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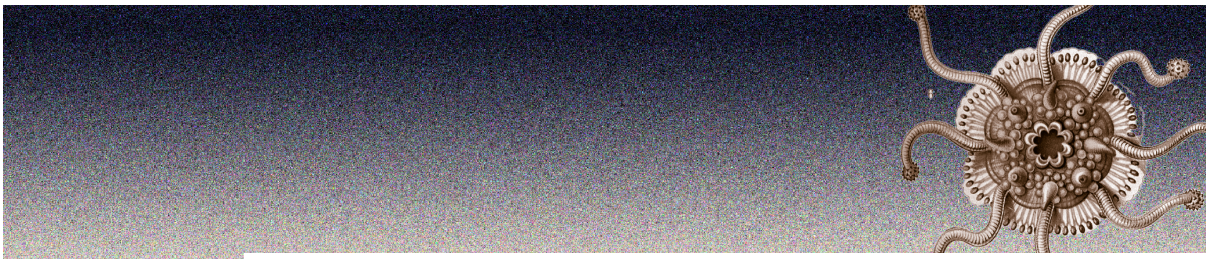

Adaptation to microenvironmental stress in glioblastoma

Mechanistic studies and potential targets

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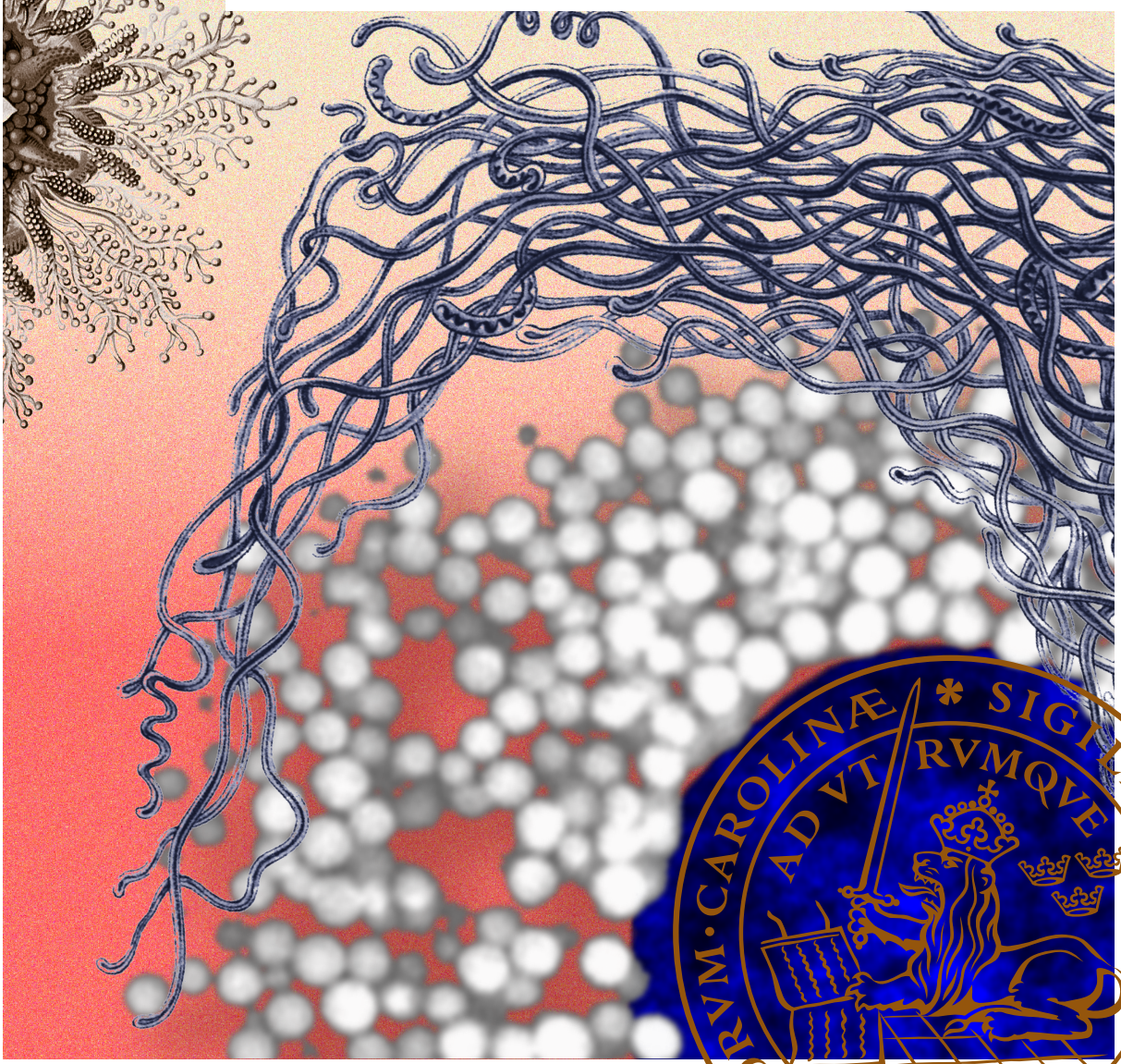

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Myriam Cerezo Magaña

LUND
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Abstract

Although the overall mortality in cancer is steadily decreasing, major groups of patients still respond poorly to available treatments. The key clinical challenge addressed in the present thesis work relates to the inherent adaptive capacity of glioblastoma (GBM) tumors, resulting in treatment resistance and dismal prognosis. GBM represents the most common and lethal primary CNS tumor in adults. Tumor hypoxia and associated metabolic acidosis are main traits of GBM, and the adaptive responses include aberrant, intracellular lipid accumulation in lipid droplets (LDs), which associate with GBM and other aggressive cancers.

Papers I and II show that scavenging of extracellular vesicles (EVs) may contribute to stress adaptation by transforming tumor cells into the LD+ phenotype. On a mechanistic level, hypoxia-induced EV uptake depended on heparan sulfate proteoglycan (HSPG) endocytosis, preferentially via the lipid raft pathway. In Paper II, we studied glioma cell adaptation to chronic acidosis, which triggered a robust induction of chondroitin sulfate (CS) in *in vitro* models and patient samples. As a functional consequence, lipid particle scavenging was decreased. Hence, CS induction together with LD loading may be a targetable protective mechanism to avoid lipotoxicity. In Paper III, we investigate whether necrosis, a histological hallmark of GBM, is a scavengeable nutrient source for glioma cells. Preliminary results show efficient transfer of protein, DNA and lipids via cell debris. Robust induction of LDs following debris scavenging may dampen the response to irradiation. LD+ cells were hypersensitive to drugs inhibiting LD biosynthesis or utilization, which deserves further exploration.

In Paper IV, we developed a methodology for tumor surfaceome mapping (TS-MAP) from intact tumor specimens. Importantly, the TS-MAP method allows to specifically identify the internalizing activity of a given target, an essential feature for cytotoxic drug delivery using antibody-based therapies.

Altogether, this thesis work contributes to mechanistic understanding of stress adaptation in GBM and presents new avenues for target identification strategies.

Key words:
Glioblastoma; Tumor microenvironment; EVs; Proteoglycans; Tumor surfaceome

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Myriam Cerezo Magaña

Coverphoto by Myriam Cerezo Magaña. *Gradients, EVs and PGs.*

Digital composite. Confocal microscopy image of a LD-loaded GBM patient cell. Parts of drawings and lithographies from Ernst Haeckel (*Siphonophorae* and *Discomedusae,* "*Kunstformen der Natur*", 1904, Public domain. Modified).

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To my family

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List of original papers

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I. **Cerezo-Magaña M**, Christianson H C, van Kuppevelt T H, Forsberg-Nilsson K & Belting M.

Hypoxic Induction of Exosome Uptake through Proteoglycan-Dependent Endocytosis Fuels the Lipid Droplet Phenotype in Glioma.

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II. Bång-Rudenstam A, **Cerezo-Magaña M**, Gonçalves de Oliveira K, Kjellén L, Tykesson E, Malmström A, van Kuppevelt T H, Forsberg-Nilsson K, Bengzon J & Belting M.

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III. **Cerezo-Magaña M**, Governa V, Forsberg-Nilsson K, Bengzon J, Talbot H[*], & Belting M.[*] [*]Shared last authorship

Glioblastoma tumor necrosis: A smörgåsbord of scavengeable nutrients as potential fuels for the lipid droplet phenotype, increased proliferation and radioresistance.

Manuscript unpublished

IV. Governa V[*], Talbot H[*], Gonçalves de Oliveira K[*], **Cerezo-Magaña M**, Bång-Rudenstam A, Johansson MC, Månsson AS, Forsberg-Nilsson K, Marko-Varga G, Enríquez Pérez J, Darabi A, Malmström J, Bengzon J, Welinder C & Belting M. [*]Equal contribution

Landscape of surfaceome and endocytome in human glioma is divergent and depends on cellular spatial organization.

Proc Natl Acad Sci USA. 2022 Mar 1;119(9):e2114456119. doi:10.1073/pnas.2114456119. PMID: 35217608

Reprints of Papers I and IV are made with permission.

Related papers not included in the thesis

Indira Chandran V, Månsson AS, Barbachowska M, **Cerezo-Magaña M**, Nodin B, Joshi B, Koppada N, Saad OM, Gluz O, Isaksson K, Borgquist S, Jirström K, Nabi IR, Jernström H, Belting M. **Hypoxia attenuates Trastuzumab Uptake and Trastuzumab-Emtansine (T-DM1) Cytotoxicity through Redistribution of Phosphorylated Caveolin-1**. Mol Cancer Res. 2020 Apr;18(4):644-656. doi: 10.1158/1541-7786.MCR-19-0856.

Cerezo-Magaña M, Bång-Rudenstam A, Belting M. **The pleiotropic role of proteoglycans in extracellular vesicle mediated communication in the tumor microenvironment**. Semin Cancer Biol. 2020 May;62:99-107. doi: 10.1016/j.semcancer.2019.07.001.

Indira Chandran V, Welinder C, Gonçalves de Oliveira K, **Cerezo-Magaña M**, Månsson AS, Johansson MC, Marko-Varga G, Belting M. **Global extracellular vesicle proteomic signature defines U87-MG glioma cell hypoxic status with potential implications for non-invasive diagnostics.** J Neurooncol. 2019 Sep;144(3):477-488. doi: 10.1007/s11060-019-03262-4

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Abbreviations

AA	Acidosis adapted
ACAT	Acyl-CoA cholesterol acyltransferase
ADC	Antibody-drug conjugate
ADCC	antibody-dependent cellular toxicity
AF	Alexa Fluor
ATP	Adenosine 5′-triphosphate
BBB	Blood-brain-barrier
BSA	Bovine serum albumin
BTB	Brain-tumor barrier
CA9	Carbonic anhydrase 9
CAR-T	Chimeric antigen receptor (CAR)-T
CE	Cholesterol ester
CNS	Central nervous system
CS	Chondroitin sulfate
CSF	Cerebrospinal fluid
CSPG	Chondroitin sulfate proteoglycan
DAMP	Damage-associated molecular pattern
DGAT	Diacylglycerol-acyltransferase
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelium-to-mesenchymal transition
ER	Endoplasmic reticulum
EV	Extracellular vesicle
FAK	Focal adhesion kinase

FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GalNAc	N-acetylgalactosamine
GBM	Glioblastoma
GlcA	Glucuronic acid
GlcNAc	N-acetylglucosamine
HSPG	Heparan sulfate proteoglycan
IdoA	Iduronic acid
LD	Lipid droplet
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase
MLKL	Mixed Lineage Kinase Domain Like Pseudokinase
MMP	Matrix metalloproteinase
mTOR	Mechanistic target of rapamycin
MVB	Multivesicular bodies
NA	Non-adapted
NecDeb	Necrotic cell debris
PAPS	Phosphoadenosine phosphosulfate
PBS	Phosphate buffer saline
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphoinositide 3-kinase
PNP-xyl	4-nitrophenyl β-D-xylopyranoside
PTEN	Phosphatase and tensin homolog
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
scFv	Single chain variable fragment
SCS	Single cell suspension
SDC1	Syndecan-1

SREBP1	Sterol regulatory element-binding transcription factor1
TAG	Triacylglyceride
TCA	Tricarboxylic acid
TERT	Telomerase reverse transcriptase
TF	Tissue factor
TGF-β	Transforming growth factor-β
TIM4	T cell immunoglobulin and mucin domain-containing protein 4
TS	Tumor surfaceome
TS-MAP	TS mapping
VEGF	Vascular endothelial growth factor
vsv-G	Vesicular stomatitis virus glycoprotein
WHO	World Health Organisation

Abstract

Although the overall mortality in cancer is steadily decreasing, major groups of patients still respond poorly to available treatments. The key clinical challenge addressed in the present thesis work relates to the inherent adaptive capacity of glioblastoma (GBM) tumors, resulting in treatment resistance and dismal prognosis. GBM represents the most common and lethal primary CNS tumor in adults. Tumor hypoxia and associated metabolic acidosis are main traits of GBM, and the adaptive responses include aberrant, intracellular lipid accumulation in lipid droplets (LDs), which associate with GBM and other aggressive cancers.

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Altogether, this thesis work contributes to mechanistic understanding of stress adaptation in GBM and presents new avenues for target identification strategies.

Popular summary

Glioblastoma (GBM) is the most common and most aggressive type of brain tumor in adults. It affects around three people in every 100 000. We do not know exactly yet why some develop GBM, it is often a matter of "bad luck". Since we cannot prevent it, it is essential to have effective treatments. GBM patients undergo surgery and receive chemo and radiotherapy. However, these treatments fail to cure the disease and the tumor often reappears months after. How can GBM cells hide or be stronger than therapy? We need to understand this better so that more effective therapies can be developed.

Tumors are constituted by many individual cells, which multiply uncontrollably. Cancer cells behave as a smart community. They exchange *messages*, they build *roads* to reach further, they *fight* against the immune system, an army of cells that normally defends us from pathogens but can also attack tumors. Cancer cells even recruit normal, non-cancerous neighboring cells and *convince* them to promote the growth of the tumor. When the population of cells outgrows the supplies of nutrients and oxygen, it leads to chaos. Part of this *chaos* involves low oxygen levels, hypoxia, as well as accumulation of metabolic waste products that leads to what we call acidosis. Among this chaos, *survival of the fittest* applies. Cells that fail to adapt to this *chaos* die, and the *graveyard* of cancer cells that we find in tumors is called necrosis.

In this PhD thesis, I have studied how the chaos of hypoxia, acidosis and necrosis contributes to the aggressiveness of cancer cells. In **Paper I** we wanted to understand why areas of hypoxia in the tumor accumulate fat. What substances lead to this fat accumulation in the tumor? And why in hypoxia? We found that cells retrieve *footprints* from other cells. We call these footprints "extracellular vesicles" (EVs), and they are bubbles that contain material of the cell which secretes them. In hypoxia, cells retrieve more EVs than when there is normal oxygen, and EVs accumulate as fat. We found evidence of how cells retrieve EVs; through their interaction with certain proteins at the cell surface, called proteoglycans, because they are importantly modified by chains of glycans, some molecules very similar to sugar.

In **Paper II**, we found that cells adapt to acidosis by changing the type of glycans that decorate proteoglycans, which goes hand in hand with fat accumulation inside cells. We believe that by changing these glycan chains, GBM cells retrieve less EVs.

But what does this remodeling mean? We think that GBM cells protect themselves from toxic fat overload by closing this glycan-coated *gate*. What would happen if we opened the *gate* again by readjusting the glycans? Uncontrolled entry of EVs and other nutrients may lead cells to succumb to toxicity by extreme fat storage.

It is important to mention that although we find fat in GBM, there is no proven evidence that diet influences its incidence, nor an association between dysregulated metabolism such as obesity, and GBM. Cancer cells find their way to obtain nutrients in very imaginative ways. And further exemplifying this, in **Paper III,** we show that parts from dead cells from the necrosis *graveyard* can be retrieved by living cells. This, again, led to fat accumulation and less effect of radiotherapy in experiments using GBM patient derived cells grown in the lab. Here, we tested different molecules, which prevent fat accumulation inside cells. These treatments efficiently killed GBM cells in the lab and motivates us to continue our research to better understand the clinical relevance of fat-targeting drugs.

Finally, in **Paper IV,** we optimized a strategy to unravel the specific proteins that decorate GBM cell's surface, and which of these proteins are *gate-openers* into the interior of the cell. Drugs should be targeted against proteins that are much more abundant, or ideally, exclusively present in cancer cells, to avoid unwanted effects in normal tissues. Our strategy allows to identify surface proteins and *gate-openers* directly from patient tumor pieces right after surgery. Our results show that brain tumors differ substantially even within the same diagnosis. Further developments on this technique may contribute to better tailored treatments for GBM patients.

Populärvetenskaplig sammanfattning

Glioblastom (GBM) är den vanligaste och mest aggressiva formen av hjärntumör hos vuxna. GBM drabbar runt tre personer per 100 000. Vi vet inte exakt varför vissa människor utvecklar GBM, det är ofta en fråga om "otur". GBM-patienter opereras samt genomgår kemoterapi och strålbehandling. Dessa behandlingar misslyckas dock med att bota sjukdomen och tumören dyker upp igen efter ett antal månader. Hur kan GBM -celler gömma sig eller stå emot behandlingen? Vi måste bättre förstå hur detta sker så att vi kan utveckla mer effektiva behandlingar.

