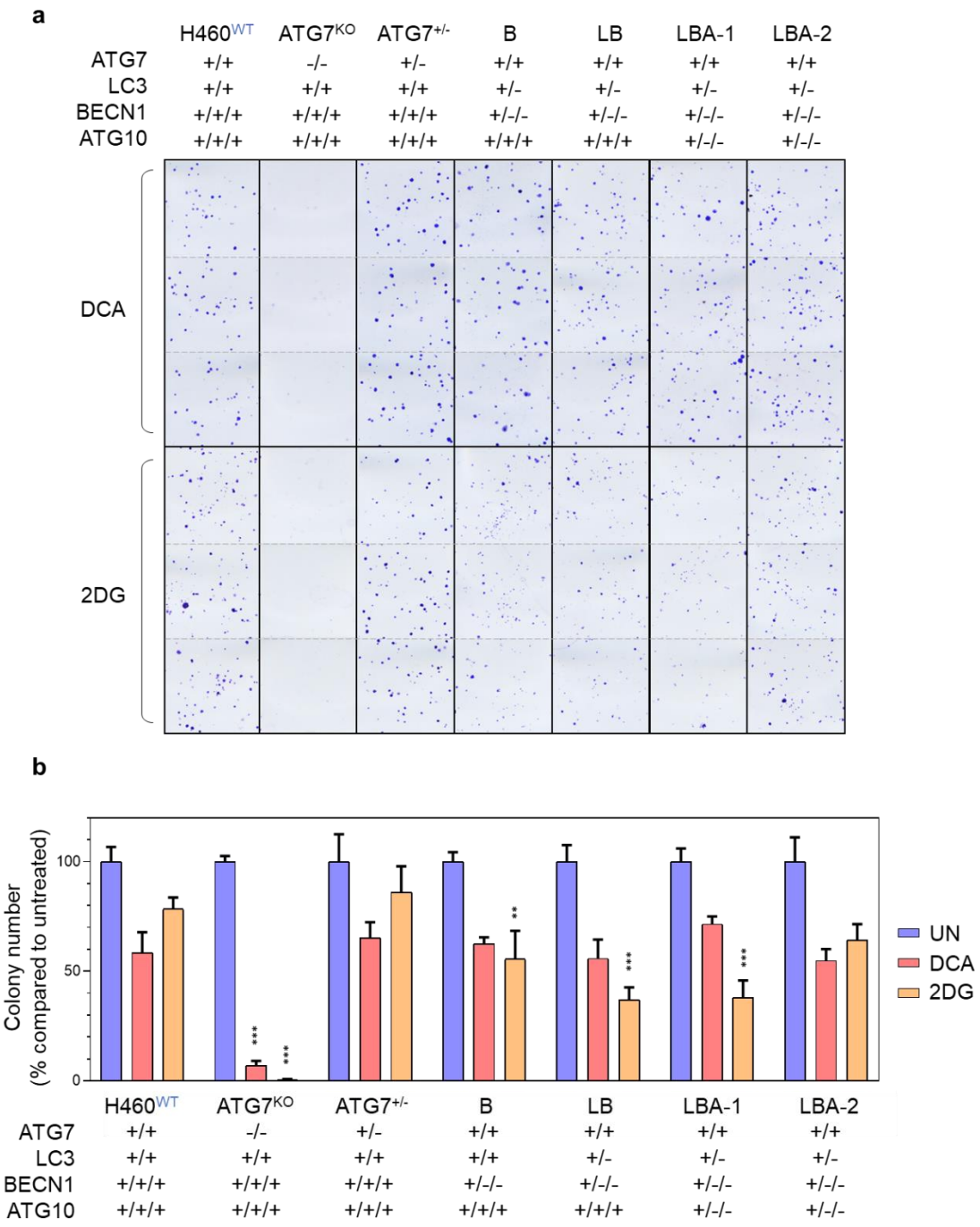


Fig. 27 Cells with accumulation of heterozygous deletions on multiple autophagy genes do not show hypersensitivity to metabolic inhibitors: quantitative clonogenic assay

a) Representative images of the quantitative clonogenic assay. DCA= 40mM; 2DG= 10mM.

b) Percentage of colony number compared to the correspondent untreated group. Mean ± SD. n =3 with two-way ANOVA analysis with Dunnett's multiple comparisons test for statistical significance. ***p<0.001 compared to WT ctrl. DCA= 40mM; 2DG= 10mM.

