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Temozolomide induces autophagy in primary and established glioblastoma cells in an EGFR independent manner

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Abbreviations

ALDH1	Aldehyde dehydrogenase isoform 1
Atg	Autophagy-related gene
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
DAPI	4‘,6-Diamidin-2-phenylindol
DMSO	Dimethyl sulfoxide
(D)PBS	(Dulbecco’s) phosphate-buffered saline
ECL	Enhanced chemiluminiscence
EGFR	Epidermal growth factor receptor
FCS	Fetal calf serum
GBM	Glioblastoma multiforme
HBEGF	Heparin binding EGF like growth factor
IDH	Isocytrate Dehydrogenate
IF	Immunofluorescence
IP	Immunoprecipitation
LC3	Microtubule associated protein light chain 3
MTIC	Methyltriazen imidazol carboxamide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MGMT	O6-methylguanine-DNA methyltransferase
NSCLC	Non-small cell lung carcinoma
o.n.	over night
PFA	Paraformaldehyde
pGBM	primary Glioblastoma multiforme
PVDF	Polyvinlyidene difluoride
Rcf	Relative centifugal force

RT	room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TKI	Tyrosine kinase inhibitor
TMZ	Temozolomide
TNFα	Transforming growth factor alpha
TP53	Tumor Protein 53
VEGF	Vascular endothelial growth factor
WB	Western-Blot
WHO	World Health Organization
Wt	Wild-type

Genes mentioned in this thesis are named after the official Human Protein Atlas, http://proteinatlas.org.

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1 Introduction

Glioblastoma multiforme (GBM) is the most common brain tumor in adulthood. Uniformly fatal, it arises from astrocytic cells and provides the ability to proliferate extensively. Maximum treatment leads to a median survival of primary GBM of 14 to 15 months. (Lee et al., 2017) Its infiltrative character combined with molecular heterogeneity hallmark the tumor's aggressiveness. (Huang et al., 2015) Despite substantial efforts in order to identify novel therapeutic strategies, tumors invariably recur after surgery. (Ellis et al., 2015)

GBM is comprehensively delineated in its genomic characteristics. However, the transfer to effective treatment options has failed to appear until now. (Furnari et al., 2015) The high recurrence rate despite multimodal therapy might partially be explained by a subpopulation of resistant cells. (Lan et al., 2017) This makes research in the field of cellular resistance mechanisms particularly relevant.

In 2016, Yoshinori Ohsumi was honored with the Nobel prize for his groundbreaking discoveries about autophagy since the 1990's. (The Nobel Assembly of Karolinska Institutet, 2016) Autophagy was known long before as a non-selective bulk degradation process. Yoshinori Ohsumi drew attention to its complex task in maintaining cellular integrity. In the following years, it was discovered that autophagy is a tightly regulated cytoplasmic recycling mechanism.

The role of autophagy in the dismal outcome of GBM has not been clarified yet. (Yan et al., 2016) However, the energy supply due to its recycling function might help to resist cancer therapy. Our lack of knowledge about the complex molecular background of autophagy in the context of GBM has encouraged this study.

1.1 Glioblastoma multiforme

1.1.1 Histopathology

Histologic morphology of GBM is unequivocal. Growth factors like vascular endothelial growth factor (VEGF) stimulate endothelial cells to form blood vessels resulting in highly vascularized tumor fields. (Giusti et al., 2016) Thrombotic events accumulate due to the upregulation of cellular initiators of thrombosis. (Rong et al., 2006) Even the highly increased angiogenesis cannot provide sufficient nutrients for the fast-growing cancer, leaving a necrosis zone in its core. Cells neatly line up around necrotic areas. This phenomenon is termed 'pseudopalisades'. (Wippold et. al., 2006) Histologically, GBM shows a polymorphic pattern as illustrated in Figure 1.

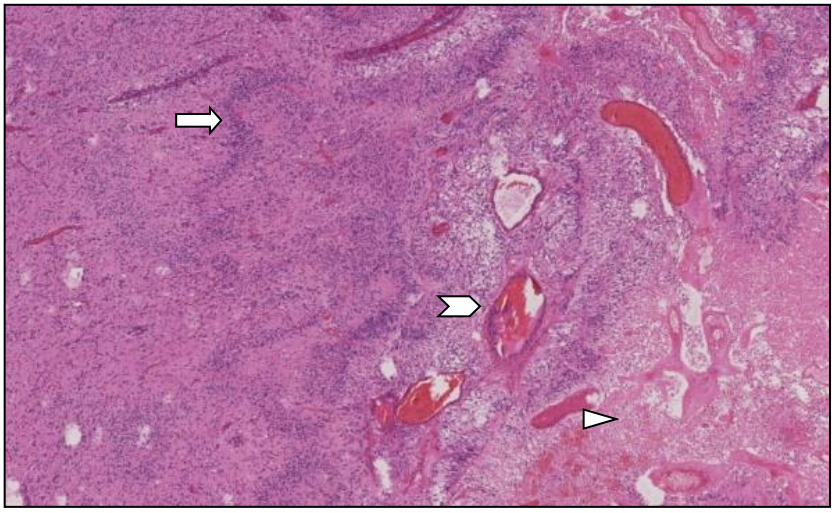


Figure 1: Hematoxylin-eosin staining of GBM.
⇨ Cell palisading on the right side,
⋙ epithelial proliferation and intravascular thrombosis, adjacent to ▻ widespread necrotic foci. Modified after Meditum viewer, Technische Universität München, with permission from Prof. Dr. med. J. Schlegel.

Different subclasses can be distinguished. Primary GBM arise de novo whereas secondary GBM develop from low-grade or anaplastic astrocytoma. It is now assumed

that primary and secondary GBM develop from different neural progenitor cells. GBM are in most cases (>90%) of primary origin. Secondary GBM progress mainly in younger patients and are associated with a better prognosis. (Louis et al., 2016)

Histology cannot clearly distinguish those two entities but they frequently express different genetic alterations. For instance, Nobusawa et al. described in 2009 that Isocytrate Dehydrogenate (IDH) mutations were found in approximately 70% of secondary GBM but very rarely in primary glioblastoma. This makes IDH a very important genetic marker for secondary GBM. (Nobusawa et al., 2009) The data of Etxaniz et al. suggest using the absence of IDH mutations as a risk factor for unfavorable outcome. (Etxaniz et al., 2017) Testing for this marker can be performed by immunohistochemistry with an antibody targeting the most common IDH mutation (p.R132H on IDH1) or by gene sequencing. (Schlegel et al., 2015) Yamashita et al. suggest non-invasive methods for predicting IDH mutations by MRI analyzes of blood flow and necrotic areas. (Yamashita et al., 2016)

In 2016, the World Health Organization (WHO) incorporated molecular patterns including IDH mutations in the classification of tumors of the central nervous system for the first time. (Louis et al., 2016) Figure 2 summarizes important astrocytic and oligodendroglial tumors.

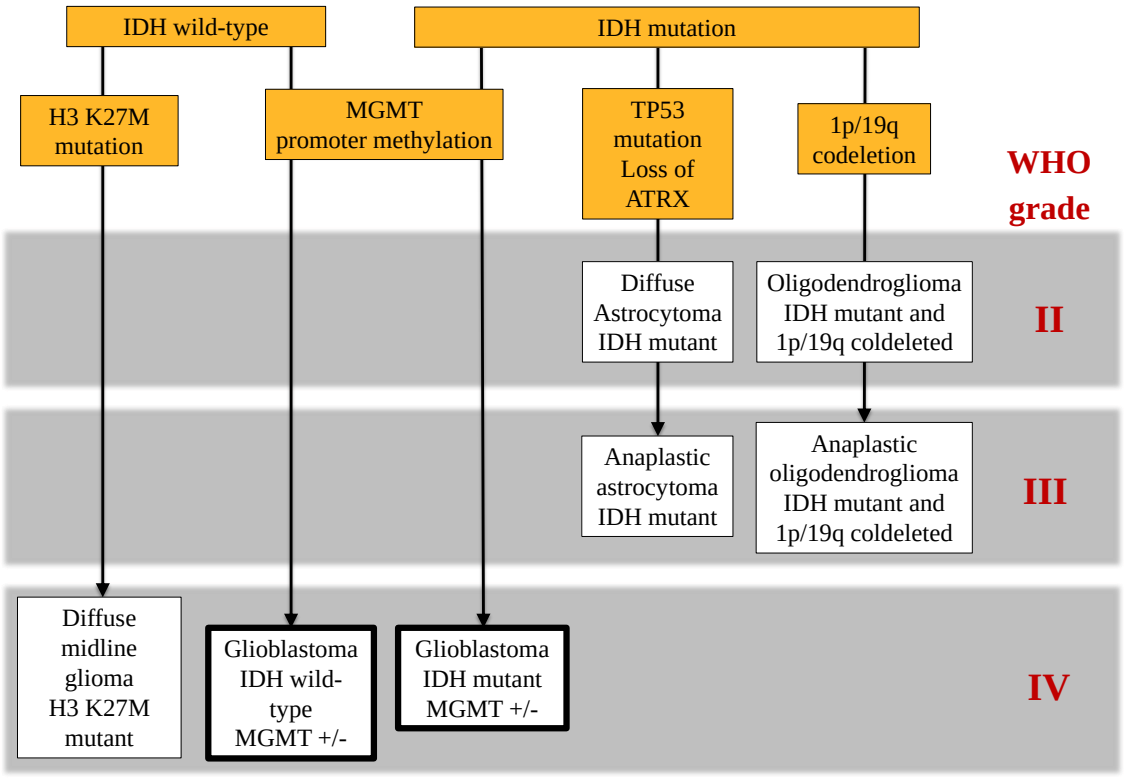


Figure 2: Diffuse astrocytic and oligodendroglial tumors. Genetic alterations are not definitively connected to the stated tumor entity but provide an indication. A selection of different typical genetic alterations is shown in orange. WHO-grades are given in red. Glioblastoma as the main subject of this thesis are bold-framed.
Graph self-derived, based on Schlegel, J., Herms, J., and Schüller, U., WHO-Klassifikation der Tumoren des Nervensystems, in *Manual Hirntumoren und spinale Tumoren*, 2016; and Louis et al., The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary, in *Acta Neuropathologica*, 2016.

- Brain tumors located near the midline with a mutation of the histone H3 gene are classified as diffuse midline glioma H3 K27M mutant. This malignant cancer predominantly occurs in adolescents. (Schlegel et al., 2016)
- In Glioblastoma, the most important predictive molecular biomarker is O6-methylguanine-DNA methyltransferase (MGMT). This protein averts DNA damages by removing methylations. (Hegi et al., 2005) Implications for therapy and outcome are detailed in the next section.

- Mutation of the gene encoding for Tumor Protein 53 (TP53, a regulator of cell cycle) and loss of ATRX combined with IDH1 mutations predestine for astrocytoma development. (Schlegel et al., 2016)
- Together with IDH1 mutation, 1p/19q loss is a characteristic finding in oligodendroglioma. Astrocytoma and oligodendroglioma are designated as WHO II/III grade according to their histological features. (Louis et al., 2016)

1.1.2 Diagnosis and therapeutic approach

GBM may lead to different symptoms depending on the cancer location. These can range from headaches to optical abnormalities. GBM is diagnosed by MRI or CT showing a typical annular contrast enhancement around the necrotic tumor mass. The diagnosis may be verified by stereotactic biopsy. (Chandana et al., 2008)

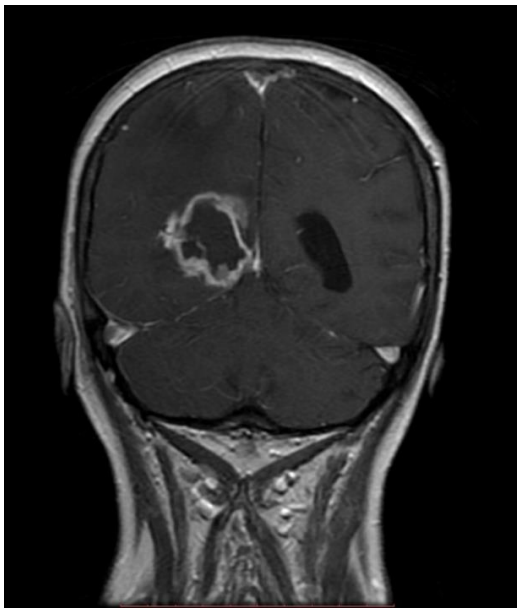


Figure 3: MRI image showing GBM in the right cerebral hemisphere. T1 post contrast. Inhomogeneous annular contrast enhancement around the necrotic core. Kindly provided by Radiologisches Zentrum München-Pasing, August 2015.

Therapy options are adjusted individually. However, standard therapy includes brain surgery and adjuvant radiotherapy combined with the chemotherapeutic agent Temozolomide (TMZ). (De Moraes et al., 2017)

In 1984, Stevens et al. identified TMZ as an oral anti-cancer chemical. It is administered for patients with GBM or brain metastases of melanoma. Its lipophilic character permits to cross the blood-brain barrier. TMZ is hydrolyzed into its active metabolite MTIC (methyltriazen-imidazol-carboxamide) when it gets in contact with tissues. A part of MTIC is the methyldiazonium ion, which, in the end, is the active component of TMZ therapy. This ion methylates guanine-residues in the DNA generating O6- or N7-methylguanine. Especially O6-methylguanin is toxic because it leads to double strand breaks when targeted by mismatch enzymes. Overall, TMZ inhibits correct DNA duplicating and leads to apoptosis. Especially highly proliferative cells like cancer cells are affected. (Sanjiv et al., 2000)

1.1.3 Mechanisms of chemoresistance

A subset of glioblastoma exhibits the protein MGMT, which abrogates the effects of TMZ by removing DNA methylations. If the corresponding promoter gene is methylated, MGMT is not expressed. This promoter methylation occurs in about 50% of glioblastoma and goes in line with a favorable prognosis regarding TMZ therapy and overall survival. (Hegi et al., 2005; Wojciech et al., 2017) However, some tumors expressing low levels of MGMT protein still exhibit chemoresistance, implying that additional mechanisms are involved in TMZ resistance and tumor recurrence. (Wick et al., 2014) MGMT protein expression is marked with $MGMT^{+}$ for the remainder of this study.

Aldehyde dehydrogenase (ALDH) 1 is a catalysator of the oxidation of intracellular acetaldehyde to acetate and furthermore a marker for stem cells. (Rasper et al., 2010, Nakano, 2015) Nakano suggests that the subtype ALDH1A3 indicates stem cell characteristics in mesenchymal glioma stem cells. (Nakano, 2015) Schäfer et al. showed by analysis of primary and established glioblastoma cell lines and retrospective immunohistochemistry that ALDH1A1 overexpression is linked to chemoresistance and poor prognosis. (Schäfer et al., 2012)

1.2 Autophagy

Mammalian cells feature different possibilities to prevent accumulation of superfluous cellular components. A well-known mechanism is the proteasome system for degradation of proteins using ubiquitin as a specific marker. (Myung et al., 2001) Another mechanism was found in 1967 by the Nobel prize winner Christian de Duve called autophagy (from Greek self-eating). (Feng et al., 2014) Autophagy is an intracellular mechanism to recycle proteins and organelles like mitochondria. It is highly conserved and thus, it can be found in most eukaryotic cells. (Yorimitsu and Klionsky, 2005) Three major types of autophagy are identified: macroautphagy, microautophagy and chaperone-mediated autophagy. (Yoshii and Mizushima, 2017) If not stated otherwise, the term autophagy refers to macroautophagy in the course of this thesis.

During the last decades, autophagy was thought to be a non-selective bulk degradation process. In contrast, the scientific community detected a highly selective character of autophagy in the last years. (Feng et al., 2014) Connected with a broad field of molecular pathways, autophagy is crucial from embryonic development to anti-aging. Particularly new findings are mentioned not only for Glioblastoma but also for neurodegenerative diseases and development of diabetes. (Quan et al., 2012; Ghavami et al., 2014; Kim et al., 2017; Guo et al., 2017) It is not yet clarified if autophagy operates as pro-survival or pro-death mechanism in adverse cellular conditions. (Jin et al., 2017) Notably, in cancer origin and progression this controversy is most important to study regarding therapeutic possibilities.

The autophagic process is shown in Figure 4.

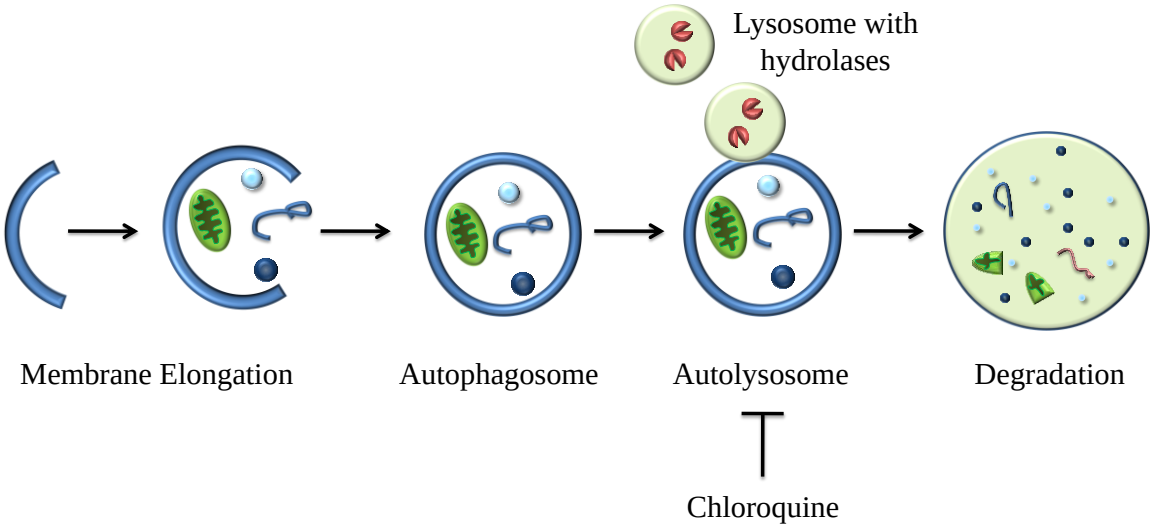


Figure 4: Schematic model of macroautophagy in mammalian cells. Graph self-derived, based on Mizushima et al., Methods in Mammalian Autophagy Research, in *Cell*, 2010; and Jin et al., SnapShot: Selective Autophagy, in *Cell*, 2013.