Tumörer består av många enskilda celler som okontrollerbart förökar sig. Cancerceller agerar smarta tillsammans. De utbyter *meddelanden*, de bygger *vägar* för att nå längre, de *kämpar* mot immunsystemet, en armé av celler som finns i vårt blod och försvarar oss mot patogener, men som också kan attackera tumörer. Cancerceller rekryterar till och med normala, icke-cancerösa grannceller och *övertygar* dem om att aktivera tumörens tillväxt. När cellpopulationen växer sig större än tillgången av näringsämnen och syre, uppstår *kaos*. Detta *kaos* innefattar bland annat låg syrehalt (hypoxi) och ansamling av avfallsprodukter som leder till så kallad acidos. Här, i *kaoset*, är det de starkaste som överlever. Celler som misslyckas med att anpassa sig till detta *kaos* dör och tumörens *kyrkogård* av döda celler kallas nekros.

I denna doktorsavhandling har jag studerat hur detta kaos av hypoxi, acidos, och nekros bidrar till aggressiviteten hos cancerceller. I **Delarbete I** ville vi förstå varför områden av hypoxi i tumörer ackumulerar mer fett. Vilka ämnen leder till denna fettackumulering i tumören? Och varför under hypoxi? Vi fann att cellerna använder *fotspår* från andra celler. Vi kallar dessa fotspår för ”extracellulära vesikler” (EVs) och de är bubblor som cellerna släpper ut och som innehåller cellmaterial. Vid hypoxi konsumerar cellerna mer EVs än när det finns normalt med syre, och detta ansamlas som fett. Vi hittade bevis för hur cellerna tar upp EVs: genom vissa proteiner på cellytan, vilka modifieras av glykan- eller sockerkedjor (därav deras namn, proteoglykaner).

I **Delarbete II** fann vi att celler anpassar sig till acidos genom att ändra de typer av sockerkedjor som finns i proteoglykaner, vilket sker samtidigt som fettansamling inuti cellerna. Genom att ändra dessa sockerkedjor hämtar GBM-celler färre EVs från omgivningen. Men vad innebär denna omformning av sockerkedjor? Vi tror att GBM-celler skyddar sig från en toxisk överbelastning av fett genom att stänga denna

sockertäckta grind. Vad skulle hända om vi öppnar grinden igen? Kommer cellerna ge efter för okontrollerade fetthalter p.g.a. toxicitet?

Det är viktigt att nämna att även om vi hittar fett i GBM, så finns det inga bevis för att kosten påverkar GBM. Det finns inte heller något påvisat samband mellan obalanserad ämnesomsättning, t.ex. vid fetma, och GBM. Cancercellerna hittar sin väg för att erhålla näringsämnen på väldigt fantasifulla sätt. För att ytterligare exemplifiera detta visar vi i **Delarbete III** att rester från döda celler kan, från nekrosens *kyrkogård,* tas upp av levande celler. Detta ledde återigen till fettansamling och minskad effekt av strålbehandling i experiment med laboratorieodlade celler från GBM-patienter. Här testade vi olika läkemedels förmåga att förhindra fettackumulering inuti cellerna. Dessa behandlingar tog effektivt död på GBM-celler i laboratoriet. Resultaten motiverar oss att fortsätta vår forskning för att bättre förstå läkemedlens kliniska relevans. Är det fettätande GBM-celler, vilka framträder under svåra tumörförhållanden (hypoxi, acidos), som uppstår i återkommande tumörer, och där nya behandlingar bör riktas?

Till sist, i **Delarbete IV** utvecklade vi en strategi för att utreda vilka specifika proteiner som finns på ytan av GBM-celler och vilka av dessa proteiner som är *grind-öppnarna* till cellens insida varifrån läkemedlen aktivt kan döda dem. Läkemedel bör riktas mot ytproteiner som är betydligt mer förekommande, eller helst enbart i cancerceller, för att undvika oönskade effekter i normala celler. Vår strategi gör det möjligt att direkt identifiera dessa *grind-öppnare* i patienttumörer direkt efter operationen. Våra resultat visar att hjärntumörer skiljer sig mycket åt, även inom samma diagnosgrupp. Detta uppmuntrar oss att bygga vidare på denna teknik, vilken kan bidra till bättre skräddarsydda behandlingar för varje enskild GBM-patient.

Resumen simplificado

El glioblastoma es el tipo de tumor cerebral más común y agresivo en adultos y afecta a unas tres personas por cada 100.000. Todavía no sabemos exactamente qué factores promueven el desarrollo de glioblastoma, a menudo es una cuestión de "mala suerte". Dado que no podemos prevenirlo, es esencial disponer de tratamientos eficaces. Los pacientes con glioblastoma son operados y reciben quimio y radioterapia. Sin embargo, estos tratamientos no consiguen curar la enfermedad y el tumor suele reaparecer meses después. ¿Cómo pueden las células del glioblastoma *esconderse* o ser más fuertes que la terapia? Tenemos que entenderlo mejor para poder desarrollar tratamientos más inteligentes.

Los tumores están constituidos por muchas células individuales que se multiplican sin control. Estas células cancerosas se comportan como una comunidad *inteligente*. Intercambian *mensajes*, construyen *caminos* para llegar más lejos y *luchan* contra el sistema inmunitario (el ejército de células que nos defiende de los agentes patógenos, pero que también puede atacar a los tumores). Incluso reclutan células vecinas normales, no cancerosas, y las *convencen* para que promuevan el crecimiento del tumor. Cuando los tumores crecen más allá del suministro de nutrientes y oxígeno que llega por sangre, se produce el *caos*. Parte de este *caos* implica bajos niveles de oxígeno, llamado hipoxia, así como la acumulación de productos de desecho que conduce a lo que llamamos acidosis. En medio de este *caos*, se aplica la supervivencia del más fuerte. Las células que no se adaptan a este *caos* mueren. En glioblastoma, la acumulación de células muertas se llama necrosis.

En esta tesis doctoral he estudiado cómo el *caos* de hipoxia, acidosis y necrosis contribuye a la agresividad de las células cancerosas. En el **Artículo I** queríamos entender por qué las zonas de hipoxia en el tumor acumulan más grasa. ¿Qué sustancias conducen a esta acumulación de grasa en el tumor? ¿Y por qué en hipoxia? Descubrimos que las células interaccionan y atrapan *burbujas* provenientes de otras células. Llamamos "vesículas extracelulares" (VEs) a estas burbujas, y contienen material de la célula que las segrega. En hipoxia, las células ingieren más VEs que cuando hay oxígeno, y esto se acumula como grasa. En cuanto al mecanismo, descubrimos que las células atrapan las VEs a través de su interacción con ciertas proteínas de la superficie celular, llamadas proteoglicanos. Los proteoglicanos están constituidos por cadenas glicanos (de ahí su nombre), unas moléculas muy parecidas al azúcar, que son esenciales para su función.

En el **Artículo II**, descubrimos que las células se adaptan a la acidosis cambiando el tipo de glicanos que decoran su superficie, lo que va unido a la acumulación de grasa en el interior de las células. Al cambiar estas cadenas de glicanos, las células de glioblastoma no pueden atrapar VEs. Pero ¿qué significa esta remodelación? Creemos que las células de glioblastoma se protegen de la sobrecarga de grasas cerrando esta *puerta*. ¿Qué pasaría si abriéramos de nuevo la *puerta* reajustando los glicanos? La entrada incontrolada de VEs y otros nutrientes podría llevar a las células a sucumbir a la toxicidad por su almacenamiento.

Es importante mencionar que, aunque encontremos grasa en el glioblastoma, no hay evidencia probada de que las grasas provenientes de la dieta influyan en su incidencia, ni una asociación entre un metabolismo desregulado, como la obesidad, y el glioblastoma: las células cancerosas encuentran su manera de obtener nutrientes de las formas más imaginativas. Y para ejemplificar aún más esto, en el **Artículo III** mostramos que partes de células muertas de la necrosis pueden ser recuperadas por células vivas. Esto, de nuevo, conduce a la acumulación de grasa y a un menor efecto de la radioterapia en experimentos con células de pacientes de glioblastoma cultivadas en el laboratorio. Aquí, probamos diferentes moléculas que impiden la acumulación de grasa en el interior de las células. Estos tratamientos mataron eficazmente las células de glioblastoma en el laboratorio y nos motivan para continuar nuestra investigación y comprender mejor si tienen relevancia clínica.

Por último, en el **Artículo IV** optimizamos una estrategia para desentrañar las proteínas específicas que decoran la superficie de las células de glioblastoma, y cuáles de estas proteínas son las que *abren la puerta* al interior de la célula. Los fármacos deben diseñarse contra proteínas mucho más abundantes o, idealmente, exclusivas de células cancerosas, para evitar efectos indeseados en los tejidos normales. Nuestra estrategia permite identificar las proteínas de la superficie directamente a partir de tejido tumoral del paciente obtenido durante la cirugía. Nuestros resultados preliminares muestran que los tumores cerebrales difieren mucho incluso dentro del mismo diagnóstico. El desarrollo de esta técnica podría contribuir a mejorar los tratamientos personalizados para los pacientes con glioblastoma.

Glioblastoma

This PhD thesis work has focused on gaining better understanding of the cellular stress adaptation mechanisms in glioblastoma (GBM) and on exploring novel treatment targets. GBM is the most common and lethal malignant central nervous system (CNS) tumor in adults. Currently, GBM patients and their families face the dismal prognosis of a median overall survival of approximately 15 months and a 5-year survival rate of only 6.8% (1).

In the last decades, there has been extensive knowledge advancements on GBM biology, especially its heterogeneity and molecular characteristics. Despite this, current treatment protocols inexorably lead to tumor recurrence, occurring in median less than 7 months after diagnosis. Clearly, a new therapeutic arsenal is needed to tackle recurrent GBM cells, which have undergone adaptation to environmental stress factors and become irresponsive to therapy. Deciphering the molecular mediators of cellular stress adaptation and novel treatment targets are key challenges in the management of GBM.

Classification, epidemiology and etiology

GBM represents approximately 50% of all primary, malignant brain tumors in adults, with an overall age-adjusted incidence of 3.2 cases per 100 000 people (1). The median age for GBM diagnosis is 65 years, having a higher incidence in males than females (2). According to the 2021 WHO classification of CNS tumors, GBMs are classified as IDH wildtype, grade IV astrocytomas. IDH mutant astrocytomas, although having histological grade IV criteria, are no longer classified as GBM, since their prognosis is significantly more favorable. Several association studies have failed to identify what are the environmental factors that favor GBM development, with the only established risk factor of exposure to ionizing radiation and a protective factor, atopic diseases and allergy.

GBM patients succumb to the disease owing to the highly invasive capacity of GBM cells, which commonly infiltrate distant regions, such as basal ganglia, corpus callosum, and the brainstem (3).

Diagnosis, histopathology and molecular drivers

GBM patients are diagnosed when neurological symptoms appear due to displacement or infiltration to brain structures or increased intracranial pressure.

GBM symptoms can include progressive headaches, epileptic seizures or other neurological or behavioral changes, depending on the affected region of the brain (4). Contrast enhanced MRI manifests GBM lesions as enhancing, necrotic-appearing tumors with a rim of non-enhancing infiltrative tumor and edema (Figure 1).

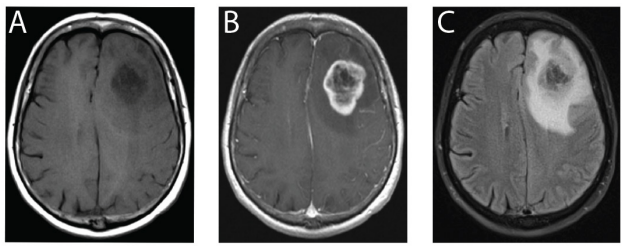

Figure 1. MRI is the primary diagnostic tool for GBM.
Axial T1-weighted GBM MRI appearance (A) and after gadolinium contrast enhancement (B).The central irregular hypo-enhancement is characteristic and reflects necrosis. (C) Axial T2-weighted FLAIR MRI. Signal intensity beyond enhancing region can represent edema or infiltration of lower grade tumor components. Reprint with permission from Tan A. C. et al. Ca Cancer J Clin 2020;70:299-312 (5)

Histopathology of GBM tissue biopsies is still the confirmatory tool for diagnosis, and virtually a requirement to allow oncological treatment decisions. Main characteristics of GBM include microvascular proliferation, vascular thrombosis, and extensive areas of pseudopalisading necrosis owing to its hypoxic nature and highly dense cellularity (Figure 2). Common genetic alterations in GBM affect cellular processes involved in initiating sustained growth [Ras/MAPK pathway *e.g*. EGFR (gene amplification, constitutively active EGFRvIII, or point mutations), PDGFRα, NF1; PI3K/mTOR pathway *e.g* PTEN (mutated or lost with chr 10q), PI3K3CA mutations], evading senescence (TP53, RB1) and enabling replicative immortality (TERT, mutated in approximately 85% of GBMs).

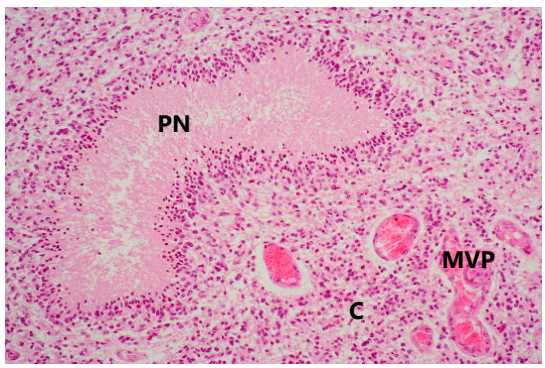

Figure 2. Classic histology of GBM
Classic histologic features of GBM include microvascular proliferation (MVP), pseudopalisading necrosis (PN) and dense celularity (C). Reprint with permission from Prof. Edward C. Klatt (webpath.med.utah.edu) (Modified).

Subclassification of GBM for better therapeutic tailoring has been a great focus of research over the last decade. Verhaak *et al.* proposed in 2010 a model of three transcriptional subtypes (proneural, mesenchymal and classical) with different molecular drives (6). However, despite their value as a framework for further research, the clinical relevance of these molecular subtypes is unclear. Single-cell RNA-sequencing studies have revealed that the complexity of GBM likely surpasses our ability to subclassify it, and there is evidence for multiple clones with different genetic, epigenetic and transcriptomic profiles coexisting within a single tumor (7–9).

To explain this degree of complexity, well-established models of tumor evolution include the clonal evolution model and the cancer stem cell (CSC) model. Although rare, a fraction of CSCs can be identified in GBM, largely based on the expression of CSC markers such as CD133, CD44 or Nestin and by their self-renewal potential (10–13). Importantly, CSCs reside in specific niches of GBM, namely the perinecrotic and the perivascular niches, which are pivotal for the maintenance of their stemness potential. However, the CSC model is challenged by numerous studies showing similar *in vivo* tumor forming potential, multipotency and self-renewal in GBM cells regardless of their CSC marker expression (14, 15), indicating that the CSC hierarchy model does not fully represent GBM. Currently, cellular states are seen as malleable and interchangeable, and GBM cell plasticity is importantly highly dependent on TME cues (16).

Current and novel therapies: Challenges and new avenues

First line treatment of GBM includes maximal safe resection, followed by chemotherapy with the DNA alkylating agent temozolomide (TMZ) and concomitant radiotherapy of 60 Gy in 2 Gy fractions over 6 weeks, followed by TMZ alone for 6 months (known as the "Stupp protocol") (2). Silencing of the enzyme O^6-methylguanine-DNA-methyltransferase (MGMT) gene expression by methylation of its promoter or loss of the second allele of chr 10 are predictive biomarkers of response to TMZ, which otherwise has limited or no effect. Since the Stupp protocol establishment (17) there have been few therapy improvements for GBM patients in the last decades. The implementation of tumor treating fields (Optune) (*i.e.* low intensity, intermediate frequency, alternating electric fields with anti-mitotic effects), which was added to first line treatment in Sweden in 2018 prolongs survival on average 4 months as compared to the Stupp protocol alone (18). The anti-angiogenic drug bevacizumab, targeting VEGF, despite the strong pro-angiogenic drive of GBMs, has no significant effect on overall survival (19).

Some of the major hurdles that dampen GBM therapy improvements include its anatomical location, which sometimes entails unresectable lesions in vital brain regions. Moreover, the blood-brain-barrier (BBB) further isolates the CNS and restricts the delivery of many therapeutic components to the brain. Over the last years, immunotherapies including chimeric antigen receptor (CAR)-T cell therapy

and checkpoint inhibitors have provided new hopes for several cancer types, including leukemia, malignant melanoma and lung cancer. For GBM, immune-based therapies in the neoadjuvant setting may have minimal survival benefit, but phase III trials with checkpoint inhibitors in addition to Stupp treatment have been negative (20–22). This can be explained by the relatively "cold" GBM tumor immune landscape, with negligible cytotoxic T cell infiltration (23), and an extensive innate immune cell infiltration. Immunosuppressive resident microglia and bone marrow-derived macrophages can constitute up to 40% of the tumor mass, and prevent immune cell targeting to GBM cells, hence presenting the innate immune cells as appealing GBM targets (23). As mentioned, one of the primary GBM treatment challenges resides in its inter and intratumor heterogeneity (24) with few treatments being effective for all patients with the same diagnosis (25). This highlights the difficulty of finding type-specific therapies and motivates our efforts for personalized approaches.

Importantly, the biggest challenge in the clinic resides in the handling of recurrent tumors, which often exhibit different transcriptional profiles than their primary counterparts (16, 26), and whose cells are survivors of strong selective pressures and refractory to chemo and radiotherapy. The harsh environmental characteristics of GBM are intrinsic selective pressures that are linked to several aspects of disease progression and a major focus for this thesis. They will be further presented in the next chapter.

