UNIVERSITY OF MOLISE

Department of Biosciences and Territory - DiBT

DOCTORAL THESIS

“BIOSCIENZE E TERRITORIO”
Curriculum “Bio-ambientale”

Cycle XXXII - 2018/2019 – S.S.D. BIO/06

Analysis of autophagy role in glioblastoma biology

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Firmato digitalmente da:Giovanni Fabbrocino
Organizzazione:UNIVERSITÀ DEGLI STUDI DEL MOLISE/92008370709
Limite d'uso:Explicit Text: Il titolare fa uso del presente certificato solo per le finalità di lavoro per le quali esso è rilasciato. The certificate holder must use the certificate only for the purposes for which it is issued.
Data:06/04/2020 18:52:29

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Analysis of autophagy role in glioblastoma biology

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DOCTORAL THESIS
Pesche (IS) 2020

Analysis of autophagy role in glioblastoma biology

Abstract

Autophagy is one of the main intracellular degradation systems, through which long-lived protein and other cytoplasmic compounds are included in double-membrane vesicles, named autophagosomes, that fuse with lysosomes to be degraded.
Several literature data demonstrate the involvement of autophagy in tumorigenesis, highlighting its dual role depending on tumor stage and on specific microenvironmental conditions.
Despite advances in tumor treatments, glioblastoma (GBM) is a still incurable cancer and the relation with autophagy is controversial.
In this regard, this doctoral thesis was aimed at analyse the role of autophagy in GBM physiopathology.
We started from the evidence that autophagy induces mesenchymal to epithelial transition-like in GBM cells through down-regulation of SNAI factors, resulting in cadherin up-regulation. Based on these observations, we found an impairment of Wnt/β-catenin signalling upon autophagy induction in GBM cells. Unlike other types of systems, we also observed a significant re-localization of β-catenin in sub-membranes areas, associating with N-cadherin. We subsequently investigated the expression of EGFR and EGFR-mediated signalling pathways in GBM cells upon autophagy and mTOR pathway inhibition. EGFR is frequently overexpressed in GBM, enhancing an aberrant cell proliferation. We observed that mTOR inhibition results in the EGFR internalization within the cell, delivering the receptor to lysosomes for degradation. As a consequence, an impairment of MAPK/ERK pathway, one of the main signalling pathways involved in cell proliferation, occurred. Finally, we found Src as a key player in EGFR trafficking. In fact, an increase of Src activation, that is commonly required for EGFR phosphorylation, occurs upon mTOR inhibition and is responsible for EGFR internalization.
Taking together, the results collected in this thesis showed that forcing GBM cells to undergo autophagy leads to an impairment of intracellular signalling pathways typically hyperactivated in GBM and induces cells to acquire epithelial-like phenotype.
Thus, we support the idea that autophagy/mTOR inhibition could be employed to contrast GBM tumorigenesis.

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List of publications

This thesis is based on the work contained in the following papers:

I. **Autophagy induction impairs Wnt/β-catenin signalling through β-catenin relocalisation in glioblastoma cells**
Barbara Colella, Fiorella Faienza, Marianna Carinci, Giuseppina D'Alessandro, Myriam Catalano, Antonio Santoro, Francesco Cecconi, Cristina Limatola, Sabrina Di Bartolomeo *(2019) Cellular Signalling, 53: 357–364.*

Review: EMT Regulation by Autophagy: A New Perspective in Glioblastoma Biology
Barbara Colella, Fiorella Faienza, Sabrina Di Bartolomeo. *(2019) Cancers, 11(3), 312.*

II. **mTOR inhibition leads to Src-mediated EGFR internalization and degradation in glioma cells**
Barbara Colella, Gianna Iannone, Mayra Colardo, Cristina Saiz-Ladera, Claudia Fuoco, Guillermo Velasco, Marco Segatto, Sabrina Di Bartolomeo
(Paper submitted for publication)

Other contributions

The list includes author's contributions not related to the thesis work.

I. **Native and iron-saturated bovine lactoferrin differently hinder migration in a model of human glioblastoma by reverting epithelial-to-mesenchymal transition-like process and inhibiting interleukin-6/STAT3 axis**
Antimo Cutone, Barbara Colella, Andrea Pagliaro, Luigi Rosa, Maria Stefania Lepanto, Maria Carmela Bonaccorsi di Patti, Piera Valenti, Sabrina Di Bartolomeo, Giovanni Musci. *(2020) Cellular Signalling, 65, 109461*.

II. **Inhibition of bromodomain and extraterminal domain 2 (BET) proteins by JQ1 unravels a novel epigenetic 3 modulation to control lipid homeostasis**
Claudia Tonini, Mayra Colardo, Barbara Colella, Sabrina Di Bartolomeo, Francesco Berardinelli, Giuseppina Caretti, Valentina Pallottini, Marco Segatto. *(2020) Int. J. Mol. Sci., 21, 1297*

III. **UNC-51-Like Kinase 1 is negatively regulated in GBM and correlates with tumor aggressiveness**
Barbara Colella, Mayra Colardo, Eleonara Sgambati, Marco Segatto, Guillermo Velasco, Sabrina Di Bartolomeo
(Manuscript in preparation)

Abbreviations

4EBP1	*eIF4E Binding Protein 1*
AMBRA1	*Activated Molecule in BECLIN1-dependent Autophagy*
AMP	*Adenosine MonoPhosphate*
AMPK	*AMP-activated protein Kinase*
ATG	*AuTophagy-related Genes*
ATP	*Adenosine TriPhosphate*
Bcl-2	*B-cell lymphoma 2*
CMA	*Chaperone-Mediated Autophagy*
CNS	*Central Nervous System*
DFCP1	*Double FYVE-Containing Protein 1*
DRAM1	*DNA damage-Regulated Autophagy Modulator 1*
EGF	*Epidermal Growth Factor*
EGFR	*Epidermal Growth Factor Receptor*
EMT	*Epithelial to Mesenchymal Transitions*
ER	*Endoplasmic Reticulum*
ERK	*Extracellular-signal-Regulated Kinase*
FIP200	*Family Interacting Protein of 200 kDa*
GBM	*Glioblastoma*
GSMs	*Glioma Stem Cells*
IDH	*Isocitrate Dehydrogenase*
IM	*Isolation Membrane*
IR	*Ionizing Radiation*
LAMP2	*Lysosome-Associated Membrane Protein 2*
LC3	*Light Chain 3*
MAPK	*Mitogen Activated Protein Kinase*
MGMT	*O6-metilguanina DNA metiltransferasi*
mTOR	*Mammalian Target Of Rapamycin*
mTORC1	*mTOR Complex 1*
mTORC2	*mTOR Complex 2*
NF1	*Neurofibromin1*
p70S6K	*Protein S6 Kinase beta-1*

PAS	*Pre-Autophagosomal Structure*
PDGFRA	*Platet-Derived Growth Factor Receptor-α*
PI3K	*Phosphoinositide 3-kinases*
PI3P	*Phosphatidylinositol 3-phosphate*
PTEN	*Phosphatase and Tensin homolog*
ROS	*Reactive Oxygen Species*
SQSTM1	*Sequestosome 1*
THC	*Tetrahydrocannabinol*
TMZ	*Temozolomide*
TP53	*Tumoral Protein 53*
ULK1	*Unc51 Like Kinase1*
VEGFR	*Vascular Endothelial Growth Factor*
VPS	*Vacuolar Protein Sorting*
WHO	*World Health Organization*

Introduction

1. Autophagy

Autophagy is a highly conserved intracellular process that, along with ubiquitin-proteasome system, represents the main intracellular degradation system in eukaryotes (Mizushima, 2018).
The word “autophagy” derives from the Greek “autòs” (self) and “phagèin” (to eat) and represents a straightforward mechanism by which a cell degrades cytoplasmic organelles, proteins and other macromolecules. This ubiquitary process normally works at basal levels. However, stressful conditions such as starvation, hypoxia and infection can stimulate autophagy, playing an important role in cell survival (Wesselborg and Stork, 2015).

1.1 Types of autophagy

“Autophagy” is normally referred to macroautophagy, the most studied of the three types of autophagy in eukaryotic cells, including microautophagy and Chaperone-Mediated Autophagy (CMA) (Fig.1).

Macroautophagy consists of double membrane vesicles formation, named autophagosomes, that engulf cytoplasmic material and then fuse with lysosomes for degradation, forming autolysosomes. Subsequently, amino acids and other simple compounds are recycled after lytic enzyme action (Yang and Klionsky, 2010).
This kind of non-selective degradation can be discriminated from selective autophagy, which leads to degradation of mitochondria (mitophagy), peroxisomes (pexophagy), endoplasmic reticulum (reticulophagy), nucleus (nucleophagy), lipids (lipophagy) and microorganisms (xenophagy) (Wirawan et al., 2012).

Microautophagy, instead, is a non-selective process in which cytoplasmic compounds are directly incorporated into lysosome by lysosomal membrane invaginations (Li et al.., 2012).

On the contrary, CMA is a highly selective process in which cytosolic chaperones recognise the specific motif KFERQ on target proteins. Upon interaction, the targeted protein is escorted by

chaperones to the lysosomal membrane. Substrate proteins are then translocated into the lysosomal lumen by LAMP2 receptor, to finally undergo degradation (Arias and Cuervo, 2011) (Fig.1).

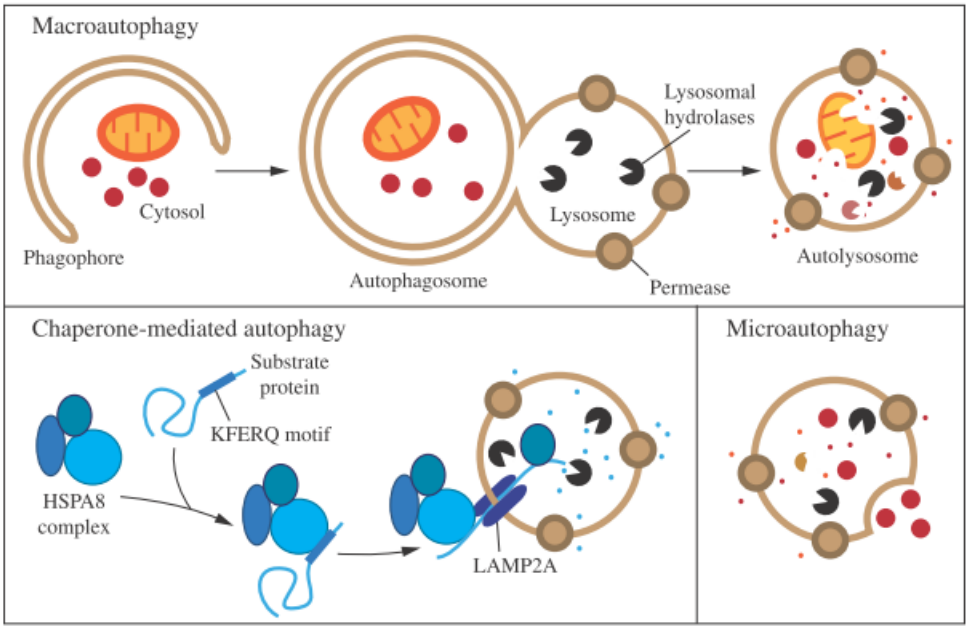

Figure 1. Three types of autophagy. Macroautophagy involves the formation of autophagosomes that engulf cargo and carry it to lysosomes forming autolysosome. Microautophagy leads to engulf of cytosolic materials directly to lysosome thanks to membrane invaginations. Chaperone-mediated autophagy involves KFERQ recognition from chaperones (such as HSPA8) that lead proteins to lysosomes, involving LAMP2 on lysosomal membrane (Parzych and Klionsky, 2014).

1.2 Autophagosome formation

Autophagosome formation requires several steps: induction, nucleation, elongation, maturation and fusion, and finally degradation and recycle (Gallagher et al. 2016; Wirawan et al., 2012).

Each step is highly regulated by ATG proteins, coded by *Atg* (*AuTophagy-related Genes*) genes, whose homologues in yeast have been identified for the first time in 1993 from Tsukada e Oshumi in *S. cerevisiae* (Tsukada and Ohsumi, 1993).

Nowadays 35 Atg proteins in yeast are known, 15 of these are required for autophagosome formation. Functionally, these proteins are divided in six protein complexes: ULK1–ATG13–FIP200–ATG101 kinases protein complex, PtdIns3K class III with protein core VPS34, VPS15, Beclin 1, ATG9A transmembrane protein complex, WIPI/ATG18–ATG2 complex, ATG5/ATG12 coniugation protein complex and ATG8/LC3 coniugation protein complex (Fig.2) (Wesselborg and Stork, 2015).

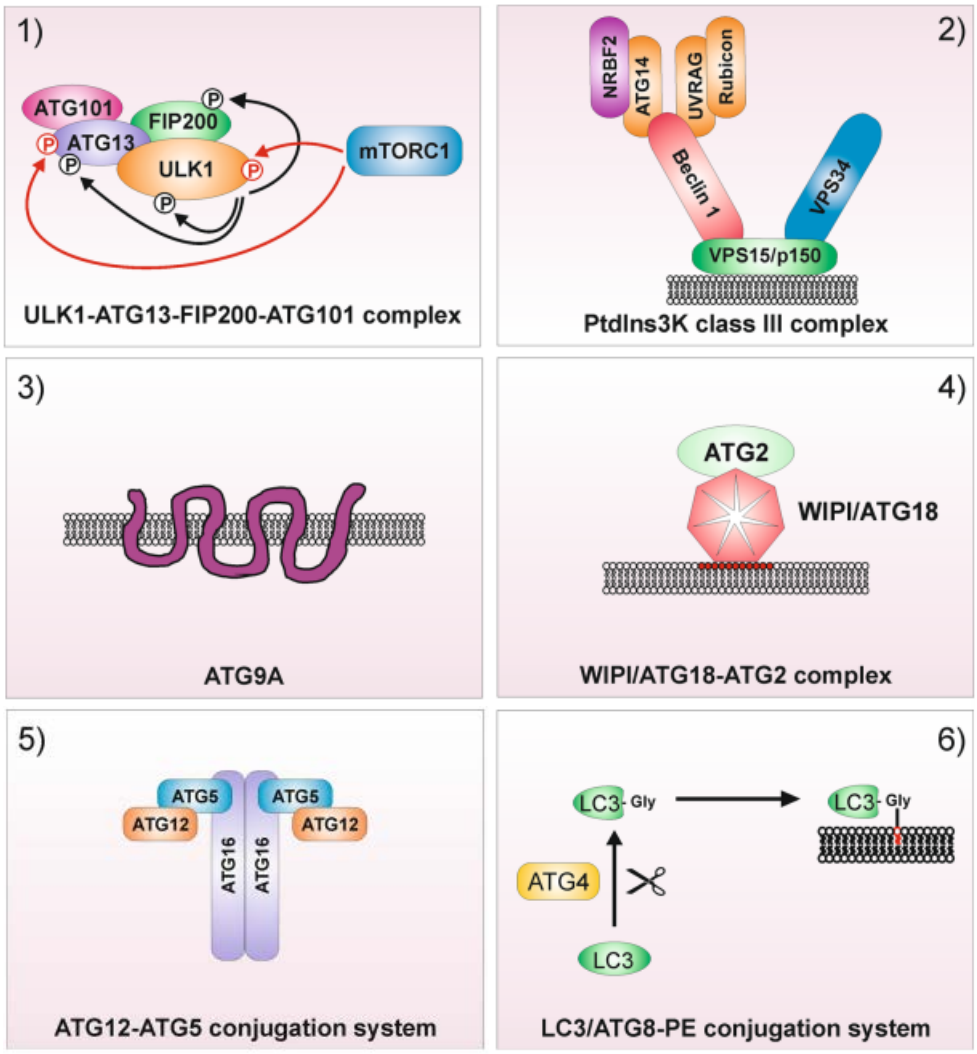

Figure 2. ATG protein complexes involved into autophagosome formation. ULK1–ATG13–FIP200–ATG101 kinases protein complex, PtdIns3K class III whit protein core VPS34, VPS15, Beclin 1, ATG9A transmembrane protein complex, WIPI/ATG18–ATG2 complex, ATG5/ATG12 coniugation protein complex and ATG8/LC3 coniugation protein complex (Wesselborg and Stork, 2015).

1.2.1 Induction and nucleation

Autophagy is generally maintained at basal levels in physiological conditions, but its activation can be strongly enhanced by a plethora of stimuli such as starvation, growth factors shortage, increase of intracellular calcium concentration, hypoxia, ATP decrease, and misfolded proteins (Fig.3).

These signals promote the activation of AMP-activated protein kinase (AMPK), which is responsible for the blockade of mTOR (mammalian Target Of Rapamycin) pathway. Notably, the inhibitory phosphorylation operated by AMPK on mTOR leads to its dissociation from ULK (Unc51 Like Kinase, Atg1 homologous) complex culminating in ULK activation (Wirawan et al., 2012).

Active ULK phosphorylates Ambra1 (Activating molecule in Beclin1 regulated autophagy 1) that binds PIK3C3 (Phosphatidil Inositol 3 Kinase Class III) and microtubule associated dynein complex, causing the release of the complex from microtubules and its repositioning to the endoplasmic reticulum (Fig. 3) (Wirawan et al., 2012; Di Bartolomeo et al., 2010).
The Isolation Membrane (IM), also known as phagophore, is first membrane needed for autophagosome formation and it is thought to be derived from the endoplasmic reticulum (ER). This structure starts as a single peri-vacuolar membrane in yeast, and is called PAS (Pre-Autophagosomal Structure) (Dunn, 1990; Reggiori and Klionsky, 2013).
Chiefly, DFCP1 (Double FYVE Domain Conteining Protein 1) localizes to ER areas enriched in phosphatidyl-inositol-3-phosphate (PI3P); this event originates membrane structures with a Ω form, called omegasomes. For this reason, DFCP1 is considered a key regulation of IM initiation (Axe et al., 2008).

Despite the involvement of ER membrane in IM formation, a number of scientific reports demonstrates that IM could also originate from other structures, such us mitochondria, TGN, plasma membrane and nucleus (Axe et al., 2008; Hailey et al., 2010; Ravikumar et al., 2010; English et al., 2009).
During autophagosome nucleation step, PIK3C3 generates PI3P from phosphatidyl-inositol (PI), leading to other ATG proteins recruitment: ATG12 binds E1/ATG7 complex and moves to E2/ATG10 before binding ATG5 to form ATG12-ATG5 complex (Fig. 3) (Mizushima et al., 2003; Kuma et al., 2002).

1.2.2 Elongation, fusion and degradation

ATG12-ATG5 is a crucial complex for the autophagosome maturation and closing as it can interact with ATG16L, forming a multimeric complex (Mizushima et al., 2003; Kuma et al., 2002).
This complex is involved in LC3 (microtubule-associated light chain-3) lipidation. LC3 (also called LC3-I) is the homolog of Atg8 and it is both cleaved and conjugated to phosphatidylethanolamine (PE) to produce LC3-PE (also called LC3-II), by the combined action of ATG7, ATG3 and the ATG16L1 complex (Fujita et al., 2008). LC3-II is normally used as autophagy-specific marker as it is, in contrast to LC3-I, specifically localized to the autophagosomal membranes (Kabeya et al., 2004) (Fig.3).
Once autophagosome is completely formed, it moves to lysosome to fuse its membrane with lysosomal membrane, releasing its contents into the lysosomal lumen where is degraded by lysosomal

hydrolases. Fusion take place by several proteins action, such as LAMP2, Rab (Ras-related GTP binding protein), SNARE and LC3 (Tong et al., 2010).

After the lytic action of hydrolases, resulting amino acids, fatty acids and nucleosides are recycled and re-used into the cytosol (Green and Levine, 2014) (Fig.3)

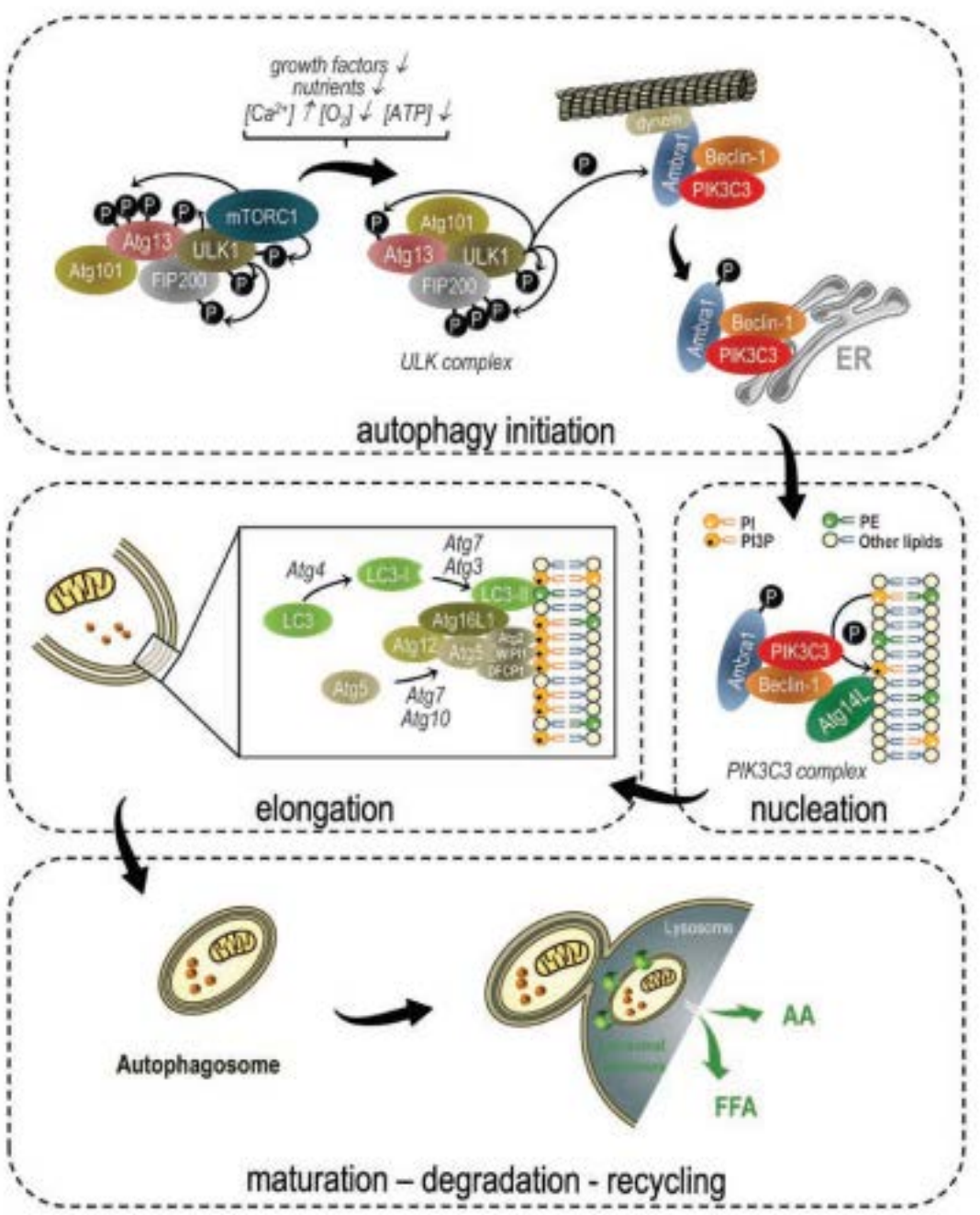

Figure 3. Autophagosome formation. Autophagosome formation takes place in several steps, starting to autophagy induction due to starvation, growth factors shortage, increase of calcium intracellular concentration, hypoxia, decrease of ATP, misfolded proteins. mTOR inhibition leads to ULK complex activation that let the autophagosome formation from the endoplasmic reticulum (ER). For nucleation, PIK3C3 generates PI3P recruiting ATG12-ATG5 complex involved in LC3-II formation. After closure, autophagosome moves to lysosome to be degraded by lysosome hydrolases and the resulting molecules, such as amino acids (AA) and free fatty acids (FFA) are recycling (Wirawan et al., 2012).

