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GOLPH2's Role in Innate Immunity

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Frau Mélanie Buchi, Frau Dr. Heike Liewen, Herr Norbert Markuly, Herr Dr. Pawel Pelczar, Herr, Prof. Hans Schlößer, Herr Prof. Frank Stenner, Herr Prof. Alfred Zippelius

Weitere Personen waren an der Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe einer Promotionsberaterin/eines Promotionsberaters in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertationsschrift stehen.

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Die in dieser Arbeit verwendete GOLPH2-/- 57BL/6 Mauslinie wurde ohne meine Mitarbeit im Labor des Department Biomedizin von der Arbeitsgruppe von Dr. Pawel Pelczar (head of the center for transgenic models (CTM) University of Basel) und in Zusammenarbeit mit der Arbeitsgruppe von Herrn Prof. Alfred Zippelius etabliert. Die Genotypisierungsuntersuchungen der Tiere sind nach entsprechender Anleitung durch Frau Mélanie Buchi teils durch sie und teils von mir ausgeführt worden.

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Zur sprachlichen und grammatikalischen Überprüfung habe ich die englischsprachige Version von ChatGPT4.0 genutzt.

Bei der statistischen Auswertung meiner Daten wurde ich von Frau Dr. Heike Liewen unterstützt.

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LIST OF ABBREVIATIONS

%: Percent
 °C: Degree Celsius
 AF: Alexa Fluor
 AOM: Azoxymethane
 APC: Allophycocyanin
 ATCC CRL 1573: American type culture collection catalog reference line 1573
 B16-F10: Murine melanoma cell line B16-F10
 BCA: Bicinchoninic acid
 Bis-Tris: Bis-(2-hydroxy-ethyl)-amino-tris(hydroxymethyl)-methane
 bp: Base pair
 BRAF: B-Raf proto-oncogene, serine/threonine kinase
 BRAF^{V600E}: B-Raf proto-oncogene, serine/threonine kinase (BRAF) substitution of valine (V) with glutamic acid (E) at position 600 in the B-Raf protein (V600E)
 BSA: Bovine serum albumin
 C-terminus: Carboxy-terminus
 C57BL/6NRj: Mouse strain C57 black 6 National Institutes of Health R Janvier (synonym: C57/B6)
 CAC: Colitis-associated carcinoma
 Cas9: Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9
 CD: Cluster of differentiation
 cDNA: Complementary deoxyribonucleic acid
 CI: Confidence interval
 cm²: Square centimeters
 CO₂: Carbon dioxide
 CRC: Colorectal cancer
 CRISPR: Clustered regularly interspaced short palindromic repeats
 CT: Cytoplasmic tail
 CTLA4: Cytotoxic T-lymphocyte associated protein 4
 CTM: Center for transgenic models
 D4M-3A: Murine melanoma cell line D4M-3A
 DAPI: 4',6-diamidino-2-phenylindole
 DBM: Department of Biomedicine of the University of Basel in Switzerland
 DBZ: Dibenzazepine
 DCs: Dendritic cells
 DMEM: Dulbecco's Modified Eagle's medium
 DNA: Deoxyribonucleic acid
 DPBS: Dulbecco's Phosphate Buffered Saline
 DSS: Dextran sodium sulfate
 ECL: Enhanced chemiluminescence
 EDTA: Ethylenediaminetetraacetic acid
 EGFR: Epidermal growth factor receptor
 EMT: Epithelial-mesenchymal transition
 ER: Endoplasmic reticulum
 ESE-1: Epithelium-specific Erythroblast Transformation Specific transcription factor-1 (synonym: epithelium-specific ETS transcription factor-1)
 ESE-1: Epithelium-specific ETS 1
 ETS: Erythroblast Transformation Specific
 ETS: Erythroblast Transformation Specific (synonym: E26 transformation-specific)
 F1: First filial
 F2: Second filial

FACS: Fluorescence-activated cell sorting
 FBS: Fetal bovine serum
 G: Birmingham gauge
 G: Glycine
 g: Gram
 GCH: Giant cell hepatitis
 GL261: Murine glioma cell line GL261
 GOLM1: Golgi membrane protein 1 (synonym GOLPH2, GP73)
 GOLPH2 KO: GOLPH2 knockout (synonym: GOLPH2^{null}, GOLPH2 -/-, Golm1 -/-)
 GOLPH2: Golgi phosphoprotein 2 (synonym GOLM1, GP73)
 GP73: Golgi protein 73 (synonym GOLM1, GOLPH2)
 gRNA: Guide RNA
 GRP78: Glucose-regulated protein 78
 HCC: Hepatocellular carcinoma
 HCV: Hepatitis C virus
 Hep3B: Human hepatocellular carcinoma cell line Hep3B
 HepG2: Human hepatocellular carcinoma cell line HepG2
 Huh7: Human hepatoma cell line Huh7
 I/O: Immunotherapy
 ICIs: Immune checkpoint inhibitors
 IECs: Intestinal epithelial cells
 IFN- β : Interferon beta
 IFN- γ : Interferon gamma
 IFN- λ 1: Interferon lambda 1
 IgG: Immunoglobulin G
 IL-12: Interleukin 12
 IL-12A: Interleukin-12 subunit alpha
 IL-1 β : Interleukin-1 beta
 IL-6: Interleukin 6
 IPA: Isopropyl alcohol
 ISG56: Interferon-stimulated gene 56
 K: Lysine
 kDa: Kilodalton
 KO: knockout
 L: Leucine
 LC3: Microtubule-associated protein 1A/1B light chain 3
 LDS: Lithium dodecyl sulfate
 LICMGs: Lung adenocarcinoma immune cell marker genes
 LID score: Lung adenocarcinoma immune difference score
 LIR: LC3-interacting region
 LPS: Lipopolysaccharide
 M: Methionine
 MAVS: Mitochondrial antiviral-signaling protein
 MC38: Murine colon adenocarcinoma cells
 MDSCs: Myeloid-derived suppressor cells
 MFI: Mean Fluorescence Intensity
 MFI: Mean fluorescence intensity
 mg: Milligram
 MHCC97H: Highly-metastatic human hepatocellular carcinoma cell line
 min: minute(s)
 ml: Milliliter

mm: Millimeter
 mM: millimolar
 mm²: Square millimeter
 mm³: Cubic millimeter
 MMP-7: Matrix metalloproteinase-7
 MMP13: Matrix metalloproteinase-13
 MMPs: Matrix metalloproteinases
 MOPS: 3-(N-morpholino)propanesulfonic acid
 mRNA: Messenger ribonucleic acid
 N-terminal: Amino-terminus
 n: Number
 N2ICD: NOTCH2 intracellular domain
 NICD: Notch intracellular domain
 nm: Nanometer
 NSCLC: Non-small-cell lung cancer
 p-Smad2: Phosphorylated Smad homolog 2
 p-Smad3: Phosphorylated Smad homolog 3
 p: Probability value
 PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
 PAM: Protospacer adjacent motif
 PBMCs: Peripheral blood mononuclear cells
 PBS-T: Phosphate Buffered Saline (PBS) supplemented with 0.1% Tween 20
 PBS: Phosphate Buffered Saline
 PCR: Polymerase chain reaction
 PD-1: Programmed cell death protein 1
 PD-1: Programmed cell death protein 1
 PD-L1: Programmed death-ligand 1
 PTEN^{null}: Phosphatase and tensin homolog
 R: Arginine
 RBC: Red Blood Cell
 RIPA: Radioimmunoprecipitation assay
 RNA: Ribonucleic acid
 rpm: Revolutions per minute
 RPMI medium: Roswell Park Memorial Institute medium
 RTK: Receptor tyrosine kinases
 RTKs: Receptor tyrosine kinases
 S: Serine
 s.c.: Subcutaneous
 SDS: Sodium dodecyl sulfate
 sGOLPH2: Soluble GOLPH2
 shGOLPH2: Short hairpin RNA targeting GOLPH2
 SK-Hep-1: Human hepatic adenocarcinoma cell line established at Sloan Kettering-Institute
 Smad: Small body size mother against decapentaplegic
 T cells: Thymus-derived lymphocytes
 t-test: Hypothesis test statistic
 TAM: Tumor-associated macrophage
 TBE: Tris(hydroxymethyl)aminomethane boric acid ethylenediaminetetraacetic acid (synonym: Tris/Borate/EDTA)
 TBS-M: Tris buffered saline methanol
 TBS: Tris buffered saline
 TC-1: Murine lung epithelial cells tissue culture-1

TC: Tissue culture
Tg: Thapsigargin
TGF- β : Transforming growth factor beta
TGF- β : Transforming growth factor-beta-induced
TGF- β 1: Transforming growth factor beta 1
TGN: Trans-Golgi network
TH9 cell: T helper 9 cell
Tm: Tunicamycin
TMD: Transmembrane domain
TNF- α : Tumor necrosis factor alpha
TNF: Tumor Necrosis Factor
TRAF6: Tumor Necrosis Factor Receptor-Associated Factor 6
Tris-MOPS-SDS: Tris(hydroxymethyl)aminomethane - 3(N-morpholino)propanesulfonic acid - sodium dodecyl sulfate
U/ml: Units per milliliter
UPR : Unfolded protein response
UVM: Uveal melanoma
V: Valine
V: Volt
wt: Wild type
Y: Tyrosine
 μ l: microliter
 μ m: micrometer
 μ M: micromolar

1. SUMMARY (Deutsche Zusammenfassung)

Diese Promotionsarbeit liefert neue Erkenntnisse über die funktionelle Rolle des potenziell onkogenen Proteins Golgi Phosphoprotein 2 ((GOLPH2); auch bekannt als Golgi-Membranprotein 1 (GOLM1) oder Golgi Protein 73 (GP73)). GOLPH2 ist bei diversen entzündlichen Erkrankungen und malignen Tumoren überexprimiert. Seine genaue Rolle in der Tumorentstehung ist bislang nur unvollständig erforscht.

Das Hauptziel dieser Arbeit war es, die funktionelle Bedeutung von GOLPH2 bei der Entstehung und Progression von Tumoren zu untersuchen. Hierzu wurden in subkutanen murinen Tumormodellen Mäuse mit fehlender GOLPH2-Expression (GOLPH2-Knockout) mit Wildtyp-Kontrollen verglichen. In einem Melanom-Modell zeigten GOLPH2-defiziente Mäuse eine deutlich veränderte Tumorstadiumsdynamik und ein verbessertes Gesamtüberleben im Vergleich zu den Wildtyp-Mäusen, was die Hypothese einer protumorigenen Funktion von GOLPH2 unterstützt. Diese Ergebnisse konnten jedoch im Glioblastom-Modell nicht reproduziert werden, dort wurden keine messbaren Unterschiede beobachtet. Hier könnte die geringere Immunogenität des Tumors, Glioblastom gegenüber Melanom, eine Rolle gespielt haben, was allerdings noch zu überprüfen wäre.

Analysen auf molekularer Ebene zeigten eine Hochregulation des Tumor-Nekrose-Faktor-Rezeptor-assoziierten Faktors 6 (TRAF6) in GOLPH2-Knockout-Mäusen. TRAF6 ist ein multifunktionales Adapterprotein, das eine entscheidende Rolle in Signalwegen spielt, die mit Entzündung, angeborener Immunität und Onkogenese assoziiert sind. Die erhöhte Expression von TRAF6 bei fehlendem GOLPH2 weist auf eine mögliche kompensatorische oder regulatorische Wechselwirkung hin, die Unterschiede in der Tumorkontrolle zwischen GOLPH2 defizienten und Wildtyp Mäusen im Melanom-Modell erklären könnte.

Zusammenfassend liefern die Ergebnisse dieser Arbeit neue Hinweise auf eine funktionelle Rolle von GOLPH2 in der Tumorbilogie und deuten darauf hin, dass GOLPH2 über TRAF6 wichtige immunvermittelte Signalwege modulieren könnte. Diese Arbeit kann als Grundlage für zukünftige Studien dienen, die die physiologische und pathologische Rolle von GOLPH2 bei Entzündung, Autoimmunität und Krebs weiter aufklären.

1. SUMMARY

This study presents novel insights into the functional role of the putative oncogenic protein Golgi phosphoprotein 2 (GOLPH2; also known as Golgi membrane protein 1 (GOLM1) or Golgi protein 73 (GP73)). GOLPH2 has been previously reported to be upregulated in various inflammatory conditions and malignancies, yet its precise role in tumorigenesis remains incompletely understood.