The tumor microenvironment (TME)

Solid tumors develop in complex milieus that share common traits among different tumor types. This set of defining characteristics is known as the tumor microenvironment (TME), where malignant cells embedded in the extracellular matrix (ECM) coexist with tumor-supportive stromal cells, immune cells, endothelial cells and other cell populations. Beside oncogenic events, stress factors such as low oxygen tension (hypoxia) and low extracellular pH (acidosis) often arise and further drive tumor progression.

Hypoxia and acidosis

Sustained proliferation and resistance to cell death are likely the most characteristic hallmarks of cancer (27). Tumor cells rapidly outgrow their metabolite supply from the often malfunctioning tumor vasculature, leading to low oxygen availability, or **hypoxia**.

Reflecting the importance of oxygen availability for cellular normal functioning, the hypoxia inducible factor (HIF) family of transcription factors are well-conserved molecular sensors of intracellular O_2 that mediate overarching adaptive responses to hypoxia. The HIFs are heterodimeric proteins consisting of an O_2 sensitive alpha subunit and a constitutive beta subunit. Three HIF members have been characterized: HIF-1, HIF-2 and HIF-3, being HIF-1 and 2 responsible for the majority of the responses to hypoxia, while HIF-3 is much less studied. Transcriptional responses to HIF-1 and -2 greatly overlap, however, it has been proposed that the temporal dynamics of HIF-1 and HIF-2 activation may not coincide. HIF-1 is conceived to mediate acute responses to short-term hypoxia, while HIF-2 dominates responses to chronic hypoxia (> 24 h) (28, 29).

The combined work from Drs Kaelin, Ratcliffe and Semenza (30–32) and others described the presence of the HIF sensing pathway and its molecular components. In presence of oxygen, prolyl hydroxylases (PHD1-3) and one asparaginyl hydroxylase, factor inhibiting HIF (FIH), add hydroxyl groups to proline and asparagine residues of the HIFα subunit. This reaction is directly dependent on cytoplasmic O_2, oxoglutarate and Fe^{2+} ions. Hydroxylated HIFα subunits are diverted to proteasomal degradation by the specific binding of the von Hippel-

Lindau protein (pVHL) and further action of ubiquitin ligases (33). Under low oxygen tensions (< 2% O_2), HIFα is instead stabilized and translocates to the nucleus where it dimerizes with the β subunit, binding to hypoxia responsive elements (HRE) of target genes together with transcriptional co-activators (Figure 3). Importantly, the HIFs can be activated under non-hypoxic conditions, a phenomenon known as "pseudohypoxia". Redox stress may lead to HIF stabilization by ROS-mediated NFkappaB activation (34, 35), and extracellular acidosis boosts HIF responses by pH-dependent pVHL nucleolar sequestration (36). Finally, certain genetic defects similarly stabilize the HIFs. The most well-established example is the loss-of-function mutation of the VHL tumor suppressor gene, which provides constitutive HIF stabilization. This syndrome increases the vulnerability to develop cancer including clear cell renal cell carcinoma (ccRCC), further highlighting the pro-tumorigenic functions of the HIF cascade. HIF inhibitors are presently evaluated in clinical trials of various tumor types, and the HIF-2α inhibitor belzutifan was recently approved by the US Food and Drug Administration (FDA) for the treatment of patients with VHL loss-of-function mutations (37).

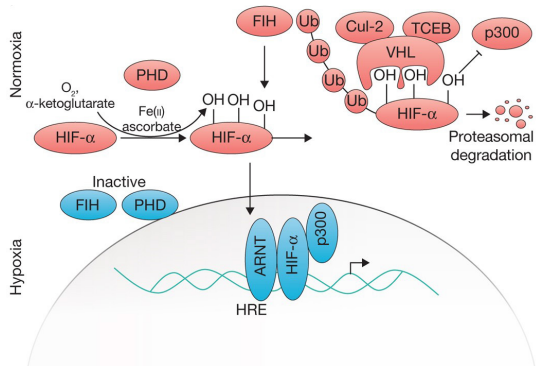

Figure 3 The HIF regulatory pathway in normoxia and hypoxia.
Reprint with permission from LaGory et. al Nat Cell Biol 18, 356–365 (2016) (38).

The transcriptional response to hypoxia is vast and involves the induction of angiogenic signaling, as the cell attempts to reestablish blood perfusion (*e.g.* VEGF), as well as promigratory and epithelium-to-mesenchymal transition (EMT) genes to escape hypoxic stress, maintenance of stem cell-like phenotypes (*e.g.* CD44), and immunosuppressive responses (*e.g.* Foxp3, PDL-1, CD47).

Of especial relevance for this thesis, hypoxia triggers a global metabolic rewiring involving induction of glycolysis genes (*e.g.* Glucose transporters to increase glucose import, pyruvate dehydrogenase kinase, blocking acetyl CoA (AcCoA) synthesis and tricarboxylic acid (TCA) cycle inhibition) (39) and lipid metabolism genes (*e.g.* CD36, FABP4, VLDLR, SR-B1 (40)) to maintain the cell's energetic

demands. In hypoxia, electron transport chain malfunctions, since O_2 is the last electron acceptor, and NADH accumulation leads to mitochondrial TCA cycle inhibition. As a result, the glycolysis end-product pyruvate is instead diverted for reduction to lactic acid by the enzyme lactate dehydrogenase. Lactic acid is mobilized to the extracellular compartment by monocarboxylate transporters (MCT-1,4), where it dissociates to lactate and a proton. Insufficient extracellular byproduct clearance leads to H^+ accumulation and low pH, or **acidosis** (Figure 4).

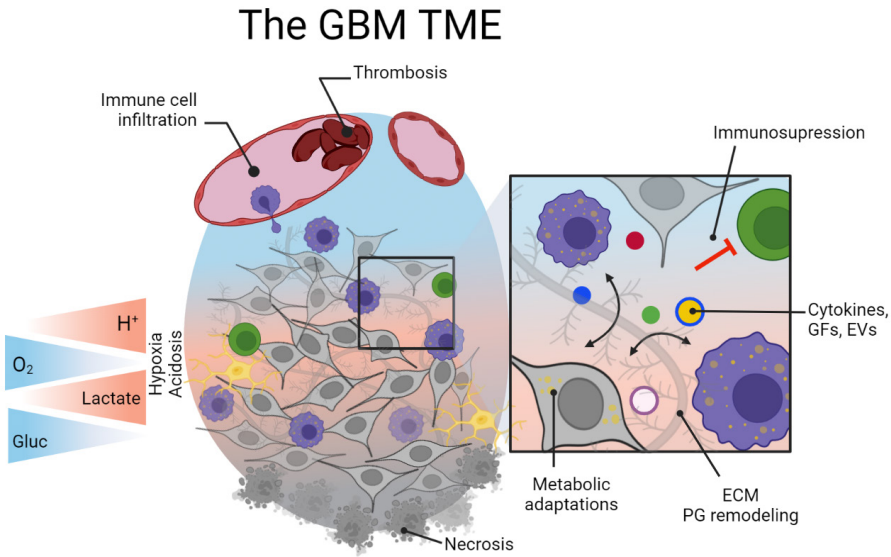

Figure 4: The tumor microenvironment in glioblastoma.
The GBM TME is characterized by areas of extreme hypoxia and acidosis (red) and neighboring pseudopalisading necrosis (grey). An interplay between tumor cells and other cell populations is mediated by soluble factors and EVs, and greatly orchestrates responses to TME stress including metabolic adaptation and ECM remodeling, vasculature proliferation or immune cell recruitment. Created with BioRender.com.

Notably, acidosis is not exclusive to hypoxic areas, the ability of cancer cells to preferentially use glycolysis for energy production even in aerobic conditions, known as the Warburg effect, can lead to extracellular acidification in well-perfused areas of the tumor. In fact, extracellular acidification is not only mediated by lactic fermentation and the glycolytic drive. In better perfused areas of the tumor, CO_2 produced from mitochondrial respiration diffuses to the extracellular space and its hydration by membrane carbonic anhydrase 9 (CA9) to bicarbonate and a proton ($CO_2 + H_2O \rightarrow HCO_3^- + H^+$).

Tumor acidosis is linked to malignant phenotypes including stimulation of metastasis, as we recently reviewed in Bång-Rudenstam *et al.* (41). Mechanistically, changes in intracellular pH during acidosis are linked to increased protease excretion by lysosomal traffic towards the plasma membrane (42). Matrix

metalloproteinases (MMPs) and cathepsins, whose activity is favored at acidic pH (43), enable migration and local invasion. Moreover, a transition towards EMT phenotypes is induced after *in vitro* culture in acidic pH in models of lung cancer (44) and melanoma, by induction of transforming growth factor-β (TGF-β) signaling (45). Important work from Corbet *et al*. has linked the acidosis promalignant phenotype to metabolic reprograming. Specifically, a link was established between the acidosis-driven EMT and metastatic potential to changes in lipid metabolism leading to intracellular lipid accumulation (46, 47)(47). We expand on this concept in **Paper II**, by revealing increased lipid accumulation and an EMT transcriptional profile in acidosis adapted GBM cells, concomitantly with increased tumor-forming capacity *in vivo*.

Lipid metabolism in the TME

Increasing evidence points towards a strong lipid dependency in cancer, especially linked to metastasis progression and resistance to therapies (41, 48, 49). This can be partly explained by the high energy density of lipids (full aerobic oxidation of 1 palmitate molecule leads to 129 ATP molecules) as compared to *e.g.* glucose (38 ATP molecules/glucose in aerobic conditions, only 2 ATP molecules in anaerobic conditions). Moreover, lipids are needed to build new cellular membranes and are involved in signaling. Since cytoplasmic free FAs and cholesterol are highly toxic, augmented lipid synthesis and/or scavenging leads to lipid accumulation in intracellular organelles known as lipid droplets (LDs), which store and regulate availability to cholesterol esters (CE) and triacylglycerols (TAGs).

Increased LD accumulation has been observed in several tumor types including prostate (50), kidney (51) and colorectal cancer (52). In GBM, magnetic resonance imaging revealed increased lipid accumulation compared to low grade gliomas or normal brain (53, 54), and LD abundance in patient GBM samples may correlate with poor patient survival (55, 56). The mechanisms through which LDs mediate pro-tumorigenic effects are multifaceted. LDs may protect cells from harmful saturated FAs (51) and may provide radiotherapy resistance by protecting against ROS (49).

Understanding the source for lipid substrates may constitute the first step towards the targeting of this cancer metabolic dependency. In GBM, increased *de novo* lipogenesis mediated by SREBP1 (downstream of PI3K/Akt/mTOR pathway, commonly altered in GBM) has been documented (56). TME characteristics can further promote lipid synthesis from AcetylCoA derived from reductive glutamine metabolism, a process increased by hypoxia (57). Importantly, acetate and lipid scavenging from the extracellular milieu provides readily available lipid species at a much lower energetic cost.

Pascual *et al.* showed that overexpression of lipid metabolism genes, including the hypoxia induced, FA-scavenging receptor CD36 correlates with higher metastatic potential, and specific antibody blocking lead to metastasis remission *in vivo* (58). Another hypoxia induced protein involved in lipid transport, fatty acid binding protein 4 (FABP4), associated with enhanced tumor growth by promoting FA internalization from adipocytes (59). Further emphasizing the role of hypoxia, elegant work from Bensaad *et al.* characterized a HIF1a dependent mechanism for FA uptake and LD storage in breast and GBM models (60). This becomes highly relevant in the context of cycling hypoxia, *i.e.* the condition of fluctuating O_2 levels according to shifting access to blood flow, since under reoxygenation conditions LDs were utilized and rendered a promalignant phenotype (60).

Other studies, including work from our lab have provided further evidence on the TME influencing lipid scavenging from higher molecular weight particles, namely lipoproteins (40) and extracellular vesicles (EVs) (**Paper I**). Increased LDL uptake after acute hypoxia or acidosis led to a LD-high phenotype with pro-metastatic behavior (40) and further motivated this thesis work. In **Papers I** and **II,** we investigate how hypoxia and acidosis modulate lipid scavenging from EVs, and in **Paper III** we investigate whether necrotic cell debris constitutes a source of metabolites, specifically lipids, in GBM cells.

Therapeutic potential of LD blockade

The numerous implications of the LD phenotype in cancer progression justify investigations of LD-targeting strategies as anti-cancer therapies. In GBM, targeting the enzyme ACAT1 blocks cholesterol esterification for storage in LDs and leads to cytotoxicity by ER-cholesterol accumulation and antitumor effects in *in vivo* models (56). In a later study by the same authors, Cheng *et al.* investigate the antitumor effects by blockade of diacylglycerol-acyltransferase 1 (DGAT1), *i.e.* a key enzyme in LD biogenesis that catalyzes the last addition of an acyl CoA to diacylglycerol (DAG), leading to TAG storage in LDs. DGAT1 expression appears increased in GBM, and pharmacological inhibitors or genetic silencing leads to cell killing by increased oxidative stress and lipotoxicity (61). In another, conceptually interesting recent study, Niticò *et al.* investigate LD pathway blockade for radiotherapy sensitization in breast cancer models. A population of LD-high cells irresponsive to irradiation were rendered sensitive when treated with DGAT2 inhibitors (62).

All in all, these and other studies motivate ongoing efforts to find therapeutic approaches that tackle GBM metabolic vulnerabilities. Unraveling what context-dependent cues lead to LD-dependencies in GBM (**Papers I-III**) will likely aid for improved drug design.

Necrosis

One of the direct consequences of poor blood perfusion leading to extreme hypoxia and starvation is the surge of necrotic tumor tissue. The procoagulant profile of GBMs due to *e.g.* high tissue factor (TF) expression in the TME further associates with necrotic establishment. Necrosis invariably correlates with more aggressive phenotypes and its potential as a diagnostic tool has been studied for several cancer types (63), including GBM (64).

Tissue necrosis is a histological trait identifiable in hematoxylin-eosin stainings by low cellularity and eosinophilic areas consisting of cellular fragments. Its presence has been the unique criterion for diagnosis of GBM *vs.* other CNS tumors for decades. GBM necrosis presents a serpentine-like morphology with a margin of live cells arranged side by side in structures resembling a palisade, conjunctively named as "pseudopalisading necrosis". Intriguingly, the interplay between GBM cancer cells and necrotic tissue is poorly understood. In fact, necrosis and hypoxia are intertwined processes, and their influence on cancer cells may be conjunctive.

Different cell death mechanisms can contribute to tissue necrosis. Besides well-described, caspase mediated apoptosis, necrosis has historically been conceived as an unprogrammed type of cell death that results in membrane breakdown and release of cell contents and damage-associated molecular patterns (DAMPs). Molecularly regulated forms of necrosis have been characterized, including necroptosis (MLKL mediated), pyroptosis (highly inflammatory, triggered by pathogens) or ferroptosis (iron mediated, triggered by lipid peroxidation).

Anticancer therapies reduce tumor burden by specific cancer cell killing leading to accumulation of dead cell components. Paradoxically, dead cells may provoke pro-tumor effects by proinflammatory signaling, *e.g.* co-incubation of colorectal cancer cells with necrotic cell debris led to increased tumor formation *in vivo*. Mechanistically, this was linked to osteopontin signaling to macrophages (65). More directly, work from Jayashankar and Edinger (66) recently showed that necrotic cell debris scavenging by tumor cells via macropinocytosis results in increased resistance to therapy. These and other studies stimulate the thinking that the pro-tumorigenic effects by necrotic debris may not exclusively be explained by immune-mediated responses, but also by direct metabolic rescue.

Several mechanisms for cell engulfment of cell parts or even whole cells (entosis) have been described (67); however, non-immune cells are not commonly conceived as prone to "cannibalism". In **Paper III,** we investigated whether necrotic material could constitute a source of nutrients for neighboring GBM cells.

The extracellular matrix

While the diversity of cellular constituents in GBM and other tumors has been profiled in great detail, the composition of the ECM and its exact role in cancer remains far less studied. This is mostly due to the biochemical complexity of the ECM and associated methodological challenges. With the increasing awareness of the central role of the ECM in cancer and other pathological conditions, more sophisticated approaches such as tissue decellularization have been developed. The brain ECM is substantially different from that of other tissues. Common ECM components including collagens, laminins and fibronectin are not as abundant in the brain, while it is mainly constituted by glycoproteins, and proteoglycans (PGs) such as heparan sulfate proteoglycans (HSPGs) and the glycosaminoglycan (GAG) hyaluronic acid (HA).

Alterations of the ECM composition are common in cancer (68) including GBM, where ECM components such as HA (69) and chondroitin sulfate (CS) PGs (70–73) are upregulated. ECM composition is as well tightly linked to maintenance of stem cell niches, including CSCs (74). Tumor hypoxia and acidosis influence ECM composition by direct induction of ECM components, including HIF1-upregulation of HA, or by favoring the release and extracellular activity of ECM-modifying enzymes (*e.g.* MMPs, cathepsins, lysyl oxidase) with acidic pH optimums (75). This has direct consequences for cell migratory and invasive capabilities. In addition, cancer cell-ECM interactions also influence outside-in signaling by mechanotransduction via *e.g.* focal adhesion kinase (FAK). Importantly, GBM ECM stiffness directly correlates with tumor grade *via* the hypoxic induction of Tenacsin C (76), and the response to radiation therapy can influence ECM deposition by GBM-associated astrocytes, supporting glioma cell stemness (77).

The intertwined relation between TME cues and the ECM and peri-cellular matrix composition by *e.g.* PG remodeling, as investigated in **Paper II**, likely orchestrates what extracellular signals reach the cell and potentially also the area of influence of the secreted cues.

Proteoglycans

PGs are highly complex macromolecules where a core protein is covalently decorated with sulfated GAG chains[1] of diverse length and composition. PGs are involved in numerous physiological processes such as embryonic development (78, 79) and serve as key structural ECM components in pathology, including atherosclerosis, infectious disease and ECM remodeling in cancer. Even if PG core proteins can also participate in protein-protein and protein-glycan interactions (80), the highly sulfated, polyanionic GAG chains have an overarching role as binding partners for a plethora of bioactive compounds, spanning from morphogens and growth factors to pathogens and lipid particles.