1.3 Autophagy regulation

Autophagy is strongly regulated by two upstream protein complexes: AMPK and mTOR complex, the major energy-sensing kinases in the cell (Kroemer et al., 2010).
AMPK is a protein kinase activated by high AMP/ATP levels: with low energy levels, AMPK directly or indirectly inhibits mTOR, by phosphorylation (Parzych and Klionsky, 2014).
mTOR includes two different multimeric protein complex: mTORC1 and mTORC2 (Laplante and Sabatini, 2012). They have different functions and rapamycin sensitivity: mTORC1 is inhibited by rapamycin and growth factors, controls protein synthesis and it is the major nutrient sensing protein. The main mTORC1 targets are the ribosomal protein P70S6 kinase (P70S6K) and Eukaryotic translation initiation factor 4E- binding protein 1 (4EBP1) (Jacinto and Lorberg, 2008; Ma and Blenis, 2009).
When mTORC1 is active, phosphorylates 4EBP1, inactiving it; conversely, P70S6K phosphorylation activates the kinase and stimulates protein synthesis (Ma and Blenis, 2009).
mTORC2, instead, is insensitive to rapamycin treatment, even though some studies show that chronic exposition to rapamycin can inhibit *de novo* mTORC2 complex formation (Li et al.., 2014). In addition, mTORC2 is involved in cellular metabolism and cytoskeleton formation by F-actin synthesis (Laplante and Sabatini, 2013).

AMPK and mTORC1 lead to ULK1 phosphorylation, with opposite effects: AMPK operates the activating phosphorylation on ULK1, whereas mTORC1 phosphorylation negatively affects its activity by preventing ULK1-AMPK interaction (Kim et al., 2011).
In shortage of amino acids and growth factors in extracellular environment, inhibition of mTORC1 complex by AMPK leads to its dissociation from ULK1 complex, promoting ULK1 activation and Atg13 hyperphosphorylation and, therefore, autophagy activation (Jung et al., 2009) (Fig.4).

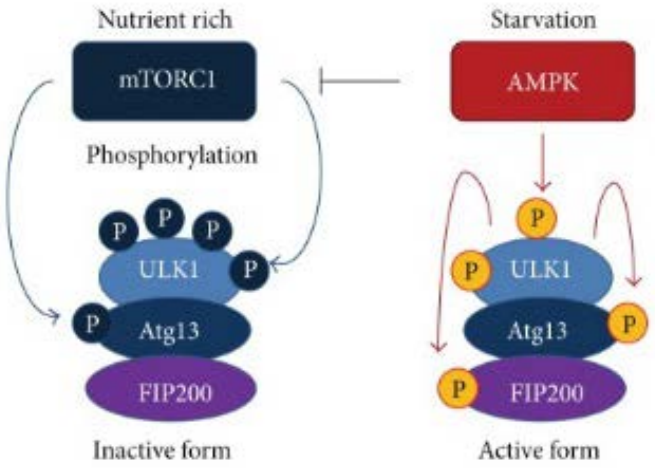

Figure 4. Autophagy regulation. Under nutrient rich conditions, active mTORC1 phosphorylates ULK1inhibiting it and, as a consequence, inhibiting the autophagy. In contrast, under starvation conditions, active AMPK inhibits mTORC1 and phosphorylates ULK1, activating it and leading to Atg13 hyperphosphorylation and autophagy induction (Adapted from Yamahara et al., 2013).

1.4 Autophagy role in pathology

As described above, autophagy leads misfolded proteins and damaged organelles to degradation in order to maintain the normal cellular homeostasis. As a consequence of a dysfunctional autophagy, accumulation of abnormal protein aggregates and organelles could cause several diseases. Furthermore, several studies have shown that mutations in most ATG genes have pathogenic roles in human diseases, most of which are neurodegenerative in nature (Komatsu et al., 2006; Hara et al., 2006). Accordingly, autophagy defects can be lethal or can leads to severe phenotypes in the vertebrates, depending on whether the mutated gene is involved in early or later stages of development. For instance, Atg5 -/- and Atg7 -/- mice show protein aggregates and inclusion bodies in hepatocytes (Atg7 -/-), cardiomyocytes (Atg5 -/-) and neural cells (Atg5 -/- or Atg7 -/-) (Wirawan et al., 2012; Komatsu et al., 2005; Hara et al., 2006). Atg5 autophagy deficient mice can survive to embryogenesis but are going to die during perinatal period because of an incapacity to feed (Di Bartolomeo et al., 2010; Kuma et al., 2004) (Fig.5). As shown in figure 5, Ambra1 is one of the main autophagy genes whose homozygous mutation leads to embryonic lethality and several defects in the central nervous system such us hyperproliferation of the neuroepithelium, midbrain and hindbrain exencephaly and defective closure of the neural tube (Di Bartolomeo et al., 2010).

The role of autophagy in pathophysiology of some disorders is controversial: in the case of Huntington's and Parkinson's diseases, elevated autophagy level seems related to the pathology onset. Conversely, Alzheimer's disease is linked to altered autophagy due to an autophagy flux engulfment (Petibone et al., 2018).

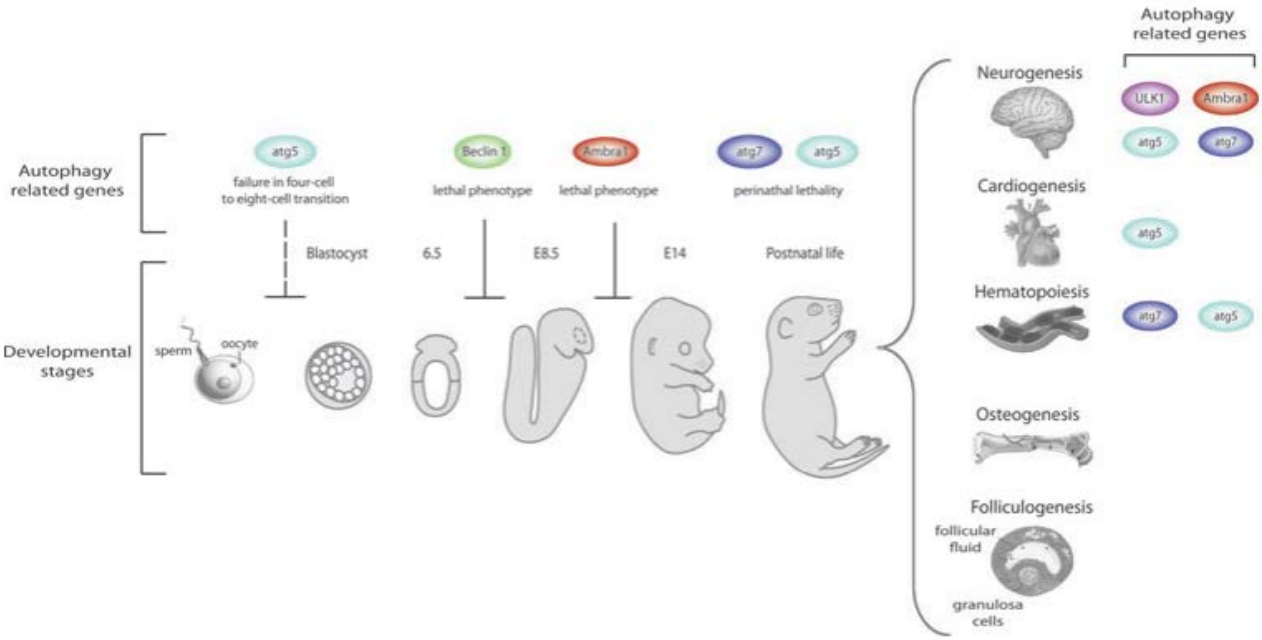

Figure 5. Autophagy in mammals development. The involvement of some of the main autophagy genes is shown. Depending on the developmental stage, autophagy deficient mice can die before birth or during perinatal period or show defects about different organogenesis (Di Bartolomeo et al., 2010).

1.5 Autophagy and cancer

The relation between autophagy and cancer is still controversial. Several works show that autophagy plays a dual role in cancer depending on tumor stage. In the early stages of tumorigenesis, autophagy seems to have an oncosuppressive role. On the other hand, autophagy is likely involved in tumor protection during tumor establishment. This is because, during tumor onset, autophagy avoids genotoxic molecules accumulation, such as aggregates of ubiquitinated proteins and reactive oxygen species (ROS) produced by dysfunctional mitochondria (Mathew et al., 2009). Moreover, autophagy is also required to maintain the physiological metabolic homeostasis preventing accumulation of damaged organelles and to contrast malignant transformation and genome instability (Green et al., 2014).

Furthermore, Beclin1, Atg4, Atg5 and other autophagy markers, have been identified as tumor suppressors (Mariño et al.., 2007; Yousefi et al.., 2006). Finally, it is known that the oncosuppressors P53 and PTEN are autophagy inducers (Yoon et al., 2012), whereas the oncogene Bcl-2 directly interacts with Beclin-1, leading to autophagy inhibition.

With the establishement of the tumor mass, autophagy is used to promote cells survival in a microenvironment characterized by several stresses such as hypoxia and nutrients deprivation, in

order to escape the response to cancer and drugs (Guo et al.., 2013). For instance, some studies have shown that growth factor deprivation can lead to autophagy increase, demonstrating that this process supports the survival of tumor cells to starvation conditions (Palumbo et al., 2014).

2. Glioma

2.1 Classification of Glioma

"Glioma" is a term used to indicate glial cell tumors, mainly originating from astrocytes and oligodendrocytes (Chen et al., 2017). To date, the classification by World Health Organization (WHO) is the international standard for glioma nomenclature and diagnosis, relying on histomorphological criteria. WHO classifies astrocytic glioma in: grade I (Pilocytic astrocytoma, rarely causes death), grade II (Diffuse astrocytoma), grade III (Anaplastic astrocytoma) and grade IV (Glioblastoma, also known as Glioblastoma multiforme).

Glial cell tumors originated from oligodendrocytes are classified in Oligodendroglioma (grade II) and Anaplastic oligodendroglioma (grade III). Finally, tumor mass originated from both types of glial cells are oligoastrocytic tumors, classified in Oligoastrocytoma (grade II) and Anaplastic oligoastrocytoma (grade III) (Louis et al., 2007) (Tab.1).

Table 1. Glioma classification

Phenotype	Subtype	Grade
Astrocytic tumors	Pilocytic astrocytoma	I
	Diffuse astrocytoma	II
	Anaplastic astrocytoma	III
	Glioblastoma	IV
Oligodendroglial tumors	Oligodendroglioma	II
	Anaplastic oligodendroglioma	III
Oligoastrocytic tumors	Oligoastrocytoma	II
	Anaplastic oligoastrocytoma	III

2.2 Glioblastoma multiforme

Glioblastoma Multiforme (GBM) is the most common brain tumor in the adult. Compared to women, white men are more affected and the median age of patients is 64 years. Despite ongoing research, patient life span remains lower than 15 months after diagnosis (Ostrom et al., 2018).
GBM is often located in cerebral hemispheres, commonly in the supratentorial region, and less often in brainstem, in the cerebellum and in the spinal cord (Hanif et al.., 2017).
It is characterized by a high grade of heterogeneity, vascularization and an infiltrating behaviour. Indeed, tumor mass shows poorly delineated margins, that makes it not distinguishable from normal tissue. Moreover, it is not common to find distant metastases from site of tumor development, both within and outside the Central Nervous System (CNS) (Louis et al., 2007).

2.2.1 GBM subtypes

GBM can be divided in two subtypes, primary and secondary GBMs, that usually evolve in patients at different ages. Primary subtype arises *de novo* in older patients, and represents the most common GBM with less aggressive features. It shows several genetic characteristics such as Epidermal Growth Factor Receptor (*EGFR*) amplification, *p16* deletion and *PTEN* mutations. At the other hand, secondary GBM is less common than the previous one and slowly arises in younger patients from lower grade of astrocytoma. It can show the same features of primary GBM but also p53 gene mutations. In both of them, these genetic mutations can lead to an uncontrollable cells growth (Ohgaki and Kleihues, 2007).
GBM can be also classified into four subtypes by gene expression profile: Classical, Mesenchymal, Proneural and Neural (Verhaak et al., 2010).
The first subtype, also named Proliferative subtype, shows chromosome 7 amplification paired with chromosome 10 loss, the most frequent event in GBMs. It is often defined by *EGFR* amplification and mutation (mainly vIII EGFR mutation) and *PTEN* loss. Neural precursors and stem cell markers are highly expressed. Furthermore, Classical subtype shows astrocytic features in culture and responsiveness to the radio and chemo therapies, that leads to a significantly decrease in mortality upon treatments (Verhaak et al., 2010; Van Meir et al., 2010).
The Mesenchymal type has usually Neurofibromin1 (*NF1*) gene mutations but also *PTEN* and *TP53* inactivation. As a consequence of Epithelial to Mesenchymal Transitions (EMT), it shows high levels of mesenchymal and astrocytes markers such as *CD44*. This tumor responds to current chemotherapy and shows a decrease in mortality as well as the previous one. Furthermore, most of immortalized

glioma cell lines display markers patterns similar to the Mesenchymal subtype (Verhaak et al., 2010; Van Meir et al., 2010).

The Proneural type is characterized by *PDGFRA* (Platet-Derived Growth Factor Receptor-α), *IDH1* (Isocitrate Dehydrogenase 1 gene) and *TP53* mutations. It typically shows oligodendrocytic but not astrocytic features and usually arises in younger patients, with no survival rate alteration after treatments (Verhaak et al., 2010; Van Meir et al., 2010).

Finally, the Neural type is defined by the expression of neuron markers, with low infiltration capabilities and oligodendrocytic and astrocytic differentiation (Verhaak et al., 2010; Van Meir et al., 2010).

2.3 Therapeutic approaches

To date, standard GBM therapy consists of surgery resection that, when possible, is used to reduce the tumor mass size even though, generally, it cannot completely eliminate it. Surgery is usually followed by radiotherapy, to kill tumor cells remained after resection, and chemotherapy (Stupp et al., 2005). Temozolomide (TMZ) is the most common chemotherapeutic drug now used for GBM treatment. It is an oral alkylating agent able to cross the blood-brain barrier and to cause DNA methylation in the O^6 position of guanine, leading to genomic instability and apoptosis (Daniel et al., 2019). On one hand this treatment can increase patient lifespan but, on the other hand, TMZ could lead to resistance phenomenon, caused by MGMT (O6-methylguanine–DNA methyltransferase) overexpression (Hegi et al., 2005).

Novel approaches are necessary to contrast GBM development. Several studies, indeed, have demonstrated that EGFR, PDGFR and VEGFR, as receptors, can be an effective target into GBM therapy as well as mTOR/PI3K related targets that could avoid resistance phenomena occurrence in GBM (Tabernero, 2007).

3. Autophagy and glioma

Several contradictory studies have shown the dual role of autophagy in tumorigenesis even in glioma. It is known that some important autophagy players, such as *BECLIN1* but also *LC3*, are less expressed in high-grade gliomas compared to low-grades (Pirtoli et al., 2009; Huang et al., 2010). Furthermore, Palumbo and colleagues, in 2012 and in 2014, observed that autophagy has a role in enhancing cell radiosensitivity after both Ionizing Radiation (IR) and IR/TMZ treatments, especially when EGFR is inhibited (Palumbo et al., 2012; Palumbo et al., 2014). Finally, in 2015, Catalano et al. demonstrated

that an autophagy induction via nutrients starvation or mTOR pathway inhibition, induces a strong impairment of both migration and invasiveness in immortalized and primary GBM cell lines, leading to a reversion of EMT phenotype (Catalano et al., 2015).

On the other hand, in 2012 Galavotti et al. reported that some autophagy associated genes, such as *DRAM1* and *SQSTM1*, are highly expressed in GBM tumors, leading to a modulation of migration and invasion capabilities in Glioma Stem Cells (GSMs) (Galavotti et al., 2012).

Further investigations about the link between autophagy and glioma are also required in order to clarify the possible use of autophagy as trigger in GBM therapy. Indeed, TMZ induces autophagy in GBM cell lines but this seems to lead to resistance phenomena, suppressing its anti tumoral effect. It has been shown that TMZ cytotoxic effect is recovered by using pharmacological inhibitors of autophagy late stages in co-treatments (Kanzawa et al., 2004). On the contrary, a combination of TMZ and mTOR inhibitors, that are known to lead to autophagy induction, as well as several compounds, for instance cannabinoids (THC), results in autophagy-dependent cell death (Josset et al., 2013; Torres et al., 2011).

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Objectives and articles

The aim of this doctoral thesis was to deepen the knowledge on GBM biology with a particular focus on the autophagy role in signalling pathways involved in cell proliferation and invasion in GBM models.

Although alterations of some autophagy regulators in GBM and their correlation to invasion and migration of GBM cells have been already observed, little is known about the autophagy impact on GBM onset and progression. Furthermore, it has been recently demonstrated that autophagy modulation reverses EMT in GBM cells, switching from a mesenchymal to an epithelial-like phenotype through a down-regulation of EMT regulators SNAI.

Moreover, it is well known that brain tumors, including GBM, frequently show altered autophagy-associated molecules, such as EGF receptor, resulting in autophagy dysregulation.

Based on this evidence, we speculated that the autophagy machinery could be alterated in GBM and, that autophagy modulation could represent and effective tool to contrast GBM progression.

To verify this hypothesis, specific goals of the project were:

- To analyze the effect of autophagy induction on Wnt/β-catenin signalling, a pathway often alterated in several cancers, including GBM, and also correlated with EMT (research article I and review article, published).
- To investigate the effects of autophagy modulation on EGFR expression and sub-cellular localization and, subsequently, on EGFR-mediated signalling pathways in GBM models (manuscript II, submitted).

I

Autophagy induction impairs Wnt/β-catenin signalling through β-catenin relocalisation in glioblastoma cells

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1. Introduction

Macroautophagy (hereafter referred as autophagy) is an evolutionary conserved and finely regulated process, mediating lysosomal degradation of cytoplasmic material, long-lived and damaged proteins, and entire organelles [1,2]. During autophagy, double-membrane vesicles, named autophagosomes, form and engulf cytoplasmic material, which is then delivered to lysosomes for degradation [3]. In shortage of nutrients, mainly aminoacids, the nutrient sensor mTOR is inhibited and Ulk1 complex can drive a massive autophagosome formation [4]. Nevertheless, in physiological conditions, a basal level of autophagy contributes to maintain the proper cell homeostasis, both during embryogenesis and adulthood, and indeed its deregulation has been associated to many human diseases, including cancer [5–7]. We have recently demonstrated that autophagy modulation regulates the migration and the invasion capabilities of glioblastoma multiforme (GBM) cells, by down-regulating epithelial-to-mesenchymal transition (EMT) factors SNAIL and SLUG, and, subsequently, up-regulating cadherin expression [8].

β-catenin is a multitasking and evolutionary conserved protein that plays a crucial role in a plethora of developmental and homeostatic processes [9,10]. It is, in fact, a structural component of the cell-cell adhesion structures, and as a component of the canonical Wnt pathway, it regulates cellular proliferation, adhesion, and movement [9,11]. Deregulation of Wnt signalling is often associated with initiation, proliferation, and invasion of several cancers, including GBM [11,12]; β-catenin and other components of the Wnt pathway are, in fact, commonly overexpressed and the pathway is constitutively active in GBM [13,14]. The Wnt/β-catenin pathway plays also a role in EMT, by promoting transcription and stabilization of the EMT activators SNAIL and ZEB1 [15,16], or by other mechanisms [17,18].

Few literature evidence exist on the relationship between autophagy and Wnt/β-catenin signalling [19–21]. It has been shown that Wnt/β-catenin activity represses autophagy and

the expression of the autophagic adaptor p62, and indeed that nutrient deprivation induces β-catenin degradation via autophagic machinery [22]. In 2014, Gao et al. demonstrated that, in colorectal cancer cells, autophagy negatively regulates Wnt signalling by promoting the degradation of the pathway mediator Dishevelled [23]. Moreover, it has been recently shown that the anti-helminthic drug Niclosamide induces autophagy and down-regulates the Wnt and other pro-survival signal transuction pathways in GBM cells [24].

Our findings indicate, for the first time, that autophagy induction attenuates the Wnt signalling in GBM cellular models and that it leads to β-catenin relocalisation within the cell. These findings further support the idea that autophagy modulation could represent a potential therapeutical strategy to contrast GBM proliferation and invasiveness.

2. Material and methods

2.1 Cell culture

Human GL15 cells were kindly provided by Dr. E. Castigli, Perugia University, Italy. Primary human GBM130 cells were obtained, as previously described [25], after surgery at Policlinico Umberto I (Rome) from GBM patients that gave a written informed consent to the research proposals. The study was approved by the Institutional Ethics Committee of Sapienza University and by Ministry of Health. Histopathological typing was done according to the WHO criteria resulting as grade IV. Human GL15, U87MG cells and GBM130 were cultured in DMEM (Lonza, Basel, Switzerland), supplemented with 10% heat-inactivated FBS (Euroclone, Milan, Italy) and 1% penicillin/streptomycin solution (Euroclone, Milan, Italy). Cells were grown at 37 °C in a 5% CO_2 humidified atmosphere. For autophagy induction, cells were cultured in Earle's Balanced Salt Solution (Sigma Aldrich, Milan, Italy) or in the presence of 250 nM Torin 1 (Sigma Aldrich, Milan, Italy) for 18 h. In order to analyse the autophagic flux, 20 μM Chloroquine(CQ) was added to the culture media.

shBeclin and sh-Ctr cells were prepared by lentiviral infection as previously described [8]. GL15 cells were infected by incubation with lentivirus containing supernatant for 6 and 8 h in presence of 4 μg/ml poly-brene.

2.2 qPCR

Total RNA was isolated with the RNeasy mini kit (QIAGEN, Hilde, Germany). 1 μg RNA was retrotranscribed using M-MLV enzyme and oligodT (Promega, Madison, Wisconsin, USA). qPCR was performed using SYBR GREEN PCR Master Mix Applied Biosystems (Thermo Fisher Scientific, Waltham, Massachusetts, USA), with the QuantStudio 12K Flex Applied biosystem. The following pairs of primers were used: axin2 (5′-AGTGTGAGGTCCACGGAAAC-3'; 5´-CTTCACACTGCGATGCATTT-3'), cyclin D1: (5´-CTGGCCATGAACTACCTGGA-3′; 5′-CTCCGCCTCTGGCATTTTGG-3´); c-myc: (5′-AGCGACTCTGAGGAGGAACA-3′; 5′-CTCTGACCTTTTGCCAGGAG'); β2m (5′-CTCCGTGGCCTTAGCTGTG-3′; 5′-TCTCTGCTGGATGACGTGAG-3′). mRNA expression level for the genes of interest was calculated by means of the ddCt method, with β2m expression as standard. The mRNA levels were then expressed as variations in respect to the control condition. Reported values are the means of three independent biological replicates.

2.3 Western blotting and antibodies

Proteins were separated by means of SDS-PAGE and electroblotted onto nitrocellulose (GE Healthcare, Life Sciences, Little Chalfont, Buckinghamshire, UK). Membranes were incubated with primary antibodies diluted in PBS/5% non-fat dry milk/0.1% Tween-20 overnight at 4°C. Detection was achieved using horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Milan, Italy) and visualized with ECL plus (GE Healthcare, Life Sciences, Little Chalfont, Buckinghamshire, UK). The following primary

antibodies were used: anti-p62 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LC3B (Cell Signaling, Danvers, MA, USA), anti-BECN1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-β-catenin (BD Biosciences, Franklin Lakes, New Jersey, USA), anti-active β-catenin (S37/T41) (Millipore, Billerica, MA, USA), anti-Dvl2 (Cell Signaling, Danvers, MA, USA), anti-Lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti- β-Actin (Sigma Aldrich, Milan, Italy).

2.4 Cell lysis and subcellular fractionation

The appropriate amount of Nucleus Buffer (1 mM K_2HPO_4, 14 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA supplemented with 0.1 mM DTT, 0.3% Triton and protease inhibitor cocktail) were added to pelletted cells and, after incubation on ice for 30 min, centrifuged at 850 g for 10 min, to isolate the nuclear fraction. Nuclei were washed twice with fractionation buffer, centrifuged as above and resuspended in RIPA Buffer (50Mm Tris HCl pH 7.4, Triton 1%, Na Deoxycholate 0.25%, SDS 0.1%, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$ supplemented with protease inhibitors cocktail). Samples were then briefly sonicated (Hold 6 constant, 30''). Proteins concentration was determined using Lowry protein assay (Bio-Rad, Hercules, CA, USA).