Macroautophagy compromises several sequestration steps beginning with a membrane also called the phagophore. Following elongation of the phagophore the double-membraned autophagosome is built. Fusion with the lysosome allows acidic hydrolases to degrade the inner components of the 'autolysosome'. (Mizushima et al., 2010) Chaperone-mediated autophagy requires Hsp70 chaperones that recognize specifically marked proteins. In bulk microautophagy, proteins nearby to the lysosomal membrane are incorporated directly. After degradation, particles are emitted to the cytoplasm and can be reused. (Mehrpour et al., 2012)

In 1997, the first autophagy-related gene (Atg) was discovered. (Yang and Klionsky, 2010) The homologue of Atg8 in mammals is called LC3 ('microtubule associated protein light chain 3'). This protein binds to the autophagic membrane and can be detected by immunoblot. In detail, pro-LC3 is split by the Atg4 protease to form LC3-I prior to binding to phosphatidylethanolamine. This lipidated form of LC3 is called LC3-II and is located at the autophagosome cytosolic and intralumenal membrane. After fusion with the lysosome, it can be degraded. The conversion from LC3-I (approximately 16-18kDa) to LC3-II (approximately 14-16kDa) can be monitored by immunoblotting. LC3 and especially LC3B is one of the most reliable proteins to

inspect the autophagic flux. (Mizushima et al., 2010) Due to the mostly faint appearance of LC3B-I in Western blotting, it is recommended to use the lipidated form, LC3B-II, for comparison. (Yoshii and Mizushima, 2017)

Beclin-1 is a pivotal protein positively controlling autophagy. It was first detected as a binding partner to the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2). Aside, Beclin-1 binds to an autophagy initiating complex called core complex containing the phosphatidylinositol 3-kinase VPS34. This core complex is essential to launch the autophagic pathway. (Sinha and Levine, 2009)

For the purpose of intervening in the autophagic process, the agent Chloroquine may be applied. Chloroquine is a medical drug used for the treatment of malaria and rheumatism. Besides, *in vitro* it inhibits the last step of autophagy, which is the fusion of the autophagosome with the lysosome (see Figure 4). Thus, LC3-II cannot be hydrolyzed and subsequently accumulates. (Yoon et al., 2010)

Based on a lot more interacting proteins and pathways, autophagy is an exceedingly complex mechanism. (Galluzzi et al., 2017)

1.3 Epidermal Growth Factor Receptor

1.3.1 Molecular alterations

EGFR (Epidermal Growth Factor Receptor) is a member of the ErbB family, which includes important tyrosine kinase receptors. It is a trans-membrane receptor known to promote cellular growth and proliferation. (Wee and Wang, 2017) Several ligands bind to EGFR, for instance EGF, transforming growth factor alpha (TNFα), and heparin binding EGF like growth factor (HBEGF). (Cuneo et al., 2015) Stimulation leads to homo- or hetero-dimerization with other ErbB family members. Subsequently, the intracellular tyrosine kinase domain is autophosphorylated, inducing activation of downstream pathways. (Holcman and Sibilia, 2015) Most important cascades include the PI3/Akt, ras/raf/MAPK and JAK/STAT pathway. These pathways are not merely linear but interrelated. (Wee and Wang, 2017)

EGFR deregulation is detected in many tumor entities. Some of them can be treated with anti-EGFR therapy like tyrosine kinase inhibitors (TKI). In GBM, efficiency of anti-EGFR therapy remains poor. (Azuaje et al., 2015)

Several alterations of the EGF receptor exist. In GBM, EGFR is amplified in nearly half of the cases which, however, is difficult to maintain in cell culture. (Furnari et al., 2015; Liffers et al., 2015) The most common mutation of EGFR is an aberrant form, called $EGFR^{vIII}$ or ΔEGFR(2-7). The outer part of this receptor is missing due to an in-frame deletion of exon 2-7. External stimuli cannot bind any longer to the receptor and it is continuously activated. (Padfield et al., 2015) $EGFR^{vIII}$ occurs in 20-30% of GBM and in 50-60% of tumors with EGFR amplification. (Gan et al., 2009) Most studies describe a negative prognostic outcome for $EGFR^{vIII}$. (Jutten and Rouschop, 2014)

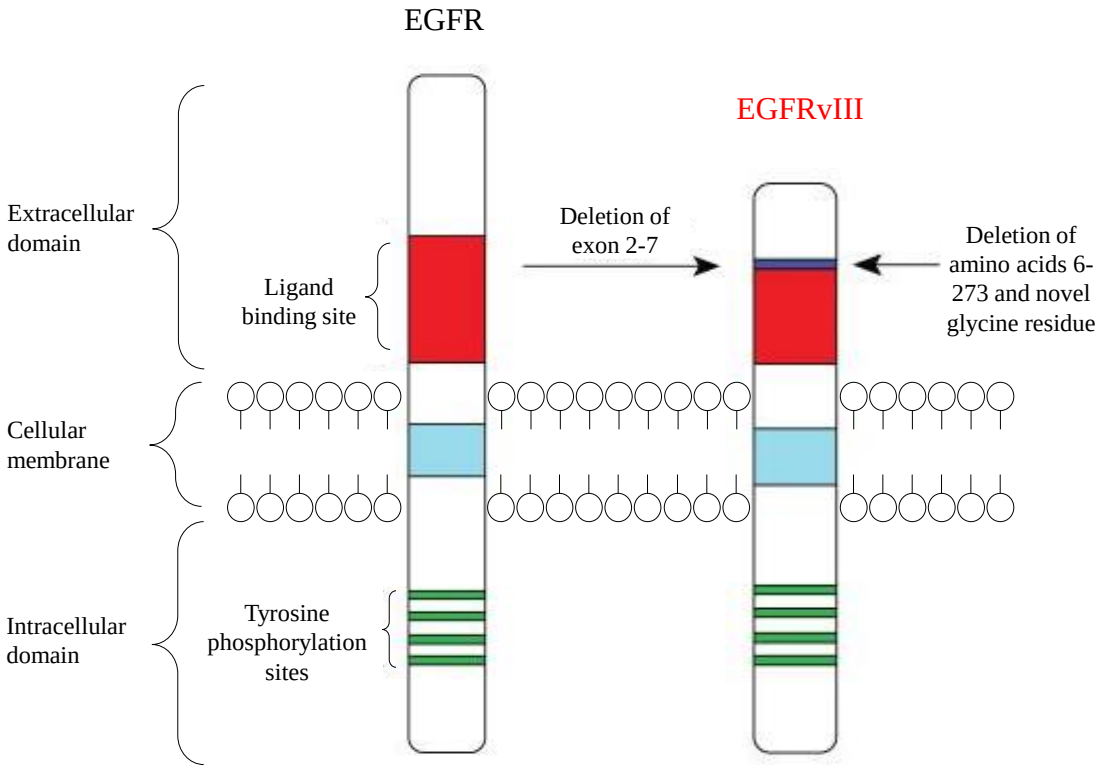


Figure 5: Wild-type EGFR and $EGFR^{vIII}$. Deletion of exon 2-7 leads to the loss of amino acids 6 to 273 and a novel glycine residue in the former ligand binding site. Adapted after Babu and Adamson, Rindopepimut: an evidence-based review of its therapeutic potential in the treatment of EGFRvIII-positive glioblastoma, in *Core Evidence*, 2012.

1.3.2 Interaction with autophagy

In 2013, Wei et al. published an important connection of EGFR to autophagy in Non-small cell lung cancer (NSCLC) cells. Active EGFR was detected to bind Beclin-1, inhibiting the initiation of autophagy by the Beclin-1-VPS34 complex. This led to decreased autophagy levels. (Wei et al., 2013) Cui et al. described several lines of evidence in different tumor entities indicating that co-targeting autophagy and EGFR might be a potent approach in cancer treatment. (Cui et al., 2014) Recently, this was confirmed for metastatic colorectal cancer. (Koustas et al., 2017)

2 Aims of this study

Glioblastoma is the most common malignant neoplasm of the brain. Despite substantial efforts prognosis remains poor. A broader understanding of the underlying chemoresistance mechanisms is essential to provide solid promises for clinically relevant success in the near future.

The main chemotherapy option with TMZ leads to cancer cell apoptosis by DNA methylation. (Lee, 2017) If cells cannot renew their genetic material this might also lead to excessive internal cell-waste. Cells might try to fight this deregulation with mechanisms to get rid of the cell-waste. This might be connected to autophagy, which is an effective recycling machinery. (White, 2015) Autophagy might be a potential approach to overcome the tumor's strategies of chemoresistance. Therefore, it is very important to assess a connection between autophagy and TMZ treatment. The first aim of this study is to investigate the regulation of autophagy by TMZ in primary and established GBM cells.

Interacting and regulating pathways of autophagy have to be explored to a greater extent prior to evaluating autophagy as a treatment possibility. EGFR is supposed to be an important factor in GBM development and maintenance. (Furnari et al., 2015) Wei et al. discovered a connection of the autophagic protein Beclin-1 to active EGFR in NSCLC cells. (Wei et al., 2013) Thus, the second aim of this study is to examine the interaction of EGFR with Beclin-1 for GBM cells.

3 Materials and methods

3.1 Materials

All consumables were used in accordance to their specific protocols. High-quality sterile plastic ware was obtained from Sigma-Aldrich, Munich, Germany.

Cell proliferation was analyzed with Roche Life Science's Cell Proliferation Kit I (Roche, Penzberg, Germany). The Pierce Classic IP Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for Co-immunoprecipitation. Protein quantification was measured by Bradford Protein Assay (Bio-Rad, Hercules, CA, USA).

3.1.1 Antibodies

All antibodies were stored and applied as recommended. HRP-linked anti-mouse and anti-rabbit antibodies from Cell Signaling Technologies were used as secondary antibodies in SDS-Page procedure (sodium dodecyl sulfate polyacrylamide gel electrophoresis). All other antibodies were applied only as primary antibodies for immunoblotting except indicated as 'IF' or 'IP'.

Antibody	Dilution in WB	Company	Order Number
ALDH1 (IF)	1:500	BD Bioscience, San Diego, CA, USA	611195
ALDH1A3 N-terminal	1:500	Sigma-Aldrich, Munich, Germany	SAB1300932
Anti-mouse IgG HRP-linked Antibody	1:10 000	Cell Signaling Technologies, Cambridge, UK	7076S
Anti-rabbit IgG HRP-linked Antibody	1:10 000	Cell Signaling Technologies, Cambridge, UK	7074P2
Beclin-1	1:1 000	Cell Signaling Technologies (Autophagy Antibody Kit), Cambridge, UK	4445S
Beclin-1 (H-300) (IF)	1:200 (only IF)	Santa Cruz Biotechnology, Dallas, TX, USA	SC-11427
Beclin-1 (IP)	1:100	Cell Signaling Technologies, Cambridge, UK	3495
EGFR (1005) (IF)	1:200 (only IF)	Santa Cruz, Dallas, TX, USA	SC-03
EGFR (Ab12) Cocktail R19/48 (IF)	1:500	Thermo Scientific, Waltham, MA, USA	MS400P1
EGFR (IP)	1:1 000	Cell Signaling Technologies, Cambridge, UK	2232S
GAPDH	1:10 000	Sigma-Aldrich, Munich, Germany	G8795
LC3A	1:1 000	Cell Signaling Technologies (Autophagy Antibody Kit), Cambridge, UK	4445S
LC3B	1:1 000	Cell Signaling Technologies (Autophagy Antibody Kit), Cambridge, UK	4445S

MGMT	1:1 000	Cell Signaling Technologies, Cambridge, UK	2739
P-Beclin-1 (Ser15)	1:1 000	Cell Signaling Technologies, Cambridge, UK	13825
P-EGFR (Y1068)	1:2 000	Invitrogen, Carlsbad, CA, USA	44788G
P-EGFR (Y1068) (D7A5)	1:1 000	Cell Signaling Technologies, Cambridge, UK	3777P

Table 1: Antibodies

3.1.2 Specific reagents

Chemical / Reagent	Abbrev.	Company
B27-Vitamine A		Life Technologies, Carlsbad, CA, USA
Chloroquine (dilutet in ddH_2O)		Sigma-Aldrich, Munich, Germany
Epidermal Growth Factor	EGF	PeproTech Inc., Rocky Hill, CT, USA
Fetal Calf Serum	FCS	Life Technologies, Carlsbad, CA, USA
Geneticin	G418	Life Technologies, Carlsbad, CA, USA
Western Blotting Substrate Luminol Reagent	ECL	Life Technologies, Carlsbad, CA, USA
Western Blotting Substrate Peroxid Solution	ECL	Life Technologies, Carlsbad, CA, USA
Insulin-Transferrin-Selenium	ITS	Sigma-Aldrich, Munich, Germany and Life Technologies, Carlsbad, CA, USA
N2 supplement		Life Technologies, Carlsbad, CA, USA

Non Essential Amino Acids	NEAA	Life Technologies, Carlsbad, CA, USA
Penicillin/Streptomycin	P/S	PAA Laboratories GmbH, Pasching, Austria
Polyhydroxyethylmethacrylate	Poly-Hema	Sigma-Aldrich, Munich, Germany
Staurosporine		Sigma-Aldrich, Munich, Germany
StemPro Accutase		Life Technologies, Carlsbad, CA, USA
Temozolomide (dilutet in DMSO)	TMZ	Sigma-Aldrich, Munich, Germany
β-mercaptoethanol		Carl Roth GmbH + Co. KG, Karlsruhe, Germany
0,05% Trypsin-EDTA		Life Technologies, Carlsbad, CA, USA
20% BIT100		Pelobiotech GmbH, Planegg/Martinsried, Germany
EmbryoMax 0.1% Gelatin Solution		Merck Millipore, Billerica, MA, USA
Geltrex Reduced Growth Factor Basement Membrane Matrix		Life Technologies, Carlsbad, CA, USA

Table 2: Specific reagents

3.1.3 Solutions and buffers

Buffer/ Solution	**Ingredients**	**in**
10x SDS running buffer	25mM Tris, 192µM Glycin, 0.5% SDS	ddH_2O
5x Laemmli (Loading Dye)	60mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol-blue	ddH_2O
BSA	5% BSA	T-BST

Cell lysis buffer	20% L-Buffer, 2% PMSF	
ECL solution	50% HRP Substrate Luminol Reagent, 50% HRP Substrate Peroxid Solution	/
Immunofluorescence blocking buffer	1% BSA, 0.1% TX100, 0.01% Tween20, 0.02% NaN2, 2.5 % Goat-Serum, 2% Cold Fish Skin Gelatin	DPBS
Milk	5% non-fat dry milk powder	T-BST
Protein lysis buffer	2% PMSF, 20% L-Buffer	ddH_2O
Semi-dry blot transfer buffers:		
Anode I	0,3M Tris, 20% Methanol	ddH_2O
Anode II	25mM Tris, 20% Methanol	ddH_2O
Cathode	25mM Tris, 20% Methanol, 40mM Amino-n-caprioic-acid	ddH_2O
Tris Buffered Saline with Tween20 (TBS-T)	10% TBS, 0,01% Tween20	ddH_2O

Table 3: Solutions and buffers

3.1.4 Specific technical devices

Device	Model	Producer
CO2 incubator	HERAcell® 150, 150i	Thermo Fisher Scientific, Waltham, MA, USA
Microplate reader	Infinite F200 PRO	Tecan Group Ltd., Männedorf, Switzerland
Microscopes	Axioimager 1	Carl Zeiss AG, Jena, Germany
	Eclipse TS100	Nikon, Düsseldorf, Germany
Microscope Camera	DS-U3	Nikon, Düsseldorf,

Control Unit		Germany
Pump compressor for hypoxic chamber	N022AN18	KNF Neuberger GmbH, Freiburg, Germany
Sterile Bench	HERA Safe	Thermo Fisher Scientific, Waltham, MA, USA
X-ray film processor	Konica SRX-101A	Konica Minolta GmbH, Langenhagen, Germany

Table 4: Specific technical devices

3.1.5 Software

Software	**Company**
Axiovision	Carl Zeiss AG, Jena, Germany
Citavi Free.4	Swiss Academic Software, Wädenswil, Switzerland
ImageJ, version 1.51	National Institute of Mental Health, Bethesda, MD, USA
NIS Elements F 3.2	Nikon Instruments Inc., Melville, NY, USA
R Studio, version 3.2.3	R Studio, Boston, MA, USA
Tecan i-control for Infinite Reader 1.9	Tecan Group Ltd., Männedorf, Switzerland
Windows Office Excel 2007, 2016	Microsoft, Redmond, WA, USA
Windows Office Word 2007, 2016	Microsoft, Redmond, WA, USA

Table 5: Software

3.2 Methods

3.2.1 Cell culture

3.2.1.1 Cell lines

The established glioblastoma cell line LN18 ("Lausanne18") was a kind gift from Dr. van Meir, Lausanne, Switzerland. LN18 cells are well characterized since 1981. (Ishii et al., 1999) U87 was derived from a malignant glioblastoma resection in the 1970s and was obtained from ATCC (Manassas, VA, USA). To investigate EGFR alterations, transfected LN18 with the constitutively active $EGFR^{vIII}$ variant ($LN18^{vIII}$) or overexpressed wild-type EGFR ($LN18^{wtEGFR}$), as well as $U87^{vIII}$ were created by Dr. Andrea Schäfer. $U87^{vIII}$ stably expresses $EGFR^{vIII}$ whereas U87 is expressing EGFR at a very low level. (Piao et al., 2008) All clones were maintained in the presence of the selection antibiotic G418 and their stable expression of $EGFR^{vIII}$ or EGFR-WT was routinely analyzed by SDS-PAGE.

