Regulation of Brain Tumour Stem Cell Fate by Galectin1

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Abstract

Brain tumour stem cells (BTSCs) are a rare population of glioblastoma cells that have properties to evade ionizing radiation (IR) and chemotherapy, survive and replenish themselves, and spur the growth of new tumour cells. Galectin1, encoded by *LGALS1* gene, is a carbohydrate-binding protein with its expression highly upregulated in BTSCs. Here, we report that galectin1 plays important cell intrinsic roles in BTSCs via regulation of cell cycle, proliferation, and self-renewal. We also show that deletion of galectin1 sensitizes the response of chemoresistant BTSCs to chemotherapy. Beginning with mRNA-Seq analysis on patient-derived BTSC73 and *LGALS1* KO BTSC73, we identified a large panel of genes involved in the regulation of cell cycle and cell division. I thus, employed RT-qPCR analysis in multiple patient-derived BTSCs to validate the gene expression profile of galectin1. Next, to study the impact of galectin1 on cell proliferation, I performed immunostaining on *LGALS1* KO BTSCs and BTSCs using the proliferation markers, KI67 and phospho-Histone H3 (PH3). Interestingly, I found a significant decrease in percentage of KI67 and PH3 positive BTSCs upon deletion of *LGALS1*. Importantly, in limiting dilution assays, I found that galectin1 regulates BTSC self-renewal and confers resistance of BTSCs to chemotherapy with Temozolomide. Finally, in follow up studies, I identified a large panel of galectin1 downstream candidate genes that are involved in the regulation of mitotic spindle assembly, chromosome segregation, multinucleation, and cytokinesis. This proposes that galectin1 may promote cell cycle and self-renewal via regulating mitosis. Taken together, our data suggests that targeting of galectin1 could be a new avenue in overcoming therapeutic resistance in brain tumour stem cells.

Résumé

Les cellules souches de tumeurs cérébrales (BTSCs) sont une petite population de cellules de glioblastome qui ont la propriété d'échapper aux rayonnements ionisants (IR), à la chimiothérapie, de survivre et de permettre la croissance de nouvelles cellules tumorales. Galectine1, codé par le gène *LGALS1*, est une protéine de liaison aux glucides dont l'expression est fortement augmentée dans les BTSCs. Ici, nous montrons que la galectine1 joue des rôles intrinsèques importants dans les cellules BTSCs via la régulation du cycle cellulaire, la prolifération et l'autorenouvellement. Nous avons également montré que la délétion de galectine1 sensibilise la réponse des cellules BTSCs chimiorésistantes à la chimiothérapie. En commençant par l'analyse de séquençage d'ARN sur les BTSC73 dérivés de patient et les *LGALS1* KO BTSC73, nous avons identifié un large éventail de gènes impliqués dans la régulation du cycle cellulaire et de la division cellulaire. J'ai donc validé par RT-qPCR le profil d'expression génique de la galectine1 dans plusieurs BTSCs dérivés de patients. Ensuite, pour étudier l'impact de la galectine1 sur la prolifération cellulaire, j'ai effectué une immunofluorescence sur les cellules BTSC contrôles et *LGALS1* KO BTSCs en utilisant des marqueurs de prolifération, KI67 et phospho-histone H3 (PH3). J'ai trouvé comme résultat intéressante, une diminution significative du pourcentage de cellules positives pour KI67 et PH3 dans les BTSCs dépourvues de *LGALS1*. De plus, par test de dilution limitante, j'ai trouvé que la galectine1 régule la capacité d'autorenouvellement et confère aux BTSCs la résistance à la chimiothérapie au Témozolomide. Finalement, dans des études complémentaires, j'ai identifié un grand nombre de gènes candidats régulés par galectine1 qui sont impliqués dans la régulation de l'assemblage du fuseau mitotique, la ségrégation chromosomique, la multinucléation et la cytokinèse, suggérant que la galectine1 pourrait favoriser le cycle

cellulaire et l'autorenouvellement via la régulation de la mitose. Ainsi, nos données suggèrent que le ciblage de la galectine1 pourrait être une nouvelle stratégie pour contrer la résistance des cellules souches de tumeurs cérébrales à la chimiothérapie.

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Contributions

The *LGALS1* knockout (KO) BTSC lines were generated by Dr. Ahmad Sharanek, and subjected to RNA-Seq analysis. The bioinformatics analysis was performed in collaboration with Dr. Hamed Najafabadi's lab. Immunofluorescence (IF) staining of KI67 protein in BTSC73 and *LGALS1* KO BTSC73 was performed in collaboration with Dr. Ahmad Sharanek. I performed all the RT-qPCR experiments and analyses including, confirmation of loss of *LGALS1* mRNA expression in *LGALS1* KO BTSCs and validation of RNA-seq data in both BTSC73 and BTSC147 cell lines. I also performed the IF staining of galectin1, PH3 and α-tubulin in BTSCs. I performed the bioinformatics analysis using the database for annotation, visualization and integrated discovery (DAVID). Moreover, I performed the limiting dilution assays (LDA) and the measurement of the sphere sizes in BTSC73 and *LGALS1* KO BTSC73. Furthermore, I performed the Immunoprecipitation (IP)/Western blot analysis of galectin1 and its binding proteins in BTSC73. I also wrote the thesis with editorial input from my supervisor, Dr. Jahani-Asl.

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List of Abbreviations

BTSCs: Brain Tumour Stem Cells

IR: Ionizing Radiation

LGALS1: Lectin galactoside binding soluble 1

RNA-Seq: Ribonucleic acid Sequencing

KO: Knockout

KD: Knockdown

RT-qPCR: Quantitative reverse transcription polymerase chain reaction

PH3: Phospho-Histone H3

IP-WB: Immunoprecipitation-Western blot

IF: Immunofluorescence

DAVID: Database for annotation, visualization and integrated discovery

LDA: Limiting dilution assay

GB: Glioblastoma

CBTRUS: Central Brain Tumor Registry of the United States

WHO: World Health Organization

CRD: Carbohydrate recognition domain

EGFR: Epidermal growth factor receptor

AP1: Activator protein 1

IDH1/2: Isocitrate dehydrogenase enzyme 1/2

TMZ: Temozolomide

G-CIMP: Glioma- cytosine guanine nucleotides island methylator phenotype

ATM: Ataxia telangiectasia mutated

C/EBPα: CAAT (Cytidine-Cytidine-Adenosine-Adenosine-Thymidine) / enhancer binding protein α

HIF-1: Hypoxia-inducible factor-1

NF-κB: Nuclear factor kappa light chain enhancer of activated B cells

kDa: Kilodaltons (molecular weight)

TCGA: The Cancer Genome Atlas

FGF-2: Fibroblast growth factor-2

VEGFR2: Vascular endothelial growth factor receptor 2

PCDH24: Protocadherin-24

JNK: c-Jun NH2-terminal kinases

ERK1/2: Extracellular signal-regulated kinases 1/2

RAS: Rat sarcoma

GTP: Guanosine-5'-triphosphate

PI3K: Phosphoinositide 3-kinase

MEK: Mitogen-activated protein kinase / Extracellular signal-regulated kinase

MAPK: Mitogen-activated protein kinase

ERK: Extracellular signal-regulated kinase

G1: Gap phase 1

S: Synthesis phase

G2: Gap phase 2

M: Mitosis phase

G0: Resting phase

NEBD: Nuclear envelope breakdown

CDKs: Cyclin-dependent kinases

CDKIs: Cyclin-dependent kinase inhibitor proteins

K-MTs: Kinetochore microtubules

XKCM1: Xenopus kinesin catastrophe modulator-1

XMAP215: Xenopus microtubule associated protein 215

A-MTs: Astral microtubules

nK-MTs: non-kinetochore microtubules

K-fibres: Kinetochore fibres

ECM: Extracellular matrix

siRNA: Small interfering Ribonucleic acid

shRNA: Short hairpin Ribonucleic acid

VEGF: Vascular endothelial growth factor

BEX2: Brain expressed X-linked gene 2

CD45: Cluster of differentiation 45

TADCs: Tumour-associated dendritic cells

EC: Endothelial cell

ABC transporters: Adenosine triphosphate - binding cassette transporters

DMSO: Dimethyl sulfoxide

TRIzol: Total Ribonucleic acid Isolation

PBT: 1X-PBS / 0.3% Triton-X /0.5% Bovine serum albumin

PBS: Phosphate buffered saline

RIPA: Radio immunoprecipitation assay buffer

PVDF: Polyvinylidene fluoride

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

Cas9: CRISPR associated protein 9

gRNA: Guide Ribonucleic acid

BSA: Bovine serum albumin

GFP: Green fluorescent protein

RFP: Red fluorescent protein

FACS: Fluorescence activated cell sorting

ANOVA: Analysis of variance

GUSB: Beta-glucuronidase precursor

GSEA: Gene Set Enrichment Analysis

NuMA: Nuclear mitotic apparatus protein

CIT: Citron Rho-interacting serine/threonine kinase

Co-IP: Co-immunoprecipitation

NSCLC: Non–small cell lung cancers

PLA: Proximity ligation assay

BioID: Proximity-dependent biotin identification

CEP55: Centrosomal protein 55

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

A. Introduction

A.1.Glioblastoma (GB)

Glioblastoma (GB) is the most aggressive and frequent primary brain and central nervous system tumour which has been designated Grade IV by World Health Organization (WHO) (1, 2). According to the 2019 Central Brain Tumor Registry of the United States (CBTRUS) there are 3.22 of GB new cases per 100,000 population per year, which is the highest incidence rate among malignant primary brain tumours (3). Moreover, in 2015, the incidence rate of GB in Canada was 4.50 per 100,000, according to the Canadian Cancer Registry (4). GB development is more common in males, and in white and non-Hispanics (5). GB will primarily form in the cerebral hemisphere with 95% of the tumours being located in the supratentorial region. However, GB has been detected in the brain stem, spinal cord, and cerebellum as well (6). GB remains an incurable disease and the current treatment consists of the surgical removal of the tumours along with post-surgical treatments with the chemotherapy drug Temozolomide (TMZ) and ionizing radiation (IR) (7). Even with these treatments, the GB prognosis is very poor and the median survival rate for patients is ~18 months after diagnosis (1, 7). This poor prognosis is attributed to an incomplete understanding of the key signalling pathways that drive different subtypes of GB. After surgical resection of the tumours, there is a high possibility of tumour recurrence within 2–3 cm of the original location (8). Moreover, the response to temozolomide treatment is not adequate and the radiation therapy has limitations due to the damage it causes to healthy brain tissue and the ability of the tumour to develop a resistance to the ionizing radiation (8). Therefore, studying the basic biology of GB is necessary to discover novel targets for therapy and develop more effective treatments.

Many prognostic molecular markers are associated with GB (Table 1), including overexpression and amplification of epidermal growth factor receptor (EGFR), mutation of isocitrate dehydrogenase enzyme 1/2 (IDH1/2), methylation status of the gene promoter for O^6-methylguanine-DNA methyltransferase (MGMT)), mutation of tumour protein (TP53) (60% to 70% of secondary GBs, 25% to 30% of primary GBs), glioma-CpG island methylator phenotype (G-CIMP), genetic losses on chromosome 10, loss of the long arm of chromosome 19, and the short arm of chromosome 1 (19q /1p) (5).

Table 1. Genetic and epigenetic alterations in GB (9).

Name	**Function**	**Expression status**	**Prevalence**	**Prognosis**
EPHA3	Regulation of adhesive and repulsive mechanisms including cell motility and adhesion	Overexpressed	40–60%	Poor, over-expression common in recurrent GBM
EGFR	Regulation of processes involved in cell growth, division and survival	Overexpressed	40–60%	Poor
MGMT	Prevention of mismatch errors	Methylated	40–60%	Favourable
CDKN2A	Regulation of cell cycle and retinoblastoma activation	Decreased	49–52%	Poor
PTEN	Regulation of cell signalling. Involved in cell proliferation and survival	Deleted and/or mutated	34%	Poor
PIK3CA	Regulation of processes involved in cell growth, division and survival	Overexpressed and/or mutated	15%	Poor; can predict recurrence
PDGFRA	Regulation of processes involved in cell growth, division and survival	Overexpressed	13%	Poor
IDH1	Production of NADPH	Mutated	5–10%	Favourable
MDM2	Regulation of p53 activity	Overexpressed	8–9%	Unclear
MET	Regulation of proliferation, survival and motility	Overexpressed and/or mutated	4–6%	Poor
SF/HGF	Activating ligand for HGFR/c-MET. Tumour growth and angiogenesis	Overexpressed	1.6–4%	Poor
VEGF	Promotion of angiogenesis	Overexpressed and/or mutated		Poor

This table was retrieved from Taylor et al., Frontiers in Oncology, 2019 (9).

Primary and secondary glioblastomas are two distinct disease subtypes that arise from different genetic pathways and are more common in patients from different age groups (10). Primary GB, also termed de novo glioblastoma, represents 80% of GB and is more prevalent in older patients, with the median age of tumour development being 62 years (5, 11). Primary glioblastoma develops rapidly, and most patients develop the tumour without any evidence of a malignant precursor lesion (11). In contrast, secondary glioblastoma develops slowly from less severe astrocytoma or anaplastic astrocytoma cancers, and is typically seen in younger patients averaging at 45 years of age (5, 11).

The most common genetic alterations in primary GB are loss of heterozygosity of 10q, EGFR amplification, and PTEN mutations. Whereas in secondary GB, mutations of TP53 are frequently detected (11). Moreover, the mRNA and protein expression profiles in these two subtypes of glioblastoma are different (11). Considering these differences is important in evaluating the response of GB to the present standards of care including radiation and chemotherapy (11).