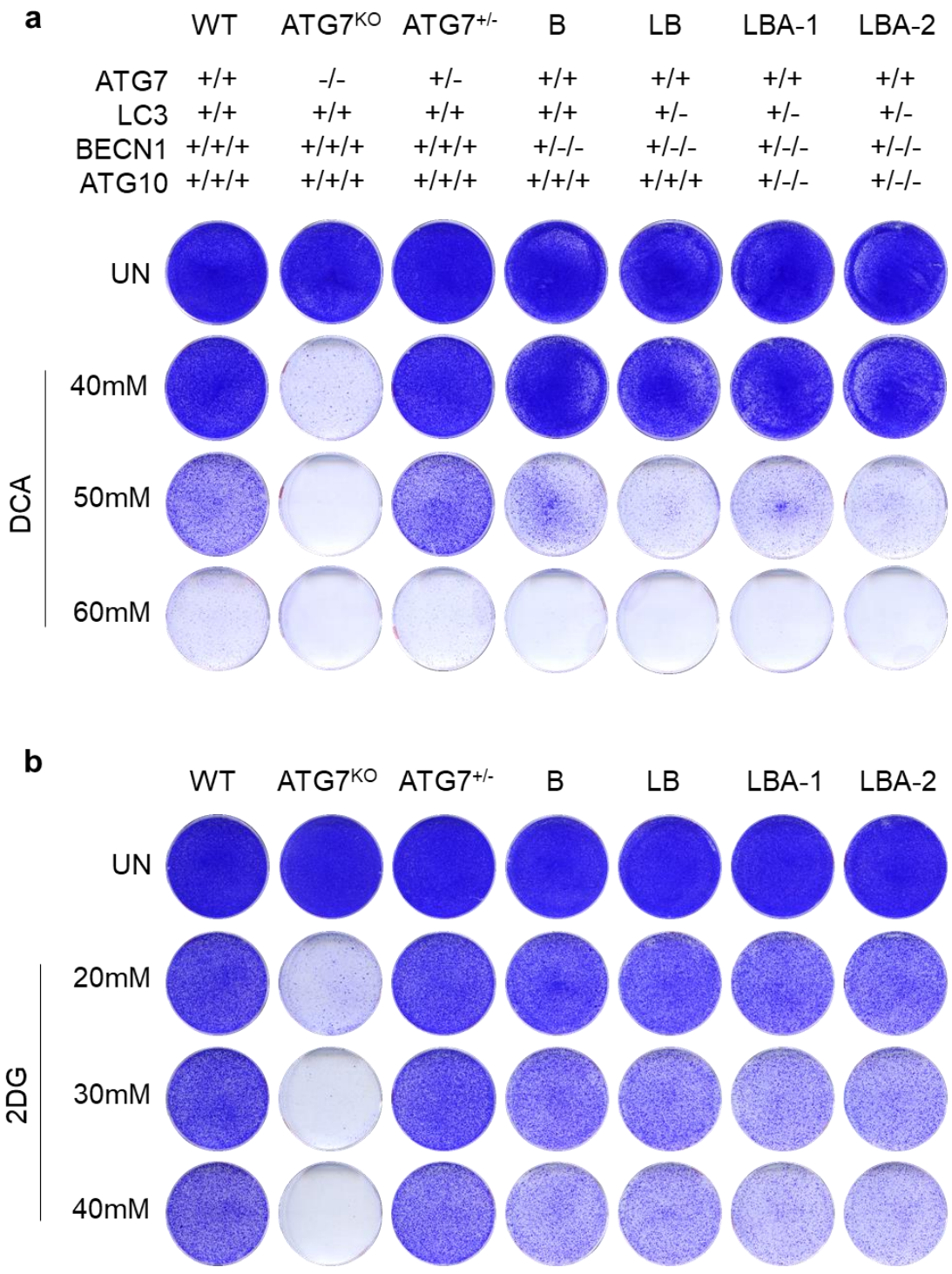


Fig. 28 Cells with accumulation of heterozygous deletions on multiple autophagy genes do not show hypersensitivity to metabolic inhibitors: clonogenic assay, extended drug concentrations

a) Clonogenic growth after treatment with three increasing concentrations of DCA.
b) Clonogenic growth after treatment with three increasing concentrations of 2DG.

4. Discussion

Since the first relevant discoveries on autophagy-related genes (ATG) in yeast in the early 1990s, autophagy has gained an increasing level of attention. The study and the findings on the topic granted Yoshinori Ohsumi the Nobel Prize in Medicine in 2016 ("The Nobel Prize in Physiology or Medicine 2016" n.d.). In the last decades, autophagy's molecular mechanisms became better understood, also thanks to the availability of several models in which the various ATG genes were manipulated, and it became evident its important role in the cell's physiology. More importantly, studies exposed the involvement of autophagy in several human pathologies such as neurodegenerative diseases, metabolic disorders, and cancer (A. M. K. Choi, Ryter, and Levine 2013). Especially for cancer research, autophagy draws considerable attention. Indeed, it is known that autophagy is a double-edged sword for what concerns cancer. On one side it acts as a tumor-preventive function, and its deregulation can be among the causes of cancer onset. On the other side, in already established tumors, autophagy acts as a survival mechanism. It sustains cancer viability in face of nutrient shortages due to the high proliferation rates and poor vascularization, and even acts as a resistance mechanism against chemotherapy treatment (Eskelinen 2011; White, Mehnert, and Chan 2015). Therefore, it can be easily understood why autophagy is increasingly often at the center of numerous translational studies and anticancer strategies, including this work. Specifically, in our case, the aim was to identify the metabolic vulnerabilities caused by autophagy defects already present in cancer and exploit them for metabolic therapy. This would open new therapeutic windows, increase treatment selectivity, and allow to contrast drug resistance phenomena that commonly arise in cancer treatment. To this end, we took into consideration two cases in which autophagy might be defective in cancer cells, and we investigated the responsiveness of such cells to metabolic drugs. In the first case, we studied a model in which chemoresistance to cisplatin is driven by an upregulation of mTOR activity, but at the same time, it causes autophagy inhibition. In the second case, we tested whether non-homozygous loss of multiple ATGs would cause a critical reduction of the proteins involved in the autophagic machinery, and consequently provoke a block of the autophagic flux.

4.1. mTOR upregulation: a double effect

mTOR is one important regulator of several processes including cell growth and survival, but also protein biosynthesis, energy sensing, and autophagy (Saxton and Sabatini 2017). In this work, we showed that in chemoresistant H460 cells ($H460^{Res}$), hyperactive mTOR signaling is responsible for chemoresistance to DNA crosslinking chemotherapeutics such as cisplatin via FANCD2 upregulation. These findings are in line with the knowledge that upregulation of the mTOR-axis can confer resistance against many anticancer drugs in several types of malignancies (Guri and Hall 2016; Easton and Houghton 2006; B.-H. Jiang and Liu 2008). For example, the PI3K/Akt axis was found to mediate the expression of the multidrug resistance-associated protein 1 membrane transporter in leukemia (Tazzari et al. 2007), while in breast cancer was found to mediate the resistance against trastuzumab (Berns et al. 2007). Similarly to our study, mTOR has also been implicated in the mechanisms of resistance to DNA-damaging drugs through regulation of the Fanconi-anemia pathway (F. Guo et al. 2013; Shen et al. 2013; Wanzel et al. 2016). In addition, an association between cisplatin resistance and an upregulation of the AKT-mTOR pathway has been already found in tumor samples from lung cancer patients (L.-Z. Liu et al. 2007). Strikingly, despite being resistant to cisplatin, our group found $H460^{Res}$ cells to be hypersensitive to metabolic/glycolysis inhibitors such as dichloroacetate (DCA) and 2-deoxy-D-Glucose (2DG). Furthermore, of the two mTOR complexes mTORC1 and mTORC2, we demonstrated that mTORC1 upregulation was not only required but sufficient to generate the beforementioned double effect.