2.5 Immunocytochemistry and confocal analysis

Cells were grown on coverslips and fixed with 4% PFA in PBS, followed by permeabilization with 0.1% Triton X-100 in PBS. β-catenin (BD Biosciences, Franklin Lakes, New Jersey, USA) and N-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies were incubated overnight at 4°C and visualized by means of Alexa Fluor (Invitrogen, Carlsbad, CA, USA). Coverslips were mounted in antifade (SlowFade; Invitrogen, Carlsbad, CA, USA) and examined under a confocal microscope

(TCS SP8; Leica, Wetzlar, Germany), equipped with a 40x 1.40–0.60 NA HCX Plan Apo oil BL objective at RT.

Regarding ImageJ software analysis, the pixels of two 8-bit images (red and green channels of each image) are considered colocalized if their intensities are higher than the threshold of their channels (set at 50) and if the ratio of their intensity is higher than the ratio setting value (set at 50%). Colocalization was assessed by calculating the Pearson's correlation coefficient *r* of at least 10 cells analyzed in two independent experiments. The Pearson's correlation coefficient was expressed as mean ± SD. The fluorescence intensity of each fluorochrome was simultaneously analyzed and plotted.

2.6 Statistical analysis

All experiments were performed at least three times. Sigma Plot software was used for statistical analysis. Statistical significance was determined by using the Student's *t*-test. P value ≤ 0.05 was considered significant.

3. Results

3.1 Wnt/β-catenin signalling is negatively regulated by autophagy in GBM cells

In order to analyse the effect of autophagy induction on Wnt signalling in GBM cells, autophagy was induced by means of 18 hours aminoacid- and serum-starvation (EBSS treatment) in GL15 and U87MG cells, as previously described [8] and as shown in Fig. 1a. qPCR analysis has been performed to measure the relative mRNA levels of the β-catenin target genes *axin2*, *cyclinD1* and *c-myc* in both control and in EBSS-starved cells. We observed a significative reduction in *axin2* and *cyclinD1* mRNA levels in both EBSS-treated GL15 and U87MG cell lines, in respect to control cells, grown in nutrient-rich medium (Fig. 1b). Similar results were obtained when autophagy was induced by culturing GBM cells in the presence of the mTOR inhibitor Torin1 (Torin) (Supplementary Figure

S1a and b). Differently from other cellular systems, we could not detect any significative variation of *c-myc* mRNA levels in cells grown in EBSS or in Torin presence (Fig. 1b and not shown). In order to prove the role of the autophagic process in promoting β-catenin de-activation upon nutrient starvation, we knocked down the autophagy regulator Beclin1, by means of specific siRNA. GL15 cells lacking of Beclin1 showed a reduced basal autophagy levels, and a decreased capability to accumulate LC3II and to degrade p62 substrate upon nutrient deprivation (Fig. 1c). In addition, the reduction of β-catenin target genes mRNA, that we observed in control cells upon nutrient deprivation, was prevented by Beclin1 silencing, almost completely for *axin2* and, at lesser extent for *cyclinD*, that is much more responsive to starvation (Fig. 1d). Moreover, a slight, but significative, increase of the basal level of *cyclinD* mRNA was observed in Beclin1-defective cells in nutrient-rich conditions thus further suggesting role for autophagy in modulating Wnt/β-catenin signalling (Fig. 1d).

3.2 Dishevelled protein is degraded upon autophagy induction

Dishevelled (Dvl) protein is a component of the protein family that mediates Wnt pathway by binding to Frizzled receptor and by inhibiting the APC-containing destruction complex [26]. Negative modulation of Wnt signalling has been linked to Dvl degradation upon autophagy induction in HEK293 and in colorectal cancer cells [23].

We analyzed the expression level of Dvl2 and, similarly to what observed in HEK293 cell line, both GL15 and U87MG cells showed an appreciable reduction of the protein upon autophagy induction (Fig. 2a and Supplementary Figure S1c). The same result was obtained

in GBM130 primary cells grown in EBSS medium (Fig. 2a).

On the other hand, no variation in Dvl2 protein levels was observed in Beclin1-devoid cells, thus demonstrating that autophagy induction is necessary for Dvl2 degradation (Fig. 2b).

3.3 The expression level of the transcriptional active β-catenin decreases upon autophagy induction

In 2013, Petherick et al. have demonstrated that nutrient deprivation leads to proteasome-independent and autophagosome-mediated β-catenin degradation in colorectal carcinoma cells, that was likely responsible for Wnt/β-catenin signalling inactivation [22]. Moreover, Cheng et al. have been shown that Niclosamide induces autophagy and β-catenin degradation in GBM cells [24] . To test if β-catenin was degraded upon nutrient deprivation in glioma models, we analysed β-catenin amount by western blot, and we did not observe any difference in the total protein levels between control and 18 h-starved GL15 and U87MG cell lines. However, by means of an antibody specifically directed against the de-phosphorylated (S37/T41) β-catenin, we observed a significative decrease of the nuclear transcriptionally active protein pool in autophagic glioma cells in respect to control cells (Fig. 3a and Supplementary Figure S1c). This reduction of the active β-catenin pool was not observed in Beclin1-devoid cells, thus suggesting a direct involvement of Beclin1-dependent autophagy in β-catenin transcriptional activity (Fig. 3b).

3.4 Autophagy induction induces β-catenin relocalisation in GBM cells

In line with the so far obtained results, we observed, by means of subcellular fractionation experiments, a significative reduction of the β-catenin nuclear fraction and a slight increase in the cytosolic one, upon 18 hours of EBSS- and Torin- culturing in the cell lines analyzed (Fig. 4a and Supplementary Figure S1d). Interestingly, this starvation-dependent reduction

in the β-catenin nuclear fraction upon starvation was completely prevented in GL15 cells lacking of Beclin1 protein although no difference in the β-catenin nuclear basal level was observed (Fig. 4b). In order to verify a possible re-localisation of the cytosolic β-catenin pool, we analyzed the protein's expression pattern in GL15 and in U87MG glioblastoma cells by immunocytochemistry. Widespread expression of the protein within the cells was observed in control conditions (Fig. 4c, upper panels), whereas a relevant accumulation of β-catenin in sub-plasma membrane areas was observed in cells cultured for 18 hours in EBSS (Fig. 4c, bottom left and middle panels) or in Torin presence (Supplementary Figure S1e, middle panels and not shown), but not in Beclin1-silenced GL15 cells (Fig. 4c, bottom right panel). Strikingly, we observed a clear enlargement of the cells and the appearance of cell-cell contacts, highlighted by β-catenin signal, that were almost completely absent in control cells (Fig. 4c and Supplementary Figure S1e).

3.5 β-catenin associates with N-cadherin upon autophagy induction

Based on the evidence of β-catenin relocalisation in sub-plasma membrane areas, that suggested a possible interaction with membrane proteins involved in cell-cell interactions, we then analyzed N-cadherin localisation within glioma cells in our experimental conditions. Confocal analysis showed a diffuse N-cadherin intracellular localization in U87MG and GL15 control cells (Fig. 5, left upper panels). Conversely, in EBSS-cultured cells, N-cadherin staining overlapped with β-catenin signal in correspondence of cell-cell contacts (Fig. 5). The same result was observed in Torin-treated U87MG (Supplementary Figure S1e, bottom panels). This result suggested that autophagy induction promotes the association between β-catenin and N-cadherin in glioma cells, and that this event is likely responsible for newly formed N-cadherin-mediated cell-cell junctions.

4. Discussion

A number of studies report Wnt signalling as aberrantly activated in GBM, thus promoting tumour growth and propagation, by transcriptional activation of multiple target genes, involved in both proliferation and invasion [13]. In the recent years, it has been reported that in HEK293 and in colorectal cancer cells, Wnt signalling could be attenuated under nutrient deprivation by accelerating autophagy-mediated Dvl degradation [23]. In addition, in nutrient-stressed colorectal cancer cells, β-catenin can be per se a direct target of autophagic degradation, in a proteasome-independent manner [22]. β-catenin down-regulation and Wnt pathway inhibition has been also observed in a GBM cellular model treated with the anti-helminthic drug Niclosamide, that is also able to induce autophagy, although the mechanism of action remains to be elucidated [24].

Here we demonstrate that autophagy induction negatively regulates Wnt/β-catenin signalling in GBM cellular models. We report, for the first time, that nutrient starvation and m-TOR inhibition strongly attenuate *cyclinD1* and *axin2* transcription, through an autophagy-dependent mechanism, as suggested by the rescue observed in Beclin1 silenced cells. Conversely, *c-myc* transcription (and *n-myc* too, data not shown) is not affected by nutrient starvation or by m-TOR pharmacological inhibition, thus suggesting a Wnt-independent regulation of *myc* genes in GBM cells. The decrease in Dvl and in axin2 expression that we observed could explain, at least partially, the impairment of Wnt/β-catenin signalling obtained.

We also found that autophagy activation by nutrient deprivation or by m-TOR inhibition does not result in β-catenin degradation in GBM cells, as observed in a colorectal model, but, surprisingly, we observed that autophagy promotes β-catenin relocalisation within the cell. In fact, we observed a decrease of the β-catenin nuclear fraction, coupled to an accumulation of the cytosolic one. In particular, immunofluorescence experiments

demonstrated a different signal distribution of the protein among nutrient rich- and nutrient poor- conditions. Upon autophagy induction, β-catenin appears mainly localized in sub-membrane areas where it decorates the cell-cell contacts which are much more evident than in control cells. The appearance of an epithelial-like phenotype of these cells was confirmed by the co-localisation of β-catenin with N-cadherin, in correspondence of the cell-cell contacts.

By our previous observations, we know that N- and R-cadherin mRNA level increase in glioma cells upon autophagy induction, likely as consequence of Snail1/2 down-regulation [8]. The role of N-cadherin in EMT is not completely understood. In epithelial tumours (carcinomas), in fact, the cadherin switch, from E- to N-isoform, is thought as a hallmark of EMT occurrence [27,28], but the contribution of cadherin isoforms in glioma EMT is less documented. It has been demonstrated that expression of N-cadherin inversely correlates with the invasive behaviour of GBM cells [29,30], and the instability and disorganisation of cadherin-mediated junctions are likely associated to GBM invasive behaviour [31,32]. Our data are consistent with a newly acquired role for β-catenin in supporting the EMT reverse process (mesenchymal-to-epithelial-like transition, MET) observed in glioma cells when they are forced to undergo autophagy [8].

The role of autophagy in GBM pathogenesis is still a matter of debate [33,34]; autophagy induction has been observed in GBM in response to radio- and temozolomide-based therapy and even though a number of clinical trials aimed at inhibiting autophagy execution, mainly by Chloroquine, have been launched, others directed to inhibiting mTOR pathway and thus activating autophagy, are ongoing [35,36]. Although further studies are necessary to better dissect autophagy role in GBM progression, our findings support the idea that autophagy modulation could represent a potential therapeutical strategy to contrast GBM invasiveness.

5. Conclusions

GBM is the most common and lethal adult brain tumour and it is characterized by a highly invasive behaviour. An aberrantly activated Wnt/β-catenin signalling is thought responsible for GBM invasion capability, beside for tumour proliferation, and stemness maintenance.

We have found that autophagy negatively regulates Wnt/β-catenin signalling in glioblastoma cellular models. Moreover, we observed that β-catenin relocalises within the GBM cell and associates with N-cadherin, thus contributing to mesenchymal-to-epithelial-like transition. The findings reported here add important clues for the definition of possible therapeutical strategies, targeting the autophagic process, to contrast GBM progression.

Author contributions

S.D.B. conceived the project. B.C. and S.D.B. designed experiments. M.C.[2] designed and performed Real Time experiments. B.C. and F.F. carried out biochemical and immunofluorescence experiments and prepared figures. G.D. and M.C.[3,4] obtained primary cells and contributed to design and analyze experiments. A.S. provided tumour biopsies for primary cultures. B.C. and S.D.B. wrote the paper with input from all authors. C.L. and F.C. supported the research and critically read the paper.

Conflict of interest declaration

The authors declare no conflict of interest.

Acknowledgments

We thank E. Castigli for kindly providing us with GL15. We also thank G.M. Fimia and M. Antonioli for shBeclin and shCtr viruses, and C. Rodolfo for helpful discussion and critical reading. This work was supported by grants from PRIN (2015 E8EMCM) and AIRC

(IG2015-16699) to CL, and from FFABR (Italian Ministry of University and Research) to SDB. CL is member of the Twinning project H2020-692340 "Synanet".

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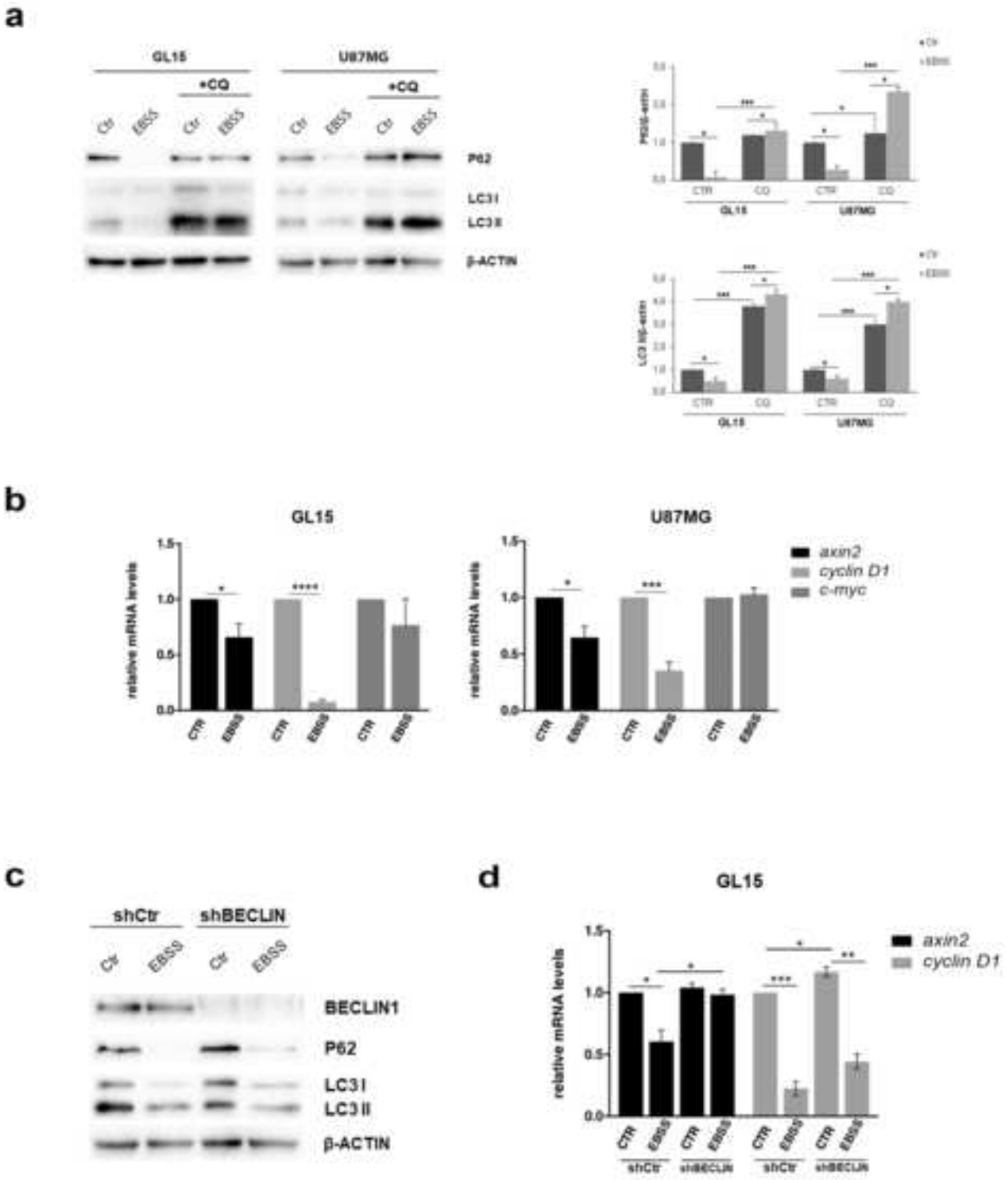

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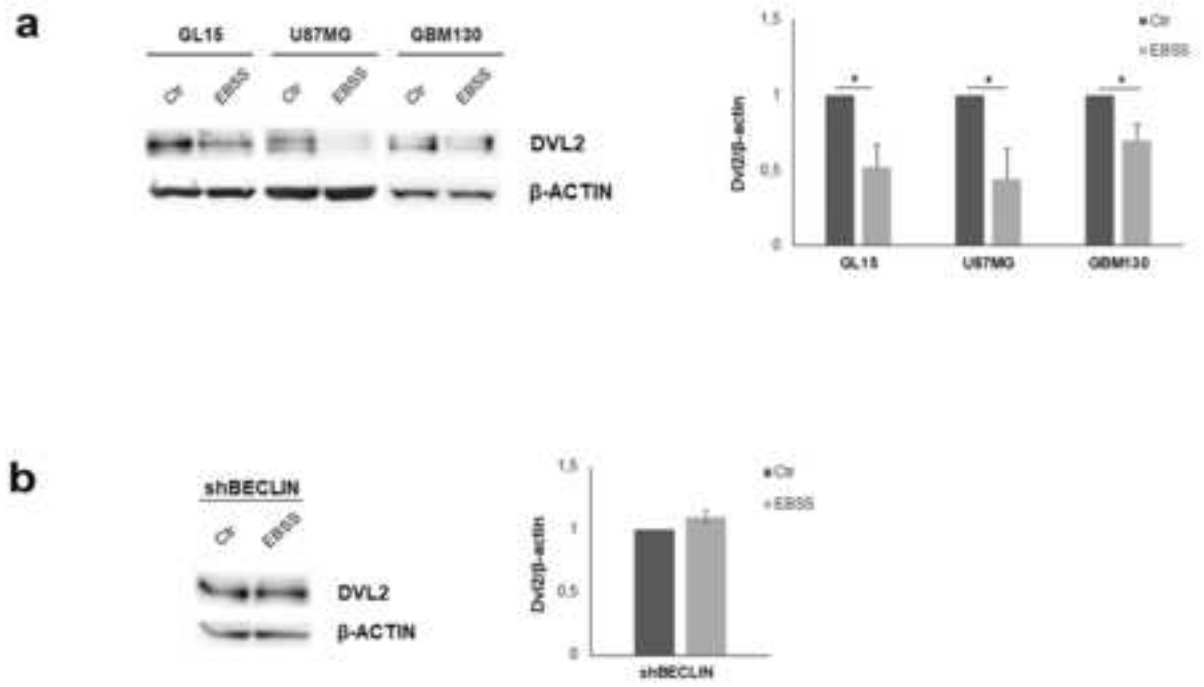

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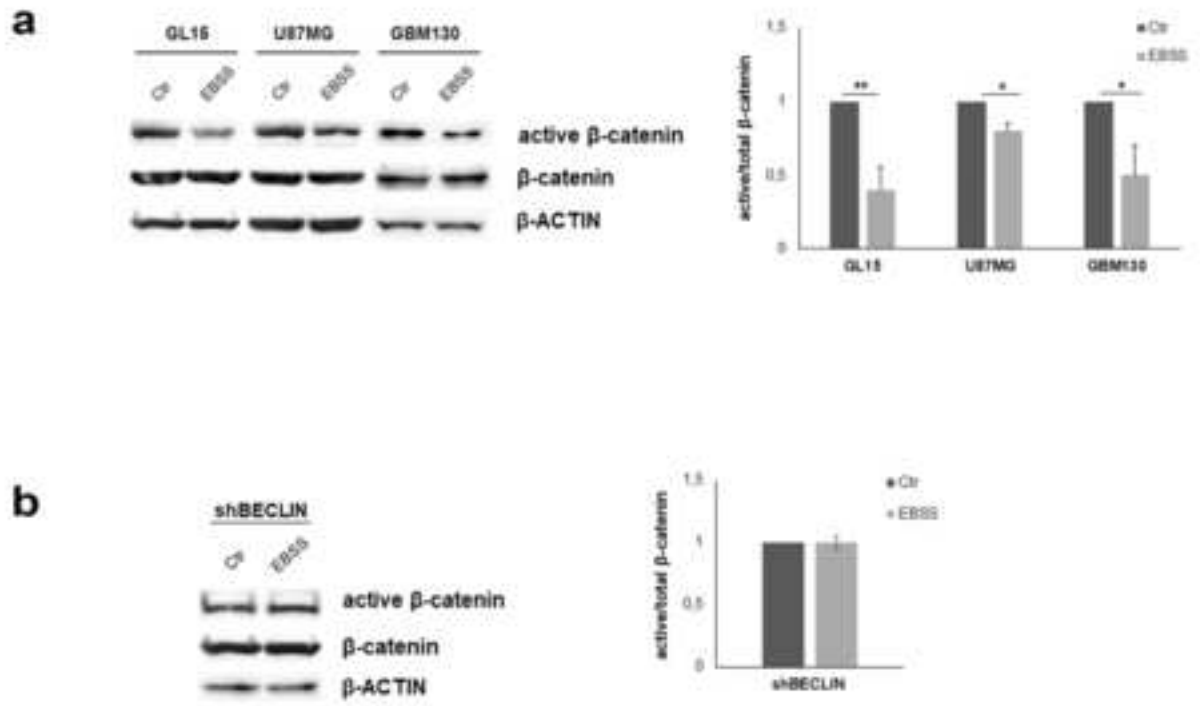

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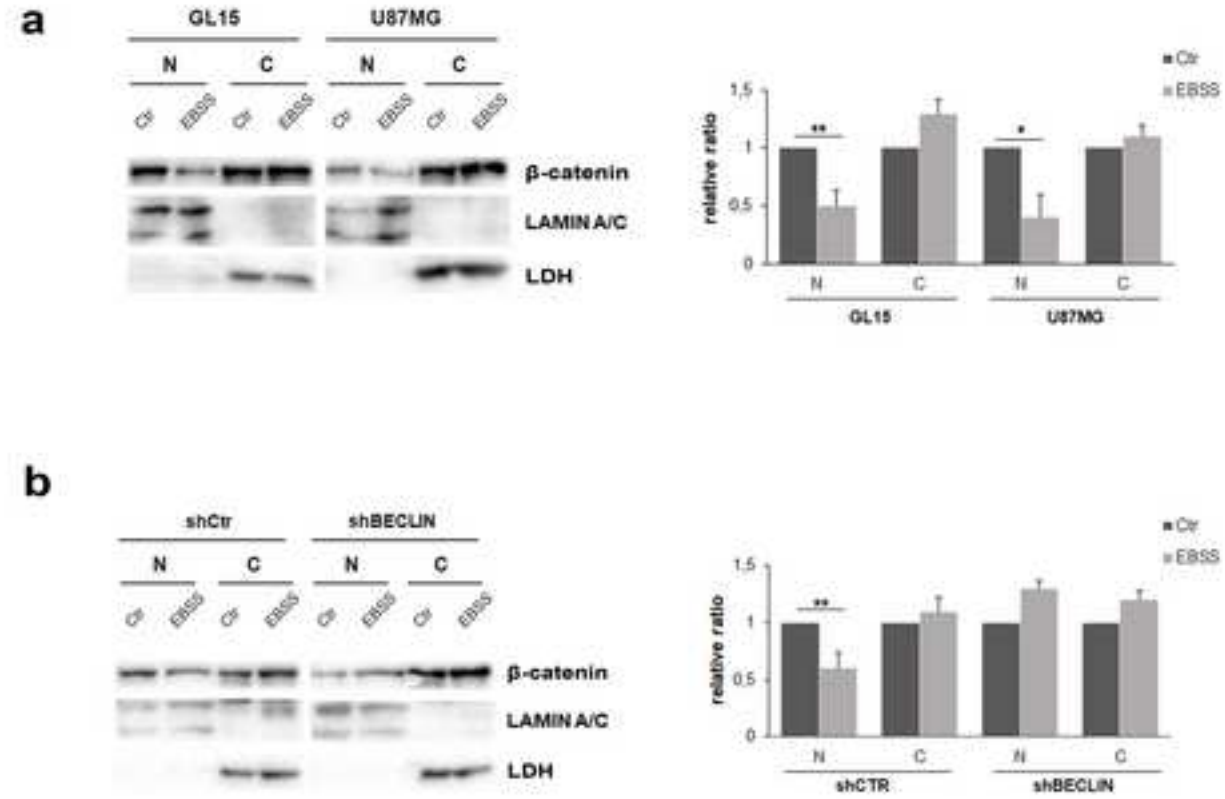