The primary aim of this work was to explore the contribution of GOLPH2 to cancer development and progression. To investigate this, murine subcutaneous tumor models were employed by using genetically engineered mice deficient in GOLPH2 expression (GOLPH2 knockout (KO)) alongside wild type (wt) controls. In a melanoma model, GOLPH2-deficient mice exhibited significantly altered tumor growth kinetics and improved overall survival compared to their wild type littermates, indicating a potential tumor-promoting function of GOLPH2. These findings, however, were not detected in a glioblastoma model, where no measurable differences were observed. This difference may be attributed to the lower immunogenicity of the latter tumor model, although this hypothesis remains to be proven.

Mechanistic analysis revealed upregulation of tumor necrosis factor receptor-associated factor 6 (TRAF6) in GOLPH2 knockout mice. TRAF6 is a multifunctional adaptor protein critically involved in signaling pathways associated with inflammation, innate immunity, and oncogenesis. Its increased expression in the absence of GOLPH2 suggests a potential compensatory or regulatory interaction that may underlie the observed phenotypic differences.

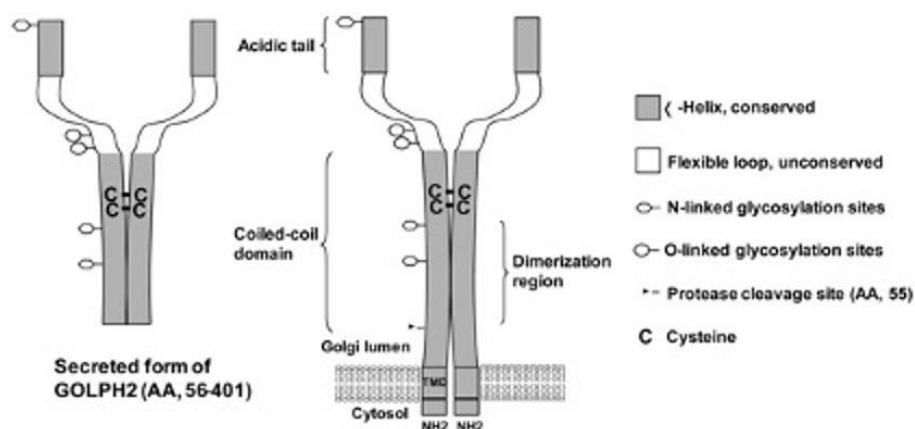
In summary, the findings of this work provide novel evidence for a functional role of GOLPH2 in tumor biology and suggest that GOLPH2 may modulate key immune-related signaling pathways via TRAF6. This work lays the groundwork for future studies aiming to elucidate the physiological and pathological relevance of GOLPH2 in inflammation, autoimmunity, and cancer.

2. INTRODUCTION

In 2000 Kladney and colleagues discovered Golgi protein 73 ((GP73); also known as Golgi membrane protein 1 (GOLM1) or Golgi phosphoprotein 2 (GOLPH2))¹. Their work on giant cell hepatitis (GCH), a form of hepatitis with plausible viral etiology, led them to compare complementary deoxyribonucleic acid (cDNA) from a GCH liver biopsy to a normal adult liver library. They found differentially expressed clones one of which showed similarity to a known partial cDNA identified from a different viral context. Focusing on this clone Kladney et al. fully sequenced the 3042 base pair (bp) messenger ribonucleic acid (mRNA), only omitting one of the two start methionines, and listed it in GenBank on February 16th 2000 under AF236056. Further analyzing the encoded protein, they showed its Golgi localization using immunofluorescence in human embryonic kidney 293 cells (american type culture collection catalog reference line 1573 (ATCC CRL 1573)) overexpressing full length protein tagged at the carboxy-terminus (C-terminus) with a V5 epitope. The protein colocalized with the cis- and medial-Golgi subcompartment marker giantin². These findings were confirmed by immunohistochemistry of GCH liver tissue samples utilizing rabbit polyclonal antiserum against the protein of interest. Detecting its protein band at 73 kilodalton (kDa) in Western blot analysis, they named it Golgi protein 73 or GP73. GP73's actual molecular mass is 45 kDa. Thus, I will be referring to GP73 as Golgi phosphoprotein 2 (GOLPH2) from here on.

GOLPH2 is a transmembrane protein with 401 amino acids and it is organized in 5 regions. The amino-terminus (N-terminal) amino acids 1-12 are located in the cytoplasm. The transmembrane domain (TMD) comprises amino acids 13-35. The protein's intraluminal part is made of a coiled-coil region (amino acids 36-205), an acidic region (206-348) and a conserved C-terminus (349-401). GOLPH2's C-terminus faces the Golgi lumen. The C-terminal ectodomain represents 87 percent (%) of the total protein and is released into the serum after furin cleavage³. At the N-terminus in the cytoplasmic tail (CT) Shao et al. identified the amino acids 7 – 12 (Glycine, Arginine, Arginine, Serine, Methionine, Lysine (GRRSMK)) as epidermal growth factor receptor (EGFR) binding domain⁴. The coiled-coil region at amino acids 131 - 134 (Tyrosine, Glycine, Arginine, Leucine (YGRL)) is a presumed interacting region with microtubule-associated protein 1A/1B light chain 3 (LC3), an important degrading protein in autophagy. This LC3-interacting region (LIR) of GOLPH2 appears to be responsible for selective autophagy of EGFR.

GOLPH2's protein organization is shown in a simplified cartoon in scheme 1⁵. GOLPH2, an "early Golgi protein", cycles from the trans-Golgi network (TGN) to the cell surface and back to the Golgi. After extensive analysis of GOLPH2 truncation mutants Hu and colleagues concluded that the N-terminal cytoplasmic tail and transmembrane domain are most relevant for Golgi localization and necessary for the transport on the cell surface⁶. Bachert and colleagues identified a furin cleavage site near the TMD of the protein's intraluminal part composed of the core sequence Arginine, Valine, Arginine, Arginine (RVRR). Here, GOLPH2 can be cleaved between Arginine 55 and Alanine 56 releasing its ectodomain comprising amino acids 56-401⁷. This ectodomain has been coined soluble GOLPH2 (sGOLPH2).



Scheme 1: A proposed model of a GOLPH2 dimer

Furthermore, Bai and colleagues recently found that the coiled-coil domain of GOLPH2 can form a tetramer in solution. This diversity of GOLPH2 protein forms might broaden its function in physiology and disease⁸.

2.1 GOLPH2 in physiology

GOLPH2 is a single pass type II transmembrane Golgi protein⁹ expressed in epithelial cells throughout the body¹⁰. “Type II” refers to its orientation within the Golgi’s membrane. GOLPH2’s N-terminus is within the cytoplasm, its C-terminus is extracellular or within the lumen of the cellular organelle. GOLPH2 spans parts of Golgi’s membrane, it is found in the cis and medial-Golgi cisternae¹⁰. Cisternae are flattened, membrane-bound sacs that are organized in compartments such as the cis-, medial- and trans-Golgi network. Proteins or lipids from the endoplasmic reticulum (ER) enter at the cis face and exit at the trans face, which is closest to the plasma membrane. Modification and sorting happen within this passage through the Golgi apparatus. Proteins and lipids are transported to their final destination within the cell or secreted outside the cell¹¹. Golgi phosphoproteins contribute to the structure, function and cellular processes of the Golgi apparatus.

Until now, the functions of GOLPH2 have been incompletely understood. However, its essential role in recycling EGFR¹², its expression in cancer cells¹³ and immune cells^{10,14,15} has been shown. Detection of a soluble GOLPH2 form in the serum led to further studies of sGOLPH2. During endosomal trafficking, GOLPH2 is cleaved by the proprotein convertase Furin and sGOLPH2 is released into the extracellular space⁷. Hence, GP73 produces a traceable biomarker for hepatocellular carcinoma.

GOLPH2 located in the Golgi apparatus, is an intracellular protein. However, sGOLPH2 has been detected in the serum of patients with viral liver disease and liver cancer, lung cancer and in the urine of prostate cancer patients^{16,17,18,19}. This property makes GOLPH2 a potential serum marker for the respective diseases.

2.2 GOLPH2 in pathology and disease

2.2.1 GOLPH2's role in ER stress

Functional proteins are folded into unique three-dimensional structures. Misfolding of proteins appears under cellular stress including mutations and aging. Misfolded proteins and protein aggregation are associated with neurodegenerative diseases like amyotrophic lateral sclerosis and Alzheimer's disease²⁰.

When misfolded proteins accumulate in the ER, endoplasmic reticulum stress occurs. Unfolded protein response (UPR) is a response to ER stress and includes a reduction of protein synthesis, an acceleration of protein degradation, an increase in protein folding. In case of unsuccessful resolution, e.g. clearance of misfolded proteins, affected cells undergo apoptosis.

Glucose-regulated protein 78 (GRP78) helps initiate UPR responses. Wei and colleagues showed enhanced secretion of GOLPH2 under ER stress *in vitro* in human hepatocellular carcinoma cell line HepG2 (HepG2), highly-metastatic human hepatocellular carcinoma cell line (MHCC97H), human hepatocellular carcinoma cell line Hep3B (Hep3B) and primary human hepatocyte cells after treatment with ER stressors thapsigargin (Tg) or tunicamycin (Tm)²¹. Also, GOLPH2 secretion was observed *in vivo* in mouse liver tissue and mouse plasma after intraperitoneal tunicamycin (Tm) injection²¹.

Further, secreted GOLPH2 stimulates ER stress activation by binding to GRP78 on the cell surface of neighboring macrophages. The macrophages then release cytokines and chemokines and show a tumor-associated macrophage (TAM)-like phenotype²¹. By immunohistochemistry of hepatocellular carcinoma (HCC) samples a positive correlation between GOLPH2 levels and TAM density could be demonstrated. Furthermore, a correlation between GOLPH2, TAM density and adverse prognosis of patients was noted²¹. Based on these findings GOLPH2 appears to be a promising immunotherapeutic target.

2.2.2 GOLPH2 in inflammation

High levels of GOLPH2 have been described in patients with acute hepatitis B. Wang and colleagues provided insight into the potential mechanism of GOLPH2's upregulated expression in hepatocellular carcinoma cells, especially under inflammatory conditions²². They have identified epithelium-specific erythroblast transformation specific transcription factor-1 (ESE-1) as a mediator of GOLPH2 transcription. Erythroblast transformation specific (ETS) is a family of transcription factors that include the ESE subfamily with the epithelium-specific ETS transcription factor-1 (ESE-1). Immunohistochemical analysis of human HCC tissue regions with higher ESE-1 expression also displayed higher GOLPH2 expression compared to lower expression of both in para-carcinoma tissue²². In the HCC model cell lines, human hepatoma cell line Huh7 (Huh7) and Hep3B direct binding of ESE-1 to GOLPH2's promoter activates GOLPH2 expression whereas GOLPH2 levels decrease when ESE-1 lacks its binding ETS domain²². Both ESE-1 and GOLPH2 expressions were enhanced in response to inflammation shown *in vitro* by interleukin-1 beta (IL-1 β) stimulation of Huh7 and Hep3B cells and *in vivo* by intraperitoneal lipopolysaccharide (LPS) and D-galactosamine injections in a mouse liver model. However, a delay between ESE-1 and GOLPH2 induction suggests the involvement of further factors to drive GOLPH2 transcription²².

2.2.3 GOLPH2 in the context of cancer

Cancer is the second most common cause of death worldwide and imposes a substantial and growing burden on public health. In 2020 it accounted for approximately 10 million deaths worldwide. Most of these cancer deaths were due to lung cancer (18%), colorectal (9.4%), liver cancer (8.3%), stomach cancer (7.7%) and female breast cancer (6.9%). Our present understanding of its complexity and tumorigenesis is summarized by Hanahan and Weinberg's "hallmarks of cancer", where they describe „rules to make a cancer cell“^{23,24}. These fundamental characteristics are shared by most, if not all, human cancers. The hallmarks are functional capabilities and allow cancer cells' survival, proliferation and dissemination. In contrast, enabling characteristics are mechanisms that facilitate the acquisition of the hallmarks of cancer.

GOLPH2's association with cancer is multifaceted. GOLPH2 is highly expressed in various tumors such as liver cancer, lung cancer, colorectal cancer, stomach cancer, breast cancer, esophageal cancer, pancreatic cancer, prostate cancer, cervical cancer, melanoma, bladder cancer, brain cancer and brain tumor¹³. Often, GOLPH2 is associated with tumor progression, poor prognosis and overall survival, stage, size, grade and metastasis, recurrence, immunosuppression and immune escape.

GOLPH2 has been described to play a role in the following aspects described by Hanahan and Weinberg: Activating invasion and metastasis, sustaining proliferative signaling, avoiding immune destruction, tumor-promoting inflammation and reprogramming cellular metabolism⁴.