mTOR is a well-known negative regulator of autophagy (J. Kim et al. 2011; Russell, Yuan, and Guan 2014), and we hypothesized that the reason for $H460^{Res}$ sensitivity to metabolic drugs was due to the inhibition of the autophagic pathway and the consequent loss of its cytoprotective effects against nutrient depletion. As a matter of fact, several studies suggest the modulation of autophagy as a strategy to increase cancer cell sensitivity towards a variety of metabolic inhibitors and anticancer drugs (Gąsiorkiewicz et al. 2021; N. Zhou et al. 2022; Levy et al. 2014; Rebecca et al. 2014), however, the majority of these studies focus on the chemical targeting of autophagy as a mean of combinational therapy. In our case, we sought to exploit the intrinsic autophagy inhibition caused by the elevated

mTORC1 activity. Indeed, our group found that in $H460^{Res}$ cells autophagy is inhibited in an mTOR-dependent manner via ULK1 phosphorylation.

To test whether disruption of the autophagic pathway would cause such metabolic vulnerability, we generated from $H460^{WT}$ autophagy-deficient cells with the CRISPR-Cas9 system by knocking out the *ATG7* gene. $ATG7^{KO}$ cells were sensitive to metabolic drugs and exhibited signs of energy stress. These effects were remarkably similar to those observed in $H460^{Res}$ cells which are also autophagy defective, corroborating the hypothesis that an autophagy impairment would contribute to the vulnerability to metabolic inhibitors.

Inhibition of glycolysis has been already proposed as a strategy to overcome chemoresistance mechanisms (Ganapathy-Kanniappan and Geschwind 2013; C. Liu, Jin, and Fan 2021; Wang et al. 2018), and both DCA and 2DG have been reported to enhance the effects of chemotherapeutics (Zeng, Liang, and Guan, n.d.; F. Zhang and Aft 2009; Park, Chung, and Kim 2017; Y. Liang et al. 2019). However, our findings identify a novel dimension to this topic: we identified a most peculiar role of mTOR which confers a dual nature to cancer cells. Namely, higher mTORC1 activity can confer chemoresistance, but simultaneously, hypersensitivity to glycolysis inhibitors. More importantly, we provided a mechanistic explanation for the sensitivity to metabolic drugs, identifying autophagy inhibition as the underlying cause.

4.2. Mechanisms involved in cytotoxic effects of metabolic drugs in chemoresistant cells

4.2.1. Energy stress

After establishing that the block of autophagy and consequently loss of its cell-protective effect can sensitize cells to the use of anti-Warburg drugs, we tried to identify the reasons for cell death in such cells. One of the most straightforward and probable possibilities is that cells die because of a severe energy deficit. Both DCA and 2DG are known to cause metabolic deficiencies and ATP depletion and they can induce an autophagy response (Pajak et al. 2020; Xi et al. 2011; Tataranni and Piccoli 2019; Lin et al. 2014). Their interference in the cell's metabolism can be compensated by the autophagy-mediated recycling of nutrients (Jia et al. 2017), however, in autophagy-compromised cells, this is not possible. Indeed, in both $H460^{Res}$ and ATG-KO cells, but at a lesser degree in $H460^{WT}$, metabolic inhibitors not only caused apoptosis and reduced proliferation but displayed increased levels of the energy-stress markers $pACC^{S79}$ and $pAMPK^{T172}$. Energetic stress is one known cause of cell death (H. Lee et al. 2020; M. Liang et al. 2022), and more importantly, it has been already reported that nutrient deficit in cells with impaired autophagy leads to cell death. For example, HeLa cells die when they are subjected to a nutrient-depleted medium upon chemical inhibition of autophagy (Boya et al. 2005), while breast cancer cells with defective autophagy were found more susceptible to metabolic stress compared to their autophagy-competent counterpart (Karantza-Wadsworth et al. 2007). It has also been reported that autophagy inhibition via AKT activation sensitizes immortalized epithelial cells to metabolic stress (Degenhardt et al. 2006). Therefore, we concluded that after treatment with DCA/2DG, cells with defective autophagy would die because of severe energetic stress as the most probable cause.

