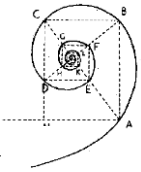

UNIVERSITÀ DEGLI STUDI DI MILANO

DOTTORATO IN MEDICINA MOLECOLARE E TRASLAZIONALE

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TESI DI DOTTORATO DI RICERCA

V-ATPASE AND AUTOPHAGY PREVENT GLIOMA GROWTH IN A *DROSOPHILA* MODEL SYSTEM

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SOMMARIO

Introduzione: *Il glioblastoma (GBM), il tipo più comune di glioma maligno, è un tumore incurabile del sistema nervoso. GBM ha origine nelle cellule gliali ma si diffonde rapidamente in tutto il tessuto cerebrale. Il trattamento del glioblastoma prevede la resezione chirurgica, radioterapia e chemioterapia, tuttavia queste aumentano la sopravvivenza fino a soli 14-15 mesi. Il GBM è un tumore altamente infiltrante, questo complica la resezione chirurgica, inoltre le cellule tumorali spesso sviluppano chemioresistenza al trattamento standard con la temozolomide (TMZ). Al momento non è disponibile una "targeted therapy" per il GBM. Tutti questi fattori determinano una sopravvivenza dei pazienti con glioblastoma di solamente il 5% a 5 anni dalla diagnosi. In molti campioni di GBM sono state riscontrate mutazioni a carico del recettore per i fattori di crescita dell'epidermide (EGFR) e mutazioni a carico della via di segnalazione pro-oncogenica del fosfoinoside 3-chinasi (PI3K). Entrambe queste vie di segnale regolano la crescita cellulare e sono altamente conservati nei moscerini della frutta. Per questo motivo abbiamo deciso di utilizzare Drosophila melanogaster come sistema modello per studiare in vivo lo sviluppo dei gliomi. La glia di Drosophila, come quella umana, è costituita da vari tipi di cellule. Queste cellule svolgono molteplici funzioni tra cui la formazione della barriera ematoencefalica e la fagocitosi di detriti neuronali. Abbiamo espresso nei precursori delle cellule gliali di Drosophila una forma costitutivamente attiva di EGFR e di p110, la subunità catalitica di PI3K. Tale manipolazione genetica causa una forte iperplasticità del tessuto gliale della larva. Per capire come le due vie attivate ectopicamente contribuiscono all'iperplasia, abbiamo analizzato la funzione dei lisosomi e dell'autofagia. L'autofagia è un processo catabolico conservato nell'evoluzione, che le cellule utilizzano per ottenere nutrienti attraverso la degradazione lisosomiale di componenti citoplasmatici. Il ruolo dell'autofagia nella crescita tumorale è al momento controverso e oscuro.*

Scopo: *Il nostro scopo è utilizzare larve di Drosophila come un modello di glioma in vivo, per capire che ruolo riveste l'autofagia e la degradazione lisosomiale nella crescita tumorale. In questo modo vogliamo identificare dei potenziali nuovi target terapeutici che possano essere traslati nella pratica clinica in modo da migliorare il trattamento della neoplasia, migliorando in futuro la sopravvivenza dei pazienti.*

Materiali e metodi*: Per caratterizzare la crescita tumorale e valutare il coinvolgimento dei processi autofagico-lisosomiali abbiamo utilizzato i cervelli di Drosophila al terzo stadio larvale. Le larve sono state fatte crescere in tubini con del cibo fino al raggiungimento del terzo stadio larvale, i cervelli sono stati quindi isolati dalle larve e utilizzati per gli esperimenti successivi. Per estrarre l'RNA abbiamo usato il kit della Qiagen Mini-RNeasy, mentre per l'estrazione proteica abbiamo usato RIPA buffer. Per disaggregare i cervelli larvali abbiamo usato la soluzione di Rinaldini e l'azione enzimatica delle proteasi. Per osservare il tessuto cerebrale, i cervelli sono stati dissezionati e processati usando anticorpi specifici per la microscopia a fluorescenza. Per diminuire l'espressione dei geni di interesse abbiamo usato delle linee di RNA-interferenza in Drosophila.*

Resultati: *L'eccessiva crescita gliale si accompagna a riduzione e severa compromissione del tessuto neurale. Inoltre, la crescita tumorale altera i comportamenti fisiologici delle larve e ne determina la morte. Il tessuto tumorale mostra aumentati livelli proteici e trascrizionali dell'adattatore dell'autofagia ref(2)P, l'omologo di p62 nei moscerini. Tuttavia, l'autofagia è controllata anche da altri geni che non risultano deregolati. Tra questi ci sono anche quelli trascritti da Mitf, l'unico omologo di TFEB in Drosophila. Questo risultato suggerisce che nel tumore l'autofagia non può essere attivata per sopperire ai bisogni della cellula. Inoltre, il compartimento lisosomiale è espanso nei gliomi, pur conservando la sua funzionalità. Abbiamo osservato anche che la diminuzione di espressione di un componente della pompa*

vacuolare V-ATPasica (V-ATPasi), fondamentale per l'acidificazione lisosomiale, è in grado di ridurre la crescita tumorale. Inaspettatamente, la diminuita espressione del componente della V-ATPasi comporta livelli di espressione di ref(2)P paragonabili a quelli controllo, sia a livello proteico che trascrizionale. Inoltre, abbiamo scoperto che la riduzione è anche in grado di prevenire la crescita tumorale andando a diminuire l'attività del PI3K pathway.

Conclusioni: *In nostri dati indicano che i processi autofagico-lisosomiali sono in grado di limitare la crescita tumorale e che la V-ATPasi può essere un promettente target per il trattamento del GBM.*

ABSTRACT

Introduction: *Glioblastoma (GBM), the most aggressive form of malignant gliomas, is an incurable tumor of the central nervous system which starts in glial cells, and rapidly diffuses through the brain. Surgical resection, radiotherapy and chemotherapy are the standard of care of GBM, improving the survival rate up to only 14-15 months. The high infiltrating nature of the tumor hampers surgical resection and tumoral cells often develop chemoresistance to the widely-used temozolomide (TMZ). Currently, a targeted therapy is not available. Therefore, the 5 years survival rate of these patients is extremely poor and does not exceed 5%. Most GBMs result from constitutive activation of the epidermal growth factor receptor (EGFR) and mutation in the pro-oncogenic phosphoinositide 3-kinase (PI3K) pathways, which are known regulators of cell growth. These pathways are extremely conserved in fruit fly: Because of this, we used Drosophila melanogaster as a model system to recapitulate in vivo certain tumoral features of GBMs. As the human one, Drosophila glia is constituted of several types of glial cells, which are involved in the formation of blood-brain-barrier and in phagocytosis of neural debris. In Drosophila glial precursor, we over-express a mutated form of the human EGFR and of Dp110, a regulator of PI3K signaling. Such manipulations result in massive hyperplasticity of the larval brain, due to an expansion of the glial compartment. Autophagy is a conserved catabolic process that provides nutrients by degradation of cellular constituents in lysosomes. How autophagy acts in tumor growth is currently unclear and the focus of intense investigation.*

Aim: *We used a Drosophila glioma model to understand in vivo the role of the autophagy-lysosomal pathway in tumor growth and to identify new potential therapeutic targets suitable for clinical translation.*

Materials and methods*: Drosophila 3rd instar larval brains were used to assess tumor growth and to monitor the autophagy-lysosomal pathway.*

Larvae were grown on standard fly food until the 3rd instar stage and larval brains were isolated and processed for the subsequent experiments. RNA was extracted by Mini-RNeasy kit from Qiagen, while proteins were extracted using RIPA buffer. Disaggregation of larval brains was carried out using Rinaldini's solution and proteases. For immunofluorescence acquisitions, brains were isolated from larvae and processed with specific antibodies. Down-regulation of a targeted gene was performed using RNAi fly lines.

Results: *The excessive glial growth affects non-cell autonomously the neural tissue, which appears reduced and morphologically altered. Moreover, tumor growth impairs larval behavior and leads to death. Interestingly, we found that compared to control, the hyperplastic tissue displays a transcriptional and protein up-regulation of the autophagy adapter ref(2)P, the Drosophila p62/SQSTM1. However, canonical regulators of autophagy, controlled by Mitf, the Drosophila TFEB, are not deregulated in the glial tumor, compared to control. This suggests that gliomas might not have the ability to induce the autophagy-lysosomal pathway to supply to their growth needs. Moreover, we observed that the lysosomal compartment is expanded but functional. In contrast, we found that down-regulation of a component of the vacuolar-H+ATPase (V-ATPase), which is required for lysosomal acidification, reduces tumor growth. Surprisingly, ref(2) protein and transcriptional levels are restored back to physiology in tumoral brain in which the component is down-regulated. Importantly, we found that depletion prevents most of glia overgrowth and decreases activity of the PI3K pathway.*

Conclusion: *Collectively, our data indicate that autophagy might be limiting for tumor growth and that components of the V-ATPase proton pump might be novel targets for treatment of GBM.*

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LIST OF ABBREVIATIONS

4EBP Eukaryotic Translation initiation factor 4E binding protein

AED after eggs deposition

AMBRA1 BECN1-regulated autophagy protein 1

AMP-K AMP-activated protein kinase

Atg1 Autophagy-related protein 1

bam bag of marbles

BBB blood-brain barrier

Bcl-2 B-cell lymphoma 2

brat Brain tumor

CLEAR Coordinated Lysosomal Expression and Regulation

CMA Chaperone-Mediated autophagy

CNS Central Nervous system

EGFR Epidermal Growth Factor Receptor

eIF4E eukaryotic translation initiation factor 4E

ERK1/2 extra-cellular signal-regulated kinase ½

EVs Extracellular vesicles

FIP200 the focal adhesion kinase family-interacting protein of 200 kD

FOXOs Forkhead family of transcription factors of the O subgroup

GBM Glioblastoma

GSC Glioblastoma stem cells

GSK3 Glycogen synthase kinase 3

HIF1a Hypoxia-inducible factor 1-alpha

IDH1/2 isocitrate dehydrogenase-1/2

IRS1/2 insulin receptor substrate 1/2

JNKs c-Jun N-terminal kinases

LC3 microtubule-associated protein 1 light chain 3

lgl Lethal giant larvae

MAPK mitogen-activated protein kinase

MGMT O6- Methylguanine-DNA methyltransferase

Mitf Microphthalmia-associated Transcription Factor

MMPs matrix metalloproteinases

MP1 MAPK scaffold protein 1

mTOR mammalian target of rapamycin

mTORC1/2 mammalian target of rapamycin complex 1/2

NBR1 Neighbor of BRCA1 gene 1 protein

OPTN optineurin

p62/SQSTM1 p62/sequestrome 1

PAS Phagophore Assembly Site

PI3Ks phosphoinositide 3- kinases

PINK1 PTEN-induced kinase 1

PIP3 phosphatidylinositol-3,4,5-trisphosphate

PKA protein kinase A

PTEN phosphatase and tensin homolog

RAVE Regulator of the V-ATPase of the vacuolar and endosomal membranes

ref(2)P Refractory to Sigma P

Rheb Ras homolog enriched in brain

RNAi RNA interference

RTKs Tyrosine Kinase Receptors

S.D. Standard Deviation

S6K p70S6 kinase

SEM Standard Error of Mean

SH sinomenine hydrochloride

siRNA short interfering RNA

SNAP (Soluble NSF Attachment Protein) Receptor (SNAREs) proteins

TCGA The Cancer Genome Atlas

TERT Telomerase reverse transcriptase

TFEB Transcription factor EB

TMZ Temozolomide

TSC1/2 Tuberous sclerosis complex 1/2

UBA Ubiquitin-associated domain

Ulk 1/2 unc-51-like kinase 1/2

UPL Universal Probe Library

V-ATPase Vacuolar-ATPase

VEGF vascular endothelial growth factor

VNC ventral nerve cord

WHO World Health Organization

Wnt Wingless related integration site

1. INTRODUCTION

1.1 Adult Brain cancer: malignant gliomas

1.1.1 Epidemiology of gliomas

Malignant gliomas are the most common and studied central nervous system (CNS) neoplasia of adults. The global incidence is of 5 cases per 100,000 population (Hanif, Muzaffar, Perveen, Malhi, & Simjee, 2017; Omuro & DeAngelis, 2013). Malignant gliomas are characterized by poor prognosis. They are named after their brain glial origin. However, they can also originate from pluripotent precursors. Gliomas are among the most devastating tumors, owing to their localization (Delgado-López & Corrales-García, 2016; Louis, Pomeroy, & Cairncross, 2002). The incidence of gliomas is higher in males than females and in white than black population (Omuro et al., 2013). The development of malignant gliomas is usually associated with the exposure to ionizing radiations. Only 5% of patients with glioma have a family history of these tumors. However, malignant gliomas can arise in combination with genetic diseases like neurofibromatosis type 1and 2, and Li-Fraumeni and Turcot syndromes (Wen & Kesari, 2008).

1.1.2 Diagnosis and treatment of gliomas

Clinically, typical features of malignant gliomas are headaches, nausea, seizures, focal neurological deficits or a combination of these symptoms (DeAngelis, 2001). To diagnose malignant gliomas, magnetic resonance (MR) is the gold standard. For the treatment of malignant gliomas, surgical resection is essential but it is not curative, due to the infiltrating ability of tumors that often leads to recurrence. Adjuvant therapy includes radiotherapy and oral chemotherapy with Temozolomide (TMZ), prolonging survival after surgical resection. Moreover, Carmustine, an alkylating chemotherapeutic, can be inserted in brain tissue after surgical resection.

The most common complications of this treatment with such an aggressive drug are lymphopenia, nausea, vomiting, and impairment in the neuroendocrine system (Omuro et al., 2013; Wen & Kesari, 2008). Despite this, no targeted therapy is currently available.

1.1.3 Prognosis of gliomas

Survival depends on many variables, ranging from the biology of the tumor to response to therapy. Prognostic staging is based on age, tumor grading and treatment responses. Younger patients tend to have a better prognosis, as is the case of lower grade patients, in contrast to higher grade patients. Positive prognostic factors are usually the possibility of massive surgical eradication, of radiotherapy and the chemosensitivity. Among negative prognostic factors, there are old age and chemoresistance.

1.2 Classification of gliomas

Malignant gliomas are classified by 2007 WHO as astrocytomas, oligodendrogliomas, and oligoastrocytomas. Based on histology, WHO divides them in grades: II, III, or IV. Astrocytomas grade IV are commonly known as glioblastoma (GBM). The last update of the World Health Organization (WHO) classification of malignant gliomas dates to 2016 (Louis et al., 2016). For the first time, the classification is not only based on tumor histology, but also on the molecular characteristics of the tumor, such as the mutational status of the metabolic enzymes isocitrate dehydrogenase 1/2 (IDH 1/2).

The Cancer Genome Atlas (TCGA) initiated a project to catalogue genetic alterations and expression patterns of gliomas to improve the characterization of malignant gliomas. This project is identifying a constantly increasing number of glioma markers. Among these markers, are: The mutational status of IDH1 or IDH2 gene, the methylation status of the O6-

Methylguanine-DNA methyltransferase (MGMT) promoter, mutation of telomerase reverse transcriptase (TERT) promoter, amplification of chromosome 7, deletion of chromosome 10, codeletion of 1p/19q chromosomes and amplification or mutation of the Epidermal Growth Factor Receptor (EGFR). A mutation common to all the different subclasses of gliomas is often the loss of the phosphatase and tensin homolog (PTEN) (E C Holland, 2000; Read, 2011; Verhaak et al., 2010).

1.3 Glioblastoma

Glioblastoma (GBM) is the most lethal tumor of the CNS accounting for 60-70% of all brain malignant tumors (Thakkar et al., 2014). Even when treated GBM has a dismal prognosis, indeed the 5-year survival rates are of only 5% (Hanif et al., 2017; Ostrom et al., 2014). Glioblastoma is often referred to as “multiforme”, not only for its histological variability but also for its genetical variety (E C Holland, 2000). Patients with positive prognostic factors, maximum surgical resection and adjuvant chemoradiotherapy show only 14.6 months of overall median survival (Hanif et al., 2017). Unfortunately, an early diagnosis does not ameliorate the clinical outcome of the patients (Omuro et al., 2013). A detailed table of the GBM prognostic factors is listed below.

Table 1 Prognostic factors related to glioblastoma survival

Prognostic factor	Comment
Age, sex, KPS	Younger females with good preoperative status live longer. Long-survivors (over 3 years) mostly belong to this group (with accompanying MGMT hypermethylation). They are all independent factors for increased survival
Extent of resection	Resections over 70–80 % on the enhancing mass and especially over 90 % (or residual tumor less than 5 cm^3) significantly improves survival
Grade	Anaplastic glioma (WHO grade III) associates longer survival compared to glioblastoma (WHO grade IV)
	Oligodendroglial component within tumor mass provides better prognosis and overall survival
Molecular GBM Cancer Genoma Atlas expression subtypes	*Classical* Enriched in gene expression patterns of astrocytes, mostly EGFR overexpression and loss of chromosome 10
	Mesenchymal Enriched in gene expression of astrocytes, mesenchymal markers and mutations in genes NF1 and PTEN deletions. Aggressive chemoradiation significantly decreases mortality in patients with classical or mesenchymal subtypes, but not in proneural subtype
	Proneural Enriched in patterns seen in oligodendrocytes. Alterations in: p53, PDGFR and IDH1. Youngest age at diagnosis. Typically seen in secondary GBM. Better prognosis and overall survival compared to the other subtypes
	Neural Enriched in gene expression seen in normal neurons
Mutations in biological markers	MGMT promoter hypermethylation (significantly increases survival), 1p19q (codeletion increases survival especially in tumors harboring oligodendroglial component), IDH1 and IDH2 (most common mutation in grade II and III gliomas; better prognosis), p53, TERT (most common mutation in GBM; worse prognosis), EGFR (overexpression of EGFR is either an unfavorable predictor or inconclusive prognostic factor), PDGFR, PTEN
Preoperative MRI parameters	Tumor-enhancing volume and eloquent location negatively affect survival. Size of edema/invasion (on FLAIR sequence) over 85,000 mm^3 significantly increases mortality
Volume of cases	Improves survival for all brain cancer patients in general. Not specifically studied for glioblastoma

Table 1. The main prognostic factors involved in the survival of patients with GBM. Adapted from David N. Louis et al., 2002

1.3.1 Glioblastoma Classification

Transcriptomics studies identified three transcriptional subclasses of GBM: Classical, mesenchymal and proneural (Q. et al., 2017). Based on genetic alterations, GBMs are divided into those with EGFR amplifications and those with TP53 mutations (Ohgaki & Kleihues, 2007). EGFR amplifications are typical of "*de-novo*" or "primary GBMs", that develop from grade IV astrocytoma and are more aggressive, more common and less responsive to radiotherapy than GBMs with TP53 mutations. These are defined as "secondary GBMs" because they tend to evolve from a lower grade astrocytoma and generally they affect young adults. IDH 1/2 are mutated in the 80% of secondary GBMs, conversely, only 6% of primary GBMs show these mutations. IDH 1 or IDH2 mutations are mutually exclusive (de Botton, Mondesir, Willekens, & Touat, 2016). A summary of reported genetic alterations is shown in **Figure 1**. The median age for primary glioblastoma

diagnosis is around 64 years, while for secondary glioblastoma is around 50-54 years.

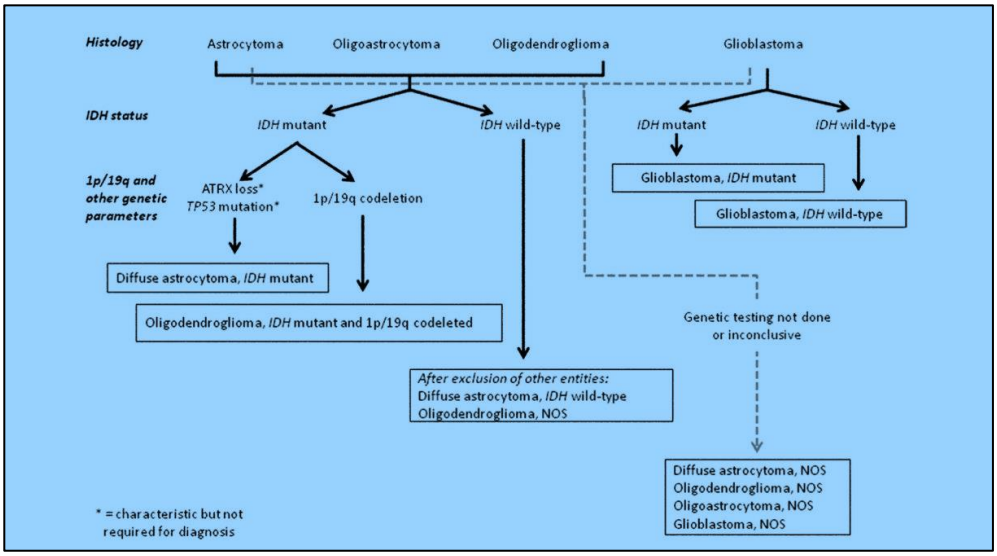

Figure 1. 2016 WHO classification of malignant gliomas based on histological and genetic features. The classification of malignant gliomas takes count of several mutations, highlighting the status of IDH 1/2. Adapted from David N. Luis et al., 2016

1.4 Genetic alterations in gliomas

1.4.1 EGFR pathway

Among Tyrosine Kinase Receptors (RTKs), EGFR is the most frequently altered in glioblastoma. EGFR was the first molecule associated with gliomagenesis (Downward et al., 1984), while the pathway is dysregulated in many types of cancers (Seshacharyulu et al., 2012). Around 40-50% of primary GBMs show amplifications or overexpression of components of the pathway (McLendon et al., 2008; Wen & Kesari, 2008). EGFR belongs to the ErbB tyrosine kinase family, constituted by transmembrane glycoproteins that regulate cell proliferation, survival and growth by binding growth factors (Normanno et al., 2005). After activation, EGFR interacts with different

effectors to propagate signals. The main characterized effectors are Ras and PI3K, whose signaling is shown in **Figure 2**. The most commonly mutated form of the receptor, which is found in 50% of GBMs, is the EGFRvIII. This mutated form lacks the extracellular inhibitory domain, due to an in-frame deletion of exons 2-7 (Normanno et al., 2005; Wen & Kesari, 2008). EGFR is considered a prognostic marker and an optimal target for GBM therapy (Binder et al., 2018). However, specific drugs to block the excessive EGFR signaling (Gefinitib, Erlotinib, Bevacizumab) are not very effective. This is because of prominent adaptation of tumor cells, which leads to resistance, as well as for the presence of the blood-brain barrier (BBB), that limits drug delivery. (Raizer et al., 2010; Westphal, Maire, & Lamszus, 2017).

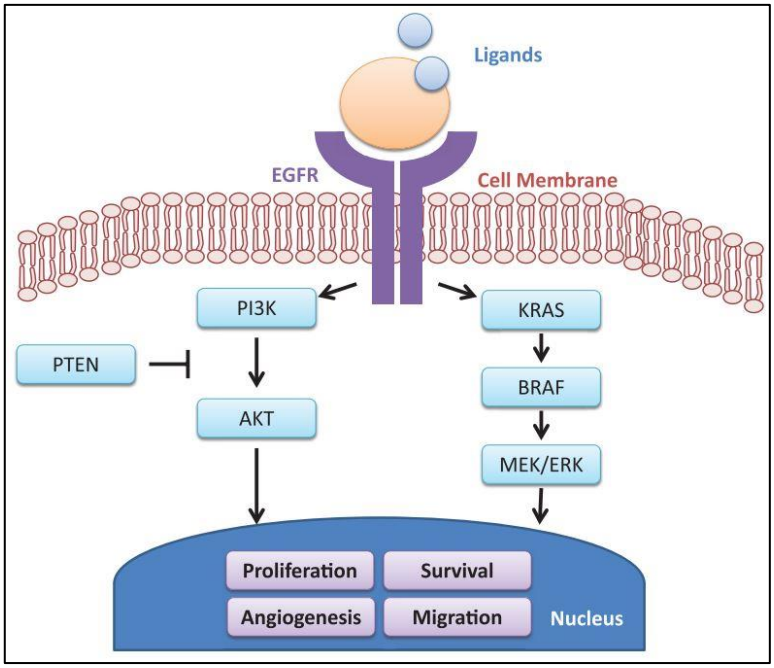

Figure 2. Schematic overview of the EGFR pathway and the main downstream effectors: Ras and PI3K pathways. After the binding with growth factors, the EGFR receptor activates PI3K-Akt and Ras-ERK pathways. The transduction of both signals leads to cell proliferation. Adapted from Hubbard and Alberts 2013.

1.4.2 PI3K pathway

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases acting downstream of several growth receptors such as the EGFR **(Figure 3)**. These kinases regulate cell proliferation, differentiation and survival. In GBM, the PI3K pathway is found mutated in 20% of tumors (Delgado-López and Corrales-García 2016; Wen and Kesari 2008b), additionally, PI3K can be overactivated due to amplification or activating mutations in EGFR, and several of the downstream effectors of this pathway are involved in tumorigenesis.

Based on the structural characteristics and on their substrates, PI3Ks are classified into three different classes. Class I PI3K enzymes are directly activated by different cell surface receptors and are composed by a p110 catalytic subunit and a p85 regulatory subunit. This class is further divided into class IA and IB, both regulating the formation of the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3), a phospholipid that resides on the plasma membrane leading to the activation of downstream targets. Specifically, only class IA is activated by RTKs, and several studies demonstrate that p110α is the major isoform driving proliferation in glioma cells (Q. W. Fan et al., 2006; Höland et al., 2014; McLendon et al., 2008).
Class II PI3K can be activated by RTKs, cytokine receptors and integrins. This class is composed of a single catalytic subunit. The specific role of this class in signal transduction is still unclear. Class III PI3K has a single catalytic subunit, Vps34, regulating membrane trafficking in a mammalian target of rapamycin (mTOR)-dependent manner. Vps34 is also involved in regulation of the autophagy pathway (Backer, 2008).

PI3Ks produce PIP3, whose cellular levels are strictly regulated by transformation in the inactive form PIP2, which is mediated by the tumor-suppressor PTEN. Thus, PTEN antagonizes the PI3K pathway and is inactivated in 40 to 50% of patients with GBMs (Baeza, Weller, Yonekawa,

Kleihues, & Ohgaki, 2003; Wen & Kesari, 2008). PIP3 leads to the translocation of the serine/threonine kinase Akt from the cytoplasm to plasma membrane. Due to conformational changes, Akt exposes two phosphorylation sites (S473 and T308). The kinase activation leading to phosphorylation of Glycogen synthase kinase 3 (GSK3), the Forkhead family of transcription factors (FOXOs) and other targets involved in the regulation of protein synthesis and metabolism. Akt mutations are seldom observed in malignant gliomas (Wen & Kesari, 2008).

In addition, downstream of PI3K and Akt, the serine/threonine kinase mTOR plays a fundamental role in the regulation of cell growth. The mTOR complex is constituted by two distinct complexes, mammalian target of rapamycin complex 1/2 (mTORC1) and mTORC2. The downstream targets of mTORC1 are p70S6 kinase (S6K) and 4E-binding protein (4E-BP), both of which are involved in cancer. The first one drives cell growth, while the second one inhibits translational processes. Consistent with this, mTOR overexpression is associated with poor prognosis and survival of GBM patients (Hu et al., 2005; Pelloski et al., 2006). The PI3K pathway has a pivotal role in cancer and is an attractive target for the development of specific therapies. However, the inhibition of a single component of the PI3K pathway has proved not effective (Cloughesy et al., 2008). Recently, a combined approach using PI-103, a p110α-specific inhibitor, is currently under preclinical evaluation, aiming at blocking both PI3K and mTOR pathways (Q. W. Fan et al., 2006; Koul et al., 2017).