Plasma membrane associated PGs include transmembrane PGs (syndecan family, CSPG4, CD44, CA9, among others) and glycosylphosphatidylinositol (GPI)-anchored PGs (glypicans), which together with other glycoconjugates such as glycolipids and glycoproteins form the pericellular matrix or glycocalyx. Extracellular GAGs and PGs (HA, versican, *etc*.) are major components of the ECM. Overall, pericellular and extracellular PGs largely influence the cell's availability to stimuli and nutrients from the extracellular milieu, and regulate other cell processes such as adhesion and migration.

PG complexity lies not only in their dual composition of protein and glycan, their diverse functions and abundant ligand partners, but also in the fact that the central dogma of molecular biology does not apply to their biosynthesis. From one protein coding gene, different PGs can arise depending on their GAG substitution and sulfation pattern, which is the net result of multiple enzymes. Furthermore, PGs cannot be described in solitude since their end function is greatly impacted by the environment, *i.e.,* the extracellular electrostatic charge distribution, which may influence ligand binding kinetics; or the availability of partner receptors for signal transduction. One example of this is the well-established HSPG-FGFR symbiosis (81–83), where FGF is presented to its receptor via interaction with GAG chains of cell-surface PGs (84). Moreover, GAG modifications may occur in other proteins outside of *bona fide* PG core proteins giving rise to so-called "part-time" PGs. GAG

[1] Hyaluronan (HA) is considered a PG, however, it is not covalently bound to a core protein and exists as a free GAG that binds other PGs through their N-terminal domains. Notably, HA is not sulfated.

substitution of CA9 has been shown to influence its subcellular localization and endocytic profile with implications for antibody-drug conjugate delivery (85).

PG diversity and implications in cancer

An orchestra of more than 20 glucosyltransferases, O- and N-sulfotransferases, and epimerases in the ER and Golgi (86), as well as intra and extracellular sulfatases (87) generates the finely tuned and diverse GAG chains. According to their saccharide composition, different families of GAGs exist: CS, dermatan sulfate (DS), keratan sulfate (KS), HS, heparin and HA. Major PG categories of special relevance for this thesis are the HSPGs and CSPGs, which harbor GAG backbones composed of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) and GlcA, respectively (Figure 5).

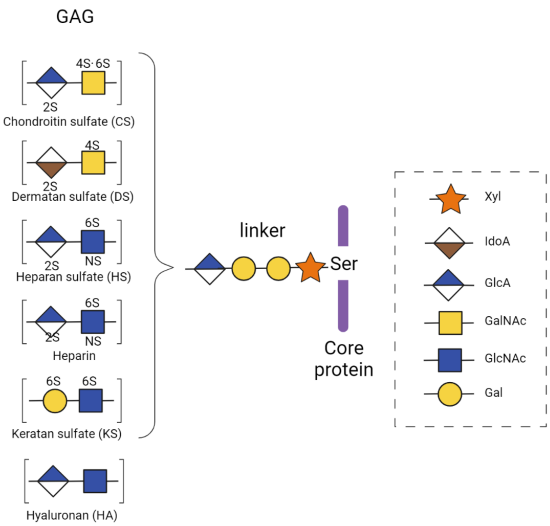

Figure 5. Composition of major GAGs
Xyl, xylose; IdoA, iduronic acid; GlcA, glucuronic acid; GalNAc, N-acetyl galactosamine; GlcNAc, N-acetyl glucosamine; Gal, galactose; S, sulfate; Ser, serine residue. Created with BioRender.com.

PG remodeling in cancer is well-documented, although not in great detail, and contributes to different aspects of cancer pathogenesis (88, 89). Core protein expression changes have been studied as biomarkers for various tumor types, including in GBM where Syndecan-4 (SDC-4) was proposed as a marker of therapy response (90). Work from our group showed that the levels of another member of the syndecan family, SDC-1, present in circulating EVs could differentiate between GBM and low grade gliomas (WHO grade II) (91). The TME largely influences PG expression, and hypoxia-induced HA associates with higher proliferation, migration and immune evasion in GBM (92), and favors stem like cell properties (93), possibly by the interaction with its receptor and stem cell marker CD44 (94). Importantly, a

preferential GAG substitution by CS associates with increased tumor aggressiveness and poor prognosis in breast and renal cancer (95, 96), and induction of DS epimerase (DSE), the enzyme that converts CS into DS, is common in several tumor types (97, 98).

PGs as scavenging receptors

The involvement of PGs in ligand internalization is well established both as facilitators of uptake, *i.e.* by binding and presenting to relevant uptake receptors, but also as direct mediators of internalization. Our lab found first solid evidence for HSPGs as sole uptake receptors using an array of single chain fragment variable (scFv) antibodies directed to different HS epitopes. Anti-HS uptake was somewhat epitope specific and HS antibodies co-localized with HSPGs in endosomal compartments (99). The mechanisms that control HSPG internalization are not fully understood, but HSPG clustering is necessary for endocytic vesicle formation in cholesterol rich membrane microdomains *i.e.* lipid rafts. Therefore, HS epitope uptake selectivity may be partly explained by a differential ability to trigger PG clustering in lipid rafts.

Several functionally relevant ligands enter cells via PGs, and both glypican and syndecan family members can mediate nanoparticle uptake (100). A well-studied example is the clearance of lipoproteins (specifically LDL) from circulating blood by the liver, which is mediated by LDL binding to HSPG receptor SDC-1 and presentation to LDL receptor. This was shown *in vivo* as KO mice for HSPG accumulate remnant LDL in the circulation (101–103). Additionally, internalization of various pathogens including bacteria (104) and viruses like the herpes virus simplex, human immunodeficiency virus (105, 106) or coronavirus such as the SARS-CoV-2 (107) depend on HSPG for efficient cell entry. Other polybasic compounds, including polyamines (108) and cell penetrating peptides (109) have similarly proven to depend on HSPG for their internalization.

Especially relevant for this thesis, work from our lab unraveled how HSPGs are major internalizing receptors of EVs in cancer cells (110). However, how TME characteristics influence HSPG-mediated EV uptake remained unclear. In **Papers I** and **II,** we investigate how cellular responses to hypoxia and acidosis influence EV uptake by cancer cells.

Several questions regarding PG remodeling and function in cancer remain open. *What are the cell intrinsic (e.g. signaling, metabolic status) and extrinsic (e.g. environmental stress, matrix interactions) cues that regulate PG remodeling?* Besides the established hypoxic induction of HA and CD44 (111, 112), transmembrane PG core protein expression and cell surface HS/CS abundance do not seem to be altered by short term hypoxia in glioma cells (8; **Paper I**). In **Paper II,** we found that more long-term adaptation to low pH globally alters GAG synthesis enzymes leading to a strong induction of CS expression. *How do changes*

in GAG composition and sulfation pattern affect ligand interaction, signaling and uptake? And most importantly, how do these changes translate functionally? It is conceivable that changes in GAG composition and charge distribution can lead to altered ligand distribution and availability to cells. As a relevant example, CS chain digestion by recombinant chondroitin ABC lyase expression in GBM tumors improved TMZ penetrance to cancer cells and increased cell killing in *in vivo* models (113). Whether cell-surface CS eradication contributes to cell toxicity through other mechanisms cannot be ruled out from the mentioned study.

Extracellular vesicles

Eukaryotic cells are characterized by a sophisticated endomembranous system including the nucleus, Golgi, ER, endolysosomes and an intricate vesicle system for intercommunication between the different compartments and with the extracellular milieu (Figure 6). According to the endosymbiotic theory, endocytosis stands at the very origin of the eukaryotic cell, being one of the most well-conserved and robust mechanisms in eukaryotic cells. Several vesicular pathways defined by different molecular mediators coexist to ensure retrieval of nutrients and signals from the environment. Of relevance for **Papers I** and **II** of this thesis work, the endomembranous system expands beyond the plasma membrane, by the secretion of exosomes and other EVs to the extracellular milieu.

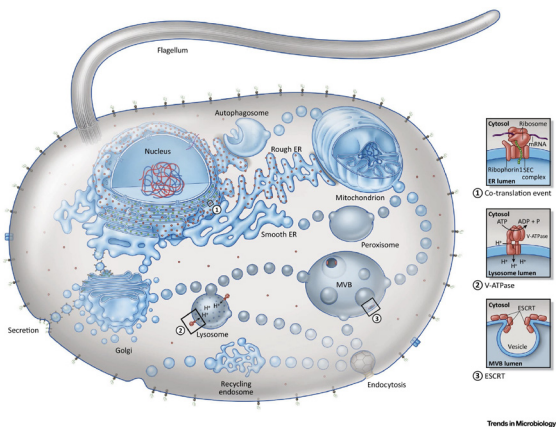

Figure 6. The eukaryotic cell
An intricate endomembranous system defines eukaryotic cells. Licensed reprint from Gould *et al.* Trends in Microbiology, July 2016, Vol. 24, No. 7 (114)

The description of EVs challenges the definition of eukaryotic cells being delimited by their plasma membrane. Instead, cell compartmental diffusion via EVs broadens our understanding of cell evolution towards a more advanced network of intra and extra-cellular vesicle exchange.

Cell to cell communication allows individual cells to reach global responses in physiology and disease by coordinating signaling processes and effector molecules. Besides soluble molecules and cell-cell interactions, compartmental exchange by

EVs is a pivotal type of intercellular communication, with special relevance in cancer.

EVs define a highly diverse group of lipid bilayer enclosed vesicles secreted through different mechanisms and with varying sizes and properties. They carry intracellular cargo, reaching to the secreting cell's vicinity and also distant cells through systemic circulation (115, 116).

Increased EV secretion has been reported in several cancer types including melanoma, breast and prostate (117–119) and further fostered by TME hypoxia (120) and acidosis (121). Interestingly, EV composition mirrors that of the secreting cell and cancer cell derived EVs can transfer oncoproteins and RNA leading to tumor promoting effects in recipient cells.

EV diversity: Biogenesis and main characteristics

EVs can be categorized based on size and density, parameters often used in experimental EV purification protocols (122), or according to their biogenesis pathway, mainly microvesicles/ectosomes , originating after outward budding of the plasma membrane, and exosomes, which originate from the endolysosomal compartment after multivesicular body (MVB) fusion with the plasma membrane. Apoptotic bodies result from cell blebbing of apoptotic cells (Figure 7). The cell surface HSPG SDC1 is involved in exosome biogenesis via the interaction of its intracellular domain with syntenin. Further docking of Alix in the so-called SDC1-syntenin-alix axis triggers membrane invagination, and together with ESCRT components in MVBs lead to intraluminal vesicle (ILV) generation for further excretion as exosomes (123). SDC1 HS chain digestion by extracellular heparanase favors SDC1 clustering and enhances EV biogenesis via the SDC1-syntenin-alix axis (124).

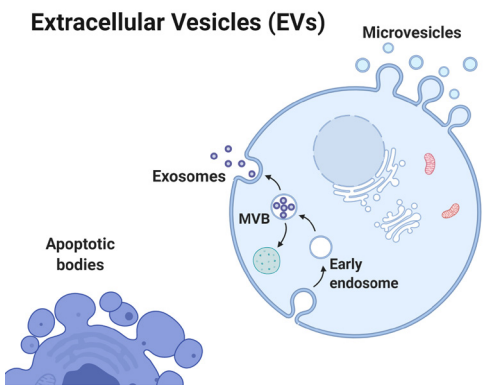

Figure 7. Major types of extracellullar vesicles.
Major EV subtypes include exosomes, derived from multivesicular body (MVB) fusion with the plasma membrane, microvesicles originated by outward membrane budding, and apoptotic bodies, encapsulating cellular material of dying cells.Created with BioRender.com.

The different EV populations are enriched in specific cargo molecules, providing molecular markers to distinguish them (Table 1). Exosomes have been extensively studied as a major type of EVs, however, it is generally hard to distinguish them among other small EV populations. For these reasons, when endosomal origin cannot be confirmed, the term EV is encouraged.

Table 1. EV categories and their markers
EV categorization is challenging due to overlapping characteristics and suboptimal currently available purification methods.When EV subtype cannot be confirmed, the term EV is recommended.

EV type	Density (g/mL)	Size (nm)	Biogenesis	Enriched cargo
Exosomes	1.13-1.19	20-100	Inward budding of late endosomes leads to MVB biogenesis. MVBs fuse with plasma membrane, releasing exosomes.	Tetraspanins (CD9, CD81, CD63), Syntenin, TSG101
Microvesicles	1.03-1,07	50-1000	Outward budding from plasma membrane.	CD40 ligand, ARF6, VAMP3, integrins
Apoptotic bodies	1.16-1.28	50-5000	Apoptotic cell membrane budding.	Phosphatidylserine, annexin V, nuclear vesicles, whole cell organelles

EV functions in cancer: Waste products, signalosomes and nutrient carriers

EVs are packed with bioactive molecules including miRNAs, proteins, lipids and PGs with signaling transduction potential. Therefore, the role of EVs as signalosomes has been the major focus in the field. First, EVs can mediate signaling pathway activation by direct contact with surface receptors in target cells. The signaling potential of EVs may not be restricted to EV docking at the cell surface, and after eventual internalization, EVs may potentially fire signal transduction even at the endosomal compartment (125). Second, transfer of functionally active molecules via EVs can occur after endosomal escape of molecules from internalized EVs. An example of this is the early discovery of EV-associated mutated EGFR, EGFRvIII, transfer to recipient glioma cells, maintaining its oncogenic signaling properties (126). Of special relevance for this thesis, EVs are known to mediate cell adaptation to stress in the TME. Hypoxic cell derived EVs mediate pro-angiogenic signaling to endothelial cells, in a PAR2 dependent manner (127). Big part of the EV research community has focused on studying the effects of specific EV-miRNAs influencing transcriptional profiles in recipient cells. However, stoichiometric studies indicate that the amount of miRNAs carried and transferred by EVs is likely insufficient to lead to physiologically relevant concentrations of miRNAs after EV uptake (128). A plausible explanation for the observed responses may be that they are product of cumulative effects from cargo transfer and EV-signaling processes.

As opposed to the canonical model of signal transduction by one ligand-receptor pair, EV mediated responses can result in more complex signaling cascades. First, because of their size, EVs may facilitate receptor oligomerization in plasma membrane microdomains, enhancing downstream kinase signaling. Moreover, the presence at the EV surface of agonists, antagonists, chaperone proteins and GAG-associated molecules likely fine-tunes the cues delivered. These different levels of complexity hinder targeting of EV signaling in the TME.

The ability of EVs to mediate paracrine signaling and cargo transfer in the cell's vicinity and at distant sites motivates the evolutionary drive that has perpetuated EV-communication in eukaryotic cells. Furthermore, EVs may represent a way to avoid cell toxicity by sorting out harmful or unnecessary intracellular material. This is supported by some studies that identified metabolic waste products and misfolded proteins in EVs (129). The sorting of beta-amyloid fragments in EVs during Alzheimer's disease progression may be a defensive mechanism to avoid cell toxicity, which instead may further promote the disease in a prion-like manner (130).

Given that a big portion of the EV content that is internalized by endocytosis is digested in lysosomes, a conceivable hypothesis is that EV contents can provide substrates for anabolic processes or, depending on the cell's metabolic status, be metabolized for energy obtention.

In **Papers I** and **II**, we investigate the metabolic potential of EV content by cancer cells under tumor microenvironment conditions. In **Paper I** we find that hypoxic cancer cells upregulate EV uptake leading to lipid transfer and accumulation in LDs. Understanding which parameters dictate whether EV content will be directed for lysosomal degradation or for endosomal escape should motivate further research.

EV uptake

Many of the functions of EVs are exerted by the transfer of content to recipient cells, which can occur via several different mechanisms (122). These include direct membrane fusion at the cell surface (131), macropinocytosis (132) and receptor mediated endocytosis (110, 133, 134). The presence of overlapping redundant mechanisms for EV uptake demonstrates that it is a well-conserved mechanism for eukaryotic cell homeostasis, while its robustness complicates the development of targeting approaches for efficient inhibition.

The TME influences several aspects of EV biology such as EV secretion (120) and content sorting (135), and in this thesis work, we hypothesized that TME characteristics may likely also influence EV uptake. In **Papers I** and **II,** we investigated how tumor hypoxia and acidosis affect EV uptake and its functional consequences.

EV uptake receptors and ligands

Several cell receptors have been proposed to mediate EV internalization, likely owing to the vast array of proteins exposed at the EV surface. These include integrins (136), phosphatidylserine receptor Tim4 (137) and HSPGs (110), which may be of varying relevance in different cell types. Work by Christianson *et al.* showed that EV uptake depends on docking to HS chains, while CSPGs seemed not to trigger internalization. PG members of both glypican and SDC families could mediate EV uptake. Despite different cell receptors are proposed to mediate EV uptake, the identification of protein partners on the surface of EVs remains a challenge, and several proteins harboring polybasic domains are likely to interact and mediate HSPG-dependent EV uptake. Several groups have suggested a "sandwich" model for EV uptake where glycosylated EV proteins and PGs bind intermediate proteins (sometimes referred to as protein corona) during dispersion or circulation that then can bind to cell receptors for uptake (138). Several groups have proposed that the biodistribution and homing of cancer-derived EVs may be explained by differential EV cargo such as integrins (136) or tetraspanins (139).

EVs as biomarkers

Overall, it is conceived that EV cargo mirrors that of the secreting cell and even if the machinery that participates in molecular cargo sorting to EVs is not fully understood it is somewhat established that sorting can be specific (122).