Activating invasion and metastasis: Abnormal cells that divide uncontrolled grow beyond their boundaries, invade and spread and become metastatic. These processes are complex and cancer cells cross several barriers by mechanisms involving changes in the physical connection of cells and activation of extracellular proteases. Matrix metalloproteinases (MMPs) are endopeptidases that can degrade the extracellular matrix and make way for cancer cells' invasion and metastasis²⁵. The Golgi protein GOLPH2's interacts with intracellular matrix metalloproteinase-7 (MMP-7) and induces invasion and metastasis in hepatocellular carcinoma²⁶. This happens by GOLPH2's vesicular trafficking where MMP-7 is brought to the extracellular spaces.

Further, GOLPH2 regulates matrix metalloproteinase-13 (MMP13) and thereby drives invasion of hepatocellular carcinoma cells²⁷. GOLPH2 also positively regulates metastasis via MMP13 in gastric carcinoma²⁸. In non-small-cell lung cancer (NSCLC) GOLPH2 activates MMP13, again leading to progression and aggressiveness of the disease²⁹. Reduced GOLPH2 expression and consecutive changes in its modulation of the MMP13/epithelial-mesenchymal transition (EMT) axis shows enhanced chemo-sensitivity of cervical cancer to methotrexate³⁰. These observations suggest the potential clinical value of targeting GOLPH2.

Sustaining proliferative signaling: EGFR is a growth receptor on the surface of cells. Upon binding to its ligand, the epidermal growth factor, its intracellular tyrosine kinase domain is activated. Activation is followed by a cascade of signals. When the pathway is dysregulated, it is commonly associated with uncontrolled cell growth and proliferation.

GOLPH2 is embedded within the membrane of the Golgi apparatus, a cellular organelle that has responsibility in processing, sorting and transporting proteins. GOLPH2 recycles EGFR back to the cell membrane, prolonging activation of subsequent kinases and thereby driving metastasis in hepatocellular carcinoma¹².

Avoiding immune destruction: The immune system serves as a critical defense mechanism, utilizing both innate and adaptive responses to monitor and combat pathogens and cancer during their development and progression. Immune surveillance, in theory, represents a perpetually vigilant system that identifies and eliminates abnormal or emerging cells upon detection.

GOLPH2 supports prevention of immune destruction by upregulation of immunosuppressive proteins such as programmed cell death protein 1 (PD-1), programmed death-ligand 1 (PD-L1), cytotoxic T-lymphocyte associated protein 4 (CTLA4), by downregulation of cytokines such as interferon beta (IFN- β), interferon lambda 1 (IFN- λ 1), interleukin 6 (IL-6), interferon-stimulated gene 56 (ISG56), tumor necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ) and interleukin-12 subunit alpha (IL-12A) and by recruitment of inflammatory immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs).

The upregulation of immunosuppressive proteins by GOLPH2 was shown in uveal melanoma³¹ and hepatocellular carcinoma³² resulting in an unfavorable prognosis.

The downregulation of cytokines was shown in hepatitis C virus (HCV) infection³³, gastric cancer³⁴ and hepato-cellular carcinoma³³.

The recruitment of inflammatory immunosuppressive cells such as MDSCs by GOLPH2 has been shown in colorectal cancer³⁵.

GOLPH2 is also expressed and secreted by immune cells^{14,15}. Interestingly, when expressed in TAMs, cancer specific overall survival in cutaneous melanoma is prolonged¹⁴.

However, immune responses appear to be suppressed by secreted GOLPH2. B cell lymphoma cells secrete GOLPH2 which inhibits dendritic cells' (DCs) interleukin 12 (IL-12) production and thymus-derived lymphocytes' (T cells) IL-12 induced IFN- γ production thereby suppressing T cell immune response¹⁵. Moreover, GOLPH2 appears to promote the functional phenotype of M2 macrophages or "alternatively activated" macrophages¹⁵. Macrophages are innate immune cells and their functional phenotype forms in response to their microenvironment. This process is called polarization and polarized macrophages can be described as "classically activated" M1 and "alternatively activated" M2. The latter being of anti-inflammatory nature³⁶.

Tumor-promoting inflammation: Tumor-associated inflammatory conditions and infiltration by immune cells promote cancer formation and progression. GOLPH2 represses innate immunity by downregulating cytokines such as IFN- β , IFN- λ 1, IL-6, ISG56, TNF- α and IFN- γ and IL-12A^{15,34}. Conversely to these down-regulated cytokines, over-expression of GOLPH2 leads to increase of IFN- γ in uveal melanoma³¹.

Lastly, the involvement of immune cells through GOLPH2 was suggested by the fact that it may promote tumor progression by facilitating the recruitment of MDSCs in colorectal cancer (CRC)³⁵.

Reprogramming cellular metabolism: Deregulated proliferation in cancer comes with altered metabolism. The Warburg effect describes cancer cells' aerobic glycolysis, which can be explained by metabolic reprogramming directed by oncogenes to support rapid cancer growth and proliferation. When metabolites accumulate, they themselves can have oncogenic properties and contribute to tumorigenesis³⁷. Oncometabolites are metabolites found more abundantly in cancer cells and that can promote tumorigenesis by alteration of the epigenome³⁸. Although cholesterol is not one of the nine famous oncometabolites, alterations in cholesterol metabolism were shown to influence GOLPH2's function in hepatocellular carcinoma. Cholesterol suppresses GOLPH2's autophagic degradation of receptor tyrosine kinases (RTKs)⁴.

2.3. GOLPH2's oncogenic role

2.3.1. Hepatocellular carcinoma

Yang et al. further investigated GOLPH2's role as an oncogene driving growth and metastasis in hepatocellular carcinoma both *in vitro* and *in vivo*³⁹. GOLPH2 overexpressing HepG2/GOLPH2 cells promoted invasion and migration, knockdown of GOLPH2 in human hepatic adenocarcinoma cell line established at Sloan Kettering-Institute (SK-Hep-1)/short hairpin ribonucleic acid (RNA) targeting GOLPH2 (shGOLPH2) (SK-Hep-1/ shGOLPH2) cells decreased invasion and migration abilities. Mice injected with HepG2/GOLPH2 cells into the tail vein had more metastatic lung foci, whereas mice injected with SK-Hep-1/shGOLPH2 cells exhibited less lung metastases. They further described GOLPH2's role in HCC invasion and metastasis by concentrating on transforming growth factor beta (TGF- β) induced EMT. TGF- β signaling plays an important role in EMT and TGF- β can be added to epithelial cells in culture to induce EMT⁴⁰. In the TGF- β pathway, the ligand transforming growth factor beta 1 (TGF- β 1) binds to a TGF- β cell-surface receptor hereby activating the receptor which then phosphorylates small body size mother against decapentaplegic (Smad) proteins that form complexes and translocate into the nucleus leading to the regulation of genes and thereby regulation of cellular processes including cell proliferation, differentiation, apoptosis, cell plasticity and migration⁴¹. EMT describes transition from tightly connected epithelial cells to more mobile mesenchymal cells. EMT occurs during stages of embryonic development or wound healing, but when it occurs in cancer it can allow cancer cells to become invasive and spread⁴². Yang et al. described that GOLPH2 overexpression enhances phosphorylated Smad homolog 2 and 3 (p-Smad2, p-Smad3) levels and promotes TGF- β 1-induced EMT and invasion in HCC cells⁴³. When HepG2 and SK-Hep-1 cells were treated with TGF- β 1, p-Smad2 and p-Smad3 activation was described⁴³. In GOLPH2 overexpressing HepG2/GOLPH2 cells p-Smad2 and p-Smad3 levels were enhanced whereas in GOLPH2 knockdown SK-Hep-1/shGOLPH2 cells p-Smad2 and p-Smad3 levels were decreased. The GOLPH2 overexpressing HepG2/GOLPH2 cells had more significant mesenchymal features and more invasive abilities, whereas the GOLPH2 knockdown SK-Hep-1/shGOLPH2 had less mesenchymal morphological changes and less invasive abilities. EMT and invasion decreased when GOLPH2 was downregulated or when the TGF- β pathway was blocked with a TGF- β receptor inhibitor (SB431542)⁴³. However, blocking the TGF- β pathway with the TGF- β receptor inhibitor, GOLPH2 upregulation still exhibited enhanced EMT and invasion which suggests involvement of further mechanisms in GOLPH2's oncogenic role³⁹.

2.3.2. Uveal melanoma

To regulate immune response, immune checkpoints, such as receptors on T cells, prevent destruction of healthy cells. PD-1 and CTLA4 are such inhibitory immunoreceptors. Immune checkpoint inhibitors in treatment of cancer can drive T cells to kill cancer cells. Immune checkpoint inhibitors (ICIs) have become a new standard cancer treatment strategy in addition to surgery, radiotherapy and chemotherapy but only limited number of patients have benefited from these drugs due to resistance⁴⁴. Because mechanisms such as: Loss of tumor antigens, dysregulation of antigen processing and presentation, upregulation of other immune suppressive receptors and ligands and inhibition of the interferon IFN signaling pathway can/may not fully explain resistance to ICIs, Kobayashi et al. suggest that oncogenic signaling pathways might play a role in regulating immune checkpoints and resistance to ICIs⁴⁴.

Liang et al. stated that GOLPH2 acts as an oncogene after identifying it as an oncogene associated with poor prognosis in 32 types of cancer³¹. In uveal melanoma (UVM), an intraocular malignancy, higher GOLPH2 expression was also correlated with poor prognosis. Further, it was also associated with increased expression of PD-L1, PD-1, CTLA4 and IFN- γ and with a favorable response to anti-PD-1/ PD-L1 and anti-CTLA4 therapies in UVM patients. GOLPH2 is positively associated with a favorable response to anti-PD-1/ PD-L1 and anti-CTLA4 therapies in UVM patients³¹. Liang et al. identified GOLPH2-low and GOLPH2-high expression groups where high GOLPH2 expression was correlated with poor prognosis and increased expression of PD-L1, PD-1, CTLA4 and IFN- γ . GOLPH2 could predict overall survival as well as response to anti-PD-1/ PD-L1 and anti-CTLA4 therapies in UVM patients³¹.

2.3.3. Lung cancer

Along with eight other genes, GOLPH2 has been identified by Liu et al. to potentially play a role in oncogenic processes of lung cancer⁴⁵. They utilized lung adenocarcinoma immune cell marker genes (LICMGs) that included GOLPH2 to predict patient prognosis and identify patients that would most likely benefit from immunotherapy and/ or chemotherapy. Compared to normal tissues, they described increased GOLPH2 levels in lung adenocarcinoma tissue samples. Nine genes including GOLPH2 were identified that were related to prognosis and were utilized to construct a lung adenocarcinoma immune difference score (LID score). High expression of genes that were predictors of poor survival including GOLPH2 resulted in a higher score. High LID score was associated with lower survival rate but higher responsiveness to immunotherapy and higher sensitivity to chemotherapy than low LID score⁴⁵.

2.3.4 Animal models addressing the role of GOLPH2

The first reported animal model was a transgenic model by Kladney et al.⁴⁶. In this transgenic mouse model GOLPH2's C-terminus was truncated. Overall survival in GOLPH2tr/tr mice was decreased and they developed hepato-renal pathology such as glomerulosclerosis and steatohepatitis. These effects were observed to be more pronounced in female mice⁴⁶. These results suggested that the acidic C-terminus of GOLPH2 is critical for its normal function and for overall survival and integrity of renal and hepatic tissues.

Zhang and colleagues generated a GOLPH2 knockout (KO, GOLPH2^{null}) mouse strain and showed that these mice were viable and did not seem to be strained¹⁵. On closer examination the researchers found IL-12 to be constitutively upregulated, resulting in a pronounced LPS-hypersensitivity, with an increased mortality compared to wild type (wt) littermates¹⁵.

In contrast to the commonly reported oncogenic role of GOLPH2, Pu et al. demonstrated that GOLPH2 acts as a suppressor of colorectal tumorigenesis by preserving the integrity of the intestinal epithelial barrier⁴⁷. Their study utilized a mouse model of colitis-associated carcinoma (CAC) induced by azoxymethane (AOM) and dextran sodium sulfate (DSS). In this model, AOM, a procarcinogen, triggers mutagenesis, while DSS, a heparin-like polysaccharide, causes epithelial damage in the colon. The combination of AOM and DSS creates an environment that mimics inflammation-driven colon cancer, highlighting GOLPH2's protective role against tumor-promoting inflammation⁴⁸.

GOLPH2 knockout mice showed more susceptibility to mucosal inflammation and colitis-induced epithelial damage compared to wild type mice. Pu et al. further described how GOLPH2 depletion

led to aberrant Notch pathway activation with consequent effects such as epithelial lesions and inflammation which were alleviated by pharmacological inhibition with dibenzazepine (DBZ) of this Notch pathway⁴⁷. The cell-surface receptor Notch binds ligands such as Delta-like or Jagged on neighboring cells. The Notch intracellular domain (NICD) is then cleaved and released and travels to the nucleus to regulate transcription. This way, the Notch pathway regulates the division, proliferation, fate, differentiation and death of cells⁴⁹ and co-workers observed upregulation of Notch pathway key genes in GOLPH2 deficient mice and further described increased nuclear translocation of NOTCH2 intracellular domain (N2ICD) in GOLPH2 deficient intestinal epithelial cells (IECs)⁴⁷.