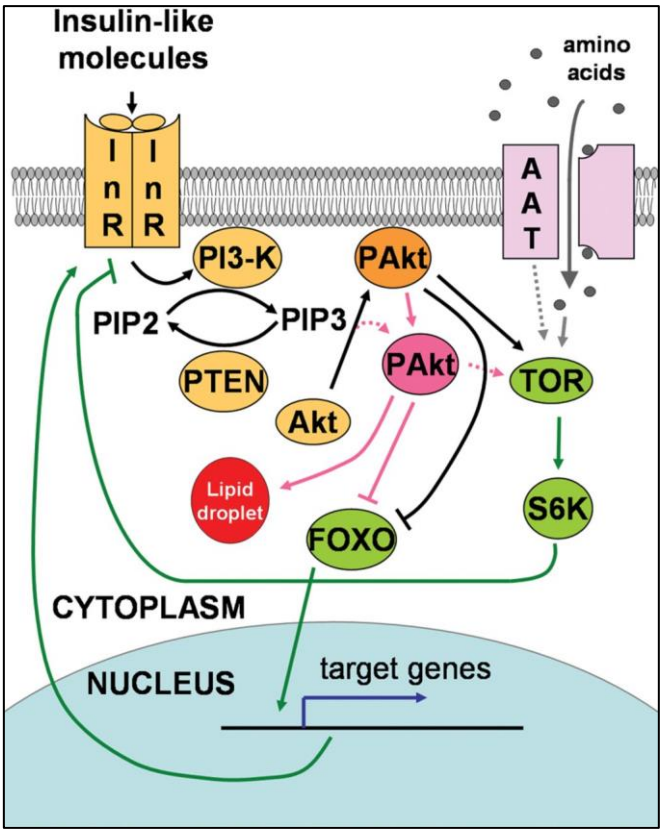

Figure 3. PI3K pathway. PIP3 generation is mediated by PI3K and leads to Akt phosphorylation and subsequent activation. This prevents FOXO nuclear translocation while mTOR can activate cell growth. Adapted from Wilson et al. 2007

1.5 Glioblastoma Treatment

Current GBM therapies consist of surgical resection, radiation and chemotherapy. The natural history of *de novo* GBMs, despite the radiotherapy, is limited to 6 months. Surgical resection improves survival of 6 months, while the combination of surgery and radiotherapy increases survival up to 12 months. Finally, the adjuvant therapy with TMZ extends survival up to 14.6 months (Delgado-López & Corrales-García, 2016; Hanif et al., 2017). Such aggressive treatments are critical to extend patient survival. However, these therapies often lead to irreversible damage to

patients. Among intracranial tumors, study of GBM is the most founded by the America National Institute of Health (Young, Jamshidi, Davis, & Sherman, 2015). Despite this, the development of new therapies has been inconclusive and new therapeutic approaches are in dire need. Recurrent failures in therapy development are due to the infiltrative and heterogeneous nature of the GBMs, the high proliferative rate, the resistance to chemotherapy and the encumbrance of the BBB. A recent study demonstrates that the BBB is not altered in all GBMs, however the development of new drug treatments have to circumvent the barrier to drug delivery to the CNS represented by the BBB (Sarkaria et al., 2018). Overall, a deeper knowledge of these aspects of GBM tumor biology are likely to be required for future therapy improvements.

1.5.1 Promising therapies in glioblastoma

Thanks to recent insights into glioblastoma biology, promising therapies are currently under development to increase patient survival and to reduce the adverse effects of chemotherapy. Targeted therapies towards modulation of EGFR, PI3K/Akt/mTOR and the vascular endothelial growth factor (VEGF) are under investigation (X. Li et al., 2016). Additionally, several virus-based therapy approaches, such as transfer of the tumor suppressor p53, or of the tumor-selective oncolytic viruses (Lang et al., 2018) or viral-stimulation of brain immune response (Desjardins et al., 2018) are currently in clinical trials.

1.6 Glioma models

Most improvements in the field of neuro-oncology are due to the ability to model tumor development using transgenic technologies. Models have proven useful to dissect pathways involved in glial tumorigenesis and to

identify potential therapeutic targets (Holland, 2001). For these reasons, several models, both *in vitro* and *in vivo*, have been developed.

1.6.1 *In vitro* models

GBM cell lines were extensively used in the past. Indeed, several glioblastoma cell lines are available, such as U87, T98G, U251. Albeit these cell line provided several insights about GBM biology, they often do not fully recapitulate the genetic alterations observed in patients with GBM. Because of the serum, the transcriptome of these cells is likely to not faithfully echo the features of primary tumors. However, Glioblastoma stem cells (GSCs), which are responsible for tumor initiation and relapse, can be isolated from surgical specimens and grown as spheres (usually named neurospheres) in serum-free culture. These cells possess more genetic similarity to the original tumors than to established cell lines. Moreover, a biobank of GSCs cell lines and their transcriptional characterization is available for the community (Y. Xie et al., 2015). Both cell lines and neurospheres can be transplanted into *in vivo* models such as mice. Despite this, xenograft of glioblastoma cell lines does not recapitulate features of tumor growth, while xenografts of GCSs lines reflect better tumorigenesis. Nevertheless, even GSCs are not able to mimic the complex environment of the tumor in the CNS. The most recent advance in *in* vitro modeling is the possibility of using transplantable human cerebral organoids (Ogawa, Pao, Shokhirev, & Verma, 2018).

1.6.2 *In vivo* models

Several engineered models are currently available to mimic, *in vivo*, the complex interplay between the tumor and the host. Indeed, *in vivo* models are better suited for observing the overall effects of experimental procedures. The study of tumor growth in the complexity of the brain tissue yields knowledge of glioma biology, that would be difficult to obtain with *in vitro*

approaches. Albeit glioma models do not reflect all the features of human glioma, they are useful especially for the identification of non-cell autonomous molecular mechanisms, expanding the possibility of discovery new targets for future development of new drug therapies (Huszthy et al., 2012). Researchers generated and studied many GBM *in vivo* model in vertebrates, such as mice, rat and recently in zebrafish (Chia, Hamilton, Mazzolini, Astell, & Sieger, 2018; Mayrhofer et al., 2017), as well as in invertebrates such as *Drosophila melanogaster*. The most studied *in vivo* models are those developed in rodents. In mice, a tumor can be propagated by xenografts of cell lines or fresh surgical samples. A tumor can also be induced by viral transfections or by transgenesis. Moreover, a tumor can be generated by chemicals (Kijima & Kanemura, 2017). The widely used glioma mouse models display alterations in oncogenic pathways such as Ras, PI3K and EGFR (Miyai et al., 2017). Despite the fact that *in vivo* models collectively recapitulate the main aspects of gliomagenesis, GBM is still essentially incurable in humans. The identification of new genes and new therapeutic targets is indispensable. However, genetic and pharmacologic screening using mouse models remains very expensive and time-consuming.

1.6.3 *Drosophila* genetic tools for disease modeling

Drosophila Melanogaster is an inexpensive model system that has been very useful for the discovery of components of signaling pathways (Clemens et al., 2000). In addition, its advanced genetics allowed identification of many genes involved in tumorigenesis (Brumby & Richardson, 2005). Finally, a lot of knowledge about neurodevelopment and neuronal wiring has been acquired using flies (Bellen, Tong, & Tsuda, 2010).

Screen for genes encoding signaling components or to identify new therapeutic compounds in flies are facilitated by their short life cycle, large progeny and by a well-described anatomy, which can be investigated with

numerous markers. Among the genetic tools available, the GAL4-UAS system is universally used because it allows over-expression or down-regulation of genes at will. The latter ones rely on RNA interference (RNAi) or on targeted mutagenesis, including that initiated by CRISPR/Cas9. The availability of large transgenic fly collections (Bloomington Stock Center, Vienna stock Center), facilitates *in vivo* modulation of most fly genes.

Thus, modeling human diseases using *Drosophila melanogaster* offers several advantages: The expression of multiple genes can be controlled in space-time and amounts, using cell type-specific drivers, inducible activators or site-specific integrations (Venken, Simpson, & Bellen, 2011). Such approach is especially useful to mimic complex tumor environments *in situ* and to speed-up dissection of complex genetic interactions and identification of gene functions.

1.6.3.1 *Drosophila* CNS

The *Drosophila* CNS is constituted by a bilobed brain and by a ventral nerve cord (VNC). As the mammalian brain, the *Drosophila* one originates from the ectoderm. Flies have different subtypes of glia: Surface glia (perineural and subperineurial), cortex glia, ensheathing glia and astrocytes-like glia, all of them fulfilling a specific role **(Figure 4)**. For instance, the ensheathing glia constitutes the glial phagocytic machinery, the fly counterpart of vertebrate astrocytes. Glial tissue constitutes around 10% of the total cells in the CNS (Doherty, Logan, Taşdemir, & Freeman, 2009).

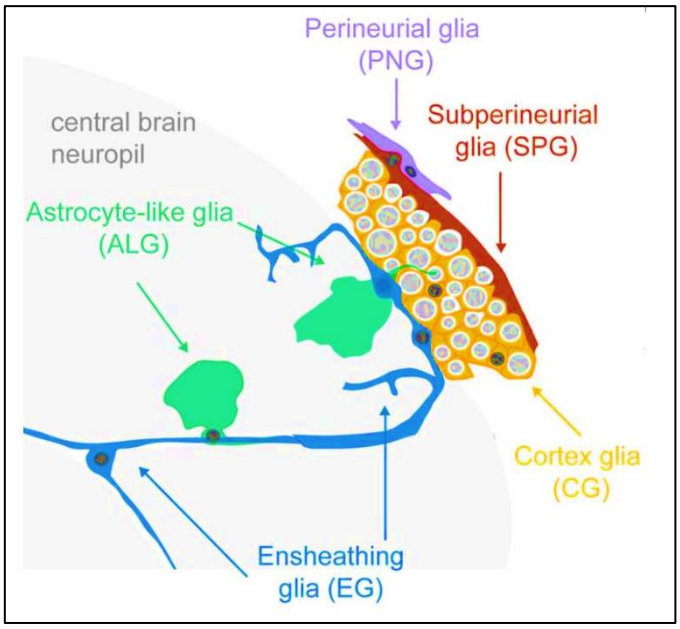

Figure 4. Glia structures in the 3rd instar larval brain. All the different types of glia are indicated using different colors. From the outside to the inside there are: Perineural and subperineurial glia, forming the BBB, cortex glia that envelopes neural cell bodies, astrocyte-like glia and ensheathing glia, the latter is the fly counterpart of astrocytes.

The neurogenesis in *Drosophila* can be divided in two distinct moments known as “waves”: The embryonic and the post-embryonic neurogenesis waves. The second one leads to neuron and glial cell differentiation. Mutations in genes involved in the neurogenic process are able to elicit neoplastic transformation and some of the genes mutated have a mammalian homologue, such as Lethal giant larvae (*lgl)* (Lgl1 and 2) and Brain tumor (*brat*) (TRIM32, TRIM3) (Klezovitch, Fernandez, Tapscott, & Vasioukhin, 2004; Schwamborn, Berezikov, & Knoblich, 2009). Thus, a search for genes able to reduce tumoral glia overgrowth in *Drosophila* is in principle a useful way to identify new therapeutic targets. Several studies uncovered evolutionarily conserved neurodevelopmental mechanisms in fruit flies and humans (Hirth & Reichert, 1999), with a high number of

neurodevelopmental genes first discovered in *Drosophila* (Karim et al., 1996; Simon, 2000; C. W. Wilson & Chuang, 2010). Indeed, *Drosophila* has been successfully used for the characterization of circadian rhythms (Jeffrey C. Hall, Michael Rosbash and Michael C. Young received the 2017 Nobel Prizes for this discovery) (Huang, 2018), learning and memory (Roman & Davis, 2001) and aggressiveness (S. Chen, Lee, Bowens, Huber, & Kravitz, 2002). Albeit the Drosophila brain shares many similarities with the human brain, notable differences include the absence of a specific immune response, making the model unsuitable for modeling diseases such as Multiple Sclerosis. Moreover, brain infarct and hemorrhage cannot be modelled due to the absence of vessels (Jeibmann & Paulus, 2009). Conversely, a strong advantage of *Drosophila* is the very high conservation of most proteins involved in diseases (Hirth & Reichert, 1999).

1.7 Renee Read's *Drosophila* glioma model

A *Drosophila* glioma model in which glia overgrowth is caused by ectopic activation of EGFR and PI3K pathways exists. Such model allowed identification of kinases that appear to mediate glioma growth (Read, 2011; Read, Cavenee, Furnari, & Thomas, 2009)**.** *Drosophila* encodes a single homologue for the core components of these pathways: EGFR (*dEGFR*), Raf (*dRaf*), PIK3CA (*dp110*), PTEN (*dPTEN*), and Akt (*dAkt*) (Read, 2011). In addition, the EGFR and PI3K pathways have a high degree of functional conservation between flies and mammals. To elicit overgrowth in the fly model, the promoter of the pan glial-specific gene *repo* is used as a diver for the expression of a constitutively active mutated form of Egfr (Egfrλ), lacking the extracellular domain. Moreover, to constitutively activate the PI3K pathway, a membrane-associated form of the catalytic subunit *Dp110α* known as CAAX (*Dp110*-CAAX) (Leevers, Weinkove, MacDougall, Hafen, & Waterfield, 1996) is used. The co-expression of Egfrλ and *Dp110*-CAAX

pathways lead to gliomagenesis and recapitulates around the genetics of 80% of malignant gliomas (Delgado-López & Corrales-García, 2016; Louis et al., 2002; Wen & Kesari, 2008). The model shows cooperation between oncogenes: Indeed, only co-expression of the two transgenes leads to gliomagenesis, a feature observed not only in *Drosophila* but also in mouse and to human cell models (E C Holland, 2000; Marumoto et al., 2009; Read, 2011). Tumor development in flies requires misexpression specifically in glial cells. In fact, the same transgenes expressed in neurons do not elicit tumor growth (Read, 2011). In addition, the constitutive activation of *Egfrλ* and *Dp110-CAAX* has to be sustained in time, as transitory expression is insufficient to induce glioma development. Furthermore, genetically-induced fly gliomas are transplantable as they are able to grow in the abdomen of fly hosts, causing early death (Read, 2011; Read et al., 2009). As a result of the study of the *Drosophila* glioma model, Read et al., 2009 demonstrated that inactivation of *dTor* and *dAkt* strongly suppresses tumor growth. On the contrary, glioma growth is reduced by activation of *FoxO*. In addition, they found that overexpression of *Thor* (4EBP in mammals), a *FoxO* target which inhibits the eukaryotic translation initiation factor 4E (eIF4E), leads to a reduction of excessive glial growth.

2 Autophagy

2.1 Autophagy in eukaryote cells

Autophagy (Greek for self-eating) is an evolutionary conserved catabolic process that regulates cell turnover and energy homeostasis. This process degrades heterogeneous cellular components into lysosomes, such as aggregated proteins, damaged organelles, macromolecular complexes and microorganisms (He & Klionsky, 2009; Lamb, Yoshimori, & Tooze, 2013; Mizushima & Komatsu, 2011). After degradation, macromolecules are

released into the cytoplasm and are available for recycling (He & Klionsky, 2009; Mizushima & Komatsu, 2011).

Autophagy is an adaptative response covering many types of cellular stresses (Z. Xie & Klionsky, 2007), indeed the pathway is compromised in several pathologies such as neurodegenerative diseases, infectious diseases, aging and cancer (Saha, Panigrahi, Patil, & Bhutia, 2018). Intense study of autophagy led to the identification of three main types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) **(Figure 5)** (Mizushima, 2007). Macroautophagy, differently from microautophagy and CMA, requires the formation of intermediate structures that have to fuse with lysosomes to degrade their content. Microautophagy is generally used during nutrient deprivation, with cytoplasmic material engulfed directly by invagination of the lysosomal plasma membrane (W. Li, Li, & Bao, 2012). In CMA, cytosolic proteins that require degradation are delivered towards lysosome surface by Chaperon proteins (Kaushik & Cuervo, 2012). Macroautophagy (hereafter named simply autophagy) can be non-selective or selective. The latter is named after the type of cargo that has to be degraded: xenophagy for bacteria or viruses, mitophagy for mitochondria, aggrephagy for protein aggregates (Galluzzi et al., 2008, 2017). Furthermore, autophagy can be distinguished into “basal autophagy” and “induced autophagy”: The first one regulates the physiological turnover of cellular components, while, the second one maintains the production of amino acids during starvation (Mizushima, 2007).

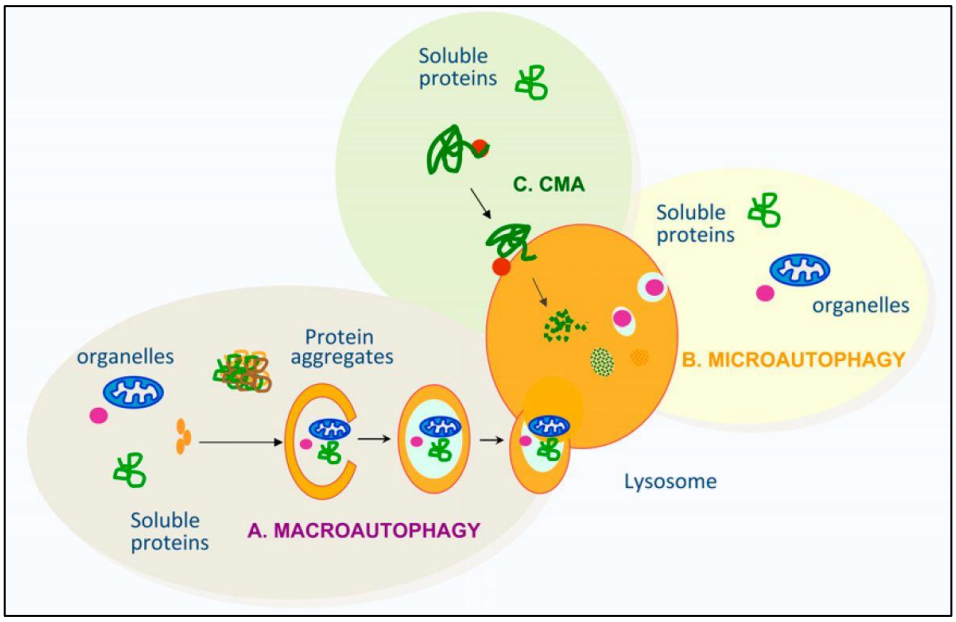

Figure 5. Macroautophagy, microautophagy and CMA. In Macroautophagy (A) cytoplasmic material is engulfed by autophagosome, then this organelle fuses with lysosomes to breakdown cargoes. During microautophagy (B), invagination of lysosomal membrane engulfs soluble proteins. In CMA (C), soluble proteins are transported toward lysosomal surface using Chaperones proteins. Adapted from Eloy Bejarano &Ana Maria Cuervo, 2010.

2.2 Brief history of autophagy

Autophagy was first observed by Keith R. Porter and Thomas Ashford. In hepatocytes of rat, they discovered that addition of the hormone glucagon leads to an increase in number of lysosomes containing organelles such as mitochondria (Ashford & Porter, 1962). Independently, Hruban and colleagues described three consecutive processes driving the engulfment of cytoplasmic material in injured and normal cells (Hruban, Spargo, Swift, Wissler, & Kleinfeld, 1963). Finally, De Duve and colleagues defined glucagon an inducer of autophagy in rat liver (Deter & De Duve, 1967). The liver tissue was widely useful for later studies on autophagy (Berg, Fengsrud,

Strømhaug, Berg, & Seglen, 1998; Øverbye, Fengsrud, & Seglen, 2007; Samari, Møller, Holden, Asmyhr, & Seglen, 2005).

The initial characterization of the different stages of the autophagy pathway has been done in the yeast *Saccharomyces cerevisiae* (Takeshige, Baba, Tsuboi, Noda, & Ohsumi, 1992; Thumm et al., 1994; Tsukada & Ohsumi, 1993). Following this works, Yoshinori Ohsumi received the 2016 Nobel prize (Ke, 2017).

2.3 Autophagy events

In yeast, autophagy pathway starts with the recruitment of Atg (Autophagy-related) proteins to the Phagophore Assembly Site (PAS), while in eukaryotes it starts with a double-membrane structure called phagophore or isolation membrane (Lamb et al., 2013). The elongation of the phagophore, which engulfs cytoplasmic components for the degradation, generates a double-membrane vesicle called autophagosome. Using microtubules, the autophagosome is transported towards vacuoles, the fusion between these organelles leads to cargoes degradation **(Figure 6).** This fusion step is tightly regulated by SNAP (Soluble NSF Attachment Protein) Receptor (SNAREs) proteins. In *Drosophila* and mammals, SNARE proteins promote the membrane fusion between the autophagosome and lysosome, generating an autolysosome (Itakura, Kishi-Itakura, & Mizushima, 2012; Morelli et al., 2014; Takáts et al., 2013). Hydrolases mediate the breakdown of cargos and the release of degradation products into the cytoplasm.

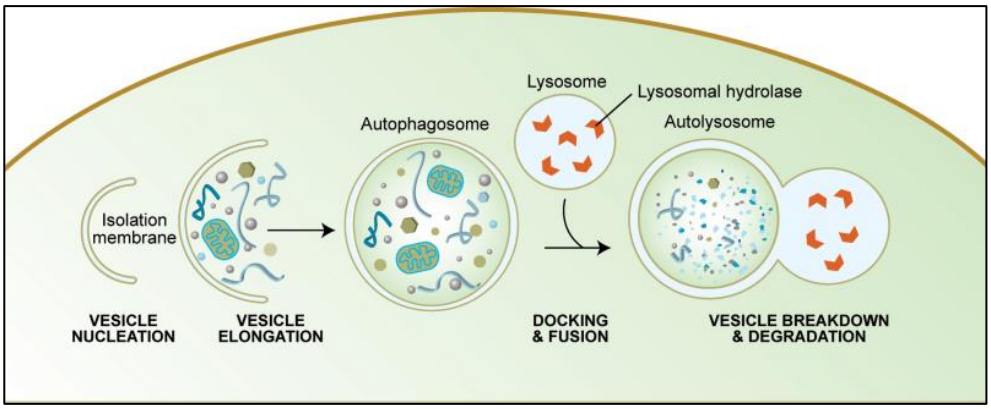

Figure 6. Overview of the steps of autophagy. Autophagy begins with the formation of the isolation membrane, a process called vesicle nucleation. The recruitment of Atg proteins on the isolation membrane drives its elongation into an autophagosome, leading to the engulfment of cytoplasmic cargoes. When the outer membrane of the autophagosome fuses with a lysosome, it forms an autolysosome. In the last step, cargoes are degraded and recycled. Adapted from Alicia Meléndez & Beth Levine, 2005.

2.4 Molecular machinery of autophagy

Screens using yeast and autophagy-altered organisms allowed to decipher molecular mechanisms that control this pathway. At steady state, autophagy levels are low but under stress condition the process is fastly induced by several stimuli such as amino acids starvation, oxidative stress, energy deprivation. These stimuli elicit a rapid increase in the number of autophagosomes attracting Atg proteins (Mizushima & Komatsu, 2011). These proteins, originally identified in yeast are evolutionarily conserved in all eukaryotes (Meijer, Van Der Klei, Veenhuis, & Kiel, 2007).

In *Drosophila*, the initiation of autophagy requires the Atg1 kinase (unc-51-like kinase 1/2 (Ulk 1/2) in mammals) at the phagophore. This kinase is crucial for the recruitment of other Atg proteins, indeed Atg1, Atg13 and the Focal adhesion kinase family-interacting protein of 200 kD (Fip200) are required for autophagy induction. The initiation of the phagophore formation

requires a complex composed by five molecules: the lipid kinase Vps34, Vps15 (p150 in mammals), Atg14 and Atg6 (Beclin1 in mammals). Atg6 can be sequestered by the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2), inhibiting autophagy induction, thus linking this pathway with apoptosis (He & Klionsky, 2009). Moreover, Atg6 promotes the production of PIP3 by Vps34, which is used for the elongation of phagophore (Mizushima, 2007).

Two ubiquitin-like conjugation complexes are required for the maturation of the autophagosome, the first one is the Atg5, Atg12, Atg16 complex, while the second one is Atg8a-PE (mammalian microtubule-associated protein 1 light chain 3, LC3). After the cleavage by Atg7, Atg12 is attached to Atg5. The Atg5-Atg12 dimer interacts with Atg16 and together they bind the phagophore. Atg8a is cleaved in Atg8a I by the protease Atg4 and Atg7. Upon the induction of autophagy, Atg8a is lipidated to form Atg8a-PE (Atg8a II). Atg8a II is targeted to the inner and outer membrane of the autophagosome by Atg12-Atg5-Atg16 complex. Atg8a marks the autophagosome until the fusion with a lysosome. Therefore, Atg8a is commonly used to monitor the induction of the autophagy flux (Mauvezin, Ayala, Braden, Kim, & Neufeld, 2014). The autophagic machinery is shown in **Figure 7**.

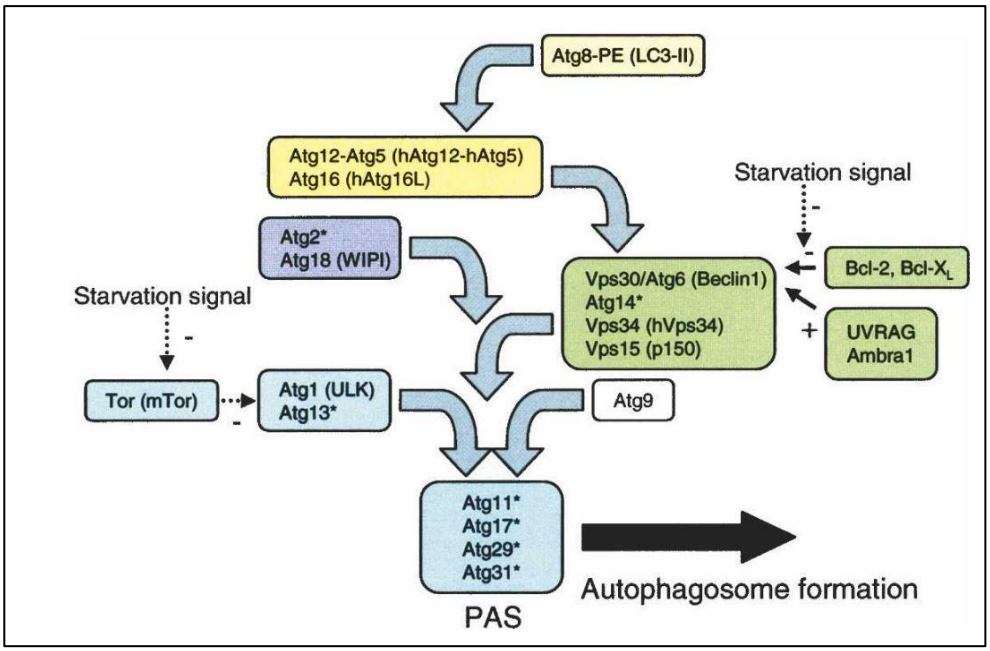

Figure 7. The autophagic machinery. The autophagy induction is regulated by mTOR. Its inhibition allows the recruitment of Ukl1/2 at PAS. Several proteins are required for autophagosome formation. The two ubiquitin-like systems are essential for the autophagosome formation. Indeed, the Atg12-Atg5-Atg16 system and LC3-II system allows the autophagosome maturation and enclosure. Adapted from Noburu Mizushima, 2007

2.5 Autophagy adapters

Autophagy can be a selective process when cargoes that have to be degraded are recognized by autophagic adapter and receptors. In *Drosophila*, the removal of ubiquitinated aggregates is mediated by the adaptor Refractory to Sigma P (ref(2)P). It was originally characterized in a screen for modifiers of sigma virus multiplication (Carré-Mlouka et al., 2007). ref(2)P recognizes mono and poly-ubiquitinated proteins through its Ubiquitin-associated (UBA) domain. Additionally, using a specific motif, ref(2)P can also bind Atg8a, which is considered an autophagy receptor. This allows the internalization of ubiquitinated cargoes during the autophagosome elongation. An impairment in the autophagic flux forces ref(2)P accumulation,

originating toxic aggregates. Indeed, ref(2)P positive aggregates have been found in *Drosophila* adult brains and are associated with aging and neurodegeneration (Kinghorn et al., 2016; Nezis et al., 2008). Moreover, ref(2)P is widely used to monitor the induction of autophagy flux (Mauvezin et al., 2014). In mammals, the homologue of ref(2)P is p62/sequestrome1 (SQSTM1). Others common mammalian autophagy adapters are Neighbor of BRCA1 gene 1 protein (NBR1) and Optineurin (OPTN). While p62 and NBR1 are involved in recognition of ubiquitinated cargoes, conversely OPTN is involved only in mitophagy (Kirkin, McEwan, Novak, & Dikic, 2009; Wong & Holzbaur, 2014). Also, in mammals p62 positive aggregates are pathological features of disorders such as neurodegenerative disease and cancer (Mizushima & Komatsu, 2011).