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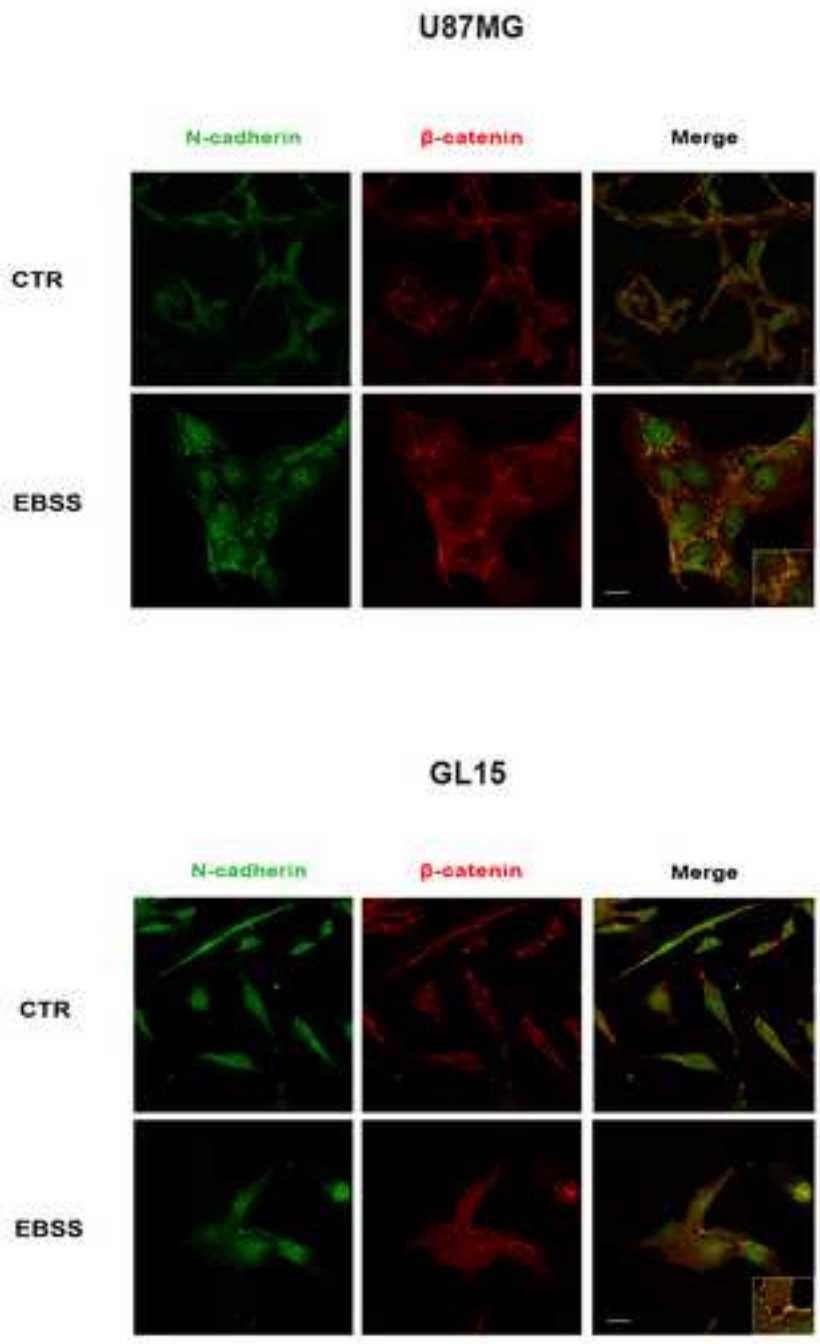

Fig. 5

Figure legends

Figure 1 Autophagy induction reduces Wnt signalling. **(a)** GL15 and U87MG cells were cultured in DMEM (Ctr) and aminoacid- and serum- free medium (EBSS) for 18 h, in presence or not of 20 μM Chloroquine (CQ). Western Blot analysis of autophagy markers P62 and LC3 I/II was used to check autophagy induction. β-ACTIN was used as loading control. The blots are representative of three independent experiments. **(b)** mRNA expression levels of *axin2*, *cyclin D1* and *c-myc* obtained by qPCR on GL15 (left panel) and U87MG (right panel) cultured in DMEM (Ctr) and aminoacid- and serum- free medium (EBSS) for 18 h. The graphs represent the mean ± SE of three different experiments. Statistical significance: * P≤ 0.05; *** P≤ 0.001; **** P≤ 0.0001. **(c)** GL15 were transduced with a scramble RNA (shCtr) or with a BECN 1-directed shRNA (shBECLIN) and were grown in DMEM (Ctr) or EBSS for 18 h. Western Blot analysis of autophagy markers P62 and LC3 I/II was used to check autophagy induction. An antibody specific for BECLIN1 was used in order to check the silencing efficiency. β-ACTIN was used as loading control. The blots are representative of three independent experiments. **(d)** mRNA expression levels of *axin2* and *cyclin D1* obtained by qPCR on GL15 cells after lentiviral infection with scramble RNA (shCtr) or BECN-1-directed RNA (shBECLIN) grown in DMEM (Ctr) or EBSS for 18 h. The graph represents the mean ± SE of three different experiments. Statistical significance: * P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001.

Figure 2 Autophagy regulates Dishevelled protein levels in GBM cell lines. Western blotting of protein extracts from GL15, U87MG and GBM130 **(a)** and shBECLIN1 GL15 cells **(b),** cultured in DMEM (Ctr) or EBSS, was performed by using a specific antibody for Dishevelled 2 (DVL2). β-ACTIN was used as loading control. The graphs represent the mean ± SE of three different experiments. Statistical significance: * P<0.05 Student *t*-test.

Figure 3 β-catenin activity is reduced in GBM cells upon autophagy induction. Western blotting of protein extracts from GL15, U87MG, GBM130 **(a)** and shBECLIN1 GL15 cells **(b),** cultured in DMEM (Ctr) or EBSS was performed by using specific antibodies for the total (β-catenin) and the dephosphorylated (S37/T41) form of β-catenin (active β-catenin). β-ACTIN was used as loading control. The graphs indicate the relative ratio between active/total β-catenin and represent the mean ± SE of three different experiments. Statistical significance: * P<0.05; ** P≤ 0.01 Student *t*-test.

Figure 4 Autophagy induces β-catenin subcellular relocalisation. (a) After subcellular fractionation experiments in GL15 and U87MG cultured in DMEM (Ctr) or EBSS for 18 h, Western blotting of protein extracts was performed by using a specific antibody for β-catenin. Lamin A/C and LDH were used as loading controls for nuclear and cytosolic fractions, respectively. The graph indicates the ratio between β-catenin and Lamin A/C (for nuclear expression) and β-catenin and LDH (for cytosolic expression) in EBSS compared to control cells in three independent experiments. **N**, nuclei; **C**, cytosol. Statistical significance: * P<0.05; ** P≤ 0.01 Student *t*-test. **(b)** The same experiment was performed in GL15 transduced with a scramble RNA (shCtr) or with a BECN 1-directed shRNA (shBECLIN) grown in DMEM (Ctr) or EBSS for 18 h. The graph indicates the ratio between β-catenin and Lamin A/C (for nuclear expression) and β-catenin and LDH (for cytosolic expression) in EBSS compared to control cells in three independent experiments. **N**, nuclei; **C**, cytosol. Statistical significance: ** P≤ 0.01 Student *t*-test. **(c)** U87MG, GL15 and shBECLIN1 GL15 cells grown in DMEM (Ctr) or EBSS medium were subjected to immunocytochemistry and confocal analysis for β-catenin localisation (red). Arrowheads indicate cell-cell contacts. Scale bar, 30 μM.

Figure 5 β-catenin and N-cadherin colocalise upon autophagy induction. U87MG and GL15 cells grown in DMEM (Ctr) or EBSS medium were subjected to immunocytochemistry and confocal analysis for N-cadherin (green) and β-catenin (red) localisation. Inset containing higher magnification views of the merge images are also shown. Scale bar, 30 μM. Colocalisation index was assessed by calculating the Pearson's correlation coefficient *r* of at least 10 cells analysed in two different experiments (mean *r* : Ctr, 0.20 ± 0.02; EBSS, 0.72 ± 0.07 for U87 and mean *r* : Ctr, 0.15 ± 0.02; EBSS, 0.66 ± 0.05 for GL15).

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Review

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Review

EMT Regulation by Autophagy: A New Perspective in Glioblastoma Biology

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Received: 30 January 2019; Accepted: 1 March 2019; Published: 6 March 2019

Abstract: Epithelial-to-mesenchymal transition (EMT) and its reverse process MET naturally occur during development and in tissue repair in vertebrates. EMT is also recognized as the crucial event by which cancer cells acquire an invasive phenotype through the activation of specific transcription factors and signalling pathways. Even though glial cells have a mesenchymal phenotype, an EMT-like process tends to exacerbate it during gliomagenesis and progression to more aggressive stages of the disease. Autophagy is an evolutionary conserved degradative process that cells use in order to maintain a proper homeostasis, and defects in autophagy have been associated to several pathologies including cancer. Besides modulating cell resistance or sensitivity to therapy, autophagy also affects the migration and invasion capabilities of tumor cells. Despite this evidence, few papers are present in literature about the involvement of autophagy in EMT-like processes in glioblastoma (GBM) so far. This review summarizes the current understanding of the interplay between autophagy and EMT in cancer, with special regard to GBM model. As the invasive behaviour is a hallmark of GBM aggressiveness, defining a new link between autophagy and EMT can open a novel scenario for targeting these processes in future therapeutical approaches.

Keywords: autophagy; epithelial-to-mesenchymal transition (EMT); glioblastoma (GBM); cadherins; Wnt/β-catenin signalling

1. Introduction

Glioblastoma (GBM) is the most malignant and frequent form of glioma. This brain tumor is derived from glial cells and is characterized by high proliferation rate and local dissemination. Despite the improvement of chemo- and radio-therapies obtained in last decades, GBM prognosis is very poor with a median survival time that rarely exceed 18 months. Notably, GBM is characterized by resistance to apoptosis and high invasiveness, driving the search for novel targets useful to design effective therapeutical strategies.

The Epithelial-to-Mesenchymal Transition (EMT) programme is considered to be crucial for the acquisition of an invasive phenotype in epithelial cancer cells. Although some differences have been outlined, glioma cells also undergo an EMT-like process, through the activation of specific transcription factors and signalling pathways. Considering the critical role played by EMT on GBM dissemination, resistance to apoptosis and maintenance of cancer stem cells staminality, a number of preclinical studies have been launched to target the process as therapeutic approach.

In addition, the role of autophagy in tumor onset, progression is acquiring increasing clinical inerest. For instance, it has been recently shown that, besides modulating cell resistance or sensitivity to therapy, autophagy can also modulate GBM invasion.

In this review, we examined the current understanding of the role of autophagy in regulating the EMT and EMT-like programmes and in directing GBM cells to a more or less invasive phenotype. Moreover, we provided some clues to argue that blocking autophagy for therapeutic purposes requires careful consideration.

2. Epithelial-to-Mesenchymal Transition

EMT is a biological process by which epithelial polarized cells undergo various biochemical modifications that convert them in mesenchymal, isolated, and not-polarized cells. A lot of evidence suggests that EMT and its reverse process (mesenchymal-to-epithelial transition, MET) are crucial for tissue remodeling during development, wound repair and the initiation of cancer metastasis. In the early 1980s, Elizabeth Hay described epithelial-to-mesenchymal phenotype changes in the primitive streak of chick embryos [1]. Initially named as "epithelial to mesenchymal transformation", this process is now known as "epithelial-to-mesenchymal transition" to emphasize its transient nature and to distinguish it from the neoplastic transformation. To acquire a mesenchymal phenotype, epithelial cells undergo morphological and biochemical changes, reorganize their cytoskeleton, and activate a specific transcriptional programme. Indeed, epithelial cells are characterized by an apical-basal polarity, a polygonal shape and various kind of junctions. The latter tightly holds epithelial cells against each other and anchors them to the basement membrane, thus ensuring the structural integrity of epithelial sheets within the body tissues [2]. Conversely, mesenchymal cells exhibit a spindle-like morphology, do not have any polarity or connection with other and they are loosely anchored to the extracellular matrix (ECM) by focal adhesions.

Transcription factors (TFs) belonging to the SNAI family, named Snail and Slug, the zinc-finger E-box-binding homeobox (ZEB)1/2, and Twist1/2 are recognized to be the master regulators of EMT execution, since they promote the transcription of genes normally expressed in mesenchymal cells, such as N-cadherin, vimentin, and fibronectin. On the contrary, they suppress the expression of the epithelial markers E-cadherin, claudins, occludins, and cytokeratins. Loss of E-cadherin, in turn, promotes Wnt signaling and β-catenin accumulation in the nucleus, where it activates Tcf/LEF-dependent transcription of genes promoting proliferation and migration [3].

Cells undergoing EMT lose their apical–basolateral polarization and acquire a fibroblast-like morphology that allow them to degrade the underlying basement membrane and to migrate from the epithelial layer in which they originated [4]. Metalloproteases are also activated during EMT, and favor cell migration by degrading the membrane basement and the extracellular matrix components [2].

Furthermore, it has been shown that noncoding microRNAs play a role in EMT, by regulating the translation of EMT players, as extensively addressed in Abba et al. [5].

EMT can be classified in three different subtypes according to EMT meetings discussion: the so-called "type 1" EMT occurs during implantation, embryogenesis and organogenesis, the "type 2" EMT is associated to wound healing, tissue regeneration and organ fibrosis, and the "type 3" EMT characterizes neoplastic cells during metastatization. For a comprehensive overview of type 1 and type 2, refer to Kalluri et al., 2009 [4]. The activation of a "type 3" EMT programme (hereafter referred to as EMT) has been proposed to be pivotal for the acquisition of a malignant phenotype by cancer cells [6], as discussed below in detail.

2.1. Signals Stimulating Epithelial-to-Mesenchymal Transition in Cancer

Epithelial carcinoma cells, and typically those present at the external part of the tumoral mass, can acquire a mesenchymal phenotype upon specific stimuli. The microenvironment surrounding the primary tumor (TME) is characterized by inflammation, hypoxia, ECM components and tissue-specific soluble factors [7]. Notably, tumor cells recruit activated fibroblasts and immune cells that secrete cytokines, that, in turn, can activate the EMT programme. TGF-β is secreted by stromal fibroblasts, platelets and tumor cells themselves, and is considered the main EMT activator as it induces the expression of specific TFs in different cancer models [8–10].

Among cytokines, Tumor Necrosis Factor-α (TNFα) is crucial for EMT induction, and its effects are mediated through NFκB signaling pathway activation [11–13]. It has been also described that interleukins, particularly IL6, released by TME cells, can contribute to EMT stimulation. Other EMT-inducing signals originated from the tumor stroma are represented by growth factors such as HGF, EGF, PDGF that are able to activate EMT-specific TFs [14–17].

Furthermore, tumor microenvironment is characterized by hypoxia that promotes EMT via hypoxia-inducible-factor-1α (HIF1α) activation [18,19]. HIF1α stimulates inflammatory cytokines [20] and cooperates with Wnt/β-catenin signaling to enforce the EMT induction [21]. Moreover, during hypoxia, mitochondria increase the production of reactive oxygen species (ROS) that further contribute to EMT activation via both stimulating NFκB signaling [22], ECM regulation and cytoskeleton remodeling [23].

Once they have acquired a mesenchymal phenotype, cancer cells can dissociate from the primary mass, migrate and eventually enter the blood vessels by intravasation to initiate the metastatic process. Following extravasation and micrometastases formation, invading cells activate a MET programme and form macroscopic metastases resembling the epithelial features of the originating primary tumor, although the molecular mechanisms of MET have been less investigated if compared to those regulating EMT. However, both EMT and MET activation seem to be highly tissue-specific and strictly dependent on the local microenvironment encountered [2,6,24,25].

2.2. Epithelial-to-Mesenchymal Transition in Glioblastoma Dissemination

EMT has been mainly characterized in carcinoma models, and the role of EMT in glioma has only recently been investigated [26–28]. Candidate cells for originating gliomas (cells of origin) are mostly neural stem cells (NSCs), normally present in the adult brain, and oligodendrocyte precursor cells (OPCs) [29–32]. However, the involvement of more differentiated cells cannot be completely excluded [33]. As a consequence of the neurodevelopmental process, neural cells assume a mesenchymal phenotype, different from the epithelial one typical of the ectodermal cells they derive from. Therefore, gliomas do not undergo the classical EMT programme during tumorigenesis, and the terms "EMT-like" or "glial-to-mesenchymal transition (GMT)" have been proposed to indicate this peculiar process [34]. Although it is not clear whether cells of origin undergo or not significant modifications toward more mesenchymal features, glioma cells show a high plasticity in terms of EMT-like/MET-like conversion, likely mediated by epigenetic alterations induced by the tumor microenvironment [35,36]. Based on The Cancer Genome Atlas (TCGA) classification, GBMs can be differentiated into four genetic subtypes: Mesenchymal, Classical, Neural and Proneural [37]. Verhaak results suggest that Proneural GBM patients do not have a survival advantage from aggressive therapeutical treatments, unlike Classical and Mesenchymal GBM patients [37]. Role of EMT in each GBM genetic subtype has been investigated by Zarkoob et al. in 2013 [38]. A significant overlap between the genes that are up-regulated in the EMT signature and those that are up-regulated in each of the GBM subtypes exists, although, among all, the mesenchymal subtype has the highest number and expression levels of up-regulated genes [38]. Indeed, GBMs belonging to the mesenchymal subtype are characterized by an elevated invasive potential, poor clinical prognosis, and significantly shortened time to recurrence following initial treatment, compared to the other subtypes. The most commonly used glioma cell lines also present a predominant mesenchymal signature.

GBM cells in the invasive front, differently from those of the inner mass, commonly execute an invasion programme characterized by detachment from the mass, direct adhesion and degradation of ECM (lack of basement membrane) and widespread dissemination in the surrounding brain tissue. Remarkably, unlike other tumors, GBMs only rarely form metastases outside CNS, even though a hallmark of their aggressiveness is the infiltration and the diffuse growth in the surrounding parenchyma.

Large-scale genetic analyses have suggested that signaling networks activated during the physiological neural development are also employed by GBM cells to promote tumor growth and

invasion [39–41]. In detail, pathways mediated by Wnt/β-catenin, TGF-/β, Tyrosine kinase receptors and SDF/CXCR4 have been involved in the activation of EMT-like related genes to promote GBM dissemination [26,27]. Kahlert et al. found that the Wnt/β-catenin pathway is predominantly activated within cells located at the invasive peritumoral front of patient specimens belonging to the mesenchymal subtype. Chiefly, it induces the expression of Zeb1, Twist1 and Slug, thus promoting the migratory capability of GBM cells in vitro [42].

Regarding TGF-β pathway, a number of evidence demonstrated its critical role for the promotion of invasive properties of glioma cells [43–45], although the molecular mechanisms involved need to be further investigated. Interestingly, TGF-β signaling is known to be crucial in the maintenance of the mesenchymal stem-like population in GBM [45,46]. ZEB1 seems to be the pivotal mesenchymal transcription factor activated by TGF-β signaling since, differently from Snail, Slug and Twist, it accumulates in the nucleus of GBM cells [47].

The Hepatocyte growth factor (HGF) binding to the tyrosine kinase receptor c-MET is another crucial event highly activated. c-MET is overexpressed within GSC populations [48] and in patient-derived GSCs belonging to the mesenchymal subtype [49]. Accordingly, elevated c-MET signaling enhances GSC migration by activating EMT TFs [49,50] and is associated with poor survival and increased tumor invasiveness in patients [51–53].

The majority of molecules involved in the classical EMT have also been shown to play also a role in the EMT-like process. An increased activity of the TFs that mainly orchestrate the typical EMT, such as SNAI proteins, ZEB1/2, and Twist, promotes the invasion of GBM cells [54–57]. For instance, SNAIL silencing reduces invasion, migration and proliferation in GBM cell lines [58,59] and overexpression of Slug correlates with GBM grade [56]. ZEB1 and ZEB2 expression is also correlated with invasive features and with survival of GBM patients; ZEB1 knock-down cells formed less invasive and more drug-sensitive masses than wild type cells when inoculated in mouse brain [54,60]. Moreover, Twist1 and Twist 2 expression, besides affecting stemness properties, has been associated to the invasive properties of GBM cells as it mediates the expression of crucial EMT-related genes such as metalloproteinase 2 (MMP2), Slug and HGF among others [57].

It is worth mentioning that the classical cadherin switch, which is widely accepted as an EMT hallmark in carcinomas, is a controversial matter in GBM. Differently from carcinomas, E-cadherin expression is almost absent in neural tissues, where its expression appears limited to GCSs cells and to a subset of highly aggressive GBM cells. Otherwise, N-cadherin is absent in epithelial tumors before the EMT execution, whereas is highly expressed in astrocytes, where it contributes to regulate cell polarity and migration and in GBM cells, that show a faster and less-directed movement to respect to astrocytes [54,61]. It was found that N-cadherin expression is inversely correlated with the invasive behaviour of GBM, and its ectopical expression reduces cell migration and restores polarity in GBM cells [62,63]. Notably, it has also been shown that differences in N-cadherin distribution rather than in its expression levels are responsible for different motility behaviours [64,65].

In addition, several studies showed that the treatment of primary GBMs with radiation therapy or with the anti-angiogenic agents Bevacizumab promotes the acquisition of a mesenchymal phenotype in recurrent tumors [34,66–68]. Indeed, glioma cells that have acquired radioresistant properties following treatment exhibit a gene expression profile enriched for genes involved in EMT-related processes [34,69,70], and the pathways promoting EMT result strongly upregulated in these cells, thereby resulting in an increased invasion capability [71,72]. An in vivo study by Halliday et al. demonstrated that proneural GBM cells rapidly shifted their gene expression pattern towards a mesenchymal phenotype in response to radiation therapy in a tumor-bearing mouse model [66]. As radiation is a universal component in the treatment of GBMs, this subtype shift poses an important clinical challenge, especially considering that cells shifted to a mesenchymal subtype display an increased radioresistance [73]. If this shift is due to changes in the microenvironment or to clonal selection of mutant therapy-resistant cells is controversial, but both the hypotheses seem to be possible.

3. Autophagy

The term "autophagy" was coined by the discoverer of lysosomes Christian de Duve and it means, in Greek language, "self-eating" [74–76]. From the first description of the process in 1960s, many studies described the process of self-degradation by a morphological point of view, until in 1993 a genetic screen led to isolation of some yeast autophagy-defective mutants and to identification of the so-called AuTophaGy-related (ATG) genes [77]. This seminal work allowed Yoshinori Oshumi to be awarded the Nobel Prize in Physiology and Medicine in 2016. Oshumi's screen identified 15 genes involved in autophagy regulation in yeast undergoing nutrient deprivation, but today, more than 40 genes have been described in yeast, many of them having orthologues in vertebrates [76]. Three main types of autophagy have been described: macroautophagy, microautophagy and Chaperone-Mediated Autophagy (CMA).

3.1. Mechanisms and Molecules

During macroautophagy (hereafter referred to as autophagy), double-membrane vesicles named autophagosomes form and engulf cytoplasmic material, including long-lived proteins and old or damaged organelles which are then delivered to lysosomes for degradation and recycling [78]. Always activated at basal level within the cell, autophagy can be modulated by several signals, mainly by nutrient signaling, growth factors, energy status, oxidative or ER stress and pathogen infection. The input from these upstream signals is integrated by the serine/threonine protein kinase mTOR (mechanistic or mammalian target of Rapamycin), which acts upstream of the ATG genes, thus controlling autophagy activation [79]. mTOR belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family, and, in mammalian cells, works as the catalytic subunit of two multi-protein complexes known as mTORC1 and mTORC2 [80]. Under nutrient deprivation, mTOR is inhibited and Ulk1/Atg13-FIP200 complex initates and drives a massive autophagy activation [81]. Autophagosome formation requires the formation of a multi-protein complex, composed by class III PI3K, Beclin1 and p150, although other proteins such as UVRAG, Ambra1 and Atg14L are able to bind and regulate the complex [82–86]. Elongation and maturation of autophagosomes involve two ubiquitin-like conjugation systems, both requiring Atg7, which catalyze the covalent linkage of ATG5 to ATG12 and ATG16-like 1, and the attachment of phosphatidylethanolamine to proteins of the microtubule-associated protein 1 light chain 3 (MAP1LC3 or LC3) family [87].