In summary, according to the hitherto data it appears most likely that GOLPH2 is an oncogenic driver and that it might restrict tumor only under certain circumstances, e.g. chronic bowel inflammation.

2.4. Subject and aim of this work

GOLPH2 is upregulated in a variety of malignancies and has emerged as a potential target for anti-cancer therapy.

Despite its association with tumor biology, the role of GOLPH2 in modulating the tumor microenvironment - and particularly its interaction with the host immune system - has not been thoroughly investigated to date.

This study is based on the hypothesis that GOLPH2 expression within the tumor microenvironment contributes to immune evasion by suppressing host immune responses, thereby promoting tumor progression. Accordingly, if the immune system plays a meaningful role in the control of a given tumor type, the absence of GOLPH2 would be expected to result in altered tumor growth dynamics and improved overall survival. A comparison of tumor progression between GOLPH2 knockout mice and wild type littermates thus provides a functional framework to assess this effect.

The primary aim of this work was to explore the immunological interactions between the tumor and its surrounding stroma in the context of GOLPH2 expression. By utilizing multiple syngeneic murine tumor models, this study sought to investigate the potential immunosuppressive role of GOLPH2 and to delineate its contribution to tumor-immune dynamics *in vivo*.

3. MATERIAL AND METHODS

3.1 Material

Deoxyribonucleic acid (DNA) extraction and polymerase chain reaction (PCR) reagents

Thermocycler	Biometra Professional TRIO
KAPA Express Extract	Sigma Aldrich KK7100
1.5 milliliter (ml) microcentrifuge tube	Fisher Scientific 10154671
0.2 ml thin-walled 8 tube & domed cap strips	Thermo Scientific AB-0266
KAPA2G Fast HotStart Genotyping	Sigma Aldrich KK5621
Primer 377: GGT TGC CTT CCT GTG GAT AC	Microsynth Oligo synthesis
Primer 378: CTG TCT TAC ACT GCA GGG TG	Microsynth Oligo synthesis
Primer 381: CTA GTG TAC ATT GTC TCA GG	Microsynth Oligo synthesis
Primer 382: CTG GAA TGT GAG AAC ATC C	Microsynth Oligo synthesis
Agarose	Huber Lab 9012-36-6
Redsafe	INTRON Biotech. 21141
Biorad gel chamber	Sub-Cell GT
Biorad power supply	Power Pac 3000
Rotiphorese 10x tris(hydroxymethyl)aminomethane boric acid	Carl Roth 3061.1
ethylenediaminetetraacetic acid (Tris/Borate/EDTA (TBE)) buffer	
Bench Top 100 bp deoxyribonucleic acid (DNA) ladder	Promega G8291

Western Blot reagents

Disposable syringe Omnifix with Luer-Lock fitting 10 ml	Carl Roth Scheiz C542.1
Red Blood Cell (RBC) Lysis Buffer (10X)	Biologend 420302
Radioimmunoprecipitation assay (RIPA) lysis and extraction buffer	Fisher Scientific 10017003
Halt Protease and Phosphatase Inhibitor Cocktail	Thermo Scientific 10127963
Pierce bicinchoninic acid (BCA) protein assay	Thermo Fisher 23225
4x lithium dodecyl sulfate (LDS) Sample Buffer	Genscript M00676-250
SurePAGE bis-(2-hydroxy-ethyl)-amino-tris(hydroxymethyl)-methane (Bis-Tris) 4-12% 12 well	WITEC for Genscrip M00653
Tris(hydroxymethyl)aminomethane-3(N-morpholino)propanesulfonic acid-sodium dodecyl sulfate (Tris-MOPS-SDS) Running Buffer Powder	Genscript M00138
Precision Plus Protein all blue prestained protein standards	Bio-Rad 1610373
Tween 20, 100 ml	FisherScientific 10113103
Blotting Paper Rotilabo 1.5 millimeter (mm) thickness	Carl Roth CL75.1
Nitro-cellulose membrane	Biorad Roll, 0.45 micrometer (µm), 1620115
Bis-Tris transfer buffer	Sigma Aldrich PPB019-20Pak
Tris buffered saline (TBS)	Sigma Aldrich PPB011-20Pak
Clarity Western enhanced chemiluminescence (ECL) Substrate 200 ml	Bio-Rad 1705061
Albumin fraction V > 98% powder	Carl Roth Schweiz 8076.2
50 ml graduated centrifuge tube	Life Systems D. E1450-0100
0.5 ml thin walled tube with attached flat cap	Life Systems D. 11405810
Eppendorf centrifuge tubes 1.5 ml	Thermo Scientific 10398031
TipOne Pipette Tip 1-200 microliter (µl)	Life Systems D. S1111-0706
TipOne Pipette Tip 10 µl	Life Systems D. S1121-2710
TipOne Pipette Tip 1000 µl	Life Systems D. S1126-7810
Methanol	Carl Roth Schweiz 0082.2

Anti GP73 rabbit polyclonal antibody	Proteintech 15126-1-AP
Anti Cyclophilin B antibody	Sigma Aldrich, SAB4200201
Peroxidas-conjugated AffiniPure Goat anti rabbit immunoglobulin G (IgG)	Jackson Europe 111-036-047
Anti-TRAF6 antibody polyclonal rabbit antibody	ABCam ab137452
Anti-Golm1/GOLPH2 Antibody (BOSTER2)	BosterBio SKU: A02975-2

Cell Culture

Murine melanoma cell lines B16-F10, D4M-3A and murine glioma cell line GL261 were cultured in Dulbecco's Modified Eagle's medium (DMEM) (ingredients see below). For the experiments cells were used at approximately 70% confluency.

Trypsin EDTA

Sera Pro fetal bovine serum (FBS) low endotoxin	Bioswisstec S 40500E
Roswell Park Memorial Institute (RPMI) medium with L-Glutamine	BioConcept 1-41F03-I
DMEM high Glucose with L-Glutamine	BioConcept 1-26F03-I
1 millimolar (mM) sodium pyruvate	Sigma Aldrich S8636
100 micromolar (µM) nonessential amino acids	Sigma Aldrich TMS-001-C
100 units per milliliter (U/ml) penicillin and streptomycin	BioConcept 4-01F00-H
L-Glutamin	Sigma Aldrich TMS-002-C
0.05 mM 2-mercaptoethanol	Bioconcept 5-69F00-E
Tissue culture (TC) flask 75 square centimeters (cm ²) filter cap	Bioswisstec REF70074
Serological pipettes Cellstar Greiner Bio-One	Huber Lab 7.607180
Dulbecco's Phosphate Buffered Saline (DPBS) 500 ml	Lucernachem P04-36500
Ethanol 70% denatured with isopropyl alcohol (IPA)	Huber Lab 9891.5000
Carbon dioxide (CO ₂) incubator	Heraeus Hera Cell
Refrigerated centrifuge (tubes)	Eppendorf 5415
Refrigerated centrifuge (plates)	Heraeus Megafuge 40R
Serological pipettes and pipet aid	Integra Pipetboy 2
Cell counter	DeNovix CellDrop BF
Brightfield microscope (inverted)	Zeiss Primovert
Trypan blue	Huber Lab CH 72-57-1

Flow Cytometry reagents

TruStain FcX Plus	Biolegend 156604
(anti mouse cluster of differentiation (CD) 16/32 antibody)	
Rabbit anti GOLPH2	Boster Bio A02975-3
Rabbit IgG isotype control	Biolegend Poly29108
Anti-rabbit Alexa Fluor (AF) 647	Biolegend Poly4064
4',6-diamidino-2-phenylindole (DAPI)	Fisher Scientific 10184322
Reagent reservoir for multichannel pipettes.	Integra 4312

Other materials

Heat lamp	Animal Infrared light
Veterinary anesthesia Isoflurane evaporator	ASA LT, UK
26 Birmingham gauge (G) 0.45 x12.7 mm needle	BD Plastipak 1 ml Sub-Q
Sterile swabs	
Paper towels	
100 µl -1000 µl pipette tips	Starlab S1111-6700
200 µl pipette tips	Starlab S1120-8710

Ear puncher
Caliper
Hair shaver

VWR Animal Ear Punch
Mannesmann digital caliper
Moser ChroMini Pro

Animals

7-12 week old mouse strain C57 black 6 National Institutes of Health R Janvier (C57BL/6NRj) wt and 2321-Golm1_KO mice

3.2 Methods

3.2.1 Detailed description of genetically modified animals

GOLPH2^{null} (knockout, GOLPH2 ^{-/-}, Golm1 ^{-/-}) mouse strain Basel

The project started out characterizing newly generated genetically GOLPH2^{null} mice. There have been descriptions of GOLPH2^{null} in the literature¹⁵ but the respective groups were not able or willing to share their GOLPH2^{null} mice with our group. Thus, in collaboration with Dr. Pawel Pelczar, head of the center for transgenic models (CTM) at the University of Basel, a novel GOLPH2^{null} strain was created at the CTM. By using clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR-associated protein 9 (Cas9) technology the complete GOLPH2 gene was deleted following the protocol outlined in Modzelewski AJ and colleagues paper⁵⁰. GOLPH2 guide ribonucleic acid (RNA) and CAS9 protein were delivered in zygotes of mouse strain C57 black 6 National Institutes of Health R Janvier (C57BL/6NRj) mice by electroporation. The guide RNA (gRNA) target sequences were:

ATG proximal - ggccctggttctaagatgat(ggg)

STOP proximal - gagtcacagtgaacccccgt(agg)

with the protospacer adjacent motif (PAM) in brackets and underlined. The electroporated pronuclear-stage embryos were implanted into the ampulla of pseudopregnant female mice within 24 hours after electroporation to generate live animals. Pups were genotyped by polymerase chain reaction (PCR). Animals with a GOLPH2 deletion were then backcrossed to C57BL/6. From the resulting first filial (F1) generation those with GOLPH2^{+/-} genotype were intercrossed. Animals of this second filial (F2) generation were characterized and those with GOLPH2^{null} genotype were selected for mating to obtain GOLPH2^{null} lines. In each offspring of those lines the genotype was tested by a newly designed GOLPH2 PCR, described in detail in the method section 3.2.1.2. The novel mouse strain was deposited in the CTM database as ID: 2321 C57BL/6J Golm1<em1(null)Ctmb>. For uniformity the name of the strain in this manuscript is GOLPH2^{null} or GOLPH2^{-/-}.

3.2.2. Genotyping by DNA extraction and PCR

GOLPH2 knockout mice, GOLPH2^{null}, were bred in the animal facility of the Department of Biomedicine, University of Basel, Switzerland. To ensure identification toe marking of each newborn GOLPH2^{null} pup was done. Leftover mouse tissue from this toe marking was used for deoxyribonucleic acid (DNA) extraction and PCR analysis.

DNA extraction

The leftover toe tissue was stored in 1.5 milliliter (ml) microcentrifuge tubes at -20 degree Celsius (°C) until processing. DNA extraction from this tissue was done using the KAPA Express Extract Kit. The samples were sorted into racks and arranged in ascending numerical order according to their animal number. A corresponding number of 0.2 ml tubes was prepared and labelled accordingly. For each sample 40 microliter (µl) of extraction mix was used. The extraction mix consisted of 35.2 µl PCR-grade water, 4 µl KAPA Express Extract Buffer, 0.8 µl KAPA Express Extract Enzyme. For 22 samples a master mix for 25 extractions, calculating a surplus of 3, was prepared with the components as follows: 880 µl PCR-grade water, 100 µl KAPA Express Extract Buffer, 20 µl KAPA Express Extract Enzyme.

A volume of 40 µl of the master mix was aspirated with a 100 µl pipette and using this pipette the toe tissue sample was picked up and transferred to the correspondingly labelled 0.2 ml tube. It was ensured that the tissue sample was correctly immersed, then the cap was closed. The strips were placed in a Biometra trio PCR block. A three step program was used, consisting of 10 minutes (min) at 75°C for lysis, 5 minutes at 95 °C for enzyme activation and a final hold at 4°C.

PCR

Two pairs of primers were designed, primers 377 and 378 to yield a 375 bp product in GOLPH2^{-/-} or GOLPH2^{+/-} DNA, primers 381 and 382 to yield a 639 bp product in a GOLPH2^{+/+} or GOLPH2^{+/-} sample.