The presence of cancer-associated proteins and miRNAs in EVs has raised considerable interest in studying the potential of EVs as biomarkers for non-invasive cancer diagnosis. Cancer EVs reach systemic circulation and other bodily fluids including cerebrospinal fluid (CSF) (140) and urine (141), thus being accessible non-invasively. Despite better isolation techniques are needed to purify tumor-EVs among the circulating EV pool, tumor-EVs may still be overrepresented in plasma due to the increased secretion by cancer cells, and several studies propose EVs as early diagnostic tools (142). Work from our group found that SDC1 levels in circulating EVs were able to differentiate between GBM and low grade gliomas (WHO grade II) (91) (Figure 8). Circulating EVs may appear as dynamic tools for monitoring of tumor aggressiveness or response to treatment. We found that a hypoxia profile is found in EVs in *in vitro* models (143) and levels of cancer-EV markers decrease after tumor resection (91). As current proteomic techniques keep improving, we may see in the future whether tumor specific antigens are sorted to and detectable in circulating EVs, aiding in the search for personalized treatments.

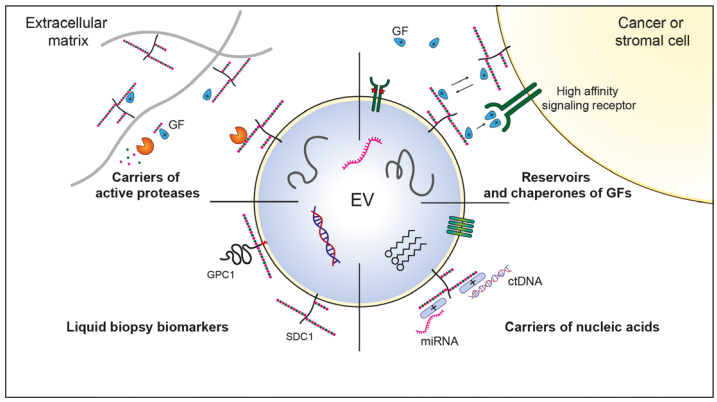

Figure 8 EV-PGs as molecular carriers and biomarkers.

The highly anionic glycosaminoglycan (GAG) chains of PGs present on the EV surface represent low affinity, high capacity binding surfaces for a wide variety of proteins containing polybasic domains. Different matrix degrading enzymes like heparanase and matrix metalloproteases (MMPs) have been found present on EVs, either as transmembrane proteins or electrostatically bound to PGs. EV-associated matrix proteases have the potential to remodel the tumor extracellular matrix, and regulate growth factor signaling, which could have consequences in tumor cell invasiveness and proliferation (*top left*). HSPGs on the surface of EVs can act as dynamic molecular reservoirs of *e.g.,* growth factors (GFs) and other signaling molecules (*top right*). Similarly, polybasic proteins bound to EV-PGs can serve as carriers for nucleic acids such as miRNA and circulating tumor DNA (ctDNA) that can trigger intracellular signaling responses and have biomarker potential (*bottom right*). Finally, EV-PGs have themselves great potential as cancer biomarkers for liquid biopsy diagnostics (*bottom left*). GPC1, glypican-1; SDC1, syndecan-1.Text and image from Cerezo-Magaña *et. al*. Semin Cancer Biol. 2020 May;62:99,(ref. 144), licensed under a CC-BY-NC-ND 4.0 license. http://creativecommons.org/licenses/BY-NC-ND/4.0/

Surface target identification and novel cancer therapies

The endomembranous system has evolved along with different intercellular pathogens such as bacteria and viruses that hijack this machinery to infect cells (145). In the case of viruses, key steps of their cycle occur in intracellular membrane compartments, finally leading to secretion to the extracellular space. As described above, EVs constitute an endogenous source of secreted membrane compartments for intercellular communication. Several endocytic pathways are altered in cancer (146) and are further influenced by the TME as well as oncogene activated signaling pathways and the metabolic status of the cell (147, 148). Importantly, membrane transport mechanisms may be harnessed to deliver cytotoxic payload for cell killing with special relevance in cancer therapy development.

A key step for specific delivery of cytotoxic drugs to tumor cells is to identify which surface proteins should be targeted for an efficient intracellular delivery of the cargo. This is of special relevance in the development of novel cancer therapeutics such as antibody-drug conjugates (ADCs) and antibody-radionuclide conjugates where monoclonal antibodies directed to tumor specific surface antigens are internalized for cytotoxic payload delivery.

Since the fist approval of the ADC gemtuzumab ozogamicin for the treatment of acute myeloid leukemia in 2000, 14 ADCs have been approved for the treatment of solid and hematological tumors, and over 100 ADCs are currently at different stages of clinical trials (149).

In addition, unraveling the TS has the potential to aid in the development of other cancer therapies including CAR-T cells, nanoparticles, engineered EVs (“trojan EVs”), oncolytic viruses, and other modalities for cell killing including antibody-dependent cellular toxicity (ADCC). Our work in **Paper IV** describes the development of a method to unravel the tumor surface proteome (tumor surfaceome, TS) in intact tumor tissue, further allowing the measurement of its endocytic activity.

The present investigation

This PhD thesis was aimed at gaining a better understanding of the mechanisms that mediate glioma cell adaptation to environmental stress with the overall goal to identify potentially targetable vulnerabilities. A focus is put on elucidating novel scavengeable nutrient sources that can fuel the pro-malignant LD+ phenotype in stressed glioma cells. Finally, with a clearer therapeutic aim, we set out to develop a methodology for surface target identification using *in vitro* models and intact mouse and patient brain tumor specimens.

Specific aims of the Thesis

- To study the impact of tumor hypoxia and acidosis on EV uptake in malignant GBM cells. - **Papers I** and **II**.
- To mechanistically describe EV uptake regulation under hypoxia and acidosis. - **Papers I** and **II**.
- To characterize the changes in PG expression and turnover under hypoxia and acidosis. - **Papers I** and **II**.
- To elucidate the role of EVs and necrotic cell-derived debris as nutrient sources for GBM cells. - **Papers I-III**.
- To develop a method for surfaceome and endocytome target characterization from intact tumor specimens. - **Paper IV**.

Main results and conclusions

The rationale behind **Papers I-IV** and their main results are summarized here. See **Papers I-IV** in appendix for detailed results and data interpretation.

Paper I

Hypoxic induction of exosome uptake through proteoglycan-dependent endocytosis fuels the lipid droplet phenotype in glioma

The contribution of EVs in metabolic adaptation to hypoxia is not well understood, and we hypothesized that EVs, in addition to their signalosome function, can serve as a nutrient source. In **Paper I**, we show for the first time that scavenging of GBM cell derived EVs can lead to the pro-malignant lipid droplet loaded (LD+) phenotype, often found in GBM but not in low grade gliomas (40). Importantly, EV-mediated LD accumulation was dependent on the symbiosis with environmental hypoxia, a common characteristic of GBM. We found that hypoxic GBM cells internalized more EVs and we could show by confocal imaging that upon internalization, FAs from EVs were sorted into intracellular LDs (Figure 9).

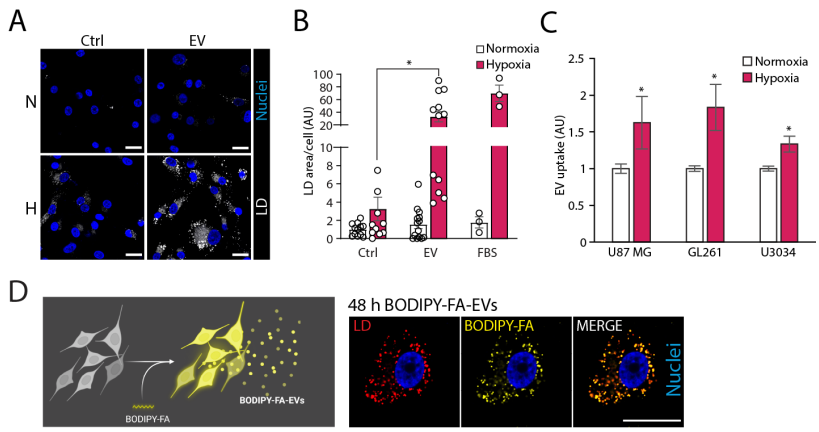

Figure 9. Increased EV uptake triggers the LD+ phenotype in hypoxic GBM cells.
A) LD staining after 48 h incubation with EVs (50 µg/mL) in normoxia (N) or hypoxia (H, 1% O_2), and (**B**) corresponding LD area quantification from n >150 cells, compared to 10% FBS. **C**) PKH67-EV uptake quantification in U87MG, GL261 and U3034 cells. **D**) EV-derived FAs are homed into LDs in recipient cells. *=p<0.05.

Mechanistically, EV uptake in hypoxia was mediated by cell surface HSPGs and, regardless of the strategy used, HSPG inhibition resulted in a decreased EV uptake magnitude and reversal of the hypoxic induction. Notably, basal anti-HS antibody uptake was also increased by hypoxia, indicating an overall upregulation of HSPG internalization in stressed glioma cells. Hypoxic cell EV uptake occurred mostly in a lipid-raft dependent manner and we could show that internalized EVs co-localized with the major internalizing HSPG SDC1 and lipid raft endocytosis marker CtxB, indicating an involvement of this pathway in hypoxic EV scavenging (Figure 10).

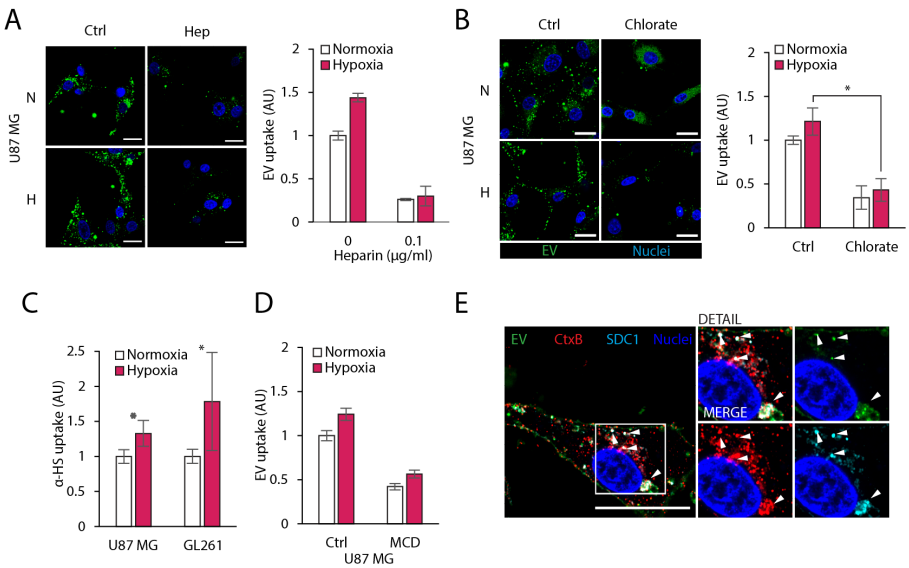

Figure 10. HSPG and lipid-raft mediated EV uptake in hypoxia
The increased EV uptake in hypoxia is reversed by HSPG competitor heparin (Hep) (**A**) or HSPG sulfation inhibitor sodium chlorate ($NaClO_3$) (**B**), as indicated by confocal microscopy and flow cytometry quantification. **C**) Anti HS scFv antbody complex uptake quantification in U87MG and GL261 cells. **D**) EV uptake quantification after 2 h treatment with the cholesterol depleating drug MCD. **E**) U87MG cells were incubated on ice with primary anti-SDC1 antibody for surface SDC1 labeling, and then PKH67-EVs (50 µg/mL) and CtxB-AF546 were added at endocytosis-permissive conditions. After 30 min, cells were extensively washed and fixed, and co-localization was assessed by confocal microscopy.

Paper II

Tumor acidosis metabolic adaptation involves the induction of a chondroitin sulfate shield against lipid particle scavenging

Paper II is focused on the adaptive responses of cancer cells to long-term extracellular acidosis, which together with hypoxia is a feature of malignant solid tumors. Global transcriptomic analysis of acidosis adapted (AA) GBM and pancreatic cancer cells revealed that adaptation to acidosis entails changes in PG expression, including GAG modifying enzymes and core proteins, as well as altered expression of lipid metabolism related transcripts. The observed changes in mRNA expression of major CS-related enzymes prompted us to investigate CSPG expression in AA cells, finding a robust CS induction in 2D and 3D models. Importantly, the CS phenotype coincided with LD+ cells, *in vitro* and in patient GBM material (Figure 11).

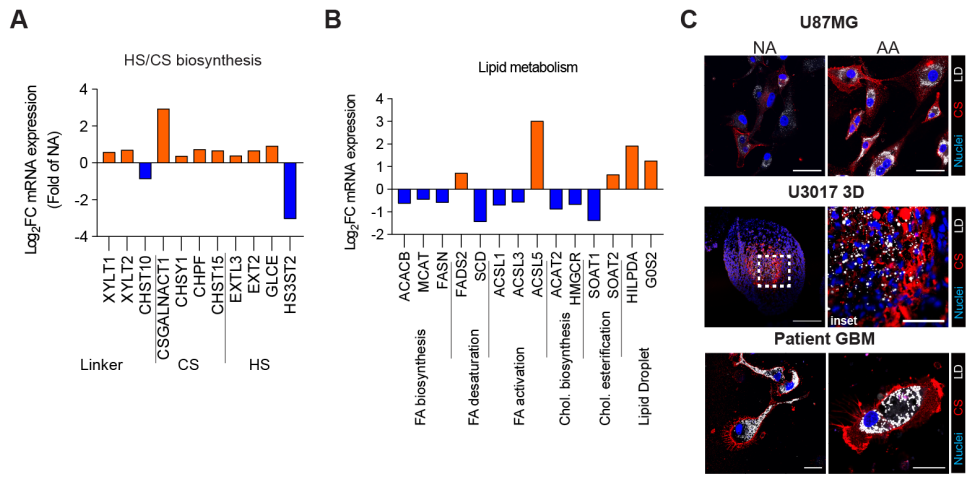

Figure 11. Transcriptional changes during acidosis adaptation lead to a LD+ CS-high phenotype
Bar graph of significantly (p<0.05) up or down-regulated expression of genes involved in HS/CS biosynthesis (**A**) or lipid metabolism (**B**), represented as Log2FC in AA *vs* NA U87MG cells (cutoff ± 0.5 log2 Fold change). **C**) CS and LD staining of NA and AA U87MG 2D cultures, U3017 3D spheroids or patient derived GBM single cell suspensions. Scale bars, 20 µm (top and lower panel); 200 µm (50 µm inset) (middle panel).

Additionally, we found downregulation at the mRNA and protein level as well as decreased uptake of the major internalizing HS/CSPG SDC1. Together, the acquired CS-high, LD+ phenotype in AA cells translated into a decrease in lipid particle (LP) uptake, including EVs and LDL. We introduce the hypothesis that cell build a "CS shield" to avoid lipotoxicity at acidic conditions. Finally, pharmacological normalization of CS expression in AA cells reverted the LP uptake levels, hence suggesting a direct link between the CS-high and LP uptake-low phenomena (Figure 12).

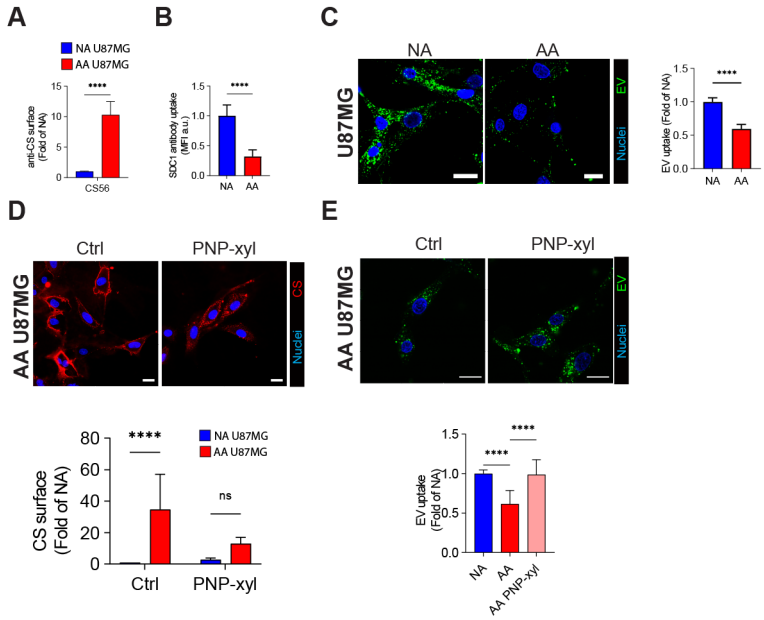

Figure 12. The CS-high phenotype leads to decreased exogenous lipid particle uptake
FACS quantification of anti-CS antibody cell-surface staining (**A**) or SDC1 antibody uptake (**B**) in U87MG NA and AA cells. **C**) Decreased EV-PKH67 uptake in AA U87MG cells visualized by representative confocal images and quantified by FACS. **D**) CS staining in AA U87MG cells after PNP-xyl treatment assessed by confocal (upper panel) and quantified by FACS (lower panel). **E**) EV uptake visualization by confocal (upper panel) and quantified by FACS (lower panel) after treatment with PNP-xyl, as in **E**. AA, acidosis adapted; NA, non-adapted. Scale bars, 20 µm.