Lipidated LC3 protein is then recruited to the autophagosome membrane that docks and fuse with lysosome, resulting in the formation of a single membrane vesicle named autophagolysosome or autolysosome. Lysosomal hydrolases, ultimately, degrade and recycle the content of autolysosomes.

Although originally suggested to be just a nonspecific and bulk degradation mechanism, autophagy is now recognized as a highly regulated process, enabling cells to sense and promptly respond to a plethora of stimuli, thereby conferring adaptation to the ever-changing environment. Nevertheless, even though a basal level of autophagy contributes to maintain the proper cell homeostasis both during embryogenesis and adulthood in physiological conditions, it is now ascertained that autophagy is deregulated in various human pathologies, including cancer [88–90].

3.2. Autophagy Role in Tumorigenesis

The observation in 1999 that the gene encoding Beclin1 is monoallelically deleted in a high percentage of human breast, ovarian and prostate cancers provided the first evidence of the involvement of the autophagic process in tumorigenesis [91]. Disruption of Beclin1 in mice results in an increased proliferation and in the spontaneous development of various malignancies, confirming Beclin1 as an haploinsufficient tumor suppressor gene [92,93]. In a similar way, mice lacking one copy of the Beclin1 interactor Ambra1 exhibit a higher incidence of spontaneous tumors than their wild type littermates, and cells depleted of the gene are characterized by a hyperproliferative phenotype [94]. Notably, *Ambra1* homozygous disruption in mouse leads to a strong hyperproliferation and lethal

defects in the developing nervous system during embryogenesis [82]. Mice bearing systemic or tissue-specific deletion of Atg5 and Atg7 also develop tumoral masses a higher frequency than the wild type counterparts [95] and are more prone to develop cancers upon carcinogen-induced stimuli [96–98].

Several mechanisms have been proposed to explain the oncosuppressive functions of autophagy [90]. First of all, the autophagy-mediated clearance of proteins and organelles ensures the proper cellular homeostasis, avoiding the accumulation of genotoxic molecules, such as reactive oxygen species (ROS) produced by dysfunctional mitochondria, as well as aggregates of ubiquitinated proteins [99,100]. An intact autophagic machinery is also required to deal with cytotoxic stress and to maintain genome stabilization, although further investigation is required to underlie the mechanisms involved [101,102]. Moreover, autophagy counteracts the metabolic switch accompanying malignant transformation by eliminating old and damaged mitochondria, thus preserving the optimal bioenergetic needs and maintaining the physiological metabolic homeostasis [103,104].

Other potential mechanisms through which autophagy acts as an oncosuppressive process are linked to its role in the regulation of immune response [105], maintenance of the staminal niches [106], defens of the organism against pathogen infections and degradation of oncogenic proteins, like mutant (but not wild-type) TP53 [107].

On the other hand, it is well accepted that, in an established tumor, cancer cells use autophagy as a strategy to overcome microenvironmental stresses, including nutrient deprivation, hypoxia and drugs. Advanced tumors sometimes exhibit an increased autophagic flux and ex-vivo cell lines in which BECN1 or ATG5 have been down-regulated are virtually unable to survive within the metastatic niche [108]. Analogously, autophagy-defective tumoral cells appear more sensitive to pro-apoptotic stimuli than autophagy-proficient cells [109–112].

Due to this dual function, autophagy has been defined a 'Janus-faced' player in cancer progression [113]: in the early stages of tumorigenesis it plays onco-suppressive functions by limiting cell proliferation, DNA damage and tumor progression; on the contrary, when the tumor mass is established, it helps cells to counteract the stressful conditions characterizing the tumor microenvironment.

3.3. Autophagy and Glioblastoma: Friends or Foes?

It was demonstrated that high-grade gliomas exhibit lower expression of some autophagy related proteins with respect to low-grade ones, and that the progression of astrocytomas toward higher grades is accompanied by a decrease in autophagic proficiency. Pirtoli et al. observed that both BECN1 mRNA (encoding for Beclin1) and protein levels are lower in GBM tissue than in low-grade and healthy brain tissue [114]. Accordingly, following Karnofski classification, high Beclin1 levels have been positively correlated with patient survival and performance status, whereas low Beclin1 expression correlates with an increase of proliferation [114]. Similarly to Beclin1 expression, also LC3B II expression (index of autophagy activation) is low in high-grade astrocytomas, thus suggesting an impairment of the autophagic process in these tumors [115]. On the other hand, in 2012, through a proteomic screening, Galavotti et al. found that some genes involved in autophagy regulation are highly expressed in the GBM mesenchymal subtype [116]. Among these, the autophagy associated genes DRAM1 and SQSTM1 encoding for the key regulator p62 are highly expressed in Glioma stem cells (GSCs), and modulate their migration and invasion capabilities [116]. Although these studies suggest that autophagy may regulate gliomagenesis, a systematic and comprehensive investigation of autophagy role among the GBM subtypes is missing, but needed. Indeed, a different expression of autophagy regulators across GBM genetic groups could be responsible for a different susceptibility to autophagy modulation.

In addition to the growing evidences showing a direct involvement of autophagy-regulating genes in GBM progression, several autophagy-associated molecules are frequently altered in brain tumors. As an example, the tyrosine kinase EGF receptor is often amplified in gliomas, and suppresses autophagy through both kinase-dependent and -independent mechanisms [117]. PTEN, is commonly

mutated in gliomas, and positively regulates autophagy by inhibiting the PI3K/Akt pathway, although PTEN and NF1 co-mutation determines an autophagy suppression through the hyperactivation of the PI3K/Akt signalling [118].Furthermore, the oncosuppressor p53, frequently mutated in gliomas, may have a dual role in autophagy regulation, as the nuclear protein is able to promote autophagy through the transcriptionally regulation of autophagy-related genes, whereas cytoplasmic p53 suppresses autophagy [119]. Further investigation are needed to define whether autophagy machinery may be considered as a novel useful prognostic and/or therapeutic marker of glial tumors.

4. Autophagy and Epithelial-to-Mesenchymal Transition

Autophagy was only recently connected to EMT. In the last years, some observations indicated that an intricate relationship exists between these two important processes in cancer [120]. According to its dual role in tumorigenesis, the effect of autophagy on EMT appears controversial and strictly dependent on the cellular type and on the stimulus employed for activating or inhibiting autophagy, as summarized in Table 2 and in Figure 1.

Literature data highlights that an autophagy stimulation of metastatization could be merely the consequence of its pro-survival activity against the apoptotic signals coming from changes in adhesion and cytoskeleton reorganization [108]. A number of compounds or microenvironmental conditions that are able to activate the EMT programme, can also induce an autophagic response in different types of cultured cancer and non-cancerous cells; autophagy inhibition in these models impairs EMT (Table 2).

However, emerging evidence also indicates that autophagy activation can induce a reversion of the EMT phenotype in different healthy and cancer models and that several anticancer compounds that induce autophagy also inhibit EMT [121–123] (Table 2). Selective degradation of EMT players seems to be a general mechanism by which autophagy can modulate EMT process [124]. Notably, in ATG3, ATG5, ATG9 and ATG12 KO mice, p62 accumulation accumulation determines stabilization of Twist1, which is normally degraded by both proteasome and autophagosomes [125]. This regulation can be crucial in those tumors characterized by p62 up-regulation, as observed so far in human squamous cell carcinoma and in melanoma (Table 2). Autophagy deficiency reduces the expression of epithelial markers and increases that of mesenchymal ones also in ATG7 KO hepatocytes [126] (Table 2).

Table 1. Autophagy and EMT.

Cell/Tissue	Autophagy Modulation	Effect on EMT on EMT	Mechanism	References
HCC cells	Induction by starvation	Induction	activation of TGFβ/Smad3-dependent and cAMP/PKA/CREB signalling	[108,127–129]
	Inhibition by ATG KD or CQ	Inhibition		
Colorectal cancer cells	Induction by mTOR inhibition or by ALS treatment	Inhibition	decreased activation of RhoA and Rac1	[122,130,131]
	Inhibition by BECN1 knockdown	Inhibition	unknown	
Ovarian cancer cells	Induction by Danu treatment	Inhibition	unknown	[123]
Non tumorigenic hepatocytes	Induction by starvation + TGF β_1	Inhibition	Snail degradation	[126]
	Inhibition by BECN1 or ATG7 KD	Induction	unknown	
NPC cells	Induction by Cisplatin	Induction	unknown	[132]
NSCL cells	Induction by TGFβ_1 treatment	Induction	unknown	[133]

Table 2. Autophagy and EMT.

Cell/Tissue	Autophagy Modulation	Effect on EMT on EMT	Mechanism	References
Lung adenocarcinoma cells	Induction by MSCs co-colture	Induction	Snail up-regulation	[134]
Endometrial cells	Induction by Hypoxia	Induction	unknown	[135]
Uroepithelial cells	Induction by DBP exposure or starvation	Induction	E-cadherin degradation or TGFβ1/Smad3 pathway activation	[136,137]
Kidney podocytes	Inhibition by V-ATPase	Inhibition	Reduction of p62 phosphorylation	[138]
MEFs, keratinocytes, melanoma cells	Inhibition by ATG KD	Induction	p62-mediated Twist stabilization	[139]
Breast	Activation by DEDD overexpression	Inhibition	Snail and Twist degradation	[140]
Gastric cancer cells and tissue	Inhibition by BECN1 KD	Inhibition	ROS-NFκB-HIF-1α pathway activation	[141]

3-MA, 3-methyladenine; ALS, Alisertib (Aurora kinase A inhibitor); ATG, autophagy related gene; Baf, bafilomycin; BECN1, Beclin1; cAMP, cyclic adenosine monophosphate; CQ, chloroquine; CREB, cAMP responsive element binding; Danu, Danusertib; DBP, n-butyl phthalate; DEDD, death effector domain containing; DRAM1, DNA damage-regulated autophagy modulator 1; EMT, epithelial to mesenchymal transition; HCC, hepatocarcinoma cells; HIF-1α, hypoxia-inducible factor 1; KD, knockdown; MEFs, mouse embryonic fibroblasts; MSC, mesenchymal stem cell; NF- κB, nuclear factor kappa beta; NPC, nasopharyngeal carcinoma; NSCL, Non-small cell lung; PI3KC3, phosphatidylinositol 3-kinase; PKA, protein kinase A; Rac1, Ras-related C3 botulinum toxin substrate 1; RhoA, Ras homolog gene family, member A; ROS, reactive oxygen species; Smad3, small mother against decapentaplegic3; SQSTM1, sequestosome 1; TGFβ, Transforming growth factor beta; V-H-ATPase, vacuolar-type H^+-adenosine triphosphatase.

Taken together, these observations show a complex crosstalk between autophagy and EMT processes. It is conceivable that, at the early stages of metastatization, autophagy acts as oncosuppressive signal, tending to inhibit the EMT programme mainly by destabilizing EMT crucial players. Later on, metastatic cells could require a sustained autophagy to survive to environmental and metabolic stressful conditions encountered [113].

4.1. Autophagy Role on Glioblastoma Dissemination

To date, only a few studies correlate autophagy to GBM cells capability to migrate and spread toward surrounding tissues, and, similarly to what observed in other tumor models, they highlight two opposite point of views, as detailed below, in Section 4.1.1, and as illustrated in Figure 1.

4.1.1. Autophagy Promotes Glioblastoma Dissemination

In 2012, Galavotti et al. showed that some autophagy players are up-regulated in GBM mesenchymal subtype, and that their modulation modifies the migration properties of GBM cells. They observed that GSCs require autophagy activation to migrate, and down-regulation of the autophagy-associated factors DRAM1, p62 and ATG7 limit their invasion capabilities potentially through the regulation of energy metabolism [116] (Figure 1A). In line with these observations, a study conducted by using an 3D organotypic model of GBM showed that ATG12 RNA silencing reduced cellular invasion, although no modifications of cellular migration capabilities was observed [142] (Figure 1A). More recently, another couple of studies have correlated autophagy activation induced by a combination of different stimuli with an enhanced mesenchymal phenotype in GBM cells through various mechanisms [143,144]. Lastly, inhibiting the late stages of autophagy by using Chloroquine, Liu et al. showed a potentiation of the effect of the multi kinase inhibitor Sorafenib in reducing cell proliferation and migration, through a further stimulation of apoptosis [145] (Figure 1A).

4.1.2. Autophagy Impairs Glioblastoma Dissemination

In spite of the previous mentioned evidence, we and other groups have recently demonstrated a direct effect of autophagy modulation on migration and invasion capabilities of GBM cells, as illustrated

in Figure 1B. Autophagy induction by nutrient deprivation or by mTOR inhibition determines a reversion of EMT phenotype in immortalized and primary GBM cells [146–149]. In 2014, Palumbo et al. observed that ATG7 RNA silencing restored clonogenic ability of irradiated GBM cells with inactive EGFR and, conversely, that rapamycin-mediated autophagy further impaired their clonogenic and migration capabilities [148]. Later on, we demonstrated that autophagy induction in GBM immortalized and primary cells, obtained by nutrient starvation or by mTOR pharmacological inhibition, induced a drastic impairment of both migration and invasiveness. On the contrary, autophagy deficiency, obtained by silencing the autophagy master genes ATG5, ATG7 or BECN1, stimulated cell motility [146], similarly to what observed in highly metastatic breast, colon and hepatocellular cancer models [122,124,130]. We correlated the migration properties of the cells analyzed with a molecular shift from a mesenchymal to an epithelial-like phenotype (Figure 1B). Upon autophagy induction, in fact, we found a down-regulation of the EMT players Snail and Slug, likely due to a general impairment of protein synthesis mediated by mTOR inhibition, rather than to the autophagosome-mediated degradation [146]. As an outcome of SNAI down-regulation, the up-regulation of N- and R-cadherin mRNA and protein expression was observed, whereas no significant differences in other cadherin family members were found (Figure 1B). Remarkably, as above discussed, although a "cadherin shift" from the E- to the N- isoform is actually believed a hallmark of carcinoma cells undergoing EMT, the role of cadherins in non-epithelial tumors is much less documented and elucidated. N-cadherin overexpression or re-distribution has been associated with the recovery of the cell polarity and the inhibition of migration of GBM cells [62–65]. Notably, we have recently found that, upon autophagy induction, the Wnt signaling effector β-catenin relocalises within the cell and associates to N-cadherin in sub-membrane compartments to form epithelial-like cell-cell adhesion structures [147], thus contributing to a mesenchymal-to-epithelial-like transition of GBM cells (Figure 1B). Similarly to other tumors, Wnt pathway is constitutively active in GBM and its deregulation is likely responsible for initiation and/or progression of the disease [150–153]. β-catenin translocation to the nucleus characterizes the mesenchymal and invasive phenotype of tumoral cells as it promotes SNAIL and ZEB1 transcription and mediates EGFR pathway-induced EMT [154–156].

In summary, a complicated interplay between autophagy process and EMT/MET activation in GBM is recently emerging. Contradictory results could be explained by the different stimuli and different models employed. Notably, autophagy induction obtained through mTOR inhibition or nutrient deprivation always results in migration/invasion reduction in independent experiments and different GBM cell lines. Conversely, autophagy activation resulting by the employment of drugs like TMZ plus low glucose, or by TGF-β, or by AEG overexpression is associated to promotion of migration capabilities of GBM cells, maybe suggesting that different and complex mechanisms contribute to the migratory phenotype in those contexes.

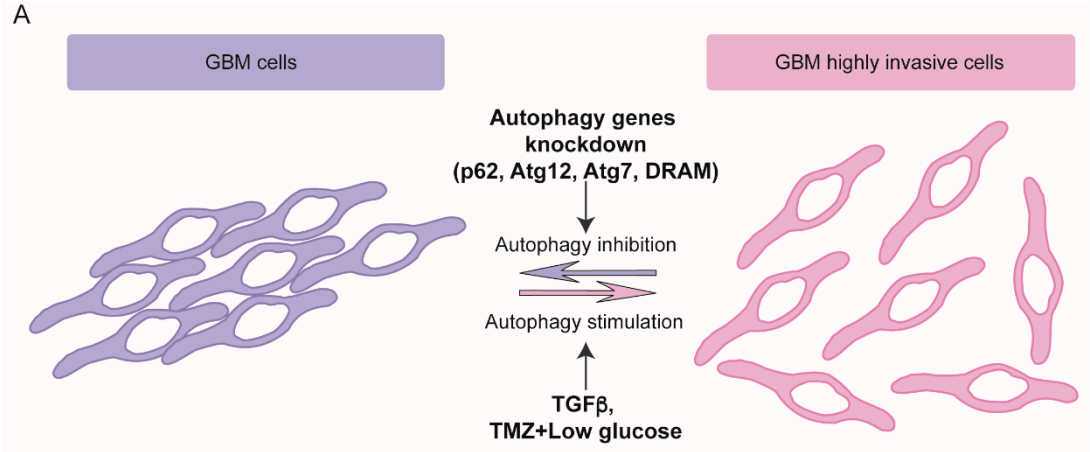

Figure 1. *Cont.*

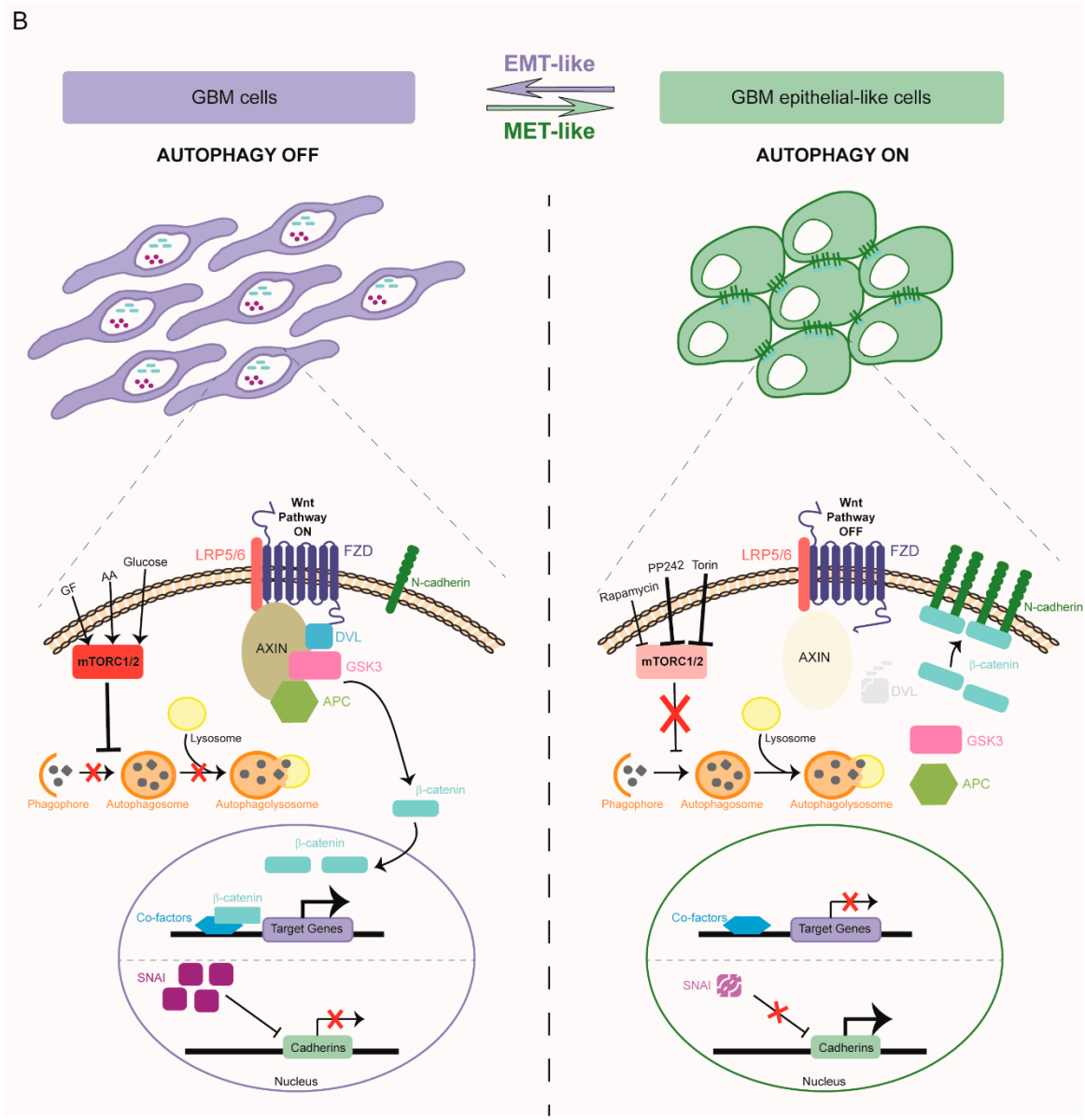

Figure 1. Effects of autophagy modulation on GBM migration/invasion capabilities from opposite point of views. (**A**) Autophagy induction promotes GBM cells invasiveness and viceversa. Upon different stimuli, autophagy is activated and a more invasive phenotype is observed in some models of GBM cells (right). Conversely, when autophagy is impaired by genetic knockdown of some autophagy-related genes, a less invasive phenotype is obtained (left). TGFβ, Tumor necrosis factor β; TMZ, Temozolomide. (**B**) Autophagy modulation promotes EMT/MET-like shifts in GBM cells. In nutrient-rich conditions, hyperactivation of the mTORC1/2 complexes impairs autophagy and Wnt pathway is active thus allowing β-catenin translocation to the nucleus where it promotes the transcription of pro-invasive molecules. In this condition, EMT players of the SNAI family express and repress cadherins expression. The genetic knockdown of autophagy related genes exacerbates the mesenchymal phenotype and enhances the cell migration capability. Upon autophagy induction, shown on the right, Dishvelled (DVL) is degraded and Wnt pathway inactivated leading to β-catenin accumulation into the cytosol. In autophagic cells, SNAI factors are down-regulated and, consequently, N-cadherin accumulates and binds β-catenin, thus promoting cell-cell adhesion.

Effects observed following autophagy inhibition also require attention, although Chloroquine effects on cells often depend on autophagy-independent mechanisms, as better illustrated in the

next section. More controversial, although conducted in different model systems, are the results on GBM invasiveness obtained by inactivating ATG genes. In light of such contradictory results, further in-depth investigation is, for sure, required to better unravel the question.

4.2. Targeting Autophagy in Glioblastoma Therapy

In the last decade, the standard therapeutic regimen for GBM has been surgical resection, if feasible, followed by radiation therapy (IR) and temozolomide (TMZ)-based chemotherapy. Both TMZ and IR are able to induce an autophagic response in GBM cell lines, likely through DNA damage induction, but the outcome of the autophagic response is so far inconclusive (Figure 2). In 2004, Kanzawa et al. reported that TMZ treatment induced autophagy instead of apoptosis in GBM cells and that the co-treatment with a pharmacological inhibitor of the late stages of autophagy restored TMZ-induced cytotoxicity, although the opposite effect was obtained by blocking autophagy initiation [157]. In line with this observation, the combination between standard therapies and autophagy flux inhibitors Chloroquine (CQ) or its analog hydroxychloroquine (HCQ) is a promising therapeutic approach in GBM treatment, and combined therapies including these molecules are currently employed in ongoing phase III clinical trials (Figure 2). Indeed, CQ and its derivatives are, in fact, the only autophagy inhibitors already approved by the USA Food and Drug Administration due to their anti-inflammatory and anti-malarial properties. Similar to other tumoral settings, CQ-treated GBM patients exhibit a better median survival if compared to placebo-treated individuals [158]. However, despite the potential promising beneficial effect, a trial combining HCQ with TMZ and IR showed that the maximum tolerated HCQ dose was ineffective in inhibiting autophagy, suggesting that CQ effect could be due to autophagy-independent mechanisms [159]. Notably, it has been recently shown that, in addition to DNA intercalation properties and ROS production, CQ also induces a strong autophagy-independent disorganization of the Golgi apparatus and of the endo-lysosomal system in mice tissues, thus suggesting caution in interpreting results obtained with this drug [160,161].