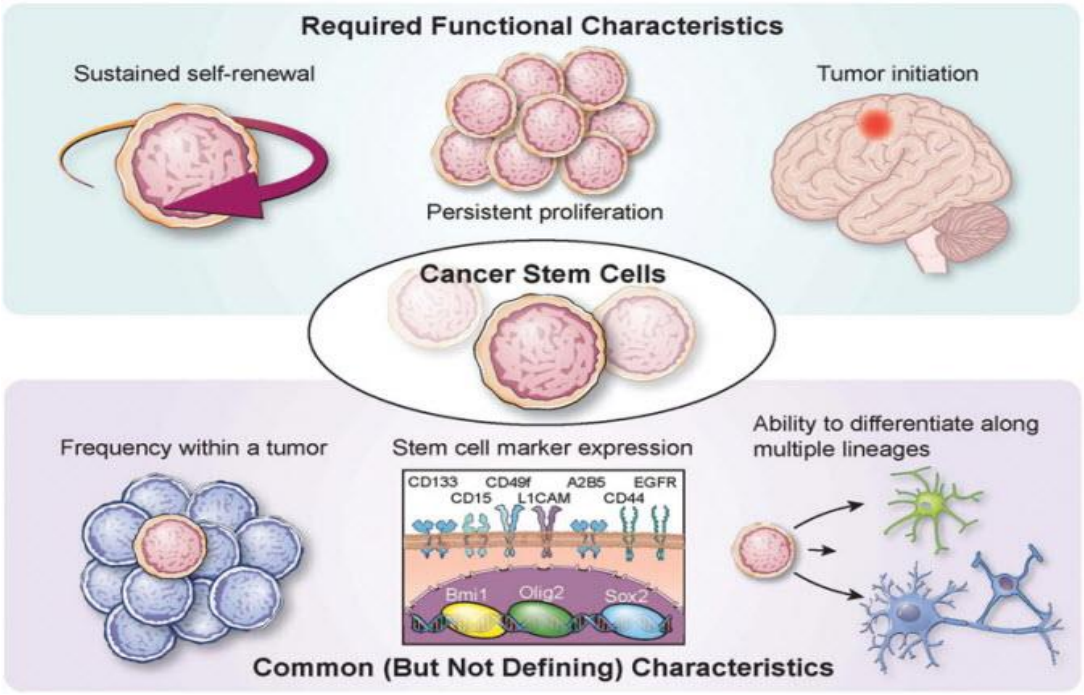
There are two hypothetical models that can explain tumour initiation and development, stochastic and hierarchical models (12). The stochastic model proposes that all tumour cells can be heterogeneous and have tumourigenic potential due to the acquisition of random mutations. In contrast, the hierarchical model predicts that only a rare subpopulation of tumour stem cells have significant proliferation capacity and sustain the growth and progression of a neoplastic clone (12). The hierarchical hypothesis advocates the cancer-stem-cell theory which is now strongly supported by abundant studies (12-17). In particular, several studies have strongly suggested that human brain tumour cells develop from a rare population of cells that have tumourigenic ability, a feature not seen by the majority of cells populating the tumour (18, 19).

A.2. Brain Tumour Stem Cells (BTSCs)

At the cellular level, GB possesses a rare population of self-renewing multipotent cells, termed Brain Tumour Stem Cells (BTSCs) (19, 20). BTSCs are characterized by the ability to evade treatment, survive and replenish themselves, and spur the growth of new tumour cells (21, 22). Previous studies have shown that BTSCs are more resistant to apoptosis caused by ionizing radiation compared to their non-stem counterparts (21). BTSCs can obtain this radioresistance by the activation of a number of DNA damage checkpoint proteins, such as the cell cycle checkpoint protein Rad17, the ataxia telangiectasia mutated (ATM), and the checkpoint kinases Chk1 and Chk2 (21). These DNA damage repair mechanisms can assist in repairing DNA damage and confer tumour resistance (21). For example, Chk1 and Chk2 inhibition has been shown to reduce the tumourigenic property of BTSCs and promote tumour sensitivity to radiation therapy (21).

There are some common features between GB stem cells and normal neural stem and progenitor cells, including formation of neurospheres, expression of neural stem cell markers, long term proliferation, and an extensive self-renewal capability. The neural stem cell markers which are also expressed on GB stem cell surface includes CD133 (the most common marker), A2B5, CD44, CD171 (L1CAM), CD15 (SSEA1), CD49f (integrin α_6), Musashi, Nestin, Nanog, Oct4, Sox2 and EGFR (Figure 1) (8, 19).

In contrast, GB stem cells show critical differences from normal stem cells which includes chromosomal abnormalities, abnormal expression of multiple differentiation genes, and the ability to form tumours (8).



This figure was retrieved from Lathia et al., Genes & development, 2015 (19).

Figure 1. Brain tumour stem cell characteristics. BTSCs can self-renew, proliferate extensively, and when transplanted will form a new tumour. They also express stem cell markers and possess the ability to differentiate down different lineages (19).

Given that BTSCs are responsible for tumour propagation as well as resistance to conventional therapy, a better understanding of how BTSCs are regulated at the molecular level is urgently needed and can help in designing novel strategies for GB treatment.

A.3. Galectins

The galectins are part of a family of carbohydrate-binding proteins that have an affinity for β-galactosides and share a highly conserved amino acid sequence in their carbohydrate recognition

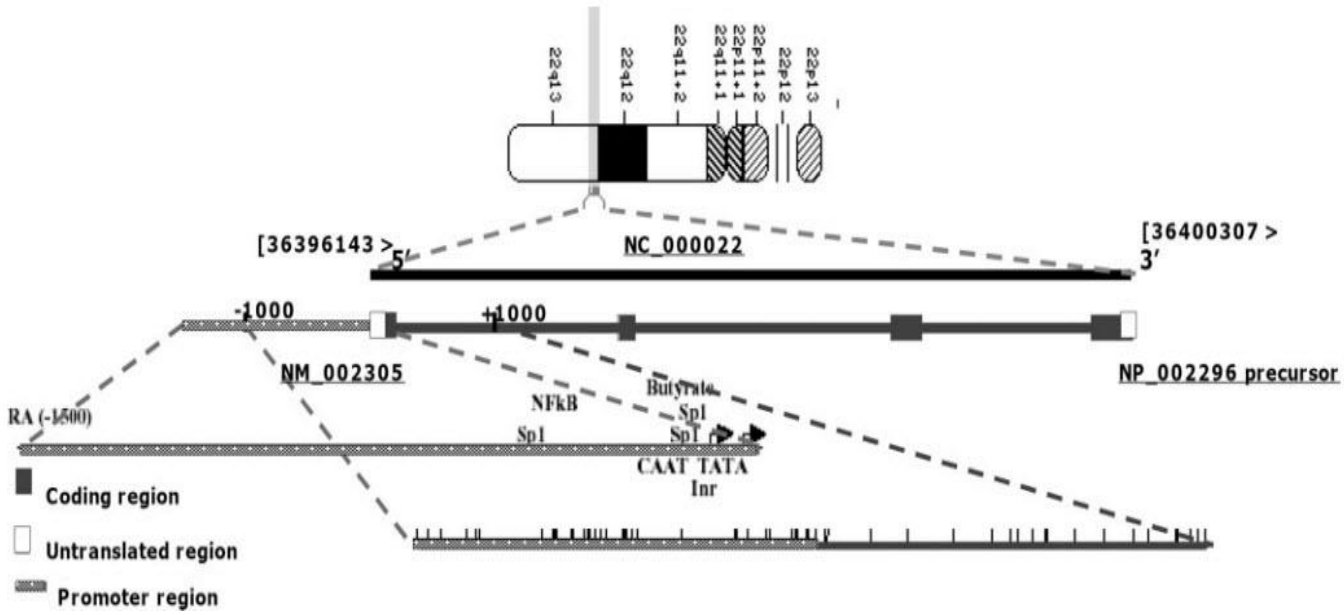
domain (CRD). The CRD, composed of approximately 130 amino acids, recognizes β-galactosides with a particular affinity for N-acetyllactosamine-containing glycans (23). Currently, 15 mammalian galectins have been identified. These proteins are classified into three groups based on their structure. Galectins 1, 2, 5, 7, 10, 11, 13, 14, and 15 are part of the first group and their common structural feature is that they all possess only one CRD. In group two, we have galectins 4, 6, 8, 9, and 12, and they have two homologous CRDs that are connected with a peptide linker. The third group has only one member, galectin 3, and is demarked by a single CRD along with a unique N-terminus (24). Recent studies suggest that galectins, particularly galectin1, have critical roles in maintaining hallmarks of cancer, including angiogenesis, assisting in evading the immune system, promoting cell migration, tumour cell adhesion, and conferring resistance to chemotherapy (25).

A.3.1. Galectin1

LGALS1 (lectin galactoside binding soluble 1) is a protein-coding gene that is located on chromosome 22q12 (26) (Figure 2). Several transcription factors are reported to regulate the expression of this gene including hypoxia-inducible factor-1 (HIF-1), CAAT/enhancer binding protein α (C/EBPα), activator protein 1 (AP1), and nuclear factor κB (NF-κB) (23). Moreover, the methylation of its promoter heavily regulates the expression of this gene (27).

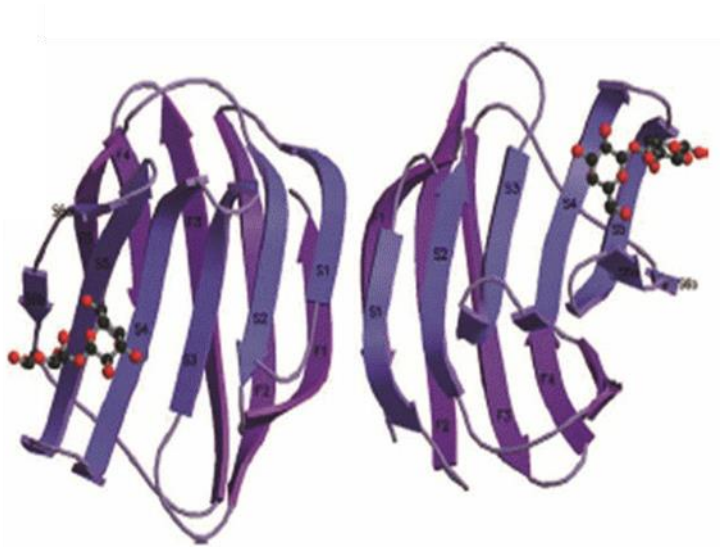
The protein encoded by the *LGALS1* gene (galectin1) is a 14 kDa monomer, and these monomers can dimerize. The dimerization is through hydrophobic interactions at the monomeric interface and the protein's hydrophobic core (28) (Figure 3). The monomeric units are positioned in a way in the dimer that their two CRDs will be on opposite ends of a quaternary structure. By having two CRDs, the homodimer is appropriate for the facilitation of cell adhesion, initiating signal

transduction events, and forming multivalent lattices with the cell surface by binding with glycosylated proteins (26).



This figure was retrieved from Camby et al., Glycobiology, 2006 (26).

Figure 2. Galectin1 gene map on the human chromosome 22q12. The initial transcription sites are shown by the curved arrows. The four black boxes represent the coding regions (exons) which result in the 0.6 kb transcript and the final protein with 135 amino acids (26).



This figure was retrieved from Camby et al., Glycobiology, 2006 (26).

Figure 3. Homodimeric structure of galectin1. Galectin1 (represented by purple color) with lactose molecules (red and black) bound in the two carbohydrate recognition domains found on the opposite ends of the homodimer (26).

Galectin1 is involved in multiple cellular and physiologic processes such as neural stem cell growth (24), and the differentiation of hematopoietic and muscle stem cells (24, 26). Moreover, overexpression of galectin1 was detected in a wide range of cancers encompassing melanoma, bladder, head–neck, colorectal, prostate, ovarian, thyroid, lung, and breast cancers. Its increased expression was associated with poor patient prognosis (29). Analysis of patient databases from TCGA and REMBRANDT reveals a significant correlation between elevated expression of galectin1 and poor prognosis in glioblastoma patients (30) (Jahani-Asl et al., *unpublished*). The mechanisms by which galectin1 regulates BTSCs and glioblastoma pathogenesis remain unclear.

A.3.2. Galectin1 subcellular localization and secretion

It is reported that galectin1 is found both intracellularly and extracellularly, and has intracellular and extracellular functions (26). Once galectin1 is synthesized, it can localize to the cytosols, nucleus, and the inner wall of the cell membrane (26, 31). Similar to other galectins, galectin1 can also be secreted into the extracellular space (32) and can then localize to the outer cell membrane and the extracellular matrix of both healthy and neoplastic tissues (26). However, its secretion and extracellular functions has been questioned since galectin1 has no recognizable secretion signal sequences required for standard endoplasmic reticulum/Golgi pathway, and has characteristics of cytoplasmic proteins, such as possessing the archetypal acetylated N-termini, and a lack of glycosylation (28, 32).

It has been reported that galectin1 is secreted using an export machinery that allows for direct translocation across the cell membrane, reminiscent of what is seen with regards to fibroblast growth factor-2 (FGF-2) secretion (26, 33). It has been proposed that this export machinery would use β-galactoside-containing surface molecules, a known binding target of galectin1. These molecules will only recognize properly folded and functional galectin1 and allow it to cross the membrane. This will provide another quality control checkpoint whereby it prevents the non-functional galectin1 to leave the cell (26, 33). In addition, a study showed that galectin1 has different molecular weights inside and outside cells, whereas the extracellular protein will weigh 15 kDa, and the intracellular galectin1 will weigh 14 kDa. This would imply that the secreted galectin1 undergoes further post-translational modifications (23, 34). A study suggested that these post-translational modifications are necessary for galectin1 secretion (34). Secretion of galectin1 in normal cells is minimal, and in these cells galectin1 is typically retained in the cytoplasm and nucleus. In contrast, cancer cells secrete a comparatively larger amount of galectin1 (23, 35, 36).

A.3.3. Galectin1 targets

Galectin1 recognizes N-acetyllactosamine residues on a number of glycoproteins and glycolipids in the extracellular compartment and is also responsible for mediating protein–protein interactions intracellularly (26). High galectin1 expression has been correlated with cellular aggregation, tumour formation, metastasis, and angiogenesis. These biological activities of galectin1 can happen through its interaction with different binding partners (28). A summary of galectin1 binding partners and their roles in cancer can be found in Table 2.

Table 2. Galectin 1 binding partners (28).

Localization	Binding partner	Biological activities	Cell type	Impact on tumourigenesis
Intracellular	H-Ras	H-Ras/MEK/ERK cascade activation	Bladder cancer	+
	Pro-24	β-catenin signaling inhibition	Colon cancer	-
	Gemin-4	Pre-RNA splicing modulation	Cervical cancer	unknown
Extracellular	90K/ Mac-2BP	Homotypic cell adhesion	Melanoma	+
	Mucin 1	Cell adhesion	Prostate cancer	+
	Laminin	Cell-ECM adhesion	Endothelial	+
	Fibronectin	Cell-ECM adhesion	Endothelial	+
	Neuropikin-1	Proliferation, migration, and adhesion induction	Endothelial	+
	VEGFR	Neovascularization activation	Endothelial	+
	CD45	Membrane redistribution, and T cell death induction	T cell	unknown
	CD43	Membrane redistribution, and T cell death induction	T cell	unknown
	CD7	T cell death induction	T cell	unknown

This table was retrieved from Cousins et al., International journal of molecular sciences, 2016(28).

A.3.4. Intracellular and extracellular galectin1

Intracellular galectin1 is a scaffold protein for signalling pathways, and functions by binding to proteins, either in the cytoplasm or nucleus, that are involved in numerous biological functions. Relevant to cancer cell biology, intracellular galectin1 participates in protein–protein interactions with H-Ras, protocadherin-24, and Gemin4 in a carbohydrate-independent manner (28). H-Ras and galectin1 interaction leads to cell transformation and proliferation thus promoting tumourigenesis (37). Protocadherin-24 (PCDH24), a tumour suppressor, regulates the localization of β-catenin to the cell membrane via inhibition of galectin1 and 3 activity in HCT116 human colon cancer cells (38). Therefore, one of the mechanisms by which PCDH24 acts as a tumour suppressor in HCT116 cells is by anchoring galectin1 and 3 at the cell membrane, thereby depleting them from the cytosol (38). Moreover, galectin1 and galectin3 are involved in spliceosome assembly *in vivo* through interacting with Gemin 4 (39). Spliceosome assembly is an important post-transcriptional process in eukaryotic mRNA synthesis, permitting the excision of introns and exon ligation (39, 40).