Paper III

Glioblastoma tumor necrosis: A smörgåsbord of scavengeable nutrients as potential fuels for the lipid droplet phenotype, increased proliferation and radioresistance

Tumor necrosis is a histopathological hallmark of GBM, which arises after nutrient starvation and hypoxia due to poor blood perfusion and thrombosis. The interplay between necrotic cell debris, *i.e.* a potentially recyclable nutrient source, and the neighboring, pseudopalisading viable cells is virtually unknown. In **Paper III,** we postulated that necrotic cell debris could constitute a reservoir of scavengeable nutrients for GBM cells. We optimized a protocol to separately label nucleic acids, proteins and lipids in necrotic debris and track them after internalization by recipient cells.

GBM cells actively internalized necrotic cell debris containing proteins, nucleic acids and lipids and this may promote cell proliferation in established and

patient-derived primary GBM cell lines (Figure 13). Interestingly, we observed that pre-incubation with necrotic debris may protect GBM cells from irradiation, a first line treatment for GBM. Since the LD phenotype stands in the nexus between lipid scavenging and aggressiveness including therapy resistance, we then investigated LD content after debris uptake. Our data suggested a robust LD induction by debris, which varied among different *in vitro* models.

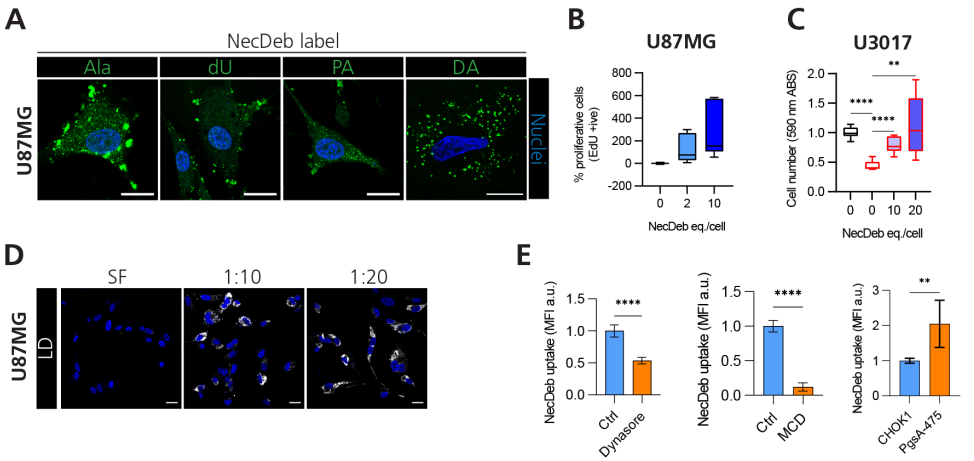

Figure 13 NecDeb uptake characterization
A) Necrotic debris (NecDeb) internalization detection after 2 h in U87MG. **B**) Flow cytometry quantification of percentage of proliferative cells by DNA EdU incorporation after 24 h NecDeb treatment. **C**) Cell proliferation analysis by crystal violet staining (590 nm absorbance, relative to sham without NecDeb) of HGCC cells after 2 Gy irradiation (red outline) following 24 h pre-incubation with indicated ratios of NecDeb. **D**) Confocal imaging of neutral lipid staining with LipidTox after 48 h incubation with increasing amounts of NecDeb in U87MG. **E**) NecDeb uptake (1 h) after 2 h pre-treatment with Dynasore (50 µM), MCD (2.5 mM) (in U87MG cells, left and middle panels) or in CHOK1 or PgsA-475 cells.

Mechanistically, necrotic debris uptake followed an endocytic route that was inhibited by MCD and Dynasore, pointing at an involvement of lipid raft endocytosis. High levels of necrotic debris uptake in PG mutant cells and after treatment with HSPG inhibitors point against an involvement of HSPG uptake (Figure 13).

We then investigated whether pharmacological targeting of the LD+ phenotype could revert the radioresistant effects. In fact, LD loaded cells appeared hypersensitive to inhibition of key enzymes of the LD-pathway, which in combination with irradiation led to synergistic cell killing effects in spheroid formation and proliferation assays (Figure 14).

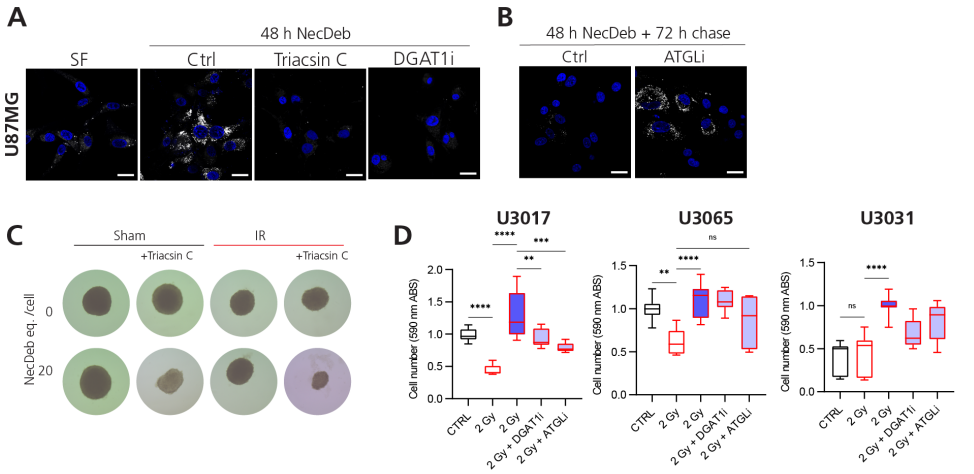

Figure 14. NecDeb scavenging and LD accumulation render GBM cells hypersensitive to LD pathway inhibition
A)LD formation in control U87MG cells (20 NecDeb eq./cell) or in cells treated with Triacsin C (1.25 µM) or A922500 (DGAT1i, 20 µM) prior and during NecDeb incubation. SF, no NecDeb. **B**) LD staining after 48 h NecDeb-LD induction followed by removal of NecDeb and 72 h LD consumption in the absence (ctrl) or presence of ATGL inhibitor Atglistatin (ATGLi, 100 µM). **C**) Representative spheroid images 48 h after irradiation (10 Gy) of cells pretreated with NecDeb with or without Triacsin C (2.5 µM) for 24 h. **D**) Following 24 h pre-incubation with NecDeb alone or with DGAT1i, cells were irradiated (2 Gy, red outline) and proliferation was assessed after additionsl 72 h; ATGLi was added immediately after irradiation. CTRL, no irradiation.

Paper IV

Landscape of surfaceome and endocytome in human glioma is divergent and depends on cellular spatial organization

Another major line of research of our group aims at finding novel cell surface targets as portals of entry for GBM therapeutics. In **Paper IV,** we set out to develop a method for direct characterization of the tumor surfaceome (TS) in the search for novel druggable targets, using intact tumor specimens. Our approach for TS mapping (TS-MAP) is based on surface protein biotinylation of tumor specimens, coupled to affinity purification by streptavidin column HPLC, and identification by LC-MS/MS. Bioinformatics analysis using the SURFME classifier containing 3 317 curated surface proteins led to the identification of available proteins at the cell surface.

We found extensive differences between 2D and 3D cultures, and our results indicate that tissue architecture may play an important role in orchestrating the TS repertoire. Furthermore, using a pilot cohort of 10 glioma patients with varying histological grades, we found extensive differences in the TS, without an apparent association to tumor grade or other histological characteristics. Importantly,

TS-MAP enables to decipher which TS targets are prone to endocytosis, which is enticing for antibody-drug conjugate (ADC) therapeutic development (Figure 16).

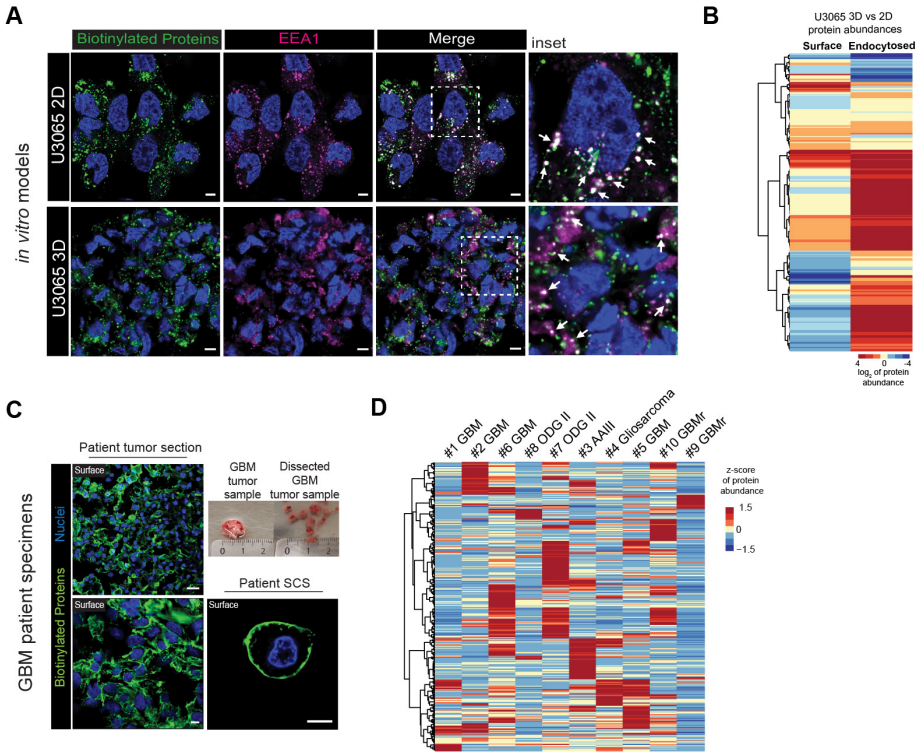

Figure 15.TS-MAP allows for deciphering of TS from *in vitro* models and fresh brain tumor specimens
A) Airyscan super-resolution imaging of endocytosed biotinylated proteins (green) and the early endosome marker EEA1 (magenta) in GBM cells grown in 2D or 3D, as indicated, after 1.5 h of endocytosis. (Scale bars, 5 µm, and 2 µm for insets). **B**) Surfaceome protein abundance heatmap demonstrates divergent surfaceome and endocytome in 3D *vs.* 2D GBM cell cultures. **C**) Confocal microscopy shows biotinylation (green) of intact patient tumor specimens. Higher magnification (Bottom) indicates surface labeling, which is further supported by Airyscan imaging of dissociated single cell suspension, SCS (Bottom Right), showing specific plasma membrane labeling. Top right shows an example of fresh tumor dissection into pieces of 0.3 to 0.5 cm in diameter prior to biotinylation (ruler scale in cm). (Scale bars, 20 µm [Top], 5 µm [Bottom]) **D**) TS-MAP was applied in a pilot cohort of 10 freshly resected patient tumors, including low grade oligodendroglioma, (ODG, WHO grade II), high grade anaplastic astrocytoma (AA, WHO grade III), primary GBM (WHO grade IV), recurrent GBM (GBMr), and gliosarcoma (WHO grade IV). Heatmap of SURFME protein abundance demonstrates divergent expression profile among patient tumors. Figure from Governa, Talbot, Gonçalves de Oliveira, *et al.,* Proc Natl Acad Sci USA. 2022;119(9):e2114456119. doi:10.1073/pnas.2114456119. Distributed under Creative Commons Attribution License 4.0 (CC BY) https://creativecommons.org/licenses/by/4.0/

Discussion and future directions

Glioma cell metabolic stress adaptation via proteoglycan endocytosis and remodeling

Our results from **Papers I** and **II** provide further evidence on the role of PGs in extracellular lipid particle scavenging and emphasize the importance of environmental conditions like hypoxia and acidosis in orchestrating their function. Previous studies described a role for HSPGs in facilitating uptake of lipoproteins, another source of exogenous lipids with notable similarities to EVs (*e.g.* size and density). HSPG-mediated lipoprotein uptake was transiently increased under short-term (2-24 h) hypoxia and acidosis in glioma cells (40). In **Paper I**, we observe a similar effect with cancer derived EVs, which can constitute a local source of lipids in the TME. Importantly, our observed increase in the internalization of anti-HS antibodies *per se*, while hypoxia had no apparent effect on HSPG surface abundance, points at a direct modulation of HSPG turnover from the plasma membrane into endocytic vesicles under hypoxia. The observed increase in lipid-raft mediated internalization in the acute phase of hypoxia (**Paper I**) coincided with increased HSPG turnover and may explain the increased lipid particle uptake.

The hypoxic induction of EV uptake was reversed by HSPG inhibition, and internalized EVs co-localized with SDC-1, together suggesting that HSPGs directly mediate EV uptake, as opposed to merely facilitating EV binding to alternative receptor mechanisms.

In **Paper II**, we describe a strong phenotypic change in PG expression driven by more long-term acidosis and resulting in the expression of what we define as a CS shield that may protect against uncontrolled levels of intracellular EV and lipoprotein particles. Accordingly, lipid particle uptake was impaired and we could show by targeting the CS phenotype that this is reversible. Hence, an increased lipid particle uptake under stressed conditions, like acute acidosis, could result in the induction of a CS shield concomitantly with increased lipid storage into LDs.

Previous results from our lab indicated an induction of EV and lipoprotein uptake after acute acidosis (40). However, in **Paper II** we show that prolonged treatment in low pH decreases EV uptake. It may be hypothesized that during tumor development, initial responses to hypoxia and acidosis involve increased lipid

particle uptake to fulfill acute metabolic needs. At prolonged stress conditions, rewired metabolism and lipid deposition into LDs can then trigger an adaptive response that involves CS remodeling and decreased EV and lipoprotein uptake.

Alternative sources and routes for nutrient supply in cancer cells

The endocytic pathway

Nutrient availability declines due to abnormal vasculature and increased competition in highly proliferative tumors. As a consequence, cancer cells rewire nutrient import from the extracellular milieu via upregulation of membrane transporters and modulation of endocytic pathways. A canonical example of this is the increased import of glucose via GLUT1 in hypoxic cells via HIF1 signaling (150). In **Paper I**, we describe an increased EV scavenging in hypoxic cells via HSPGs following a lipid-raft dependent pathway. Hypoxic cells may also upregulate macropinocytosis for nutrient scavenging in liver cancer (151). However, despite the fact that EVs can enter cells via macropinocytosis (152), our results showed no significant differences in macropinocytosis in glioma cells, suggesting tumor specific regulation of certain endocytic mechanisms. Therapeutic targeting of specific endocytosis pathways has been attempted *in vivo* (153) and may constitute an interesting approach to specifically attenuate certain metabolic dependencies. However, the universality of endocytic pathways in virtually all cell types makes it difficult to avoid unwanted secondary effects.

Necrotic cell clearance by tumor cells

Over the last decade, the definition of what substrates constitute a source of nutrients during metabolic adaptation in cancer cells has been broadened. Besides *bona fide* nutrient substrates such as FAs, glucose and amino acids, complex structures such as ECM components and EVs typically related to other functions can fuel the cell's nutrient demands (5, 6, **Paper I, Paper II**). Interestingly, our work from **Paper III** revealed that glioma cells are able to internalize necrotic material to retrieve amino acids, nucleic acids and lipids. This is of special relevance in GBM since tumor necrosis is one of its histological hallmarks, and the interplay with cancer cells is poorly understood. Until now, necrotic and apoptotic cell scavenging has been mostly studied in professional phagocytes (156), and the mechanisms that may orchestrate necrotic debris clearance by cancer cells remain elusive. A recent study pointed at a role for macropinocytosis in necrotic debris uptake in a breast cancer

model (66); however, from our results with macropinocytosis inhibitors and tracers, we cannot conclude that necrotic debris uptake follows this route, further emphasizing the complexity of the process.

In **Paper III**, we study necrotic debris uptake effects in terms of lipid loading and response to radiotherapy in glioma. Given the effects in cytokine release by phagocytes after necrotic cell clearance, a possible hypothesis is that necrotic clearance by glioma cells can impact cytokine production and the paracrine interplay with the immune cell compartment. In this manner, glioma cell recycling of necrotic material may provide a link between metabolic rewiring and immunoregulation in the TME.

The lipid droplet phenotype: An Achilles heel for aggressive tumor cells?

Aberrant lipid metabolism leading to the LD+ phenotype is linked to tumor aggressiveness in different tumor models (50, 52, 60, 157) including GBM (158, 159) by fueling energy production and promoting proliferation and metastasis. Of special relevance for this thesis work, tumor hypoxia and acidosis coincide with increased LDs as an adaptive response to stress (40, 46, 60). In **Papers I** and **II**, we identify EVs as important lipid providers in the stressed niche. Importantly, the LD+ phenotype has been linked to resistance to chemotherapy (52) and irradiation (62). In **Paper III**, we observe a potential radioprotective effect in cells by necrotic debris uptake, concomitant with induction of the LD+ phenotype. The observed radioprotective effect by debris could be due to *e.g.* increased energetic and metabolic fitness, including a more favorable redox situation, or more directly by inadequate DNA damage by free radicals that could be buffered by LD lipids or even debris-derived DNA (160).

Irrespective of the protective mechanism, different therapeutic approaches to target the LD pathway have been attempted. Increased oxidative stress in hypoxic cells and cells undergoing aerobic glycolysis, together with irradiation induced ROS production may have deleterious effects for the cell. Specifically, Diacylglycerol O-Acyltransferase 1 (DGAT1) catalyzes FA inclusion into LD TAGs, and DGAT1 inhibition was shown to induce FA-dependent mitochondrial stress in GBM (61). In this scenario, the buffering effect of LDs is disrupted, resulting in the release and modification of toxic lipid species that elicit cell death and tumor growth inhibition.