2.6 Signaling pathways that regulate autophagy

2.6.1 mTOR-mediated regulation of autophagy

In all eukaryotes, autophagy is tightly and rapidly regulated to face different cellular stresses. mTOR is the main negative regulator of autophagy, while AMP-activated protein kinase (AMP-K) and Ulk1/2 are positive regulators of the pathway. Importantly, the anabolic process of growth and the catabolic process of autophagy are regulated in opposite way. The kinase mTOR activates mTORC1, which is strictly involved in the regulation of nutrient status of the cell. Plenty of amino acids signal to activate cell growth. Consequently, mTORC1 inhibits autophagy by phosphorylating Ulk1. In contrast, nutrient deprivation prevents mTORC1 activation, with Ulk1 fully able to phosphorylate FIP200 and Atg13 (Hosokawa et al., 2009) to promote autophagosome formation. The activity of Ulk1 is also stabilized by the Activating Molecule in BECN1-Regulated Autophagy protein 1 (AMBRA1)

(Nazio et al., 2013). Also in *Drosophila*, the mTor homolog dTor modulates phosphorylation of Atg1 (Lee et al., 2007).

2.6.2 mTORC1-V-ATPase-Mitf axis regulates autophagy

Recent observations reveal another mechanism that controls mTORC1-mediated autophagy. The cell growth regulator can be also activated or inhibited by Rag guanosine triphosphatases (GTPases) (Kim, Goraksha-Hicks, Li, Neufeld, & Guan, 2008), according to the amino acid content sensed by lysosomal proton pump vacuolar-ATPase (V-ATPase). Specifically, when mTORC1 is active it resides on the lysosomal surface and prevents nuclear translocation of the Transcription factor EB (TFEB) (Sardiello et al., 2009). Starvation or inhibition of cell growth activates Rag GTPases that release mTORC1 from the lysosomal surface. Thus, TFEB is free to translocate into the nucleus to modulate expression of genes involved in autophagy or in lysosomal biogenesis, such as those encoding subunits of the evolutionary conserved V-ATPase proton pump **(Figure 8)** (Peña-Llopis et al., 2011). Therefore, TFEB is a positive regulator of autophagy contributing to autophagosome formation and fusion with the lysosome (Settembre et al., 2012; Settembre, Fraldi, Medina, & Ballabio, 2013). *Drosophila* Microphthalmia-associated Transcription Factor (*Mitf)* initially associated specifically with eye development is the unique homologue of TFEB (Hallsson et al., 2004). Mitf has been recently involved in regulation of the lysosomal autophagic signaling pathway, similarly to TFEB (Bouché et al. 2016; Zhang et al. 2015). Indeed, down-regulation of *Mitf* impairs lysosomal biogenesis, autophagosome maturation and response to starvation. Furthermore, nuclear translocation of Mitf increases expression of V-ATPase (Bouché et al., 2016), suggesting that the proton pump is strictly involved in regulation of the autophagy pathway also in flies (Bouché et al., 2016; Tognon et al., 2016).

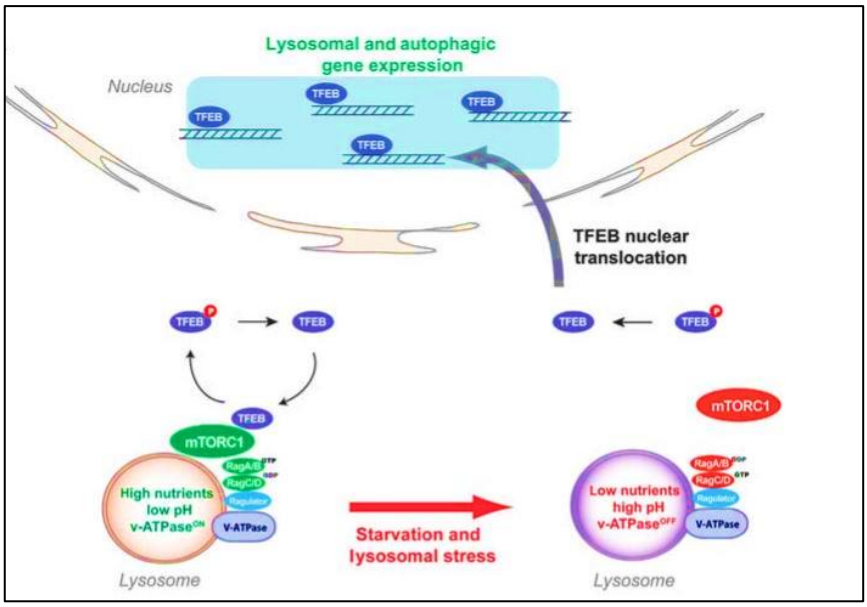

Figure 8. Lysosomal mTORC1 regulates TFEB. V-ATPase senses lysosomal pH. When pH is low Rags anchor the growth kinase on lysosomal surface, preventing the nuclear translocation of TFEB. When pH is high, Rag proteins mediate the release of mTORC1 form lysosomal surface, TFEB translocases into the nucleus driving the transcription of autophagy and lysosomal-related genes. Adapted from Settembre et al., 2012

2.6.3 Hormonal control of autophagy

Autophagy can also be regulated by hormones such as Insulin and Insulin-like growth factors. These hormones mediate induction of the anabolic pathway using a molecular mechanism that, as for nutrient-induced autophagy, converges on mTOR (He & Klionsky, 2009). Growth factors bind their receptor allowing phosphorylation of tyrosine residues which recruit the Insulin Receptor Substrate 1 and 2 (IRS1 and IRS2). These activate the lipid kinase class I PI3K, that in turn generates PIP3 and leads to the phosphorylation and activation of Akt. The activity of the Akt kinase is negatively regulated by the phosphatase PTEN, which reverts PIP3 in PIP2 resulting in positive regulation of the autophagy. As shown in **Figure 9**, the activation of Akt prevents association of Tuberous Sclerosis Complex 1 and

2 (TSC1/2), allowing the GTPase Ras homolog enriched in brain (Rheb)-mediated activation of mTOR (Inoki, Li, Xu, & Guan, 2003). The kinase modulates cell growth and autophagy by acting on its downstream targets S6K and 4EBP1 (Fingar, Salama, Tsou, Harlow, & Blenis, 2002), a circuit that is present also in *Drosophila* (Gao & Pan, 2001; Lee et al., 2007).

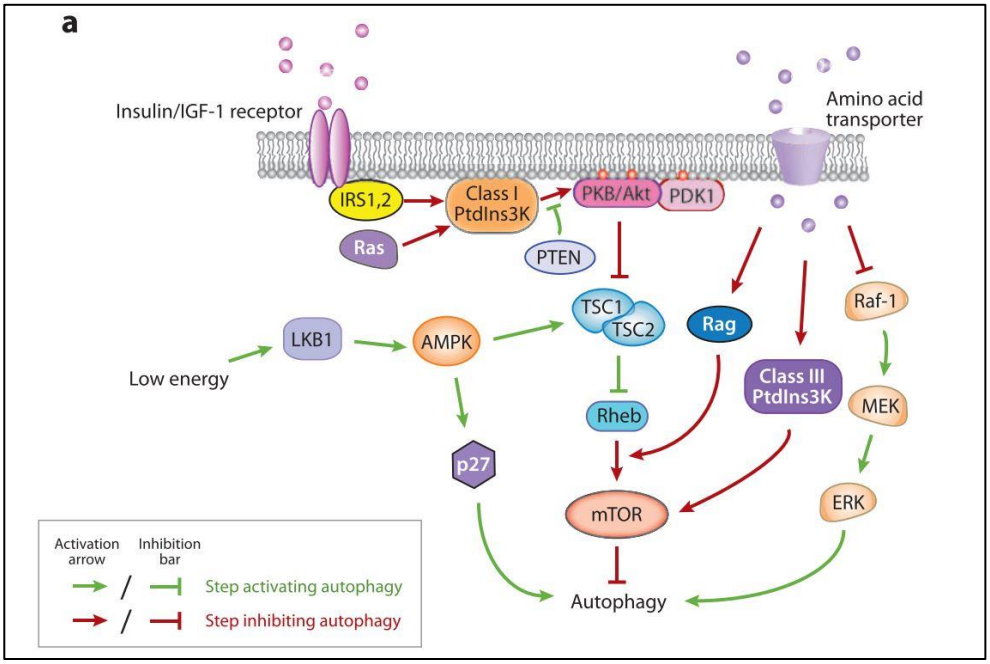

Figure 9. Regulation of autophagy by growth factors. Growth factors bind their receptor leading to the activation of several molecules, such as Class I PI3K, Akt and lastly mTOR. The activation of several kinases converges on mTOR, demonstrating its pivotal role in cell homeostasis. Adapted from Congcong et al., 2009.

2.6.4 Ras involvement in autophagy

The small GTPase Ras is an additional intracellular transducer of growth factor receptors. The protein has a dual role in autophagy: With PI3K is a negative regulator of the process. Conversely with Raf-1 is a positive regulator of autophagy (Furuta, Hidaka, Ogata, Yokota, & Kamata, 2004). Upon nutrition, Raf 1 senses amino acids level and prevents phosphorylation

of the downstream effectors Mitogen-activated protein kinase (MAPK) ERK kinase 1/2 (MEK1/2) and Extracellular signal-regulated kinase 1/2 (ERK1/2), inhibiting autophagy. Conversely, amino acids deprivation leads to phosphorylation of ERK1/2, inducing of autophagy.

2.6.5 AMPK regulation of autophagy

Another regulator of the autophagy pathway is the protein kinase AMPK, which monitors cellular levels of ATP. Hence, a decreased ATP/AMP ratio leads to AMPK-mediated activation of TSC1/2 complex, that inhibits mTOR and induces autophagy. Furthermore, AMPK prevents cell cycle arrest, allowing autophagy to restore energy levels during moments of cell stress. Also, activation of cell-cycle inhibitor $p27^{Kip1}$ by AMPK protects from cell death (Liang et al., 2007).

2.6.6 Transcriptional regulation of autophagy by FOXO

Another transcriptional regulator of autophagy, initially characterized in *Drosophila,* is *FoxO* (Zinke, Schütz, Katzenberger, Bauer, & Pankratz, 2002), *which* up-regulates levels of *Atg8a* during starvation-induced autophagy (Juhász et al., 2007). Indeed, in *Drosophila* muscles*,* autophagy is triggered by overexpression of *FoxO,* which activates *Thor*, preventing the formation of protein aggregates which could cause muscle loss during aging (Demontis & Perrimon, 2010). Furthermore, increased expression of *FoxO* ameliorates aggregate accumulation in *Drosophila* models of Parkinson induced by alterations in the activity of the serine/threonine kinase PTEN-induced kinase 1 (PINK1) (Koh et al., 2012). The constitutive expression of *FoxO* in neurons and fat body leads to death in *Drosophila* larvae, highlighting its involvement in suppression of cell growth (Hwangbo, Garsham, Tu, Palmer, & Tatar, 2004). The subsequent discovery of Mitf-mediated control of autophagy demonstrates that transcriptional regulation of autophagy is not only limited to FoxO. In mammals, the FOXO family has several components: FOXO1,

3, 4 and 6. Similar to *Drosophila*, FOXO cellular localization is controlled by Akt (Brunet et al., 1999), which prevents FOXO translocation into the nucleus (Settembre et al., 2013; Tzivion, Dobson, & Ramakrishnan, 2011). Moreover, nuclear translocation of FOXO3 up-regulates LC3, as observed in muscular dystrophy (Mammucari et al., 2007).

2.7 Autophagy and cancer

The role of autophagy in cancer is controversial. During tumor initiation, autophagy has been shown to play a tumor suppressive role, while once the tumor is established autophagy could have a positive impact on tumor survival by increasing metabolic activity to support cell proliferation (Fulda, 2017; Kimmelman, 2011). Furthermore, tumors need autophagy to survive to hypoxia, at least until angiogenesis is established (Kimmelman, 2011). The involvement of the autophagy pathway in cancer has emerged only recently (Fulda, 2017; He & Klionsky, 2009; Levy, Towers, & Thorburn, 2017) and understanding of its regulation is contradictory with examples in which autophagy is involved in both cell survival and cell death (Sui et al., 2013), suggesting that the biological functions of autophagy are strictly context-dependent (Bhutia et al., 2013).

2.7.1 Autophagy and cancer: a dual role

A clear example of the bimodal involvement of autophagy in cancer development has been demonstrated in *Drosophila*. In eye epithelial tissue, autophagy promotes growth when *Ras* is mutated (Pérez, Das, Bergmann, & Baehrecke, 2015a). This suggests an interdependence between autophagy and *Ras*-driven tumors. This crosstalk was demonstrated also in human and mouse cell lines, in which depletion of autophagy core components reduces *Ras*-mediated tumor growth (Guo et al., 2011).

However, in eye epithelial tissue of *Drosophila*, autophagy can also decrease tumor growth driven by c-Jun N-terminal kinases (JNKs) mutation and Notch pathway activation (Pérez et al., 2015a). Additionally, in a mouse model of lung cancer, down-regulation of Atg5 and Atg7 prevents tumor initiation mediated by Ras. However, down-regulation of these Atg genes does not influence tumor growth when Ras is mutated and p53 is lost (Rao et al., 2014). This data highlights that autophagy involvement in tumor growth is strongly influenced by the genetic alterations of a given tumor. Interestingly, a recent work by Nadja S. Katheder and colleagues points the attention on the effect of the tumor microenvironment on autophagy. In fact, in eye imaginal discs, *Ras*-induced tumor growth leads to non-cell-autonomous autophagy in both the tumor microenvironment and tissues like the fat body, gut and muscles. In this case, inhibition of autophagy prevents tumor growth (Katheder, Khezri, O'Farrell, Schultz, Jain, Schink, et al., 2017). In *Drosophila* it was also recently found that autophagy is involved in regulating occupancy of stem cell niches. Indeed, ovarian stem cells mutant for Bag of marbles (*bam*), a gene involved in gametogenesis, shown increased autophagy. The latter correlates with occupancy of the stem cell niche by cancer stem cells, revealing another mechanism by which autophagy could be involved in tumor progression (Zhao, Fortier, & Baehrecke, 2018). Conversely, in mouse liver, down-regulation of Atg5 and Atg7 promotes the formation of hepatomas (Takamura et al., 2011), while loss of the gene encoding for Beclin1 in mice tissues leads to development of several spontaneous tumors (Cicchini et al., 2014; Yue, Jin, Yang, Levine, & Heintz, 2003). Moreover, human primary melanomas, compared to benign nevi, show a decreased expression of Atg5, a finding that correlates with poor prognosis (H. Liu et al., 2013).

2.7.2 Autophagy and glioblastoma

Interestingly, the most effective drugs used in glioma therapy, the widely used TMZ, is able to induce autophagy (Kanzawa et al., 2004; Sui et al., 2013; Yuanliang Yan et al., 2016). TMZ treatment often fails because cells acquire chemoresistance (Yuanliang Yan et al., 2016). In lymphomas, autophagy is used as an adaptive mechanism to survive during chemotherapy (Amaravadi et al., 2007). Inhibition of autophagy, using the antimalarial drug quinacrine hydrochloride increases susceptibility of glioblastoma stem cells to TMZ treatment, inducing cell death (Buccarelli et al., 2018). Furthermore, the blockade of autophagy using short interfering RNA (siRNA) against core components of the pathway such as Atg7, Atg13 and Ulk1 in mouse models arrests glioma development and leads to cell senescence (Gammoh et al., 2016). Conversely, in glioblastoma cell lines treated with sinomenine hydrochloride (SH), an alkaloid agent used as an anti-inflammatory for rheumatoid arthritis, autophagy is induced while cell growth is inhibited (Jiang et al., 2017). The autophagy induction mediated by SH leads to ROS generation, inhibition of both Akt and mTOR and activation of the JNK pathway. Additionally, SH increases lysosomal biogenesis via TFEB both *in vivo* and *in vitro* (Jiang et al., 2017). Given the role of autophagy in cancer progression, this pathway might yield candidate targets for glioblastoma treatment that need more investigation.

3 V-ATPase

Initially characterized in yeast, V-ATPase is a ubiquitously expressed proton pump involved in acidification processes (Ohsumi & Anraku, 1981). Cellular pH is tightly regulated to enable physiological processes such as regulation of growth, metabolism and trafficking (Forgac, 1999). V-ATPase has been found associated with subcellular compartments such as lysosomes, endosomes, the Golgi apparatus, secretory vesicles and with the plasma

membrane of specialized cells (Nishi & Forgac, 2002). The regulation of pH in intracellular organelles is a well-characterized function of V-ATPase. Indeed, control of the lysosomal pH by the vacuolar proton pump is critical to energize endocytic processes, ensuring proper function of the organelles involved in autophagy and lysosomal degradation.

3.1 Structure of V-ATPase

The proton pump is composed of 15 subunits, arranged in two sectors: the cytoplasmic V1 sector of 640 kDa (A-H subunits), that is mainly involved in ATP hydrolysis, and the membrane-embedded V0 sector of 260 kDa (a, d, e, c and c'' subunits) that mediates the transport of protons across the membrane, using a rotary mechanism. The V1 sector is composed by three repetitions of A and B subunits, forming a hexameric cylinder in which ATP hydrolysis takes place, specifically using the three catalytic domains located between the A and B subunits (Cotter, Stransky, McGuire, & Forgac, 2015). The V1 sector also includes the peripheral stalk, which is composed of three different E-G dimers, preventing the rotation of A and B subunits during ATP hydrolysis. The last component of the V1 sector is the central stalk (subunits C, H, D, F), that functions as a rotor. V1 and V0 can detach from each other when activity is downregulated. Apart from this, subunit C can be released independently from V1 and V0 sectors, *in vivo* (Nishi & Forgac, 2002). Subunit C and H, due to their position, mediate the interaction between V1 and V0 sectors (Inoue & Forgac, 2005; Wilkens, Inoue, & Forgac, 2004), while subunit D and F constitute the central rotor interacting with the subunit d of the V0 sector. The subunit d is located on the proteolipid ring which is made by 5 or 6 copies of subunit c and c'' **(Figure 10)**. The highly hydrophobic proteolipid ring contains several Glu residues that are protonated during proton transport, upon rotation of ring subunits after hydrolysis of ATP (Nishi & Forgac, 2002). In high eukaryotes, the V0 sector

lacks the c' subunit that is replaced by the accessory subunit Ac45 (Supek et al., 1994). The V0 subunit a is composed by a N-terminus, that regulates targeting of the proton pump to certain compartments, and a C-terminus that regulates proton transport. During ATP hydrolysis, protons are conveyed to a hemichannel in subunit a. Subunit a and e are fixed by the peripheral stalk, while the rotation of the proteolipid ring leads to the translocation of protons.

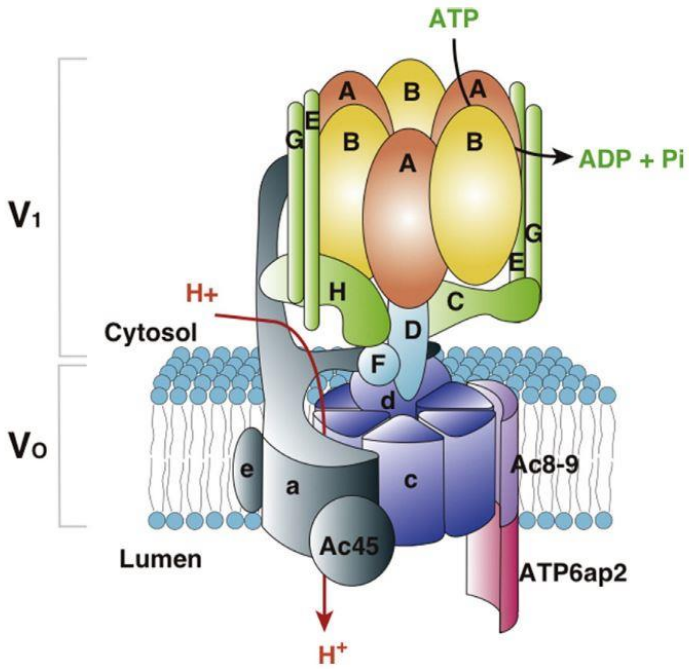

Figure 10.The structure of V-ATPase. The proton pump is composed by V1 and V0 sectors. The first one hydrolyzes ATP while the second one leading to protons translocation against the concentration gradient. The V1 sector is composed by A-H subunits, the V0 sector is composed by a, c, c'', d, e subunits. Adapted from Ge-Hong S., 2015

The V-ATPase can be inhibited by nanomolar concentration of drugs such as Bafilomycin A1, Archazolid and Concanamycin. Specifically, these drugs bind the c subunit of the proteolipid ring preventing their own rotation and the translocation of protons. These drugs act indistinctly on V-ATPase of each organism (Bowman & Bowman, 2002; Huss et al., 2002).

3.2 Assembly and targeting of V-ATPase

In vivo, the assembly of V-ATPase has been largely characterized using yeast. In such system, the V0 and V1 sectors can be assembled in a coordinated way or independently. The subunit a leads to complex formation through the contemporary binding with subunit A. Alternatively, the V0 sector is assembled by chaperones that reside in the ER while the V1 sector is added as a pre-assembled sector or by single subunits one by one (Kane, 2006). As mentioned before, the N terminus of the subunit a plays a pivotal role in the targeting of the complex. In mammals, there are several isoforms of V-ATPase subunits that are dynamically targeted in different subcellular compartments in a particular cell type. Specifically, in human the subunit a is encoded by 4 paralog genes (a1-a4). The a1 protein is expressed mostly in the brain in presynaptic axon terminal (Morel, Dedieu, & Philippe, 2003) while the a2 protein is present in endosomes. The a3 protein targets the complex from lysosomes to the membrane of osteoclasts, upon their activation (Toyomura et al., 2003). The a4 protein is specifically found in renal intercalated cells or in cells of the epididymis (Pietrement et al., 2006). Moreover, mammals have two types of subunit d, B, E, H and three types of subunits C and G. The G1 and G2 proteins are strictly associated with neurons (Murata et al., 2002), while the G3 is mainly associated with kidney (Norgett et al., 2007). The expression of different subunit genes is thought to change V-ATPase activity, so that the proton pump can cater to different cellular needs (Smith, Borthwick, & Karet, 2002). Immunoprecipitation studies have demonstrated that V-ATPase shows a specific expression pattern. Thus, the identification of physiological as well as pathological gene signatures can be used into the clinical and pharmacological fields for the development of specific drug targets (Murata et al., 2002)

3.3 Regulation of acidification

The acidification capability of the proton pump is modulated by several mechanisms, the most characterized of which is reversible dissociation. This mechanism, originally identified in yeast, leads to the dissociation of the V0 and V1 sectors when glucose uptake is decreased (Nishi & Forgac, 2002). The presence of free V0 and V1 sectors in others organisms suggests that this mechanism is commonly used to regulate V-ATPase activity and organelle acidification (Forgac, 1999). Another regulatory mechanism is the inhibition of V-ATPase functions by generating disulphide-bonds between the conserved cysteines of the catalytic domains, a feature that is used especially in oxidative stress conditions (Forgac, 1999). Other mechanisms identified are: Changes in coupling efficiency and changes in conductance **(Figure 11)**, the first one regulating cellular pH while the second one involving other cellular transporters (Forgac, 1999).

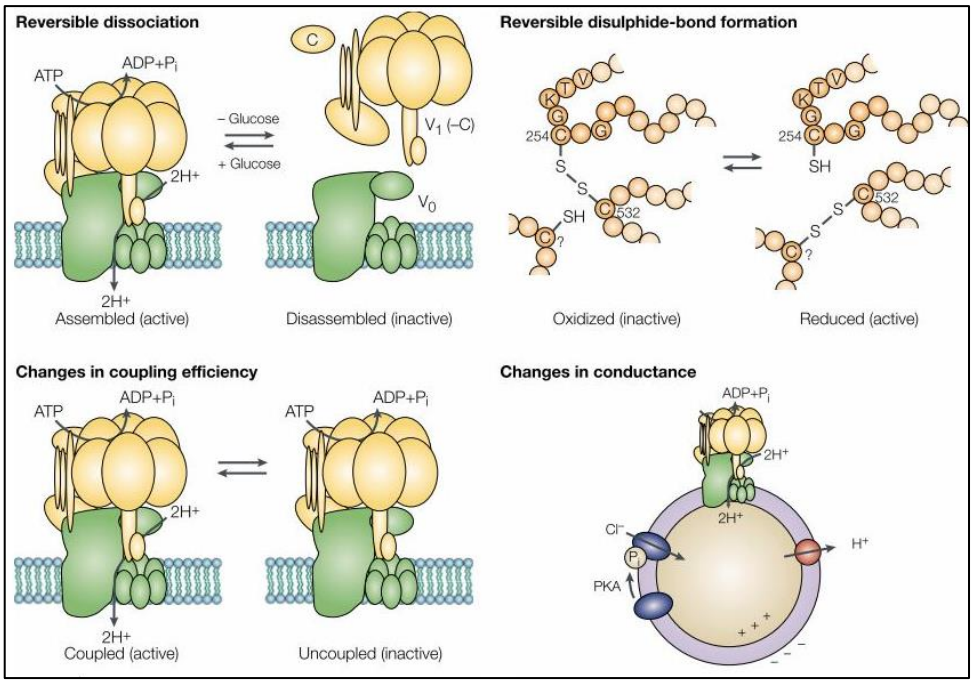

Figure 11. Regulation of V-ATPase assembly. Reversible dissociation is the most commonly used mechanism to regulate V-ATPase assembly in eukaryotes. Other mechanisms are: Reversible disulphide-bond formation, changes in coupling efficiency and changes in conductance, these mechanisms leading to inactivation of the proton pump activity decreasing the catalytic activity, the coupling efficiency and activating other cellular transporters, respectively. Adapted from Nishi & Forgac, 2002.

3.4 Molecular regulation of V-ATPase activity

In yeast, Ras/cAMP and protein kinase A (PKA) are known to increase the assembly of V-ATPase when glucose uptake is enhanced. Alternatively, low cytosolic pH triggers proton pump assembly, leading also to PKA activation (Bond & Forgac, 2008). The assembly of the proton pump is promoted by the complex Regulator of the V-ATPase of the Vacuolar and Endosomal membranes (RAVE) interacting with the V1 domain (Kane, 2012). In higher eukaryotes, the assembly of V-ATPase is regulated in several ways: by PI3K

and ERK signaling in infected cells (Marjuki et al., 2011); by PI3K when glucose levels are increased (Sautin, Lu, Gaugler, Zhang, & Gluck, 2005); by PI3K and AMPK increase lysosomal assembly of the proton pump during glucose starvation (Collins & Forgac, 2018); by EGF when abundance of amino acids induces mTORC1 activation. EGF modulation of V-ATPase can be inhibited by Bafilomycin, preventing mTORC1 activation, as demonstrated by the decreased phosphorylation of both 4EBP1 and S6K (Xu et al., 2012). An alternative mechanism used to modulate proton pump activity is the regulation of its trafficking. In intercalated kidney cells, a low cytoplasmic pH drives the PKA mediated-fusion between cytoplasmic vesicles carrying V-ATPase with the apical plasma membrane, increasing their number on the cell surface (Alzamora et al., 2010).