Extracellular galectin1 participates in β-galactoside binding activity (41). In other words, galectin1 binding to its glycoprotein, glycolipid targets, and its ECM targets- notably laminin, fibronectin, thrombospondin, vitronectin, and osteospondin, is done in both a dose-dependent and β-galactoside dependent manner (41). Interactions between galectin1 and glycoproteins in the ECM can result in the metastatic spread of cancer cells (26). Furthermore, interactions between galectin1 and cell-surface glycoproteins, including 90K/Mac-2BP and Mucin, can induce cellular aggregation and tumour formation (26). A previous study demonstrated that galectin1 in colon cancer could interact with the adhesion molecules CD44 and CD326, which are markers for breast and colon cancer stem cells (42). Galectin1 is also shown to be involved in metastasis through

binding to CD44 or CD326 proteins that promote the ability of metastatic cells to pass in and out of blood vessels by encouraging the attachment of cancer cells to the endothelium (42). Furthermore, the interaction of extracellular galectin1 and the type I transmembrane glycoprotein NRP1 induces vascular endothelial growth factor receptor 2 (VEGFR2) phosphorylation, thereby activating its downstream signalling cascades which includes the extracellular signal-regulated kinases 1/2 (ERK1/2), and c-Jun NH2-terminal kinases (JNK). This in turn leads to increased angiogenesis (43). A study by Jouve et al., 2013, demonstrated how the galectin1 interaction with CD146 protected endothelial cells from apoptosis induced by galectin1 by acting as a trap for galectin1 and preventing it from performing its other functions (44).

Conversely, the interaction between galectin1 and α5β1 integrin reduced epithelial tumour cell growth by inducing p21 and p27, which in turn causes caspase-8 activation and makes the carcinoma cells more susceptible to anoikis- a form of cell death resulting from the cells becoming unanchored (45).

A.3.5. Galectin1 oncogenic signalling:

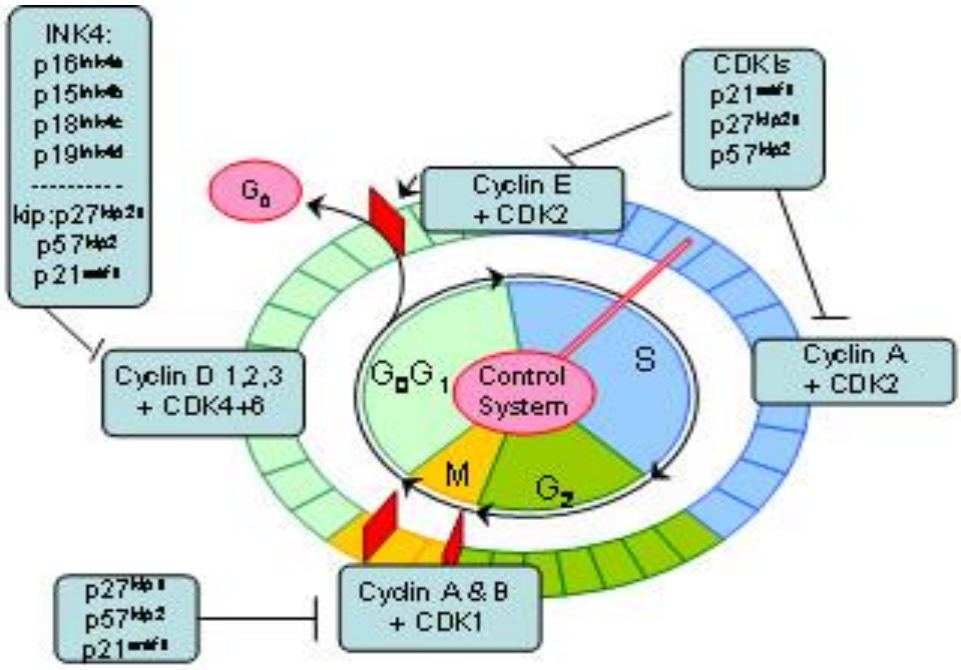
An interaction of galectin1 with H-Ras is documented in an oncogenic context. In human tumours, it's known that it's very common for Ras genes to be mutated, and Ras transformation targets can promote malignancy (37). In order for Ras transformation to occur, the membrane-associated H-Ras must be anchored to the cell membrane (37). This is achieved by its recruitment of cytosolic galectine1 to the membrane (46). Since galectin1 does not affect the localization of inactive H-Ras (H-Ras-guanosine diphosphate), the activation of Ras by binding to Guanosine-5'-triphosphate (GTP), is required for the H-Ras/ galectin1 interaction (37). By binding with galectin1, H-Ras-GTP will form a more stable connection with the cell membrane (37, 47). The hydrophobic pocket

is essential for the stable binding of H-Ras-GTP to the cell membrane, as even a single point mutation in that region will result in H-Ras-GTP being displaced from the membrane and having the active form of H-Ras inhibited (47). Furthermore, H-Ras regulates various downstream pathways, including phosphoinositide 3-kinase (PI3K) and the proto-oncogene serine/threonine-protein kinase Raf-1/mitogen-activated protein kinase MEK/ERK signalling pathways (48). A study by Elad-Sfadia et al., 2002, established that galectin1 promotes H-Ras activation and diverted H-Ras signalling towards the Raf-1 pathway at the expense of PI3K (49). In addition, when galectin1 is overexpressed, there is an increase in the number of H-Ras-GTP that are associated with the cell membrane, thereby enhancing the sites of Raf-1 recruitment. This results in a sustained activation of the MEK/ERK pathways (47). A predominant negative mutation of H-Ras or galectin1 antisense RNA was shown to inhibit cell transformation (37). These data suggest that galectin1 assists in the localization of H-Ras into the plasma membrane where it can act as a platform for cell signalling. Galectin1's ability to increase H-Ras-mediated cell transformation opens the door to a potential new strategy of targeting galectin1 in order to suppress the oncogenic Ras signalling.

A.3.6. Galectin1 in cell cycle and proliferation

The cell cycle process consists of a series of events that modulate several biological processes including DNA replication, cell division, and cell proliferation (50). The different stages of the cell cycle include: (i) synthesis (S) phase where the DNA is replicated; (ii) gap phase 2 (G2) in which production of essential proteins for mitosis occurs; (iii) mitosis (M) phase where the chromatin becomes condensed, the nuclear envelope will breakdown (NEBD), the chromatids will separate, and finally, cytokinesis will occur; (iv) gap phase 1 (G1), where the genes required for DNA

replication will become activated and there will be an accumulation of proteins required for S-phase progression; (v) resting phase (G0), which as the name implies, is the phase where the cells will exit the cell cycle and become quiescent or relatively inactive (Figure 4) (50, 51). Uncontrollable cell proliferation can occur when the cell cycle is deregulated, which is a hallmark of cancers (52). Cyclin-dependent kinases (CDKs) have important roles in cell cycle regulation (50). The CDKs are tightly regulated by both cyclins and CDK inhibitors (CDKIs) (Figure 4). Overexpression of cyclins or downregulation of CDKIs can lead to uncontrolled cell growth and cancer (53).



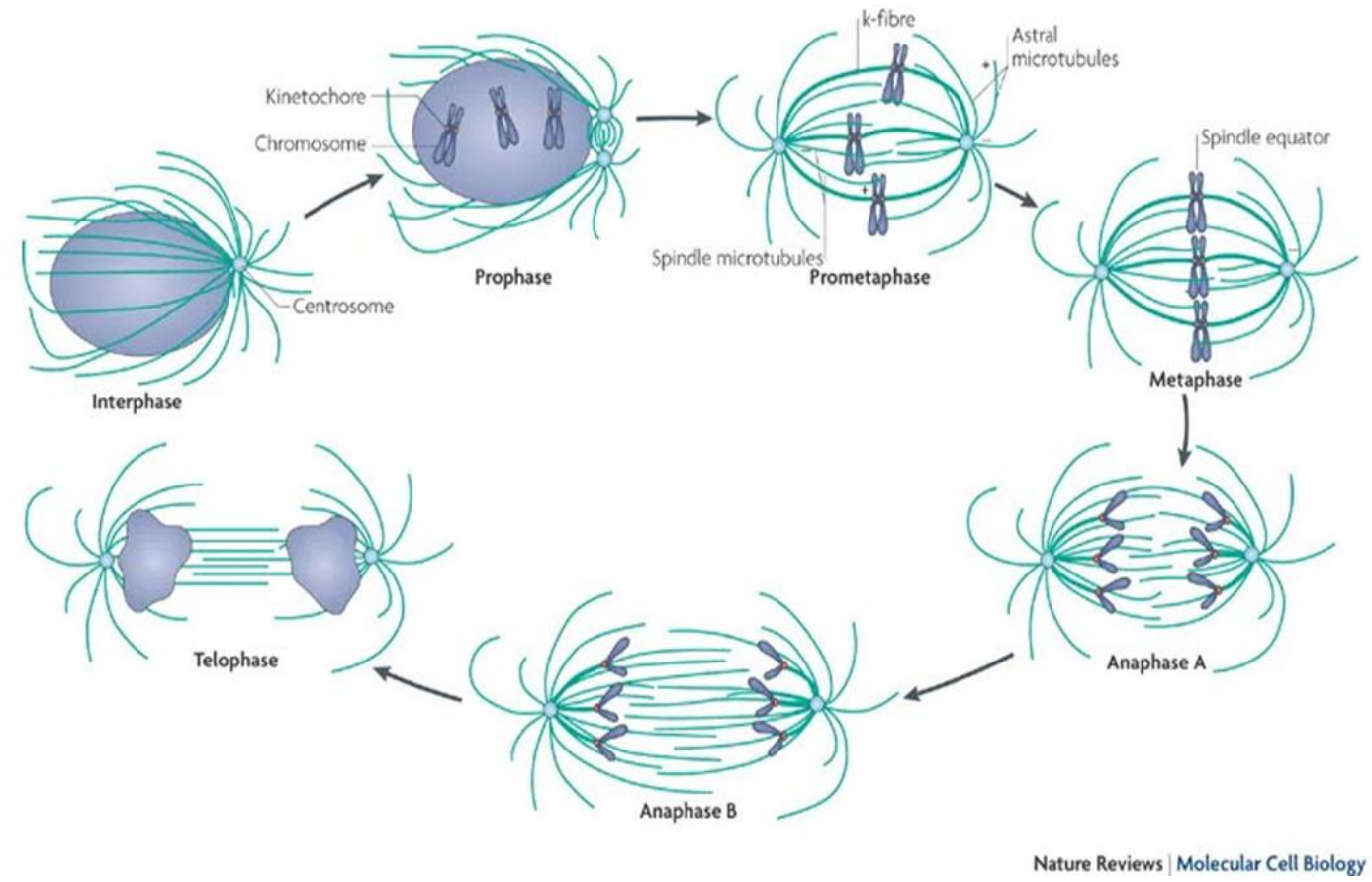
This figure was retrieved from Madhuri et al., Journal of Ovarian Research, 2012 (54).

Figure 4. Cell Cycle with various cell cycle regulatory proteins (54). S: synthesis phase, G2: gap phase 2, M: mitosis phase, G1: gap phase 1, G0: resting phase. Cyclins: a family of proteins that regulate the progression of the cell cycle, CDKs: Cyclin-dependent kinases, CDKIs: cyclin-dependent kinase inhibitor proteins, INK4: a family of cyclin-dependent kinase inhibitors.

Wells et al., 1999, found that extracellular galectin1 is able to induce cell cycle arrest in the G2 phase. This was also followed by progressive apoptotic death in mammary cancer cell lines (55). Fischer et al., 2005, reported that the interaction of extracellular galectin1 with α5β1 integrin can suppress the Ras-MEK-ERK pathway and induce the transcription of p27, a cell cycle inhibitor. This process resulted in the suppression of tumour growth in different cell lines including melanoma, hepatocarcinoma, breast, ovarian, and colon carcinoma (56). They also reported that galectin1 promotes late G1 arrest through the accumulation of p21 in colon cancer cells with high expression of α5β1 (56). Jeschke et al., 2006, showed that exogenous galectin1 can lead to inhibition of proliferation in trophoblastic cancer cells (57). Conversely, other studies showed that galectin1 can promote cell proliferation and tumour cell growth. Jung et al., 2008, showed that overexpression of galectin1 by transfecting glioma cells with a plasmid DNA that produced sense galectin-1 mRNA led to enhanced cell proliferation (58). Banh et al., 2011, indicated that galectin1 expression in Lewis lung carcinomas was essential for the growth of the tumours (59). Kim et al, 2013, described that down-regulation of galectin1 expression by small interfering RNA led to the suppression of cell growth and proliferation of cervical cancer cells (60). The exact molecular mechanisms by which galectin1 can promote or inhibit the cell cycle remains unclear. Furthermore, the role of galectin1 in cell cycle regulation in glioma stem cells remains to be investigated.