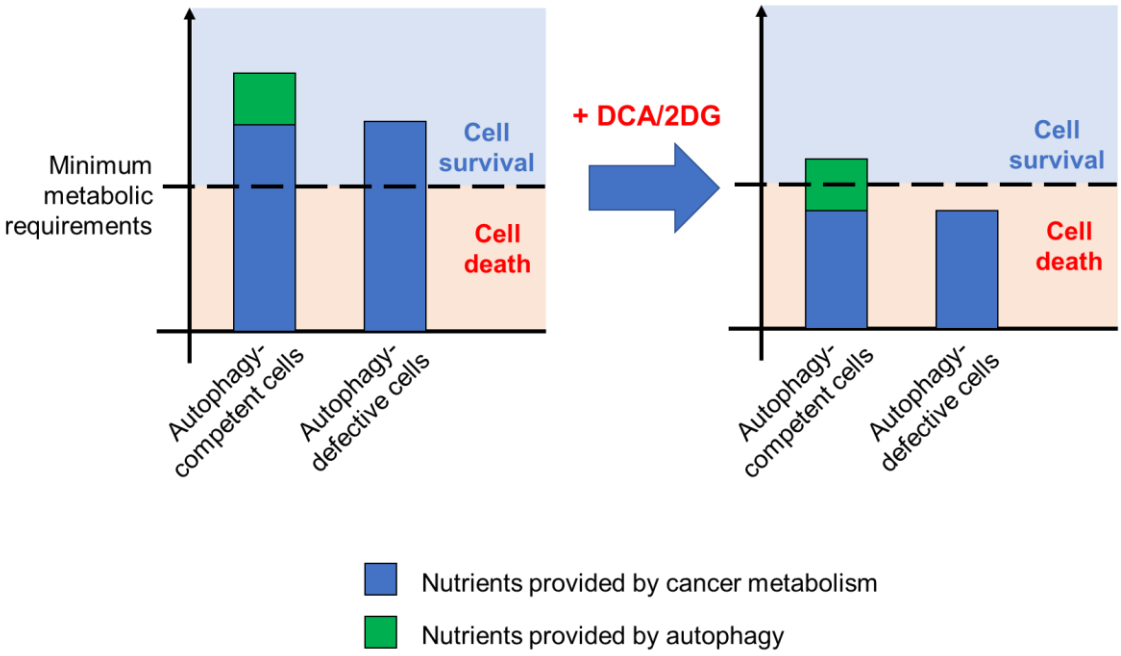


Fig. 29 Scheme for the proposed model of sensitivity to metabolic inhibitors
On the left: basal conditions. The ATP and nutrients obtained from the metabolism in cancer cells are sufficient to stay above one hypothetical minimum metabolic threshold. Nutrient levels should stay above this threshold to allow survivals of cancer cells, regardless of the autophagic status.
On the right: treatment with metabolic inhibitors DCA/2DG. The inhibitors interfere with cancer metabolism provoking nutrient deficiencies. Autophagy provides additional nutrients that allow cells to survive, but autophagy-deficient cells die due to nutrient shortage.

4.2.2. Metabolic inhibitors cause DNA damage in H460^{Res}

In addition to energy stress, we investigated whether other factors would contribute to cell death in H460^{Res} since we observed that such cells died at faster rates compared to ATG-KO cells when exposed to metabolic inhibitors. Despite having increased FANCD2 levels, which confers resilience against DNA-crosslinking agents, the H460^{Res} cells displayed DNA damage signs when treated with DCA/2DG. They exhibited increased phosphorylation of histone H2AX (γH2AX) and formation of γH2AX nuclear foci, known biomarkers for DNA double-strand breaks (Mah, El-Osta, and Karagiannis 2010). This could be a sign of replicative stress triggered by a critical depletion of the available nucleotide pool caused by the use of metabolic drugs. Indeed, it is reported that a negative balance between nucleotide synthesis and consumption in cancer with

hyperactive mTORC1 can lead to replication stress and DNA damage (Valvezan et al. 2017). Autophagy can contribute to replenishing the nucleotide pool in cancer cells (J. Y. Guo et al. 2016), but as we have demonstrated, autophagy in $H460^{Res}$ cells is inhibited. Although the presence of DNA damage signs is evident, more work would be needed to understand how much its contribution to the death mechanisms is determinant, or if the observed γH2AX is only a consequence of apoptosis (Rogakou et al. 2000).

Another hypothesis open for speculation could be that the observed γH2AX is due to an increase in reactive oxygen species (ROS) level. ROS are highly reactive molecules containing oxygen known to cause DNA damage, including double-strand breaks, and capable to induce a DNA damage response (DDR) (Srinivas et al. 2019). Our team found that basal ROS levels in $H460^{Res}$ cells are moderately higher in comparison to $H460^{WT}$, and the treatment with DCA and 2DG increases such levels even further (personal communication, Dr. M. Wanzel). One common cause for altered intracellular ROS levels can be found in the presence of defective mitochondria, as one of the main cellular ROS producers (Bonawitz, Rodeheffer, and Shadel 2006). $H460^{Res}$ cells were created through repeated exposure to cisplatin, and it is reported that this chemotherapeutic induces ROS generation, possibly by damaging the mitochondrial DNA (Y.-M. Choi et al. 2015; Marullo et al. 2013). $H460^{Res}$ cells are also autophagy-incompetent, and one of the autophagy functions is to eliminate old and dysfunctional mitochondria. If autophagy is not working properly, damaged mitochondria would accumulate, together with ROS (G. Chen, Kroemer, and Kepp 2020). There is also evidence that moderate amounts of ROS can activate mTOR through AKT (Kma and Baruah 2022; M. Li et al. 2010), and that mTORC1 can be involved in the DDR mechanisms, and be activated in presence of genotoxic stress (Ma, Vassetzky, and Dokudovskaya 2018).
If on one side ROS-activated mTOR could help to cope with DNA damage (Ma, Vassetzky, and Dokudovskaya 2018), on the other side, it promotes proliferation and increases the metabolic demand (Deleyto-Seldas and Efeyan 2021). If $H460^{Res}$ were to be found with faulty mitochondria, it is reasonable to think that they could use aerobic glycolysis as a mean to provide the needed metabolites without completely relying on oxidative respiration. Nonetheless, treatment with

DCA would force them to shift the use of glucose-carbon sources into “classic” cellular respiration, similarly, 2DG would severely hinder the glycolysis pathway (Aft, Zhang, and Gius 2002; Tataranni and Piccoli 2019). With defective mitochondria and inhibited autophagy, cells would not be able to keep up with the energy requirements and die of severe energy stress, as described in the previous chapter. On top of that, we could imagine that increased levels of oxidative respiration with damaged mitochondria would produce even more ROS, above a hypothetical critical threshold, with consequent induction of DNA damage that we detected in form of γH2AX. It is known that cells can redirect glycolysis metabolites into the pentose-phosphate pathway (PPP) to generate not only metabolic intermediates for biomass production, but also nicotinamide adenine dinucleotide phosphate (NADPH) as an important ROS scavenger (P. Jiang, Du, and Wu 2014; Mullarky and Cantley 2015; Cho et al. 2018). However, in $H460^{Res}$, the NADPH produced by the PPP might not be enough to maintain low ROS levels, since ROS are detected at higher levels compared to $H460^{WT}$. In any case, these are just speculations trying to explain the observed DNA damage, and further investigations would be required to provide support to these hypotheses.