3.5 Functions of V-ATPase in membrane transports

The modulation of cellular pH is crucial for membrane transport. Compared to the extracellular milieu, a lower pH in early endosomes promotes dissociation of ligand-receptor complexes that have been internalized. This process regulates recycling of receptors and controls their number on the plasma membrane, ensuring the correct uptake of ligands. The number of receptors is modulated also to control cellular responses to growth factors and their receptors. Additionally, endosomal acidification is required for the transport of vesicles carrying degradative cargoes from the early to the late endosomes. Acidification of late endosomes is also modulated by the vacuolar proton pump, leading to the release of lysosomal enzymes from mannose-6-phosphate receptor carriers allowing their recycling back to the Golgi apparatus **(Figure 12)**. V-ATPase is also involved in membrane fusion processes, by association with SNAREs (Bayer, Reese, Bühler, Peters, & Mayer, 2003).

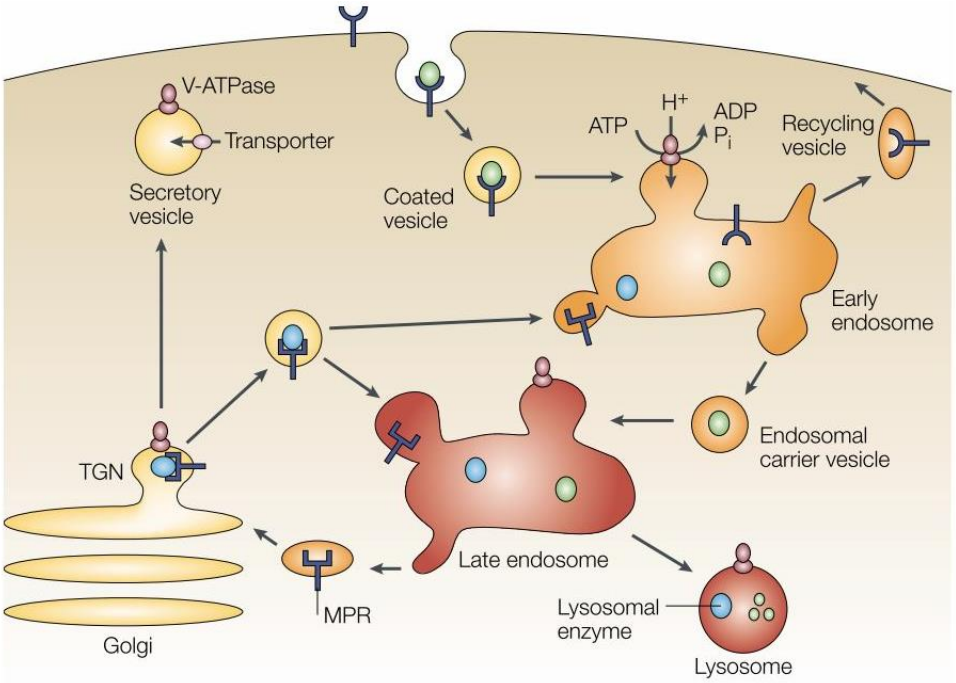

Figure 12. V-ATPase-mediated regulation of pH controls membrane transport. The proton pump drives the recycling of receptors after the internalization of ligand-receptor complex, V-ATPase increased acidification mediates the release of carrier vesicles from early to late endosomes, moreover, it mediates the recycle of mannose-6-receptor to Golgi apparatus after the release of lysosomal enzymes. From Nishi & Forgac, 2002.

3.6 Functions of the V-ATPase at the plasma membrane

V-ATPase has also been found at the plasma membranes of macrophages, renal intercalated cells and osteoclasts **(Figure 13)**. Macrophages use V-ATPase to regulate cytoplasmic pH in the acidic microenvironment generated at the site of infection or inflammation (Brisseau et al., 1996). V-ATPase molecules on the apical surface of renal intercalated cells are involved in secretion of protons into the renal fluid (Smith et al., 2000). Indeed, the first evidence of a pathology associated with defects in the proton pump was the metabolic acidosis caused by mutations in the ATP6B1 gene,

encoding the B subunit. Additionally, this mutation is associated with deafness, suggesting an involvement of the proton pump in regulation of hearing (Smith et al., 2000). Moreover, the activity of V-ATPase is required in osteoclast mediated-bone resorption. Indeed, mutations in the proton pump lead to alterations in bone turnover (Frattini et al., 2000; Y.-P. Li, Chen, Liang, Li, & Stashenko, 1999). Finally, the V-ATPase on the apical membrane of epididymis and vas deferens is also involved in regulation of sperm motility and maturation (Brown & Breton, 2000).

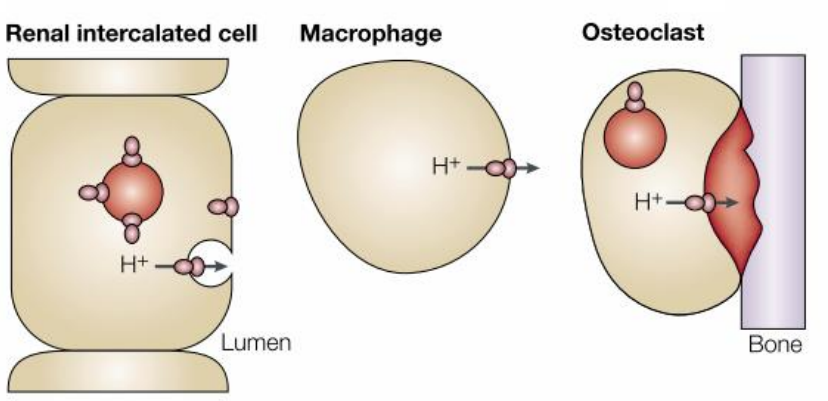

Figure 13. V-ATPase functions at the plasma membrane. V-ATPase is associated with the plasma membrane of specialized cells such as renal intercalated cells, macrophages and osteoclasts. In these cells, the proton pump is involved in functions like the regulation of lumenal pH, alkalinization of the pH of macrophages and acidification of bone matrix during bone resorption. Adapted from Nishi & Forgac, 2002.

3.7 V-ATPase-mediated intracellular signaling

V-ATPase is essential for transduction of Wnt, Notch and mTOR signaling. These signals are implicated in physiological and pathological regulation of cell proliferation. Indeed, the pharmacological or genetical inhibition of the proton pump affects their activation in several organisms (Sun-Wada &

Wada, 2015). Consistent with this downregulation of V-ATPase arrests canonical Wnt pathway-mediated anteroposterior patterning of *Xenopus* in the early central nervous system (Cruciat et al., 2010). Pharmacological inhibition of V-ATPase decreases Notch activity in both breast cells and T-cell leukemia, limiting also Akt/mTOR activity (Kobia, Duchi, Deflorian, & Vaccari, 2014). In addition, V-ATPase is majorly involved in regulation of mTORC1 by amino acids, independently of TSC1/TSC2. In fact, the hydrolysis of ATP mediated by V-ATPase is necessary for its interactions with Ragulator, a complex composed by p18, p14 and MAPK scaffold protein 1 (MP1) proteins. This complex anchors Rag GTPases to the lysosomal surface allowing recruitment of mTORC1 **(Figure 14)**. Indeed, shRNA against the ATP6V0C gene suppresses mTORC1-mediated phosphorylation of S6K (Zoncu et al., 2011). Moreover, mTORC1 modulates V-ATPase gene expression by regulating phosphorylation of the transcription factor TFEB which prevents its nuclear translocation and subsequent involvement in transcription of autophagy and lysosomal genes (Peña-Llopis et al., 2011).

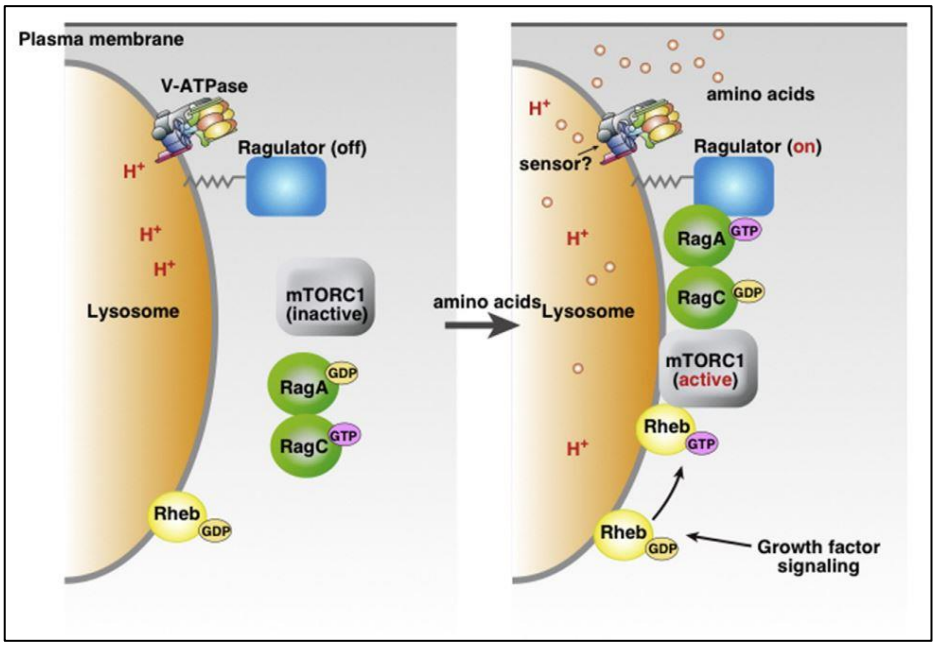

Figure 14. V-ATPase regulates mTORC1 activation during amino acids plenty. The interaction between V-ATPase and Ragulator lead to the anchor of Rag GTPases on the lysosomal surface, they recruit and activate mTORC1. Adapted from Ge-Hong S., 2015.

3.8 V-ATPase and cancer

V-ATPase is currently under intense investigation as a putative anti-cancer target (Forgac, 2018; Stransky, Cotter, & Forgac, 2016). In tumor cells, increased expression of V-ATPase at the plasma membrane has been suggested to allow cell survival in the highly hypoxic and acidic tumor microenvironment. The latter promotes invasiveness by enhancing the activity of matrix metalloproteases (MMPs) and of lysosomal hydrolases. Consistent with this, in tumoral cell lines the activation of Cathepsin B is impaired by the V-ATPase-inhibitor Archazolid (S. Fan et al., 2015; Kubisch et al., 2014). Moreover, increased proton pumping was found to support the alkaline pH necessary to activate glycolysis, leading to the Warburg effect

(Torigoe et al., 2002). Additionally, chemoresistance has been reported to be promoted by an acidic tumor microenvironment (Wojtkowiak, Verduzco, Schramm, & Gillies, 2011). Modulation of mTORC1 activation upon knock-down of the ATP6V1C1 subunit has been shown to decrease cancer breast cell proliferation and bone metastasis, (McConnell et al., 2017).

The first evidence that links proton pumps with tumor invasiveness derives from the study of breast cancer cells, in which inhibition of the proton pump using Bafilomycin leads to a decrease in their invasiveness (Sennoune et al., 2004). In cancer cell lines, treatment with the V-ATPase inhibitor Archazolid B induces cell stress, activating adaptative responses such as autophagy, expression of hypoxia-inducible factor 1-alpha (HIF-1α), and apoptosis (Von Schwarzenberg et al., 2013). In glioblastoma-derived cancer stem cells (neurospheres), expression of V-ATPase subunits is up-regulated, compared to other malignant gliomas. Moreover, ATP6V1G1 is associated with poor survival of patients. The knock-down of this subunit leads to cell death and decreases the invasiveness of neurospheres, while treatment with Bafilomycin A1 recapitulates most features of ATP6V1G1 knock-down (Di Cristofori et al., 2015).

3.9 Structure and functions of V-ATPase in *Drosophila*

Albeit the characterization of the proton pump was performed initially in yeast, the first knockout of a V-ATPase subunit was carried out in *Drosophila* (Davies et al., 1996). The structure of fruit fly V-ATPase is shown in **Figure 15.** This model system was essential for the characterization of proton pump function at plasma membranes.. An example of this is the finding that inactivation of the *Vha55* gene, encoding for the B subunit, leads to alterations of the Malpighian tubules (the fly counterpart of the kidney) and to larval lethality, suggesting a pivotal role of the proton pump in multiple cellular processes (Allan, Du, Davies, & Dow, 2005).

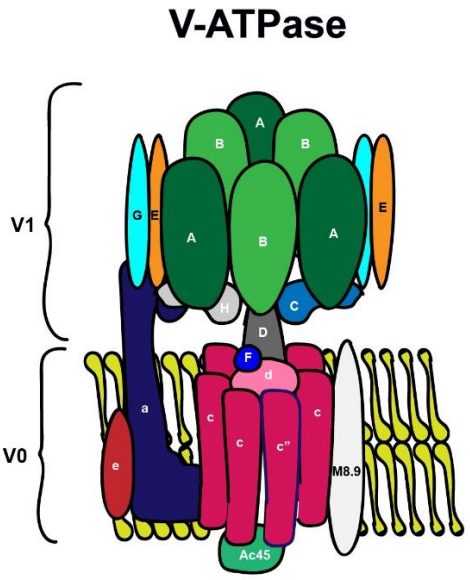

Figure 15. Schematic representation of the V-ATPase structure in Drosophila. The fly V-ATPase is conserved during evolution, the nomenclature for the V1 sector is: A for Vha68, B for Vha55, C for Vha44, D for Vha36, E for Vha26, F for Vha14, G for Vha13, H for VhaSDF. For the V0 sector: a for Vha100, c for Vha16, c'' for VhaPPA1, d for VhaAC39, e for VhaM9.7. The nomenclature of accessory subunits is: Ac45 for VhaAc45, M8.9 for VhaPPP.

In *Drosophila*, such as in human, V-ATPase subunits are encoded by multiple genes that are often spliced into several isoforms expressed in specific tissue types. A summary of the correspondence between human and *Drosophila* V-ATPase is shown in Table 2. As in the case of the human a1 subunit, fly *Vha100-1* is expressed specifically in neurons. Consistent with this, mutation of *Vha100-1* leads to accumulation of synaptic vesicles. Additionally, Vha100-1 colocalizes with SNAREs, suggesting an involvement of the V0 sector in membrane fusion events that drive exocytosis processes (Hiesinger et al., 2005). In wing discs, the accessory subunit VhaPP1 is required for planar cell polarity by modulation of non-canonical Wnt signaling, functioning as an adaptor between Wnt and V-ATPase (Cruciat et al., 2010; Hermle, Saltukoglu, Grü, Walz, & Simons, 2010). V-ATPase activity is also

strictly associated with Notch trafficking in endosomes and Bafilomycin A1 treatment decreases Notch signaling in wing discs (Kobia et al., 2014; T. Vaccari, Duchi, Cortese, Tacchetti, & Bilder, 2010; Thomas Vaccari, Lu, Kanwar, Fortini, & Bilder, 2008). In contrast, mutations in *VhaAc39*, encoding the fly subunit V0d, lead to alterations in endosomal trafficking of Notch (Yan Yan, Denef, & Schüpbach, 2009). The overexpression of Vha44, the fly C subunit, in wing epithelium impairs endosomal trafficking, leading to Notch accumulation in endosomes. Additionally, overexpression of this subunit triggers JNK-pathway mediated activation of MMP1, generating a tumor-like wing disc (Petzoldt, Gleixner, Fumagalli, Vaccari, & Simons, 2013). The correlation between V-ATPase, Notch and cancer has been demonstrated also in the adult fly brain, indeed *brat*-induction of tumoral growth is decreased upon V-ATPase and Notch down-regulation (Wissel et al., 2018).

V1	Human	*Drosophila*	V0	Human	*Drosophila*
A	ATP6V1A	*Vha68-1* *Vha68-2* *Vha68-3*	a	ATP6V0A1 ATP6V0A2 ATP6V0A3 ATP6V0A4	*Vha100-1* *Vha100-2* *Vha100-3* *Vha100-4* *Vha100-5*
B	ATP6V1B1 ATP6V1B2	*Vha55*	c	ATP6V0C	*Vha16-1* *Vha16-2* *Vha16-3* *Vha16-4* *Vha16-5*
C	ATP6V1C1 ATP6V1C2	*Vha44*	PPA1 (c'')	ATP6V0B	*VhaPPA1-1* *VhaPPA1-2*
D	ATP6V1D	*Vha36-1* *Vha36-2* *Vha36-3*	M9.7 (e)	ATP6V0E	*VhaM9.7-1* *VhaM9.7-2* *VhaM9.7-3* *VhaM9.7-4*
E	ATP6V1E1 ATP6V1E2	*Vha26*	Ac39 (d)	ATP6V0D1 ATP6V0D2	*VhaAC39-1* *VhaAC39-2*
F	ATP6V1F	*Vha14*	Accessory subunits		

G	**ATP6V1G1** **ATP6V1G2** **ATP6V1G3**	***Vha13***	**Ac45**	**ATP6VAP1**	**VhaAC45**
H	**AT6V1H**	***VhaSDF***	**M8.9**	**ATP6VAP2**	**VhaPRR**

Table 2. Summary of human and *Drosophila* V-ATPase genes. The human nomenclature for V-ATPase is ATP6 followed by the name of the gene encoding for the subunit, while in *Drosophila* is *Vha* followed by the name of the gene encoding for the subunit.

Finally, the presence of Coordinated Lysosomal Expression and Regulation (CLEAR) sequences in the promoters of fly V-ATPase subunit genes allows transcriptional modulation by Mitf, the fly homolog of TFEB family genes (Bouché et al., 2016; Tognon et al., 2016; Zhang et al., 2015). In the fly fat body and in wing discs, up-regulation or down-regulation of Mitf results in increased or decreased expression of V-ATPase genes (Tognon et al., 2016). Finally, accumulation of protein aggregates in fly brains is prevented by mTORC1-mediated activation of Mitf (Bouché et al., 2016), indicating that as in mammals the lysosomal V-ATPase, mTORC1 and Mitf constitute an axis that regulates cellular homeostasis. Overall, all function of mammalian V-ATPase are conserved in flies revealing that it could be a model system to study the effect of V-ATPase activity in tumorigenesis.

4 AIM OF THE WORK

Glioblastoma is the most aggressive brain cancer with an extremely poor patient survival (Thakkar et al., 2014). The combination of all these treatments available increases survival only up to 14-15 months. The inefficiency of the therapeutic treatments is due to massive over proliferation of glial cells, the infiltrative nature of the tumor and the frequent development of chemoresistance to temozolomide. A deeper understanding of GBM biology is urgently required to develop targeted therapies, which are currently lacking. The most common altered pathways in GBM are highly conserved during evolution making *Drosophila melanogaster* a good model to recapitulate genetic and *in vivo* features of malignant gliomas. The autophagy-lysosomal pathway has recently emerged as an important target in cancer therapy (Fulda, 2017). However, its role in regulating tumor growth is controversial. Interestingly, temozolomide is able to induce autophagy, suggesting a pivotal role in GBM. The aim of my PhD project is to use *Drosophila melanogaster* as a glioma model system to characterize *in vivo* the role of autophagy-lysosomal pathway in tumor growth. Furthermore, we aimed to identify new potential therapeutic targets that can be translated into clinic to improve future GBM treatment.

5 MATERIAL AND METHODS

5.1 *Drosophila* husbandry

Fly strains were kept and raised into vials containing standard food medium, which consist of yeast, cornmeal, molasses, agar, propionic acid, tegosept and water. All crosses were performed at 25°C and fresh yeast was added. Larvae were grown for 120–140 hrs after eggs deposition and wandering 3rd instar larvae were selected for subsequent analysis.

5.2 Genetics

Drosophila lines used in this study were provided by the Vienna Stock Center, the Kyoto stock center and by our collaborators. *repo-Gal4* UAS-*CD8mGFP/TM2*; *repo-Gal4* UAS-*Dp110-CAAX* UAS-*mCD8GFP* UAS-*hΔEGFR/TM6C TubGal-80* (kindly provide by Renee Read); 4-2 mCherry::Atg8a/Cyo (kindly provided by Gabor Juhasz); UAS-*VhaPPA1-1*^{RNAi} (VDRC 471155) and UAS-Mitf DN (a gift from F. Pignoni), YFP::Lamp1/Cyo from Kyoto/DGRC (Takáts et al., 2013).

5.3 Immunofluorescence of *Drosophila* larval brains

Larval brains are located on the anterior portion of the larva, near the mouth hooks**.** To dissect 3rd instar animals, larvae were teared in half and the posterior part was discarded. The anterior was inverted like a sleeve by pushing delicately on the mouth hooks. The brains are attached to the mouth near the salivary glands. Larval brains were kept attached to carcasses to facilitate handling. To prepare carcasses for fixation, we removed the gut, fat tissue, and salivary glands. Carcasses were transferred into a 1.5 mL tube filled with 1× PBS. Carcasses were fixed using 4% PFA for 20-30 min at room temperature. After removal of the fixative, tissues were rinsed with 0,1% TRITON X-100 diluted in PBS 1X (PBST solution) for 5 min. This step was repeated 3 times to remove all traces of fixative. Tissues were permeabilized

with 1X PBS, 1 % Triton X-100 for 30 min to improve the penetration of antibodies. After permeabilization, samples were incubated with blocking solution, composed of 4% BSA diluted in PBST. Tissues were blocked for 30 min at room temperature. After removal of the blocking solution, primary antibody was added and incubated overnight, all the antibodies were diluted in blocking solution. After three washes, the secondary antibody was added and incubated at room temperature for 2 hrs. Tissues were washed prior mounting.

Primary antibodies against the following antigens were used: Chicken anti-GFP 1:1000 (Abcam), Mouse anti-Repo 1:20 (Developmental Studies Hybridoma Bank), Rat anti-Elav 1:40 (Developmental Studies Hybridoma Bank), Mouse anti-FK2 1:250 (Enzo), Rabbit anti-ref(2)P 1:1000 (a gift from Tor Erik Rusten), Rat anti-mCherry 1:1000 (a gift from Gianluca Deflorian), Rabbit anti-Mitf 1:200 (developed by our group), Rabbit anti-Cleaved Caspase 3 1:200 (Cell Signaling), Mouse anti-Cyclin B 1:20 (Developmental Studies Hybridoma Bank). Secondary antibodies used were Alexa conjugated secondary antibodies from Invitrogen (1:400). Rhodamine-phalloidin was used to mark filaments of actin 1:100 (Sigma). Samples were mounted on slides using glycerol 70%. Confocal acquisitionS were performed using Leica SP2 microscope with ×40/NA 1.25 or ×63/NA 1.4 oil lenses or Nikon A1R confocal microscope. Measurements and fluorescence evaluation were carried out through the ImageJ Software (NIH).

5.4 RNA extraction from *Drosophila* tissue

After dissection, 3rd instar larval brains of the selected genotype were homogenized in Homogenization solution with autoclaved dispensable pestles. The RNA extraction was performed using Maxwell® RSC simply RNA Tissue Kit and the Maxwell RSC Instrument. The concentration of extracted RNA and DNA were measured using the NanoDrop 1000

Spectrophotometer. Complementary DNA (cDNA) was synthesized from RNA through reverse transcription, according to the SuperScript® VILO™ cDNA Synthesis Kit. Samples were tested for different genes by Real-time PCR (qPCR).

5.5 Protein extraction from *Drosophila* tissues

Drosophila larval brains were isolated from carcasses and tissues were homogenized with autoclaved dispensable pestles in RIPA buffer (1 mM Tris-HCl, 150 mM NaCl, 5mM EDTA, 1% TritonX, 1% Deoxycholate and 0,1% SDS) plus the addition of proteinase inhibitors 1:200 (Calbiochem) or phosSTOP-phosphatase inhibitor cocktail (Roche). After 20 minutes on ice, the homogenate was centrifuged at 13000 rpm for 20 minutes at 4°C. The supernatant was collected and quantified to determine the concentration of proteins in the sample, through the use of Bicinchoninic Acid Assay (BCA) method.

5.6 Western Blotting

Proteins were denaturated in Laemmli Buffer 6X (6.25 mM Tris-HCl pH 6.8, 1% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.0012% bromophenol blue) and boiled for 5 minutes at 98 °C. Proteins were separated by SDS gel-electrophoreses, transferred onto a nitrocellulose membrane and stained with Ponceau Red (diluted 1:10) to visualize the correct transfer of proteins. Ponceau was removed by washing with TBST. The membrane was incubated with Milk 5% or BSA 5% for 1 hour at RT to reduce the background. Then, the primary antibody of interest was added for 2 hours at RT or overnight at 4°C. After the incubation, the membrane was washed several times with TBST and then was incubated with specific secondary antibodies for 1 hour at RT. Finally, membranes were washed with TBST. To visualize HRP conjugated signal, we used Supersignal West Femto Chemiluminescent Substrate (Thermo Scientific) and Chemidoc (Biorad).

Primary antibodies used were: Rabbit anti-ref(2)P 1:1000 (from Tor Erik Rusten), Rabbit anti-Atg8a 1:5000 (from Gabor Juhasz), Mouse anti-β-tubulin 1:8000 (Amersham), Mouse anti-Repo 1:20 (Developmental Studies Hybridoma Bank), Rat anti-Elav 1:40 (Developmental Studies Hybridoma Bank), Rabbit anti-Mitf 1:200 (developed by our group), Mouse anti-Cyclin B 1:20 (Developmental Studies Hybridoma Bank), Mouse-anti Actin 1:500 (Sigma). Rabbit anti-1:1000 phospho-AKT (Cell Signaling), Rabbit anti-1:1000 AKT (Cell Signaling). Secondary antibodies used were rabbit, mouse, rat and chicken HRP-conjugated 1:8000 (Amersham).

5.7 Tissue Disaggregation

For FACS analyses, 3rd instar larval brains were dissected and collected into Rinaldini's Solution. The proteases Collagenase I and Papain were added. To allow disaggregation, samples were incubated for 1 hour at 37°C. Disaggregated cells were washed with Schneider's *Drosophila* Medium and immediately separated using FACS. After cell sorting, cells were resuspended in Eppendorf within HBSS and were pelleted using a centrifuge. The pellet was processed to extract RNA with mini RNeasy kit of QIAGEN. cDNA was synthesized from RNA template using the SuperScript® VILO™ cDNA Synthesis Kit. Gene expression levels were evaluated by qPCR analysis.

5.8 Oligonucleotides used for experiments

Oligonucleotides used for qPCR analysis were UPL (Universal Probe Library) by Roche. UPL technology is based on only 165 short hydrolysis probes, which span different organisms. Probes are labeled at the 5' end with fluorescein and at the 3' end with a dark quencher dye. Primers are designed to flank the probe, so that cleavage of the probe separates the fluorescein from the rest allowing the fluorescence emission.

Primers for qPCR		
Atg8s	CATGGGCTCCCTGTACCA	F
	CTCATCGGAGTAGGCAATGT	R
Atg1	TTTTACGCTGCCCGAACT	F
	GCTCCTGTGTCCAGCAGACT	R
Elav	CCGGCAGCACCAGTAAGA	F
	CCAGTTGGGGATTGAGGAA	R
Lamp1	GCTTTCCTTTATGCAAATTCATC	F
	GCTGAACCGTTTGATTTTCC	R
ref(2)P	AGACAGAGCCCCTGAATCCT	F
	GGCGTCTTTCCTGCTCTGT	R
Repo	GCATCAAGAAGAAGAAGACGAGA	F
	GTTCAAAGGCACGCTCCA	R
Rpl32	CGGATCGATATGCTAAGCTGT	F
	CGACGCACTCTGTTGTCG	R
VhaPPA1-1	ATCTTCGGTTCGGCCATC	F
	ATAATGGAGTGGCGAAGGAC	R
Thor	CCAGATGCCCGAGGTGTA	F
	AGCCCGCTCGTAGATAAGTTT	R

Table3. UPL primers used for qPCR analysis. The primers Forward and Revers used in this study are indicated above.