Tissues for the primary cell lines pGBM T1 and T12 were received in cooperation with the Department for Neurosurgery by Dr. Florian Ringel. Freshly resected glioblastoma specimens were enzymatically processed by Dr. Andrea Schäfer. The primary cell line GBM T67 was isolated by Dr. Fabian Schneider. The primary glioblastoma tumor stem cell line GBM X01 was a generous gift from Dr. Andreas Andoutsellis-Theotokis (Carl Gustav Carus Universität Dresden, Germany). Usage of primary cell lines was limited to early passages.

3.2.1.2 Cultivation and cryopreservation

Cells lines were cultivated under standard cell culture conditions in the presence of 5% CO2 at +37°C in a humidified incubator. Cells were grown as monolayer or sphere cultures in different media. Dishes were coated with gelatin 0.1% EmbryoMax or Geltrex (pGBM X01 and GBM T67) one hour prior to plating. Experiments were carried out in open sterile plastic vessels whereas the cell lines themselves were

maintained in filtertop flasks. Cells were passaged at 80-100% confluency every 2-3 days. Cells were washed once with pre-warmed DPBS. The DPBS was discarded and 0.02% pre-warmed trypsine (+37°C) was added to the culture vessels. After the cells had detached, they were collected in fresh medium and redistributed. ITS was administered for cell lines under reduced serum conditions (0.1 - 4% FCS). Before treating cells with chemotherapeutics, FCS was applied at a concentration of 0.1% to reduce undesired side effects. Spheres were collected by sedimentation for a minimum of 10min or centrifuged at 150rcf for 3min at RT. Sedimentation or centrifugation was repeated after a washing step with DPBS. Spheres were disassociated by pipetting up and down to allow redistribution of single cells in new culture vessels.

For cryopreservation, cells were detached with 0.02% trypsine, washed twice with DPBS, centrifuged at 300g for 3-5min and gradually cooled down in freezing medium (composition see Table 6). Vials were collected in a Mr. Frosty freezing container and put at -80°C for 4-48h before they were transferred into liquid nitrogen (-180°C) for long term storage.

Cell line	**Cell culture medium**
Adherent culture:	
LN18, T1, T12	DMEM Medium, 4-10% FCS, 1% P/S, 1% ITS, 1% NEAA
$LN18^{vIII}$, $LN18^{wtEGFR}$	DMEM Medium, 4-10% FCS, 0,6% G418, 1% ITS
$U87^{vIII}$	RPMI-1640 Dutch modified, 1% L-Glutamin, 1% P/S, 1% ITS, 4 – 10 % FCS, 1% NEAA
T67, X01	GBM cancer stem cell medium: RPMI-1640 Dutch modified, 20% BIT100, 2% L-Glutamine, 1% N1, 1% NEAA, 0,1% Primocin, 300 pg/ml TGFβ, 20 ng/ml bFGF, 1 ng/ml EGF
Sphere culture:	
LN18	RPMI-1640 Dutch modified, 1% L-Glutamine, 2% B27, 1% N2, 1% NEAA, 1% β-Mercaptoethanol, 1% BSA, 1% P/S

$LN18^{vIII}$, $LN18^{wtEGFR}$	RPMI-1640 Dutch modified, 1% L-Glutamine, 2% B27, 1% N2, 1% NEAA, 1% β-Mercaptoethanol, 1% BSA, 0,6% G418
U87	RPMI-1640 Dutch modified, 1% L-Glutamine, 1% ITS, 1% N2, 1% NEAA, 1% P/S
Starvation:	
LN18, T1, T12	DMEM Medium, 0,1% FCS, 1% P/S, 1% ITS
$LN18^{vIII}$, $LN18^{wtEGFR}$	DMEM Medium, 0,1% FCS, 0,6% G418, 1% ITS
Freezing medium:	90% FCS, 10% DMSO

Table 6: Cell line medium maintenance overview

3.2.1.3 Formation of tumorspheres

Cell lines grow in divergent shapes. Some cell lines possess the capability to grow in a spherical form based on a single cell. The spherical model is supposed to represent a more natural tumor cell draft compared to adherent cell cultures regarding form, oxygen and nutrient deprivation. Weiswald et al. classified 3D culture into four different types:

- multicellular tumor spheroids**,** a single cell-based approach in non-adherent conditions
- tumorspheres**,** which grow in a serum-free medium supplemented with growth factors
- tissue-derived tumor spheres, formed by mechanical dissociation
- organotypic multicellular spheroids, formed by cutting tumor fragments (Weiswald et al., 2015)

To allow cells to grow in 3D, tissue plate surfaces were covered with an inhibitor of cell adhesion. Polyhydroxyethylmethacrylate (Poly-Hema) was solved in 96% Ethanol to a 1X solution agitated at +60°C o.n. and subsequently sterile filtered through 0.22µm. 300-600µl/well were applied per 6 well plate well, allowing the ethanol to evaporate o.n. This procedure was repeated 3 times before cells were plated. Cells were disassociated prior to seeding. Sphere medium is described in Table 6.

Sphere medium, anti-adhesive tissue plates, low density seeding and particular cautious handling to prevent aggregation do not ensure clonal development of spheres. 3D culture arisen in this way is termed tumorsphere in this thesis. The name sphere or tumorsphere in this thesis should not be confounded with neurospheres (neural stem cell characteristics).

In 3D culture, U87 grow half-adherent and half-floating. LN18 grow in a 3D formation in sphere media. To compare, LN18 grow as a monolayer in normal medium. U87^{vIII} grow adherent but in a more astrocytic way than LN18.

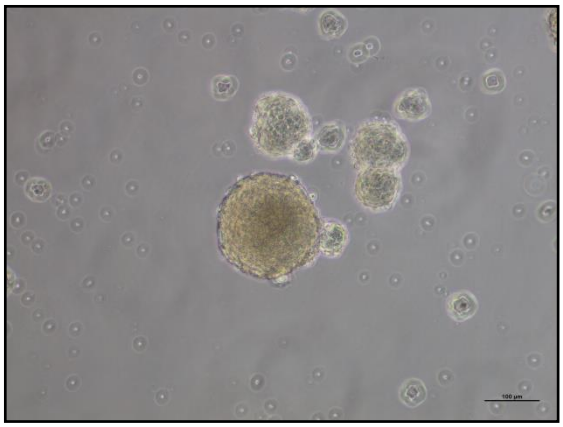


Figure 6: LN18 growing in sphere medium as free-floating sphere. (Nikon, 10x magnification)

3.2.1.4 Sphere forming assay

To evaluate the sphere forming ability of established and primary GBM cells, disassociated cells were seeded in 96 well plates at clonal density. Either 1000, 500, 100 or 10 cells per single well were plated and sphere formation was quantified after 8 days in culture.

3.2.1.5 Cellular proliferation assay

The colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay provides the ability to assess the proliferation of different cells. Only mitotic active cells metabolize the yellow tetrazolium salt MTT into purple formazan crystals.

The MTT was performed as recommended by the manufacturer's manual. In brief, cells were seeded onto a 96-well flat-bottom plate (7,500 cells/well). After 24h the MTT labeling reagent (10µl/well) was added for 4h. To solubilize the salt crystals, 100µl of the solubilization reagent was added to each well and incubated overnight. Absorbance was measured on the Tecan Infinite M200 Pro microplate reader at 595nm.

3.2.2 Immunofluorescence

Immunofluorescence is a histochemical analysis to detect antigens. It uses fluorophore-labeled secondary antibodies raised again unlabeled primary antibodies.

30,000 cells per well were seeded on a 24-well plate prepared with round glass slips (#1.5), which were coated with 0.01% gelatin. Cells were growing for 48h before different treatment options were applied. 48h after treatment cells were fixed with 4% PFA (paraformaldehyde) for 30min and washed 3 times with PBS. Blocking was conducted with antibody blocking buffer containing 2.5% goat-serum (Table 3) for 30min at RT. Primary antibodies were applied for 2h. Following washing with PBS, anti-mouse and anti-rabbit secondary antibodies (Table 1) were applied at a dilution of 1:500 in blocking buffer. Covered from light, cells were incubated for 45min. After washing, Hoechst was deployed for 15min before applying cover glasses to microscope slides.

3.2.3 SDS-Page

3.2.3.1 Protein isolation

After scratching the cells from the dish surface and spinning down by 300g at +4°C for 3min the supernatant was discarded. To obtain clear debris a washing step with ice-cold DPBS and centrifugation followed. The pellet was resuspended in freshly prepared lysis buffer adequate to the number of cells and incubated rotating at +4°C for 10min. Prior to protein quantification the lysis suspension was centrifuged at 10,000rpm for 10min and the supernatant was transferred to a new vial. Protein levels were quantified by Bradford Protein Assay comparing the sample absorption with a previously prepared

standard curve of 0 – 2,000µg/ml BSA. After quantification, 5x Laemmli buffer containing the detergent SDS was applied (1:5) to unfold and charge the proteins. Subsequently the vial was vortexed, briefly spinned down and heated for 5min at +99°C to promote denaturation.

3.2.3.2 SDS – PAGE

Acrylamide gels were prepared with their running part permeability adjusted to the protein size (7-12% gels). Alternatively, gels were purchased by Bio-Rad (Hercules, CA, USA). Gel casters were submerged in SDS running solution in an electrophoresis chamber (Bio-Rad Hercules, CA, USA). The separation through the gel matrix depending on the molecular weight (kDa) of the proteins was performed at 120-180V for 30min to 1.5h.

3.2.3.3 Semi-Dry blotting

The transfer system was set up from anode to cathode with 1-2 sheets of Whatman-paper previously plunged in anode I / II buffer, a PVDF membrane moistened with methanol, the acrylamide gel and 3 sheets of Whatman-paper plunged in cathode buffer. The transfer to the immobilizing PVDF membrane was performed at 25V for about 35min varying due to protein size.

3.2.3.4 Detection of proteins

Brief staining with Ponceau solution allowed cutting the blots at the right lanes. Blocking of unspecific binding sites was performed with 5% milk for 1h. Antibodies were diluted as recommended or tested in 5% milk or 5% BSA. Binding of primary antibodies took place rotating o.n. at +4°C. In the following, blots were incubated with secondary antibodies for 1h. Every mentioned step was followed by a triple 5min washing with PBS. To enlighten the binding sites blots were dripped with ECL solution and the chemiluminiscent reaction was visualized with an X-ray film.

3.2.4 Co-Immunoprecipitation

To detect protein-protein interactions, Pierce Classic Immunoprecipitation (IP) Kit (Thermo Fisher Scientific, Waltham, MA, USA) was applied as the manufacturer's protocol required. First, spin columns equipped with resin were prepared by using the

AminoLink Plus Coupling Resin and affinity-purified antibody. Adherent or floating cells were washed and carefully lysed by using ice cold IP lysis buffer. The immune complex was captured by adding the lysate to a column containing antibody-conjugated resin and mixing o.n. at +4°C. After a 5min incubation with Elution Buffer, the flow-through was collected and subsequently analyzed by Western Blot.

3.2.5 Hypoxic treatment

Cells were cultured under normal conditions in 6cm dishes for 24h. In the following, cells were placed in the hypoxia incubating chamber kindly provided by Dr. Daniela Schilling, Klinikum rechts der Isar. O_2 was cautiously replaced by nitrogen within eleven cycles minding the flow meter. After incubation for 24h at 1% O_2, cells were lysed at the same time as their normoxic control matches.

3.2.6 Statistical analysis

Proteins of interest on Western Blots were normalized by relative normalization control values of respective GAPDH lanes. Error bars indicate the mean densitometric value ± standard deviation. Statistical significance was examined by two-sided Student's *t*-test and Pearson's correlation with R Studio, version 3.2.3. P values < 0.05 were considered statistically significant * (< 0.01 **, < 0.001 ***).

4 Results

4.1 Autophagy in primary and established glioma cell lines is regulated via Chloroquine

Chloroquine is an approved agent to treat malaria but is also a key component in autophagy regulation techniques. (Towers and Thorburn, 2016) In the present study, it is essential to assess whether cells are responsive to autophagy regulation. Therefore, Chloroquine is applied to the established GBM cell lines LN18 and to the primary cells pGBM T1 and T12. Immunoblot analysis shows the expression of the autophagic proteins Beclin-1 and LC3B as well as an upregulation of LC3B-II in Chloroquine treated cells.

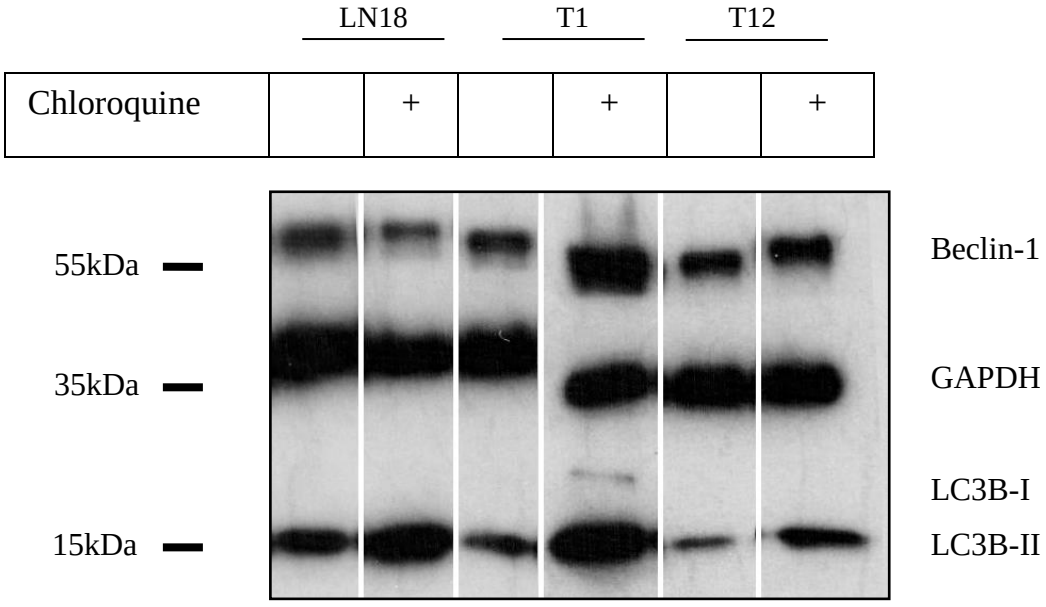


Figure 7: LC3B-II is upregulated via Chloroquine treatment in established and primary cell lines. Western Blot analysis reveals increased expression of LC3B-II after treatment with 50µM Chloroquine for 2h in GBM LN18, pGBM T1 and pGBM T12. The lanes were rearranged out of one blot as indicated.

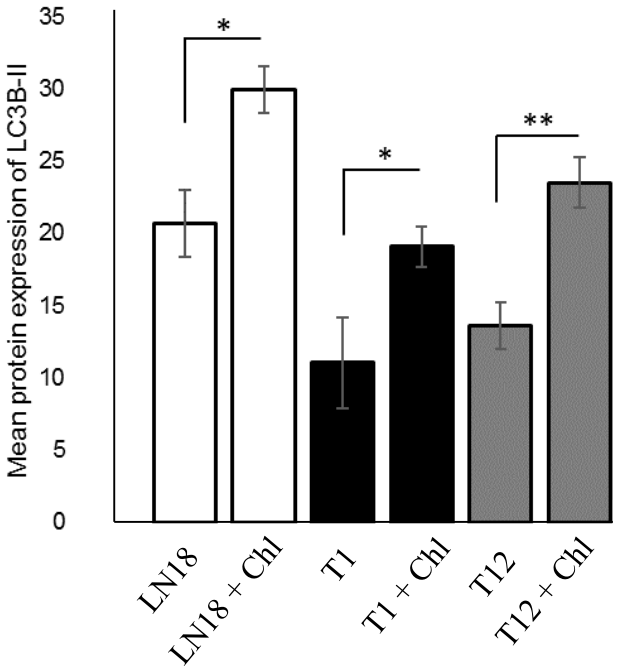


Figure 8: Densitometric analysis of LC3B-II out of three samples. LN18 – LN18+Chloroquine p=0.0127, T1 – T1+Chloroquine p=0.0499, T12 – T12+Chloroquine p=0.0043. Error bars indicate the mean densitometric value ± standard deviation.

As recommended by Mizushima and Yoshimori, LC3-II levels might be compared to monitor autophagy. (Mizushima and Yoshimori, 2007) Increased levels of LC3B-II are detected in GBM LN18, pGBM T1 and pGBM T12 following autophagy induction by Chloroquine, suggesting a block of autophagy. Especially the primary line pGBM T1 is subjected to strong turnover of autophagy marker LC3B. Levels of LC3B-I are not clearly discernable in the cell lines of this study. However, LC3B-I of pGBM T1 is slightly visible in Figure 7. Beclin-1 is ubiquitously expressed and not affected by Chloroquine treatment.

Primary and established GBM cell lines are subjected to autophagy regulation by Chloroquine. Further characterization regarding chemoresistance mechanisms and stem cell properties reveals possible reasons for differences in autophagy responses by other therapy options.

4.2 Characterization of LN18 and LN18^{vIII}

4.2.1 LN18 express high levels of ALDH1 and MGMT, the latter decreased by TMZ application

ALDH1, as well as the subtypes ALDH1A1 and ALDH1A3 are potential stem cell markers. (Rasper et al., 2010, Nakano et al., 2015, Schäfer et al., 2012) Additionally, the findings of Schäfer et al. indicate that the overexpression is linked to TMZ resistance. (Schäfer et al., 2012) The enzyme is stably expressed in LN18 at high levels (see Figure 9). TMZ, Chloroquine or the combination of both do not lead to altered expression of ALDH1.