4.3. Somatic copy number alterations affecting autophagy genes

4.3.1. Hypothesis

We have demonstrated that flaws in the autophagic pathway can make cells vulnerable to metabolic inhibitors such as DCA and 2DG. Such flaws can be caused either by the upregulation of upstream negative regulators such as mTOR or by completely deleting important autophagy genes such as *ATG7*. However, while the first case, mTOR upregulation, is a common event in many tumors (Ilagan and Manning 2016; Xu, Liu, and Wei 2014), homozygous deletions or disrupting mutations affecting autophagy genes are instead sporadic (Lebovitz et al. 2015). Nonetheless, another type of genetic defect could provoke an impairment in the autophagic pathway: somatic copy number alterations (SCNAs). In contrast to homozygous deletions, SCNAs affecting autophagy are frequently found in different types of cancers, with *BECN1, MAP1LC3B* (*LC3*), and *ATG10* among the genes most affected. Furthermore, they are characterized mostly by allelic losses rather than allelic gains. A haploinsufficiency network analysis reported that, for example, *LC3* and *BECN1* are monoallelically deleted in 94% of ovarian tumors (Joe Ryan Delaney et al. 2017). In malignancies distinguished by a particularly high rate of copy number changes, such as ovarian, breast, and lung cancer (Ciriello et al. 2013), SCNAs could affect multiple genes simultaneously. On this basis, it has been hypothesized that SCNAs affecting multiple ATG genes at the same time could have a serious impact on the autophagic flux, and make cells vulnerable to the action of autophagy-stressing drugs. In favor of this thesis, the ovarian cancer cell line OVCAR-3 characterized by monoallelic loss of both *BECN1* and *LC3* was found more sensitive to the autophagy inhibitor chloroquine than cell lines without these deletions. The sensitivity of other cell lines, IGROV1 or SKOV3, was also increased when subjected to shRNAs with modest suppression effects against *LC3* and *BECN1* to simulate SCNAs (Joe Ryan Delaney et al. 2017). Of note, the OVCAR-3 is incidentally also a cisplatin-resistant cell line (Sakhare et al. 2014) which might have upregulated mTOR. These data suggest that SCNAs affecting multiple autophagy genes might generate a metabolic vulnerability, and be used

to identify tumors that could benefit from treatment with metabolic inhibitors. However, formal proof to support of this hypothesis was missing.

4.3.2. ATGs show resilience against somatic copy number alterations

To provide a proof of concept, in this study we used the CRISPR-Cas9 technology to target the *BECN1, MAP1LC3B* (*LC3*), and *ATG10* genes in H460^{WT} cells in a cumulative manner, generating cells with an *LC3*$^{+/-}$; *BECN1*$^{+/-/-}$; *ATG10*$^{+/-/-}$ genotype. However, while the knockout of one single gene caused cells to become highly sensitive to metabolic inhibitors, when at least one copy of the targeted gene was still present cells showed little-to-no signs of autophagy block and maintained a WT-like phenotype for what concerns sensitivity to DCA/2DG. The accumulation of further heterozygous deletions of autophagy genes could theoretically make cells, in the end, metabolically vulnerable, but it is not likely that more than three mutations of this type can occur in the same tumor. Ultimately, the autophagic pathway showed remarkable resilience against non-homozygous losses.

One probable reason for such resilience could be found in the impact that such mutations have on the overall gene expression. Despite the deletion of one allele (or two in the case of *BECN1* and *ATG10*), the loss did not translate into a proportional reduction in the correspondent protein. In fact, we observed only a slight decrease in protein levels and consequently only modest signs of autophagy block. It is well known that SCNAs can alter gene expression levels (Stranger et al. 2007), and it is reported that there is a close correlation between copy number and gene expression for the majority of genes (Fehrmann et al. 2015; Shao et al. 2019). However, adaptive mechanisms often take place and can lead to compensations responses (Bhattacharya et al. 2020; R. A. Veitia, Bottani, and Birchler 2013). Cells can respond to altered gene copy numbers in different ways. For instance, genetic compensation can be mediated already at the transcriptional level with different degrees of transcriptional adaptation, allowing an increased level of genetic robustness, although the underlying mechanisms are not completely understood (El-Brolosy and Stainier 2017; R. A. Veitia, Bottani, and Birchler 2013). Post-transcriptional regulation can also play a role: for example, miRNAs and long noncoding RNAs have been identified to

target and regulate several autophagy machinery components (Füllgrabe, Klionsky, and Joseph 2014; Botti-Millet et al. 2016). Another aspect to keep in consideration is that even when a strong correlation between copy number and final protein levels exists, this does not guarantee a change in the phenotype. This is a central aspect in the study of haploinsufficient genes, where is often observed a nonlinear relationship between genotype and phenotype (Johnson, Nguyen, and Veitia 2019). Indeed, a gene coding hypothetically for an enzyme whose activity is a non-rate-limiting step in a broad pathway with several other components would show much more resilience to copy losses. Such a gene would be haplosufficient even if its protein levels drop dramatically even lower than 50%. On the contrary, other genes could be much more sensitive to even slight protein level drops. Typical examples are transcription factors since they can bind to enhancers, and multiple binding sites of a promoter, and their action can induce the transcription of a variety of target genes (R. a. Veitia, Caburet, and Birchler 2018; Wilkie 1994; Johnson, Nguyen, and Veitia 2019). For all these reasons, it is difficult to predict the actual effects of a non-homozygous deletion.