5.9 Starvation-induced autophagy

To induce starvation in larvae, we washed them in PBS 1X to remove food residues. Larvae were left for 4 hours on a Petri dish containing sucrose 20% diluted in PBS 1X. After starvation, larvae were dissected to isolate brains for the subsequent analysis.

5.10 DQ-BSA assay

DQ Red BSA is a fluorogenic substrate for proteases. Upon hydrolysis, DQ Red BSA releases fluorescent fragments that have excitation and maxima emission of ~590 nm and ~620 nm **(Figure 16)**. This artificial substrate is used to evaluate lysosomal proteolytic degradation. 3rd instar larvae were dissected in order to expose internal organs to the substrate, DQ Red BSA was used at a concentration of 120µg/µl diluted in M3 insect medium. Samples were incubated for 6 hours at RT to allow internalization of the substrate by endocytosis, then they were fixed with 4% PFA. After 3 washes with PBS 1X, brains were mounted on a slide with glycerol 70% for immediate confocal acquisitions.

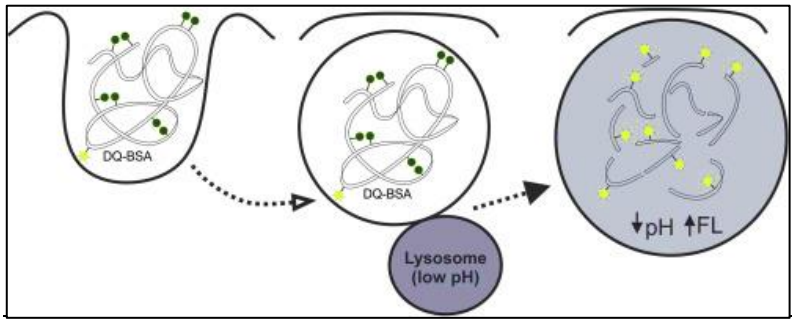

Figure16 . Schematic representation of DQ-Red BSA assay. DQ-Red BSA is endocytosed in cells and traffics through early endosomes to late endosomes

towards lysosomes. Their fusion leads to the degradation of DQ-Red BSA and subsequent de-quenching of the dye attached to this cargo. Adapted from Frost et al. 2017

5.11 Bafilomycin A1 treatment

Bafilomycin A1 was directly added to the food. The compound was used at a concentration of 25μM. Adults were kept in culturing tubes for 8 hours and then removed, while eggs were kept at 25° until 3rd instar stage. Larval brains were dissected and fixed using PFA 4%. After 30 minutes of fixation, brains were isolated and mounted with 70% glycerol on glass slides.

5.12 Lysotracker assay

Fat body was isolated from larvae, washed in PBS 1X and incubated on ice for 30 minutes in a solution of M3 insect medium with Lysotracker Red-DND-99 1:3000 (Life technologies) and DAPI. Samples were then rinsed twice with PBS1X and mounted immediately in glycerol 70% for confocal acquisitions.

5.13 Locomotor Assay

Using a brush, we moved 3rd instar larvae to a lid of a 15 cm Petri dish (pre-coated with 2% agarose). Under the lid is pasted a graph paper with a 0.2 cm^2 grid. We measured the number of the grid lines crossed in 5 minutes. This value is the linear distance from a starting point, while the crawling velocity is obtained dividing the previous value for the time.

5.14 Statistical Analysis

ImageJ (National Institutes of Health, Bethesda, USA) was used for analyzing immunofluorescence acquisitions, n> 10 brains analyzed for each experiment. Quantification of the GFP amount in larval brains was performed using a plugin of ImageJ developed by Emanuele Martini. Western blots protein levels were analyzed using Image Lab (BIORAD). Statistical analysis was performed with GraphPad Prism. Sample size used was: n≥40 brains

for Western Blot, n≥20 brains for RNA extraction, n≥100 brains for both FACS and cell sorting experiments. P* indicates a P value of 0,05, P** indicates a P value of ≤ 0.01 and P*** indicates a P value ≤ 0,0001.

6 RESULTS

6.1 In *Drosophila* larval brains, glial expression of *Dp110-CAAX* and *hΔEGFR* leads to gliomagenesis

As previously demonstrated, glial expression of a membrane tagged form of *Dp110-CAAX* (Leevers et al., 1996) and of a deleted form of *Drosophila Egfr*, both of which are constitutively active, leads to gliomagenesis in *Drosophila* larvae (Read, 2012; Read et al., 2009). To understand whether a constitutively active form of human EGFR *(hΔEGFR*) also supports glial tissue overgrowth in *Drosophila*, we co-expressed *Dp110-CAAX* with *hΔEGFR*. To this end, we took advantage of the Gal4-UAS system by using a P*(Gal4)* insertion in the *repo* locus (*repo-Gal4*) which provides pan-glial expression. To mark glial cell membranes, we also expressed UAS-mCD::GFP, a membrane-inserted GFP form (Lee T, Luo L, 1999). *In vivo*, constitutive expression of *Dp110-CAAX* with *hΔEGFR* results in larval glia overgrowth, with brains that are larger and morphologically altered, compared to controls **(Fig. 17A)**. In fact, optic lobes are elongated, the VNCs are abnormal, and the width and thickness of brains are increased **(Fig.17 B, C)**.

To quantify glial overgrowth, we measured mCD::GFP (hereafter named mGFP) signals by immunofluorescence and confocal imaging. We found that simultaneous overexpression of both constructs leads to a three-fold increase in the amount of glia in tumor brains, compared to controls **(Fig.17 D)**.

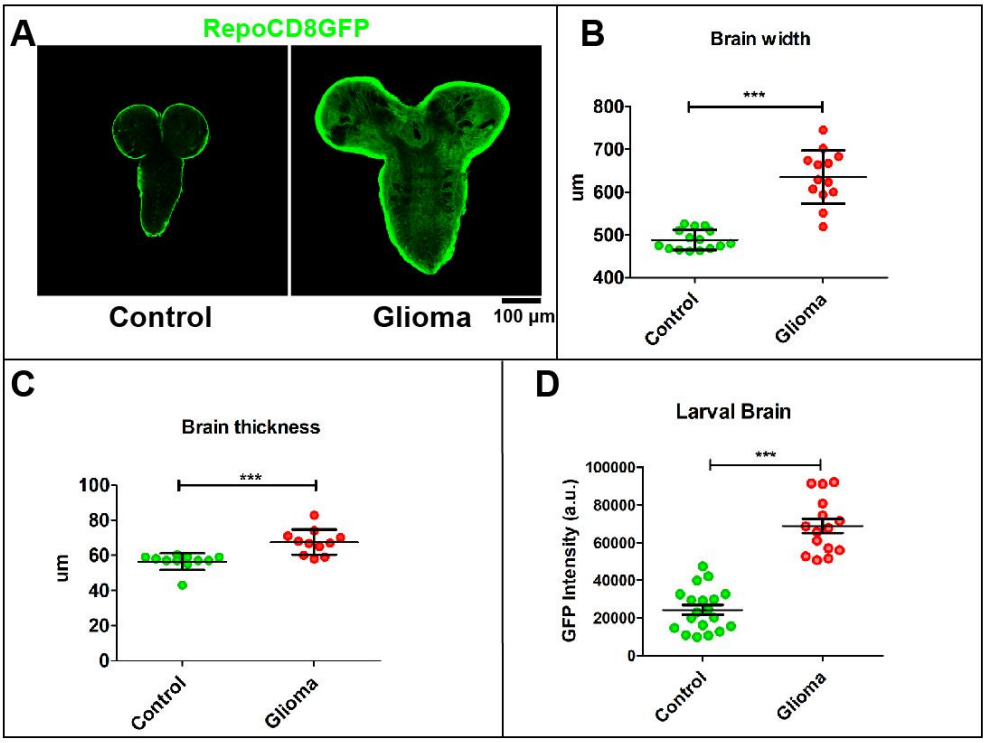

Figure 17. The overexpression of *Dp110-CAAX* and *hΔEGFR* constructs leads to glia overgrowth in *Drosophila* larval brains.

A) Single medial confocal section of whole brains from 3rd instar larvae. Dorsal view, anterior up. Glial cell membranes were labeled with anti-GFP (green) driven by the glial promoter *repo-Gal4*. Larval brains carried glioma show an increased amount of glia, an enlargement of the CNS and an abnormal VNC relative to control brain. Scale bar 100 um. **B-C).** The width and thickness (in µm) of larval brains are increased in brains carrying glioma compared to controls. n ≥10 brains per sample. The mean ± Standard Error of the Mean (SEM) are shown, and *P-values* are determined by Mann-Whitney test, P***≤ 0,0001. **D)** Glia quantification, using anti-mGFP to mark glial cell membranes. Notice the increased amount of fluorescence in gliomas compared to controls. n≥10 brains per sample. Mean ± SEM are shown, *P-value* is determined by Kruskal Wallis test, P***≤ 0,0001. All the quantifications shown in B, C and D were performed by ImageJ.

6.2 In *Drosophila*, overgrowth of glial cells impairs development of the neural system, compromises larvae behavior and leads to early death

To evaluate whether excess glial growth causes neural alterations, we measured by qPCR the mRNA of *repo,* which is specific for glial cells and of *elav*, a gene expressed exclusively in neurons. We found that *repo* levels are 3 folds increased in glioma brains compared to control brains **(Fig.18 A)**, while *elav* transcriptional levels are decreased in gliomas compared to controls **(Fig.18 B)**. We confirmed this result by assessing the levels of Repo and Elav proteins by Western blot. We also demonstrated that the presence of glial cells is strongly increased in gliomas, whereas the presence of neurons is heavily reduced compared to control brains **(Fig.18 C)**.

To characterize the morphological alterations in brains carrying gliomas, we performed an IF of *Drosophila* larval brains. To this end, we used antibodies anti-mGFP, anti-Repo and anti-Elav to mark glial cell membranes, glial cell nuclei and neurons, respectively. We found that, glial cells are randomly distributed in glioma optic lobes. Conversely, in control optic lobes, glial cells are mainly confined to surface glia. In addition, the number of Repo-positive glial cells is strongly increased in optic lobes of glioma, when compared to optic lobes of control. We also observed that the neural cells are reduced and show altered morphology and distribution **(Fig.18 D)**. Indeed, Elav-positive cells in glioma brains are located in small areas surrounded by the overgrown glia, while such morphology is not present in control brains. Overall, data reveal that excessive glia growth not only subverts larval glial organization, but it also hampers neurons, altering their normal distribution.

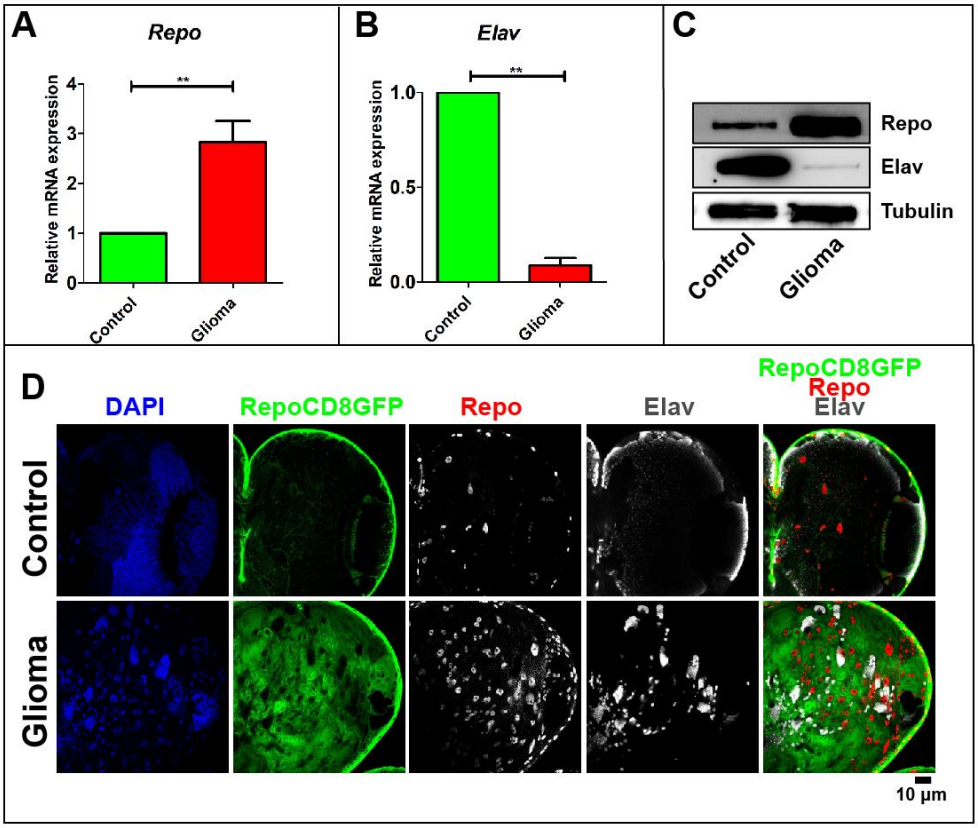

Figure 18. mRNA and protein levels of glial and neural markers reveal altered lineage organization

A-B) Evaluation of glia and neuron organization by mRNA expression (using *repo* and *elav* respectively) in 3rd instar larval brains. The mRNA levels of *repo* are strongly increased in gliomas compared to controls. Conversely, *elav* levels are strongly suppressed in gliomas compared to controls. *RpL32* was used as a housekeeping control. Each value represented the mean ± Standard Deviation (S.D.) of n≥3 independent experiments and *P-values* are determined by Mann-Whitney test, P**≤ 0.01. **C)** Repo and Elav protein levels were tested by Western blot. Repo levels are clearly increased in gliomas compared to controls, while Elav protein levels are strongly decreased in tumor compared to control brains. Tubulin was used as a loading control. **D)** Single medial confocal sections of *Drosophila* 3rd instar larval brains. Anti-mGFP (green) was used to stain glial cell membranes. These cells cover the entire optic lobe in glioma samples, while only the external surface of optic lobes in control samples. The number of glial cell nuclei, stained with anti-Repo (grey), are increased in glioma samples compared to control samples. Neurons, marked by anti-Elav (grey), show a completely subverted morphology in

glioma optic lobes compared to control optic lobes. In the merge, Repo cell nuclei are shown in red. Scale bar 10 um.

To precisely determine the extent of glial overgrowth, we used FACS to separate mGFP positive (GFP^+) glial cells from other cell types that are mGFP negative (GFP^-). As reported in literature, we observed that in controls glial cells are 10% of the CNS cells, the rest being mostly neurons **(Fig.19 A)**. In glioma brains, the number of GFP^+ cells is extremely increased, up to 7 folds compared to controls. In contrast, GFP^- cells in the overgrown brains are drastically decreased compared to controls. We conclude that co-expression of *Dp110-CAAX* and *hΔEGFR* in the *Drosophila* glia likely causes cells to adopt glial fates at the expense of neuronal fates.

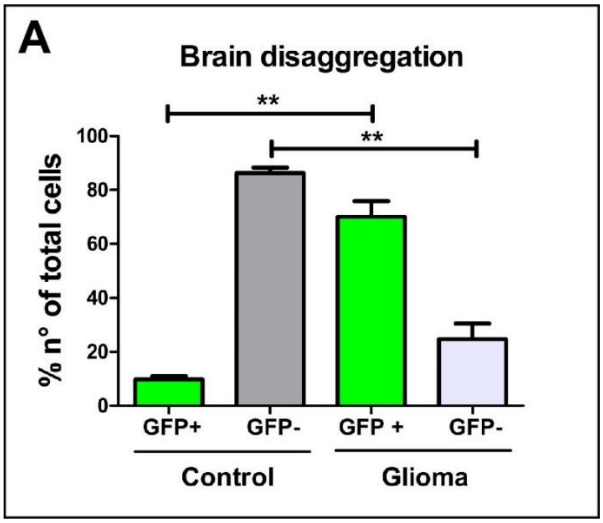

Figure 19. Glia is 7-fold more abundant in tumor brains and its overgrowth is at the expenses of neurons

A) 3rd instar larval brain cell types were quantified. After tissue disaggregation, mGFP positive glial cells (GFP^+) and mGFP negative neural cells (GFP^-) were separated by FACS. In controls, glial cells represent the 10%, while in gliomas are up to 70% of the total brain population. The overgrowth of the glia involves a strong reduction of neuronal cells that result decreased compared to control brains. Each value represented mean ± S.D. of n≥3 independent experiments and *P-values* are determined by Kruskal Wallis test. P**≤0,01.

To investigate if altered brain organization in larvae bearing gliomas has an impact on brain function, we performed two behavioral tests. We measured the crawling velocity of larvae and the linear distance that they are able to cover from a starting point. We found that both abilities are decreased in larvae carrying glioma **(Fig.20 A, B)**. Thus, genetic induction of glioma induces functional alterations in brain development and reduces the ability to perform simple neuromotor tasks. Consistent with such a major behavioral impairment, we found that all the larvae carrying a glioma die during the transition between L3 and pre-pupal stage (not shown).

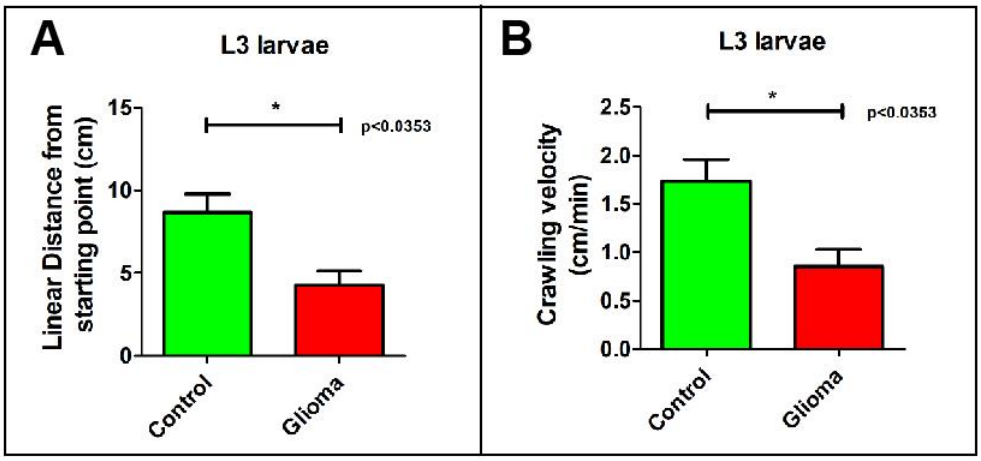

Figure 20. Glioma-induced neural alterations cause changes in larvae behavior

A-B) The abilities to cover a linear distance (cm) and the crawling velocity (cm/min) were evaluated in 3rd instar larvae. Both abilities are reduced in larvae carrying glioma compared to control larvae. Each value represented mean ± S.D. of n≥3 independent experiments and *P-values* are determined by Mann-Whitney test, P*≤ 0.05.

6.3 The expression of *Dp110-CAAX* and *hΔEGFR* in glioma brains compromises induction of the autophagy pathway

We next used our *Drosophila* model to investigate the poorly understood role of autophagy in gliomas. To detect the activity of degradative processes, we evaluated the presence of ubiquitinated proteins in the brain tissue using an anti-ubiquitin (FK2), while to monitor autophagy we used anti-ref(2)P, which marks specifically proteins that have to be eliminated by the such pathway. Remarkably, both markers accumulate and colocalize in the optic lobes of 3rd instar larvae carrying gliomas **(Fig.21 A)**. This result suggests that in gliomas the autophagic process is either impaired or strongly induced. To distinguish between these possibilities, we evaluated the autophagic flux by measuring ref(2)P and Atg8a protein levels by Western blot. Using this technique, we found that ref(2)P accumulates in glioma brains, while it does not in control brains. Despite the increased levels of ref(2)P, Atg8a levels are similar in glioma and control brains **(Fig.21 B)**. These data reveal that glioma cells accumulate autophagic cargoes that are not efficiently cleared by formation of autophagosomes.

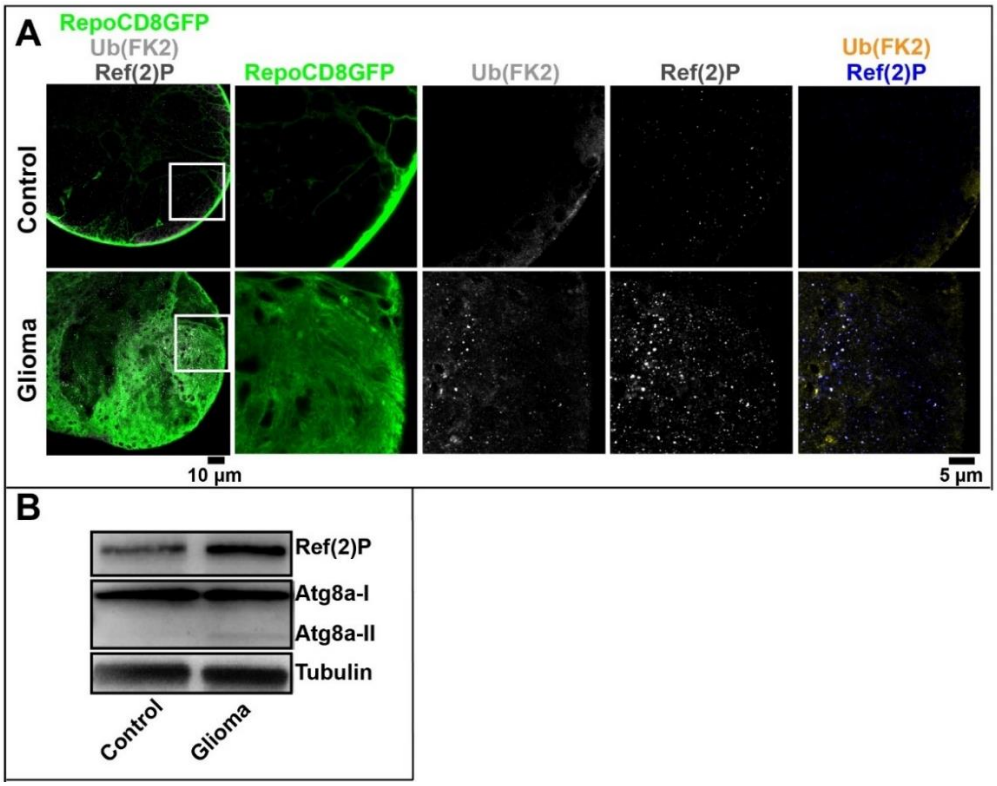

Figure 21. Gliomas accumulate ubiquitin and the autophagy adapter ref(2)P
A) Single medial confocal sections of 3rd instar larval brains shown alterations in degradative process. High magnifications of insets were shown as merge and separate channels: glial cell membranes, marked by anti-GFP are presented in green, ubiquitin FK2 and autophagy adapter ref(2)P are both in grey. Notice the increase in the signal of both markers in gliomas compared to controls. In the merge of high magnifications, FK2 (yellow) partially colocalizes with ref(2)P (blu). Scale bars 10 um and 5um. **B)** Western blot of 3rd instar larval brains. Autophagy markers were stained with anti-ref(2)P and anti-Atg8a I-II. In gliomas ref(2)P protein levels are increased compared to controls, albeit Atg8a levels are only slightly changed. Tubulin was used as a loading control.

To monitor induction of the autophagy pathway, we assessed the transcriptional levels of core autophagy-related genes *Atg1*, *Atg7*, *Atg8a* and of the autophagic adapter r*ef(2)P*. We found that in glioma samples, A*tg*

genes are down-regulated and, conversely, *ref(2)P* is up-regulated to similar levels of that of the glial marker *repo* **(Fig. 22 A)**.

To assess if the up-regulation of *ref(2)P* is occurring specifically in glial tissue, we used cell sorting to separate GFP^+ glial cells from GFP^- neurons and other cell types. We observed that the increase in mRNA levels of *ref(2)P* is for the most part occurring in tumoral glial cell **(Fig.22 B)**. In GFP^- cells of glioma samples, the transcriptional levels of *ref(2)P* are comparable to both GFP^+ and GFP^- control brains. These data reveal that the up-regulation of *ref(2)P* is strictly associated with glial cells carrying the constitutive co-activation of *Dp110-CAAX* and *hΔEGFR*.

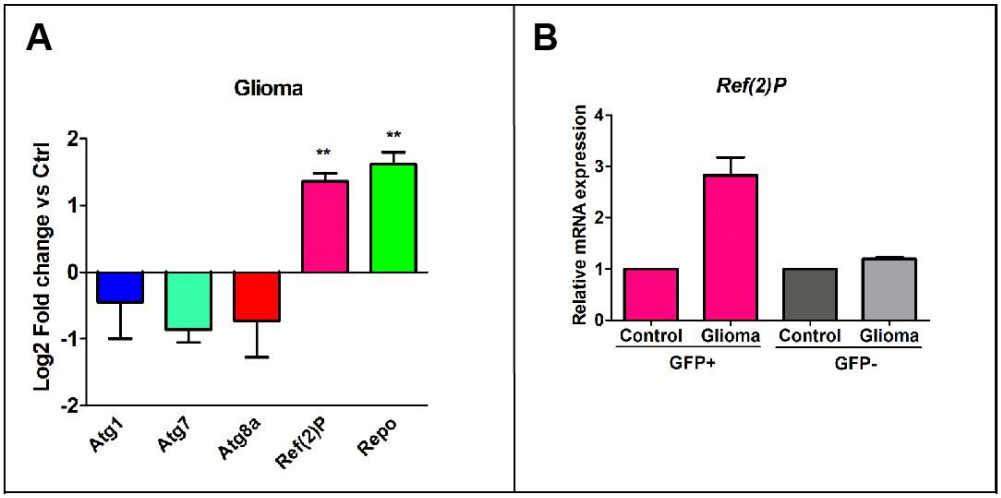

Figure 22. In gliomas, *Atg* genes are down-regulated, while *ref(2)P* is up-regulated

A) *Atg* genes expression in 3rd instar larval brains were evaluated by qPCR. In glioma samples, mRNA level of *Atg1, Atg7 and Atg8a* are down-regulated while *ref(2)P* and also the glial marker *repo*, are strongly up-regulated. Expression levels were relative to control samples. *RpL32* was used as a housekeeping control. Each value represented mean ± S.D. of n≥3 independent experiments and *P-values* are determined by Mann-Whitney test, P**≤ 0.01. **B)** *ref(2)P* expression levels in 3rd instar larval brains, after tissue disaggregation. Glial cells (GFP^+) and neural cells (GFP^-) were separated by cell sorting. *ref(2)P* mRNA expression is up-regulated only

in glial cells that belong to tumor. Normalization on GFP^+ cells of control brains. *RpL32* was used as a housekeeping. Each value represented mean ± S.D. of n≥3 independent experiments.

To assess whether autophagy can be induced in larval brains, we resorted to starvation, a stimulus known to promote activation of autophagy. To evaluate the autophagic flux in fed and starved conditions we used Western blots. As previously demonstrated by IF, fed larvae carrying gliomas accumulate ref(2)P, although Atg8a levels are the same in control and glioma brains **(Fig.23 A)**. After starvation, ref(2)P decreases in control brains, as expected for induction of autophagy. Conversely, ref(2)P accumulation is increased in gliomas. Atg8a levels are the same in both tissues **(Fig.23A)**.

We also monitored changes in autophagy by using mCherry::Atg8a, a tagged form of Atg8a, and ref(2)P. Under fed condition, a low level of mCherry::Atg8a signal is visible in controls, but not in glioma brains. Upon starvation, the signal of mCherry::Atg8a is strongly increased in control samples, while in gliomas the signal of mCherry::Atg8a is undetectable **(Fig.23 B)**.