A.4. Mitotic spindle:

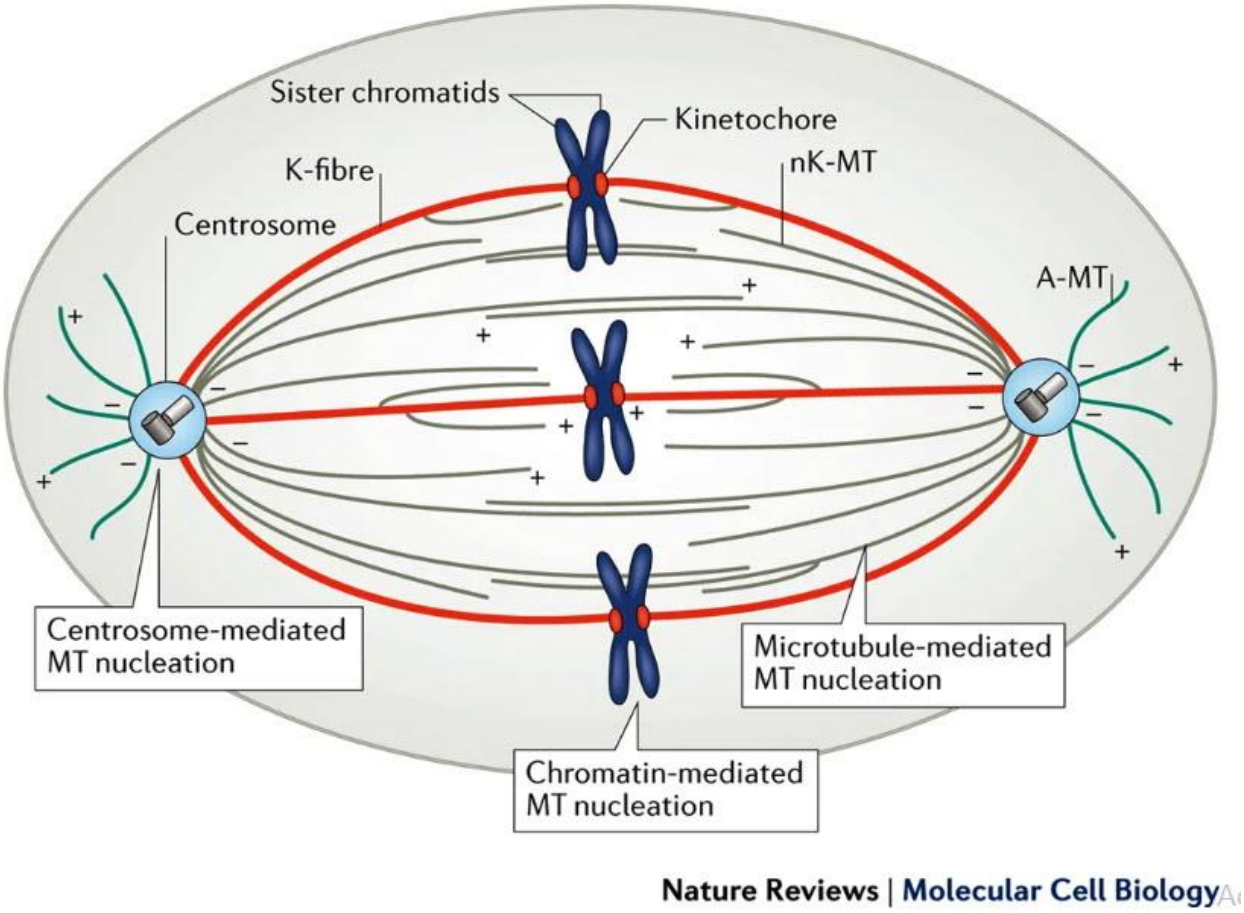
It is now established that mitosis not only regulates chromosome segregation, but can also determine cell fate and tissue architecture (61). The mitotic spindle is an interesting structure and can be described as a macromolecular machine that is able to segregate the chromosomes to opposite poles of the cell and precisely distribute the genome into the daughter cells during mitosis (Figures 5 and 6) (61). Furthermore, a large number of proteins including xenopus kinesin-related protein XKCM1 and xenopus microtubule associated protein XMAP215 family members, are involved in the regulation and formation of the mitotic spindle to ensure its correct function (62). In addition, microtubule polymers are the major structural elements of the spindle and are essential for bipolar spindle organization due to their inherent polarity and dynamic properties (61). Mitotic spindle deregulation can lead to chromosome missegregation, cytokinesis defects, and genomic instability (62).



This figure was retrieved from Walczak et al., Nature Reviews Molecular cell biology, 2010 (63).

Figure 5. Structure of the mitotic spindle in different phases of mitosis. At interphase, the individual form of the chromosomes cannot be observed due to the decondensed form of the chromatin in the nucleus. At prophase, the mitotic spindle begins to form, and chromosomes start to condense. At prometaphase, the mitotic spindle grows and binds to the chromosome to begin to organize them**.** At metaphase, all the chromosomes have been caught by the mitotic spindle and are now arranged in the middle of the cell, termed the metaphase plate. **The spindle checkpoint:** at this point the cell will ensure that all the chromosomes are present at the metaphase plate and properly attached to the microtubules. During the anaphase A, chromosomes start moving toward the poles. At anaphase B, the two spindle poles and chromosomes will be separated. During the telophase, the DNA decondensation occurs and the nuclear envelope begins to reform. These

phases will be followed by the cytokinesis which divides the cytoplasm of the daughter cells and completes the cell division (63).



This figure was retrieved from Prosser et al., Nature Reviews Molecular cell biology, 2017 (64).

Figure. 6. **Mitotic spindle schematic.** The spindle is an array of microtubules. The minus ends of the microtubules will be concentrated at the spindle poles where the centrosomes reside to organize the spindle microtubules. The microtubule plus ends migrate towards the equator of the cell, here the microtubules from each pole will overlap. The microtubules attach to the chromosomes through the kinetochores found at the centromere of each chromatid. K-MTs: Kinetochore

microtubules, nK-MTs: non-kinetochore microtubules, A-MTs: astral microtubules, K-fibres: kinetochore fibres (64).

The roles and molecular mechanism of galctin1 in mitotic spindle formation and chromosome segregation remain unknown.

A.5. Multinucleation

One of the hallmarks of cancer cells is genetic instability which varies among different cancer cells. This includes genetic alterations such as gene amplifications, translocations, deletions, point mutations, aneuploid chromosome numbers, and multinucleations (65).

Multinucleation is an event that is frequently observed in different malignant neoplasms (66). Different processes and mechanisms have been proposed to be responsible for the formation of the multinucleation, including DNA over-replication, cytokinesis failure, and entosis which is a nongenetic mechanism of cytokinesis failure (67-69).

In mononuclear cells, when nuclear division occurs without cellular division (due to failure in cytokinesis), multinucleated cells are generated (70). In most of these cases, the polyploid cells cannot effectively undergo mitosis and eventually die (71).

It has been shown that disruption of cytokinesis results from genetic alterations in mitotic pathways or checkpoints in cancer cells (67). In other words, aberrant regulations or mutations of the genes that are responsible for the regulation of mitotic progression, can result in cytokinesis failure (67). For instance, upregulation of the mitotic kinase Aurora-A can lead to centrosome

amplification and tetraploid formation in a tumour model- correlated with poor outcome and high grades of breast cancer in patients (72).

In the nongenetic process of entosis, live cells are engulfed by a neighboring cell resulting in aneuploid cell lineages and multinucleation (67).

Furthermore, Noll et al., 2012, found that mutations of p53 hotspot can lead to centrosome abnormalities such as enhanced centrosome size, loss of cohesion, and amplification of centrosome, resulting in mitotic defects and multinucleation (73). Another study investigating the formation of multinucleation suggested that multinucleation formation can occur by vulnerability and genetic abnormality in the components of the cellular cytoskeleton (74).

Rajaraman et al., 2005, introduced a theory termed neosis, which is the mode of cell escape from senescence and going through neoplastic transformation and tumour progression. In this theory, the researchers tried to elaborate on the replication mechanism of multinucleated cells. They demonstrated that multinucleated cells display transient stem cell characteristics such as self-renewal and resistance to genotoxins and drugs (75).

Although, it was long presumed that the giant multinucleated polyploid cells do not survive and are destined to undergo cell death as a result of mitotic catastrophe, recent studies reported that a small proportion of them may survive and generate viable progeny and clones (76). The molecular mechanisms behind multinucleation formation and its roles in cancer cells have not been fully understood in detail yet.

A.6. Other functions of galectin1 in the tumour microenvironment

A.6.1 Galectin1 in tumour invasion and metastasis

When cancers become metastatic, the cells undergo changes where their cell-to-cell or cell-to-ECM (extracellular matrix) adhesion is modified, and they become more capable of migrating and invading other locations. As a result, the cells can travel from their primary site and occupy a secondary site (77). Several studies have shown the involvement of galectin1 in tumour cell invasion in lung, pancreatic, epithelial ovarian, and hepatocellular tumours (23). Galectin1 appears to play a role in different metastatic processes: (i) adhesion of tumour cells to the ECM, (ii) binding to ECM glycoproteins, and (iii) enhancing proteolytic enzyme pathways (28). Homodimeric galectin1 has been shown to increase the adhesion of tumour cells to ECM and endothelial cells in ovarian cancer cells (78). Overexpression of galectin1 has also been reported at the border of glioblastoma tumour tissues (79). Furthermore, elevated expression of galectin1 has been observed in glioblastoma-invaded tissue compared to areas that see less invasion (79, 80). Moreover, high galectin1 expression was associated with enhanced invasiveness of cells that are typically poor at invading. Highly invasive oral squamous cell carcinomas had their invasiveness reduced when galectin1 was inhibited with siRNA (81). Kim et al., 2013, indicated that silencing of galectin1 by siRNA *in vitro* reduced the invasion of cervical cancer cells (60). These findings suggest a critical role for galectin1 in different cancer cells in metastasis.

A.6.2. Role of galectin1 in tumour angiogenesis

Angiogenesis is the process by which new blood vessels are formed from pre-existing capillaries and can be induced by factors secreted by the tumour cells, including vascular endothelial growth factor (VEGF). Endothelial cells are the primary component of blood vessels and they can extend

the vascular network by generating new vessels to redirect and maintain blood flow. In order for solid tumours to continue growing, they need a constant supply of oxygen and nutrients, thus, this tumour-induced angiogenesis is crucial (82). Galectin1 can upregulate proangiogenic pathways, such as VEGF signalling, and can increase the proliferation and activation of endothelial cells (23). A study reported that binding of galectin1 to VEGFR-2 increased endothelial cell (EC) signaling and preserved the angiogenic phenotype, even in the absence of VEGF-A (83). Laderach et al., 2013, showed that the expression of galectin1 was significantly higher in tumour cross-sections from advanced prostate cancers that had high levels of CD34, a known marker of endothelial cells (84). Several studies reported that galectin1 knockdown in cancer cells disrupted endothelial cell proliferation and migration, and thereby inhibited angiogenesis (85, 86). Additionally, it has been proposed that galectin1 could establish a physical connection between the endothelial cells and components of the ECM in the tumour, acting as a scaffold to promote the growth of new blood vessels and the establishment of a vascular network (28). Moreover, other studies have shown that tumour growth and angiogenesis could be significantly reduced when only tumour-derived galectin1 levels are inhibited by shRNA transfection (59, 87). Furthermore, galectin1 can control expression of genes involved in tumour angiogenesis. For instance, downregulation of galectin1 in the oligodendroglioma hs683 cell line resulted in a decreased expression of the brain expressed X-linked gene 2 (BEX2, a tumour suppressor gene), but also a reduction in glioma cell adhesion and invasion as well as a reduction in tumour angiogenesis (88). These findings confirm the role that galectin1 plays in tumour angiogenesis.

A.6.3. Galectin1 in immune system

High expression of galectin1 can lead to the tumour's evasion from the immune response in poor prognosis cases. In fact, tumours evade the immune response by secreted galectin1 which triggers apoptosis of infiltrating T-cells (26). Rubinstein et al., 2004, provided evidence to indicate that high galectin1 expression in cancer cells can protect the tumour from the host's T-cell response (89). They suppressed the inhibitory effect of galectin1 and observed a reduction of tumour growth and more tumour rejection, as well as the generation of a potent tumour-specific T1-type response (89). It has been shown that galectin1 can modulate apoptotic signaling pathways via colocalization of receptors into signaling complexes, and so far, several specific apoptotic-related receptors for galectin1 have been identified including CD45, CD43. and CD7 (90). Furthermore, other studies showed that galectin1 can affect tumour growth through its effects on CD4+ and CD8+ T-cell apoptosis, or infiltration into the tumour stroma in different types of cancers such as melanoma, breast, and Lewis lung tumours (59, 87). Moreover, it has been reported that galectin1 can stimulate tumour-associated dendritic cells (TADCs), which are capable of inhibiting the effector T-cell response (91). Furthermore, the galectin1-stimulated TADCs produced large amounts of heparin-binding EGF-like growth factor (HB-EGF), promoting cancer cell growth. In addition, when galectin1 was knocked down in Lewis lung carcinoma cells, there was a significant decrease in the expression of HB-EGF, and the mice injected with these cancerous cells had a better prognosis (91). Together, these results demonstrate that galectin1 can inhibit the immune response and promote cancer growth and survival.

A.6.4. Galectin1 in chemotherapy and radiation therapy

Galectin1 is proposed to regulate tumour cell radiation resistance. In cervical cancer cells, due to the interaction between galectin1 and H-Ras, there is a marked increase in DNA repair and proliferation after irradiation. This would imply that galectin1 is providing some resistance to irradiation (92). Huang et al., 2012, also reported that galectin1 binding with H-Ras increased the cell's resistance to radiation (92).

Moreover, galectin1 is shown to confer resistance to chemotherapeutic modalities. Cancer cells can reduce the effects of anti-cancer drugs via developing several mechanisms including reduction of drug uptake, using ATP-binding cassette (ABC) transporters to improve the efflux and flowing out of the drug, enhancing metabolism of the drug, and resistance to drug-induced apoptosis (93). A study showed that galectin1 increases resistance to chemotherapeutic agents, such as Adriamycin and Imatinib, via inducing the expression of MDR protein 1, which in turn facilitates the ability of tumour cells to pump out cytotoxic drugs (94). Anginex (b pep-25) is a synthetic peptide with anti-angiogenic ability (85). Anginex suppresses proliferation and migration in endothelial cells through binding with galectin1 (85). Moreover, administration of Anginex can lead to a significant reduction of membrane-bound H-Ras-GTP, and as a result, decreases the signalling cascade of the Raf/MEK/ERK in vascular endothelial cells (95). It was shown that administration of Anginex combined with chemotherapy and angiostatin, had synergistic effects in tumour suppression and improved chemotherapy and anti-angiogenic therapies (96, 97). In addition, significant overexpression of galectin1 was observed in chemoresistant cervical cancer patients compared to the control group, which suggested a chemotherapy resistant role of galectin1 (98, 99).

A.7. Conclusions

Galectin1 is highly expressed in different aggressive human cancers and regulates multiple cellular processes including angiogenesis, invasion and migration, proliferation, and T-cell apoptosis. Moreover, *in vivo* experiments support a key role for galectin1 in tumourigenesis (28). However, the precise function and molecular mechanisms of action of galectin1 in glioma stem cells remain to be investigated.

A.8. Hypothesis and specific objectives

We performed RNA-Seq analysis on patient-derived brain tumour stem cells to obtain galectin1 gene expression profiling. This analysis led to the identification of a large panel of genes involved in the mitotic spindle checkpoint and chromosome segregation. Among these targets were MAD2L2, NEK6, CENPM, KLHL22, NEDD9, NDC80, and SPC24.

The hypothesis of my thesis is that galectin1 regulates glioma stem cell self-renewal and glioblastoma pathogenesis via interaction with cell cycle checkpoints.

To address this hypothesis, I pursued the following specific aims:

1. To validate galectin1 gene expression profile via confirmation of RNA-Seq data by RT-qPCR.
2. To determine the impact of galectin1 loss-of-function on proliferation and cell cycle in patient-derived brain tumour stem cells.
3. To investigate galectin1 binding partners in order to identify the mechanisms by which galectin1 regulates cell cycle checkpoints.