To obtain a wider and more comprehensive view of how copy number, mRNA, protein levels, and autophagy flux actually correlate in different cancer entities, public databases can be queried. For example, the TCGA PanCancer Atlas, which contains genomic, transcriptomic, and proteomic data from thousands of patient tumors of different origins, is publicly available ("The Pan-Cancer Atlas" n.d.). Indeed, a multi-omic and pan-cancer analysis of the autophagy-related genes *BECN1* and *ATG7* performed by Prof. Dr. Stiewe revealed that non-homozygous deletions decisively correlate with decreased mRNA levels, but this is less evident at the protein level. Furthermore, p62 protein expression levels (its accumulation used as an indicator of blocked autophagy) correlate with the p62 copy number, but showed no correlation with *BECN1* and *ATG7* copy number, implying that they do not have any impact on the autophagic flux. Similarly, p62 protein expression showed no significant level variation even when multiple non-homozygous deletions of *BECN1, ATG7, ATG10*, and *LC3* were simultaneously present (Polo et al. 2022). These analyses are in line with our results obtained in the CRISPR-Cas9 engineered lines. They further demonstrate that the autophagic flux is unaffected by SCNAs since these deletions do not translate

into diminished protein levels, most probably due to post-transcriptional mechanisms that compensate for the decreased mRNA amount.

Our findings offer an opportunity to speculate about whether and how SCNAs affecting autophagy genes might contribute to tumor development: ATGs are found frequently monoallelically deleted in cancer, often affecting multiple ATGs simultaneously (Joe Ryan Delaney et al. 2017). Numerous studies have demonstrated how autophagy exerts a tumor-preventing action for example by reducing the amount of old and dysfunctional proteins and organelles (Chavez-Dominguez et al. 2020; White and DiPaola 2009). Papers even report autophagy genes such as *BECN1* functioning as haploinsufficient tumor suppressors, hinting at defects in the autophagy pathway (Yue et al. 2003; Joe R. Delaney et al. 2020). It is not questioned that copy number variations can have an impact on gene expression: these might become the drivers for tumor development when affecting main oncogenes or tumor suppressor genes (Shao et al. 2019). However, in light of our findings, it is reasonable to question if this is the case also for autophagy genes since the PanCancer Atlas analysis demonstrated that there is no correlation between ATGs copy number and a possible autophagy block (inferred from p62 accumulation) (Polo et al. 2022).
Therefore, although there is robust experimental evidence suggesting that non-homozygous losses of ATGs might contribute to tumorigenesis, data in our work does not point to a universal mechanism dependent on a reduced autophagic flux. Instead, it would be more reasonable to think of independent mechanisms linked to single genes. For example, as mentioned before, *BECN1* was reported as a haploinsufficient tumor suppressor in different studies (Yue et al. 2003; Qu et al. 2003) until it was discovered to be often co-deleted, due to its vicinity on chromosome 17q21, with the tumor suppressor breast cancer 1 (*BRCA1*) (Laddha et al. 2014). Although more recent research indicated that monoallelic deletion of *BECN1*, and not *BRCA1*, is sufficient to drive tumorigenesis and genomic instability (Joe R. Delaney et al. 2020). Other studies propose further autophagy-independent mechanisms: for instance, *BECN1* was found to induce membrane localization of the breast tumor-suppressor E-cadherin (Wijshake et al. 2021) and to have functions related to chromosomal segregation during mitosis (Frémont et al. 2013). *ATG7* hemizygosity was instead discovered to

affect tumor progression in an autophagy-independent manner (Long et al. 2022). This is in line with the notion that ATGs are also involved in non-autophagic roles such as apoptosis, signaling, transcription, secretory and transport processes, and membrane organization (Subramani and Malhotra 2013).

Despite multiple lines of evidence suggesting that copy number variation of autophagy-related genes play an important role in tumor progression, in our model such mutations did not show a clear phenotypical impact. Nevertheless, we cannot exclude the possibility that such alterations could play an important role at the early stages of tumor progression but have no effect on established tumors.

4.4. Cancer-targeting strategies: considerations

Aggressive tumors must keep up with the elevated proliferation rates of their cells with a considerable uptake of nutrients to sustain the gross amount of biomass that is constantly produced. Not only this but for the same reason they also face a reprogramming of their metabolism which includes a deregulated uptake of glucose and the use of glycolysis/TCA cycle intermediates for biomolecules synthesis and NADPH production (Pavlova and Thompson 2016). This aberrant behavior is considered one of the hallmarks of cancer, and it is since long been at the center of interest for scientists because it potentially offers one angle for effective therapy. In practice, this is still a challenging topic and the use of metabolic inhibitors is often associated with severe side effects and toxicities (Luengo, Gui, and Vander Heiden 2017). For these reasons, we must not think of metabolic therapy as a silver bullet for all types of cancer, but rather as a treatment strategy that could have remarkable potential in specific cases, but it could have also serious drawbacks depending on the situation. It is therefore of primary importance to identify such cases, as doing so could benefit the treatment through an increase in the selectivity and efficacy of metabolic drugs.

4.4.1. The use of DCA and 2DG

One example of the problems to face when employing metabolic therapy is given by DCA and 2DG themselves. Both compounds have been at the center of interest for years, but their clinical use is still restricted for several reasons. DCA

has raised concerns in the past due to its side effects including neurotoxicity (Tataranni and Piccoli 2019), and while 2DG has demonstrated instead safety in several animal and human studies (Stein et al. 2010; Pajak et al. 2020), it displayed problems related to its fast metabolization and short half-life (Hansen, Levy, and Kerr 1984), and it must be used in high doses to compete with normal glucose (Strandberg et al. 2013). Notably, the design of new analogs and new drug preparations aiming to improve delivery and reduce side effects has brought renewed interest in the use of these compounds (Pajak et al. 2020; Tataranni and Piccoli 2019).

Despite these problems, DCA and 2DG are valuable tools to dissect metabolic vulnerabilities in preclinical studies. Of note, the $H460^{Res}$ and all the ATG-KO cells showed high sensitivity to both DCA and 2DG, drugs with similar effects, but different modes of action. This suggested that the response was due to metabolic interference rather than being specific to one single drug. As a matter of fact, other experiments from members of our team found $H460^{Res}$ cells to be sensitive to another class of metabolic compounds: the antidiabetic drugs metformin and phenformin (Gremke et al. 2020), biguanides acting on the electron transport chain in mitochondria, with metformin being widely used in the clinic (Yendapally et al. 2020).