In an apparently contradictory way, other therapeutic approaches and clinical trials aimed at inhibiting mTOR pathway, inducing in turn an autophagic response, have been launched. PI3K/Akt/mTOR pathway is often deregulated in human cancers including GBM, and is involved in cancer stem cell maintenance, thus inducing an uncontrolled proliferation [162,163]. It was observed that the co-treatment of TMZ and the mTOR inhibitor RAD001 (also known as everolimus) induced cell death [164] (Figure 2). RAD001 was shown to enhance the cytotoxic effect of an oncolytic adenovirus in a viral-mediated therapy by inducing an autophagy-dependent cell death [165,166].

mTOR inhibition also promotes TMZ-induced senescence in in vitro and in vivo models [167,168]. However, the link between autophagy and senescence needs to be further clarified in order to be exploited a potential therapeutic tool.

Very recently, it has been suggested that the failure of some clinical trials targeting PI3K and mTOR, could be due to the employment of Rapalogs (rapamycin and its analogs) which are known to inhibit mTORC1, but not mTORC2 [149]. In fact, a feedback mechanism activated by mTORC1 inhibition stimulates mitogenic pathways, thus compromising the rapalog efficacy on cell proliferation [169]. In order to overcome the emerged limitations, a second generation of mTOR inhibitors, (named ATP-competitive mTOR kinase inhibitors or TORKIs) have been developed and are revealing more efficacious than rapalogs in GBM treatment [170–172]. As an example, the novel TORKI PP242, able to target both mTORC1 and mTORC2, impairs cell proliferation and reduces stemness and invasiveness properties in a group of GBM cell lines carrying different genetic alterations [149] (Figure 2). In this respect, a putative difference in the "autophagy signature" among GBM subtypes, if found, could affect the response to the treatments.

Notably, in our model, we have obtained β-catenin relocalisation and migration impairment by both nutrient deprivation and by inhibiting mTOR complexes by means of Torin 1, an ATP-competitive mTOR inhibitor able to target both the mTOR complexes [147].

Finally, a number of compounds, such as arsenic trioxide, sodium selenite and cannabinoids (THC), used in combination with traditional therapy showed beneficial effects through the induction of an autophagic response, in some cases potentiating drug-induced cell death, some other inducing mitochondrial damage or ER stress [173–176] (Figure 2). In addition, autophagy-induced cell death seems also to be the mechanism by which some compounds overcome the apoptosis-resistance typical of the anoxic cells inside tumors. For instance, the class of small molecules able to bind the BH3 domain of the anti-apoptotic protein Bcl-2, known as BH3 mimetics, were found to induce an autophagy-dependent cell death in GBM [177,178]. If these compounds are also able to trigger a beneficial or detrimental effect on GBM invasiveness remains to be investigated.

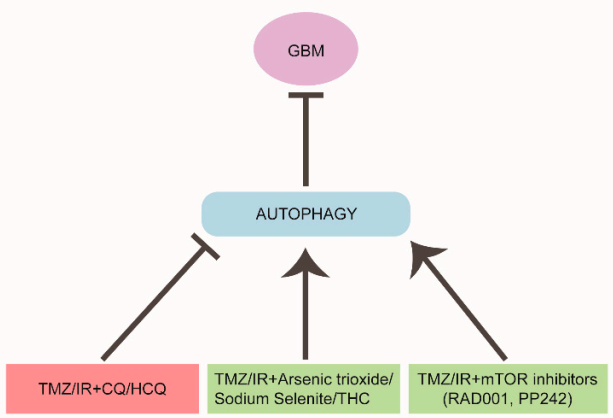

Figure 2. Targeting autophagy in GBM therapy. The effects of different therapeutic combinations on autophagy and the outcome on GBM progression in patients are shown. Chloroquine or hydroxy-chloroquine (CQ/HCQ) addition to standard protocols (TMZ/IR, temozolomide/radiation therapy) impairs the autophagy flux and sensitizes GBM to the treatment (pink box). Otherwise, several chemical compounds and mTOR inhibitors, that are able to stimulate autophagy, also increase the efficacy of the standard treatments (green boxes). THC, tetrahydrocannabinol.

5. Concluding Remarks

GBM is the most common and aggressive brain malignancy, and is characterized by a highly invasive behaviour, although the role of EMT in GBM dissemination has only been recently investigated. Although several molecules and signalling pathways mediating EMT in carcinomas play a role also in glioma invasion, further investigation will be necessary to better characterize EMT-like and its reverse MET-like processes occurring in GBM.

Targeting autophagy in GBM therapy is still a matter of debate; autophagy induction has been observed in GBM in response to radio- and temozolomide-based therapy and even though a number of clinical trials aimed at inhibiting autophagy execution, mainly by CQ, have been launched, others directed to inhibiting mTOR pathway, and thus activating autophagy, are ongoing.

Intriguingly, we observed that autophagy induction by nutrient starvation or by mTOR inhibition impairs migration and invasion of GBM cells, in line with other studies conducted on other cancer models. Further in-depth studies will be crucial to clearly dissect the autophagy role in GBM biology and to carefully evaluate autophagy modulation as therapeutic strategy to contrast GBM progression.

Funding: This work was supported by a grant from FFABR (Italian Ministry of University and Research) to SDB.

Conflicts of Interest: The authors declare no conflict of interest.

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II

(Paper submitted for publication)

mTOR INHIBITION LEADS TO SRC-MEDIATED EGFR INTERNALIZATION AND DEGRADATION IN GLIOMA CELLS

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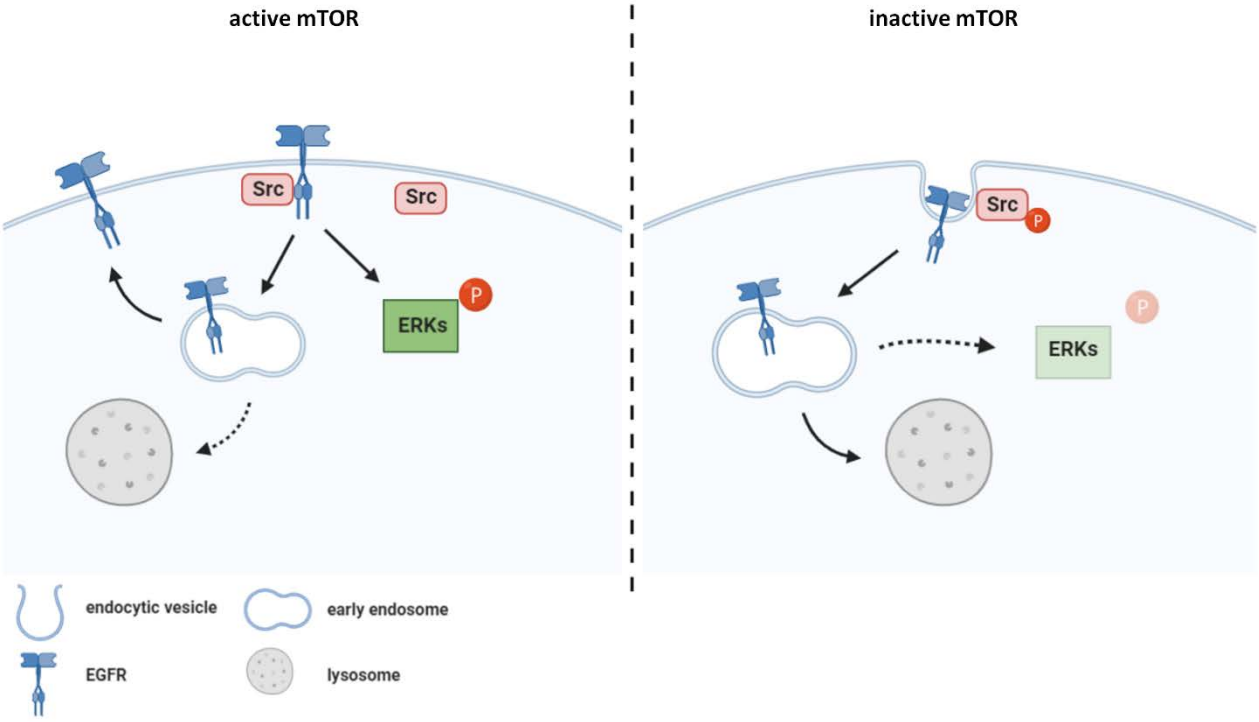
Abstract

EGFR is a tyrosine kinase receptor widely expressed on the surface of numerous cell types, which activates several downstream signalling pathways involved in cell proliferation, migration and survival. EGFR alterations, such as overexpression or mutations, have been frequently observed in several cancers, including glioblastoma (GBM), and are associated to uncontrolled cell proliferation. Here we show that mTOR inhibition mediates EGFR internalization and delivery to lysosomes for degradation in GBM cells, independently from autophagy activation. Accordingly to EGFR internalization and degradation, mTOR blockade negatively affects MAPK/ERK pathway. Furthermore, we provide evidence that Src kinase activation is required for EGFR internalization upon mTOR inhibition. Our results confirm the hypothesis that mTOR could be an effective target in GBM pharmacological treatment, as its inhibition could result in EGFR degradation and in proliferative signal alteration.



Keywords: mTOR; EGFR; Glioma; autophagy

1. Introduction

Epidermal Growth Factor receptor (EGFR), also known as ErbB1 or HER1, belongs to the tyrosine kinase receptors family (RTK) and regulates epithelial tissue development and homeostasis (1). In physiological conditions, inactive RTK receptors continuously travel through the endocytic compartment, slow internalization and recycling to plasma membrane. EGF binding stimulates EGFR activation and signal transduction inside the cell, thus regulating cell growth, differentiation, proliferation and migration (2,3). In particular, upon ligand engagement, activated receptors are subjected to clathrin-mediated (CME) or clathrin-independent (CIE) endocytosis in dependence on ligand abundance, and spend most of their remaining lifetime travelling the vesicular network of endosomes (4–8). Once internalized in early endosomes, EGFR can be recycled to cell surface or delivered to lysosomes for degradation, in a delicate balance between continuous signalling from cell surface/endosomal compartments and signal attenuation from the degradative route (4,9).

It has been demonstrated that activated RTK still retains the ability to continuously signal within the vesicular compartments until the receptors are recycled back to cell surface or taken up into lysosomes for degradation (9).

EGFR alterations have been reported in several diseases, particularly, in cancer (10). Indeed, EGFR gene is frequently mutated, amplified or overexpressed in various kinds of tumours including Glioblastoma multiforme (GBM), the most aggressive brain tumor in adults (10,11). In particular, in GBM EGFR is amplified in 40%, overexpressed in 60% and mutated or deleted in 24-67% of primary tumors (12). Deletion of exon 2-7, also known as variant III (EGFR vIII), represents the most frequently occurring mutation in GBMs, found in up to 45% of cases (13,14). Mutated EGFR are usually constitutively active, ligand-independent receptors with an altered trafficking and down-regulation, resulting in an aberrant downstream signal transduction which promotes tumour development (15,16). Receptor overexpression, which is often associated to an enhanced expression of cognate ligands (17), is also associated to receptor turnover alterations and to increased downstream signalling (18,19). mTOR kinase is part of two multi-protein complexes, known as mTORC1 and mTORC2, that acts as nutrients sensors in the cell, regulating protein synthesis through a phosphorylation cascade (20). Aberrant mTOR activation is linked to tumour proliferation and invasion. For this reason, mTOR protein is recurrently proposed as a putative pharmacological target, and several specific inhibitors, mainly directed to mTORC1, have been developed and tested in the last decade (21). mTOR also regulates autophagy, a lysosome-degradation system crucial to maintain cellular homeostasis (22). As nutrients sensor, mTOR is normally activated under fed conditions, leading to autophagy inhibition; conversely, nutrient starvation results in mTOR inactivation and in a subsequent autophagy induction.

It is now well established that RTK signalling regulates PI3K/mTOR pathway and autophagy. However, the possible existence of an opposite relation has still not been investigated (23). In this work, we evaluated the prospective modulation of mTOR on EGFR activity and turnover. We found that mTOR inhibition delocalises EGFR from plasma membranes to the cytoplasm in GBM cellular models. We also observed that the receptor is delivered to lysosomes for degradation, independently of autophagy. These events ware also accompanied by the suppression of MAPK/ERK pathway, suggesting that mTOR inhibition may affect EGFR ability to endorse GBM cell survival and proliferation.

2. Results

2.1 mTOR inhibition induces EGFR relocalisation in GBM cells

Similarly to other tumors, GBM is characterized by an aberrant EGFR expression and activation, leading to de-regulated downstream signalling pathways. In order to investigate a putative impact of autophagic stimuli on EGFR expression and signalling in GBM, we cultured U87MG and GL15 cells

in EBSS (aminoacid- and serum- free medium) or in presence of Torin1 and AZD8055, two inhibitors of both mTORC1 and mTORC2 complexes, for 18 hours. GH2 primary cells were also cultured in complete medium or in presence of Torin1 for 18 hours. As shown in figure 1A (left panels), mTOR inhibition obtained by cell exposure to the different treatments, was confirmed by the strong decrease of the phosphorylated forms of its substrate P70S6K, observed by western blotting analysis. Next, we checked for EGFR subcellular localisation by immunocytochemistry and we found, as expected, the receptor mainly localized at plasma membrane of control cells; conversely, autophagic cells did not show EGFR membrane exposure (Figure 1A, right panels). Chiefly, EGFR signal was strongly reduced 18 hours after EBSS treatment, whereas a punctate immunostaining pattern was observed in discrete areas within cytoplasm in Torin1 and AZD8055-treated cells (Figure 1A, right panels). A time-course analysis revealed that EGFR re-localization was already evident in 2 and 4 hours after mTOR inhibition with Torin1 (Fig. 1B).

2.2 EGFR de-localization is not dependent on canonical autophagy in GBM cells

In order to discriminate between an autophagy-dependent or -independent effect of mTOR inhibitors on EGFR de-localization, a Beclin1-silenced GL15 cell line was employed (24). GL15 shBeclin1 are not able to accumulate LC3II (the autophagosome-associated form of LC3) and to degrade the autophagy substrate P62, two autophagy hallmarks (25), in both basal and starved conditions, being Beclin1 indispensable for canonical autophagy (Figure 2A, left panel and (26)). Interestingly, Beclin1 silencing did not prevent EGFR de-localization upon EBSS incubation or upon Torin1 treatment for 18 hours (Figure 2A, right panels). To confirm an autophagy-independent role of mTOR on EGFR trafficking, we infected U87MG cells with a GFP-LC3 expressing retrovirus and analyzed EGFR localization upon autophagy stimulation. Immunofluorescence analysis shows the appearance of the typical GFP-LC3 dots, identifying autophagosomes, 24 hours after Torin1 treatment (Figure 2B). Notably, GFP-LC3 puncta did not co-localize with EGFR intracellular signal in Torin1-treated cells, thus excluding a re-localization of the receptor within autophagosomes (Figure 2B). These results indicate that mTOR activity is involved in EGFR internalization and trafficking, independently of its role on autophagy regulation.

2.3 mTOR inhibition targets EGFR to lysosomes

Next we investigated the subcellular compartment involved in EGFR translocation mTOR inhibition. To this aim, we performed immunofluorescence analyses by using specific subcellular markers for cytoplasmic organelles, that hare known to be involved in RTK trafficking (4).

Figure 3 A-C shows that EGFR signal is largely separated by those of endoplasmic reticulum, Golgi cisternae and mitochondria upon mTOR inhibition in U87 cells. By contrast, a great amount of EGFR was detected in correspondence to CathepsinD-positive puncta, indicating a lysosomal delivery of the receptor upon 24 hours following Torin1 treatment (Figure 3D). The occurrence of EGFR localization to lysosomes upon Torin1 administration was also observed in GL15 cells and in primary GH2 cells (Suppl. Figure S1).

2.4 EGFR expression is reduced upon mTOR inhibition in GBM cells

The obtained results prompted us to verify whether the mTOR inhibition could produce any effects on EGFR protein expression. Thus, we analysed the expression level of the receptor in U87MG and GL15 cells cultured in presence or not of Torin1. Western blotting analysis indicated a significant reduction of EGFR expression in 2 and 4 hours of Torin1 exposure (Figure 4A) which persisted up to 24 hours (Figure 4B), compared to control cells. Likewise, the reduction of EGFR protein levels was also observed in primary GH2 cells treated with Torin1 for 2, 4 and 24 hours (Figure 4C). These results suggest that the mTOR inhibition induces EGFR degradation, in line with the observation of EGFR lysosomal delivery.

2.5 mTOR inhibition leads to MAPK/ERK pathway down-regulation in GBM cells

EGFR is involved in cell proliferation, growth and differentiation, through the activation of several downstream signal transduction pathways, including MAPK/ERK cascade. Therefore, we checked whether the EGFR de-localization and degradation occurring upon mTOR inhibition also affected ERK activity. To this aim, U87MG and GL15 were grown or not in presence of Torin1 at different time points, and western blotting analyses was performed, by taking advantages of antibodies recognizing the phosphorylated, activated, forms of ERK1/2. As shown in figure 5A, ERK1/2 phosphorylation was strongly and persistently suppressed in Torin1-treated cells, being observable at 2 and 4 hours after treatment, and maintained up to 48 hours compared to control cells. ERK1/2 dephosphorylation was maintained up to 48 hours in U87MG and in GL15 (Figure 5B). Moreover, the dependence of ERK modulation on mTOR inhibition was further evaluated in GBM primary cells. Similarly to U87MG and GL15 cells, ERK1/2 activation was drastically reduced after 2 and 4 hours following Torin1 treatment, although it increased again after 24 hours exposure (Figure 5C).

2.6 SRC activity is required to EGFR internalization in GBM cells

RTK endocytosis is regulated by clathrin-dependent and -independent mechanisms. It has been shown that Src kinase plays a role in regulating EGFR endocytosis by phosphorylating clathrin heavy

chain following EGF binding to receptor (27,28). Thus, we checked whether Src activity was involved in EGFR internalization upon mTOR inhibition. First, we performed western blotting analysis by using an antibody raised against the phosphorylated and active form of Src on protein extracts of U87MG stimulated or not with EBSS, Torin1 or AZD8055 for 18 hours. As shown in Figure 6A, mTOR inhibition resulted in Src activation upon exposure to all the mTOR-inhibitory stimuli employed in this study. Src activation was already evident after 6 hours of Torin1 treatment, and was completely prevented by co-administration of Src inhibitor PP2 (Figure 6B). Notably, EGFR internalization was completely abrogated when cells were incubated with PP2 for both 6 and 24 hours, thus indicating the involvement of Src activation in mTOR-mediated receptor trafficking (Figure 6C).

3. Discussion

EGFR is one of the most studied RTK members due to its well-known oncogenic activity (10). For the same reason, during last decade, it has emerged as powerful target of multiple cancer therapies, some of them successfully introduced in clinical practice for some tumours type. A specific group of *EGFR* deletions, point mutations and amplification are frequently found in GBM (29). Specifically, overexpression of this receptor on the cell surface is found in 60% of primary GBM, and is associated with the most aggressive GBM phenotypes (12). Overexpression and oncogenic mutations of EGFR lead to spontaneous dimerization and activation of the receptor, independently on the presence of the ligand (30). In addition, beside abnormal expression and activation, dysregulated EGFR intracellular trafficking also plays a crucial role in GBM oncogenesis (9).

For the first time we demonstrated that the inhibition of the mTOR complexes obtained by drug administration or by aminoacid depletion strongly induces EGFR disappearance from the plasma membrane and its internalization within GBM cells. Receptor delocalization was still observed in cells defective for the autophagy master gene BECLIN1, thus suggesting that autophagy induction is not responsible for EGFR de-localization.

Besides the well-characterized recycling and degradative trafficking pathways, emerging evidence underlies alternative routes for EGFR endocytosis, including traffic to nucleus, mitochondria and other subcellular compartments (31,32). We did not found any co-localization with nucleus, mitochondria, ER and Golgi, whereas a strong overlapping signal was observed into lysosomes. EGFR positivity was not observed into autophagosomes, thus confirming that receptor delivery to the degradative compartment is not mediated by the autophagic network. An evident decrease in protein expression supports the evidence that mTOR blockade impairs receptor recycling and directs EGFR to the degradative route. This observation is in line with literature data demonstrating that

mTOR activity is necessary for the recycling route of Transferrin receptor and plasma membrane lipids (33). Indeed, mTORC1 inactivation reduces the expression of hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a component of Endosomal Sorting Complex Required for Transport (ESCRT), which is crucial for lysosomal targeting of ubiquitylated cargoes (33). This event leads to transferrin receptors and sphingomyelin delivery to lysosomes, independently of canonical autophagy.

In physiological conditions, EGFR phosphorylation at the plasma membrane leads to the recruitment of multiple effector proteins via recognition and binding of Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains to phosphotyrosine motifs on the receptor. Formation of the EGFR signaling complex, in turn, triggers a variety of signaling cascades involved in cell proliferation, migration, and survival. Interestingly, similar substrates are activated downstream of wt EGFR and EGFRΔIII, but with differing levels of intensity (13). These pathways include the phosphoinositide 3 kinase (PI3K), mitogen activated protein kinase (MAPK), signal transducer and activator of transcription 3 (STAT3) pathways and the non-receptor tyrosin kinase Src (17). In the tumoral masses, receptor alteration and impaired degradation are often associated to enhanced downstream oncogenic signaling (34,35). As a consequence of EGFR endocytosis and degradation, we observed a clear and rapid inhibition of ERK1/2 kinases activity in our models, thus suggesting a restriction of the proliferative potential of GBM cells. We also found that mTOR inhibitions elicits EGFR endocytosis by enhancing Src activation, as the administration of the Src inhibitor PP2 impairs the receptor internalisation when mTOR activity is suppressed. Src kinase is thought as a crucial partner for EGFR in mediating oncogenesis, as they co-localize in lipid rafts and synergize to increase the mitogenic activity of EGFRs (36,37). It has been also demonstrated that Src activity is required for clathrin phosphorylation and EGFR endocytosis upon EGF stimulation (27). Our results show that Src activity is positvily regulated by mTOR kinase in GBM cells and indicate a crucial role for mTOR/Src axis on EGFR trafficking and localization.

4. Material and methods

4.1 Cell culture

Human glioblastoma GL15 cells were kindly provided by Dr. E. Castigli, University of Perugia, Italy. Glioblastoma patient-derived culture GH2 as obtained from human GBM tumour samples from the Spanish National Cancer Center (CNIO, Madrid, Spain) biobank (GH2). Histopathological typing was done according to the WHO criteria resulating as grade IV. All procedures involving samples of human origin were performed with the approval of the corresponding ethical committees from each

institution as well as of the ethical committee of Complutense University (Madrid, Spain). Briefly, GICs cultures were obtained by using the following procedure: tumour samples were mechanically and enzymatically dissociated with 0.12 mg/ml of collagenase type Ia from Clostridium histolyticum (#C9722, Sigma) for 2 hours at 37 ºC and filtered using a 100 µm nylon filter (Millipore, Burlington, MA, USA). Cells were then plated and maintained as non-adherent neurospheres for at least 3 consecutive passages (with the aim of enriching the cultures in cells with stem-like properties) in a DMEM:Ham's F-12 media (Lonza, Basel, Switzerland) supplemented with 1% penicillin–streptomycin (Lonza), 5 mM HEPES buffer (Lonza), 2 mM ultraglutamine (Lonza), 20 ng/ml EGF and FGFb (Gibco, Carlsbad, USA), 2 µg/ml heparin sodium salt (Sigma), 1% B27 (Invitrogen, Carlsbad, USA) and 1 µg/ml leukemia inhibitory factor (LIF, Millipore).

Human U87MG and GL15 were cultured in DMEM (Lonza, Basel, Switzerland), supplemented with 10% heat-inactivated FBS (Euroclone, Milan, Italy) and 1% penicillin/streptomycin solution (Euroclone, Milan, Italy). Cells were grown at 37 °C in a 5% CO2 humidified atmosphere. shBECLIN1 and shCTR cells were prepared by lentiviral infection as previously described (24). U87MG were infected by incubation with the supernatant of GFP-LC3-expressing retroviruses for 8h in presence of 4 µg/ml poly-brene (38).