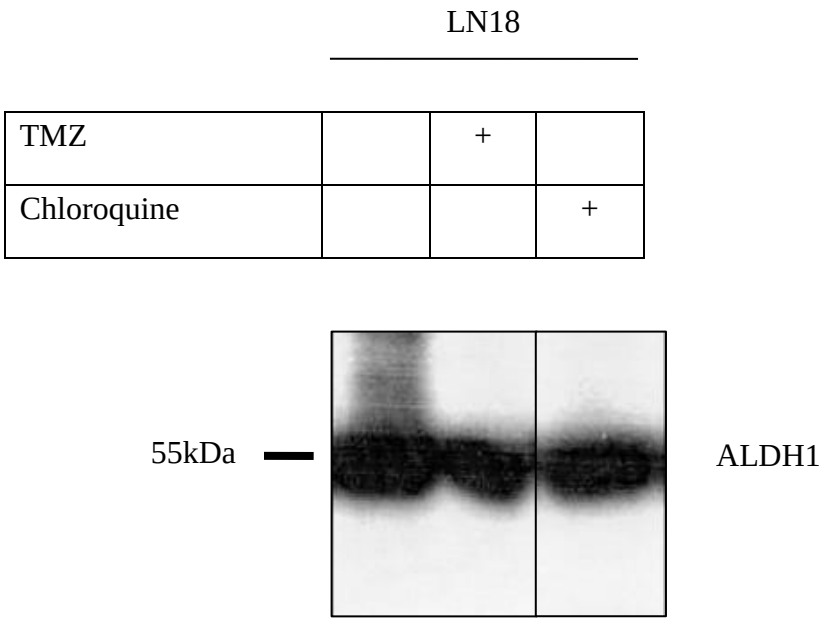


Figure 9: ALDH1 is strongly expressed in LN18. Western Blot analysis shows high levels of ALDH1 in GBM LN18, which is not modified by TMZ (200µM, 24h), Chloroquine (50µM, 2h) or combined application. The lanes are rearranged out of one blot as indicated and same protein weight (50µg) was loaded in each lane.

Temozolomide (TMZ), an alkylating agent, is applied as standard chemotherapy option for high grade GBM. (De Moraes et al., 2017) Therapeutic effectiveness of TMZ is diminished by O^6 methylguanine DNA methyltransferase gene (MGMT) positive GBM

cells. (Bobola et al., 1996) The following immunoblot shows the MGMT positive character of LN18.

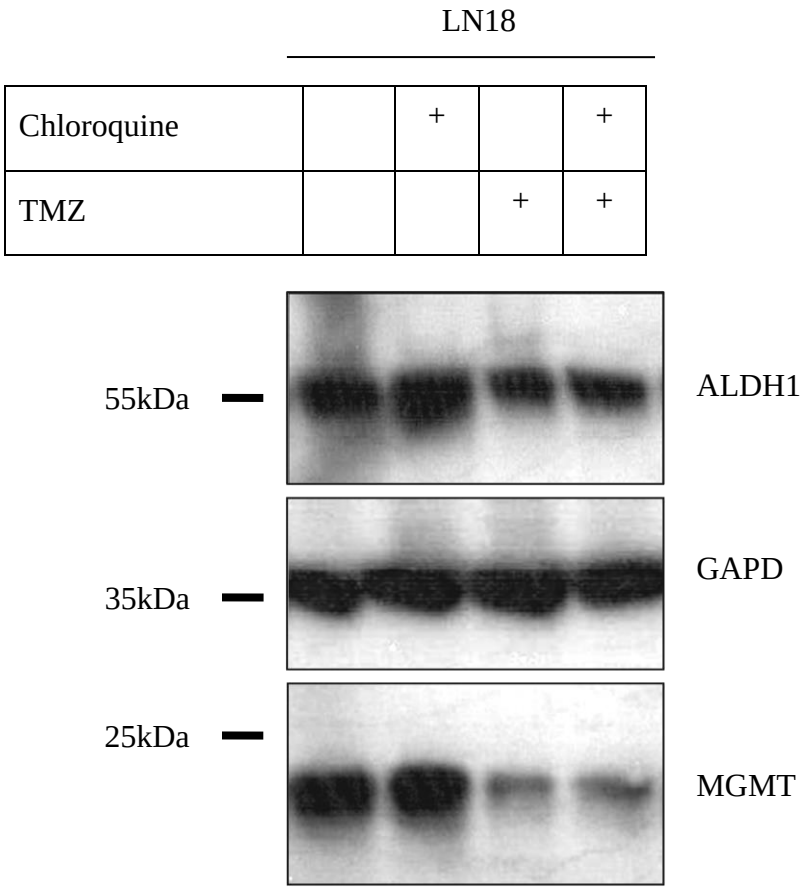


Figure 10: MGMT protein levels are decreased after TMZ treatment. LN18 are $MGMT^{+}$, which is reduced following TMZ treatment (200µM, 24h) detected by Western Blot. Chloroquine (50µM, 2h) in single or combined treatment does not modify ALDH1 or MGMT levels.

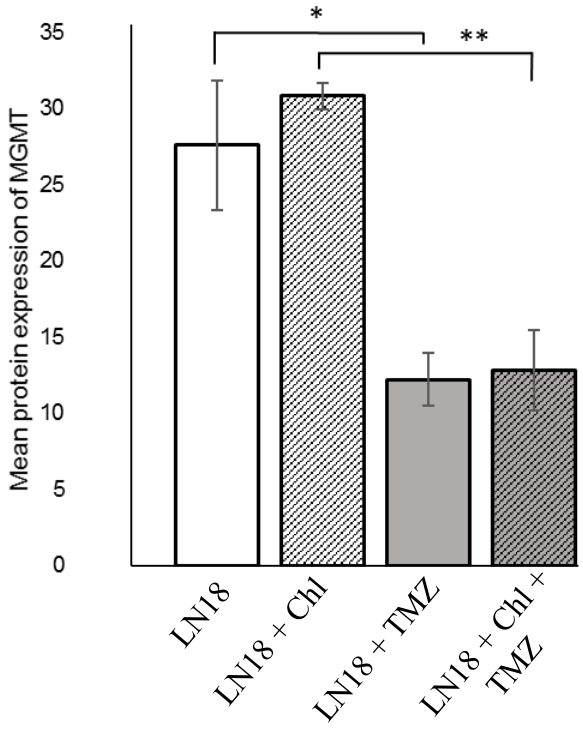


Figure 11: Densitometric analysis of MGMT. Chl = Chloroquine, TMZ = Temozolomide. LN18 – LN18+TMZ p=0.02337, LN18+Chloroquine – LN18+Chloroquine+TMZ p=0.00631. Error bars indicate the mean densitometric value ± standard deviation.

MGMT expression is highly decreased after TMZ treatment. Chloroquine has no influence on ALDH1 or MGMT expression. The slight decrease of ALDH1 is not significant, but this phenomenon has previously been assessed in Prof. Schlegel's laboratory.

LN18 can be transfected with $EGFR^{vIII}$ and wild-type (wt) EGFR to analyze different responses depending on the EGF receptor. The cell lines $LN18^{vIII}$ and $LN18^{wtEGFR}$ likewise express ALDH1 and $MGMT^{+}$ (data not shown).

4.2.2 LN18^{vIII} show the highest sphere forming capacity compared to LN18 and LN18^{wtEGFR}

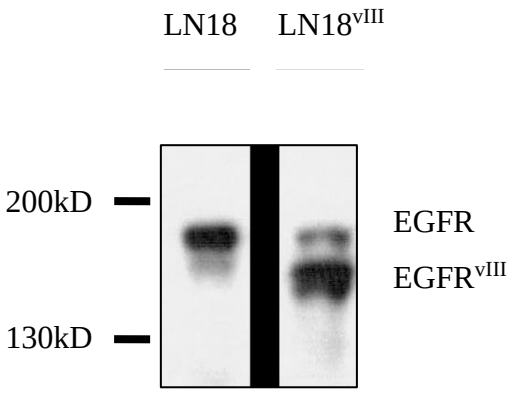
One third of all primary GBM express the truncated EGFR^{vIII}. (Heimberger et al., 2005) To investigate the differences between wild-type and aberrant EGFR form, LN18 transfected with plasmid DNA and stably expressing EGFR^{vIII} or overexpressing wild-type EGFR are taken into culture.

LN18^{vIII} clearly and constantly express the aberrant form of the EGF receptor (approximately 145kDa) whereas LN18 only feature wtEGFR (170 kDa) (Figure 12A). Li et al. presented in 2015 that the truncated EGFR is usually coexpressed with wtEGFR, as it is shown in Figure 12A. (Li et al., 2015)

Divergent findings have been described for the outcome of patients with EGFR^{vIII} expressing tumors. However, most studies suggest shorter overall survival due to EGFR^{vIII}. (Jutten and Rouschop, 2014) Heimberger et al. discovered that the EGFR^{vIII} alteration is an independent negative prognostic indicator for patients with glioblastoma surviving $\geq$ 1 year. (Heimberger et al., 2005) To detect possible reasons for this unfavorable outcome, LN18^{vIII} are closely analyzed in the following.

Resistance to chemotherapy and recurrence of glioma after surgery might be mediated by high clonogenic growth potential of a remaining subpopulation of tumor cells. A sphere forming assay shows differences in self-renewal capacity of cells. Figure 12B reveals LN18^{vIII} as the cell line with the highest sphere forming capacity. Every 23rd cell forms a sphere over 8 days in contrast to LN18 (every 84th cell) and LN18^{wtEGFR} (every 68th cell).

A



B

	Every x cell formed a sphere
LN18	83.65
LN18^{wtEGFR}	68.08
LN18^{vIII}	23.05

Figure 12A: Wild-type and truncated EGFR is detected in Western Blot. The truncated vIII form is clearly apparent at approximately 145 kDa.

Figure 12B: LN18^{vIII} displays the highest sphere forming capacity. The sphere forming assay of LN18, LN18^{wtEGFR} and LN18^{vIII} indicates that LN18^{vIII} exhibits the highest sphere forming capacity.

Differences of cell lines expressing wild-type or mutated EGFR can also be disclosed by Immunofluorescence. Fluorescent-labeled secondary antibodies detect specific primary antibodies, which bind at individual proteins and can be visualized by microscopy.

4.2.3 Immunofluorescence reveals ALDH1 expression in LN18^{vIII} and restricted response to TMZ application

ALDH1* – *EGFR* - *DAPI

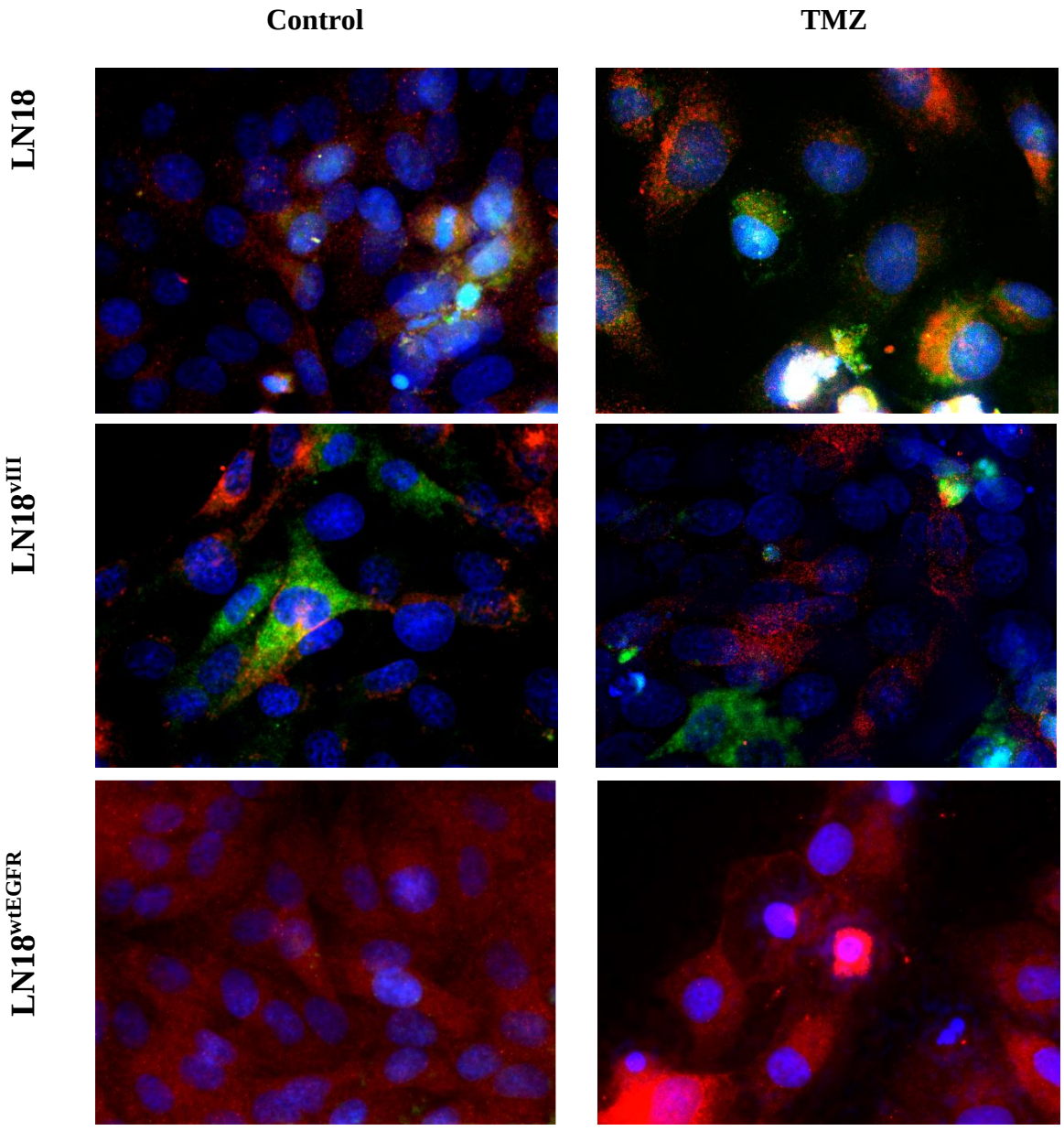


Figure 13: Immunofluorescence visualizing ALDH1 and EGFR. Especially LN18 and LN18^{vIII} show some ALDH1 positive cells. In LN18^{vIII} less wtEGFR is found. LN18^{wtEGFR} expresses most EGFR as expected. Primary antibodies: ALDH1 1:200, EGFR 1:200. TMZ (500µM) was applied for 48h, control cells were concomitantly starved. Magnification: x63.

ALDH1 is mostly found in LN18 and LN18^{vIII}. The cells of all three cell lines are heterogeneous in their ALDH1-expressing character. LN18^{wtEGFR} display highly positive cells for EGFR. Based on application of an EGFR-WT-specific antibody, LN18^{vIII} show less EGFR in IF. Application of TMZ leads to slightly restricted cell growth, mainly in LN18^{wtEGFR}. Interestingly, progression of LN18^{vIII} is not diminished severely.

The established cell line LN18 express high levels of MGMT and ALDH1, both potential mediators of chemoresistance. By contrast, primary cell lines pGBM T1 and pGBM T12 do not express MGMT. Characterization of LN18^{vIII} reveals important features distinguishing this cell line from LN18 or LN18^{wtEGFR}. Compared to LN18^{wtEGFR}, the cell line with the continuously active EGFR^{vIII} shows the higher clonogenic growth potential in the sphere forming assay, higher levels of ALDH1 in IF, and less TMZ induced growth restriction in IF.

These findings and its implication for TMZ treatment have to be considered when assessing the following results.

As previously shown, autophagy can be regulated via Chloroquine in established and primary cells of this study. LN18 response slightly to TMZ when administered in high dose (500µM) monitored by IF (Figure 13). The following results reveal a potential connection between autophagy and TMZ treatment.

4.3 TMZ has no influence on autophagy regulation in LN18 regarding clinically relevant dosing and short-term treatment

TMZ is administered orally in a dose of 75-200µM/m^2/day. Patient plasma concentrations peaks of TMZ are subsequently lower, particularly concentration affecting GBM cells in the brain. To gain insight into more natural conditions in cell culture compared to patient treatment, TMZ is applied in a concentration of 100µM and 200µM for 2h.

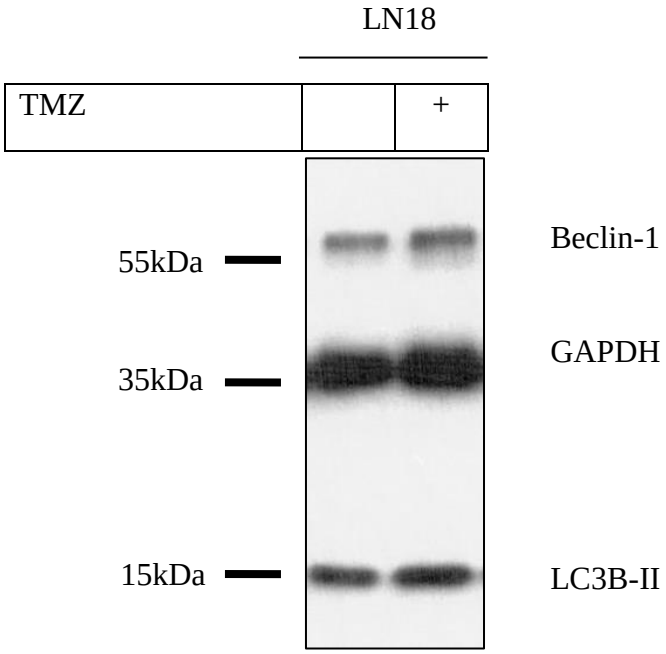


Figure 14: TMZ does not influence autophagy in LN18 after short-term treatment. Western-Blot showing LN18 cells in control or TMZ (200µM, 2h) condition.