Among others, membrane lipid peroxidation leads to a type of cell death denominated ferroptosis. Due to its tight link with the redox balance and lipid

metabolism, often unbalanced in cancer, tumor killing via ferroptosis has gained increased attention in the last years. PUFAs (polyunsaturated FAs, preferential substrates for peroxidation) are stored in LDs, hence counteracting ferroptosis, and LD utilization not only leads to ATP via beta oxidation, but also increases PUFA availability for peroxidation. Thus, sensitivity to ferroptosis is highly dependent on the metabolic cellular state. Moreover, free lipid in the cytoplasm can lead to ER stress and cell death (161, 162).

Our results from **Papers I-III** motivate further research to elucidate the potential therapeutic benefit of targeting LD metabolism by repurposing of drugs from the atherosclerosis field. LD targeting may be especially efficient in the primary setting by potentiating the response to radiochemotherapy (Figure 16).

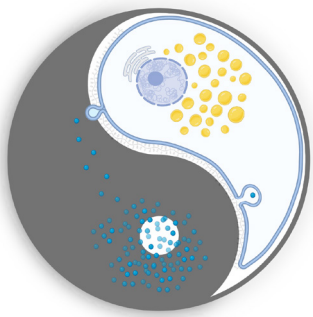

Figure 16. Targeting LD metabolism for anticancer therapy
LD metabolism rests at an equilibrium that is vulnerable to cancer intervention. The beneficial effects of lipids in cancer cells can be turned against the cell's own interests by targetting key enzymes of the LD pathway. The endomembranous system, including EVs and endocytic mechanisms, tightly mediates this equilibrium by accomodating the cell's energetic demands. Created with BioRender.com.

Changes in the tumor surfaceome and surface proteoglycans as targetable moieties in cancer

Cell surface antigens are sources for druggable targets of immunotherapies and other personalized approaches. In cancer, genetic events lead to gain and loss of function mutations or overexpression of signaling receptors that have been a major focus for cancer therapies. Changes in cell surface antigen expression occur as a response to cell intrinsic cues such as genetic events (163, 164), or cell extrinsic stress from therapy (165) or the TME (147). These changes have been mostly studied at the genomic level but are not restricted to encoded proteins (**Paper IV**). Cell surface PG expression remodeling has been previously described in cancer (166), but their anti-cancer target potential is underexplored. This thesis work identifies CSPG as an abundant cell surface antigen of acidosis adapted glioma cells (**Paper II**). Beside excellent methodological tools, anti-GAG scFv antibodies, widely used in this thesis, may also represent promising tools for anti-GAG targeting.

Comprehensive profiling of the TS revealed remarkable differences in 2D *vs.* 3D patient derived cultures, emphasizing the influence of tumor architecture in target remodeling and motivating further studies using 3D models and direct identification from patient-derived tumor tissue. Probably explained by GBM's multiclonality and phenotypical plasticity, TS interrogation revealed high intertumoral divergence. Targeting multiple TS antigens simultaneously or along temporal tumor evolution with bi or trispecific antibodies (167–169) may likely offer more efficient cytotoxic payload delivery.

The blood-tumor barrier presents altered characteristics which make it more permeable than the BBB, and antibody-drug conjugates may traverse it via transcytosis (170). *In vivo* studies have shown that small amounts of toxic payload may lead to biologically relevant effects, highlighting the importance of the target identification approach (170).

Methods

Main methodologies used in this thesis work are described below. For additional details, please see Materials and Methods sections in **Papers I-IV**.

Cell culture

Throughout **Papers I-IV**, *in vitro* GBM cell culture models grown in 2D or as spheroids were used. In **Paper II**, acidosis adaptation was also investigated in a pancreatic adenocarcinoma *in vitro* model, and for the study of HSPG function, we used GAG-mutant CHO cell lines (Table 2).

The U87MG cell line has been used in this thesis. It is the most widely used, commercially available (from ATCC) and best characterized cell line for high grade gliomas, thus allowing for confirmatory validation studies by other researchers (171). However, there is some controversy around U87MG cells (172), as cell-lines provided by vendors such as ATCC were misidentified and differ from the original cell culture established in 1968 at Uppsala University from a 44 year-old female (173). Despite its exact tumor origin is unclear (172), extensive analyses have demonstrated that it derives from a IDH-wildtype high grade glioma harboring typical GBM mutations in the NF1, PTEN and TERT genes (171, 174, 175).

In **Papers I-IV**, primary, patient derived GBM cells (U3017 to U3082) from the Human Glioblastoma Cell Culture resource (HGCC, www.hgcc.se, Uppsala University, Uppsala, Sweden) (176) were used with the advantage of displaying the diversity of GBM tumors (6), and grown in stem-like cell permissive, serum-free conditions (177). The disadvantage, however, is the sequential clonal takeover and transcriptional drift that lead to passage sensitivity (178), thus limiting the reproducibility between laboratories, which is of concern.

For spheroid cultures, GBM cells were seeded at a density of 5 x 10^3 - 10^4 cells per well in PrimeSurface® 3D Culture Spheroid plates (Ultra Low Attachment, U-bottom 96 well plate, #MS-9096UZ, S-bio), in their corresponding culture medium and cultured on an orbital shaker at 90 rpm. After 10-14 days, spheroids reached a diameter of 0.4 to 0.6 mm and were further processed for experiments.

Table 2: Cell lines and primary cultures used in this thesis work.
GBM, Glioblastoma; CL, classical, MS, mesenchymal, PN, proneural.

Cell line	Species	Origin	Type
U87MG	Human	ATCC	GBM
GL261	Mouse	ATCC	Murine glioma, GBM phenotype
U3017	Human	HGCC	GBM, CL
U3031	Human	HGCC	GBM, MS
U3047	Human	HGCC	GBM, PN
U3034	Human	HGCC	GBM, MS
U3054	Human	HGCC	GBM, MS
U3065	Human	HGCC	GBM, MS
U3082	Human	HGCC	GBM, PN
PANC1	Human	ATCC	Pancreatic adenocarcinoma
CHO K1	Hamster	ATCC	Ovary cancer, wild-type
CHO pgsA-745	Hamster	ATCC	Ovary cancer, Xylosyltransferase I mutant

Culture conditions

Cells were routinely cultured in DMEM with high glucose (HyClone, GE Healthcare) (U87MG, GL261, PANC1) or F12-K (Gibco, Life industries) (CHO) supplemented with 10% (volume/volume) fetal bovine serum (Sigma-Aldrich), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL Streptomycin (Thermo Fisher Scientific) for < 30 passages.

HGCC cells were cultured on poly-L-ornithine- and laminin-coated surfaces in serum-free neurobasal:DMEM/F12 (1:1) medium supplemented with EGF (10 ng/mL), FGF (10 ng/mL) (Peprotech, AF-100-15 and 100-18B, respectively) and stem cell supplements N2 (Gibco, 17502-048) and B27 (Gibco, 12587-010).

Primary brain tumor patient derived specimens

Clinical specimens were collected from patients referred to the Neurosurgery Department at Skåne University Hospital, Lund, Sweden. The study was carried out according to the ICH/GCP guidelines and in agreement with the Helsinki declaration, and was approved by the local ethics committee, Lund University (Dnr. 454 2018/37). Patients were diagnosed by routine MRI, and surgical and pathological procedures, received standard oncological treatment and were followed up according to local and national recommendations. Fresh samples of macroscopically viable tumor were directly processed by mechanical disruption and enzymatic dissociation with TrypLE express and DNAse I, for 10 min at 37°C to obtain a single cell suspension, which was filtered through 70 and 40 µm nylon cell strainer, and red blood cells (RBC) were removed using RBC Lysis Buffer. Cells

were seeded in 8-well chamber slides, and incubated in a humidified 21% O_2 and 5% CO_2 incubator at 37°C for 4 h prior to fixation and further analysis.

Methods to mimic the tumor microenvironment

Hypoxia: Empirical measurements of O_2 concentrations in brain tumors have showed that oxygen levels can range from 2.5 to 0.1% (179). In **Papers I** and **III**, we used 1% O_2 levels to determine hypoxia, which is known to stabilize HIF-1 and 2 already after 2 h (147). Hypoxic conditions were achieved by incubation in a humidified hypoxia station (SCI-tive N-N, Baker Ruskin) set at 1% O_2, 5% CO_2 and 94% N_2, 80% humidity and 37°C.

Other methods to trigger the HIF-mediated hypoxic response used in this thesis include treatment with desferrioxamine, a Fe^{2+} chelator (180) or dimethyloaxyglycine, (DMOG) a 2-oxoglutarate analog (181). Both drugs target cofactors of the PHDs, thus leading to HIF-stabilization.

Acidosis: Intratumoral acidification reaching pH levels of 5.6 to 6.8 has been empirically measured (182). In **Paper II**, U87MG and PANC1 cells were put through acidic culture conditions (pH 6.4-6.7), or pH 7.4, as a control, for 8-10 weeks to achieve acidosis adapted (AA) cells (or non-adapted controls, NA). Cell culture medium was supplemented with a buffering system consisting of: Hepes 20 mM, 4-Morpholineethanesulfonic acid (MES) sodium salt and 4-Morpholinepropanesulfonic acid (MOPS) (all, 20 mM). Medium pH was adjusted and sterile filtered prior to use.

Necrosis: Necrosis naturally occurred in the hypoxic cores of our 3D spheroid model systems, after reaching a diameter of 0.4-0.6 mm (**Paper III**). To study necrotic debris transfer o GBM cells, we employed an *in vitro* model of induced necrosis. To induce necrosis, U87MG cells were harvested, washed and resuspended in PBS at a concentration of 5-10 x 10^6 cells/mL, and subjected to 4 cycles of liquid nitrogen immersion (30 sec) and thawing in a 37° water bath, a method previously described to cause cell swelling and membrane bursting, characteristic of necrotic cell death (183–185). Necrotic cells were centrifuged at 10 000 x g and non-soluble necrotic cell debris (referred to as NecDeb) was resuspended in serum-free culture medium for experiments. For LD formation, unlabeled NecDeb was used at 10 or 20 NecDeb cell equivalents/recipient cell.

Metabolite tracking from NecDeb

In **Paper III**, we used two different approaches to track metabolites derived from NecDeb; amino acids, nucleotides and lipids were tracked with a click-chemistry based approach, and lipids were additionally followed using a BODIPY-conjugated dodecanoic acid.

Cells were cultured for 18 h in the presence of L-azidohomoalanine (25 µM, C10102), which incorporates into *de novo* synthesized proteins, or palmitic acid azide (25 µM, C10265, Thermo Fisher), a functionally active FA (186). Twenty-four h prior to and during L-azidohomoalanine loading, cells were incubated in methionine and cysteine-free DMEM (21013024, Gibco) to deplete "cold" amino acids. 5-Ethynyl-2′-deoxyuridine (EdU) loading (100 µM) was done in full medium during the exponential, proliferative phase to ensure high DNA incorporation of the nucleotide (Click-iT EdU cell proliferation for imaging, AF-488, C10337, Thermo Fisher). Alternatively, cell lipids were labeled by incubating donor cells with BODIPY-dodecanoic acid (DA) for 15 min at 37 °C (2.5 µM, D3822, Thermo Fisher). BODIPY-DA NecDeb was used for uptake experiments at a ratio of 2 NecDeb cell eq./cell.

After metabolite loading, NecDeb was induced by freeze-thawing cycles, as described above, and used for internalization studies. After fixation, permeabilization and blocking of unspecific signal, the detection of internalized Ala and PA-labelled NecDeb was done by the copper-catalyzed cycloaddition of azide-metabolites to biotin-alkyne (B10185) (click reaction), using the Click-iT™ Cell Reaction Buffer Kit (C10269, Thermo Fisher). To visualize metabolite uptake in recipient cells, fluorescent staining was performed for 30 min with 4 µg/mL Streptavidin-AF 488 (S32354, Thermo Fisher). EdU labelled NecDeb uptake was visualized by staining with click reaction reagent from the Click-iT EdU imaging kit as per manufacturer´s instructions.

EV isolation and characterization

U87MG-derived EVs were isolated from serum-free cell culture supernatants, to avoid contamination with serum lipoproteins, which share extensive similarities with EVs, as reviewed by us (187). Conditioned media were collected and cleared for cell debris, followed by ultracentrifugation, as previously described (110, 135, 188). The EV population secreted by U87MG cells has been extensively characterized (135, 143) and presents exosome-like characteristics, including enrichment in tetraspanins CD9 and CD63, and depletion of endosome (EEA1) or Golgi (GM130) markers. The average size of U87MG EVs peaks around 100 nm using nanoparticle-tracking analysis (Zetaview, Particle Matrix) (Figure 17).

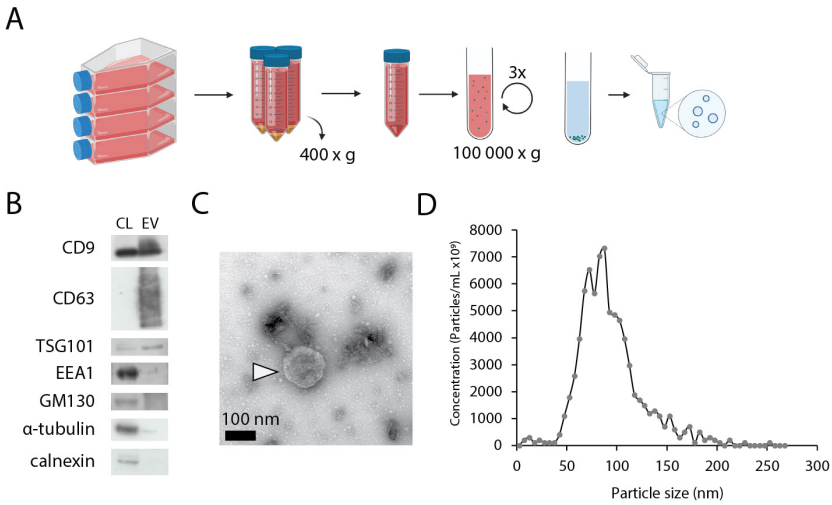

Figure 17. EV isolation and exosome characteristics
Schematic of the EV isolation protocol (A) and main molecular characteristics including enriched protein cargo (B) and size as shown by electron mycroscopy (C) and nanoparticle tracking analysis (D). A, Created with BioRender.com. B, D, Reprint with permission from Cerezo-Magaña *et* al, Mol Cancer Res (2021) 19 (3): 528–540. (188).

For uptake studies, EVs were labeled with the lipophilic membrane dye PKH (26, red, 67, green). Other methods to track EV biodistribution or uptake include expression of reporter EV-markers such as tetraspanins (189). Due to the innate heterogeneity of EVs, labeling methods using certain EV markers may lead to partial coverage of the overall population and biased or hard to interpret results. Instead, lipophilic dyes target the lipid compartment, which is present in all EV subclasses. Despite some studies reporting PKH dye-micelle artifacts (190) or overall poor efficiency of commonly used methods (191), previous work from our lab in U87MG cells showed that non-specific transfer of free-PKH to recipient cells was negligible (110).

HSPG function inhibition

To inhibit HSPG function, we employed different approaches. First, co-incubation with exogenous heparin, *i.e.* a more sulfated HS-mimetic, (H3393, Sigma Aldrich) leads to competition for ligand binding to HSPGs. Second, HS-GAG chain digestion using specific bacterial HS lyases leads to HS shedding and leaving the core protein naked. Third, incubation with 4-nitrophenyl β-D-xylopyranoside (PNP-xyl, N2132; Sigma Aldrich), competitively inhibits GAG elongation on the core protein by acting as an alternative substrate (192). Finally, given that sulfation status is pivotal for HSPG function, treatment with sodium chlorate ($NaClO_3$), an inhibitor of the sulfation donor (phosphoadenosine phosphosulfate, PAPS) synthetase, leads to under sulfated GAG chains with altered ligand binding properties (193) (Figure 18).

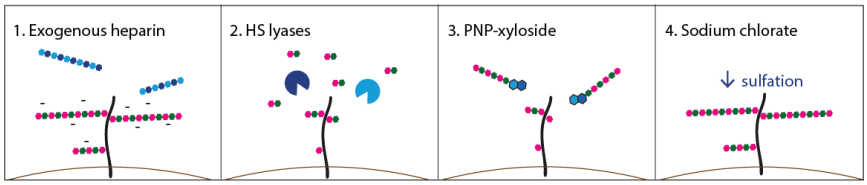

Figure 18. Methods for HSPG inhibition
HS, heparan sulfate; PNP-xyloside (4-nitrophenyl β D xylopyranoside); Sodium chlorate, $NaClO_3$. Modified with permission from Cerezo-Magaña *et* al, Mol Cancer Res (2021) 19 (3): 528–540. (188).

Endocytic pathway characterization

To characterize the involvement of the different endocytic pathways in ligand internalization, we employed specific markers and inhibitors of major endocytic mechanisms (Figure 19).

Cholera toxin subunit B (CtxB) strongly binds to monosialotetrahexosyl-ganglioside (GM1), a structural constituent of membrane domains called lipid rafts, whose integrity directly depends on membrane cholesterol abundance. The drug methyl-β-cyclodextrin (MCD) (C4555, Sigma Aldrich), inhibits membrane-raft endocytosis by depleting cholesterol from the plasma membrane (194). Alternatively, β-cyclodextrins can also be used as cholesterol donors provided that appropriate ratios of exogenous cholesterol are provided, as described in (195, 196). In **Paper I**, we used this approach to enrich for membrane cholesterol, which was further validated by increased lipid raft staining. Dynasore monohydrate (D7693, Sigma Aldrich), has been shown to inhibit the GTPase activity of dynamin, blocking constriction and fission of endocytic vesicles. Interestingly, Dynasore also is involved in cholesterol homeostasis and reduces plasma membrane cholesterol, inhibiting mobilization of the cellular membrane (197, 198). This function of Dynasore is in line with our results with MCD. Transferrin is a marker for clathrin-mediated endocytosis, since transferrin receptor intracellular domain is known to trigger clathrin-coated endosomal pits (199).