Our results reveal that brains developing gliomas are not able to fully induce autophagy, as demonstrated by applying a physiological stress such as starvation.

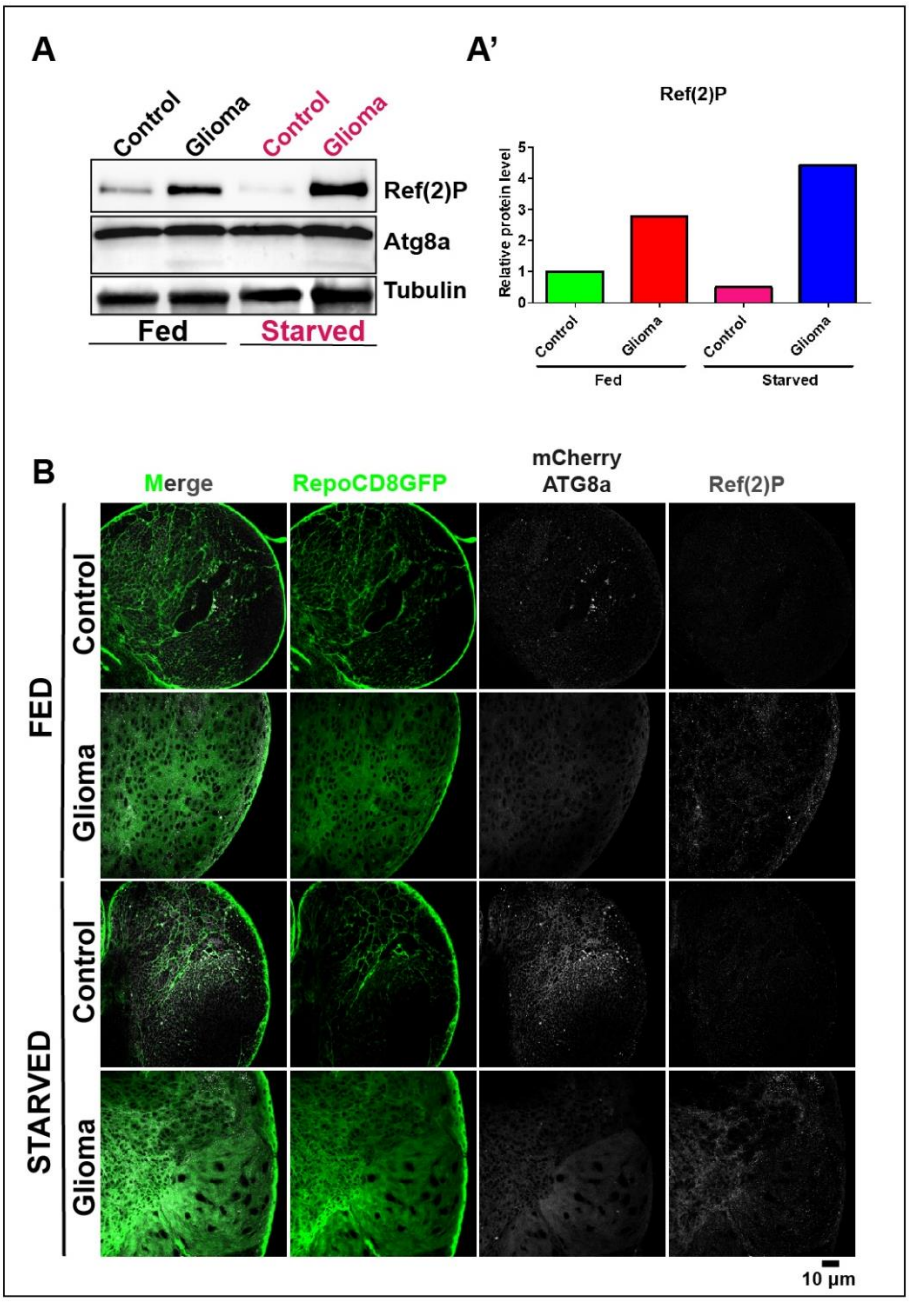

Figure 23. In tumoral tissues, starvation-induced autophagy does not occur

A) Western blot of 3rd instar larval brain extracts. Autophagy markers ref(2)P and Atg8a I-II were evaluated in fed and starved conditions. Starvation induces ref(2)P degradation in control compared to fed control, while its protein levels are increased

in glioma compared to fed glioma. Tubulin was used as a loading control. **A')** Evaluation of Western blot band intensities. The quantification demonstrates the failure to induce autophagy in glioma brains. Western blot loading differences between samples were equalized using tubulin and then normalized on fed control to obtain band intensities. **B)** Single medial confocal sections of 3rd instar larval brains reared under fed and starved conditions. Anti-mGFP (green) was used to mark glial cell membranes, anti-mCherry to detect Atg8a (grey), while anti-ref(2)P to recognize the autophagy adapter (also in grey). Notice, during starved condition, the strong increase of mCherry Atg8a in controls and the absence of mCherry Atg8a signal in gliomas. After starvation, in gliomas ref(2)P is even more accumulated. Scale bar 10 um.

6.4 Lysosomes are active and abundant in *Dp110-CAAX* and *hΔEGFR* induced-gliomas

We next decided to investigate whether lysosomes are present and active in gliomas, as inactivity could explain failure to perform autophagy. For this purpose, we evaluated both mRNA and protein levels of the lysosomal component Lamp1. Surprisingly, the transcriptional levels of *Lamp1* are two folds increased in glioma compared to control brains **(Fig.24 A)**. To determine the protein levels of Lamp1 in our model, we took advantage from a Lamp1::GFP fly line and we performed a Western blot using anti-GFP. We found that Lamp1 signal is also increased in gliomas, compared to controls **(Fig.24 B)**.

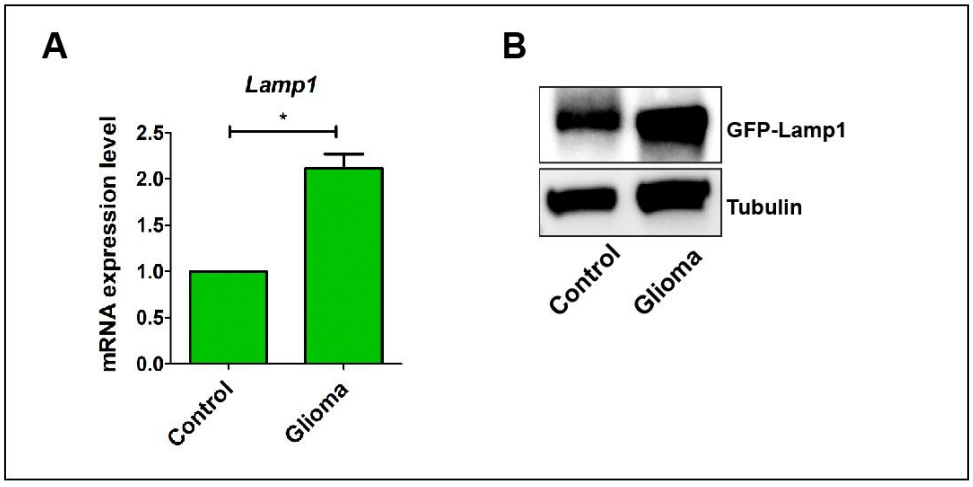

Figure 24. Lamp1, in gliomas, is increased at transcriptional and protein levels

A) The *bona fide* marker for lysosomes, *Lamp1,* was evaluated in 3rd instar larval brains. mRNA levels of *Lamp1* are strongly increased in gliomas compared to controls. *RpL32* was used as a housekeeping control. Each value represented mean ± S.D. of n≥3 independent experiments and *P-value* is determined by Paired t-test, P*≤0,05 **B)** Western blot of 3rd instar larval brain extracts. Lamp1::GFP fly line was used to mark lysosomes and an anti-GFP was used to detect lysosomes. In gliomas, Lamp1 protein levels are increased compared to controls. Tubulin was used as a loading control.

Finally, to evaluate the degradative ability of lysosomes *in vivo*, we used the dye DQ-BSA, that emits fluorescence when BSA is digested by lysosomal proteases. The experiment demonstrated that lysosomal activity is preserved in glioma and control brains. Additionally, the number of lysosomes labeled by DQ-BSA appeared increased in gliomas, compared to controls **(Fig.25 A)**. Overall, these data indicate that failed autophagy in fly gliomas is unlikely to be due to changes in lysosomal function.

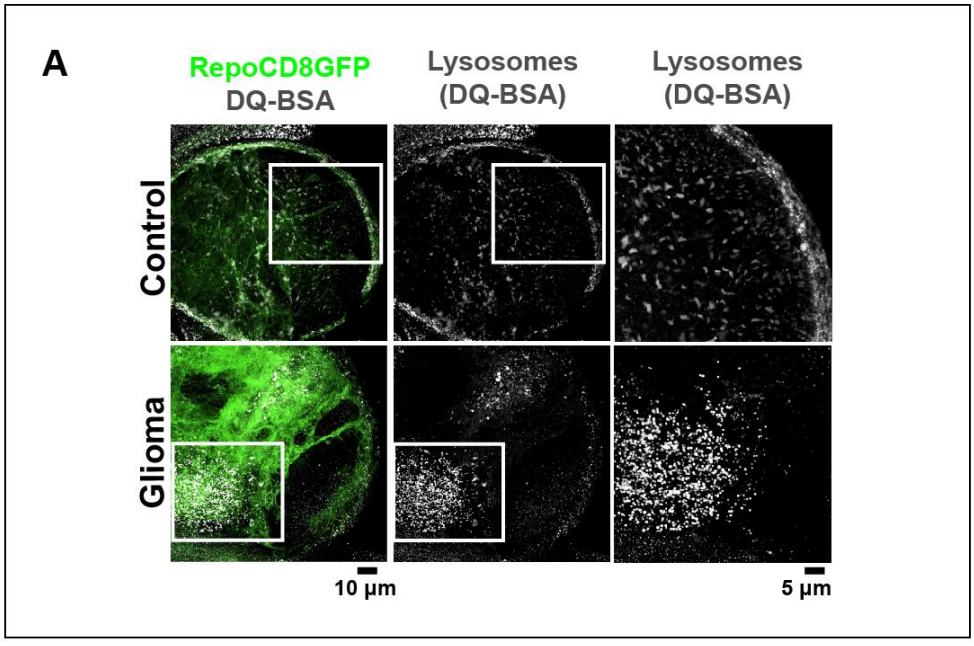

Figure 25. In gliomas, the lysosomal activity is preserved

A) Max projections of medial confocal sections of 3rd instar larval brains, after 6 hrs of incubation with DQ-BSA. Anti-mGFP stained glial cell membranes (green), while DQ-BSA stained the acidic environment of lysosomes (grey). Glioma brains preserve their lysosomal activity, due to proteolytic cleavage of the dye that allowed fluorescence emission. Furthermore, the lysosomes are increased in glioma compared to control brains. High magnifications of insets clearly shown the lysosomal expansion in gliomas. Scale bar 10 um and 5 um.

6.5 The transcription factor *Mitf*, a known regulator of lysosomal biogenesis, is up-regulated in glioma brains

Considering the failure in the induction of autophagy and the high number of lysosomes in gliomas, which could be due to activation of TFEB, we explored whether the *Drosophila* homolog Mitf is modulated in glioma brains by using an anti-Mitf antibody previously developed by our group. Confocal images show a strong increase of the Mitf signal in glioma tissue compared to controls, which is consistent with the observed lysosomal expansion **(Fig.26 A)**. However, in gliomas Mitf is mainly present in the cytoplasm and is rarely visible in cell nuclei, where it is known to be active as a transcription factor. To confirm this observation, we evaluated Mitf protein levels by Western blot. We found that Mitf is slightly more expressed in gliomas than in controls **(Fig.26 B)**.

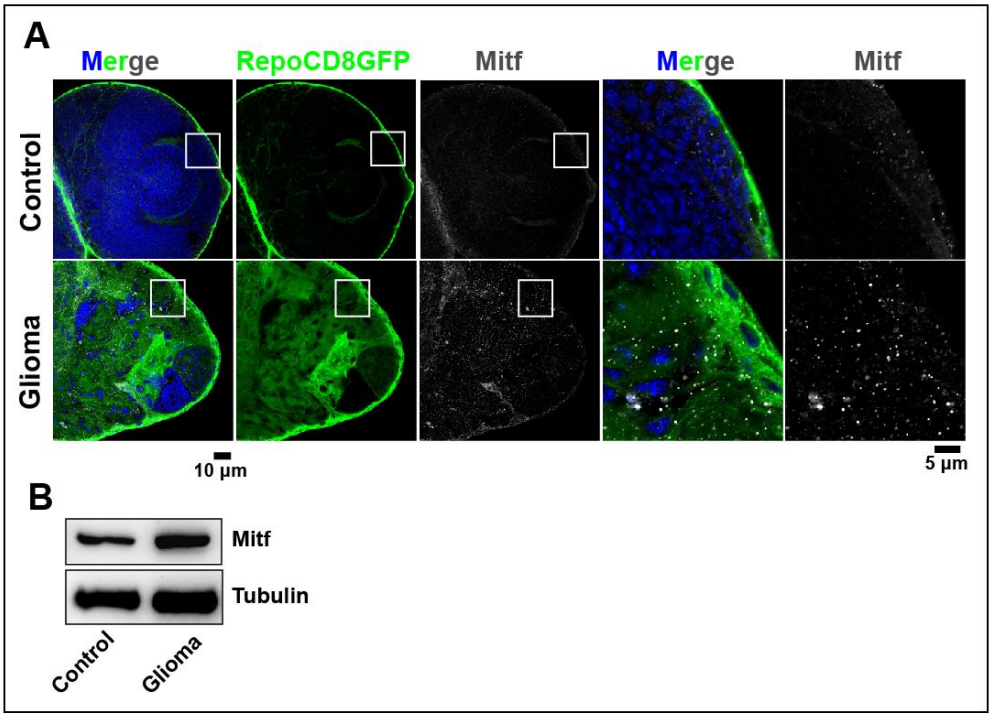

Figure 26. The development of gliomas leads to Mitf overexpression, however, the transcription factor is not active in gliomas

A) Single medial confocal sections of 3rd instar larval brains. Nuclei were stained with DAPI (blue), glial cell membranes were stained with anti-mGFP (green), the transcription factor was stained with anti-Mitf (grey). Mitf is heavily accumulated in glioma optic lobes compared to control optic lobes. Mitf accumulation can be better appreciated in higher magnifications of insets. Notice that in gliomas, Mitf is almost exclusively in the cytoplasm, the transcription factor is seldom associated with nuclei. Scale bar 10 um and 5 um. **B)** Western blot of 3rd instar larval brain extracts. Anti-Mitf reveals increased protein levels in gliomas compared to controls. Tubulin was used as a loading control.

6.6 The transcription factor *Mitf* is dispensable for tumor growth in gliomas

To test whether *Mitf* is important for growth in *Dp110-CAAX* and *hΔEGFR* induced-gliomas, we used a fly RNAi line to down-regulate *Mitf* expression. We confirmed down-regulation by qPCR, in both glioma brains, relative to

control **(Fig.27A)**. We then measured the amounts of glial cells by quantifying the signal intensity of mGFP. We found that gliomas developing with low expression of *Mitf* (glioma>*Mitf*^{RNAi}) possess less glia than gliomas without down-regulation, but this difference is not statistically significant **(Fig.27 B)**. In conclusion, the down-regulation of *Mitf* impacts only partially the excessive glia growth observed in gliomas.

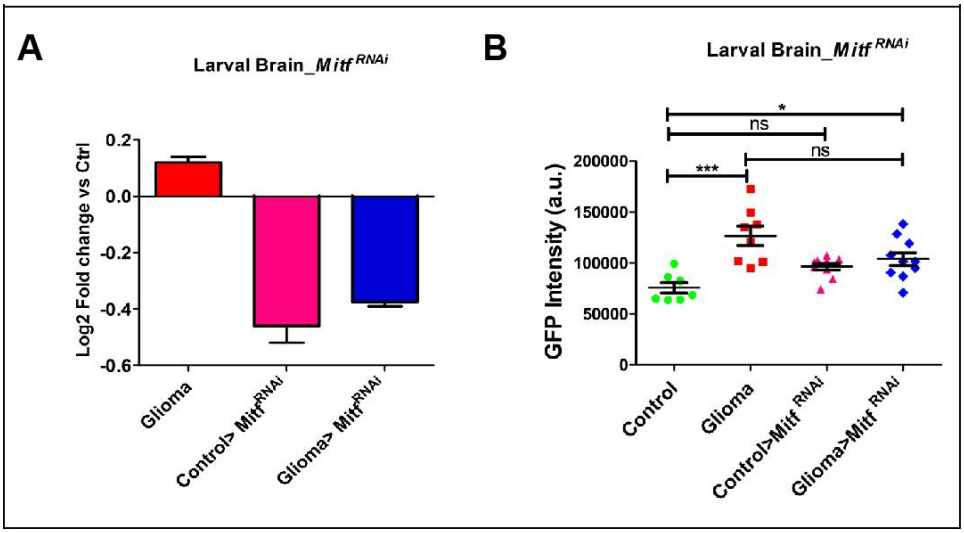

Figure 27. The down-regulation of *Mitf* does not influence tumor overgrowth
A) The transcriptional levels of *Mitf* of the indicated genotypes were evaluated by qPCR. Samples carrying the down-regulation of *Mitf* shown a reduced expression of the gene. Notice that mRNA levels of *Mitf* are up-regulated in gliomas compared to controls. *RpL32* was used as a housekeeping control. Each value represented mean ± S.D. of n≥2 independent experiments. **B)** Glia was quantified using anti-mGFP. The down-regulation of *Mitf* in gliomas decreases glia overgrowth but this difference is not statistically significant, albeit is less evident. Mean ± SEM are shown, *P-values* are determined by Kruskal Wallis test, P*≤ 0,05, P***≤ 0,001. Quantification in B was performed by ImageJ.

6.7 The V-ATPase subunit VhaPPA1-1 plays a crucial role in glia overgrowth induced by the expression of *Dp110-CAAX* and *hΔEGFR*

To understand the role of V-ATPase in glioma growth, we down-regulated the expression of *VhaPPA1-1,* encoding the V0 sector subunit VhaPPA1-1. The evaluation of *VhaPPA1-1* transcriptional levels by qPCR demonstrated efficient down-regulation. Furthermore, qPCR results revealed that this V-ATPase subunit is more expressed in gliomas than control brains, consistent with reports in mammalian cells indicating upregulation of V-ATPase subunits in GBM (Di Cristofori et al., 2015; Von Schwarzenberg et al., 2013) **(Fig.28 A)**. To assess the effect of *VhaPPA1-1* down-regulation on tumor growth, we again visualized glial tissue using mGFP. Surprisingly, down-regulation of *VhaPPA1-1* prevented most of the morphological defects observed in glioma brains. Indeed, brains with down-regulation of *VhaPPA1-1* show similar morphology to that of controls **(Fig.28 B)**. We further evaluated the intensity of mGFP upon down-regulation the *VhaPPA1-1* subunit in gliomas and found that glial cells amounts are similar to that of controls **(Fig.28 C)**.

To characterize more precisely *VhaPPA1-1* expression in gliomas, we used cell sorting. We observed that the mRNA levels of the gene are strongly decreased in non-glial tissue compared to glial tissue **(Fig.28 D)**, indicating that V-ATPase expression is normally higher in larval glia than in other CNS cell types.

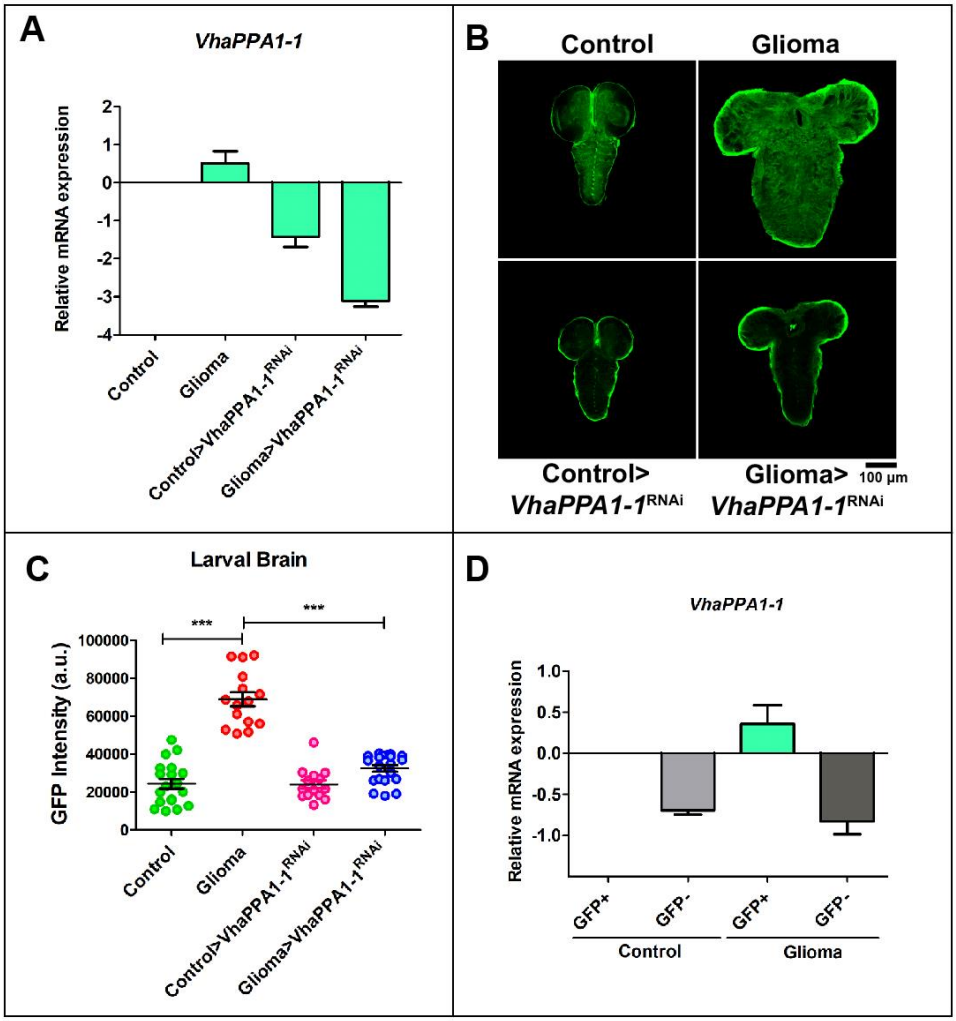

Figure 28. The down-regulation of *VhaPPA1-1* prevents gliomas overgrowth

A) *VhaPPA1-1* transcriptional levels were measured by qPCR. The gene results down-regulated in genotypes carrying the down-regulation. Notice the up-regulation of *VhaPPA1-1* in gliomas. *RpL32* was used as a housekeeping control. Each value represented mean ± S.D. of n≥3 independent experiments. **B)** Single medial confocal sections of whole brains from 3rd instar larvae. Dorsal view, anterior up. Glial cell membranes are labeled with anti-GFP (green). The excessive growth of the glia, observed in gliomas, is restored to control levels in gliomas>*VhaPPA1-1*^{RNAi}. Scale bar 100 um. **C)** Glia quantification, using mGFP. Tumors show excessive glia compared to controls. In gliomas>*VhaPPA1-1*^{RNAi}, glia has the same expression of controls. For each genotype n≥ 10 brains. Mean ± SEM are shown, and *P-values*

are determined by Kruskal Wallis test, P***≤ 0,001. Quantifications were performed by ImageJ. **D)** Evaluation of the *VhaPPA1-1* expression in 3rd instar larval brains, after tissue disaggregation. Glial cells (GFP^+) are separated by cell sorting from the other cell types (GFP^-). *VhaPPA1-1* mRNA expression is up-regulated only in glial cells that belong to the tumor while is down-regulated in neural cells. Normalization on GFP^+ cells of control brains. *RpL32* was used as a housekeeping. Each value represented mean ± S.D. of n≥3 independent experiments.

6.8 The role of apoptosis in glioma development

To investigate whether glial downregulation of *VhaPPA1-1*^{RNAi} causes cell death, we evaluated the presence of cleaved-caspase 3. We found that in control and glioma brains apoptotic cells are not prominent. Conversely, in control>*VhaPPA1-1*^{RNAi} there is a strong induction of apoptosis, marked by cleaved-caspase 3 **(Fig.29 A)**. Furthermore, nuclei show clear picnotic morphology **(Fig.29 A insets)**. This result reveals that the decreased brain size observed in glioma>*VhaPPA1-1*^{RNAi} is not due to cell death. Consequently, the activity of *VhaPPA1-1* is essential in normal glial cells, but not in tumor cells.

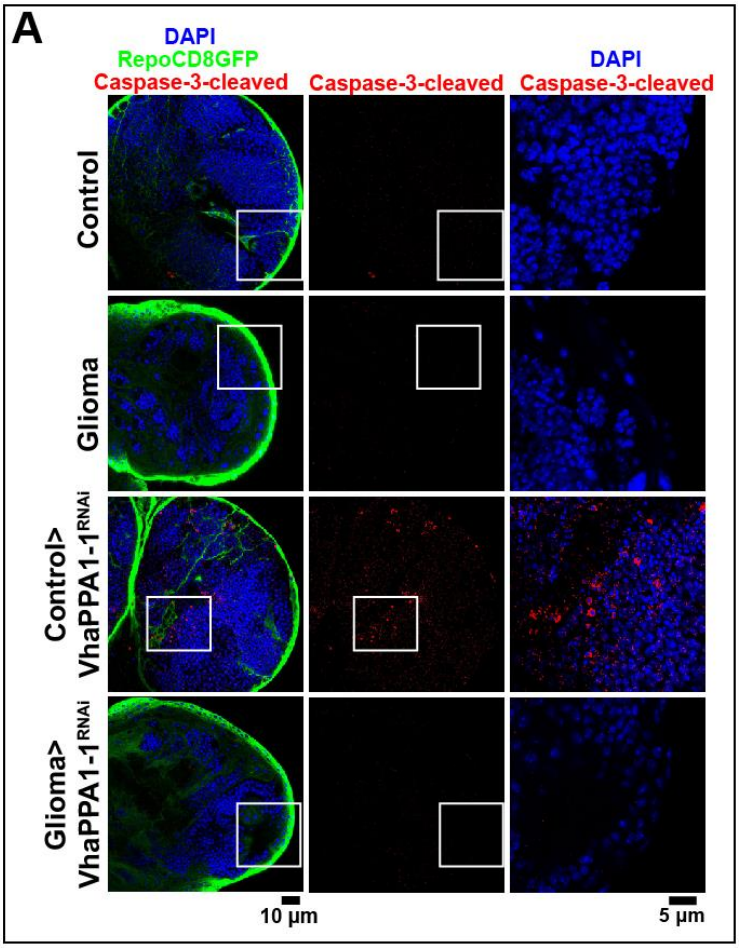

Figure 29. The down-regulation of the V-ATPase subunit leads to apoptosis in controls>VhaPPA1-1^{RNAi} but not in gliomas>*VhaPPA1-1^{RNAi}*

A) Single medial confocal sections of *Drosophila* 3rd instar larval brains. Cell nuclei were stained with DAPI (blue), glial cell membranes were stained with anti-mGFP (green), apoptotic cells were stained with anti- cleaved-caspase 3 (red). Only in control>*VhaPPA1-1^{RNAi}* cells are positive for anti-cleaved-caspase 3 and nuclei show an apoptotic morphology. High magnifications of insets revel apoptosis in control>*VhaPPA1-1^{RNAi}*. Scale bar 10 um.