For autophagy induction and mTOR inhibition, cells were cultured in Earle's Balanced Salt Solution (Sigma Aldrich) or in presence of 250 nM Torin 1 (Sigma Aldrich), or 100 nM AZD8055 (Sigma Aldrich). For SRC kinase inhibition, 20 µM PP2 (Sigma Aldrich) was added to the culture medium.

4.2 Cell lysis and Western blotting

Protein extracts were prepared by lysing cells with the appropriate amount of RIPA Buffer (50 Mm Tris HCl pH 7,4, Triton 1%, Na Deoxycholate 0,25%, SDS 0,1%, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl 2 supplemented with protease inhibitors cocktail). After incubation on ice for 30 min, samples were centrifuged at 16,000 g for 30 min. Supernatants were recovered and protein concentrations were determined using Lowry protein assay (Bio-Rad, Hercules, CA, USA). Proteins were separated by means SDS-PAGE and then electroblotted onto nitrocellulose (GE Healthcare, Life Sciences, Little Chalfont, Buckinghamshire, UK). After blocking, membranes were incubated with primary antibodies diluted in PBS/5% non-fat dry milk/0,1% Tween-20 overnight at 4°C. Detection was obtained by using horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Milan, Italy) and visualized with ECL plus (GE Healthcare, Life Sciences, Little Chalfont, Buckinghamshire, UK). The following primary antibodies were used: anti- P-p70SK, anti-p70SK, anti- P-mTOR, anti- mTOR, anti-LC3B, anti- P-ERK1/2, anti- ERK1/2, anti- Src, anti- P-Src (Y416) (Cell Signaling, Danvers, MA, USA), anti-p62, anti-BECLIN1, anti-HSP90 (Santa Cruz

Biotechnology, Santa Cruz, CA, USA), anti-EGFR (Upstate Biotechnology, USA), anti-β-Actin (Sigma Aldrich, Milan, Italy).

4.3 Immunocytochemistry and confocal analysis

Cells were grown on coverslips and fixed with 4% PFA in PBS, followed by permeabilization with 0.1% Triton X-100 in PBS. EGFR (Upstate Biotechnology, USA), Calnexin, Tomm-20, Cathepsin-D (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Giantin (Abcam, Cambridge, UK) primary antibodies were incubated overnight at 4 °C and visualized by means of Alexa Fluor secondary antibodies (Invitrogen, Carlsbad, CA, USA).

After nuclear staining with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA), coverslips were mounted in antifade (SlowFade; Invitrogen, Carlsbad, CA, USA) and examined under a confocal microscope (TCS SP8; Leica, Wetzlar, Germany), equipped with a 40 × 1.40–0.60 NA HCX Plan Apo oil BL objective at RT.

Co-localization was assessed by calculating the Pearson's correlation coefficients of at least 10 cells analyzed in three independent experiments. The Pearson's correlation coefficient was expressed as mean ± SD.

4.4 Statistical analysis

All experiments were performed at least three times. Sigma Plot software was used for statistical analysis. Statistical significance was determined by using the Student's t-test. P value ≤ 0.05 was considered significant.

5. Conclusions

In our previous works, we demonstrated that nutrient starvation and mTOR inhibition strongly impaired EMT process and Wnt/β-catenin signalling in GBM cells, through β-catenin colocalisation with N-cadherin in sub-membrane areas (24,26,39). Here, we provide the first evidence for an involvement of mTOR/PI3K pathway in RTK trafficking and expression, thus enforcing the proof of concept that mTOR complexes may be considered as potential tools for pharmacological interventions in cancer, including GBM (40).

EGFR is currently considered a promising therapeutic target in cancer treatment; since its alterations show an homogenous and tissue-specific distribution across the tumour mass and are scarcely detectable in the healthy brain and in other tissues, making it an attractive target for therapeutic intervention. In this context, the clinical benefits of EGFR inhibitors have been observed in some

kinds of tumour. For instance, both monoclonal antibodies and EGFR-directed inhibitors are employed and continuously optimized as antitumorals. However, the occurrence of a multitude of adaptive mechanisms often leads to the appearance of drug resistance (41,42). Moreover, the effectiveness of EGFR-based therapies has not yet been validated in GBM. The observations here reported may help to set the bases for design novel therapeutic strategies to fight GBM, based on mTOR pharmacological inhibition.

Author Contributions: Conceptualization, Sabrina Di Bartolomeo, Guillermo Velasco and Marco Segatto; Data curation, Barbara Colella, Gianna Iannone, Claudia Fuoco and Marco Segatto; Funding acquisition, Sabrina Di Bartolomeo; Investigation, Barbara Colella, Gianna Iannone and Mayra Colardo; Methodology, Cristina Saiz- Ladera and Claudia Fuoco; Resources, Guillermo Velasco; Software, Mayra Colardo; Supervision, Sabrina Di Bartolomeo and Cristina Saiz-Ladera; Writing – original draft, Sabrina Di Bartolomeo; Writing – review & editing, Barbara Colella and Marco Segatto.

Funding: This work was supported by a grant from FFABR (Italian Ministry of University and Research) to S.D.B and by PI18/00442 grant integrated into the State Plan for R & D + I2013-2016 and funded by the Instituto de Salud Carlos III (ISCIII) and the European Regional Development Fund (ERDF), a way to make Europe to G.V.

Acknowledgments: We thank E. Castigli for kindly providing us with GL15. We also thank G.M. Fimia and M. Antonioli for GFP-LCR expressing and viruses, Israel López-Valero for help with culture of glioma cells and use of the microscopy facility and D. Barilà, C. Cirotti and C. Contadini for helpful discussion and critical reading.

Conflicts of Interest: The authors declare no conflict of interest.

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Figure legends

Fig.1 EGFR internalizes into GBM cells upon mTOR inhibition. (A) U87MG (upper panels), GL15 (middle panels) and primary GH2 cells (lower panels) were cultured in complete DMEM (CTR) or aminoacid- and serum- free medium (EBSS) or in DMEM in presence of 250nM Torin1 or 100nM AZD8055 for 18h. Immunocytochemistry and confocal analysis for EGFR localization (red) were then performed. Hoechst 33342 was used to stain nuclei (blue). Scale bar, 30 μM. Western blot analysis of P-p70S6K and p70S6K was performed to check mTOR pathway inhibition. Hsp90 was used as loading control. The blots are representative of three independent experiments. **(B)** Immunocytochemistry and confocal analysis for EGFR localisation (red) were performed in U87MG cells, upon 2h and 4h Torin1 treatment. Western blot analysis of P-p70S6K and p70S6K was carried out to check mTOR inhibition by Torin1. Scale bar, 30 μM.

Fig.2 EGFR de-localization in GBM cells is independent on canonical autophagy. (A) shCTR and shBECLIN1 GL15 cells (24) were cultured in DMEM (CTR) or EBSS media or in DMEM in

presence of 250nM Torin1 for 18 h and subjected to immunocytochemistry and confocal analysis for EGFR localisation (red) (right panels). Hoechst 33342 was used to stain nuclei (blue). Scale bar, 30 μM. Western blot analysis of P62 and LC3 I/II was performed to check autophagy induction (left panel). A specific antibody for BECLIN1 was used to check the silencing efficiency. β-ACTIN was used as loading control. The blot is representative of three independent experiments. **(B)** U87 cells were transduced with GFP-LC3-expressing retrovirus as described in Material and Methods. Infected cells, cultured in normal medium (CTR) or in DMEM containing 250nM Torin1 for 24h, were subjected to immunocytochemistry and confocal analysis for EGFR (red) and autophagosomes (green) localization. The images showing the merge of the two signals are shown in the right panels. Scale bar, 30 μM.

Fig.3 EGFR is delivered to lysosomes upon mTOR inhibition in GBM cells. U87MG treated with Torin1 for 24h or untreated (CTR) were subjected to immunocytochemistry and confocal analysis for EGFR (red) and for different subcellular markers (green): Calnexin for endoplasmic reticulum **(A)**, Giantin for Golgi cisternae **(B)**, Tomm-20 for mitochondria **(C)** and Cathepsin D for lysosomes **(D)**. The images showing the merge of the two signals are shown in the right panels. Inset containing a high magnification view of the merge image is also shown. Scale bar, 30 μM. Colocalization was assessed by calculating the Pearson's correlation coefficient r (mean r in D: CTR, 0.2 ± 0.03; Torin1, 0.75 ± 0.06).

Fig.4 mTOR inhibition leads to a reduction of EGFR expression in GBM cells. (A, B) U87MG, GL15 were cultured in complete DMEM medium in presence (TORIN1) or not (CTR) of 250nM Torin1 and analysed at the indicated time points. Western blot analyses were performed by using a specific antibody for EGFR. HSP90 was used as loading control. **(C)** GH2 cells were cultured in complete DMEM medium in presence (TORIN1) or not (CTR) of 250nM Torin1 and analysed at the indicated time points. Western blot analyses were performed by using a specific antibody for EGFR. HSP90 was used as loading control. The graph represents the mean ± SE of three different experiments. Statistical significance: * $P < .05$ Student t-test ** $P < .01$ Student t-test.

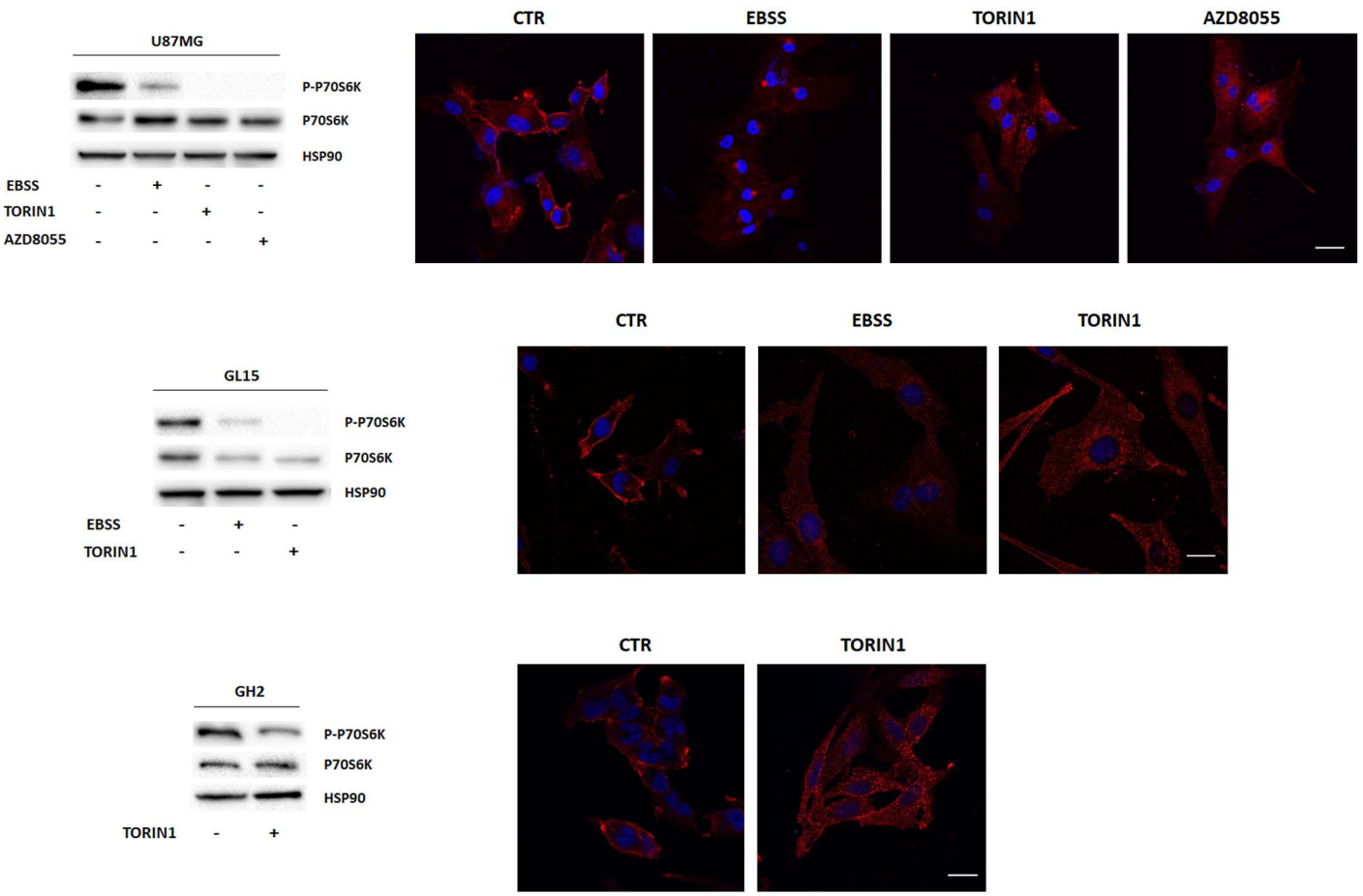
Fig.5 MAPK/ERK pathway is down-regulated upon mTOR inhibition in GBM cells. (A, B) U87MG and GL15 were cultured in complete DMEM medium in presence (TORIN1) or not (CTR) of 250nM Torin1 and analysed at 2h and 4h **(A)** and at 24h and 48h **(B)**. Western blot analysis was performed by using specific antibodies for ERK1/2 and P-ERK1/2. HSP90 was used as loading control. **(C)** GH2 cells were cultured in complete DMEM medium in presence (TORIN1) or not

(CTR) of 250nM Torin1 and analysed at the indicated time points. Western blot analysis was performed by using specific antibodies for ERK1/2 and P-ERK1/2. HSP90 was used as loading control. The graph represents the mean ± SE of three different experiments. Statistical significance: * P < .05 Student t-test ** P < .01 Student t-test, *** P < .001 Student t-test.

Fig.6 mTOR inhibition leads to SRC activation. (A) U87MG cells were cultured in complete DMEM (CTR) or aminoacid- and serum- free medium (EBSS) or in DMEM in presence of 250nM Torin1 or 100nM AZD8055 for 18h. Western blot analysis was performed by using specific antibodies for the total (SRC) and the phosphorylated (Y416) form of SRC (P-Src). β-ACTIN was used as loading control. **(B)** U87MG cells were cultured in DMEM in presence of Torin1 pre-incubating or not with 20μM PP2 inhibitor for the indicated time points. Western blot analysis was performed by using specific antibodies for the total (SRC) and the phosphorylated (Y416) form of SRC (P-Src). β-ACTIN was used as loading control. The graphs represent the mean ± SE of three different experiments. Statistical significance: ** P < .01 Student t-test *** P ≤ .001. **(C)** Immunocytochemistry and confocal analysis for EGFR localisation (red) were performed in U87MG, upon 6h and 24h of Torin1 and PP2 treatments, as indicated. Hoechst 33342 was used to stain nuclei (blue). Scale bar, 30 μM.

Supplementary Fig.S1 EGFR is delivered to lysosomes upon mTOR inhibition in GL15 and in primary GH2 GBM cells. GL15 and GH2 GBM cells, cultured in complete DMEM medium in presence (TORIN1) or not (CTR) of 250nM Torin1, were subjected to immunocytochemistry and confocal analysis for EGFR (red) and for lysosomes (green). The images showing the merge of the two signals are shown in the right panels. Inset containing higher magnification views of the merge images are also shown. Scale bar, 30 μM. Colocalization was assessed by calculating the Pearson's correlation coefficient r (for GL15: Ctr, 0.28 ± 0.01; Torin1, 0.7 ± 0.05. for GH2: Ctr, 0.2 ± 0.01; Torin1, 0.68 ± 0.02).

A


U87MG
P-P70S6K
P70S6K
HSP90
EBSS - + - -
TORIN1 - - + -
AZD8055 - - - +
CTR
EBSS
TORIN1
AZD8055
GL15
P-P70S6K
P70S6K
HSP90
EBSS - + -
TORIN1 - - +
CTR
EBSS
TORIN1
GH2
P-P70S6K
P70S6K
HSP90
TORIN1 - +
CTR
TORIN1

B

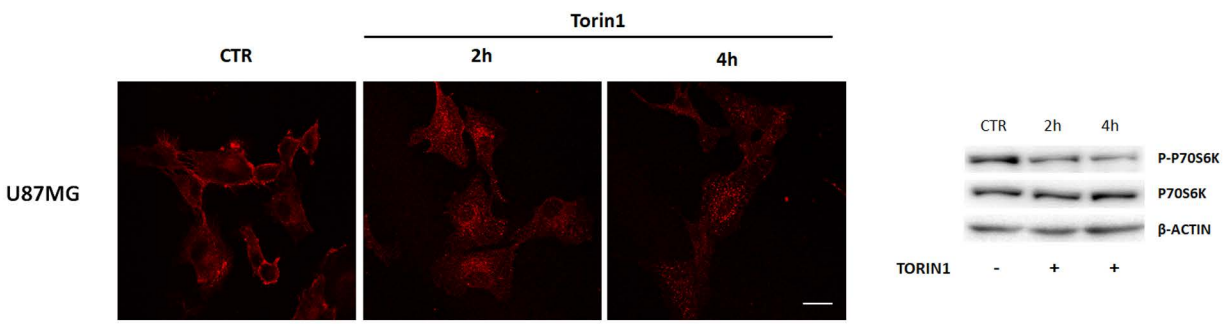
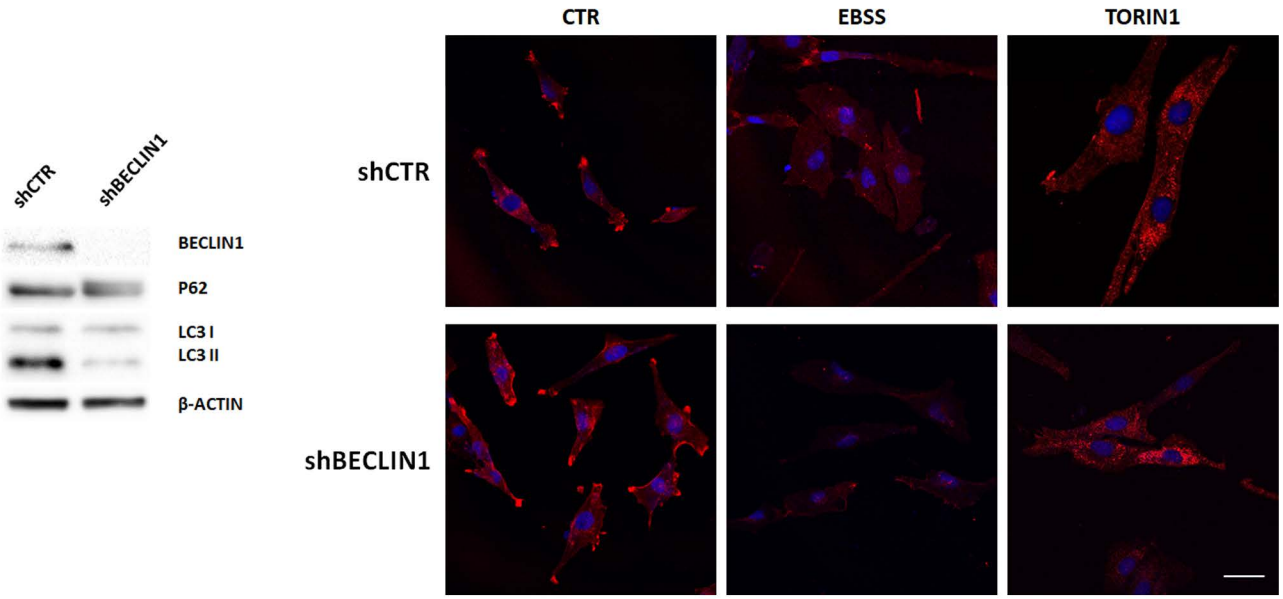