The PCR master mix was prepared with KAPA2G Fast HotStart Genotyping Mix. For 22 samples and two controls a PCR master mix for 25 reactions, calculating a surplus of 3, was prepared with the components as follows: 200 µl PCR-grade water, 250 µl 2x KAPA 2G mix and 12.5 µl of 10 micromolar (µM) working solutions of each of the four primers 377, 378, 381 and 382. PCR strips were labelled with the corresponding animal numbers or control type and 19 µl of PCR master mix was added to each tube. 1 µl per sample was transferred to its labelled PCR tube. Water was used as a negative control and a DNA sample from a wild type mouse served as a positive control. After closing the caps, the strips were vortexed, placed into a table top centrifuge and spun for a few seconds. Then the strips were placed into the PCR block and a predefined program was started. The program initialized for 2 minutes at 95°C and comprised 35 cycles of denaturation for 15 seconds at 95°C, annealing for 20 seconds at 60°C and elongation for 30 seconds at 72°C. After these 35 cycles, final elongation was performed for 2 minutes at 72°C followed by a final hold at 4°C.

TBE Gel and analysis

The side of a Bio Rad sub-cell GT gel holder was sealed with tape. A 2% agarose gel was prepared by adding 2.4 gram (g) of agarose and 120 ml of 1x tris(hydroxymethyl)aminomethane boric acid ethylenediaminetetraacetic acid (Tris/Borate/EDTA (TBE)) buffer to a glass bottle of 250 ml. The bottle was gently shaken to mix the solution and then transferred to a microwave oven and heated. This procedure of mixing and heating was repeated until the agarose powder was completely dissolved and no more visible viscous filaments could be found in the solution. The glass bottle was held under cold water until the solution had cooled down. Then 5 µl of Redsafe reagent was added, the solution was mixed until homogenized and then poured into the prepared gel holder. Combs were placed in the designated area of the gel holder. Once the gel was solid, tape and combs were removed. The gel was placed in a BioRad electrophoresis chamber. The running buffer 1x TBE was filled up so that the gel was fully submerged. Into the gel pockets 18 µl per PCR sample were loaded. In each row one pocket was filled with 5 µl Bench Top 100 bp DNA ladder. The gel was run at 120 volt (V) for 45 minutes.

The gel was placed in the drawer of the front of a Vilber Fusion Fx machine for analysis. The program Evolution Edge and imaging system Fusion FX7 Edge 18.11 SN was started. The gel

was manually positioned in the middle of the drawer centered beneath the camera under visual control at the monitor. Using the “focus” tap the capture size was selected before the protocol was run. The picture was saved, exported and inserted in a PowerPoint file to annotate animal numbers to the respective gel slots.

3.2.3. Western blot for GOLPH2^{null} phenotype confirmation

Preparation of murine blood samples

Whole blood samples from C57BL/6 mice and putative GOLPH2^{null} mice were collected in 2 ml microcentrifuge tubes. Blood drops were suspended in 500 µl Phosphate Buffered Saline (PBS) and stored at 4°C for up to 6 hours prior to processing. The samples were centrifuged at 1500 revolutions per minute (rpm) for 5 minutes, and then the supernatants were discarded. The resulting cell pellets were resuspended in 250 µl Red Blood Cell (RBC) Lysis Buffer, mixed thoroughly, and incubated at room temperature for 3 minutes. The samples were then centrifuged at 1500 rpm for 5 minutes, and the supernatants were removed. An equal volume of radioimmunoprecipitation assay (RIPA) lysis buffer was added to the pellets, and the samples were incubated on ice for 30 minutes with vortex mixing every 5 minutes. The lysates were then centrifuged at 12000 rpm for 20 minutes at 4°C. The supernatants were collected and centrifuged again at 12000 rpm for 5 minutes. The clarified supernatants were transferred to clean 1.5 ml microcentrifuge tubes for further analysis.

Quantification of protein in lysed samples

Protein amount was measured using a bicinchoninic acid (BCA) protein assay. First, the albumin standard dilutions were prepared. From one ampule of 2.0 milligram (mg)/ml albumin standard, 0.5 ml was transferred to a 15 ml centrifuge tube, diluted in 4.5 ml Dulbecco's Phosphate Buffered Saline (DPBS), and labeled “A 200 µg/ml”. From this tube A 2 ml were transferred to a fresh 15 ml centrifuge tube, diluted in 8 ml PBS and labeled “B 40 µg/ml”. From this tube B, 4 ml were transferred to a fresh 15 ml centrifuge tube, diluted in 4 ml PBS, and labeled “C 20 µg/ml”. This last 1 to 1 dilution was repeated 5 times to prepare tubes D-H which were the standards 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1 µg/ml, and 0.5 µg/ml. Lastly, tube I was filled with 4 ml PBS and served as blank.

In a 96-well plate, 150 µl of albumin standards in descending order and the blanks were pipetted per well in duplicates. From each sample, 150 µl was applied to the plate in duplicate. Then 150 µl of premixed working reagent consisting of 3750 µl reagent MA, 3600 µl of reagent MB and 150 µl of reagent MC was added to each well. The plate was sealed, incubated for 1 hour at 60°C, cooled on ice to room temperature and absorbance was read in a spectrophotometer Gemini at 562 nanometer (nm). The average 562 nm absorbance reading of the Blank standard replicates was subtracted from the 562 nm reading of all other individual standard and sample replicates.

A standard curve was prepared by plotting the average blank-corrected 562 nm reading for each bovine serum albumin (BSA) standard against its concentration in µg/ml using PRISM Graph. The protein concentration of each sample was determined in Graphpad PRISM by mouse over the standard curve at the measured absorbance value.

SDS PAGE gel run and transfer of proteins onto a nitrocellulose membrane

In a 0.5 ml centrifuge tube 30 µg protein supernatant and 5 µl of lithium dodecyl sulfate (LDS) sample buffer were mixed and boiled for 5 minutes at 95°C in a heating block. Samples were cooled on ice.

A precast 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) gel was placed in the inner chamber of a Western Blot chamber system so that the shorter plastic slide was facing inwards. To close the inner chamber a dummy plastic slide was placed on the opposite side. The inner chamber was positioned inside the Western Blot box and filled halfway with 3-(N-morpholino)propanesulfonic acid (MOPS) running buffer. Then the inner chamber was filled with running buffer up to the upper end of the shorter plastic slide of the precast gel. Each prepared sample was pipetted in the pockets of the gel in duplicate as well as 10 µl of pre-stained protein standard. The lid was mounted on the Western Blot chamber and 100 V voltage was applied. While controlling the progress of the marker bands the gel was run for about 1.5 hours.

Then the lid was removed and the inner chamber was taken out and disassembled. The plastic frame of the precast gel was cracked open, the gel was recovered and equilibrated in a small plastic box containing a transfer buffer. Six pieces of thick blotting paper about the size of the dummy plastic slide were thoroughly wetted in the bis-(2-hydroxy-ethyl)-amino-tris(hydroxymethyl)-methane (Bis-Tris) transfer buffer supplemented with 10% Methanol (Tris buffered saline (TBS) methanol (TBS-M)). Nitro-cellulose membrane was cut to the size of the gel and wetted in TBS-M for 5 minutes. In a semi dry Transfer Blotter a sandwich of 3 pre-wetted blotting paper sheets, the pre-wetted Nitro-cellulose, the gel, and again 3 pre-wetted blotting paper sheets was assembled in the middle of the lower electrode plate. The upper electrode plate was mounted by putting the lid on the machine. By connecting the electrical wires to a power supply 25 V voltage was applied. The transfer was controlled after 1.5 hours by carefully disassembling the sandwich and checking for transfer of the prestained ladder bands to the nitro-cellulose membrane.

The membrane was then washed in a plastic box in TBS for 5 minutes at room temperature on a slowly rocking shaker and cut near the marker in half to allow two different immunostaining in parallel.

The two membrane pieces were incubated in a blocking buffer containing PBS, 5% BSA, and 0.1% Tween 20 for one hour at room temperature on a shaker. Anti GP73 rabbit polyclonal antibody was prepared in a 10 ml solution of PBS and 5% BSA in a dilution of 1:4000. Anti Cyclophilin B antibody was prepared in a dilution of 1:10 000. The two membrane pieces were transferred to two 50 ml centrifuge tubes. One tube was filled with an anti GP73 antibody solution, the other with Cyclophilin B antibody solution. The tubes were placed on a rolling board located in a 4°C fridge and membranes were incubated overnight.

The next morning both membranes were washed in a plastic box three times in PBS supplemented with 0.1% Tween 20 (PBS-T) for 20 minutes on a shaker at room temperature. Then an anti rabbit horseradish peroxidase labeled goat antibody fragment was applied on both membranes in a dilution of 1:4000 in PBS 5% albumin for 1 hour shaking at room temperature. Membranes were washed again three times. Both membranes were then transferred to a pouch of a punched pocket that had been cut open at the long side. Meanwhile reagents from a Bio-Rad Clarity Western enhanced chemiluminescence (ECL) reaction kit were mixed in a 2 ml microcentrifuge tube by adding 1 ml of reagent A and 1 ml of reagent B. From this mixture 1 ml was pipetted on each membrane piece, the upper sheet of the pouch was placed on the prepared membrane avoiding bubbles while closing. The pouch with the membranes was placed in a Bio-Rad ChemiDoc and the emitted luminescence was captured as dark bands on an image.

3.2.4 Western blot for TRAF6 protein expression

Whole blood lysate from C57/B6 wild type and GOLPH2^{null} mice was boiled in SDS sample buffer, run on an SDS PAGE gel and the proteins were transferred to nitro-cellulose as described above in 3.2.3. The membranes were incubated in anti tumor necrosis factor receptor-associated factor 6 (anti-TRAF6) antibody in PBS and 5% BSA overnight at 4°C. The next morning both membranes were washed three times in PBS-T. Next, an anti-rabbit horseradish peroxidase labeled goat antibody fragment was applied on both membranes in a dilution of 1:4000 in PBS 5% albumin for 1 hour shaking at room temperature. Membranes were washed and protein bands were detected in a Bio-Rad ChemiDoc utilizing Bio-Rad Clarity Western ECL solution.

3.2.5. Cell culture

Murine tumor cell lines that were routinely used in C57BL/6 mouse models were chosen for the experimental setup^{51,52}.

The murine tumor cell lines, namely murine melanoma cell lines B16-F10, D4M-3A and murine glioma cell line GL261 were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 2 millimolar (mM) Glutamin, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 units per milliliter (U/ml) penicillin/streptomycin, 100 µM nonessential amino acids, 50 µg/ml ciproxin and 0.05 mM 2-mercaptoethanol. The cells were assumed to be in a logarithmic growth phase when a 70% confluency was documented. Then, medium was aspirated, cells were washed by covering them with PBS and gently moving the flask. PBS was aspirated and 3 ml Trypsin ethylenediaminetetraacetic acid (EDTA) was added and the flask was set lying in the incubator for 3-4 min. After control under the microscope and tapping of the side of the flask to detach all rest cells, trypsinization was stopped by adding 5 ml of DMEM medium with a serological pipette. The suspension was added to a 50 ml tube and centrifuged at 1500 rpm for 4 min. The supernatant was aspirated and cells were washed with PBS. Cells were counted using 10 µl of 10 µl of trypan blue and 10 µl of cell suspension and pipetting it onto the Cell counter DeNovix. Again, the supernatant was aspirated and the pellet was resuspended in PBS at a concentration of 1.8×10^7 tumor cells per ml. The cells were kept in a 2 ml centrifuge tube and remained on ice until injection. A volume of 50 µl of this cell suspension was injected subcutaneously into each animal.

3.2.6. Flow cytometry analysis

For flow cytometry murine lung epithelial cells tissue culture-1 (TC-1), murine colon adenocarcinoma cells (MC38), murine melanoma cell lines B16-F10, D4M-3A and murine glioma cell line GL261 cells were cultured in 75 square millimeter (mm²) flasks in a cell incubator as described above. At the day of the assay, cells were washed in PBS and detached using 0.5 mM EDTA in PBS. Detachment was stopped by adding 10 ml of Roswell Park Memorial Institute (RPMI) medium, then the cells were harvested in 50 ml tubes and centrifuged at 1500 rpm for 3 minutes. Supernatants were discarded and cell pellets were resuspended in 200 µl PBS. Cells were counted by preparing a mixture of 10 µl trypan blue and 10 µl of cell suspension and pipetting it onto the Cell counter DeNovix. According to the measured count of viable cells an appropriate volume of cell suspension was diluted in cold fluorescence-activated cell sorting (FACS) buffer at a cell concentration of 1×10^6 cells per ml. In a 96 well v-bottom plate 100 µl of the cell suspension accounting for 100.000 cells was distributed per well, five wells per cell line. From each cell line, well one was stained with anti murine GOLPH2 antibody (1 µg/100µl), well two with a rabbit isotype control antibody and wells three to five were left untouched. Upon incubation on ice for

one hour the plate was centrifuged at 1500 rpm for 5 minutes, supernatant was removed and cell pellets were washed in 200 µl FACS buffer by pipetting. This washing step was repeated twice. Then cell pellets were resuspended in 100 µl FACS buffer. Wells one, two, and three were stained with anti-mouse Alexa Fluor (AF) 647 (1 µl/100µl), wells four and five were left untouched. Incubation on ice in the dark lasted 45 minutes, then cells were washed three times and resuspended in 100 µl FACS buffer. Wells one to four were stained with 4',6-diamidino-2-phenylindole (DAPI) then the plate was transferred to a Cytoflex Flow cytometer and analyzed utilizing the CytExpert software. In the analysis of well four DAPI-positive cells were excluded by setting the gate in the PacificBlue channel accordingly. In well two DAPI negative cells were then gated to exclude isotype control positive cells, thereby setting the cutoff in the allophycocyanin (APC) channel for truly positive cells. Then well 1 was analyzed in this DAPI negative and isotype control outshining gate for truly positive anti-GOLPH2 cells.