6.9 Down-regulation of *VhaPPA1-1* in gliomas does not prevent cell division in 3rd instar larval brains

To assess for possible changes in the mitotic rate in tumors and *VhaPPA1-1*^{RNAi} downregulated tumors, we determined expression of the mitotic cyclin B in 3rd instar brains. IF shows that cyclin B is not differentially expressed in gliomas and gliomas>*VhaPPA1-1*^{RNAi}. However, Cyclin B is more expressed in glioma cells than in control samples, independent of expression of *VhaPPA1-1*^{RNAi}. Additionally, Cyclin B appears mostly present in overgrowing glial cells **(Fig.30 A)**.

We confirmed this result using Western blots that revealed that Cyclin B levels are unchanged between glioma and glioma>*VhaPPA1-1*^{RNAi} while, they are decreased in control>*VhaPPA1-1*^{RNAi} **(Fig.30 A)**. Collectively, our data suggest that reduced glia overgrowth in gliomas>*VhaPPA1-1* ^{RNAi} is likely not due to decreased mitotic rate, at least at the 3rd larval instar.

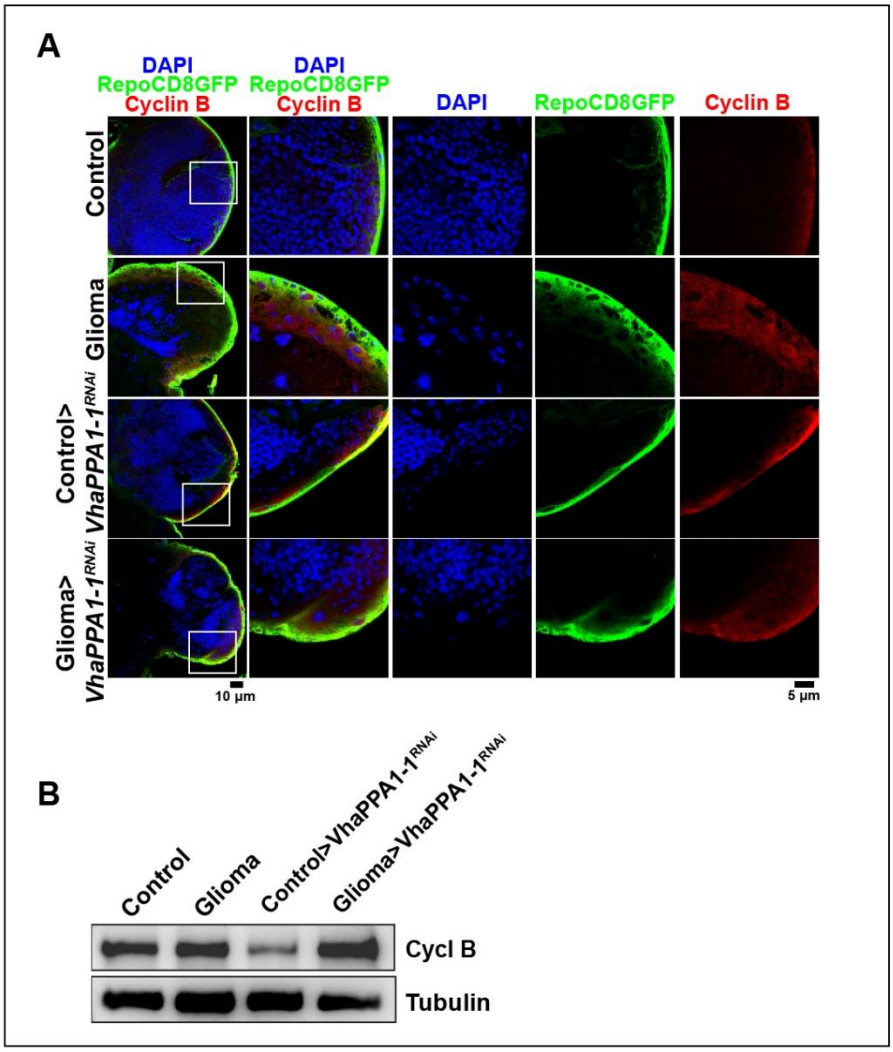

Figure 30. The mitotic rate is unchanged in gliomas>*VhaPPA1-1^{RNAi}*

A) Single medial confocal sections of 3rd instar larval brains. Nuclei were stained with DAPI (blue), glial cell membranes were stained with anti-GFP (green), anti-Cyclin B was stained in red. Cyclin B is more expressed in glioma compared to control brains. Gliomas and gliomas>*VhaPPA1-1^{RNAi}* show the same expression of Cyclin B. Scale bar 10 um and 5 um. **B)** Western blot of 3rd instar larval brain extracts. Cyclin B protein levels are comparable in all samples except for controls>*VhaPPA1-1^{RNAi}*, in which Cyclin B levels are decreased. Tubulin was used as a loading control.

6.10 Down-regulation of *VhaPPA1-1* affects CNS size and partially restores normal organization of the neural tissue

To determine the effect of *VhaPPA1-1* downregulation, we analyzed brain morphology and cell distribution using glial and neural markers (Repo and Elav respectively). We found that in gliomas>*VhaPPA1-1*^{RNAi}, the number of glial cells is increased compared to controls>*VhaPPA1-1* ^{RNAi} **(Fig.31 A)**. While the neural tissue is heavily altered in gliomas compared to controls, in gliomas> *VhaPPA1-1* ^{RNAi} neural architecture is less compromised than in gliomas developing without the downregulation. Despite this, the neural tissue does not show the same architecture observed in controls or in controls>*VhaPPA1-1* ^{RNAi} **(Fig.31A)**.

By Western blot, we confirmed that the levels of Repo are increased in gliomas, compared to control. However, expression of Repo is unchanged between gliomas and gliomas>*VhaPPA1-1*^{RNAi}. In contrast, Repo expression in controls>*VhaPPA1-1*^{RNAi} is decreased compared to controls. Finally, Elav protein levels in gliomas>*VhaPPA1-1*^{RNAi} are slightly increased compared to glioma brains **(Fig.31 B),** confirming a partial reversion of neuronal disorganization. Finally, we measured pixel surface areas of Repo positive cells to assess changes due to the down-regulation of *VhaPPA1-1*. We observed that Repo cells of gliomas are smaller compared to glial cells of controls. Furthermore, glial cells in gliomas>*VhaPPA1-1*^{RNAi} are smaller than controls **(Fig.31 C)**.

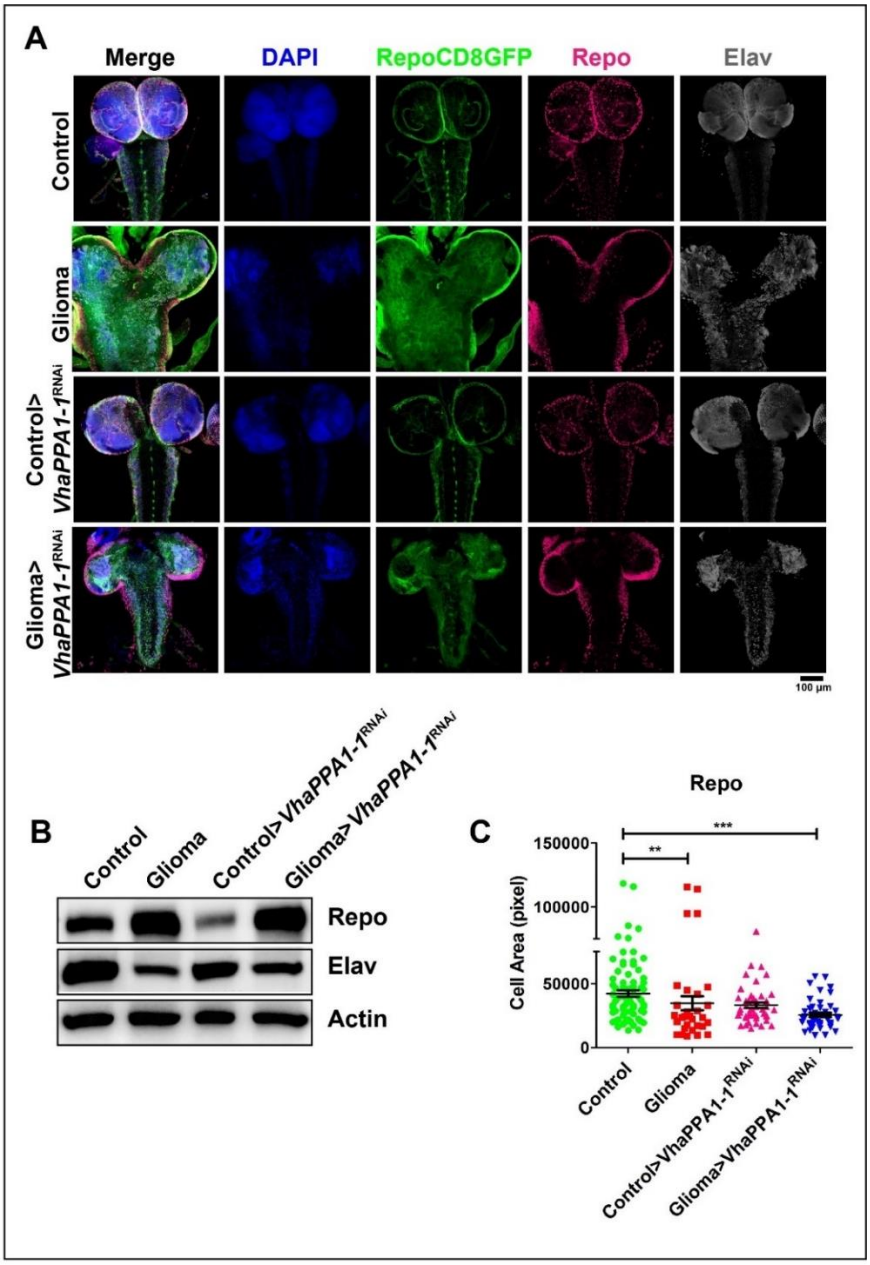

Figure 31. The down-regulation of *VhaPPA1-1* does not influence glial cells number but their size

A) Single medial confocal sections of 3rd instar larval brains. Nuclei, glial cell membranes, glial cell nuclei and neurons, were marked respectively by DAPI, GFP, Repo and Elav.

In glioma and glioma>*VhaPPA1-1*^{RNAi} glial nuclei (pink) are mainly localized in the external part of the optic lobes. Notice that the architecture of neural cells (grey) in gliomas>*VhaPPA1-1*^{RNAi} is less compromised than gliomas. Scale bars 100 um. **B)** Western blot of 3rd instar larval brain extracts. Repo protein levels are unchanged in gliomas compared to gliomas>*VhaPPA1-1*^{RNAi}, albeit Elav levels are slightly increased. In controls>*VhaPPA1-1*^{RNAi} Repo protein levels are decreased compared to controls. Actin was used as a loading control. **C)** Glia cells surface area was quantified. Glia cells are smaller in glioma compared to control brains. In gliomas>*VhaPPA1-1*^{RNAi}, glial cells are even smaller than controls. Mean ± SEM are shown, *P-values* are determined by One-way ANOVA, Kruskal Wallis test, P***≤ 0,001. Quantification was performed by ImageJ.

6.11 Down-regulation of VhaPPA1-1 partially restores the autophagy-lysosomal pathway in gliomas

To test if modulation of V-ATPase subunits can affect the lysosomal-autophagic pathway in glioma samples, we evaluated expression of the ubiquitin marker FK2 and of the autophagy adapter ref(2)P in 3rd instar larval brains. Similar to previous experiments, strong accumulation of these two markers is visible in glioma tissue, compared to control brains. In sheer contrast, in Gliomas>*VhaPPA1-1*^{RNAi}, neither FK2 or ref(2)P are accumulated **(Fig.32 A)**. We quantified ref(2)P accumulation in whole brains, confirming previous result by IF **(Fig.32 B)**. We also assessed the transcriptional levels of *ref(2)P* using qPCR and found that they are restored to control levels in gliomas>*VhaPPA1-1*^{RNAi}.

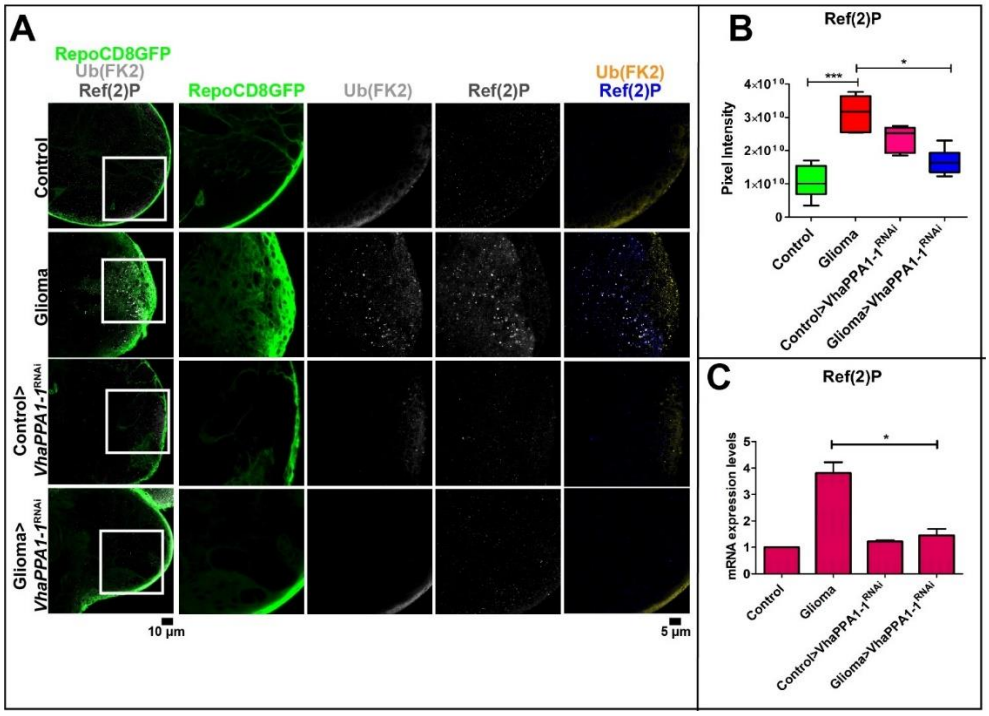

Figure 32. The accumulation of the ubiquitin marker FK2 and the autophagic adaptor ref(2)P is prevented, in gliomas, by the down-regulation of the *VhaPPA1-1* gene

A) Single medial confocal sections of 3rd instar larval brains. High magnifications of insets shown: glial cell membranes in green, ubiquitin FK2 and autophagy adapter ref(2)P both in grey. The increased signal of FK2 and ref(2)P observed in gliomas, is absent in gliomas>*VhaPPA1-1*^{RNAi}. In higher magnifications of the merge, FK2 is shown in yellow and ref(2)P in blue. Scale bars 10 um and 5um. **B)** Quantification of ref(2)P in whole 3rd instar larval brains. The increased levels of ref(2)P in gliomas are decreased in gliomas>*VhaPPA1-1*^{RNAi}. Means ± SEM are shown, *P-values* are determined by One-way ANOVA, Kruskal Wallis test, P*≤ 0.05; P***≤ 0.001. Quantification was performed by ImageJ. **C)** qPCR evaluation of *ref(2)P* in 3rd instar larval brains. In gliomas, *ref(2)P* mRNA levels are strongly up-regulated compared to controls. In gliomas>*VhaPPA1-1*^{RNAi} *ref(2)P* level are restored to control levels. *RpL32* was used as a housekeeping control. Each value represented mean ± S.D. of n≥3 independent experiments and *P-value* was obtained by Mann-Whitney test, P*≤ 0.05.

To measure the induction of autophagy following down-regulation of *VhaPPA1-1*, we carried out a starvation experiment. We then tested the expression of autophagy markers ref(2)P and Atg8a in fed and starved conditions. In fed conditions, glioma>*VhaPPA1-1*^{RNAi} shows the same levels of ref(2)P observed in controls. Upon starvation, glioma>*VhaPPA1-1*^{RNAi} shows an accumulation of ref(2)P, which is less than that observed in gliomas. Conversely, ref(2)P levels are decreased in both controls and control>*VhaPPA1-1*^{RNAi} **(Fig.33 A)**. Quantification of Western blot clearly shows the effect of starvation-induced autophagy on different genotypes **(Fig.33 A')**. Combined, our results surprisingly indicate that V-ATPase might be limiting for glioma development and that their activity in tumorigenic context might be related to the lysosomal-autophagic pathway.

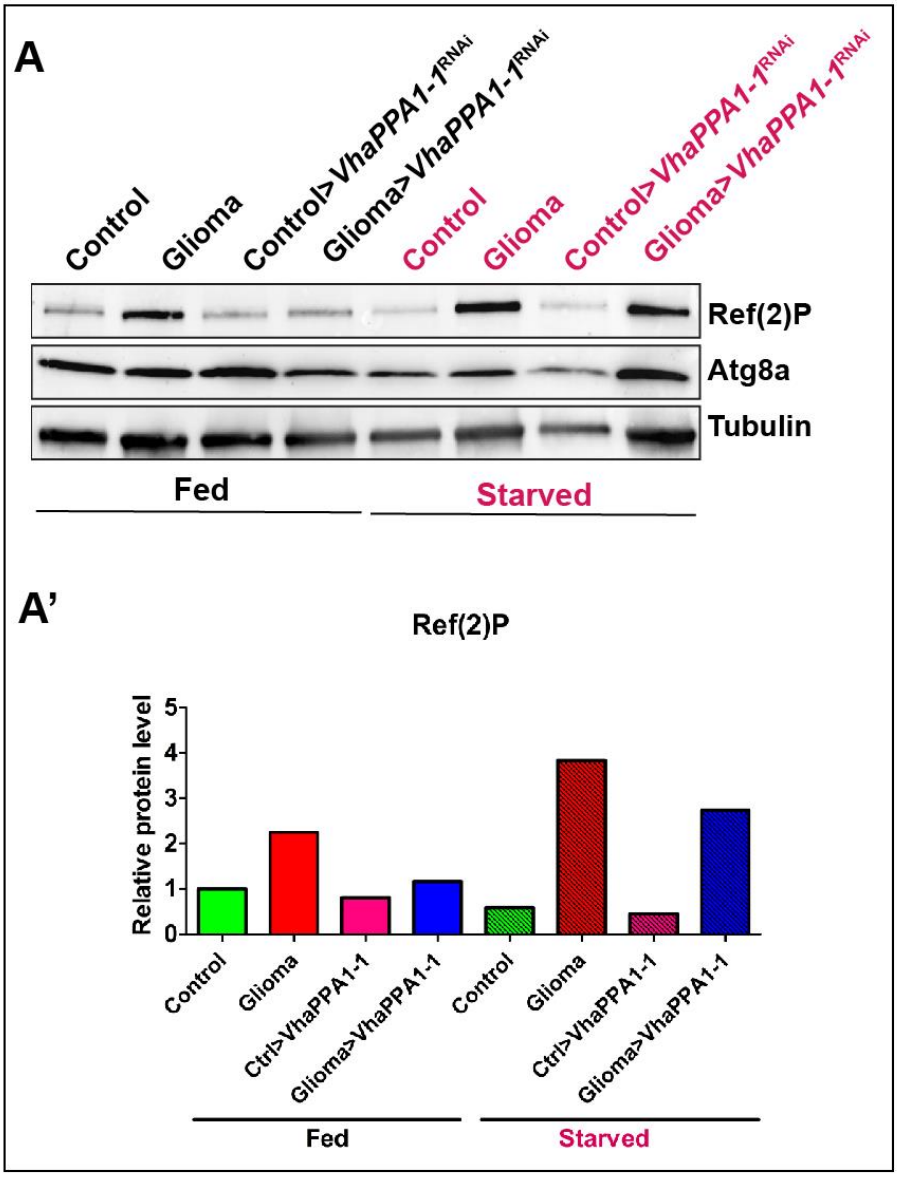

Figure 33. In gliomas>*VhaPPA1-1*^{RNAi}, the expression of ref(2)P is partially modulated by starvation induced-autophagy

A) Western blot of 3rd instar larval brain extracts. Autophagy markers ref(2)P and Atg8a I-II were evaluated in fed and starved conditions. In fed condition, the accumulation of ref(2)P observed in gliomas is not detectable in gliomas>*VhaPPA1-1*^{RNAi}. Starvation leads to ref(2)P degradation in controls and controls>*VhaPPA1-1*^{RNAi}, while in gliomas ref(2)P is even more accumulated. In gliomas>*VhaPPA1-1*^{RNAi}

the expression of ref(2)P is anyway increased. The second band of Atg8a (II) is not appreciable. Tubulin was used as a loading control.
A') Quantification of Western blot band intensities. Western blot loading differences between samples were equalized using tubulin control. Samples are normalized to obtain band intensities relative to fed control. In starved conditions, the expression of ref(2)P in gliomas>*VhaPPA1-1*^{RNAi} is lower compared to gliomas.

6.12 In gliomas>*VhaPPA1-1*^{RNAi}, the amount and activity of lysosomes and the expression of Mitf are similar to those of non-tumoral controls

We used the DQ-BSA assay to evaluate lysosomal activity following *VhaPPA1-1* down-regulation. Confocal images reveal that down-regulation of *VhaPPA1-1* does not alter the endosome-mediated delivery of cargos, as BSA is visible in lysosomes, also indicating that lysosomes maintained their degradation capability. Additionally, IF shows that the lysosomal signal observed in glioma samples is absent in glioma>*VhaPPA1-1*^{RNAi} samples (**Fig.34 A**). Thus, unexpectedly down-regulation of *VhaPPA1-1* restores physiological appearance of lysosomes.

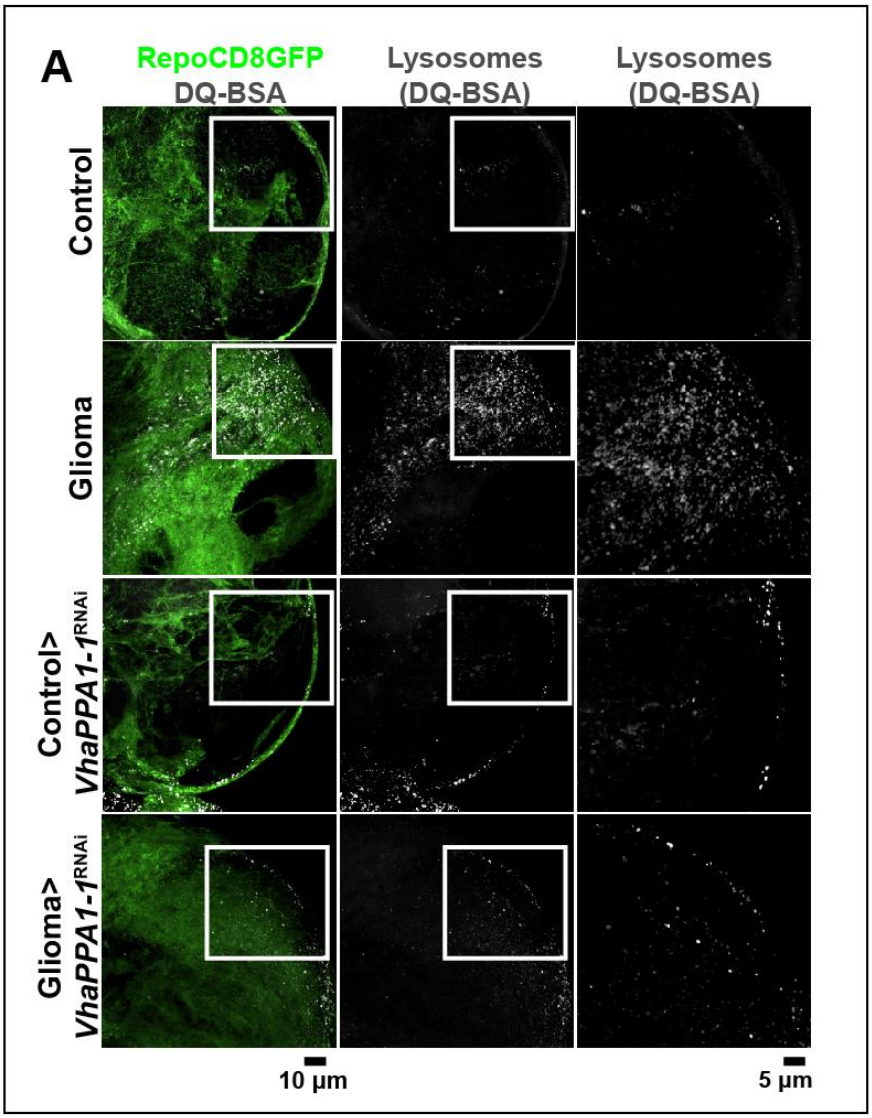

Figure 34. Down-regulation of the *VhaPPA1-1* subunit prevents lysosomal expansion in gliomas

A) Max projections of medial confocal sections of 3rd instar larval brains, after 6 hrs of incubation using DQ-BSA. Glial cell membranes were stained with anti-GFP (green), while lysosomes were stained with DQ-BSA (grey). The lysosomal expansion observed in gliomas is restored to control levels in gliomas>*VhaPPA1-1^{RNAi}*. High magnification of insets clearly shown the lysosomes of the indicated genotypes. Scale bar 10 um and 5 um.

We next analyzed Mitf expression. To this end, we performed an IF using anti-Mitf and found that in gliomas>*VhaPPA1-1*^{RNAi} expression of Mitf is reverted to control levels. We also tested Mitf levels by Western blot and confirmed the result, revealing that in genetic induced-gliomas, down-regulation of the *VhaPPA1-1* subunit prevents changes in Mitf expression **(Fig.35 A, B)**.

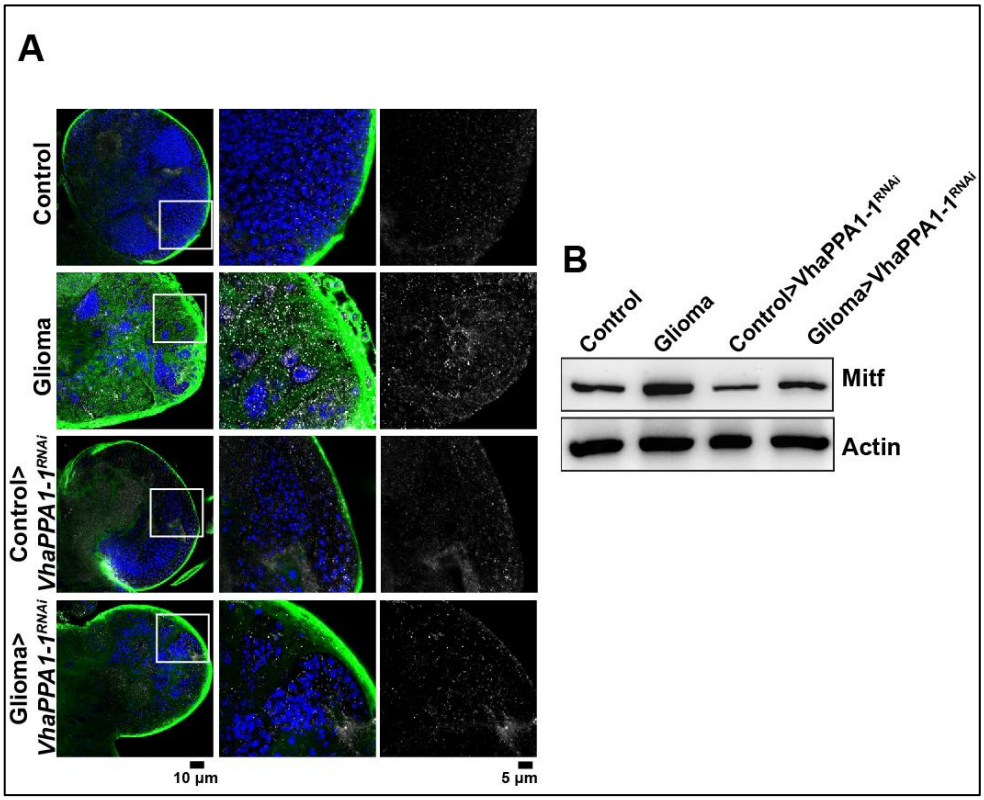

Figure 35. Mitf expression levels are decreased in gliomas>*VhaPPA1-1*^{RNAi}
A) Single medial confocal sections of 3rd instar larval brains. Nuclei were stained in blue (DAPI), glial cell membranes in green (anti-GFP), anti-Mitf in grey. The transcription factor is not accumulated in gliomas>*VhaPPA1-1*^{RNAi}. High magnifications of insets show Mitf subcellular localization, which is almost exclusively cytoplasmic in brains carrying the down-regulation of *VhaPPA1-1*. Scale bar 10 um and 5 um. **B)** Western blot of 3rd instar larval brain extracts. In gliomas,

the increased Mitf levels are restored to control following the down-regulation of *VhaPPA1-1*. Actin was used as a loading control.