Torin1
CTR
2h
4h
U87MG
CTR 2h 4h
P-P70S6K
P70S6K
β-ACTIN
TORIN1 - + +

Fig.1

A


shCTR
shBECLIN1
BECLIN1
P62
LC3 I
LC3 II
β-ACTIN
CTR
EBSS
TORIN1
shCTR
shBECLIN1

B

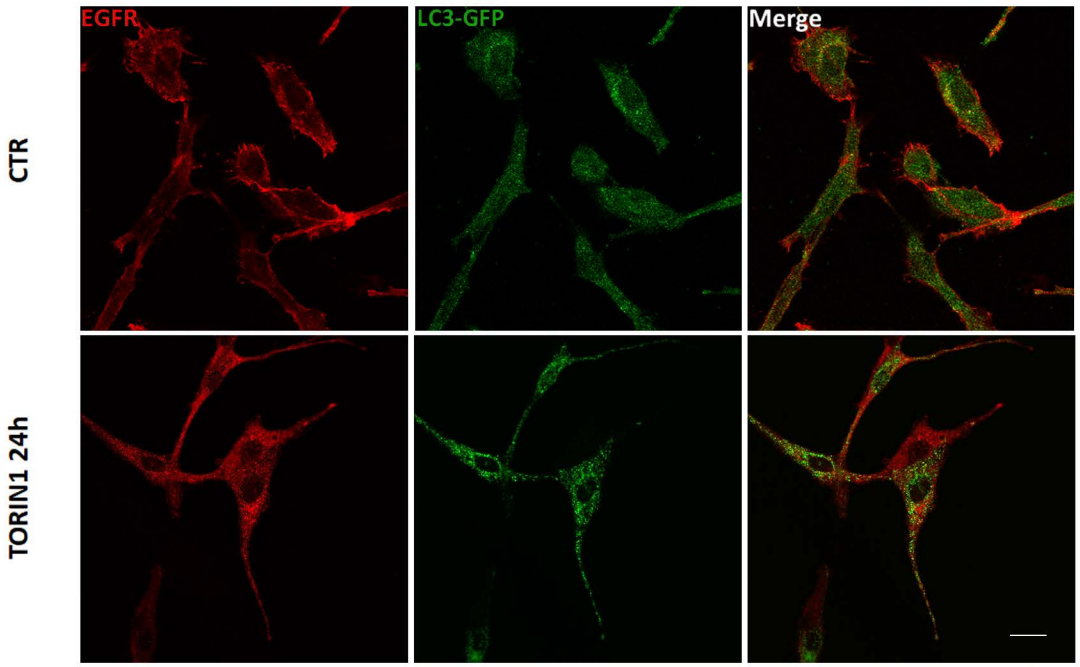

EGFR
LC3-GFP
Merge
CTR
TORIN1 24h

Fig.2

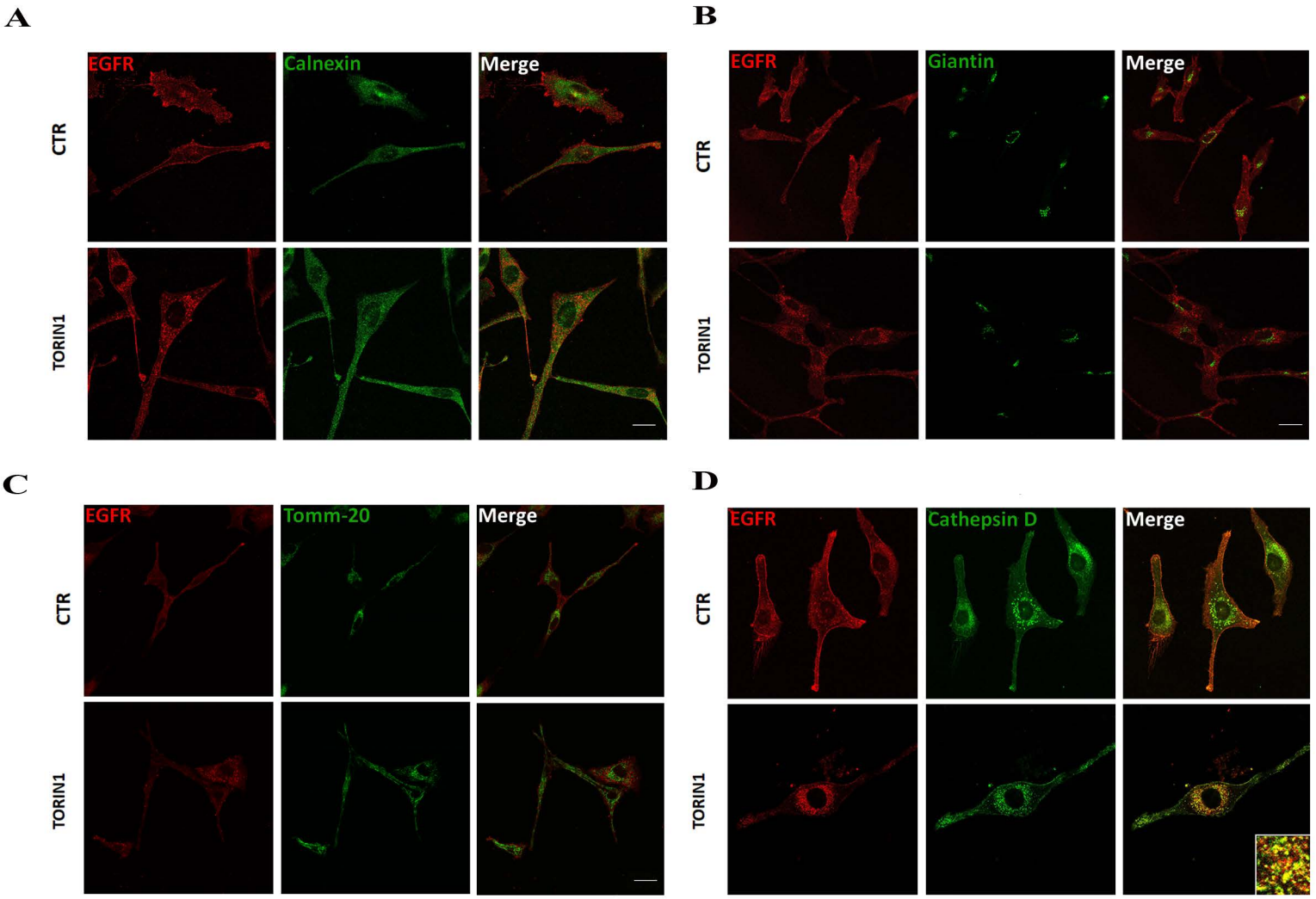
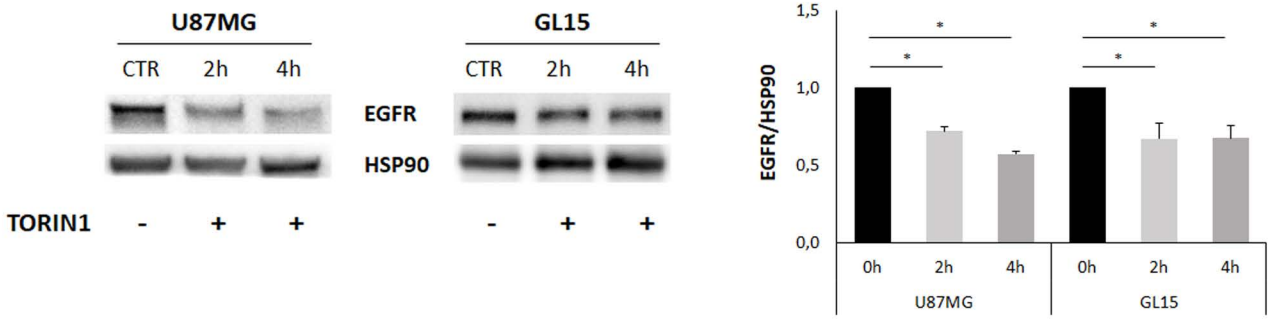
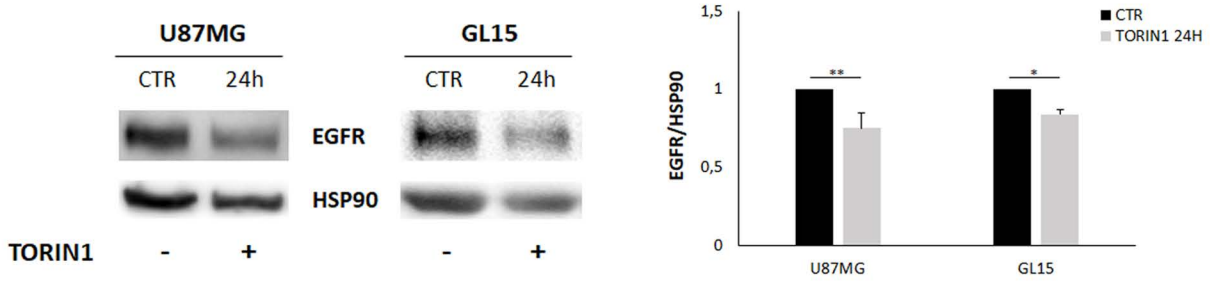

A
EGFR
Calnexin
Merge
CTR
TORIN1
B
EGFR
Giantin
Merge
CTR
TORIN1
C
EGFR
Tomm-20
Merge
CTR
TORIN1
D
EGFR
Cathepsin D
Merge
CTR
TORIN1

Fig.3

A


U87MG
GL15
CTR
2h
4h
CTR
2h
4h
EGFR
HSP90
TORIN1
-
+
+
-
+
+
EGFR/HSP90
1,5
1,0
0,5
0,0
0h
2h
4h
0h
2h
4h
U87MG
GL15

B


U87MG
GL15
CTR
24h
CTR
24h
EGFR
HSP90
TORIN1
-
+
-
+
CTR
TORIN1 24H
EGFR/HSP90
1,5
1
0,5
0
U87MG
GL15

C

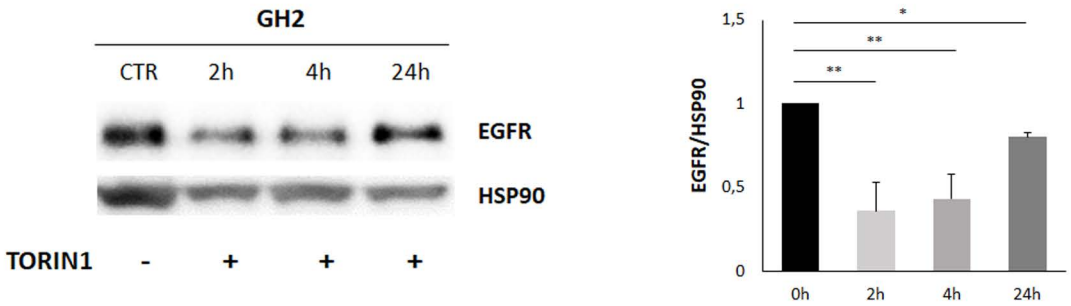
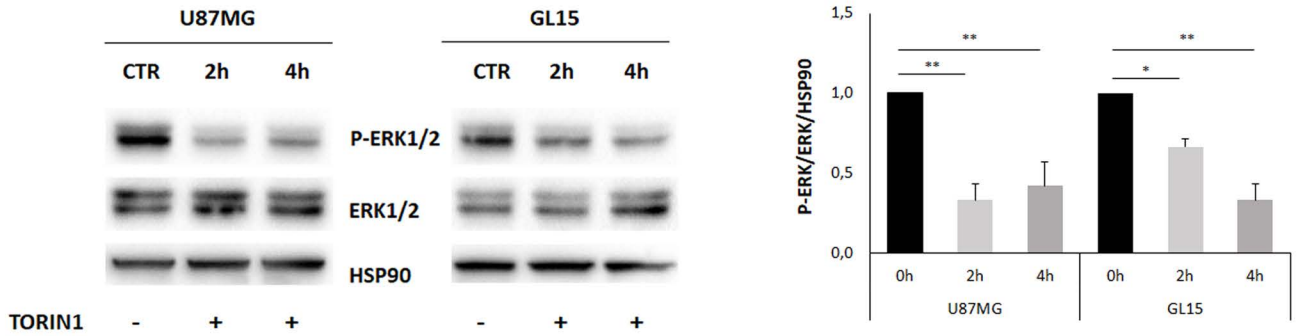
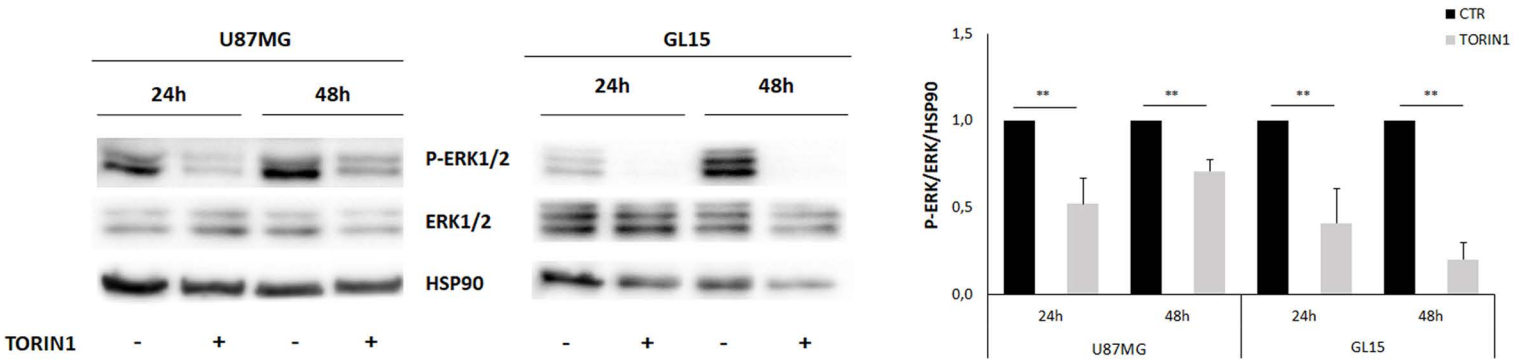

GH2
CTR
2h
4h
24h
EGFR
HSP90
TORIN1
-
+
+
+
EGFR/HSP90
1,5
1
0,5
0
0h
2h
4h
24h

Fig.4

A


U87MG
GL15
CTR
2h
4h
CTR
2h
4h
P-ERK1/2
ERK1/2
HSP90
TORIN1
-
+
+
-
+
+
P-ERK/ERK/HSP90
1,5
1,0
0,5
0,0
**
**
**
*
0h
2h
4h
0h
2h
4h
U87MG
GL15

B


U87MG
GL15
24h
48h
24h
48h
P-ERK1/2
ERK1/2
HSP90
TORIN1
-
+
-
+
-
+
-
+
CTR
TORIN1
P-ERK/ERK/HSP90
1,5
1,0
0,5
0,0
**
**
**
**
24h
48h
24h
48h
U87MG
GL15

C

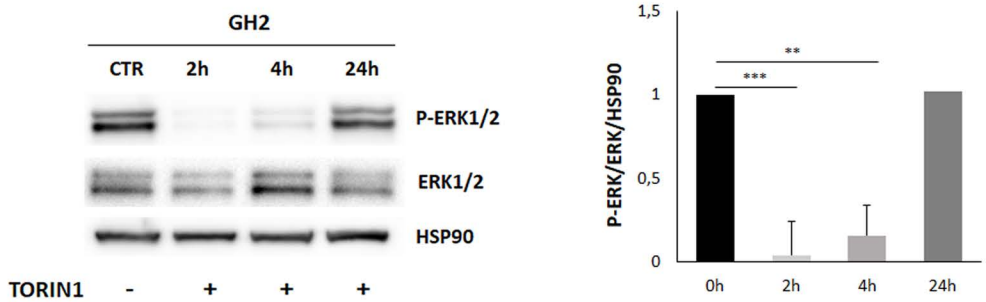
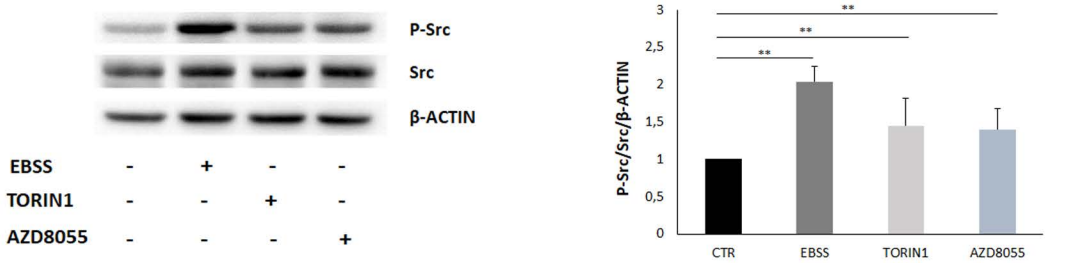
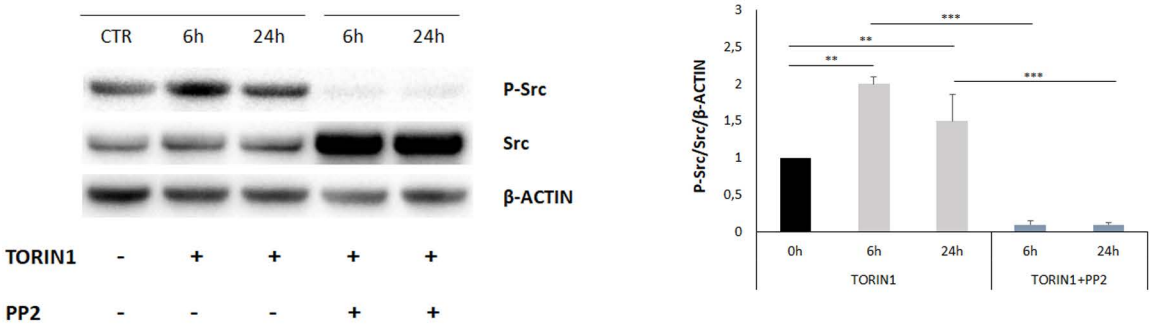

GH2
CTR
2h
4h
24h
P-ERK1/2
ERK1/2
HSP90
TORIN1
-
+
+
+
P-ERK/ERK/HSP90
1,5
1
0,5
0
**
***
0h
2h
4h
24h

Fig.5

A


P-Src
Src
β-ACTIN
EBSS - + - -
TORIN1 - - + -
AZD8055 - - - +
P-Src/Src/β-ACTIN
3
2,5
2
1,5
1
0,5
0
**
**
**
CTR
EBSS
TORIN1
AZD8055

B


CTR 6h 24h 6h 24h
P-Src
Src
β-ACTIN
TORIN1 - + + + +
PP2 - - - + +
P-Src/Src/β-ACTIN
3
2,5
2
1,5
1
0,5
0
**
**
***
***
0h 6h 24h 6h 24h
TORIN1
TORIN1+PP2

C

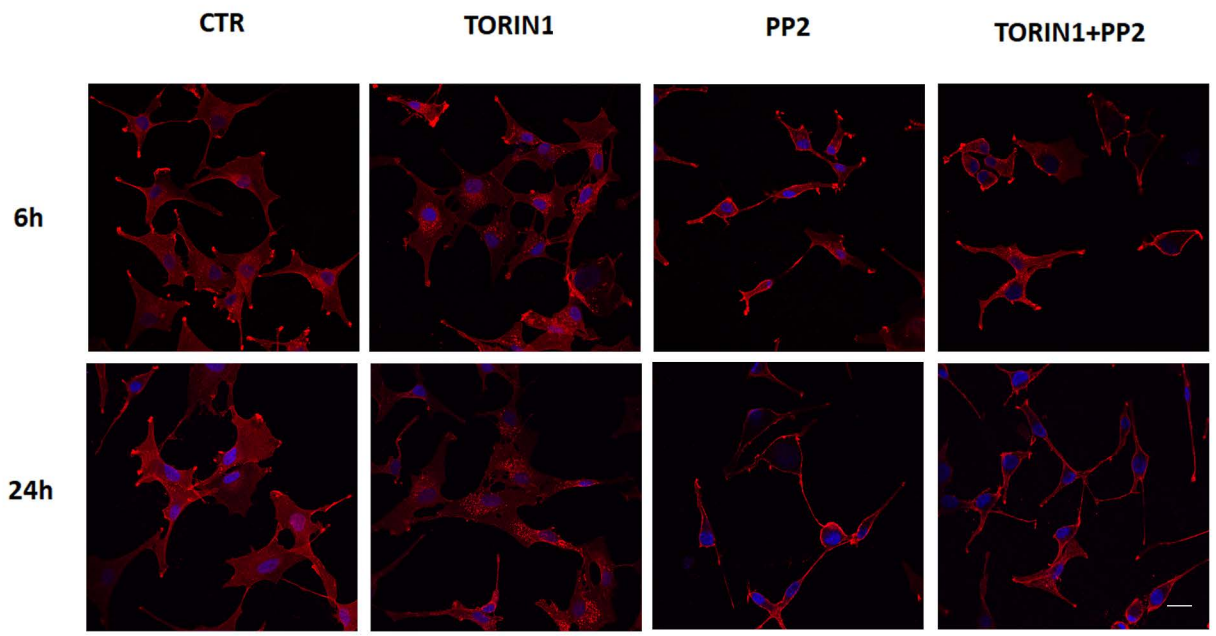
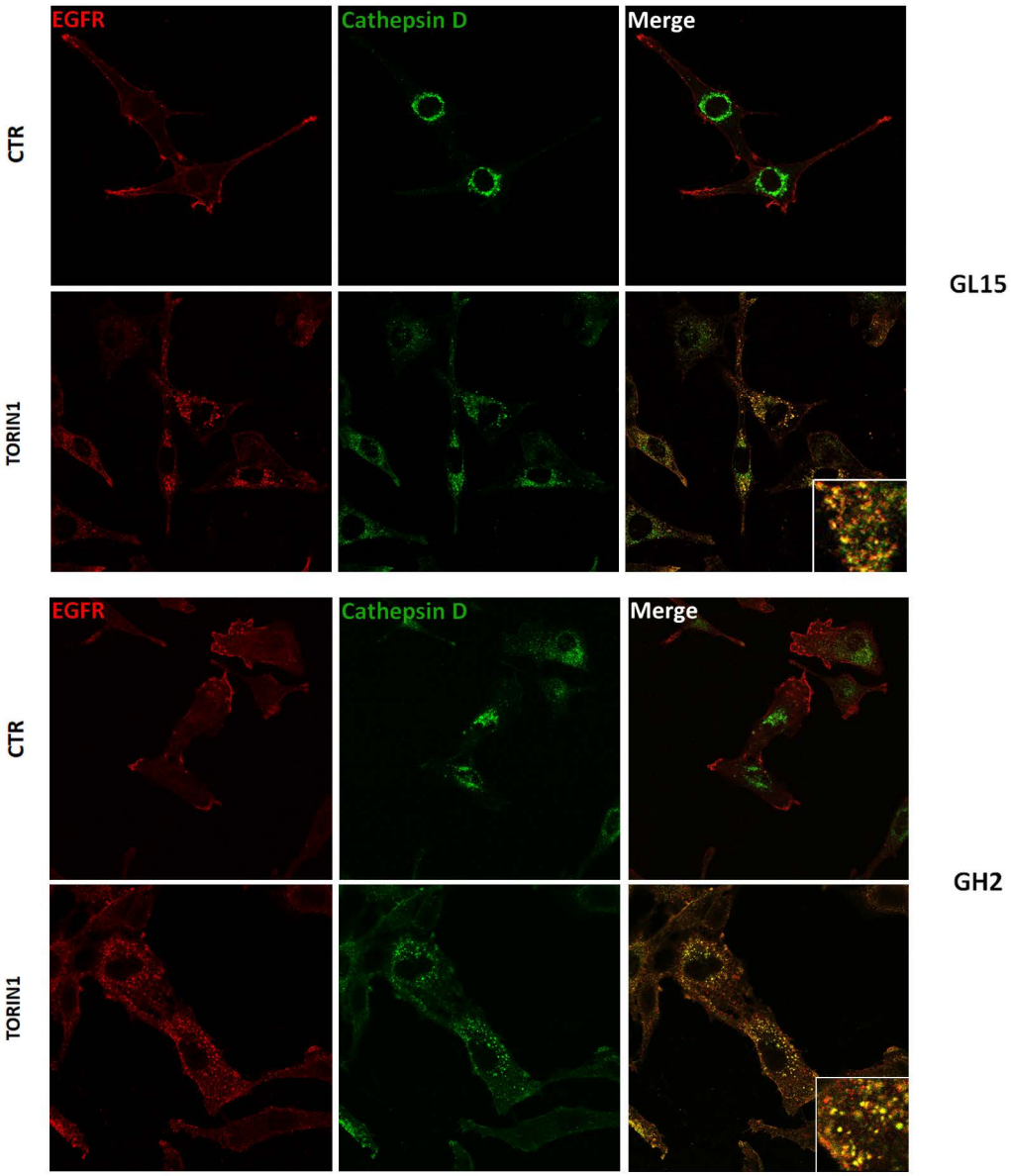

CTR
TORIN1
PP2
TORIN1+PP2
6h
24h

Fig.6



Supplementary Fig.S1

Conclusions and future perspectives

In this thesis, the role of autophagy in GBM biology was investigated through the analysis of different aspects involved in GBM pathogenesis.

The research article I illustrates the effect of autophagy induction on Wnt/β-catenin signalling in GBM cells. The main results indicated that autophagy induction leads to an impairment of Wnt/β-catenin signalling in GBM cellular models. Upon starvation and mTOR inhibition, we observed a strong down-regulation of β-catenin target genes and, unlike other models, a marked relocalization of the protein within the cell. Notably, β-catenin appears mainly localised in plasma membrane areas and co-localises with N-cadherin in correspondence of cell-cell contacts, suggesting the role of β-catenin in supporting the mesenchymal-to-epithelial-like transition. Accordingly, a decrease of the β-catenin nuclear fraction occurs, coupled to an accumulation in the cytosolic compartment.

Based on our results and on previous findings collected by Catalano and colleagues in 2015, it is now clear that autophagy induction contributes to the switch of GBM cells from a mesenchymal phenotype to an epithelial-like one.

In the manuscript II, the role of mTOR in EGFR trafficking was evaluated. Results obtained in the first research article led us to investigate EGFR expression and activity in our GBM cellular models. This receptor is often overexpressed and mutated in several cancer types, as well as in GBM, resulting in a constitutive activation of downstream proliferative signalling pathways. We observed that, upon several autophagy stimuli, EGFR is internalised within the cell, becoming unavailable for putative ligand binding on cell membrane. This delocalization results in EGFR delivery to lysosomes for degradation. According to this observation, a decrease of EGFR expression occurs in GBM cells upon the same treatments.

Canonically, EGFR lysosomal degradation is stimulated upon the interaction of the receptor with its growth factor ligands EGF. On the other hand, low concentration of extracellular ligands results in a higher recycling rate of the receptor to cell membrane.

In this work, instead, we demonstrated that EGFR is delivered to lysosomes without any EGF external stimulation.

Surprisingly, EGFR internalization appears to be independent on autophagy stimulation, but a consequence of mTOR inhibition. In fact, by using Beclin-1 silenced cells, we observed that silencing Beclin-1, one of the main autophagy regulators, does not prevent EGFR de-localization.

It is known that EGFR activation results in the induction of downstream pathways, including MAPK/ERK pathway, involved in several physiological and pathological processes such as proliferation, differentiation, growth, migration and apoptosis.

Interestingly, data obtained in the manuscript II also show that EGFR internalization leads to a negative regulation of MAPK/ERK pathway, confirmed by the strong reduction of the phosphorylated form of ERK 1/2 in cells that underwent mTOR inhibition, compared to control cells.

These results suggest that mTOR is required for EGFR trafficking and, therefore, for sustaining proliferative signalling.

Finally, we also found that the protein kinase Src is likely involved in the EGFR internalization, resulting more phosphorylated upon mTOR inhibition. To confirm that, by inhibiting both Src phosphorylation and mTOR pathway, the internalization of EGFR is prevented. The receptor appears, in fact, mainly localized in plasma membrane, as well as in control cells.

To date, EGFR is a promising therapeutic target in cancer treatment. Standard strategies used against EGFR, such as monoclonal antibodies and tyrosine kinase inhibitors, however, often lead to a multitude of adaptive mechanisms and appear ineffective in GBM therapeutical approaches. In a prospective vision, the inhibition of mTOR could be an effective alternative tool to contrast GBM progression by means of EGFR internalization and degradation.

Although controversial works about the autophagy role in cancer, as also reported in our published review, the findings shown in this doctoral thesis support the idea that targeting the autophagy process could be an effective strategy in the fight against this aggressive and still incurable cancer.