3.2.7. Animal models

Subcutaneous (s.c.) tumor models

The subcutaneous tumor models have been standardized in our laboratory and also the supervisions and observation of tumor growth are standardized. For tumor growth and animal well being, we used a score sheet that has been validated in our laboratory and has been approved by the veterinary department of Basel. Animals falling below the permissible score (eg. weight loss, pain, discomfort and ulceration) were euthanized immediately.

The tumor models in detail

To address the role of GOLPH2 in cancer immunity and its possible interference with cancer progression three syngeneic animal models were chosen for this study:

- D4M-3A, a murine melanoma cell line that recapitulates the human melanoma B-Raf proto-oncogene, serine/threonine kinase (BRAF) substitution of valine (V) with glutamic acid (E) at position 600 in the B-Raf protein (V600E) (BRAF^{V600E}) mutation,
- B16-F10, a murine melanoma cell line and
- GL261, a murine glioblastoma cell line.

The first two melanoma cell lines display a low responsiveness profile towards immunotherapy (I/O) *in vivo*. D4M-3A, a BRAF mutated and phosphatase and tensin homolog (PTEN^{null}) melanoma line and B16-F10 are resistant or only moderately sensitive to I/O therapy⁵³.

The GL261 cell, a glioblastoma model is known to be only moderately immunogenic⁵⁴. However, GL261 tumors are responsive to higher doses of immunotherapy, e.g. anti-PD-1 antibodies⁵⁵.

Animal preparation before tumor injection

For each experiment a sample size of 6 mice per group was used, six C57BL/6NRj wild type mice and six GOLPH2^{null}.

7-12 week old mice were used. C57BL/6NRj mice (wild type) and GOLPH2^{null} mice were bred in the animal facility of the Department of Biomedicine (DBM) of the University of Basel in Switzerland. Besides breeding rooms the animal facility of the DBM has dedicated experimental rooms for experimental procedures. Regular cage maintenance was done by animal facility staff ensuring fresh food, water supply and enrichment within each cage as well as regular assessment of well being of the animals. Health and tumor checks were done by me according to the protocol of the animal licence #3118.

Animals were not required to be pre-conditioned or pre-treated before the experiment. Tagging by ear punch was necessary in order to score health conditions and document tumor growth of each mouse during the respective experiments. Ear punching was very reliable and as a one time procedure was less stressful than repetitive markings (e.g. shaving or color marking). Also, ear punching was done during tumor cell injection (under an anesthetic face mask) with the marking scheme for a cage of 6 mice as follows: unmarked ($\emptyset$), Right (R), Left (L), Right/Left (RL), Right/Right (RR) and Left/Left (LL).

Experiment: Subcutaneous tumor model without treatment (survival study)

Tumors were implanted in a total of 12 mice by subcutaneous injection of 900.000 B16-F10 cells using a volume of 50 μ l PBS administered under an anesthetic face mask to the shaved right flank of each animal.

For this experiment, we utilized 7-12 week old mice. We observed a group of six C57BL/6NRj wild type mice and another group of six 2321-Golm1_KO knockout mice. These animals were bred in the animal facility of the Department of Biomedicine of the University of Basel in Switzerland. The subcutaneous injection of tumor cells was performed under general anesthesia. This allows exact placement of tumor cells in the subcutaneous tissue and significantly reduces ulcerations and complications during tumor growth. For the installment of tumor cells, only a short duration of anesthesia of approximately 2-3 minutes was required and anesthesia did not exceed 10 minutes within a cage of 6 animals. In general, subcutaneous tumors are not painful and do not require any regular analgesia. Moreover, anti-inflammatory medication might interfere with tumor growth. In case of ulceration or distress or if any single parameter of the Scoresheet equaled zero the animals were sacrificed immediately.

For general anesthesia isoflurane 2-4% was used and administered via chamber and face mask using 0,6 liter/min of oxygen as carrier gas. The mice were transferred from their cage to an anesthesia induction chamber using 3-4% isoflurane for induction and 2-3% for maintenance with a steady flow of 0,6 L/min O₂ as carrier gas. Once breathing has slowed and reached a steady pattern, the mice were taken out from the chamber and placed on a soft pad where isoflurane 3.5% was administered using an anesthetic face mask. Under this general anesthesia we performed the tumor installment while monitoring body temperature and breathing movement continuously. The body temperature was maintained stable by using the heat lamp installed above the mouse cage. Furthermore, breathing movement and foot pad pinch reflex were checked regularly during the whole procedure. To place the anesthetized mouse on the soft pad, the animal was grasped by the skin at the nape of its neck and gently placed on its left side with its nose under the face mask. Prior to tumor cell injection, the ear was marked with an ear punch and the right flank was shaved and disinfected using a wipe. Tagging by ear punch was necessary in order to score health conditions and document tumor growth of each mouse. Ear punching is very reliable and as a one time procedure less stressful than repetitive markings (e.g. shaving or color marking) and was done during tumor cell injection (under an anesthetic face mask) with the marking scheme for a cage of 6 mice as follows: unmarked ($\emptyset$), Right (R), Left (L), Right/Left (RL), Right/Right (RR) and Left/Left (LL). The epi tube with B16-F10 cells (or D4M cells and GL261 cells, respectively) was inverted several times to assure resuspension and a syringe was filled with 50 μ l of cell suspension. The tumor cells were injected into the right flank of the mouse by pinching the skin of the mouse between the index finger and thumb and pulling the skin away from the body of the mouse. 900.000 B16-F10 cells (or 1 million D4M cells and 1 million GL261 cells, respectively) in maximal volume of 50 μ l of PBS were slowly and evenly injected into the pouch created by the fingers, creating a single bubble of cells beneath the skin and avoiding too much spread of the cells. Once injected, the mice were regularly monitored from the next days onwards. These models generated palpable tumors which were measured regularly every 3x/week and when the tumor reached a critical size near 1000 cubic millimeter (mm³) every other day. Tumor volume was measured with calipers and calculated with the measurements of the

shortest (d) and longest (D) perpendicular tumor diameters using the formula: tumor volume cubic millimeter (mm^3) = $d^2 \times D/2$.

Tumor sizes were measured and documented by two persons and the person doing the tumor measurements did not know if the animal belonged to the experimental treatment or the control treatment group to reduce a potential readout bias.

On average, the experiments lasted for 3 weeks after tumor implantation. During this time tumor bearing mice were monitored and the tumor progression of C57BL/6NRj and of GOLPH2^{null} mice were documented. The primary read-out was assessed by measuring tumor growth and overall survival and time to end-point. The end-point was reached once the tumor reached a size of 1500 mm^3 (or longest diameter exceeded 20 millimeter (mm)), or when their body score fell below 6 (according to body scoring sheet) or when tumor ulceration was noted, whichever came first. If any humane endpoint was reached the mice were euthanized by carbon dioxide (CO_2) inhalation.

Humane endpoints:

- Tumor size <1500 mm^3
- Longest diameter exceeds 20 mm
- Ulceration of the tumor
- Tumor interferes with movement of mice or function of organs
- Animals are unable to eat or drink
- Animals are in distress (scoring sheet)
- Loss of 20% of the initial body weight

All animals were sacrificed at the end of the respective experiment.

Methods of euthanasia: / Method to sacrifice mice

In detail, CO_2 euthanasia was performed by inhalation of highly concentrated CO_2 from pressurized tank with compressed CO_2 in an uncrowded chamber provided by the animal facility. First, animals were put into the chamber and then the chamber was flooded with CO_2 . Animals were left in the chamber for a sufficient amount of time (4 to 5 minutes) to guarantee the death of the animals. Death was confirmed by checking the breathing and eyes of the animal.

4. RESULTS

4.1 Verification by genotyping of GOLPH2^{null} mice

The GOLPH2 genotype of each offspring was analyzed by PCR as outlined in section 3.2.

Two primer pairs, one to yield a PCR product of 376 bp in a GOLPH2^{-/-} or GOLPH2^{+/-} sample and one to yield a PCR product of 639 bp in a GOLPH2^{+/+} or GOLPH2^{+/-} were used in the same PCR.

In Figure 1 the genotype of the offsprings 60716 to 60738 can be unambiguously deduced from the visible bands in the TBE gel. For orientation, the 500 bp band in the ladder slot is the brightest. Amplification of only the 639 bp product, and therefore genotype GOLPH2^{+/+}, occurred in samples of animals 60716, 60718, 60721, 60726, 60727, 60728, 60729 60738.

Amplification of both, 375 bp and 639 bp products, and thus genotype GOLPH2^{+/-}, is apparent in samples of animals 60720, 60722, 60730, 60731, 60733 to 60737.

Genotype GOLPH2^{-/-}, has an amplification of a 375 bp product only, e.g. animals 60717, 60719, 60723 to 60725 and 60732.

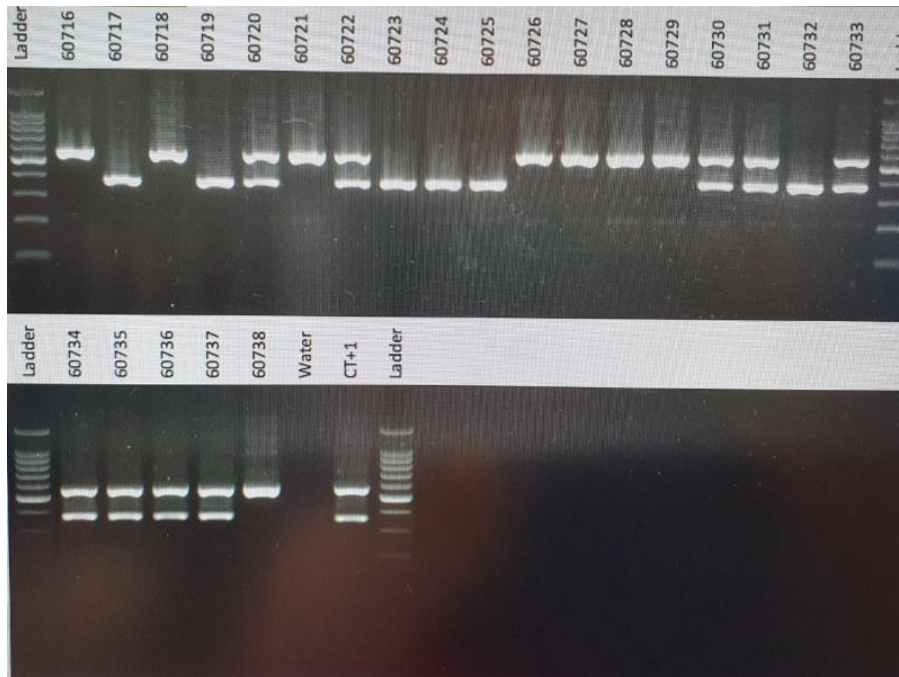


Figure 1 **Genotyping PCR for detection and confirmation of GOLPH2 mice.**

Each lane shows GOLPH2 status in one mouse. One band at 375 bp, e.g. lower band only indicates a GOLPH2^{null} phenotype. A singular band at 639 bp represents a wild type mouse.

A lane with two bands indicates a monoallelic genotype, e.g. GOLPH2 ^{-/+}, The 500kb marker in the ladder is the most prominent marker band.

4.2 Verification by Western blot of GOLPH2^{null} mice

GOLPH2 protein expression was analyzed by Western blot using blood-derived cell lysates from 2321-GOLPH2^{null} mice and C57BL/6 wild type littermates (Figure 2). A whole blood lysate from a C57BL/6 wild type mouse was included as a positive control. Samples from the genotyped GOLPH2 ^{-/-} mice 60717, 60725 and 60732 were loaded in lanes 1,3, and 5, respectively. No protein band was detected at approximately 45 kDa in these samples, indicating the absence of GOLPH2 protein expression. In contrast, clear protein bands at the expected molecular weight were observed in samples from C57BL/6 wild type mice loaded in lanes 2 and 4, respectively.