6.13 *In vivo*, the V-ATPases inhibitor Bafilomycin A1 is not able to prevent glia overgrowth

To mimic genetic down-regulation of V-ATPase subunits, we fed larvae with the V-ATPase inhibitor Bafilomycin A1 until they reached the 3rd instar larval stage. Evaluation of the size of larval brains after drug treatment revealed that glioma brains are still three folds bigger than control brains **(Fig.36 A)**. To control whether uptake of the drug by larvae is occurring, we monitored lysosomal acidification in fat bodies using Lysotracker Red and found it reduced, as expected, in treated animals **(Fig.36 B)**. Thus, *in vivo* drug treatment with BafA1 does not appear to mimic genetic down-regulation of the V-ATPase subunit expression.

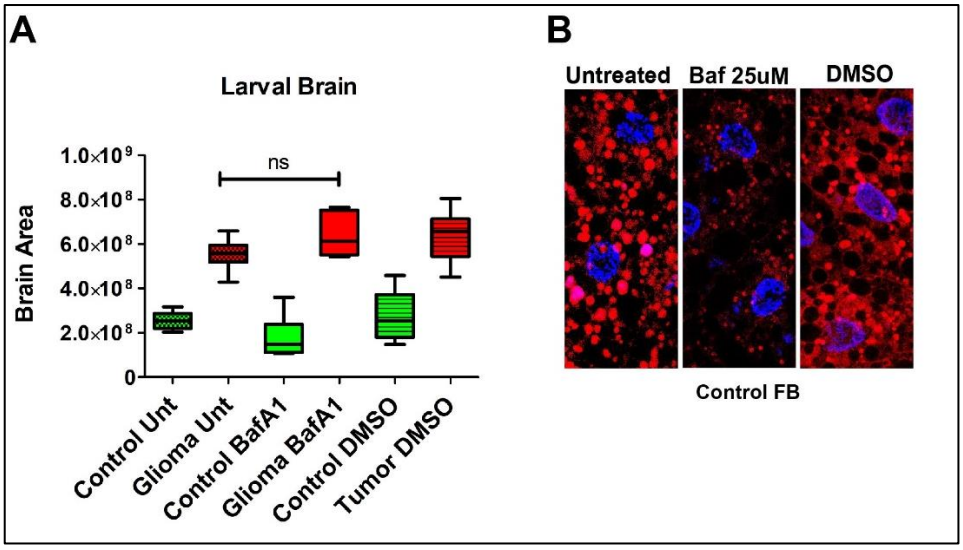

Figure 36. Bafilomycin A1 does not prevent glia overgrowth

A) 3rd instar larval brains were measured after feeding with normal food or 25uM BafA1 supplemented food. In untreated conditions, glioma brains were 3 folds bigger than control brains. In treated conditions, this difference was maintained. DMSO was used as a negative control. Mean ± SEM of n≥3 independent experiments, *P-values*

are determined by One-way ANOVA, Kruskal Wallis test. Quantification was performed by ImageJ. **B)** Larval Fat body of control samples was stained with Lysotracker Red to evaluate the correct uptake of the drug.

6.14 *VhaPPA1-1* influences glia overgrowth by modulating the PI3K-Akt pathway

To explore the molecular mechanism that prevents glia overgrowth in gliomas>*VhaPPA1-1*^{RNAi}, we investigated whether activation of the EGFR and the PI3K pathways was changed. We used p-ERK as a readout for the EGFR pathway and found no change upon down-regulation of *VhaPPA1-1* (not shown). We then detected p-Akt as a readout of the PI3K pathway. In this case, we found a strongly increased phosphorylation of Akt in glioma samples that is reduced in gliomas>*VhaPPA1-1*^{RNAi} , when compared to controls brains **(Fig.37 A)**. Total Akt (t-Akt) levels confirmed similar expression of Akt in all samples. Thus, down-regulation of *VhaPPA1-1* modulates PI3K pathway, leading to a reduction in phosphorylation of Akt.

To further investigate the functional interaction of the V-ATPase subunit with the PI3K pathway, we monitored expression of *Thor*, an inhibitor of translation that is downregulated by Akt activation. Consistent, with p-Akt findings, qPCR analysis demonstrated that *Thor* expression is up-regulated in gliomas>*VhaPPA1-1*^{RNAi} **(Fig.37 B)**, possibly explaining how cell growth could be reduced in depleted brains. Collectively, our data demonstrate that V-ATPase is able to influence glioma development by the modulation of the PI3K pathway.

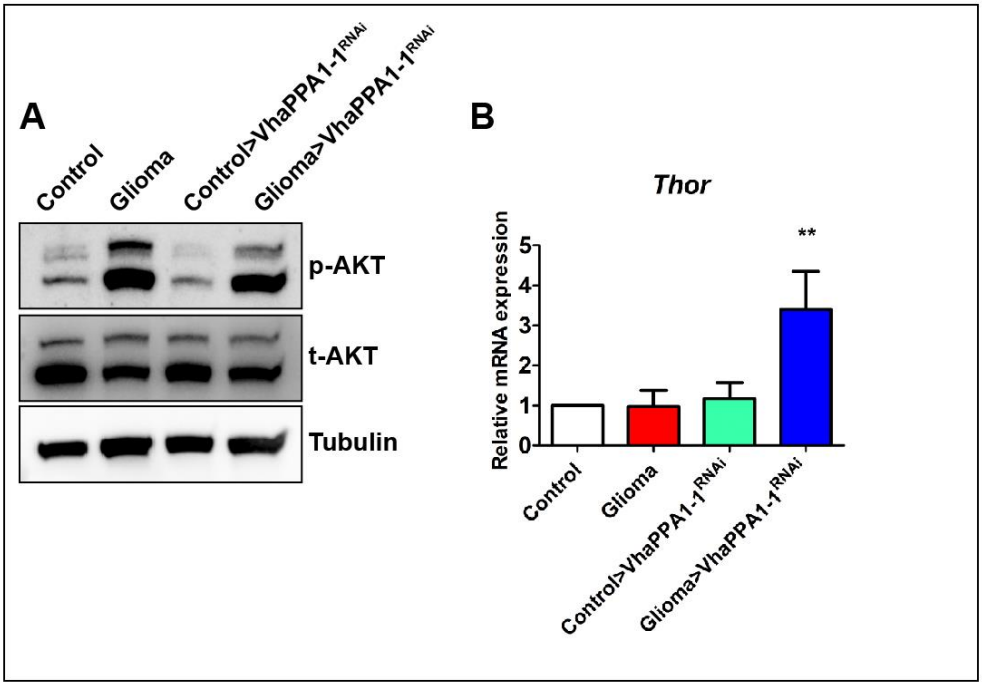

Figure 37. The down-regulation of *VhaPPA1-1* prevents glia overgrowth modulating Akt and *Thor*

A) Western blot of 3rd instar larval brain extracts. p-Akt levels shown decreased phosphorylation levels in gliomas>VhaPPA1-1^{RNAi}. t-Akt was used as a control. Tubulin was used as a loading control. **B)** The transcriptional levels of *Thor* were evaluated by qPCR in 3rd instar larval brains. mRNA levels of *Thor* are up-regulated in gliomas>*VhaPPA1-1^{RNAi}*. Expression levels were relative to control brains. *RpL32* was used as a housekeeping control. Each value represented mean ± S.D. of n≥3 independent experiments, *P-value* was obtained by One-way analysis of variance, Bonferroni's Multiple Comparison Test, P**≤0.01.

7 DISCUSSION

7.1 Drosophila is a powerful glioma model system

Our *Drosophila* glioma model allows *in vivo* assessment of effects of the genetic alterations present in most human glioblastoma. The fly model clearly shows that only simultaneous expression of both *hΔEGFR* and *Dp110-CAAX* leads to gliomagenesis, consistent with evidence from mouse models, in which tumoral expansion is also driven by combinatorial mutations (Brennan et al., 2013; Ding et al., 2003; Eric C Holland et al., 2000; Wei et al., 2006). The genetic control of our model avoided us the evaluation of other mutations commonly associated with gliomas. The *hΔEGFR* and *Dp110-CAAX* - mediated glia expansion reduces non-cell autonomously the neuronal tissue, and compromises larvae behavior and viability. Thus, thanks to its superior genetic accessibility, *Drosophila* in principle could allow easy identification of pathways of gliomagenesis (Jeibmann & Paulus, 2009; Read, 2011; Teresa Witte, Jeibmann, Klämbt, & Paulus, 2009). Finally, glial growth in the fly system can be followed by GFP expression, is highly reproducible and fully penetrant, allowing a rapid and inexpensive *in vivo* characterization of putative novel targets for future treatment of GBM.

7.2 Glioma growth impairs neuronal development

Our data uncover a largely compromised neuronal architecture and also a decrease in neuronal tissue in flies carrying gliomas. These data are in accordance with what is known on brain lineage differentiation. In *Drosophila,* glial and neural cells differentiate from the same precursors (Homem & Knoblich, 2012; Sousa-Nunes, Cheng, & Gould, 2010). Therefore, an increased rate of glial growth during lineage differentiation could explain a reduction in the neuronal population. This is supported by the strong up-regulation of *repo* and the reduction of *elav* expression. An extended

coordination between glia and neurons is pivotal for human brain development. In addition, the interaction between glial and neuronal cells in preserved in human gliomas. Indeed, patient-derived glioblastoma cells maintain the ability to undergo neuronal differentiation (Faria et al., 2006). The knowledge of a common origin for glia and neurons is today at the base of several studies focusing on reprogramming of glioblastoma cells towards a neuronal fate (Su et al., 2014; Yuan et al., 2018). The use of FACS sorting of disaggregated brains allowed us to assess quantitatively glial overgrowth and the non-cell autonomous effect on neurons. Interestingly, the latter was intensely investigated in the context of SLA and of other neurodegenerative diseases, in both human and mouse models (Di Giorgio, Carrasco, Siao, Maniatis, & Eggan, 2007; Lobsiger & Cleveland, 2007). Such an effect was also observed in a *Drosophila* model of neurofibromatosis, in which the overexpression of *Ras* in peripheral glia induces a non-cell autonomous overgrowth of the perineural glia (Lavery et al., 2007). Finally, few hours before dying, larvae show alteration of head movements (data not shown). Remarkably, among symptoms observed in terminal patients with GBM, there is the increase in intracranial pressure, cognitive dysfunctions and seizures (Sizoo et al., 2010). The absence of a skull in *Drosophila* does not allow to mimic problems due to tumor compression. However, the alteration of larvae motility, which requires the coordination of neural circuits (Clark, Zarin, Carreira-Rosario, & Doe, 2018), suggests that functional outcomes of brain carrying glioma could be further studied using flies. Overall, considering these observations, the *Drosophila* glioma model appears suitable for future investigation of the relationship between neural tissue and gliomagenesis. This could be achieved by mis-expression in neurons using a neuronal driver, such as *elav*.

7.3 Gliomas and the autophagy-lysosomal pathway

We observed that the overgrowth induced by *hΔEGFR* and *Dp110-CAAX* leads to the accumulation of ubiquitinated cargoes positive for ref(2)P, the fly homolog of the autophagic cargo adapter p62. In addition, we observed that *ref(2)P* is up-regulated at the transcriptional level exclusively in tumoral glia. However, our model shows an alteration of autophagy that is strictly cell autonomous. In addition, the absence of up-regulation of Atg genes and of Atg8 accumulation after starvation-induced autophagy reveal an inability to induce autophagy in glioma tissue. Such an impairment is remarkably distinct from what was observed in other tumor models. In fact, in *Drosophila*, the induction of autophagy promotes tumor growth in cancer stem cells of the ovary, leading to an increased occupancy of the ovarian stem cell niche (Zhao et al., 2018). Similarly, *Ras*-induced tumor growth in eye discs of *Drosophila* induces non-cell-autonomous autophagy in surrounding tissues, while the depletion of autophagy genes reduces tumor growth (Katheder, Khezri, O'Farrell, Schultz, Jain, Rahman, et al., 2017). Another model in which autophagy supports tumor growth was developed by Perez and colleagues. They found that in *Ras*-mutant eye discs, autophagy helps tumor growth, however, that tumor growth is reduced in eye discs when the tumor is induced by alterations in JNK or Notch pathways (Pérez, Das, Bergmann, & Baehrecke, 2015b). Additionally, studies in mice and mammals show that tumor growth is enhanced when the activation of autophagy is impaired (Cicchini et al., 2014; H. Liu et al., 2013; Takamura et al., 2011). Our and these other published data highlight the paramount importance of the type of genetic alteration to understand the role of autophagy in cancer.

Several works on human *in vitro* models demonstrate that autophagy is used as an adaptative stress response to energy deprivation and hypoxia in tumoral growth (Abdul Rahim et al., 2017; Von Schwarzenberg et al.,

2013). Moreover, inhibition of autophagy in glioblastoma tumor cells leads to apoptosis or other types of cell death due to chemotherapy (Buccarelli et al., 2018; Gammoh et al., 2016). In gliomas, treatment with the gold standard TMZ triggers autophagy induction (Kanzawa et al., 2004; Natsumeda et al., 2011). It was also demonstrated that the combination of TMZ with Bafilomycin A1 is able to increase cell death in malignant glioma cells (Kanzawa et al., 2004). Although our preliminary data on Bafilomycin A1 feeding of larvae did not influence tumor growth, our model can be useful for these types of study, such as evaluation of the effect of TMZ on *hΔEGFR* and *Dp110-CAAX*-induced glioma in tissues with or without *VhaPPA1-1* down-regulation, or in combination with other drug treatments. These experiments could shed some light on the efficiency of autophagy modulation in brain tumorigenesis.

There is also evidence that the compound sinomenine is able to induce autophagy and to decrease tumor growth in glioblastoma, down-regulating the PI3K-Akt-mTOR pathway (Jiang et al., 2017). Despite such diverse use of autophagy, our data suggest that persistent overactivation of the EGFR and PI3K pathways might strongly inhibit the autophagic response of glial cells. Consistent with this, we observed a partial expansion of the lysosomal compartment, that have been reported to occur also in human gliomas (Sarafian, Koev, Mehterov, Kazakova, & Dangalov, 2018). Such expansion could be triggered by the unmet need for cellular clearance induced by accumulation of cargoes (Bouché et al., 2016; Sardiello et al., 2009), or it could result from an increased need for nutrients to cope with tumoral growth, or finally, it could be a direct effect of elevation of PI3K-Akt-mTOR signaling. Importantly, we find that in glioma cells lysosomal proteases, such as cathepsins, maintain their degradative ability. Such findings confirm that autophagic clearance is blocked very upstream, at the level of pathway induction. In accordance, lysosomal enzymes have been

described to promote tumor invasiveness (Kubisch et al., 2014; Y. Liu, Zhou, & Zhu, 2012), a feature recaptured by the *Drosophila* glioma model (Read et al., 2009) that could be evaluated in future experiments.

The visualization of the lysosomal and autophagy regulator Mitf (TFEB in mammals) in fly gliomas reveled enhanced cytoplasmic accumulation, suggesting that Mitf is inactive in these tumors. Increased expression can also be explained as a compensatory mechanism, as observed previously by others (Giatromanolaki et al., 2015; Pi et al., 2017). However, the inactive status of Mitf in glioma tissue is in accordance with the absence of autophagy induction. Molecularly, the *hΔEGFR* and *Dp110-CAAX*-mediated activation of the PI3K/Akt/dTOR pathway is known to exert several effects on the Mitf homolog TFEB. The first effect involves mTORC1: Indeed, its activation prevents TFEB dephosphorylation and subsequent transcriptional regulation of genes related to the lysosomal-autophagic pathway. The second effect is that activation of the PI3K/Akt/dTOR pathway inhibits directly Atg1 activity, preventing autophagy induction. Finally, it was recently demonstrated that mammalian TFEB can be inhibited directly by Akt, in a mTORC1-independent manner (Palmieri et al., 2017). Which of these mechanisms occur also in *Drosophila* gliomas remains to be determined. However, the absence of upregulation of TFEB target genes overall confirms that the ectopic growth signaling exerts a strong inhibition on autophagy.

7.4 The role of V-ATPase in *Drosophila* gliomas

Acidification is necessary to enable a plethora of biological processes in health and disease, including cancer. Indeed, lysosomes are under intense investigation to understand their role in tumor progression (Piao & Amaravadi, 2016). In this study, we have reported for the first time that in *Drosophila* CNS, V-ATPase is differentially expressed among glia and

neurons. This echoes findings in mammalian cells and is likely to be related to the glia ability to phagocyte damaged neuronal cells (Doherty et al., 2009). Moreover, the V-ATPase subunit *VhaPPA1-1* is increased in glioma samples, compared to control, similar to what we and others have recently reported for glioblastoma (Di Cristofori et al., 2015). Additionally, the *VhaPPA1-1* (V0B in mammals) is strongly up-regulated in patients with glioblastoma (Terrasi et al., 2018, in press EBIOM). In contrast to what has been observed by others in flies (Bouché et al., 2016; Tognon et al., 2016), down-regulation of *Mitf* does not influence V-ATPase expression in our model (data not shown), probably because Mitf is mostly inactive. However, *Mitf* down-regulation partially prevents overgrowth of gliomas, indicating that residual TFEB activity might be limiting for tumor development. Remarkably, down-regulation of the *VhaPPA1-1* subunit exerts a much stronger negative effect on tumoral growth, indicating that V-ATPase is a key positive regulator of tumorigenesis. To understand the mechanism regulating tumor growth, we first evaluated the induction apoptosis. It is known that inhibition of V-ATPase activity leads to cell death (Ohta et al., 1998). There are also several studies in which apoptosis is induced in tumoral cells treated with the V-ATPase inhibitors (Morimura, Fujita, Akita, Nagashima, & Satomi, 2008; Von Schwarzenberg et al., 2013). In glioblastoma, the down-regulation of ATP6V1G1 prevents tumoral growth through the induction of caspase-mediated apoptosis (Di Cristofori et al., 2015). We observed that the down-regulation of *VhaPPA1-1* induces apoptosis only in control brain but not in glioma brains. This indicates that *in vivo* reduced cell growth might not involve cell death. This suggests that probably in the *Drosophila* model the activation of pro-oncogenic pathways that inhibit the induction of apoptosis is still present. Larvae treated with Bafilomycin A1 do not recapitulate the phenotype observed upon *VhaPPA1-1* down-regulation. Probably because the drug was administrated by feeding, and to reach the target tissue it had

to cross the intestine, to diffuse in the circulatory system and, moreover, to overcome the BBB. These problems could be avoided by *ex vivo* culturing *Drosophila* larval brains. In this scenario, the V-ATPase inhibitor could reach directly the target tissue, increasing the effectivity of treatment.

V-ATPase is also related to the activity of immune cells in tumor microenvironment. Therefore, a decreased activity of the proton pump prevents the transition between the M1/M2 phenotype of macrophages, delaying tumor growth in mice models of breast cancer (Katara et al., 2016). Tumor-associated macrophages in glioblastoma are currently strongly investigated for their involvement in tumorigenesis and tumor progression. Indeed, promising therapies aim to elicit the immune response of resident cells against glioblastoma (Z. Chen et al., 2017; Z. Chen & Hambardzumyan, 2018). Compared to mouse models, which are mostly immune-deficient, in fly model cellular immune responses are preserved to some extent (Cordero et al., 2010), allowing future study of the tumor microenvironment. Consistent with macrophage alterations, small black spots, which are caused by melanization, a well-known fly immune response (Tang, 2009), are often observable around tumoral brains (data not shown).

V-ATPase activity has been previously associated with tumor invasiveness. Several studies demonstrate that the inhibition of the proton pump reduces metastatic spreading by preventing activity of caspases and also by decreasing the localization of EGFR and p-Akt at leading age of cancer cells (Kubisch et al., 2014; Wiedmann et al., 2012). The proton pump has been also associated with internalization of EGFR receptors (Yoshimori, Yamamoto, Moriyama, Futai, & Tashiro, 1991). Thus, it will be very interesting to observe in the future if *VhaPPA1-1* down-regulation modifies EGFR localization in glial cells.

Previous evidence suggests that the release of extracellular vesicles (EV) is regulated by V-ATPase, and this has been proposed also in

Drosophila (Beer & Wehman, 2017). EVs are involved in CNS development and function (Corrigan et al., 2014)(Korkut et al., 2009). *Ex vivo* culturing of wing discs treated with Bafilomycin A1 showed a decrease in the release of Wnt in EVs (Gross, Chaudhary, Bartscherer, & Boutros, 2012). The role of these structure in cancer has emerged only recently (Desrochers, Antonyak, & Cerione, 2016). In *Drosophila*, using transgenesis, these vesicles can be labeled with CD63::GFP and can be easily tracked *in vivo*. Moreover, they can be isolated from hemolymph for co-culturing assays. Consistent with this, the study of EVs in our *Drosophila* model will be useful to determine if glial tumors, as well as tumors with V-ATPase down-regulation, release functional EV.

How could V-ATPase support tumor growth? To understand it, we first monitored the autophagy-lysosomal system. Its activity appears restored upon *VhaPPA1-1* down-regulation. Indeed, gliomas>*VhaPPA1-1*^{RNAi} do not accumulate ref(2)P and also its transcriptional levels are not up-regulated. The number of lysosomes is similar to control levels, along with *Mitf* levels. This evidence suggests that the activity of V-ATPase on tumor growth is tightly related to autophagic-lysosomal function, rather than other functions of V-ATPase. We predict that this effect is molecularly connected to the modulation of the PI3K/Akt/dTOR growth pathway. This can be due to the existence of a feedback-loop connecting Akt and V-ATPase. In fact, decreased phosphorylation of Akt is expected considering the established functions of the V-ATPase-TFEB-mTORC1 axis on the lysosomal surface (Peña-Llopis et al., 2011; Settembre et al., 2012; Zhang et al., 2015). The possibility is strongly supported by the restoration of *Mitf* levels and autophagic clearance in our model. While it remains to be tested, we hypothesize that impairment in V-ATPase activity causes inactivation and release of dTor from the lysosomal surface, leading to negative regulation of the PI3K-Akt-dTor growth pathway, that would restrain tumor growth.

The inactivation of Akt is known to induce nuclear translocation of FoxO, leading to the transcription of the target gene *Thor* (Hwangbo et al., 2004). Activation of FoxO is often associated with reduced cell growth and proliferation, especially in the *Drosophila* brain (Hwangbo et al., 2004). Our data support a role of FoxO in glioma growth because we observe a decreased size of glial cells in gliomas>*VhaPPA1-1*^{RNAi}, compared to controls. Moreover, FoxO overexpression decreases phosphorylation of Akt, ultimately reducing overgrowth of perineural glia mediated by PI3K-Akt (Lavery et al., 2007). The association between FoxO, *Thor* and the PI3K-Akt pathway was also observed by others (Junger et al., 2003; Miron et al., 2001; Webb & Brunet, 2014). Accordingly, we observe that *VhaPPA1-1* down-regulation not only decreases Akt activity, but it also results in up-regulation of *Thor*. Despite this, we did not test other important players of autophagy regulation, such as AMPK, which was recently shown to be recruited on the lysosomal surface for the regulation of cellular homeostasis (McGuire & Forgac, 2018). Thus, it would be worth to study AMPK localization and activity in the fly glioma model in the future.

8 CONCLUSION

In summary, we have developed a schematic model of glioma growth **(Fig. 38)** that points the attention on V-ATPase ability to modulate autophagy. We propose that down-regulation of a V-ATPase activity could decrease Akt function. This, in turn could reduce ectopic activation of the PI3K-Akt-dTor pathway allowing autophagy induction. Moreover, decreased activity of Akt would allow FoxO-mediated expression of *Thor*, which is known to prevent anabolic processes such as translational. We expect that both mechanisms could prevent excess cell growth in cancer glial cells. Consistent with the very well established ability of inhibitors of the PI3K pathway to reduce glioma growth (Q. W. Fan et al., 2006; Fujiwara K. and Yasuko Kondo, 2007;

X. Li et al., 2016; Sami & Karsy, 2013; Teresa Witte et al., 2009) in both *Drosophila* and humans, our work uncover the molecular rationale by which V-ATPase could represent a promising candidate gene to target for future therapies aimed at extending survival of GBM patients.

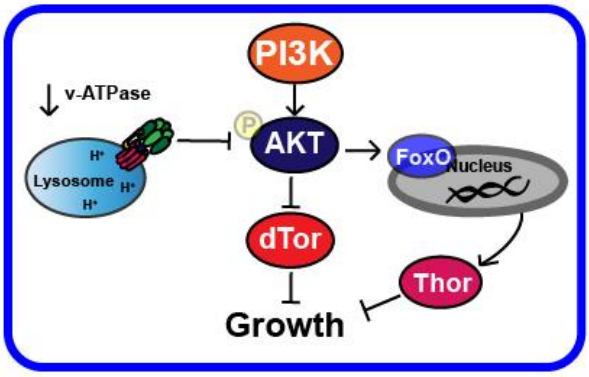

Figure 38.Prosed model for the prevention of glial growth mediated by the down-regulation of the V-ATPase subunit *VhaPPA1-1*. The Akt phosphorylation in glioma context is decreased, while *Thor* is up-regulated. This mechanism prevents overgrowth determining a decreased activity in the PI3K/Akt/dTor pathway and increasing the inhibition of translational processes.

During my PhD course I contributed to the following science communication activities:

- I was selected for a talk at PhD meeting-ABCD Congress 2017, Bologna, Italy;
- I presented a poster at ABCD Congress 2017, Bologna, Italy;
- I was selected for a talk at *Drosophila* Neurobiology Congress 2017, Cold Spring Harbor, USA;
- I presented a poster at the "Ubiquitin-assisted autophagy- from mechanisms to pathology" course 2017, at IFOM, Milan, Italy;
- I presented a poster at International Summer School on "Cancer biology and therapeutic strategies towards personalized medicine" 2018, Lipari, Italy;
- I was selected for a talk at IDRC 2018, Padova, Italy;

During my PhD my work contributed to the following publications:

- Andrea Terrasi, Irene Bertolini, Cristina Martelli, Gabriella Gaudioso, Andrea Di Cristofori, Alessandra Maria Storaci, **Miriam Formica**, Silvano Bosari, Manuela Caroli, Luisa Ottobrini, Thomas Vaccari, Valentina Vaira, 2018. Specific V-ATPase expression sub-classifies IDHwt lower- grade gliomas and impacts glioma growth *in vivo.* In press on EBioMedicine.

- Irene Bertolini, Andrea Terrasi, Cristina Martelli, Gabriella Gaudioso, Andrea Di Cristofori, Alessandra Maria Storaci, **Miriam Formica**, Paola Braidotti, Katia Todoerti, Stefano Ferrero, Manuela Caroli, Luisa Ottobrini, Thomas Vaccari, Valentina Vaira, 2018. A GBM-like V-ATPase signature directs cell-cell tumor signaling and reprogramming via large oncosomes. Under revision on EBioMedicine.

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