B. Materials and Methods

B.1. Brain Tumour Stem Cell cultures

We used the human BTSC lines 73 and 147, a kind gift from the laboratory of Dr. Samuel Weiss at the University of Calgary. BTSC lines were generated following surgery with informed consent of adult glioblastoma patients following the BWH/Partners IRB protocol for use of excess/discarded tissue. Several major mutations were characterized in the glioblastoma cells including EGFRvIII, p53, PTEN, and IDH1 (Table 3). In this study, we used the two BTSC lines, 73 and 147, that harbour EGFRvIII mutation. Prior to being used for any experiments, BTSCs were removed from liquid nitrogen, recovered from cryopreservation in 10% dimethyl sulfoxide (DMSO, Sigma Aldrich, #D8418) and 90% BTSCs medium, and cultured in Nunc ultra-low attachment flasks as neurospheres in NeuroCult NS-A medium (Stem Cell Technologies, #05750) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma Aldrich, #P4333), heparin (2µg/mL, Stem Cell Technologies, #07980), human EGF (20 ng/mL, Miltenyi Biotec, #130-093-825), and human FGF (10 ng/mL, Miltenyi Biotec, #130-093-838). All cell lines were tested negative for mycoplasma by PCR method as previously described (100).

Table 3: Characterization of brain tumour stem cell lines (Adapted from *Jahani-Asl et al., Nature Neurosci, 2016* (101).)

Line	EGFRvIII Status	P53 Status	PTEN Status	IDH1 Status
BTSC73	Positive	Mutant	Wildtype	Wildtype
BTSC147	Positive	Mutant	Mutant	Wildtype

B.2. Limiting dilution assay

BTSC73 and *LGALS1* KO BTSC73 cells were separated into the single-cell suspensions by using the ACCUMAX cell detachment solution (Stem Cell Technologies). Afterwards, we counted the number of cells and plated them in a 96-well plate. We used different cell densities starting from 200 and going down to 12 cells per well. After 24 hours, we treated BTSC73 and *LGALS1* KO BTSC73 cells with 10µM Temozolomide (TMZ) (Sigma Aldrich, T2577). We also treated BTSC73 and *LGALS1* KO BTSC73 cells with 10µM DMSO for control groups. The number of spheres were counted after 7 days of treatment. An Olympus IX83 microscope with 10X objective was used to visualize the spheres. Additionally, to measure the size of spheres, we used an Olympus cellSens imaging Software (Version 1.12). These experiments were repeated for 3 independent biological replicates.

B.3. RT-qPCR

Cells were harvested and RNA was isolated using the TRIzol method. Briefly, pelleted cells were resuspended and lysed in 1 mL of TRIzol reagent (Invitrogen, #15596026). Afterwards, 0.2 mL of chloroform (Sigma Aldrich, #288306) was added and the tubes were shaken vigorously for 15 seconds. The samples were centrifuged at 12000 x g at 4 °C for 15 minutes, and the aqueous phase was collected and transferred to a tube containing 0.5 mL isopropanol (Sigma Aldrich, #190764). The samples were incubated at room temperature for 10 minutes and then centrifuged for 10 minutes at 12000 x g at 4 °C. The supernatant was removed, and the pellets were washed with 75% ethanol, centrifuged at 7500 x g at 4°C for 5 minutes, and the supernatant discarded. The RNA pellet was left to

dry for 5-10 minutes. RNA was then resuspended in 50 µl RNase-free water and incubated at 55 ℃ for 10 minutes. cDNA was generated using the SuperScript III First-Strand cDNA synthesis system (ThermoFisher, #11904018), by adding 4 µl (200 U⁄µl) SuperScript® III Reverse Transcriptase with 1 µg RNA to a final volume of 20 µl. The PCR program was as follows: one cycle of 45–60°C for 15 min and 94 ℃ for 2 min, then followed by 40 cycles of 94 ℃ for 15 seconds, 55 ℃ for 30 seconds, and 68 ℃ for 1 min, and then extended by 68 ℃ for 5 min and hold at 4 ℃. qPCR of the resulting cDNA was then performed using SYBR Green Master Mix (Life Technologies, #A25742), a fluorescent double-stranded DNA dye, which can be used to quantify amplicon amount during the course of the PCR by tracking overall fluorescence emission. The RT-qPCR reactions mixed was made with 5 µl of SYBR Green Master Mix, 1 µl of 10 mM forward primer, and 1 µl of 10 mM reverse primer and 3 µl of cDNA. RT-qPCR was performed using the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems) with the following program: 50 °C for 2 min, 95 ℃ for 10 min, 40 cycles of 95 ℃ for 15 sec, 60 ℃ 30 sec, and 72 °C for 30 sec, followed by 72 ℃ for 10 min. mRNA expression levels were then normalized to the housekeeping gene beta-glucuronidase (GUSB). Several housekeeping genes (ACTB, B2M, GUSB, PP1A, RPL13A, and TFRC) were tested in BTSC73 and BTSC147 to determine the gene which displayed the least variation following *LGALS1* knockdown (Data not shown). GUSB was found to be the most consistent housekeeping gene.

In order to design our primers of interest to perform RT-qPCR, we first obtained sequences of our genes of interest on the Ensemble Genome Browser 97 by choosing the exons from the longest protein-coding sequences. Afterwards, we designed primers by

using Primer 3 Input (version 0.4). We considered important primer conditions, such as primer size (19 -21 bp), temperature (57- 62°C), and the percentage of primer GC content (minimum 40%). Furthermore, we re-analyzed each of the primer sets on the NCBI BLAST program and the UCSC Genome Browser database in order to assess the specificity of each primer.

The following qPCR primers were used:

Table 4. The sequence of primers used in this study.

Genes	Forward Primer Sequence	Reverse Primer Sequence
PIM2	GTGGCTGTGCCAAACTCATT	ATGCCCAGTGACCAGACAGT
E2F7	GAAAGCACCAAAGAGCCTTCT	AAGACCATGCAAGGGACACT
NEDD9	TGACTGTAGCAGCAGTGATGG	TGTTCCAGCTGCATCTTGTT
MCM5	CAGAGGCAGATGTGGAGGAG	GCTTGAGCTGCTTCTCGATG
KLHL22	CCACAATGACCTGAATGCTG	TCAGGTAATCCTCCCCTCTG
NDC80	CTGTTAACCAGGGGCTCAGT	GACCCAACATGTGTAGCAACC
GSPT2	CAAAGATATGGGCACTGTGG	GTTTTCACCTGGGGCTACAA
SPC24	CACCAGAGAGCTGGAAGAGC	TCCCTGGCTCACACTCATAA
HIRA	ACGCACGGTACCTCGTAAAC	TGTTGACTCCCACTGGCTTC
SMARCA4	GATGACAGTGAAGGCGAGGA	GGCCAAGCTTGATCTTCACTT
CENPM	TCTTGGGGAAGGTGTGTTTC	TAGAGCAGGGGGCTTTGATA

GTSE1	CAGAAGTAGCTCGGGAGGAA	CTTGCAGCATCTGGAGTGAC
MAD2L2	GCTGTACCTTCACAGTCCTGGT	ATGTCCGACGTCATGGTTTT
NEK6	GACGCCCTACTACATGTCACC	TGGCACAGGGAGAAGAGATT
SKP2	ACCTTTCTGGGTGTTCTGGA	CTGGGTGATGGTCTCTGACA
CCND3	ATTTCCTGGCCTTCATTCTG	CGGGTACATGGCAAAGGTAT
CDK6	CATTCAAAATCTGCCCAACC	GGTGGGAATCCAGGTTTTCT
CDKN2D	GTCATGATGTTTGGCAGCAC	CGTCATGGACTGGACTGGTA
ANLN	GGCATCGAAGATGGTGTGTT	TCAAAAGTGTTGCGTCTTGC
CIT	GGCGTCCTCATACCAGGATA	CTTGGTGATGTGCTCGTTGTA
KIF20B	GGTGTAAACCTGGCCACTAAGA	AATTTCCGTTTGGCTCGTTT
RNF8	GGAGAAGATGCAAGCACAGA	CTTCCGCTTCATCCATTCAT
CALM1	GTAATGGCACCATTGACTTCC	CATGACGTGACGTAGTTCTGC
CENPV	TGCAGCATTTGCAAGAAGAA	AAGCTCTGAACGCCACATCT

B.4. Immunofluorescence

Due to the non-adherent nature of the cells, prior to immunostaining, the BTSC73 and 147 were plated on a Lab-Tek II, CC2-treated chamber slide system (Thermo Fisher Scientific, #154941) in media containing 10% FBS, for 2 hours. Cells were washed two times with PBS and fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. Next, cells were washed three times with PBS and permeabilized with 0.5% Triton-X

(Sigma Aldrich, #T8787) for 30 minutes at room temperature. Then, cells were washed twice with 0.3% Triton-X and blocked with PBT (1X-PBS / 0.3% Triton-X /0.5% BSA) for 1 hour at room temperature. The cells were then incubated overnight at 4°C with primary antibodies to anti-mouse galectin1 (1:100, Cell Signaling Technology #40103), or anti-rabbit galectin1 (1:100, Cell Signaling Technology, #12936), α-Tubulin (1:1000, Sigma-Aldrich, T9026) or phospho-Histone H3(Ser10) (PH3) (1:500, Sigma-Aldrich, 09-797) diluted in PBT. Cells were washed two times with PBS/ 0.3% Triton-X and then incubated with secondary Alexa fluor 488 goat anti-mouse (1:400, Cell Signaling Technology, #4412s) and 594 goat anti-rabbit (1:400, Cell Signaling Technology, #8890) antibodies for 1 hour at room temperature. Next, cells were washed two times with 1X-PBS/0.3% Triton-X and were incubated for 20 minutes with 2µg/mL DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride, Thermo Fisher Scientific, #D1306) in PBS to detect nuclei. Coverslips were then mounted on slides using ProLong Gold Antifade Mountant (ThermoFisher, #P36930) and sealed with nail polish for subsequent imaging. Images were captured using 20X, 40X, and 60X objectives on a laser scanning confocal microscope (ZEISS LSM 800).

B.5. Immunoprecipitations - Western blot (IP-WB) and antibodies:

Four million BTSC73 cells were collected and washed two times with PBS. The cells were then lysed for 10 minutes on ice in RIPA lysis buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific, #A32959). Protein concentration was determined by Bradford assay (Bio-Rad). Lysates were cleared by centrifugation (10000 xg, 10min, 4°C) and subsequently incubated with 2 µg of antibodies to galectin1 (Cell Signaling

Technology #40103, mouse), galectin1 (Cell Signaling Technology, #12936, rabbit), galectin1 (Abcam, ab108389, rabbit) and IgG (Millipore, #12-371, and Cell Signaling Technology, #3900S) as a negative control. Primary antibodies were incubated overnight at 4°C, followed by one-hour incubation at room temperature with Dynabeads Protein G magnetic beads (Thermo Fisher Scientific, #10003D). Beads were washed three times with lysis buffer and eluted by boiling in 3% SDS sample buffer for five minutes at 95°C. Immunoprecipitates were analyzed by Western blot using the indicated antibodies. Ultimately, we had five samples to analyze in the Western blot, input (5%, 2.5% and 1%), IgG-IP and galectin1-IP.

15% gels were prepared for these Western blots. All five samples were subjected to SDS-PAGE and transferred onto Immun-Blot® PVDF membrane (BIO-RAD) using Trans-Blot® Turbo™ transfer system (BIO-RAD). Membranes were blocked in 5% non-fat milk or 5% bovine serum albumin (BSA) in TBST for 1 hour. Next, sequential probing with primary antibodies was performed and each primary antibody was incubated overnight at 4°C, followed by a 1-hour incubation at room temperature with HRP-conjugated secondary antibodies (anti-rabbit or anti-mouse depending on the primary antibody) in blocking solution. Target proteins were visualized by peroxide solution and Luminol/enhancer solution (Clarity Western ECL substrate, BIO-RAD) and imaged through the ChemiDoc Imaging System (BIO-RAD).

The following table shows the antibodies that were used for these Immunoprecipitation - Western blot (IP-WB):

Table 5. Antibodies for IP-WB.

Antibody	Species	Company	Catalogue No.
Galectin1	anti-mouse	Cell Signaling Technology	#40103
Galectin1	anti-rabbit	Cell Signaling Technology	#12936
Galectin1	anti-rabbit	Abcam	ab108389
IgG	anti-rabbit	Cell Signaling Technology	#3900S
IgG	anti-mouse	Millipore	#12-371
H-Ras + K-Ras	anti-rabbit	Abcam	EPR18713
HOXA5	anti-rabbit	Abcam	ab82645

B.6. Deletion of galectin1 expression with CRISPR.

We used CRISPR technology to genetically delete galectin1 in patient derived human BTSC73 and 147. Briefly, two gRNAs were designed using Off-Spotter software to delete exon 2-4, resulting in a 4.1kb deletion of the *LGALS1* gene. gRNA-1 and -2 were cloned into pL-CRISPR.EFS.GFP (Addgene plasmid #57818), and pL-CRISPR.EFS.tRFP (Addgene plasmid, #57819), respectively. 5μg of each construct was nucleofected into BTSC73 using an AMAXA nucleofector 2b device (Lonza, #AAB1001). The GFP and RFP positive cells were then sorted two days post-electroporation using BD FACSAria™ Fusion (BD Biosciences) and plated clonally. Three weeks after FACS sorting, genomic DNA was isolated from 20000 cells from each clone and then screened for *LGALS1*

deletion via PCR using specific internal and external primers around the site of deletion. This led to the identification of biallelic deletion clones. The knockout level of galectin1 was assessed via RT-qPCR and WB. The following gRNAs and screening primers were used for the CRISPR/Cas9 system:

gRNA *LGALS1*-1: GGAGAGTGCCTTCGAGTGCGAGG
gRNA *LGALS1*-2: GCCTCCAGGTTGAGGCGGTTGGG

B.7. Whole-transcriptome analyses (RNA-seq).

Total RNA was isolated from BTSC lines by using the Trizol RNA isolation method. Libraries for poly(A)+ RNA were prepared by following the Illumina protocol as previously described (102). The quality of libraries was assessed by bioanalyzer before sequencing. Libraries were sequenced on Illumina GAIIX Genome Analyzer or on HI-SEQ 2000 platforms. RNA-seq reads were mapped and analyzed by TopHat/Cufflinks RNA-seq analysis pipeline using default parameters. Differentially expressed genes were called by CuffDiff using default parameters. Gene ontology of expression data was done using the functional annotation module of DAVID 6.7 (http://david.abcc.ncifcrf.gov).

B.8. Statistical Analysis

Statistical analyses were performed using either ANOVA or Student's t-test, with the GraphPad 7 software. Two-tailed and unpaired t-tests were used to compare two conditions. One-way ANOVA with Tukey's or Dunnett's post hoc analyses were used for analyzing multiple groups. n = 3 biological replicates.

C. Results

C.1. Confirming loss of *LGALS1* mRNA expression in *LGALS1* KO BTSCs

To investigate the role of galectin1 in patient-derived brain tumour stem cells, our lab performed CRISPR to generate two *LGALS1* KO cells from the BTSC cell lines, BTSC73 and BTSC147. Prior to using these cells, I first confirmed the deletion of *LGALS1* in BTSC73 and 147, by RT-qPCR analysis (Figure. 7). In parallel, I performed immunostaining of BTSC73 and 147 to confirm the knockdown of galectin1 at the protein level (Figure. 8 and 9).