Concluding, despite their shortcomings, DCA and 2DG are useful tools to discover possible metabolic vulnerabilities and they are paving the way for the development of future more effective drugs, the development of new formulations, or the use of different drug classes. More importantly, they prove that targeting metabolism could be an effective strategy to improve treatment selectivity in cancer treatment.

4.4.2. Targeting mTOR

Due to its serious role in the regulation of cancer metabolism (Magaway, Kim, and Jacinto 2019), mTOR is also one appealing target for cancer treatment. Upregulation of the mTOR pathway is a common feature observed in a variety of tumors and a driver for chemotherapeutic resistance (Ilagan and Manning 2016; G. Y. Liu and Sabatini 2020). For decades, different compounds have been developed starting from rapamycin as the initial prototype drug to achieve mTOR inhibition as an anticancer approach, and up-to-date, 3 generations of mTOR

inhibitors have been developed: each new one with improved efficacy, and can inhibit not only both mTORC1 and mTORC2 complexes but also different mTOR domains simultaneously (Ali et al. 2022). Unfortunately, despite being very effective in inhibiting mTOR, their success as monotherapy against tumors has been so far limited and the effects of treatment are mainly cytostatic (Ali et al. 2022). This is in line with what we observed in our study: $H460^{Res}$ cells did not show sensitivity to treatment with either rapamycin, everolimus, or AZD8055 as a single treatment. Furthermore, because of the crucial function of mTOR, its inhibition could have destructive effects on healthy cells (Ali et al. 2022).

On this basis, it has been investigated in several studies to use mTOR inhibitors in combinational therapies to improve their overall effectiveness rather than employing them as monotherapies (Hua et al. 2019; Yardley 2013). This would be a good method to avoid the insurgence of mTOR-mediated chemoresistance mechanisms, for example against DNA-damaging agents such as cisplatin. But other studies even suggest the use of mTOR inhibitors in combination with metabolic drugs to obtain improved efficacy (Mossmann, Park, and Hall 2018). However, this last approach could give disadvantageous effects, as in our work we demonstrated how sensitivity to metabolic inhibitors is strictly bound to mTORC1 activity. mTORC1 inhibition would activate cytoprotective autophagy which in turn would nullify the effects of combinational treatment.

4.4.3. Autophagy modulation

Likewise, modulation of autophagy is also considered an appealing goal, since its induction is a common feature arising during chemotherapy. Countless studies have demonstrated the cytoprotective effects of autophagy, and for this reason, its inhibition with drugs like hydroxychloroquine (HQ) or 3-methyladenine combined with chemotherapeutic compounds has been suggested as a promising anticancer strategy (Chang and Zou 2020; Z. J. Yang et al. 2011). While the combination with chemotherapeutics may give good results, more careful consideration must be used when employing autophagy inhibitors combined with metabolic drugs. If on one hand, these therapeutic settings may increase cell death, there is a risk that this is achieved at the expense of selectivity. Indeed, $H460^{WT}$ cells can become sensitive to metabolic drugs upon inhibition of autophagy, either through a genetic intervention (KO of ATG genes)

or through pharmacological treatment (hydroxychloroquine treatment combined with DCA/2DG) (Gremke et al. 2020). This could cause nutrient depletion even in healthy cells which may lead to severe side effects when the combination is with metabolic inhibitors. It is reported that *ATG7*^{KO} mice would even suffer fatal hypoglycemia upon fasting (Karsli-Uzunbas et al. 2014). For these reasons, autophagy modulation is a strategic option that must be carefully evaluated, especially when used in combination with metabolic compounds. Nonetheless, in this study we underline how exploiting cases of pre-existing impairments of the autophagy pathway with metabolic inhibitors would be a much more effective strategy.

4.4.4. Biomarkers

As we already mentioned, although metabolic therapy when used in combination with mTOR and autophagy modulation could be a promising anticancer strategy, the possible outcome is context-dependent, and determining the metabolic and autophagic conditions before treatment is necessary. To evaluate the status of autophagy, the most widely used markers are LC3 and p62 (Schläfli et al. 2015; Klionsky et al. 2021). In general, missed conversion of LC3-I into LC3-II and accumulation of p62 are symptoms of a block in the pathway. However, accurately assessing the autophagic flux is infamously difficult and the interpretation of the results can be misleading, even more in a clinical context (Klionsky et al. 2021). Therefore, finding more reliable and, most of all, univocal biomarkers would be preferable. The results from our study demonstrated that sensitivity to metabolic inhibitors is highly correlated to mTORC1 hyperactivation and autophagy impairment. Therefore, high phosphorylation levels of p70S6K^{T389}, 4E-BP1$^{T37/46}$, and inhibitory phosphorylation of ULK1^{S757}, which stands at the edge between the mTOR and the autophagy pathways, would be a good option as markers of metabolic vulnerability (Liao, Sy, and Yen 2012). Also constitutively hyperactivating mutations of the mTOR kinase can be taken into consideration, although they are infrequent events (Grabiner et al. 2014). For what concerns the sole autophagic pathway, we demonstrated that SCNAs affecting ATGs, although being frequent events in cancer, would not induce metabolic vulnerability, not even when affecting multiple genes simultaneously. On the other hand, disruptive indel mutations of any of the main ATGs would

predict the cells' sensitivity to metabolic drugs, although these mutations are much less frequent.

5. Conclusions

Successful treatment of cancer is still one challenging goal to achieve. One of the main reasons behind this problem is the high toxicity and low selectivity of the therapies that are normally used. The second common reason is that after an initial positive response, chemoresistance phenomena emerge, and the tumor becomes refractory to the initial treatment. For these reasons, it is necessary to find new, selective therapeutic options that can increase success rates. Equally important, there is a need to find reliable biomarkers that can predict whether a certain tumor would benefit from treatment with a specific therapy.