A dosage of 100µM and an elevated dosage of 200µM does not result in a modification LC3B-II protein levels in LN18 (Figure 14). Besides, cells are not visibly affected by this low-dose and short TMZ application. Hence, higher doses and prolonged treatment time frames are tested.

4.4 Long-term treatment with high-dose TMZ increases autophagy levels in LN18

In patient treatment, TMZ is applied over weeks following a dosing scheme (Figure 26). Due to the slight response of LN18 when adding a high dose of TMZ (IF, Figure 13) and the absent response of autophagy in LN18 after TMZ in therapeutic dosage (Figure 14) it might be assessed whether high-dose and long-term treatment with TMZ influences autophagy. LN18 is treated with 500µM TMZ for 72h.

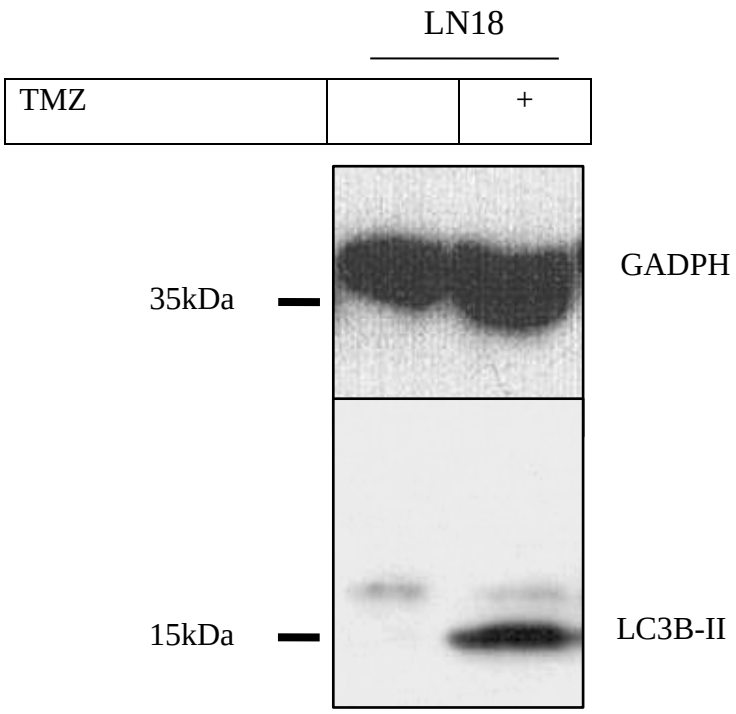


Figure 15: LN18 shows upregulation of LC3B-II following long-term TMZ treatment. TMZ was applied for 72h in a 500µM concentration.

Based on a high-dose and long-term TMZ treatment LN18 show a marked increase of LC3B-II levels. Subsequently, this TMZ induced increase is evaluated for primary, $MGMT^-$ cell lines GBM T1 and T12.

4.5 TMZ promotes autophagy induction in pGBM favoring cell lines with high autophagy turnover

Autophagy regulation in pGBM T1 and T12 is analyzed upon short-term TMZ treatment. In contrast to LN18, the level of LC3B-II significantly increases in pGBM T1 after 2h treatment with 200µM TMZ. The primary cell line pGBM T12 exhibits only minor changes of LC3B-II levels.

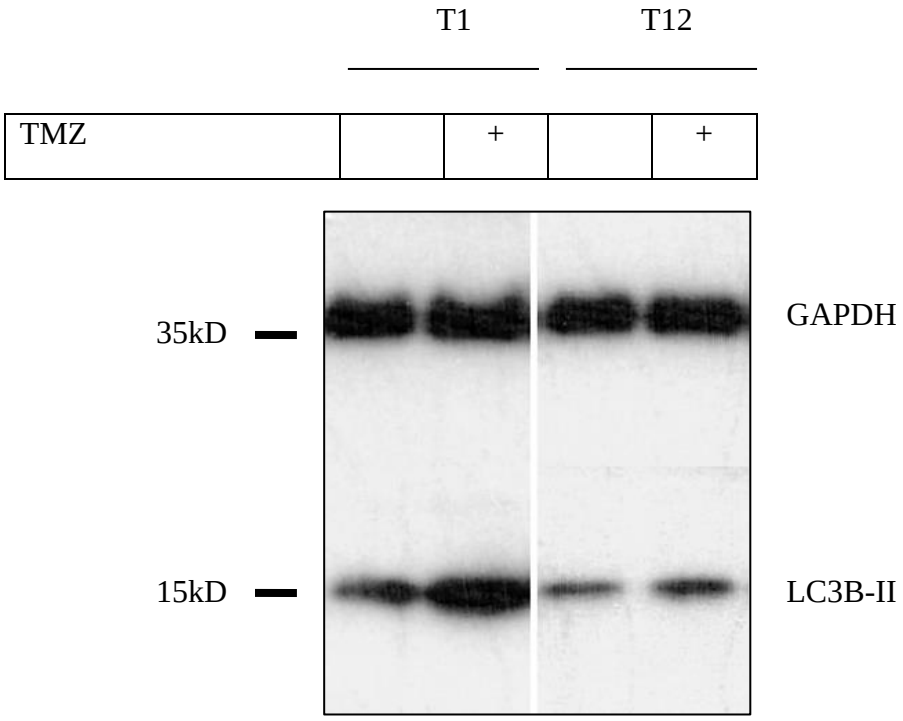


Figure 16: Autophagy levels are increased in pGBM T1 after short-term application of low dose TMZ. In pGBM T1 level of LC3B-II is upregulated after TMZ treatment (200µM, 2h). In pGBM T12 LC3B-II level is not notably modified by TMZ. Besides, LC3B-II seems to be generously attenuated in pGBM T12 in comparison to pGBM T1 levels.

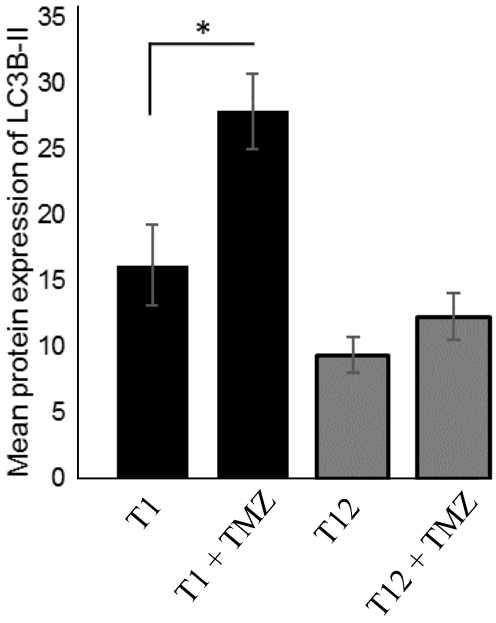


Figure 17: Densitometric analysis of LC3B-II normalized to GAPDH. T1 – T1+TMZ p=0.0168, T12 – T12+TMZ p=0.1436. Error bars indicate the mean densitometric value ± standard deviation.

TMZ treatment enhances LC3B-II levels in established and primary cell lines of this study. However, pGBM T1 responds to lower TMZ treatment in comparison to LN18. Compared to pGBM T12, T1 reflects higher autophagy turnover and concomitantly higher autophagy induction following chemotherapy with TMZ.

4.6 Cell proliferation of established and primary GBM cell lines is not modified by TMZ in clinically relevant dosage after short-term treatment

In order to prevent side-effects, the chemotherapeutic agent TMZ is not applied in high doses in patient care. To assess the toxicity of common dosages of TMZ on different glioblastoma cell lines, the established cell line LN18, the cell lines with deregulated EGFR, and the primary cell lines pGBM T1 and T67 are analyzed by the MTT assay. This method displays the proliferation after different treatment options. Negative controls include DMSO, which is used for diluting TMZ.

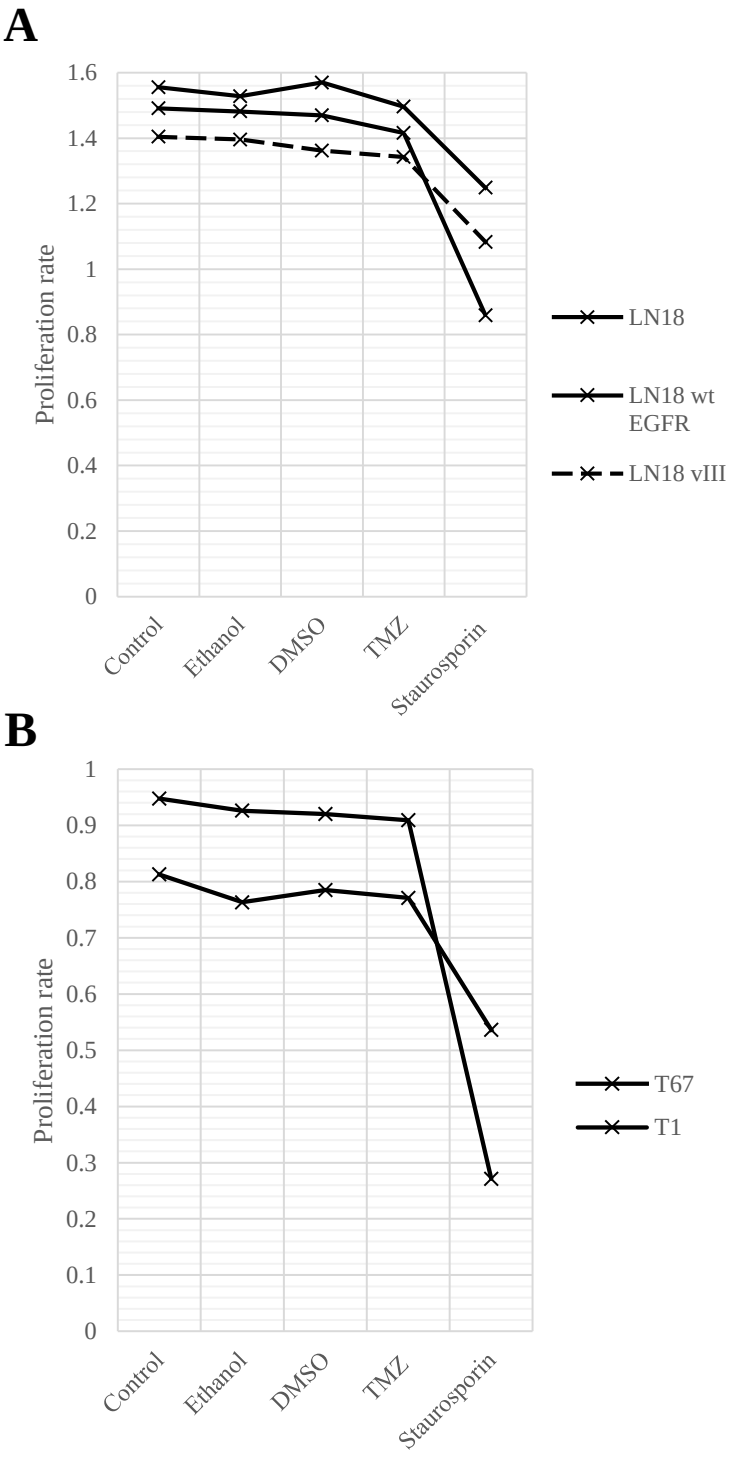


Figure 18: Analysis of MTT assays illustrate no changes in proliferation after low-dose TMZ application**.** Three negative control conditions (normal medium, 96% ethanol 1: 1,000, DMSO 1: 1,000), TMZ (100µM, 24h) and one positive control with staurosporine (5µM) are applied to established (A) and primary (B) glioblastoma cell lines. The graph is based on the mean value of three to four samples. Appendix I specifies respective standard deviations.

Cell line	TMZ	Staurosporine
LN18	-3.8%	-19.7%
$LN18^{wtEGFR}$	-5.0%	-42.4%
$LN18^{vIII}$	-4.4%	-22.9%
T1	-4.0%	-71.4%
T67	-5.1%	-34.0%

Table 7: Mean decrease of proliferation rate in different GBM cell lines. P-values are detailed in appendix II.

The three control conditions show similar results, whereas the negative control indicates the deadly effect of staurosporine on each cell type. Regarding the cytotoxicity of TMZ at low dose (100µM) for 24h, no significant difference appears in comparison to control conditions. $LN18^{wtEGFR}$ as well as the primary cell line T1 are mostly affected by staurosporine.

Presented cell lines are held in adherent culture conditions in previous tests. However, cells in sphere form display a more natural model of the tumor architecture. (Weiswald et al., 2015) For further characterization of the term sphere, please see 'classification of spheres' in the Methods section. The following examination exemplifies the comparison of adherent and serum-free sphere forming culture.

4.7 Sphere forming culture decreases level of LC3B

Even though cells growing in spheres are the same cells as adherent ones, intracellular processes can be varied. (Witusik-Perkowska et al., 2017) To distinguish between autophagy regulation in serum-free sphere culture (3D) and adherent cells (2D), both culture methods are compared.

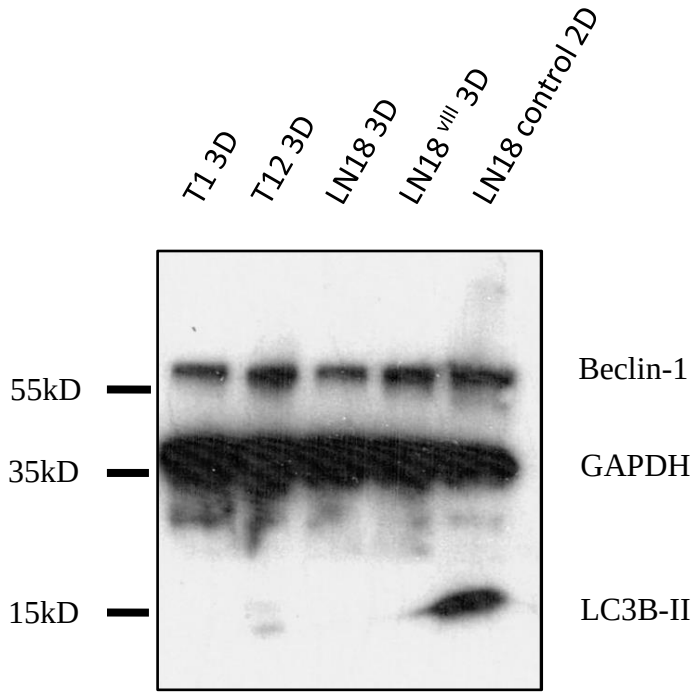


Figure 19: Sphere culture attenuates LC3B-II level. GBM cell lines T1, T12, LN18 and LN18^{vIII} express low levels of LC3B in sphere culture (3D). The last lane displays LN18 cells growing adherent (2D). To compare, see also Figure 7.

Spheres highly attenuate the autophagy protein LC3B-II. This inhibitory effect is observed in primary lines (T1 and T12, Figure 19) as well as in LN18 and LN18^{vIII} to a similar extent.

Wei et al. investigated the interaction of autophagy and EGFR, a commonly altered receptor in oncology. They discovered the phosphorylation of Beclin-1 by active EGFR and the resulting arrested autophagy flux in NSCLC cells. (Wei et al., 2013) As illustrated in Figure 12A, LN18 and LN18^{vIII} express high levels of EGFR or the

truncated version EGFR^{vIII}. Hence, these cell lines are the first to be investigated about potential EGFR – Beclin-1 interaction.

4.8 Analysis of EGFR and Beclin-1 reflects no interaction in LN18, LN18^{vIII} and LN18^{wtEGFR}

Binding of Beclin-1 to active EGFR (pEGFR) promotes multisite phosphorylation of Beclin-1. (Wei et al., 2013) Hence, Beclin-1 is kept from launching the autophagic process. Different techniques can be applied to monitor a potential interaction of Beclin-1 and EGFR. Immunoblotting reveals the phosphorylation status of Beclin-1 and EGFR. Co-Immunoprecipitation (Co-IP) indicates if two proteins bind to each other by pulling down the whole protein complex. Immunofluorescence might visualize if EGFR is adjacent to Beclin-1 in case of interaction.

4.8.1 SDS-Page reveals no phosphorylation of Beclin-1 by EGFR

Western-Blot does not detect pBeclin-1 in LN18, regardless of control condition or TMZ treatment (500µM, 72h). To minimize the effects of inactive EGFR, LN18^{vIII} and LN18^{wtEGFR} as well as the addition of EGF to all LN18 cell lines is tested, which did not result in Beclin-1 phosphorylation. Additionally, LC3B-II levels are not altered following EGF stimulation.

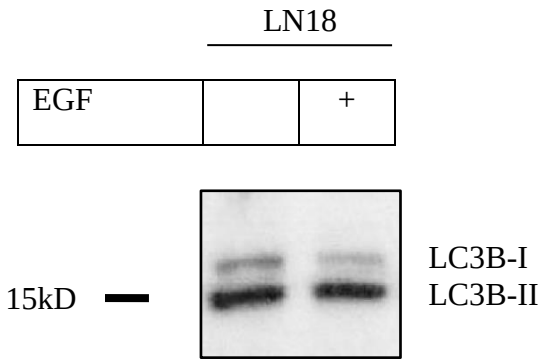


Figure 20: LC3B-II levels are not modified by EGF application

4.8.2 Co-Immunoprecipitation detects no EGFR – Beclin-1 complex

Co-Immunoprecipitation is a common tool to detect protein aggregates. A protein complex can be pulled down with one or two antibodies depending on the question whether the interaction itself or the expression of both individual proteins is being examined. In this case, Beclin-1 and EGFR are known to be constantly expressed in LN18. Hence, several Co-IPs are performed by pulling only one antibody (Beclin-1). Figure 21 illustrates the pull-down of both, EGFR and Beclin-1, indicating there is no protein-protein interaction.