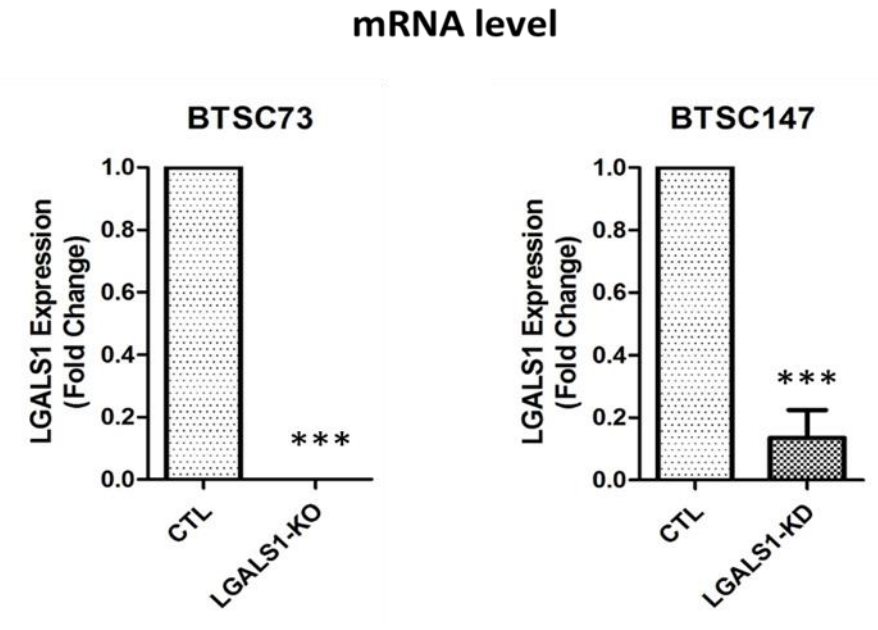


Figure 7. mRNA expression of *LGALS1* in BTSC73 knockout (KO) and BTSC147 knockdown (KD) was quantified by RT-qPCR. Expression of *LGALS1* is normalized to the housekeeping gene GUSB. Three replicates were done at different passage numbers. *LGALS1* expression results were analyzed relative to the levels found in CTL cells, set at 1. ***$P < 0.001$ compared with CTL cells.

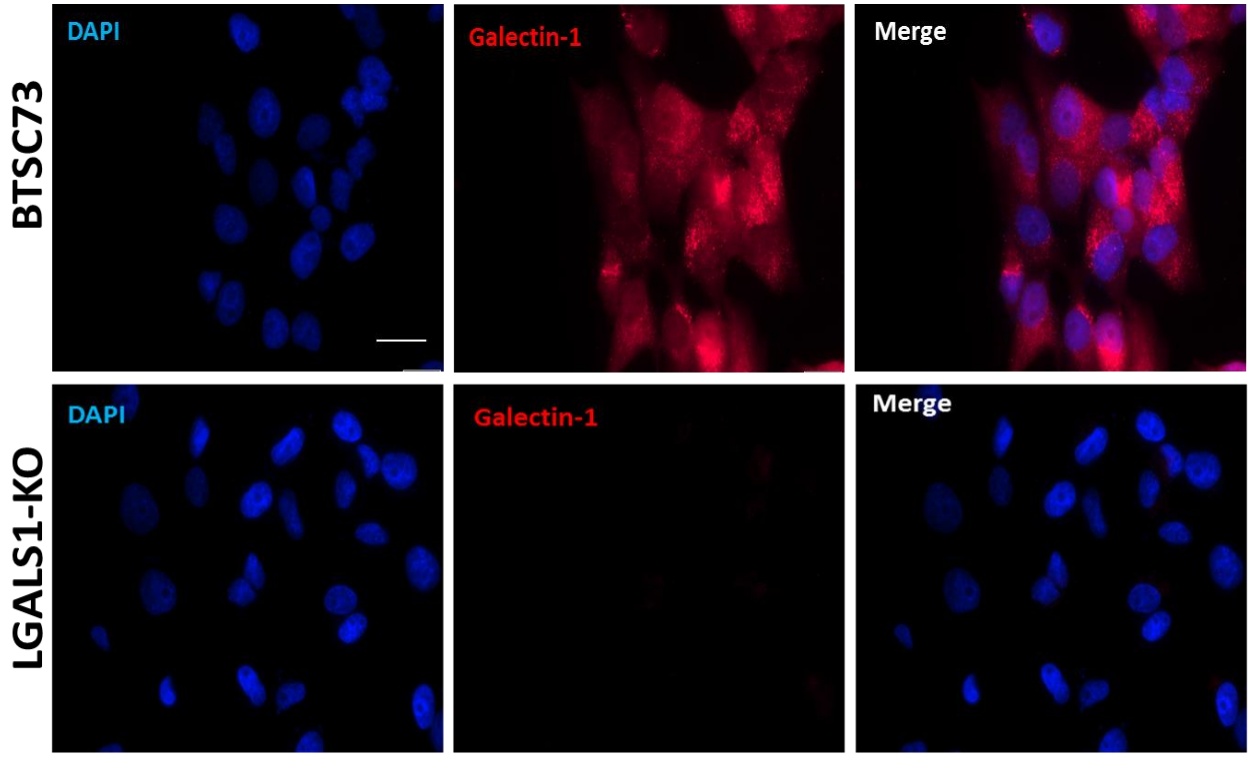


Figure 8. Immunofluorescence (IF) staining of galectin1 protein in BTSC73 and *LGALS1* KO BTSC73 was performed using an anti-galectin1 antibody. DAPI was used to stain the nuclei. Scale bar = 50 µm.

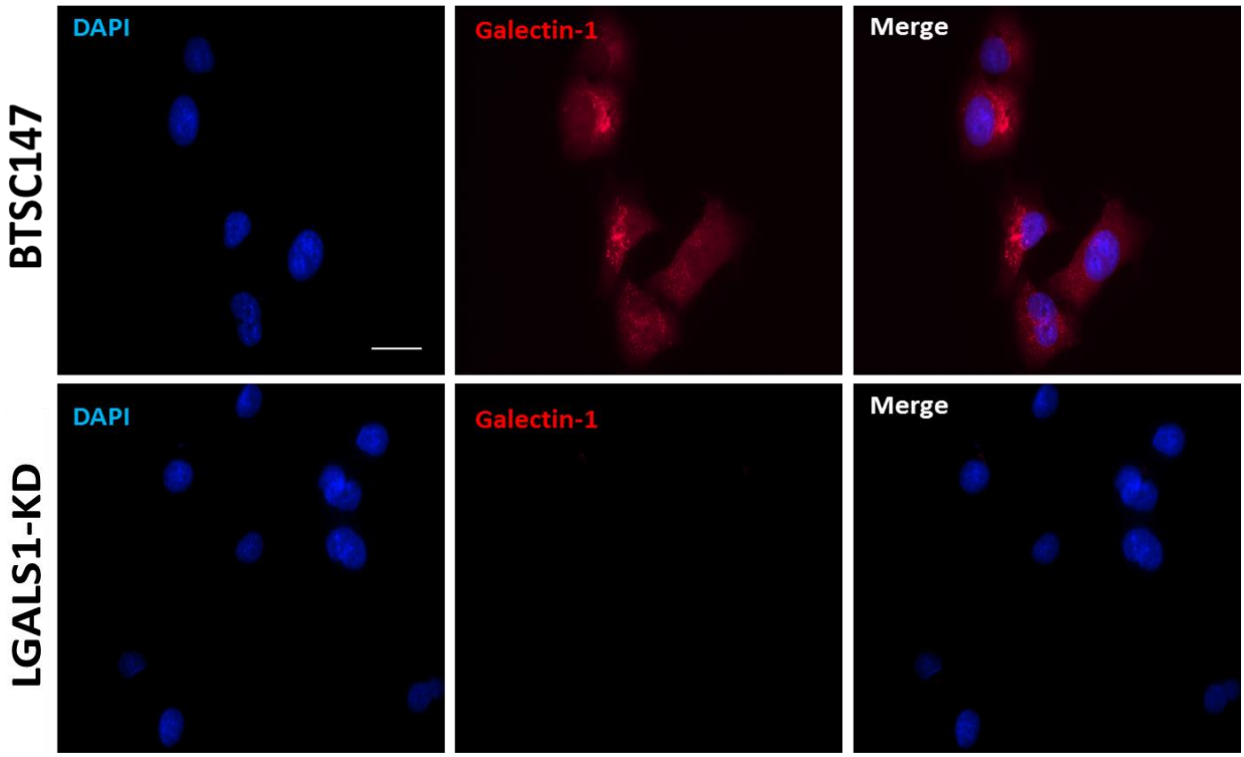


Figure 9. Immunofluorescence (IF) staining of galectin1 protein in BTSC147 and *LGALS1* KD BTSC147 was performed using an anti-galectin1 antibody. DAPI was used to stain the nuclei. Scale bar = 50 µm.

C.2. Validation of galectin1 candidate target genes

To study how galectin1 regulates BTSC gene networks, RNA-Seq analysis was performed on the *LGALS1* KO BTSC73 and BTSC73. Bioinformatics analysis of these data revealed a large number of genes that were deregulated in the *LGALS1* KO BTSC73 cells compared to BTSC73 (Figure 10).

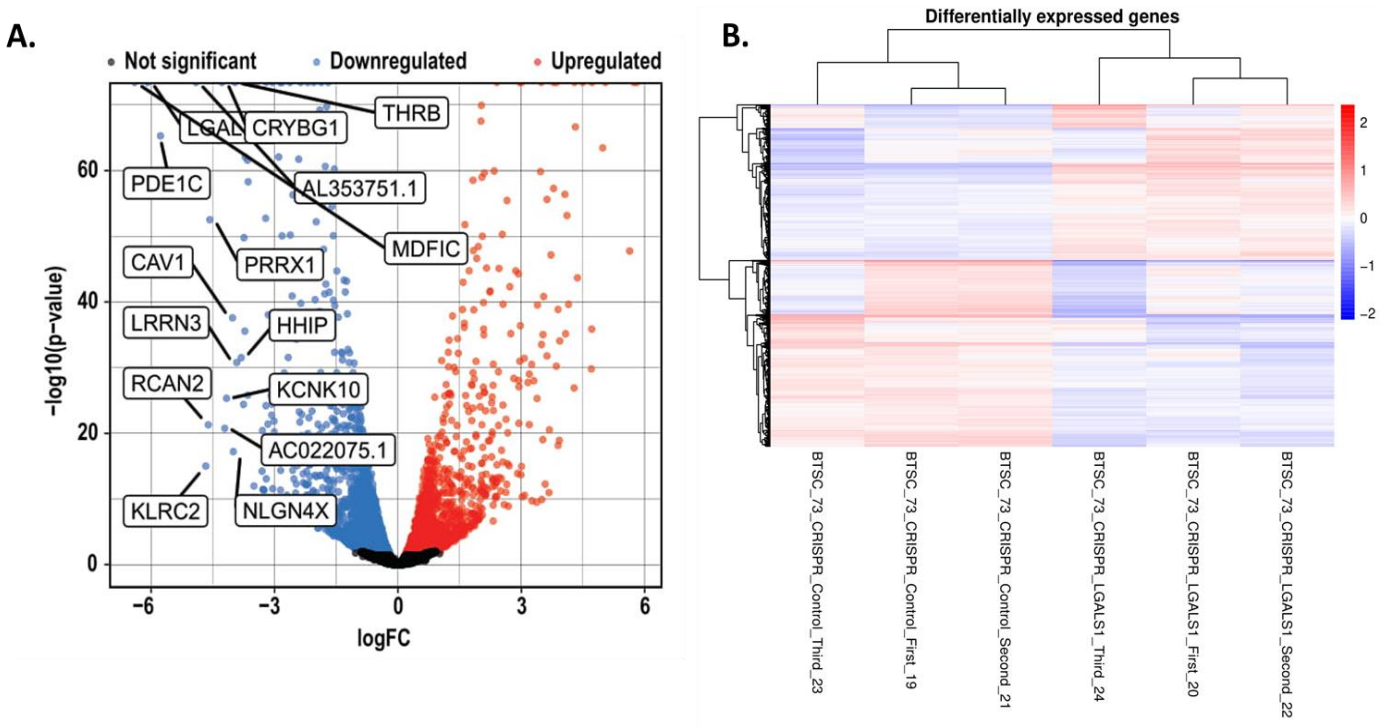


Figure 10. RNA-Seq-based transcriptome analysis of *LGALS1* KO BTSC73 and BTSC73. **A**. Volcano plots of the distribution of gene expression of the galectin1 target genes in BTSC73. Red dots correspond to genes that are upregulated, and blue dots represent downregulated genes as a result of *LGALS1* deletion in BTSC73. **B.** Heatmap shows the changes in transcriptional profiles in *LGALS1* KO BTSC73. RNA-Seq was done in 3 independent biological replicates for each group.

The Reactome pathways analysis revealed that a large panel of candidate target genes involved in proliferation, including G2/M transition and mitotic G2-G2/M phases, are significantly downregulated in response to *LGALS1* deletion (Figure. 11), leading to the hypothesis that galectin1 regulates proliferation and cell cycle mechanisms in BTSCs.