The fluid-phase marker dextran is internalized via macropinocytosis (or "cell drinking"). The Na^+/H^+ exchanger (NHE) inhibitor EIPA (5-(N-ethyl-N-isopropyl) amiloride, A3085, Sigma-aldrich) specifically blocks macropinocytosis by directly inhibiting macropinosome formation. Alternatively, macropinocytosis is susceptible to Rac1 inhibition (553502, Sigma-Aldrich), since the small GTPase is essential for macropinocytosis.

To validate endocytosis, we also employed immunofluorescence stainings against major components of early endosomes (Early Endosome Antigen 1 (EEA1), ab2900 or ab70521, 1:200-1:500, Abcam) or late endosomes (CD63, ab8219, 1:100, Abcam).

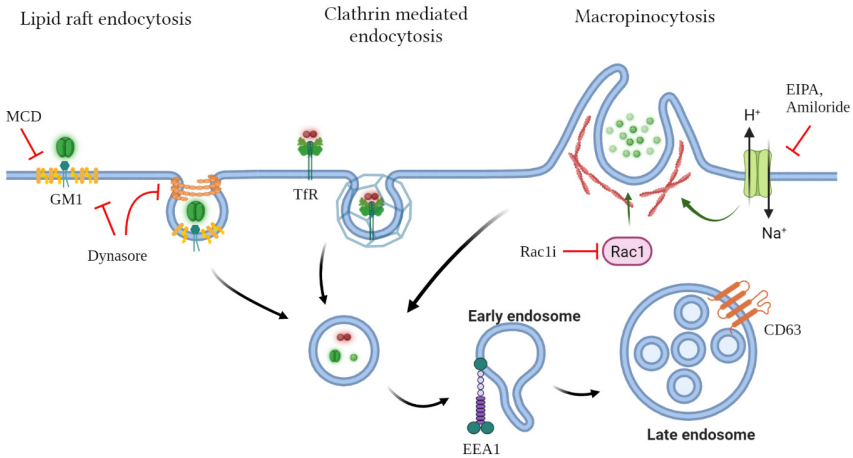

Figure 19. Major cellular endocytic pathways and corresponding tracers, markers and inhibiors. Created with BioRender.com.

Targeting the LD phenotype

In **Paper III**, we employed several drugs targeting LD formation or metabolism. Inhibitors for DGAT-1 and -2 (DGAT1i, A922500 and DGAT2i, PF-06424439) were used to inhibit the conversion of DAGs to TAGs, necessary for LD incorporation (62). The enzyme adipose triglyceride lipase, (ATGL) is necessary for FA utilization from TAGs, and its inhibition was achieved with Atglistatin (46). Triacsin C is a potent long-chain acyl-CoA synthetase (ACSL) inhibitor, shown to efficiently inhibit FA-induced LD formation in macrophages, blocking their foam cell phenotype (200). Acyl-CoA cholesterol acyltransferase (ACAT) inhibition with avasimibe inhibits cholesterol esterification for LD accumulation. Notably, many of these drugs were developed for and clinically tested in the treatment of atherosclerosis.

Glycosaminoglycan immunophenotyping

Owing to their complex biochemical structure, comprehensive GAG characterization often represents a methodological challenge. High performance liquid chromatography (HPLC) based techniques allow separation of oligosaccharides, which coupled with mass spectrometry provide rich information about chain length and sequence (201, 202). Here, we used a panel of single chain variable fragment (scFv) antibodies against specific GAG epitopes to characterize GAG expression. These scFv antibodies were developed using a phage display technology by the van Kuppevelt group (Nijmegen, The Netherlands). This

technique allows antibody generation against epitopes with low immunogenicity, such as GAGs, and they are greatly versatile tools to characterize GAG expression and/or internalization in tissues or *in vitro* cultures. A summary of the anti-GAG antibodies used in this thesis is presented in Table 3.

Table 3. Antibodies used for GAG characterization and suggested high-affinity epitopes
HS (Heparan sulfate), CS (chondroitin sulfate), DS (dermatan sulfate). 2OS, 2-O-sulfated; 3OS, 3-O-sulfated; 4/2,4 diOS, 2,4-di-O-sulfated; 6OS, 6-O-sulfated; CS-A, GlcA-GalNAc,4S; CS-D, GlcA,2S-GalNAc,6S; CS-E, GlcA-GalNAc,4S, 6S; IdoA, iduronic acid; NS, N-sulfated

Clone	HS/CS/DS	Epitope	Reference
AO4B08	HS	IdoA, NS, 2OS, 6OS	(203)
HS4C3	HS	NS, 2OS, 3OS, 6OS	(204)
CS-56	CS	CS-A, CS-D	(205)
GD3G7	CS	CS-E	(206)
IO3H10	CS	CS-A, CS-C, CS-E	(207)
LKN1V	DS	4/2,4 diOS DS	(208)
GD3A12	DS	IdoA-GalNAc4S	(209)

VSV-G tag conjugation to scFv antibodies allows for detection using a secondary antibody against VSV-G tag and a fluorescently labelled tertiary antibody (Figure 20).

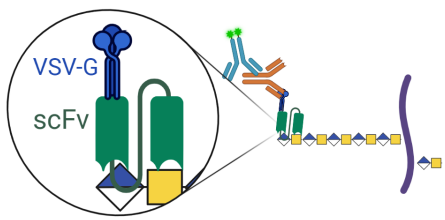

Figure 20 Schematic representation of scFv antibody staining for GAG immunophenotyping. Created with BioRender.com.

Flow cytometry

Flow cytometry was used for quantification of cellular mean fluorescence intensity (MFI) of SCS. In cell surface staining experiments, cells were detached with 2x PBS containing 0.5 mM EDTA, unspecific signal was blocked with 3% BSA and cells were incubated with corresponding primary and secondary antibodies at 4°C under agitation prior to extensive washes and flow cytometry analysis. In uptake studies, fluorescently labeled ligands (*e.g.* EVs, LDL, NecDeb or endocytosis markers) were incubated with adherent cells at 37°C for the indicated durations. Fluorescent ligands bound to the cell surface by electrostatic interactions were removed by 1 M NaCl washes and samples were further analyzed with flow cytometry. An Accuri C6 Flow Cytometer (BD Biosciences) was used with a 488 nm wavelength

excitation laser and appropriate detectors. Data was analyzed with Accuri C6 software.

Confocal microscopy

Confocal microscopy allows high resolution visualization of fluorescently labelled samples (tissue sections or adherent cultures) with better signal-to-noise ratios than other imaging techniques such as widefield fluorescence microscopy. Importantly, the optical sectioning in confocal microscopy enables colocalization studies and the visualization of subcellular localization, especially relevant for uptake pathway characterization. Here, we used a Zeiss LSM710 confocal fluorescence microscope system using Plan Apochromat 20x/0.8M27, EC Plan-Neofluar 40x/1.30 Oil DIC M27, and C-Apochromat 63X/1.20W korr M27 objectives. For super-resolution imaging, we used an Airyscan detector (Zeiss), composed by 32 detector elements which act as single pinholes for increased signal-to-noise ratio. Image processing was done with Zen software (Zeiss).

External beam irradiation

In **Paper III**, U87MG and HGCC cells were irradiated using an orthovoltage x-ray radiotherapy unit (XenX Xstrahl Ltd., UK) without collimator. Beam energy was set at 225 kV, gantry was set at 0° and source-to-target distance was 20 cm, and an irradiation dose of 19 s per gray (Gy). GBM cells were exposed to doses of 2 to 10 Gy. Sham plates were brought to the irradiation room to account for the time outside the cell culture incubator.

Tumor surfaceome mapping (TS-MAP)

Our tumor surfaceome mapping (TS-MAP) strategy was developed from previous studies interrogating the hypoxic cell surfaceome in *in vitro* cultures (147). Briefly, the procedure consisted of the following steps, as graphically summarized in Figure 21:

1. Subconfluent primary GBM cells grown in 2D monolayers or as spheroids, mouse GBM orthotopic tumors or patient GBM specimens (0.3 to 0.5 cm) (under 100 rpm orbital agitation for spheroids and tissue samples).

   (*Steps 1-5, on ice)

2. Washed twice with ice-cold PBS containing $MgCl_2$ and $CaCl_2$ (Mg/Ca–PBS), pH 8.0.
3. Biotin incubation: 1mg/mL, in Mg/Ca–PBS, pH 8.0, 30 min protected from light (EZ-Link Sulfo-N-hydroxysuccinimide-SS-biotin, membrane impermeable).
4. Free biotin quenching: Incubation with 0.1 M glycine in Mg/Ca–PBS, 10 min.

5. Washed twice with ice-cold Mg/Ca–PBS.

For profiling of endocytome**,** proceed to step to step 6, alternatively proceed to 11:

6. Addition of pre-warmed primary cell medium and incubation in a humidified 5% CO_2 incubator at 37 °C for the indicated time (1.5-4 h).
7. Stop endocytosis: samples on ice for 10 min, and remaining
8. Cell-surface biotin removal by 15 min incubation (at 4 °C in the dark, repeat twice) with the membrane impermeable, reducing agent, MesNa (200 mM, sodium-2-mercaptoethanesulfonate), in 50 mM Tris, 100 mM NaCl, 1 mM EDTA and 0.2% BSA, pH 8.6.
9. Wash with ice-cold Mg/Ca–PBS.
10. Incubation with iodoacetamide (IAA, 5 mg/mL) in Mg/Ca–PBS for 10 min in the dark.
11. Wash with ice-cold Mg/Ca–PBS.

→Endpoint for immunofluorescence staining and imaging or flow cytometry quantification, or western blot analysis. Single cell suspension (SCS) from biotinylated tumor specimens was achieved as described above and samples were further processed accordingly.

C. For LC-MS/MS analysis:

12. Additional cell-surface biotin blocking step with unconjugated streptavidin (50 µg/mL; in 1% BSA Mg/Ca–PBS), for 30 min at 4 °C.
13. Extensive washes to remove unbound streptavidin:
    13.1. 12 mL of PBS 0.1% Triton X-100.
    13.2. 10 mL RIPA: PBS 0.1% Triton X-100 1 M NaCl (1:1).
    13.3. 10 mL of PBS 0.1% Triton X 100.

    As a control of cell-surface biotin removal efficiency, we verified the absence of signal after the MesNa and streptavidin blocking steps in surface-biotinylated cells that did not undergo endocytosis.
14. SCS from biotinylated tumor specimens was achieved as described above.
15. SCSs were lysed for 20 min at 4 °C in RIPA containing 2x protease inhibitors.
    15.1. Lysates were clarified by centrifugation at 18 000 × g for 10 min at 4 °C.
    15.2. The soluble fraction was collected, and total protein was quantified using BCA Protein assay.
16. Lysates were diluted 1:4 with Mg/Ca–PBS supplemented with protease inhibitors, filtered with a 0.45-µm surfactant-free cellulose acetate syringe filter, and then

High-Affinity Chromatography Enrichment of Biotinylated Proteins

High-pressure liquid chromatography (HPLC) UPC 900 system (Amersham Biosciences) equipped with an online ultraviolet (UV) detector set at 280 nm, and

17. Column pre-equilibration: HiTrap streptavidin HP-1 mL columns were pre-equilibrated in PBS 0.1% Triton X-100 at a flow rate (FR) of 250 µL/min.
18. Sample loading to HiTrap streptavidin HP columns

19. Washes to remove non biotinylated proteins (FR of 1 mL/min)

    19.1. 10 mL of PBS 0.1% Triton X-100

    19.2. 10 mL of RIPA/PBS 0.1% Triton X-100 1M NaCl 1:1 (vol/vol)

    19.3. 10 mL of PBS 0.1% Triton X-100

20. Elution of biotinylated proteins by reduction of the protein-SS-biotin linker with 10 mL freshly prepared 150 mM MesNa in PBS 0.1% Triton X-100 (reduced FR, 125 µL/min).

21. Protein precipitation: One volume of 20% trichloroacetic acid was added to the collected eluate and incubated for 30 min on ice.

22. Centrifugation for 10 min at 18 000 × g.

23. Protein pellets were washed twice with 2% sodium acetate and resuspended in 6 M urea buffer for LC-MS/MS sample preparation. See Materials & Methods section in **Paper IV** for additional LC-MS/MS details.

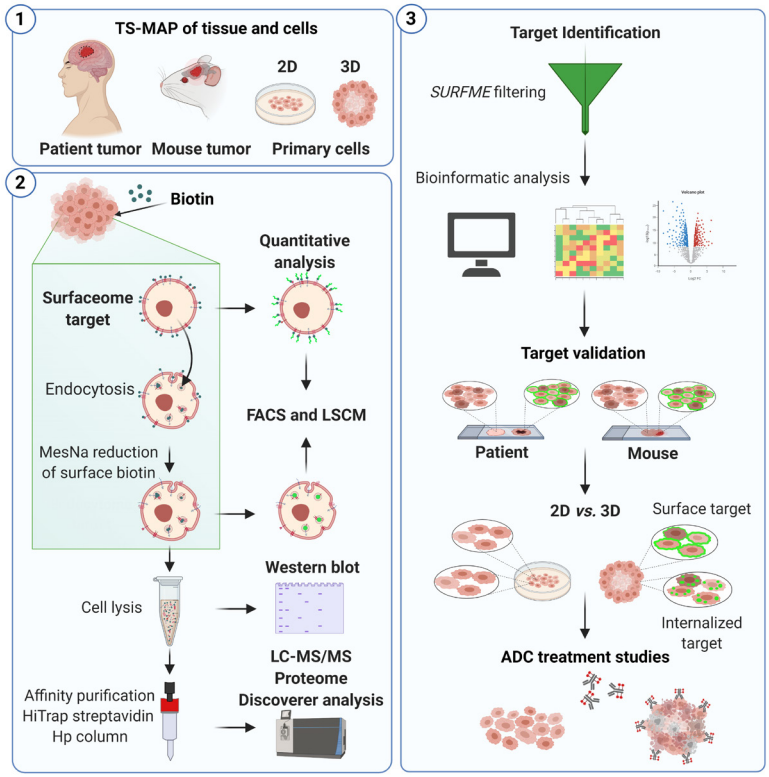

Figure 21 Tumor surfaceome mapping (TS-MAP) of patient GBM specimens, orthotopic mouse brain tumors, and 2D/3D GBM *in vitro* cultures. Figure from Governa, Talbot, Gonçalves de Oliveira *et al*. Proc Natl Acad Sci USA. 2022;119(9):e2114456119. doi:10.1073/pnas.2114456119. Distributed under Creative Commons Attribution License 4.0 (CC BY) https://creativecommons.org/licenses/by/4.0/

The SURFME filter

The SURFME filter constitutes a validated list of *bona fide* surface protein IDs. The pipeline to build the SURFME filter is depicted in Figure 22.

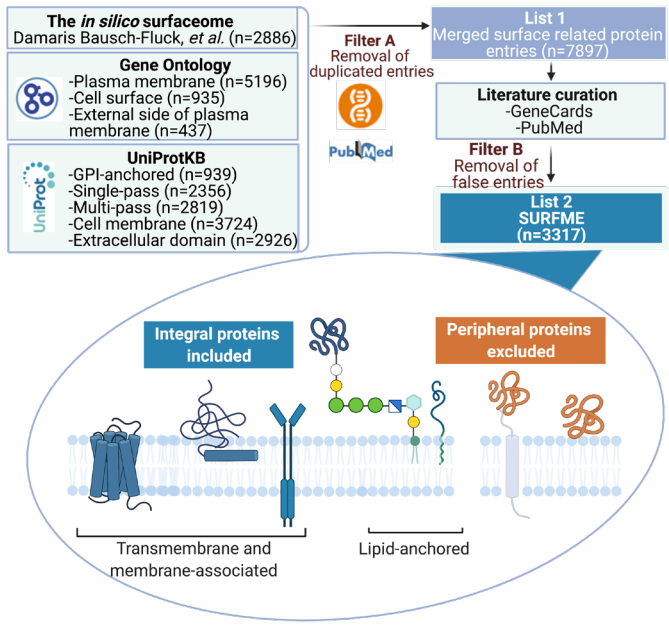

Figure 22 The SURFME filter

The "*in silico* surfaceome" described in Bausch-Fluck *et al* (39) with a reported accuracy of 93.5% was added to IDs classified under the Gene Ontology terms "plasma membrane", "cell surface", "external side of plasma membrane" and UniProt annotated "cell membrane" and "extracellular domain" protein IDs including GPI anchored proteins and multipass transmembrane proteins. After filtering out duplicated IDs, and false positive IDs (*e.g.* peripheral, membrane associated proteins), the SURFME filter consisted of 3 317 curated TS protein IDs. Figure from Governa , Talbot , Gonçalves de Oliveira *et al.* Proc Natl Acad Sci U S A. 2022;119(9):e2114456119. doi:10.1073/pnas.2114456119. Distributed under Creative Commons Attribution License 4.0 (CC BY) https://creativecommons.org/licenses/by/4.0/

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Originally from Huércanos, a small village in La Rioja, Spain, I studied Biomedicine at University of Barcelona. Driven by an interest in cancer research and extracellular vesicles, I moved to Lund for the final project of my Bachelor's thesis. After nine exciting months in the Tumor Microenvironment lab, I became further intrigued and continued my research under Mattias Belting's supervision during my Master's in Lund and later, as a PhD student.

During my PhD I have studied glioblastoma, the most devastating brain tumor in adults. Specifically, I have focused on the adaptive mechanisms to its hostile environment, that partly explain its aggressive phenotype. The work from my thesis' projects contributes to a better understanding of glioblastoma's potential vulnerabilities and presents new avenues for target identification.

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