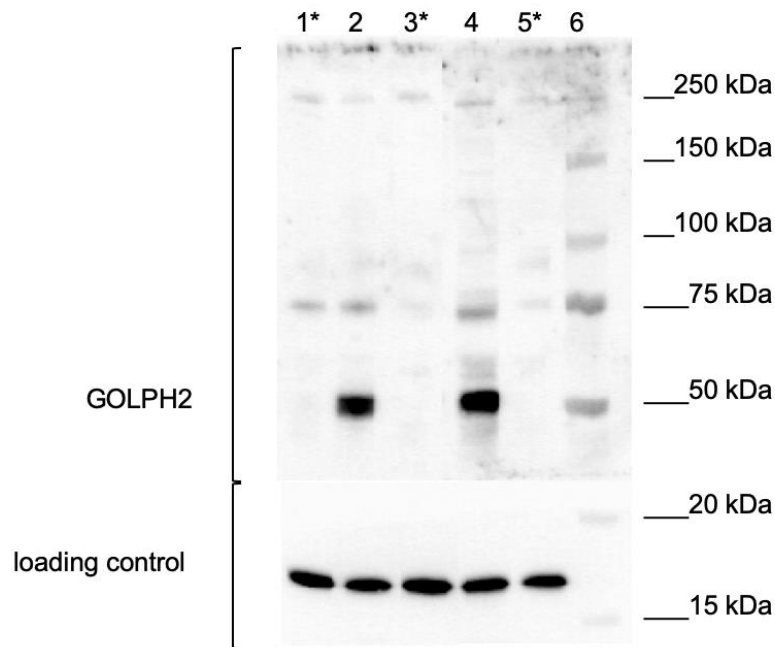


Figure 2 **Anti-GOLPH2 western blot** of peripheral blood mononuclear cells (PBMCs) from GOLPH2^{-/-} mice (Lanes 1, 3 and 5 marked with *) and age and sex matched wild type littermates are shown in lane 2 and 4.

Lane 6: Protein weight marker of the upper panel. Cyclophilin B (lower panel) served as loading control.

Note: GOLPH2 protein runs at approx. 43 kDa and the distinguishing band is only visible in lane 2 and 4. The other bands are unrelated artifacts detected by the anti-GOLPH2 rabbit polyclonal antibody.

4.3 TRAF6 protein expression in GOLPH2^{null} mice versus wild type C57BL/6NRj

The role of Golgi protein 73 (GP73/GOLM1) has been established in viral pathogenesis³³. GP73 negatively regulates the host's innate immune response, thereby aiding virus replication. This is achieved through the degradation of MAVS (mitochondrial antiviral-signaling protein) and TRAF6 (TNF receptor-associated factor 6), both crucial components in the antiviral signaling pathway. By facilitating the breakdown of these proteins, GP73 impairs the host's ability to mount an effective immune defense, highlighting a potential target for therapeutic intervention in viral infections.

Here, I examined the TRAF6 levels in GOLM1^{-/-} mice in comparison to C57BL/6 wild type mice. As shown in Figure 3 TRAF6 expression was upregulated in the peripheral blood of knockout mice.

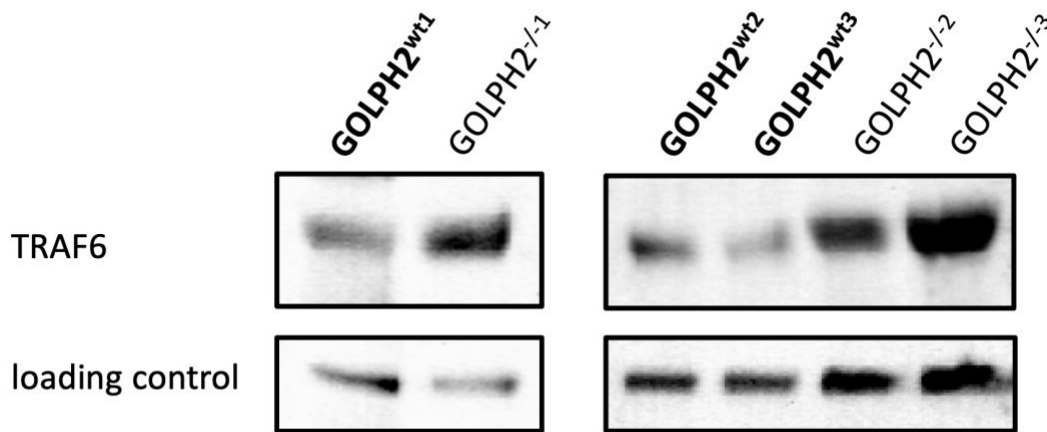


Figure 3 **TRAF6 levels in GOLPH2^{null} versus wild type mice.** Western blot analysis of peripheral blood lysates from three biologically independent GOLPH2^{null} mice and three wild type littermates showing TRAF6 expression. Cyclophilin B was used as a loading control. The upper panels show samples from GOLPH2^{null} mice, namely GOLPH2^{-/-1}, GOLPH2^{-/-2}, GOLPH2^{-/-3} and wild type littermates, namely GOLPH2^{wt1}, GOLPH2^{wt2}, GOLPH2^{wt3} (bold). The corresponding Cyclophilin B loading controls are shown in the lower panels.

4.4 GOLPH2 expression on the cell surface of various murine tumor cells

Syngeneic cell lines that are often used as tumor models in C57BL/6 mice were analysed in flow cytometry for GOLPH2 surface expression. The highest expression of GOLPH2 was found in a lung and colorectal cancer (TC-1 and MC38 cell line). As I was most interested in GOLPH2's role in the microenvironment in melanoma, the two melanoma cell lines B16F10 and D4M-3A were chosen. As a syngeneic cell line with similar GOLPH2 expression regarding the percentage of positive cells and mean fluorescence intensity (MFI) GL261 were selected. Figure 4 shows the respective GOLPH2 levels.

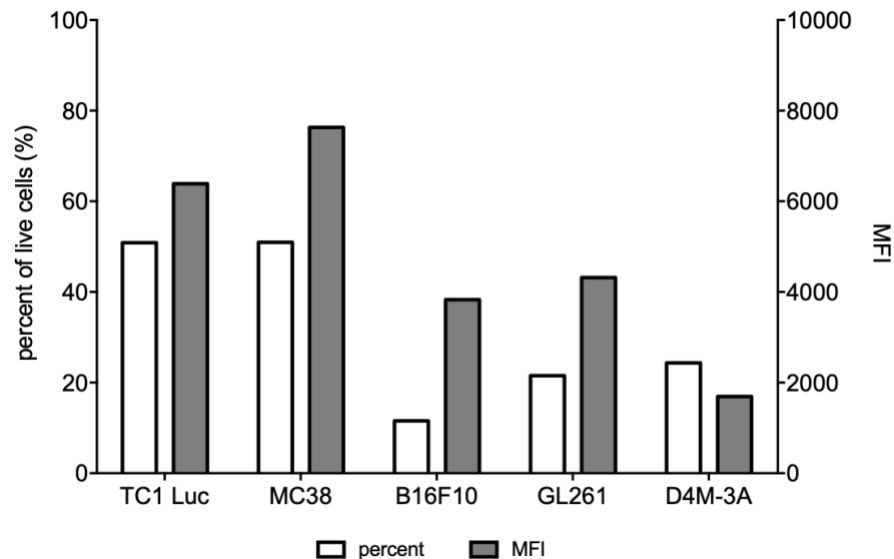


Figure 4 **Cell surface levels of GOLPH2** in the indicated tumor cell lines was determined by flow cytometry with a polyclonal antibody (BosterBio SKU: A02975-2).

In the lung cancer cell line TC-1 (here the luciferase transfected variant, named TC1 Luc is shown) and the colon cancer cell line MC38 50% of cells were GOLPH2 positive with a mean MFI of about 7000. In the melanoma cell line B16F10 12% of cells and in D4M 24% of cells were GOLPH2 positive, with the respective MFI of 3800 and 1700. In the glioblastoma cell line GL261 22% of cells were GOLPH2 positive with a mean MFI of 4300.

4.5 *In vivo* tumor models

The role of GOLPH2 in tumor propagation was studied in GOLPH2 $-/-$ versus wild type littermates. Three animal experiments were designed in three different tumor models. The respective tumor models were B16-F10, D4M-3A and GL261. The cell lines B16-F10 and GL261 were obtained from ATCC, D4M-3A were a gift from Mikael Pittet, Ludwig Cancer Research Institute (Lausanne).

It is noteworthy that all tumor models were established s.c. leading to orthotopic tumor growth in the melanoma models and heterotopic tumor growth in the case of GL261. The respective mice were bred in the animal facility of the DBM/ University of Basel, Switzerland.

To investigate the impact of GOLPH2 on tumor progression and survival, three subcutaneous murine tumor models were employed in C57BL/6NRj wild type mice and GOLPH2 knockout (GOLPH2 $^{-/-}$ or GOLPH2^{null}) mice. Tumor growth kinetics and overall survival were compared across models.

In the B16-F10 melanoma model, GOLPH2 knockout mice exhibited significantly slower tumor growth compared to wild type controls (Figure 5a). A multiple t-test on day 21 revealed a statistically significant difference (probability value (p) = 0.021). Correspondingly, median overall survival was prolonged in GOLPH2 $^{-/-}$ mice (21 days, 95% confidence interval (CI): 0.34–4.25) relative to wild type mice (17.5 days, CI: 0.24–2.95), although this difference did not reach statistical significance (Figure 5b).

A similar trend was observed in the D4M-3A melanoma model, where tumor growth was markedly delayed in GOLPH2 $^{-/-}$ mice (Figure 5c). Significant differences between groups were observed on days 7, 9, 12, and 16 ($p < 0.01$, 0.016, 0.037, 0.026, respectively). Despite this, overall survival, although prolonged in GOLPH2 $^{-/-}$ animals (21 vs. 17.5 days), remained statistically non-significant (Figure 5d).

Contrasting results were obtained in the GL261 glioma model, where GOLPH2 $^{-/-}$ mice displayed significantly accelerated tumor growth compared to wild type controls (Figure 5e). From day 53 onward, tumor size was significantly greater in GOLPH2 knockout mice ($p = 0.046$, 0.022, 0.015 on days 53, 56, and 58, respectively). Correspondingly, overall survival was shorter in the GOLPH2 $^{-/-}$ group, with all mice succumbing before day 60, while half of the wild type mice survived beyond this time point (Figure 5f). Median survival was 41 days (CI: 0.17–2.78) in GOLPH2 $^{-/-}$ mice versus 59 days (CI: 0.36–5.75) in wild type mice. However, this survival difference was not statistically significant.

Syngeneic melanoma tumor model: B16-F10 experiment:

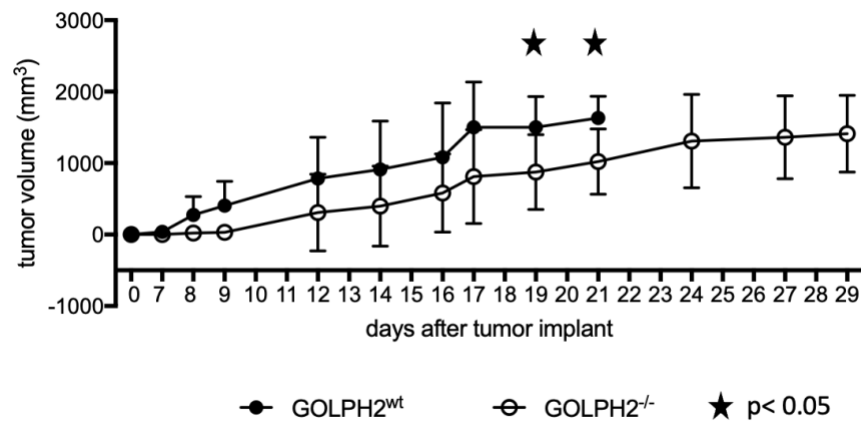


Figure 5a **Tumor growth in C57BL/6 wt vs GOLPH2^{-/-} mice (B16-F10 model)**. Slower tumor growth was observed in knockout mice compared to wild type mice. On day 21, a multiple hypothesis test statistic (t-test) revealed a significant difference ($p = 0.021$) (number (n) = 6 per group)

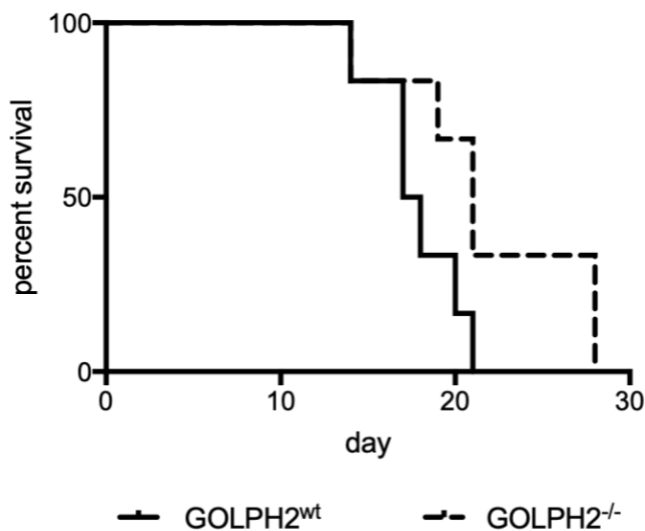


Figure 5b **Survival of C57BL/6 wt vs GOLPH2^{-/-} mice in the B16-F10 model**. Overall survival was prolonged in GOLPH2^{-/-} mice, with a median survival of 21 days (CI: 0.34–4.25) compared to 17.5 days in wild type mice (CI: 0.24–2.95) ($n = 6$ per group); this difference was not statistically significant.