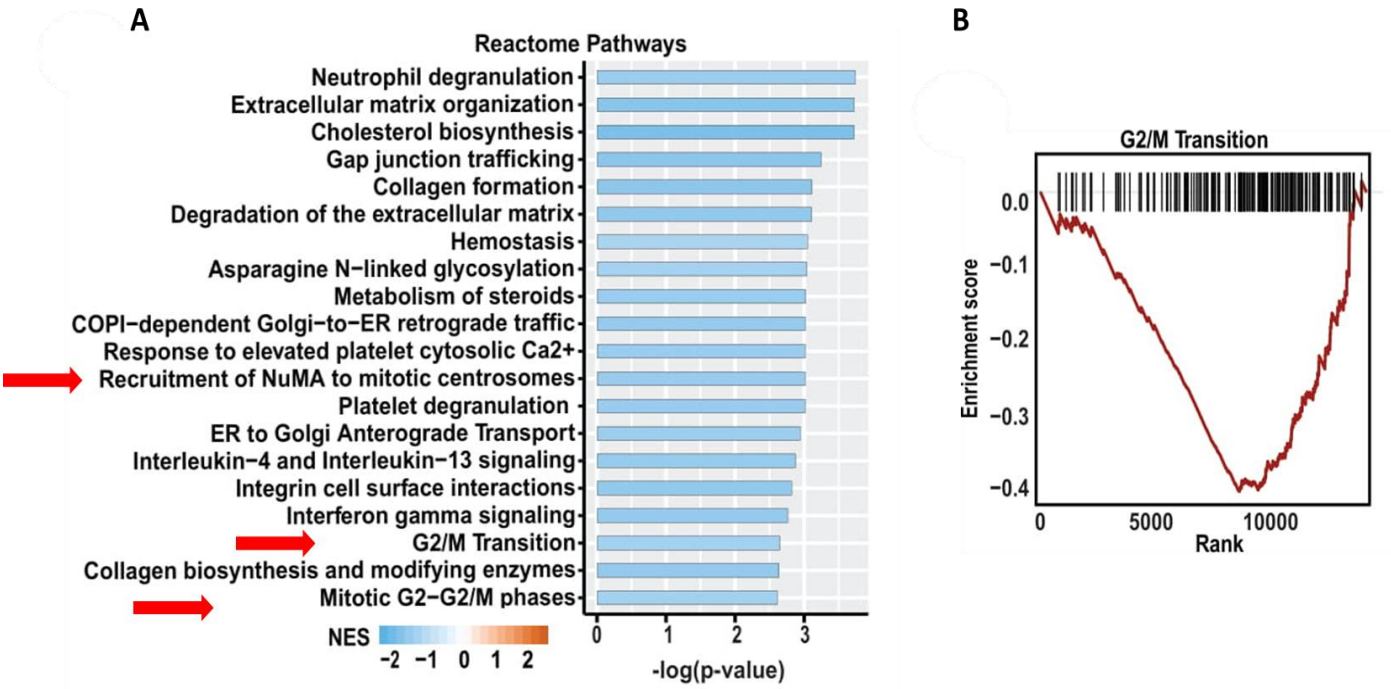


Figure 11. RNA-Seq analysis of *LGALS1* KO BTSC73 cells. A. Differentially regulated genes in *LGALS1* KO BTSC73 were subjected to enrichment analysis using Reactome pathways. The enriched pathways are labeled on the Y-axis and the P-value is represented on the X-axis. The arrows in the enrichment analysis point to the G2/M transition and mitotic G2-G2/M phases pathways which are downregulated upon deletion of *LGALS1*. **B** Gene Set Enrichment Analysis (GSEA) panel from RNA-Seq analysis demonstrates that *LGALS1* KO BTSC73 are more enriched for GO gene sets corresponding to G2/M transition pathway. Negative enrichment scores indicate enrichment with downregulated genes.

In further support of this hypothesis, I employed the Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional annotation and found that

galectin1 loss of function resulted in deregulation of a wide range of genes that are involved in the modulation of cell cycle and proliferation (Table 6). We, therefore, designed primers to validate the changes in gene expression by using RT-qPCR in both BTSC73 and 147 (Figures 12 and 13). RT-qPCR analysis indicated that some of the genes that have roles in the regulation of cell cycle and proliferation, such as E2F7, GSPT2, GTSE1, MCM5, PIM2, and CDK6, are significantly downregulated as a result of *LGALS1* KO in both BTSC73 and 147 cell lines (Figures 12 and 13). Our results support the hypothesis that galectin1 regulates BTSCs cell cycle and proliferation by regulating these target genes.

Table 6. Top downregulated genes in *LGALS1* KO BTSC73 which are involved in the modulation of cell cycle and proliferation (DAVID analysis).

Downregulated Genes:	**Fold Change (FC)**	**p-value**
AKT1 (AKT serine/threonine kinase 1)	0.8	0.010950625
BAD (BCL2 associated agonist of cell death)	0.6	0.000039
CRKL (CRK like proto-oncogene, adaptor protein)	0.7	0.000137362
HRAS (HRas proto-oncogene, GTPase)	0.6	0.000000441
JAK2 (Janus kinase 2)	0.6	0.004183513
MAPK1 (Mitogen-activated protein kinase 1)	0.6	0.00000000000686
RELA (RELA proto-oncogene, NF-κB subunit)	0.7	0.005047475
E2F7 (E2F Transcription Factor 7)	0.5	0.000000000293
GSPT2 (G1 To S Phase Transition 2)	0.4	0.0000000000226

MCM5 (Minichromosome maintenance complex component 5)	0.6	0.0000000000081
PIM2 (Proviral Integrations of Moloney virus 2)	0.5	0.00000315
HIRA (Histone Cell Cycle Regulator A)	0.6	0.0000000277
SMARCA4 (SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 4)	0.7	0.0000268
GTSE1 (G2 And S-Phase Expressed 1)	0.5	0.00000000000599
SKP2 (S-Phase Kinase Associated Protein 2)	0.5	0.000000000000000153
CCND3 (Cyclin D3)	0.6	0.000103027
CDKN2D (Cyclin Dependent Kinase Inhibitor 2D)	0.4	0.0000000708
CDK6 (Cyclin Dependent Kinase 6)	0.7	0.000123979

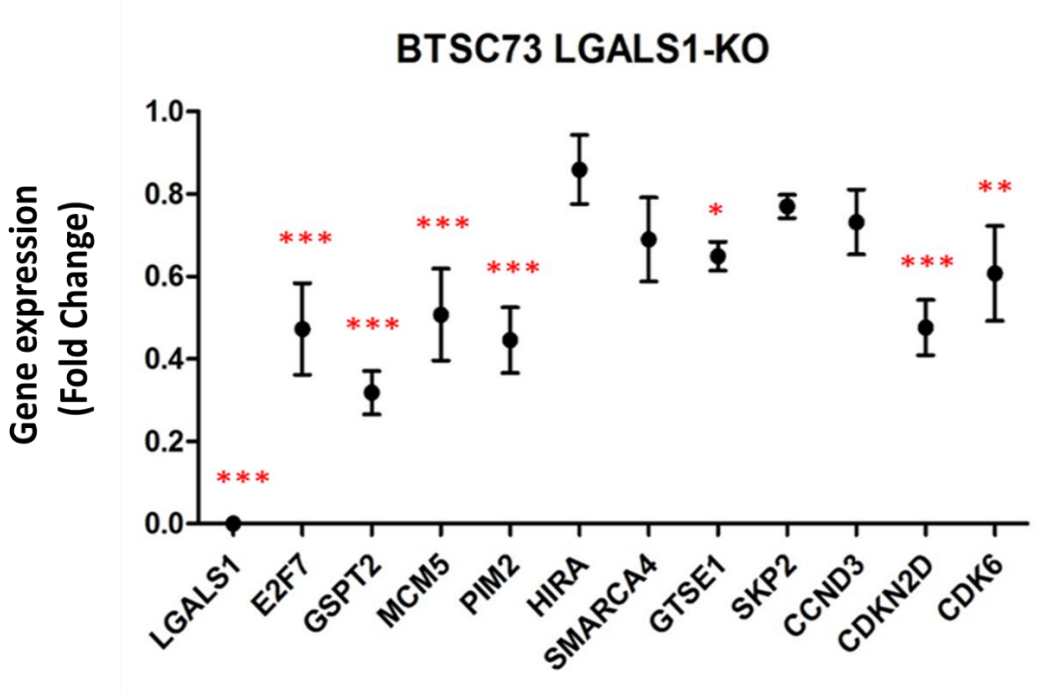


Figure 12. Validation of RNA-seq data by RT-qPCR. *LGALS1* KO is correlated with reduced expression of genes that are involved in the regulation of the cell cycle and proliferation in BTSC73. In all samples, gene expression is normalized to the housekeeping gene GUSB. (n = 3 biological replicates). Two-tailed t-test. Gene expression results were analysed relative to the levels found in CTL cells, set at 1. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with CTL cells.

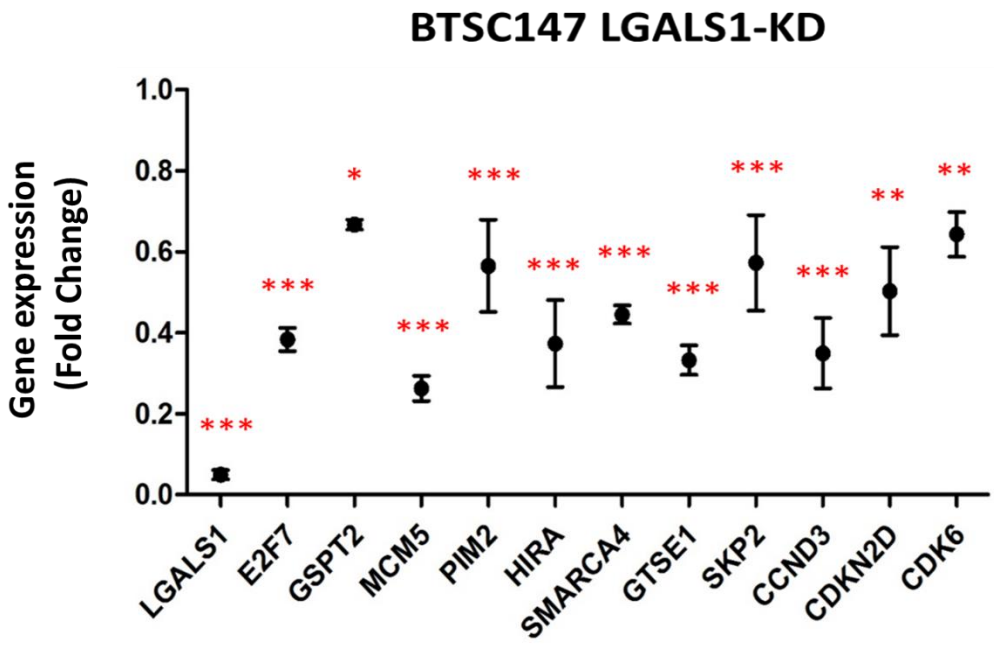


Figure 13. Validation of RNA-seq data by RT-qPCR. *LGALS1* KD is correlated with reduced expression of genes that are involved in the regulation of the cell cycle and proliferation in BTSC147. In all samples, gene expression is normalized to the housekeeping gene GUSB. (n = 3 biological replicates). Two-tailed t-test. Gene expression results were analysed relative to the levels found in CTL cells, set at 1. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with CTL cells.

C.3. The impact of galectin1 loss of function on proliferation and cell cycle in BTSCs.

In order to look more in-depth at the effects of galectin1 on proliferation, we decided to perform immunofluorescence (IF) staining of Ki67, a marker of proliferation, in BTSC73. Our data from Ki67 staining showed that 65% of the cells in the BTSC73 were positive for Ki67 compared to 45% in the *LGALS1* KO BTSC73. These results suggest that there is a decrease in proliferation upon deletion of *LGALS1* in the BTSC73 (Figure 14).

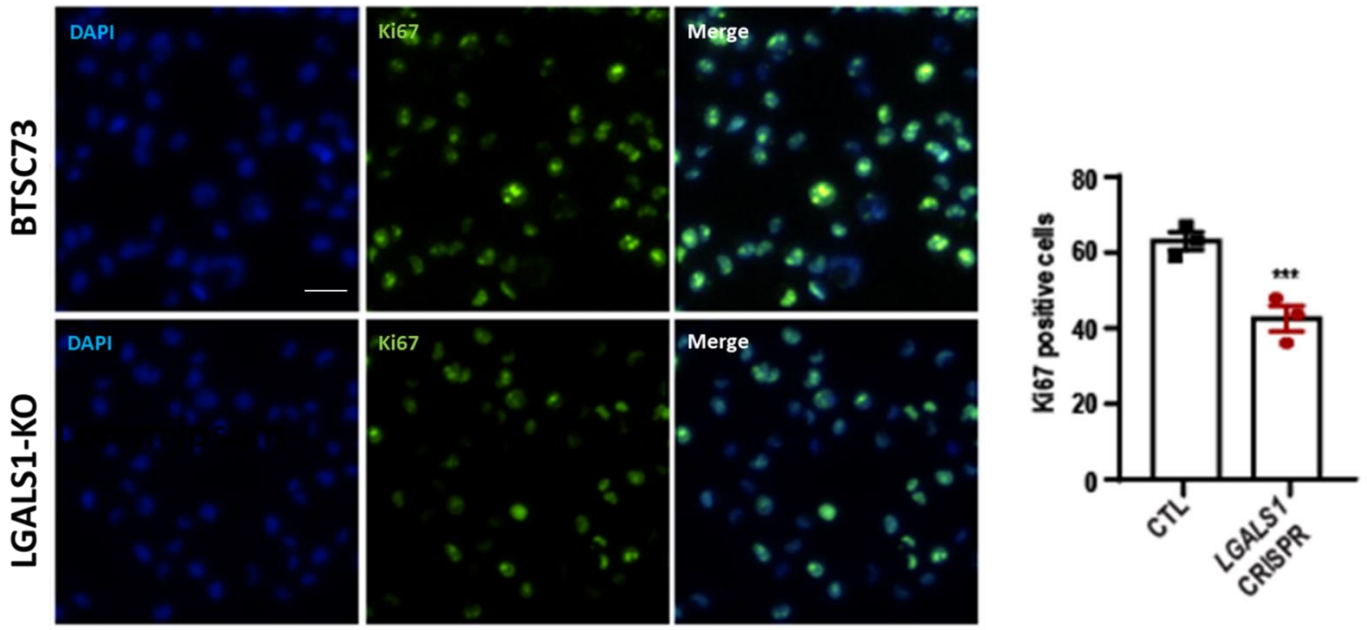


Figure 14. Immunofluorescence (IF) staining of Ki67 protein in BTSC73 and *LGALS1* KO BTSC73. This experiment was performed in collaboration with Dr. Ahmad Sharanek. Scale bar = 50 µm. (n = 3 biological replicates). Two-tailed t-test. Ki67 staining results were analyzed relative to the levels found in CTL cells, set at 100%. ***$P < 0.001$ compared with CTL cells.

Furthermore, we confirmed the results of the Ki67 staining experiment by performing IF staining of another marker of proliferation, phospho-Histone H3 (PH3), in BTSC73 and BTSC147. Correspondingly, we found that there was a difference in proliferation where the *LGALS1* KO BTSC73 cells were about 20% positive for PH3 compared to the BTSC73 cells at that were 35% positive for PH3 (Figure 15). We also observed the same results in PH3 staining of BTSC147, whereby 40% of the BTSC147 cells were positive, compared to 30% of the *LGALS1* KD BTSC147 cells (Figure 16). Altogether, these results support our hypothesis that galectin1 may regulate BTSC proliferation.