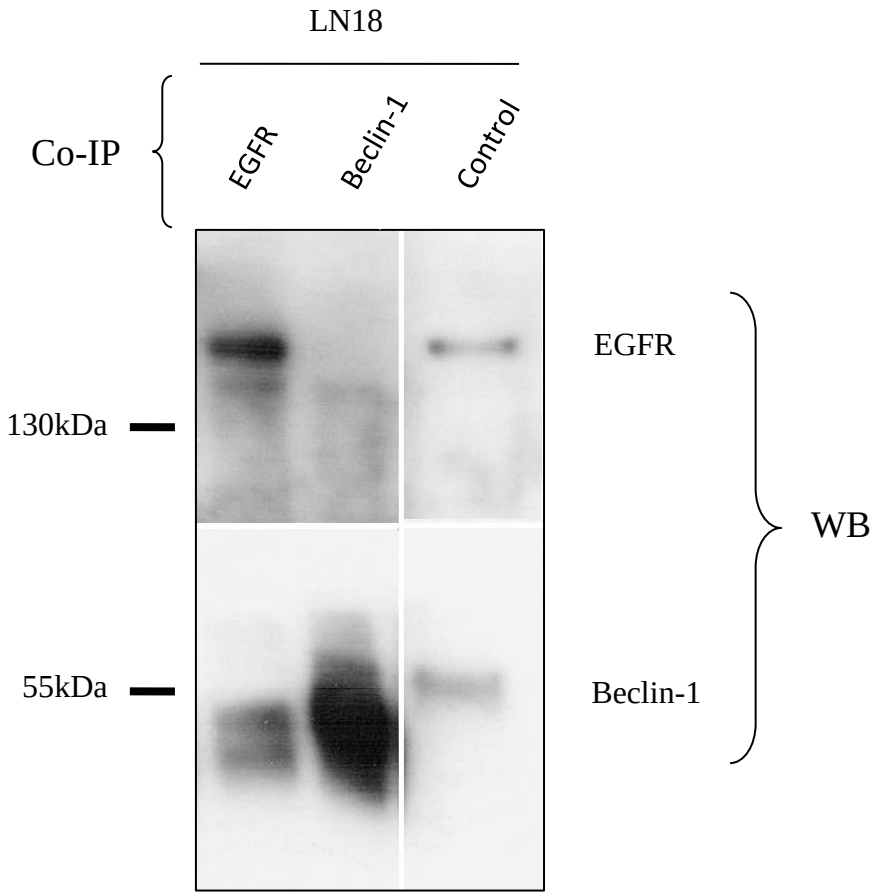


Figure 21: EGFR and Beclin-1 do not bind in LN18. The Immunoblot of the Co-IP shows the pull-down of EGFR in the first lane, the pull-down of Beclin-1 in the second lane and the original lysate in the last lane. Beclin-1 antibody causes a smear in all Co-IP blots. Compared to Beclin-1, this smear is localized at a lower lane.

LN18, LN18^{vIII} and LN18^{wtEGFR} in control or TMZ (500µM, 24h) treatment conditions do not display association of Beclin-1 and EGFR. Taking the cells in sphere conditions (control versus TMZ 500µM, 24h), EGFR and Beclin-1 does not promote coimmunoprecipitation. As no interplay of EGFR and Beclin-1 is revealed, EGF (20ng/ml, 30min) is applied to stimulate inactivated EGFR.

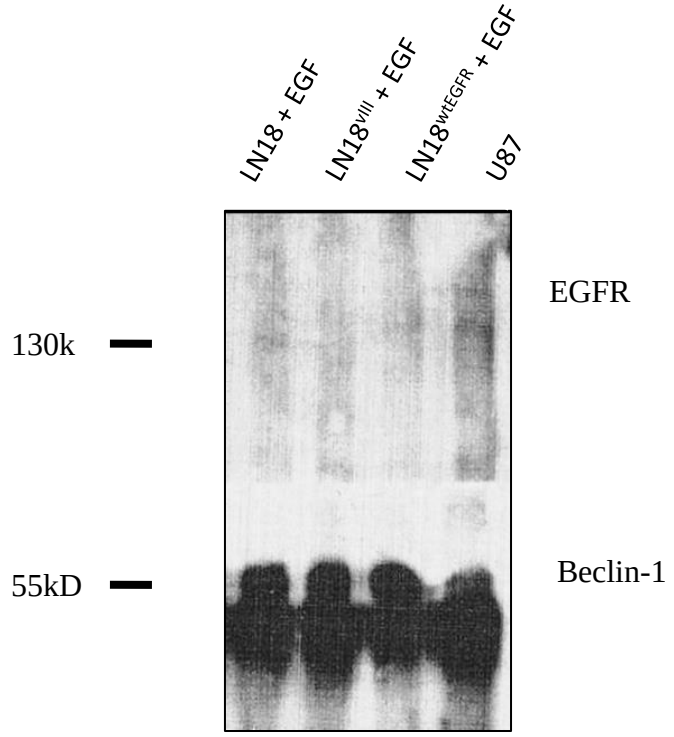


Figure 22: EGFR and Beclin-1 do not interact after treatment with EGF. Cells are treated with EGF (20ng/ml, 30min) and Co-IP is performed by pull down of Beclin-1. The last lane displays untreated U87.

LN18, LN18^{vIII} and LN18^{wtEGFR} do not display EGFR - Beclin-1 interaction following EGF treatment. To evaluate their interaction in other cells, GBM U87 cells are cultured for further analysis. Likewise, Beclin-1 and EGFR do not bind in U87 (Figure 22) or in U87^{vIII}. The same resulted for the primary pGBM cell line X01.

To investigate whether hypoxia, as found in the center of tumor masses, affects EGFR – Beclin-1 association, GBM cells U87, U87^{vIII} and X01 are taken into hypoxic culture. Hypoxia is performed in a hypoxic incubation chamber for 24h at 1% O_2. Formation of the EGFR – Beclin-1 complex is not promoted by hypoxia for 24h or by normoxic conditions in U87, U87^{vIII} or X01.

4.8.3 Immunofluorescence visualizes individual Beclin-1 and EGFR locations

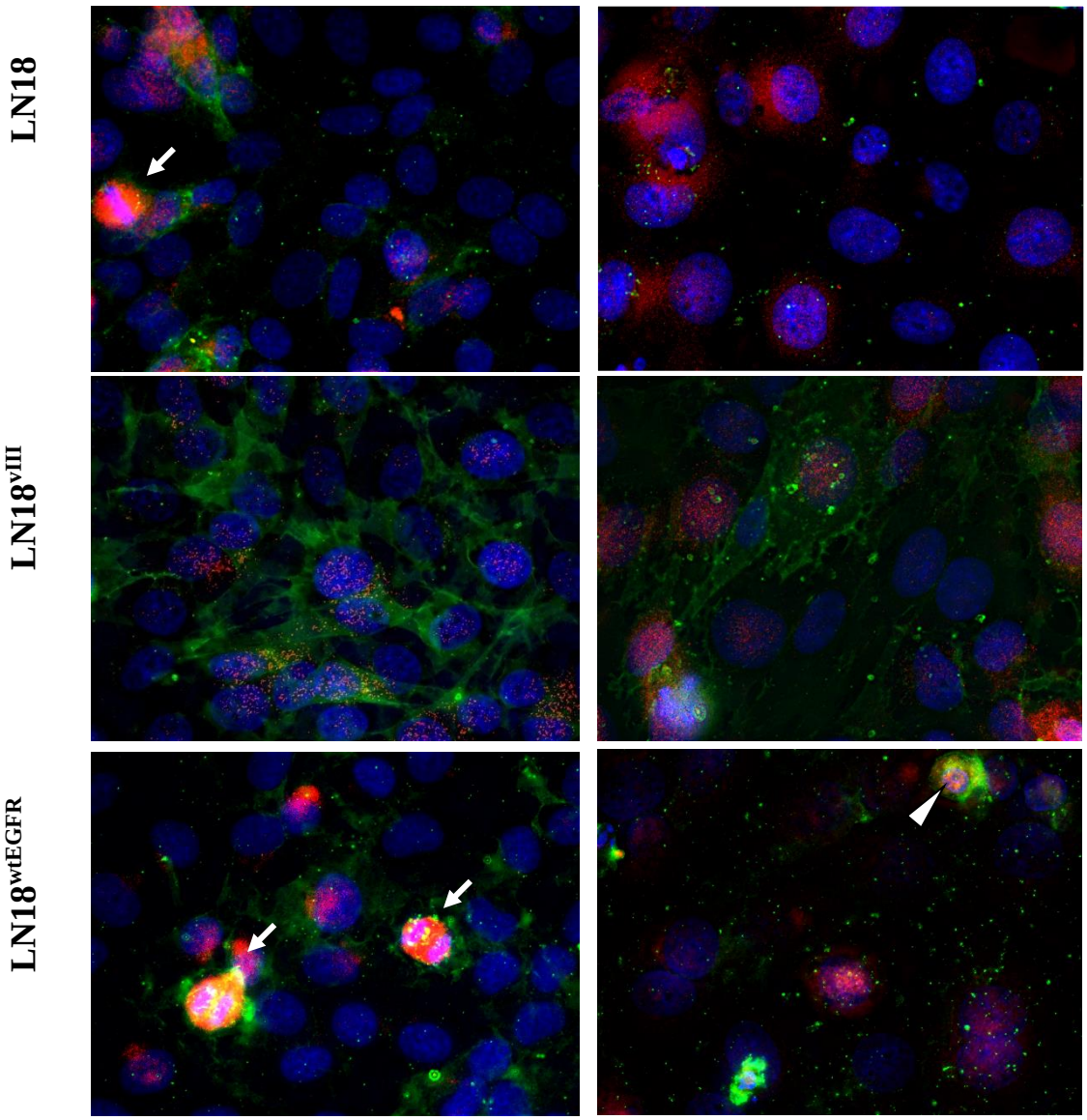


Figure 23: Immunofluorescence of Beclin-1 and EGFR. LN18^{wtEGFR} is affected by TMZ most severely whereas growth of LN18^{vIII} is only slightly attenuated. LN18^{vIII} displays most EGFR cocktail spots followed by LN18^{wtEGFR}. Beclin-1 is expressed on equal levels in all three cell lines. Primary antibodies: EGFR Cocktail 1:200, Beclin-1 1:200. The white arrows (➞) indicate mitotic cells and the small arrow (►) points at a dying cell. Going in line with Figure 13, LN18^{wtEGFR} presents a higher susceptibility towards TMZ than LN18^{vIII}. Growth of LN18 cells is more repressed than growth of LN18^{vIII}. Magnification: x63

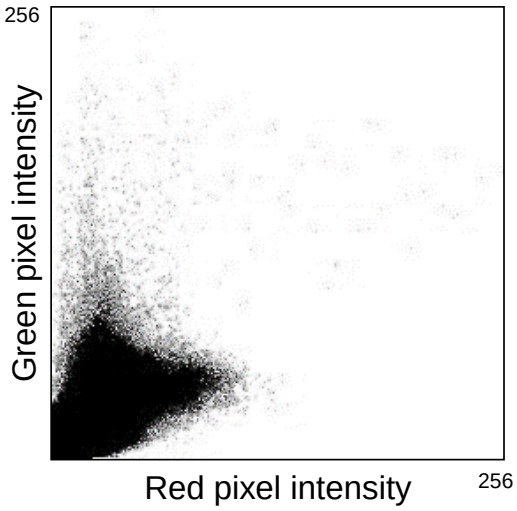


Figure 24: Colocalization map for Figure 23 IF LN18-Control. The colocalization map of Beclin-1 and EGFR shows off-diagonal elements indicating that the locations of both proteins are not interdependent.

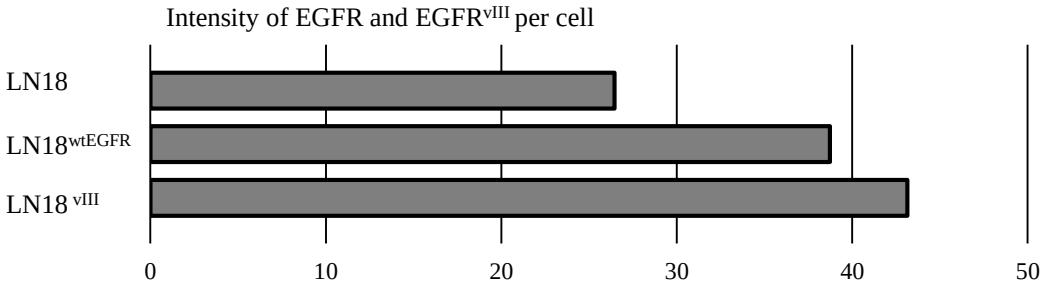


Figure 25: Mean intensity of EGFR and EGFR^{vIII} per cell in LN18, LN18^{wtEGFR} and LN18^{vIII}. Mean intensity is based on six different IF micrographs of each cell line of one experiment. Most EGFR spots are identified in LN18^{vIII} due to the EGFR Cocktail antibody detecting wild-type EGFR as well as truncated vIII-form.

Immunofluorescence double-labeling displays no co-localization of EGFR and Beclin-1 independent of EGFR status or TMZ treatment. Colocalization analyses confirm this visual result for LN18, overexpressing EGFR LN18^{wtEGFR} and LN18^{vIII}. Figure 24 reflects the lack of correlation of the two proteins of interest in LN18 control cells. Pixel-by-pixel covariance is analyzed by Pearson's correlation coefficient, which is presented in Table 8.

	Control	TMZ
LN18	0.3871	0.0000
$LN18^{vIII}$	0.0126	-0.0557
$LN18^{wtEGFR}$	0.2805	0.1429

Table 8: Correlation analyses of colocalization of Beclin-1 and EGFR (Pearson's correlation analysis by Image J). Regardless of EGFR status and TMZ treatment, no correlation is assessed for Beclin-1 and EGFR.

Due to the lack of Beclin-1 phosphorylation despite activating EGFR by the truncated vIII-form or EGF, missing evidence of co-immunoprecipitation in control, hypoxic or EGF-enriched conditions in various cell lines, and the absence of correlation in colocalization analyses of IF we suggest that both proteins do not directly interact in the GBM cell lines of this study.

5 Discussion

GBM comprises more than 80% of malignant brain tumors in adulthood. (Ranjit et al., 2015) Prognosis is poor due to its pronounced invasiveness and high recurrence rate. Standard therapy combines surgery, radiation and chemotherapy but does not lead to long-term tumor survival. Particularly the intratumor heterogeneous character poses a major challenge to therapy options. (Ellis et al., 2015)

Temozolomide remains the main chemotherapy treatment option. The heterogeneous character of GBM cells renders the evaluation of interference of TMZ with different cellular pathways difficult. Nevertheless, main influences on pathways by TMZ have to be understood to analyze adverse side effects as well as possible accompanying or individual therapy approaches. One affected pathway seems to be autophagy, a mechanism to degrade and recycle intracellular proteins. The data of this thesis showed that autophagy was induced upon TMZ application to GBM cell culture in a dose and cell line dependent manner.

In 2013, Wei et al. suggested an important role of EGFR for autophagy initiation. The data indicated that phosphorylation of Beclin-1 by active EGFR resulted in autophagy inhibition in NSCLC cells. (Wei et al., 2013) Autophagy of primary and established GBM cell lines of this study was not regulated by $EGFR^{vIII}$, overexpressed wtEGFR, or stimulated EGFR by EGF. Beclin-1 did not directly interact with EGFR in control or treatment option with TMZ. This is in favor of other regulative pathways in the heterogenous GBM cells of this study. Accumulating evidence has demonstrated that autophagy plays a tumor-facilitating and tumor-suppressing role depending on the context and tumor stage. (Ravanan et al., 2017) This bidirectional approach makes the quest for adequate therapy even more challenging. Autophagy might be an accompanying treatment option for patients with GBM when a comprehensive understanding of the process itself and interacting networks is established.

5.1 Autophagy regulation is altered via Chloroquine and TMZ chemotherapy

Autophagy is a highly conserved pathway, which removes and recycles damaged organelles and denaturated proteins, warranting cellular quality control. In 2016, the Nobel Assembly honored Yoshinori Ohsumi with the Nobel prize for his comprehensive and groundbreaking work on autophagy. Since his discoveries in the 1990's, the impact of autophagy on inflammation and carcinogenesis is more and more recognized. (The Nobel Assembly of Karolinska Institutet, 2016) By now, the mechanism is on suspicion of influencing the development of Alzheimer's, Parkinson's and Crohn's disease as well as chronic obstructive pulmonary disease. (Benito-Cuesta et al., 2017; Qian et al., 2017) The relation of autophagy and tumors might be context-depending and requires further scientific efforts.

In 2007, Mizushima et al. described the conversion of LC3, which has become the most commonly applied method to monitor autophagy. (Mizushima et al., 2007; Yoshii and Mizushima, 2017) The conversion of LC3-I to the lipidated form LC3-II in Western Blot is highly cell specific and the response in cell culture remains less than shown in yeasts. (Klionsky et al., 2012) An increase of LC3 detected by Western Blot correlates to the amount of autophagosomes. (Mizushima et al., 2007) The comparison of LC3-II between samples is more reliable than the comparison of LC3-I/II ratios because LC3-II appears more sensitive to immunoblot detection. (Yoshii and Mizushima, 2017) Therefore, LC3-II levels normalized to the housekeeping protein GAPDH were compared between control and treatment samples in this study. If LC3 is only fairly displayed in Western blot, the addition of protease inhibitors such as pepstatin A might enhance representation, which was not applied in this study.