Syngeneic melanoma tumor model: D4M-3A experiment:

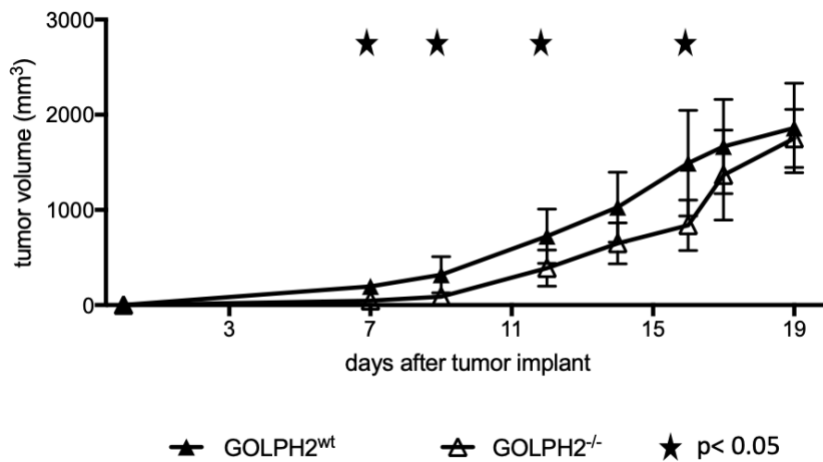


Figure 5c Tumor growth in C57BL/6 *wt* vs GOLPH2^{-/-} mice (D4M-3A model). Slower tumor growth was observed in GOLPH2 knockout mice compared to wild type mice. On day 7, 9, 12 and 16 the groups differed significantly ($p < 0.01$, 0.016, 0.037, 0.026) ($n = 6$ per group).

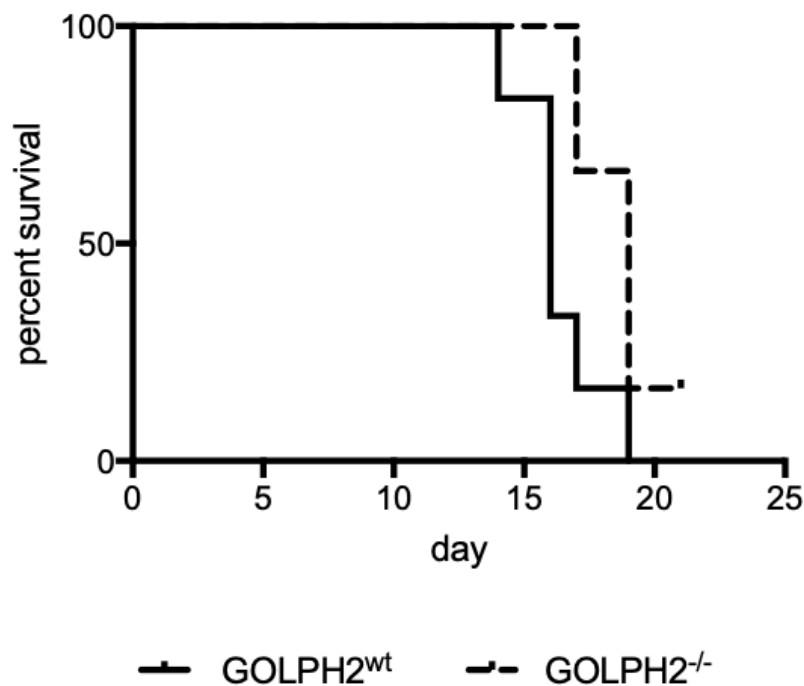


Figure 5d Survival of C57BL/6 *wt* vs GOLPH2^{-/-} mice (D4M-3A model). Overall survival was prolonged, but did not reach significance. Median survival for GOLPH2^{-/-} was 21 days (CI: 0.34-4.25) versus 17.5 days (CI: 0.24-2.95) ($n = 6$ per group).

Syngeneic glioma tumor model: GL261 experiment:

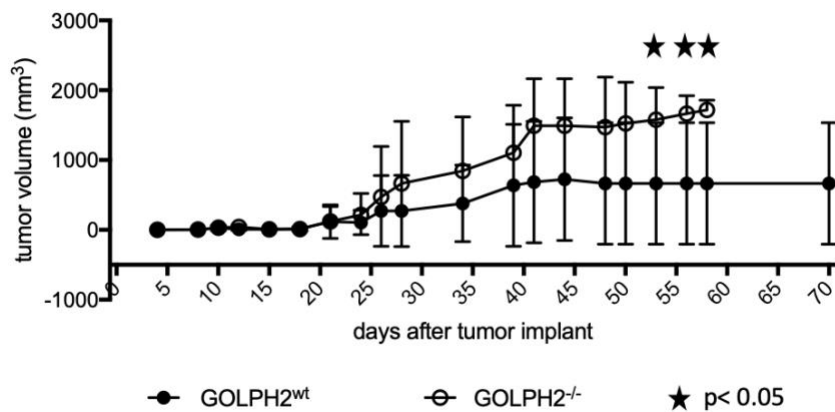


Figure 5e **Tumor growth in C57BL/6 *wt* vs GOLPH2^{-/-} mice, s.c. GL261**

Accelerated tumor growth was observed in the GOLPH2 knockout mice. From day 53 onward the groups showed a significant difference that was preserved until the end of the experiment (day 53, 56, 58) ($p = 0.046$, $p = 0.022$, $p = 0.015$) ($n = 6$ per group).

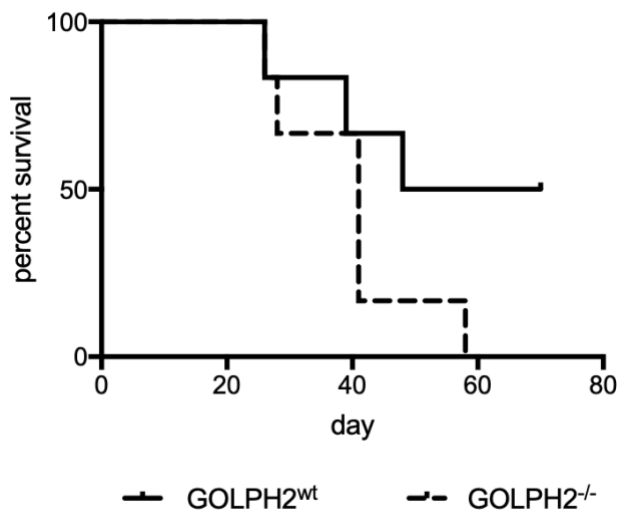


Figure 5f **Survival of C57BL/6 *wt* vs GOLPH2^{-/-} mice (s.c. GL261 glioma model)**. Overall survival was shorter in the GOLPH2 ^{-/-} mice and all mice died before day 60. In the *wt* control group, half of the mice survived beyond day 60. Median survival for GOLPH2 ^{-/-} was 41 days CI (0.17-2.78) versus 59 days CI (0.36-5.75) ($n = 6$ per group). This difference was statistically not significant.

Taken together, these data indicate that GOLPH2 may promote tumor progression in immunogenic melanoma models, as evidenced by delayed tumor growth and prolonged survival in its absence. Conversely, in a less immunogenic glioma model, loss of GOLPH2 correlated with accelerated tumor growth and reduced survival, suggesting a context-dependent role of GOLPH2 in tumor-host interactions.

5. DISCUSSION

A novel GOLPH2^{null} mouse strain has been successfully established and characterized in this study. These knockout mice thrive and reproduce normally, showing no signs of developmental abnormalities or reduced lifespan, with some animals observed for up to 18 months under standard housing conditions in our DBM facility. This makes them a robust model for investigating the functional role of GOLPH2 in adult physiology and disease.

On a molecular level, GOLPH2-deficient mice demonstrate upregulation of TRAF6 in peripheral blood cells. This alteration, as observed in this work, does not impair normal development or baseline physiological function in C57BL/6 mice. The model thus offers a unique opportunity to assess GOLPH2's role under pathological conditions, such as cancer.

To explore the role of GOLPH2 in tumorigenesis, GOLPH2-expressing tumors were implanted into GOLPH2^{null} and wild type mice, and their tumor growth and survival outcomes were assessed (Figures 5a - 5f). In two syngeneic melanoma models (B16-F10 and D4M-3A), tumors in GOLPH2^{null} mice exhibited slower growth, suggesting improved tumor control in the absence of GOLPH2. Although the differences in overall survival did not reach statistical significance - likely due to limited group sizes - a consistent trend toward better outcomes in GOLPH2^{null} hosts was observed.

In contrast, the glioma model (GL261) showed faster tumor growth and reduced survival in GOLPH2^{null} mice compared to wild type controls, suggesting that loss of GOLPH2 does not universally enhance anti-tumor immunity. This opposite effect highlights the context-dependent nature of GOLPH2's function.

Several factors may explain this discrepancy. For one, non-orthotopic implantation of glioma cells may limit the role of the microenvironment, as subcutaneous implantation does not fully recapitulate the native brain tumor milieu. Further, GL261 glioma is an immunologically "colder" tumor compared to melanoma, and may therefore be less affected by the immune modulatory functions of GOLPH2.

These findings underscore that GOLPH2's influence is shaped by the tumor's immune context. In more immunogenic tumors such as melanoma, the absence of GOLPH2 may unleash immune responses that restrict tumor growth. In contrast, in tumors like GL261, which have a more immunosuppressive profile, GOLPH2 deletion may fail to confer a protective effect - or may even exacerbate tumor progression.

The role of TRAF6 further supports this context dependency. TRAF6 has been reported to possess both pro- and anti-tumor functions, depending on the cellular and molecular environment^{56,57}. While TRAF6 can activate proliferative pathways that promote tumor growth, its upregulation in T cells has also been associated with enhanced anti-tumor immunity, notably through CTLA-4 degradation⁵⁸ and T helper 9 cell (TH9 cell) activation⁵⁹. It is tempting to speculate that in GOLPH2^{null} mice, constitutively elevated TRAF6 levels in the immune system might boost TH9-mediated anti-tumor responses, contributing to the melanoma-restricting phenotype. While direct evidence for TH9 activation is not provided in this study, the findings align with such a mechanism.

Conversely, in the glioma model, TRAF6 upregulation may not support effective immune control. The glioma microenvironment may suppress or circumvent TRAF6-driven immune responses, or

alternatively, TRAF6 may act in a pro-tumorigenic manner via innate immune pathways in this context. This aligns with previous observations that GOLPH2 impairs certain innate immune responses, and its absence may inadvertently enhance tumor-supportive inflammation in specific settings.

In summary, this work reveals a complex, tumor-type-specific role for GOLPH2 in modulating tumor-host interactions. In GOLPH2^{null} mice, the microenvironment is altered - most notably through increased TRAF6 expression. While this appears to facilitate immune control of melanoma, it may be ineffective or even deleterious in glioblastoma. These findings not only improve our understanding of GOLPH2 biology but also suggest that targeting GOLPH2 or its downstream pathways may represent a novel strategy in cancer immunotherapy, particularly in tumors with high immunogenic potential.

5.1. Conclusion

This doctoral thesis investigated GOLPH2's physiological relevance and its role in the tumor microenvironment. While GOLPH2 is dispensable for normal development and survival, its deletion leads to constitutive TRAF6 upregulation, suggesting a regulatory role in immune signaling. Given TRAF6's involvement in host defense, further exploration of the GOLPH2-TRAF6 axis is warranted. The observed tumor-suppressive effects in melanoma models point to GOLPH2 as a potential therapeutic target in cancer immunotherapy.

6. LITERATURE

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7. **APPENDIX**
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