One such potential treatment option is given by the use of metabolic inhibitors. These drugs, for example glycolysis inhibitors, are highly selective against those cancer cells possessing specific metabolism alterations. Further studies revealed that vulnerability to these compounds can be caused by a defective autophagic pathway. In this work, we evaluated two cases of potential defective autophagy that could generate druggability. In the first, we found that upregulated mTOR activity can confer resistance to the chemotherapeutic cisplatin, but at the same time inhibits autophagy, generating hypersensitivity to metabolic drugs. Cells would die due to severe energy stress. In the second case, we examined whether non-homozygous copy number deletion of multiple ATG genes, a genetic alteration often found in several types of cancer, would generate an autophagy defect and consequent druggability. To this end, we generated through CRISPR-Cas9 technology several cell lines harboring progressive allelic deletions of key ATG genes. Cells became sensitive to metabolic drug treatment after the complete deletion of even one single gene and exhibited symptoms that suggested a block of autophagy. However, the treatment had little impact on proliferation when cells harbored non-homozygous deletions, even of multiple ATGs simultaneously, showing no signs of autophagy inhibition. Therefore, the autophagy pathway proved to be resilient against SCNAs mutations, although happening with high frequency in different malignancies.

Taken together, these results showed how autophagy inhibition and mTOR-upregulated activity are enough to generate selective druggability by using metabolic inhibitors, opening a valid therapeutic window to exploit against cancer. Selecting markers that are univocally associated with autophagy deficiency or that can at least predict the effectiveness of the treatment with the

beforementioned drugs can be challenging. To this end, a multi-omic approach that takes into consideration not only genetic alterations, but also the final proteomic landscape would be necessary, with special attention to the phosphorylation of mTOR targets, and key ATG protein levels.

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Appendix

Curriculum Vitae

Personal data

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Date of birth	14 April 1990
Place of birth	Jesolo, Italy

Academic education and degrees

- May 2018 – May 2021: Doctoral study, PD Dr. Michael Wanzel, Molecular Oncology Institute (director Prof. Dr. Thorsten Stiewe), Philipps-Universität Marburg (DE)
- 2014 – 2017: Master's degree, Pharmaceutical Biotechnology, University of Padua (IT)
- 2009 – 2014: Bachelor's degree, Biotechnology, University of Padua (IT),

Professional experience

- 2022 – present: Scientific researcher, Prof. Dr. Lauth group, Zentrum für Tumor- und Immunbiologie, Philipps-Universität Marburg (DE)
- 2018 – 2021: Scientific researcher, Prof. Dr. Stiewe group, Zentrum für Tumor- und Immunbiologie, Philipps-Universität Marburg (DE)
- 2016 – 2017: Master thesis internship, Neuroblastoma research group, Institute of Pediatric Research "Città della Speranza", Padova (IT)

Training

2022: Mouse handling certificate (https://www.las-interactive.de)

List of pubblications

From the PhD thesis

- **Polo P**, Gremke N, Stiewe T, Wanzel M. **Robustness of the Autophagy Pathway to Somatic Copy Number Losses**. Cells. 2022 May 27;11(11):1762. doi: 10.3390/cells11111762. PMID: 35681458; PMCID: PMC9179279.

- Gremke, N., **Polo, P.**, Dort, A. et al. **mTOR-mediated cancer drug resistance suppresses autophagy and generates a druggable metabolic vulnerability**. Nat Commun 11, 4684 (2020). https://doi.org/10.1038/s41467-020-18504-7

Other publications

- Koeniger A, **Polo P**, Brichkina A, Finkernagel F, Visekruna A, Nist A, Stiewe T, Daude M, Diederich WE, Gress TM, Adhikary T, Lauth M. **Tumor-suppressive disruption of cancer subtype-associated super enhancer circuits by small molecule treatment.** NAR Cancer. 2023 Feb 6;5(1):zcad007. doi: 10.1093/narcan/zcad007. PMID: 36755960; PMCID: PMC9900422.

- Aveic S, Pantile M, **Polo P**, Sidarovich V, De Mariano M, Quattrone A, Longo L, Tonini GP. **Autophagy inhibition improves the cytotoxic effects of receptor tyrosine kinase inhibitors**. Cancer Cell Int. 2018 Apr 24;18:63. doi: 10.1186/s12935-018-0557-4. PMID: 29713246; PMCID: PMC5916832.

List of academic teachers

Academic teachers at the Philipps University Marburg:

- Prof. Dr. Thorsten Stiewe
- Dr. Michael Wanzel

Academic teachers at the University of Padua:

- Prof. Dr. Antonella Caputo
- Prof. Dr. Chiara Bolego
- Prof. Dr. Barbara Gatto
- Prof. Dr. Gianfranco Pasut
- Prof. Dr. Patrizia Polverino de Laureto
- Prof. Dr. Stefano Salmaso
- Prof. Dr. Dorianna Sandonà
- Prof. Dr. Andrea Sartorel
- Prof. Dr. Brbara Spolaore

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Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel „Exploiting metabolic vulnerabilities caused by autophagy defects for cancer therapy“ im Institut für Molekulare Onkologie unter Leitung von Prof. Dr. Thorsten Stiewe mit Unterstützung von PD Dr. Wanzel ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Ich versichere, dass ich sämtliche wörtlichen oder sinngemäßen Übernahmen und Zitate kenntlich gemacht habe.

Mit dem Einsatz von Software zur Erkennung von Plagiaten bin ich einverstanden.

Vorliegende Arbeit wurde in Teilen in folgenden Publikationsorganen veröffentlicht:

- Polo P, Gremke N, Stiewe T, Wanzel M. Robustness of the Autophagy Pathway to Somatic Copy Number Losses. Cells. 2022 May 27;11(11):1762. doi: 10.3390/cells11111762. PMID: 35681458; PMCID: PMC9179279.
- Gremke, N., Polo, P., Dort, A. et al. mTOR-mediated cancer drug resistance suppresses autophagy and generates a druggable metabolic vulnerability. Nat Commun 11, 4684 (2020). https://doi.org/10.1038/s41467-020-18504-7

Ort, Datum, Unterschrift Doktorandin/Doktorand

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Die Hinweise zur Erkennung von Plagiaten habe ich zur Kenntnis genommen.

Ort, Datum, Unterschrift Referentin/Referent