An increase of LC3B-II as shown in Figure 7, could be induced in primary and established GBM cell lines by autophagy regulation through Chloroquine. Chloroquine is an aminoquinoline well known as an approved antimalaria drug. In its function as a weak base Chloroquine increases the pH of acidic organelles like lysosomes. (Akpowva, 2016) Thereby, the fusion of lysosomes with autophagosomes is impaired. Autophagy is blocked at its last step and LC3B-II accumulates. (Yoon et al., 2010)

Chloroquine has been examined as an intervening variable in tumor growth, e.g. in lung cancer cells. (Fan et al., 2006) In the presence of EGFR inhibition and downstream Akt inhibition, autophagy is induced providing recycling material for tumor cells. TKIs in combination with Akt inhibitors and Chloroquine decreased NSCLC growth *in vitro* and *in vivo.* (Bokobza et al., 2014)

In GBM, Chloroquine has been studied extensively in combination with TMZ treated gliomas since increased chemosensitivity has been demonstrated *in vitro* and *in vivo*. (Golden et al., 2014) Interestingly, only late blocks of the autophagic flux seem to be a promising approach whereas early blocks decrease adverse effects of toxics. (Li et al., 2015) In clinical setting, a phase I/II study of Rosenfeld et al. revealed the toxic effect of high dose Chloroquine treatment, resulting in neutropenia and thrombopenia. (Rosenfeld et al., 2014) A recent Phase I trial aims to assess the adequate dosage of Chloroquine in combination with radiotherapy and TMZ, starting with a daily dose of 200mg. (ClinicalTrials.gov Identifier: NCT02378532, http://clinicaltrials.gov) Other quinolones similar to Chloroquine are being tested. Mefloquine and Quinacrine seem even more potent in the inhibition of autophagy compared to Chloroquine. (Yan et al., 2016)

Another protein to monitor autophagy is Beclin-1. It induces autophagy when being released from its anti-apoptotic binding partner Bcl-2. The corresponding gene, BECN1, was suspected to be tumor suppressing. (Qu et al., 2003; Miracco et al., 2007) For instance, Beclin $1^{+/-}$ mutant mice are tumor prone. (Yue et al., 2013) Recent findings clarified that the direct neighborhood of BECN1 contains the tumor suppressing gene BRCA1. Deletions of wild-type alleles of BRCA1 typically include adjacent genes, as it was shown for BECN1. By now, the independent role of BECN1 as a tumor suppressor has been severely criticized. (Amaravadi et al., 2016)

In this study, Beclin-1 amounts in immunoblots did not vary within one cell line at different autophagy experiments. Primary GBM T1 showed higher Beclin-1 levels than other cell lines as shown in Figure 7. Nevertheless, this does not implicitly reflect a higher overall autophagy level in pGBM T1.

Cells were held under adherent as well as three-dimensional culture conditions. 3D culture displays a more natural model of cancer. (Weiswald et al., 2015, see also classification of spheres in the Methods section) It mimics the tumor cells growing in every direction leaving the core deprived from oxygen and nutrients. This core is often necrotic in fast growing tumors like GBM. Due to this low nutrient supply it would be reasonable that sphere-like cells upregulate autophagy. Nevertheless, LC3B-II levels were decreased in free-floating spheres of pGBM T1, T12, and established cell lines LN18 and LN18^{vIII} in comparison to their corresponding adherent controls. A decreased level of autophagy might reflect that 3D growing cells can establish other mechanisms to recreate nutrient resources or that autophagy is suppressed by cellular pathways activated in 3D conditions. Basically, this highlights the dramatic changes in signaling pathways solely by switching cell culture conditions as also described by Weiswald et. al. (Weiswald et al., 2015) 3D culture remains a reliable standard to get a more adequate model of tumors under *in vitro* conditions. Cancer stem cell characteristics might be favored in clonal density conditions. However, sphere-forming cells in serum-free medium should not be equated with stem cells, which face a lot more features. (Pastrana et al., 2011)

The data of Witusik-Perkowska et al. reflect the importance of comparing adherent models and 3D culture in heterogenous cells like GBM. Serum-free cultured spheres presented higher sensitivity to cytotoxic agents. The extent of sensitivity varied in different GBM cell lines. (Witusik-Perkowska et al., 2017) Overall, this argues for different models in *in vitro* experiments of GBM.

The standard therapy of GBM includes TMZ, a chemotherapeutic preventing the correct duplication of DNA in highly proliferative cells through methylation. The DNA repair enzyme MGMT removes these methylated DNA adducts. The absence of its promoter methylation and the following expression of MGMT is a negative predictive factor for progression-free and overall survival. (Wojciech et al., 2017) LN18 express MGMT (MGMT^{+}). MGMT levels were highly decreased by TMZ (Figure 10), suggesting that MGMT was consumed when repairing methylated TMZ lesions. This goes in line with the findings of Gilbert et al. (Gilbert et al., 2013) Primary GBM T1 and T12 are MGMT^{-}, which can be understood as beneficial regarding TMZ treatment.

The application of 200µM TMZ for 2h in LN18 cells provided no change in LC3B-II (Figure 14). In contrast, a high-dose (500µM) and long-term (72h) TMZ treatment enhanced LC3B-II levels. This indicated a positive regulation of TMZ on autophagy. LN18 exhibited a TMZ resistant character that could be overcome with an augmented TMZ concentration. However, this high-dose is not feasible in patient care with resistant GBM due to side effects. TMZ treatment normally ranges from 75-200µM/m^2/day (see Figure 26 for patient dosing scheme) and plasma concentrations might be below these concentrations.

To evaluate differences in established and primary cell lines, pGBM T1 and T12 were compared regarding autophagy regulation. LC3B-II levels increased in TMZ-treated pGBM T1 cells at 200µM. This contrasted with GBM LN18, which did not respond to this concentration of TMZ. Primary GBM T12 did not show major regulation of autophagy following TMZ treatment. This indicates that the reason of autophagy induction by TMZ cannot be simply MGMT status or primary versus established cell lines.

Overall, TMZ induced autophagy in primary GBM cells. The data of Lee et al. in 2015 provides evidence that TMZ induced autophagy in established U87 cells. (Lee et al., 2015)

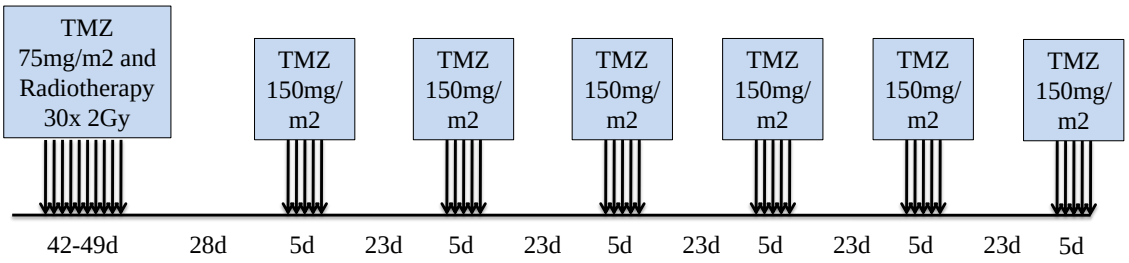


Figure 26: TMZ dosing scheme**.** Example of a TMZ dosing scheme for newly diagnosed GBM after surgery. TMZ is administered daily in the first 42-49 days. The dosage is taken orally as capsules of e.g. 75mg/m^2 body surface area. This first phase is concomitant to focal radiotherapy (2Gy for 30 days). The next 28 days represent a recovering period. Six cycles of TMZ follow. One cycle includes five days of TMZ (each day 150mg/m^2) and 23 days of recovery. The dosage and number of cycles is adapted individually. Scheme self-derived, based on Stupp et al., Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomized phase III study: 5-year analysis of the EORTC-NCIC trial, in *Lancet Oncology*, 2009.

To get a more holistic view of impacts by TMZ on established versus primary cell lines, their cell proliferation was assessed. Cell proliferation was reflected by an MTT assay staining only cells with active mitotic function. Common therapeutic concentration of 100µM for 24h suppressed proliferation of GBM LN18, LN18^{vIII}, LN18^{wtEGFR}, pGBM T1 or pGBM T67 only very slightly compared to control conditions. Decrease in proliferation was not significant. Proliferation was suppressed significantly with the cytotoxic agent staurosporine, particularly in LN18^{wtEGFR} and pGBM T1.

This study showed that depicted established and primary cell lines' intracellular pathways reacted on TMZ application by an induction of autophagy. This was dependent upon TMZ concentration and cell line. The mechanisms that underlie this effect remain still poorly understood.

5.2 EGFR interaction with autophagy is highly complex

To explore the wider context of autophagy regulation, the receptor tyrosine kinase EGFR and its connection to autophagy was investigated. EGFR aberrant signaling is widespread in cancers. Amplification or mutations like the most common one, $EGFR^{vIII}$, is encountered in many GBM. $EGFR^{vIII}$ provides constant EGFR signaling for the tumor cell. This signaling is ligand-autonomous as the extracellular regulative part is missing (see also Figure 5). (Keller and Schmidt, 2017) Enhanced activity of downstream pathways leads to proliferative advantage. However, $EGFR^{vIII}$ signaling displays not only enhanced but divergent characteristics from the wtEGFR signaling. (Bleeker et al., 2012; Eskilsson et al., 2014)

GBM LN18 transfected with $EGFR^{vIII}$ or with an increased amount of wtEGFR reflected differences compared to control LN18. The expression of the truncated vIII form was less sensitive to high doses of TMZ as seen in IF (Figure 13 and Figure 23). Additionally, clonogenic growth potential was highest in the sphere forming assay in $LN18^{vIII}$. This can be interpreted as higher tendency to stem cell characteristics, which is in line with increased TMZ resistance. (Pastrana et al., 2011; Ulasov et al., 2011) Stem cell characteristics are not to be equated with stem cells. LN18 showed other potential tumor resistant characteristics, by the expression of MGMT and ALDH1 as illustrated in Figure 10. Rasper et al. reported that ALDH1 expression indicates stem cell characteristics. (Rasper et al., 2010) This could be a reason of increased resistance to TMZ in LN18 and particularly in $LN18^{vIII}$.

Interestingly, Bleeker et al. and Talasila et al. suggested an entirely different tumor growth as a function of wtEGFR versus $EGFR^{vIII}$. (Bleeker et al., 2012; Talasila et al., 2013) They reported that wtEGFR promotes invasion of GBM independently of angiogenesis whereas $EGFR^{vIII}$ is responsible for aggressive and angiogenic progression. In addition, $EGFR^{vIII}$ is usually coexpressed together with wtEGFR, being also the case in $LN18^{vIII}$ (Figure 12A). Li et al. suggested an antagonistic relationship between wtEGFR and $EGFR^{vIII}$. (Li et al., 2015) This might favor malignancy in GBM. The prognostic impact of EGFRvIII remains controversial. Heimberger et al. discovered a reduced survival time due to $EGFR^{vIII}$ mutation in a subgroup of patients surviving more than one year after diagnosis. (Heimberger et al., 2005) In contrast, several studies

measured no prognostic relevance for $EGFR^{vIII}$. (Bleeker et al., 2012; Weller et al., 2014; Faulkner et al., 2014; Felsberg et al., 2017) Montano et al. report an increased overall survival for GBM expressing the truncated $EGFR^{vIII}$. (Montano et al., 2011) The implication of the aberrant $EGFR^{vIII}$ form has to be investigated in detail on a cellular level and in the clinical setting. Nimotuzumab, an anti-EGFR antibody, shows high activity against $EGFR^{vIII}$, which still has to be proven in clinical trials. (Nitta et al., 2016) An ongoing clinical trial about targeting $EGFR^{vIII}$ with redirected T cells will provide further insights in December 2018. (ClinicalTrials.gov Identifier: NCT02209376, http://clinicaltrials.gov; Johnson et al., 2015)

EGFR is linked to many intracellular pathways. Wei et al. discussed the inhibition of Beclin-1 by active EGFR. Unphosphorylated Beclin-1 associates to the VPS34 kinase, which initiates the autophagic flux. Phosphorylation of Beclin-1 by EGFR resulted in reduced autophagy in NSCLC cells. (Wei et al., 2013) Active EGFR means $EGFR^{vIII}$ or EGFR stimulated by EGF.

It is of great interest if EGFR in GBM regulates Beclin-1, particularly regarding the frequent amplification or mutation of EGFR in GBM. By using different methods, each with its strengths and weaknesses, the potential interaction might be elucidated. Beclin-1 was not phosphorylated by EGFR independently of TMZ treatment in LN18, $LN18^{vIII}$ and $LN18^{wtEGFR}$. Phosphorylation status of Beclin-1 remained unaffected by EGF application. To identify protein-protein interaction, Co-IP was performed, which showed that EGFR did not bind to Beclin-1 in LN18, $LN18^{vIII}$ and $LN18^{wtEGFR}$ in adherent or 3D culture, each independently of TMZ treatment. To evaluate other cell lines as well, U87, $U87^{vIII}$ and the pGBM X01 cells were analyzed showing no interaction of Beclin-1 to EGFR in Co-IP with or without hypoxia for 24h. IF revealed that Beclin-1 locations are not directly adjacent to EGFR locations in LN18, $LN18^{vIII}$ and $LN18^{wtEGFR}$, which did not vary by TMZ application (off-diagonal distribution of colocalization map in Figure 24 and correlation analysis in Table 8). These data suggested that active and inactive EGFR did not inhibit autophagy in several examined GBM cells independently of TMZ, 2- or 3D culture or hypoxia. This is in line with Zhu et al.: "It would not be surprising that Beclin1 and autophagy are independent of EGFR in GBMs and are regulated by other pathways" (Zhu and Khalid, Multiple lesions in

receptor tyrosine kinase pathway determine glioblastoma response to pan-ERBB inhibitor PF-00299804 and PI3K/mTOR dual inhibitor PF-05212384, in C*ancer Biology and Therapy*, 2014, pp. 815–822). However**,** sources of error might be a mutated Beclin-1 precluding the interaction to EGFR. Error sources might as well be of technical origin for instance Co-IP and antibody binding; however, antibody binding was fine in control conditions. Besides, hypoxia could be carried out for more than 24h imitating fast growing GBM cells deprived of oxygen supply.

Further evaluation of EGFR and of possible interactions to autophagy has to be performed. In particular, experiments might analyze the role of inactive EGFR. Tan et al. clarified in 2015 that inactive wtEGFR is essential for autophagy initiation in different cell lines. Inactive EGFR binds to the endosomal oncoprotein LAPTM4B. The complex associates with Rubicon, an autophagy inhibitor. Subsequently, Beclin-1 is released from the Rubicon – Beclin-1 interaction and autophagy is initiated. (Tan et al., 2015) Targeting not only EGFR but the complex of EGFR – LAPTM4B might be favorable. (Li et al., 2016)

It comes more and more into focus that the regulation as well as the downstream pathways of EGFR are not linear but highly complex. A very recent publication of Li et al. explained the different regulative functions of EGFR in different cellular localizations. Active EGFR located in the cellular membrane or the cytoplasm regulates autophagy by well-known downstream pathways like PI3/Akt1, RAS/RAF/MAPK and STAT3. Inactive plasma-membrane or cytoplasm located EGFR inhibits autophagy. Active endosomal EGFR inhibits autophagy, while inactive endosomal EGFR enhances the autophagic flux. Nuclear EGFR seems to inhibit autophagy. The influence of mitochondrial EGFR has not yet been clarified. (Li et al., 2017)

5.3 Autophagy regulation as a new therapeutic approach is seen critically

Figure 16 reflects the upregulation of LC3B-II due to TMZ treatment in primary GBM cells. Underlying mechanisms might be a reaction to energy depletion in fast growing

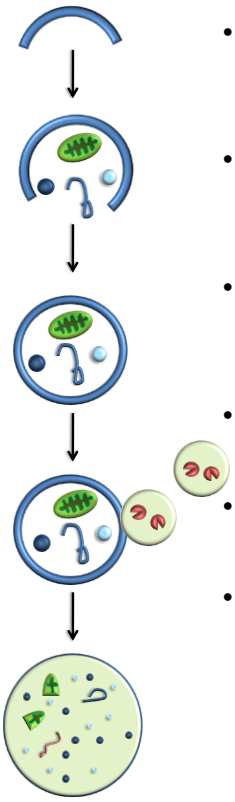
tumor cells. (Jin et al., 2017) This leads to the assumption that autophagy inhibition in combination with TMZ might be favorable in GBM therapy. However, autophagy seems to play opposing and context-dependent roles. Particularly regarding tumor suppression or progression, paradoxical roles for autophagy are vigorously debated.

Figure 27 illustrates an overview of recent findings summarizing the role of autophagy in cancer development or suppression.

Anti-cancer role

- Regulation of cell homeostasis and recycling of cell waste – reduction of damages on DNA[1]
- Inhibition of necrosis and the involved inflammation – reduction of tumor development factors[2]
- Inhibition of autophagy by oncogenes; induction of autophagy by tumor suppressor genes[3]
- Increased tumor development by deficiency of autophagy[4]
- Impaired cell migration by autophagy[5]
- Increased LC3B and Beclin-1 levels in high-grade glioma[6]
- Inhibition of tumor progression by activation of autophagy due to Itraconazole[7]
- Increased toxic effect of TMZ in combination with Thalidomide by autophagy[8]
- Impaired tumor progression induced by antidepressants combined with blood thinners leading to increased autophagy[9]

Autophagy



Pro-cancer role

- Delivery of nutrients in stressed tumor environment and fast growing cells[10]
- Increased chemosensitivity by the autophagy inhibitor Chloroquine[11]
- Induction of cell death in lung cancer cells by prevention of compensatory autophagy[12]
- Decreased toxic effect of TMZ in combination with curcumin by autophagy[13]
- Reduced tumor growth by Atg7 deficiency[14]
- Suppressed tumor initiation by Atg7 deficiency[15]
- Increased surface exposure of calreticulin by Atg5 deficiency[16]

Figure 27: Role of autophagy in cancer development and progression. The number of listed studies does not reflect the importance of the pro- or anti-cancer role. Sources: 1) Jin and White, 2007; Jawhari et al., 2016 2) Mathew et al., 2007, 3) Mizushima et al., 2008, 4) Qu et al., 2003; Yue et al., 2003, 5) Catalano et al., 2015, 6) Huang et al., 2010, 7) Liu et al., 2015, 8) Yan et al., 2016, 9) Shchors et al., 2015, 10) Yang et al., 2007; Jawhari et al., 2016, 11) Golden et al., 2014, 12) Bokobza et al., 2014, 13) Zanotto-Filho et al., 2015, 14) Amaravadi and Debnath, 2014, 15) Gammoh et al., 2016, 16) Garg et al., 2013. Graph self-derived.

Anti-cancer role:

On the one side, autophagy seems to prevent tumorigenesis by a cytoprotective function. The conserved mechanism recycles damaged cellular components, which endanger DNA stability. It reduces necrosis factors, which might lead to cancer development. (Jin and White, 2007; Mathew et al., 2007; Jawhari et al., 2016) Additionally, tumor oncogenes tend to block autophagy, whereas tumor suppressor genes induce autophagy. (Mizushima et al., 2008) BECN1 has been discussed as a tumor suppressor gene for several years. (Jin et al., 2017) However, this role is now doubted due to the co-deletion of BRCA1 as mentioned above. (Amaravadi et al., 2016) This is the reason why BECN1 is not listed as anti-cancer characteristic in Figure 27. Second evidence of the role of autophagy in tumor suppression is based on increased tumor development by a knockdown of autophagy related proteins. (Qu et al., 2003; Yue et al., 2003) Additionally, autophagy impaired cell migration of primary and established GBM cells. (Catalano et al., 2015)

Levels of LC3 and Beclin-1 are significantly decreased in ovarian cancer tissue in comparison to benign ovarian tumors. (Shen et al., 2008) In astrocytic tumors, the overall level of LC3B and Beclin-1 was positively linked to the WHO grade and to overall survival. (Huang et al., 2010) Similar results are reported for breast, and lung cancer treated with chemotherapy. However, this effect might be biased by chemotherapeutic drugs leading to upregulation of autophagy. (Bortnik and Gorski, 2017) He et al. described an inconsistent effect of Beclin-1 and LC3B for breast cancer prognosis. (He et al. 2014)

Regarding potential additional treatment options, Itraconazole, an antifungal drug, enhances the level of autophagic flux in cancer cells and thereby inhibits tumor progression *in vitro* and *in vivo*. (Liu et al., 2015) Autophagy increased the toxic effect of TMZ in combination with thalidomide *in vitro*. (Yan et al., 2016) These studies argue for an anti-cancer role of autophagy within tumor progression. This is consistent with Shchors et al., who published that antidepressants combined with blood thinners enhanced autophagy levels in GBM cells and led to increased survival time in mice with GBM. (Shchors et al., 2015)

Taken together, these data might be in favor of a tumor suppressing function of autophagy. However, several studies suggest a tumor facilitating function of autophagy.

Pro-cancer role:

Autophagy seems to sustain cell growth in tumor cells stressed by oxygen and nutrient deprivation or chemotherapeutics. (Yang et al., 2007) Jawhari et al. reported a tumor suppressing function of autophagy in the early stages by inhibiting cell proliferation and genetic damages. However, in the stage of resource scarcity, autophagy might promote tumor proliferation by its recycling function. (Jawhari et al., 2016)

As mentioned above, Chloroquine led to significantly increased chemosensitivity in GBM *in vitro* and *in vivo.* (Golden et al., 2014) Bokobza et al. showed that enhanced autophagy prevented full therapeutic efficiency of TKIs combined with Akt inhibitors for treatment of EGFR-mutated NSCLC cells. This obstacle could be overcome by inhibiting autophagy with Chloroquine *in vitro* and *in vivo*. (Bokobza et al., 2014) Regarding extended therapy options, autophagy mitigated the effects of the combination of TMZ and curcumin, a phytochemical. (Zanotto-Filho et al., 2015)

By suppressing Atg7, an important autophagy gene, growth of BRAF-driven lung cancers was enhanced. However, in another subgroup of lung cancers including KRAS-mutation, Atg7 deficiency resulted in reduced tumor growth. In the pancreas of p53 -/- mice, Atg7 knockdown accelerated the development of pancreatic ductal adenocarcinomas. This led to the assumption to include patients with pancreatic cancer presenting p53 mutation less frequently in studies with Chloroquine. (Amaravadi and Debnath, 2014)

Silencing of Atg7 by shRNA resulted in suppressed tumor initiation in GBM, which reveals an important role of autophagy in cancer development. (Gammoh et al., 2016) Interestingly, Garg et al. discovered that a knockdown of Atg5 and the subsequent inhibition of autophagy led to an increase in the surface exposure of calreticulin, signaling 'eat me' to macrophages. (Garg et al., 2013) This indicates a survival benefit mediated by autophagy in cancer cells.

Overall, these results demonstrate the dual role of autophagy in cancer suppression and facilitation. Ongoing investigations tackle the issue whether a block in autophagy by chemotherapeutics or an induction of autophagy might be beneficial in cancer treatment. Given the apparently opposite approaches, the search for autophagy regulatory compounds is even more challenging. By now, it is known that tumor therapy can be improved by both, induction and inhibition of autophagy. (Ravanan et al., 2017) Amaravadi summarizes the multiplayer autophagy as follows: "In cancer, autophagy can be neutral, tumor-suppressive, or tumor-promoting in different contexts". (Amaravadi et al., Recent insights into the function of autophagy in cancer. In: *Genes & Development*, 2016, pp. 1913–1930) The context-dependent role of autophagy might be further uncovered by integrating energy and oxygen supply, microenvironmental stress, and the effectiveness of immune responses into autophagy research. (Amaravadi et al., 2016)

5.4 Outlook

Various research projects focused on autophagy in the last years shedding light on a previously underestimated metabolic process. Accumulating evidence is in favor of a highly complex mechanism integrated in a network of cellular pathways. Concluding from the examples mentioned above autophagy has a great impact on tumor development and progression. To clarify whether autophagy regulation should be used for GBM therapy at some point, this highly complex mechanism must be understood comprehensively including the process itself and its interacting pathways in different tumor entities. For the process itself, it has to be highlighted that LC3-II immunoblots only capture a specific moment within the autophagic flux. Other techniques as the previously shown are already available, such as visualizing GFP-LC3 in transfected cells for the amount of autophagosomes and time-dependent monitoring of autophagic markers. Aside from macroautophagy, selective types of autophagy will receive more attention. Examples are aggrephagy (degradation of aggregates), ferritinophagy

(splitting of the iron-ferritin complex), lipophagy (removal of lipid droplets), and zymophagy (elimination of proenzymes). (Klionsky et al., 2016)

The interplay with other pathways possibly interacting with autophagy like the important mTOR pathway and EGFR should be clarified as well. Particularly studies about inactive EGFR in different cellular locations might help to surpass ambiguities of the EGFR – autophagy interaction. Patient material like primary cell lines held under different culture conditions might play a major role in future cellular autophagy research.

For the transfer to patient-based studies, the precise targeting of malignant tumors is essential in order to reduce collateral damage on healthy tissue and adverse systemic side-effects. For instance, the trial about redirected T cells, which target $EGFR^{vIII}$ represents a step forward in focused cancer treatment. (ClinicalTrials.gov Identifier: NCT02209376, http://clinicaltrials.gov; Johnson et al., 2015) TMZ-loaded nanocarriers are extensively studied and are solid promises for targeted chemotherapy. (Lee, 2017) Another approach is provided by MGMT inhibitors like AA-CW236. This compound has not yet been tested in GBM, but sensitized breast and colon cancer cells. (Wang et al., 2016)

Clinical trials about autophagy regulation in GBM via Chloroquine are ongoing as detailed above. Other quinolones might be beneficial as well as an addition to TMZ therapy. The induction of autophagy by TMZ is promising to serve as a novel target of GBM treatment at some point when the pro- or anti-cancer characteristics of autophagy are fully uncovered. Unfortunately, it is still highly difficult to monitor autophagy in humans. LC3-II analysis in peripheral lymphocytes is possible in mice but has not yet been established in patient-care. (Yoshii and Mizushima, 2017; Wolpin et al., 2014) No studies about inducing autophagy in cancers have been launched until now.

It might be hazardous to draw conclusions from clinical studies about autophagy regulation regarding our lack of knowledge about the molecular background. The seemingly opposing effects of autophagy in tumor suppression and promotion are challenging. Therefore, regulating pathways of autophagy have to be investigated to a greater extent. EGFR seems to inhibit autophagy in certain tumor entities despite the

lack of evidence in primary or established GBM cell lines used in this thesis. The polymorphic character of GBM poses a major obstacle to research and therapy. A multiple target approach could be promising including regulation of interacting pathways combined with in situ regulation of autophagy. Research with patient material and clinical trials in GBM research should be encouraged. Together with an interdisciplinary work including different departments it could be possible to alleviate the fatal outcome of GBM.

6 Summary

Glioblastoma multiforme (GBM) is the most common and most malignant type of primary brain tumor in adults. Current standards of chemotherapeutical treatment including the alkylating drug Temozolomide (TMZ) do not lead to long-term tumor control. (Lee 2017) The aim of this study was to analyze the role of autophagy in primary and established GBM cells and its interplay with TMZ. Autophagy is a complex intracellular mechanism to degrade dysfunctional or toxic substances. Due to its protein recycling function, the process is crucial for maintaining cell homeostasis. (Ravanan et al., 2017) To clarify whether autophagy regulation might be beneficial for cancer therapy, this highly complex mechanism must be understood comprehensively in different tumor entities. The data of this study demonstrate that TMZ treatment leads to an upregulation of autophagy in primary GBM cells. In established GBM cell lines, TMZ induces autophagy in high-dose application. Autophagy could be a significant mechanism of GBM to resist chemotherapy as it provides nutrient and energy supply in adverse conditions. However, several studies are in favor of a tumor suppressing function of autophagy, which arguments against autophagy inhibition as an adjuvant therapy. (Jin et al., 2017) This dual role of tumor facilitation and tumor suppression has to be elucidated regarding different tumor stages and contexts.

To uncover the underlying molecular background of autophagy regulation, the second aim of this study was to examine a possible direct crosstalk of the Epidermal Growth Factor Receptor (EGFR) to autophagy. EGFR is the most common amplified or mutated receptor in GBM. (Keller and Schmidt, 2017) Cells constitutively transfected with $EGFR^{vIII}$, the truncated version of EGFR, seem to provide enhanced TMZ resistance. By using different methods, each with its strengths and weaknesses, the interaction of EGFR and autophagy was examined. The data suggest that EGFR does not directly interact with Beclin-1, an important autophagy initiating protein, in established and

primary GBM cells of this study. Future studies might focus on inactive EGFR and the differentiation of EGFR in different cellular locations.

In the future, the limited efficacy of patient treatment strategies in GBM might be enhanced by autophagy regulation. Adverse effects on healthy tissue could be overcome by targeted therapy. Due to our current lack of knowledge about the multiple cellular interactions of autophagy, conclusions from clinical studies have to be interpreted with caution. Research about interacting pathways including context-dependent roles should be encouraged. Substantial effort in order to understand GBM development and progression might lead to a clinical relevant success against heterogenous glioma recurrence.

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Publications:

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Wu, Wei; Schecker, Johannes; Würstle, Silvia; Schneider, Fabian; Schönfelder, Martin; Schlegel, Jürgen (2018): Aldehyde dehydrogenase 1A3 (ALDH1A3) is regulated by autophagy in human glioblastoma cells. In: *Cancer letters* 417, pp. 112-123. DOI: 10.1016/j.canlet.2017.12.036.

Poster:

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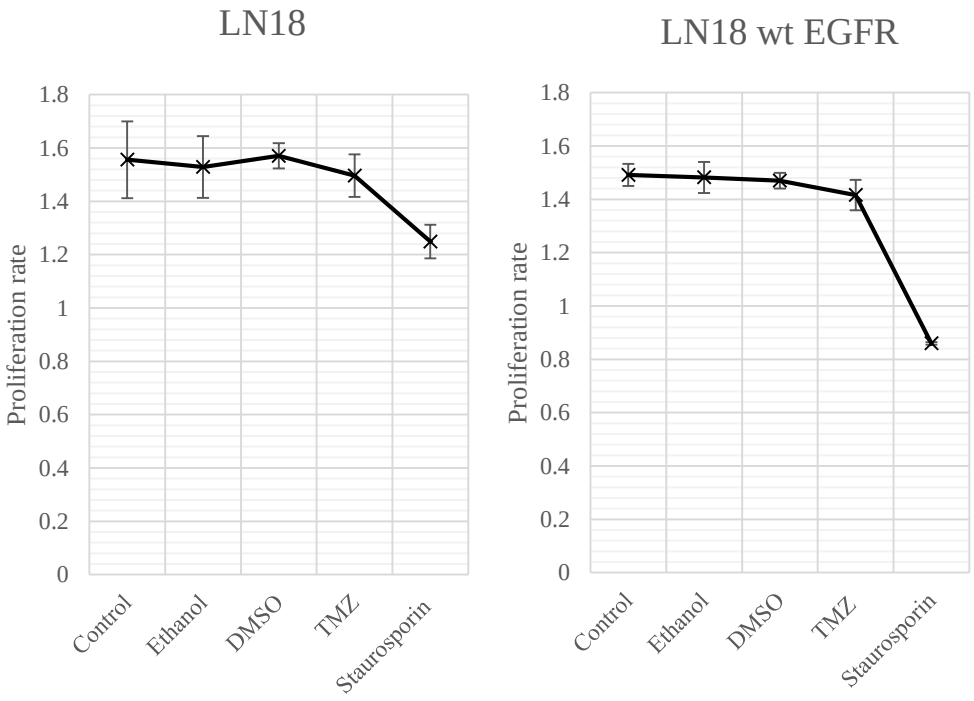
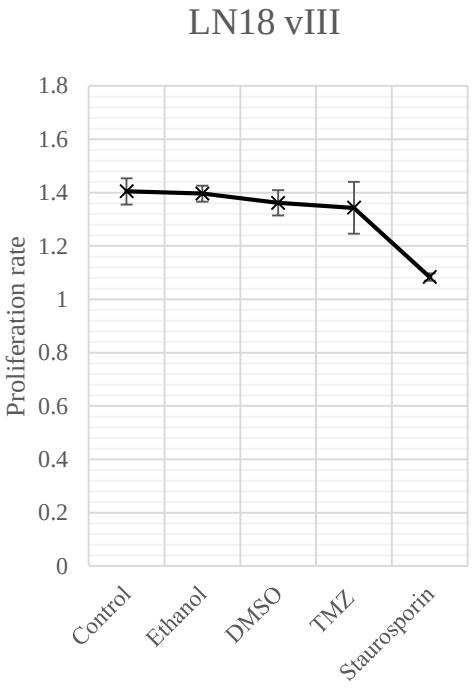
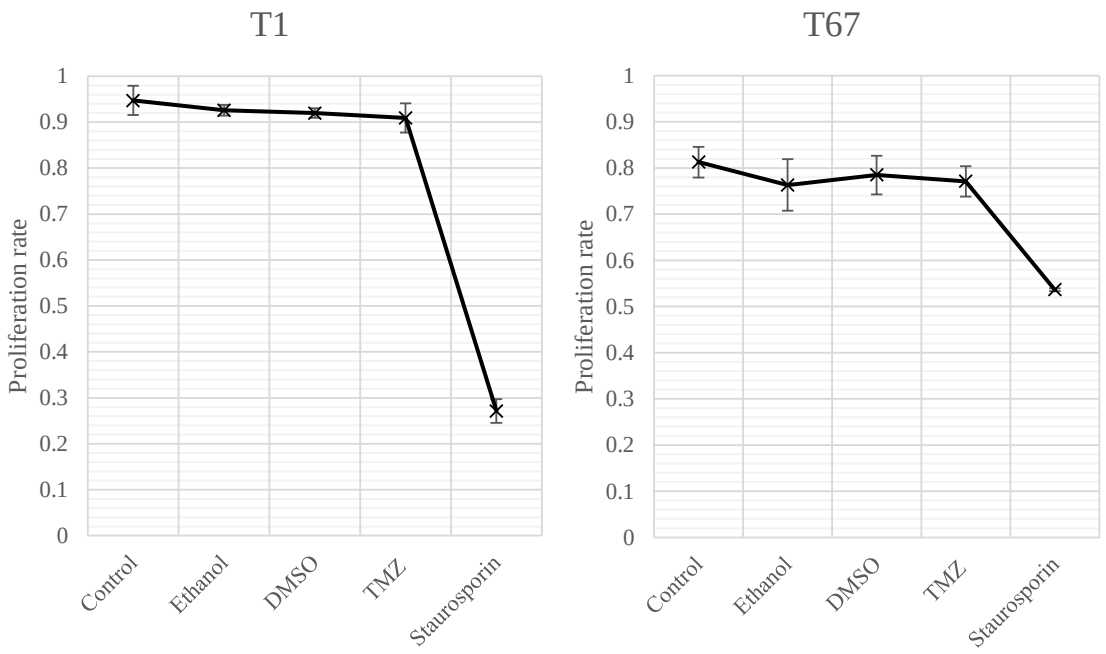
9 Supplementary data







Appendix 1: Graphs of different GBM cell lines in response to treatment options including standard deviation. The analysis is based on the MTT assay of chapter 4.6 in the Results section.

Cell line	TMZ	Staurosporine
LN18	0.4567	0.06179
$LN18^{wtEGFR}$	0.0814	6.304e-05
$LN18^{vIII}$	0.3148	0.00049
T1	0.2956	0.00116
T67	0.1867	0.00039

Appendix 2: P-values of TMZ or staurosporine treatment in different GBM cell lines compared to their respective controls. The analysis is based on the MTT assay of chapter 4.6 in the Results section.

10 Declaration – Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die der Fakultät für Medizin der Technischen Universität München zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:
"Temozolomide induces autophagy in primary and established glioblastoma cells in an EGFR independent manner"
in der Fachabteilung Neuropathologie des Instituts für Allgemeine Pathologie und Pathologische Anatomie des Klinikums Rechts der Isar unter der Anleitung und Betreuung durch Prof. Dr. med. Jürgen Schlegel ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.
Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt. Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt. Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.
Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.
Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei bei der TUM bin ich einverstanden.

München, den 15.1.18

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Silvia Würstle