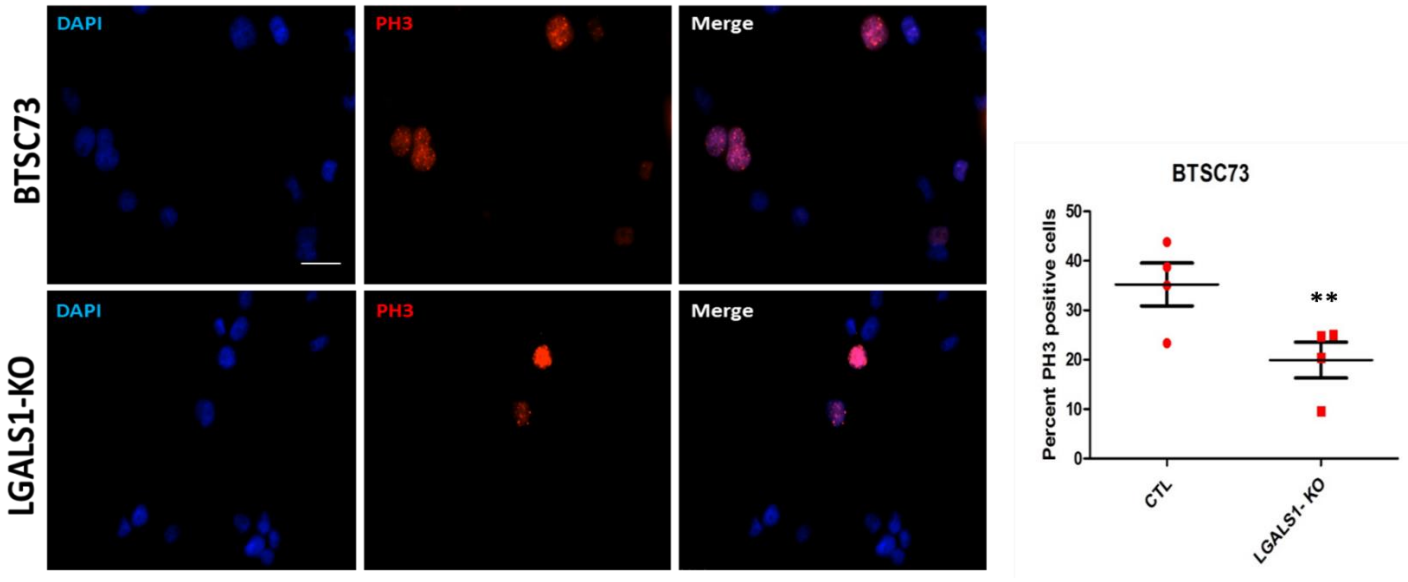


Figure 15. Immunofluorescence (IF) staining of PH3 in BTSC73 and *LGALS1* KO BTSC73. Scale bar = 50 µm. (n = 3 biological replicates). Two-tailed t-test. PH3 staining results were analyzed relative to the levels found in CTL cells, set at 100%. ***P* < 0.01 compared with CTL cells.

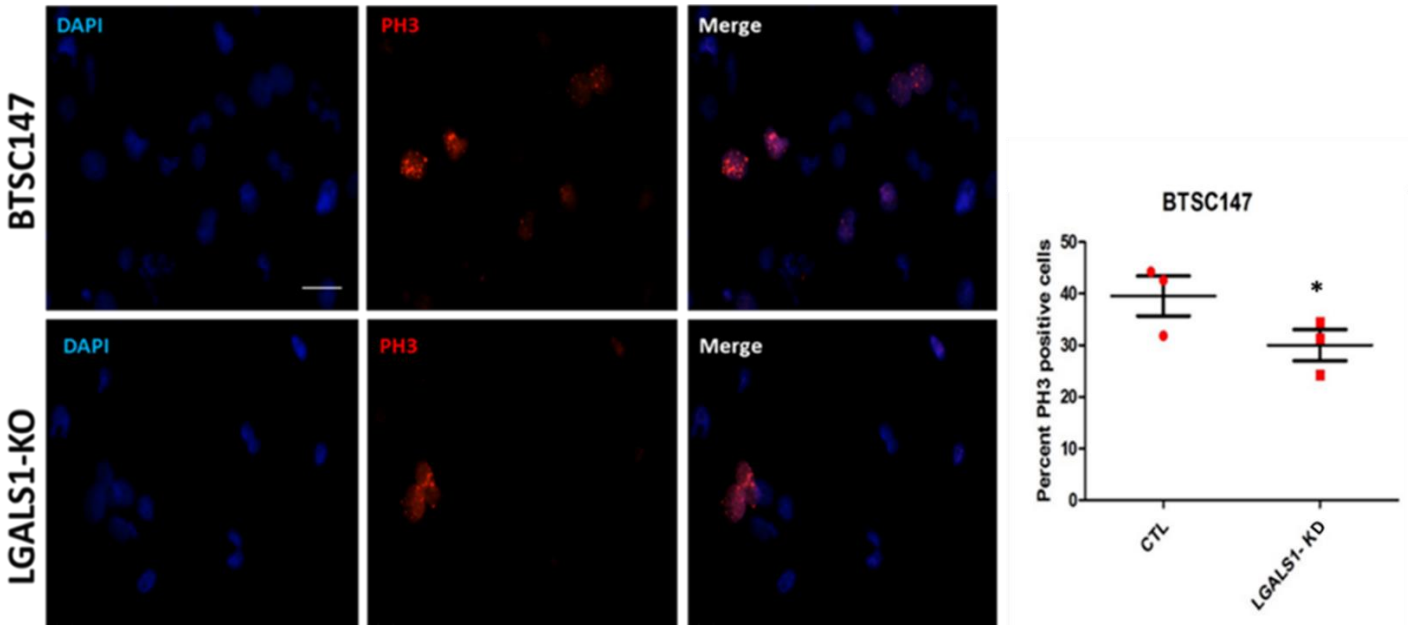


Figure 16. Immunofluorescence (IF) staining of PH3 in BTSC147 and *LGALS1* KD BTSC147. Scale bar = 50 µm. (n = 3 biological replicates). Two-tailed t-test. PH3 staining results were analyzed relative to the levels found in CTL cells, set at 100% *$P < 0.05$ compared with CTL cells.

C.4. Galectin1 confers resistance to chemotherapy and controls the self-renewal of BTSCs.

It has been suggested that galectin1 plays a chemotherapy resistance role in several types of cancers, including cervical cancer (93, 98, 99). This raises the question of whether galectin1 can be involved in the chemoresistance of BTSCs. To answer this, we performed limiting dilution assays (LDA) on BTSC73 and *LGALS1* KO BTSC73 cells to evaluate the response of these cells to Temozolomide (TMZ) treatment. Our results suggest that deletion of galectin1 combined with TMZ treatment induced a significant decrease in the sphere numbers and the mean sphere size of the BTSC73 (Figure 17). These results suggest that galectin1 may protect BTSCs against chemotherapy with TMZ.

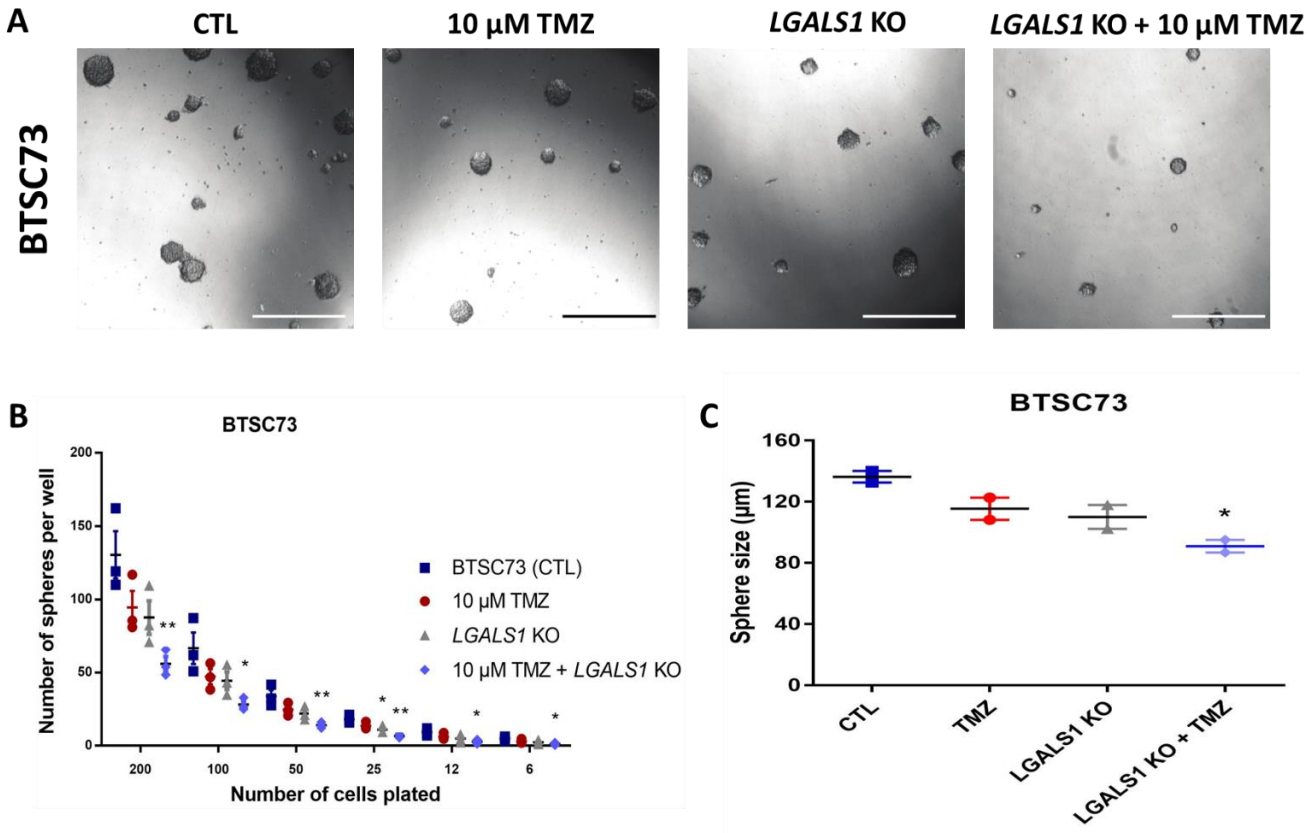


Figure 17. Galectin1 confers resistance to chemotherapy with TMZ in BTSC73. (A) Representative phase-contrast images of BTSC73 and *LGALS1* KO BTSC73 following treatment with 10µM TMZ. Scale bar = 500 µm. (B) Limiting dilution assay was performed on BTSC73 and *LGALS1* KO BTSC73 with and without treatment with 10µM TMZ. DMSO was used as a vehicle control. All the counts were performed 7 days after plating. 200 cells (**$P_{\text{CTL vs. 10 µM TMZ + LGALS1 KO}} = 0.0084$), 100 cells (*$P_{\text{CTL vs. 10 µM TMZ + LGALS1 KO}} = 0.0165$), 50 cells (**$P_{\text{CTL vs. 10 µM TMZ + LGALS1 KO}} = 0.0046$), 25 cells (**$P_{\text{CTL vs. 10 µM TMZ + LGALS1 KO}} = 0.0013$, *$P_{\text{CTL vs. LGALS1 KO}} = 0.0215$), 12 cells (*$P_{\text{CTL vs. 10 µM TMZ + LGALS1 KO}} = 0.0348$), 6 cells (*$P_{\text{CTL vs. 10 µM TMZ + LGALS1 KO}} = 0.0441$). (C). The spheres were measured 7 days following 10µM TMZ treatment in *LGALS1* KO BTSC73 and BTSC73.

*P = 0.0204. Data are presented as the mean ± SEM and one-way ANOVA followed by Tukey's test for multiple comparisons.

C.5. Galectin1 is involved in the regulation of the mitotic spindle assembly and chromosome segregation.

From bioinformatics analysis, we found that recruitment of NuMA to mitotic centrosomes was another cell cycle-related pathway that was highly affected by the deletion of galectin1 (Figures 11 and 18).

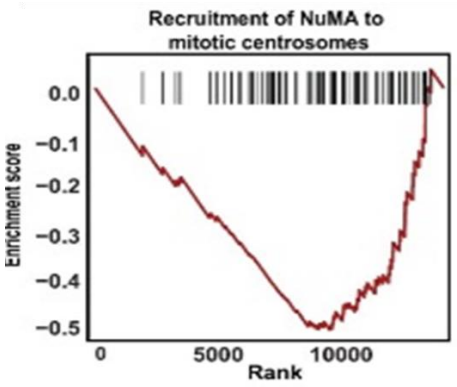


Figure 18. GSEA panel from RNA-Seq analysis (Figure 10) demonstrates that *LGALS1* KO BTSC73 are enriched for GO gene sets corresponding to recruitment of NuMA to mitotic centrosomes. Negative enrichment scores indicate enrichment with downregulated genes.

Therefore, I performed additional bioinformatics analysis using DAVID functional annotation and found a large panel of genes involved in mitotic spindle checkpoint and chromosome segregation that were deregulated upon deletion of LGALS1 (Table 7-8).

Table 7. Top downregulated genes in *LGALS1* KO BTSC73 that are involved in mitotic spindle regulations. (DAVID analysis).

Downregulated Genes:	**FC**	**p-value**
SPC24 (Spindle Pole Body Component 24 Homolog)	0.5	0.0000000000689
NDC80 (NDC80 Kinetochore Complex Component)	0.6	0.0000000000689
KLHL22 (Kelch Like Family Member 22)	0.6	0.00000503
NEDD9 (Neural Precursor Cell Expressed, Developmentally Down-Regulated 9)	0.5	0.0000000055
CENPM (Centromere Protein M)	0.5	0.00000195
MAD2L2 (Mitotic Arrest Deficient 2 Like 2)	0.6	0.0000009
NEK6 (NIMA Related Kinase 6)	0.7	0.00018496
CENPE (Centromere protein E)	0.8	0.01145296
EML1 (Echinoderm microtubule associated protein like 1)	0.4	0.00000000000000427
KIF18A (Kinesin Family Member 18A)	0.7	0.000270209
KIF20B (Kinesin Family Member 20B)	0.7	0.009912053
HAUS8 (HAUS augmin like complex subunit 8)	0.6	0.0000049

SKA1(Spindle and kinetochore associated complex subunit 1)	0.6	0.00000485

Table 8. Top upregulated genes in *LGALS1* KO BTSC73 that are involved in mitotic spindle regulations. (DAVID analysis).

Upregulated Genes:	**FC**	**p-value**
ARL8B (ADP ribosylation factor like GTPase 8B)	1.2	0.001396174
ERCC6L (ERCC excision repair 6 like, spindle assembly checkpoint helicase)	1.3	0.005136041
KLHL13 (Kelch like family member 13)	1.7	4.91E-18
ERCC6L (ERCC excision repair 6 like, spindle assembly checkpoint helicase)	1.3	0.005136041
EML4 (Echinoderm microtubule associated protein like 4)	1.2	0.001258329
KIF15 (Kinesin family member 15)	1.3	0.001207442
RCC2 (Regulator of chromosome condensation 2)	1.2	0.0000845
STAG2 (Stromal antigen 2)	1.7	0.000000000000124
TNKS (Tankyrase)	1.6	0.00000000000256

Next, I validated the RNA-Seq data by RT-qPCR and confirmed that deletion of *LGALS1* led to a significant downregulation of some of these genes such as MAD2L2, CENPM, KLHL22, NEDD9, and NDC80 in two different patient-derived BTSCs, BTSC73, and BTSC147 (Figures 19 and 20).

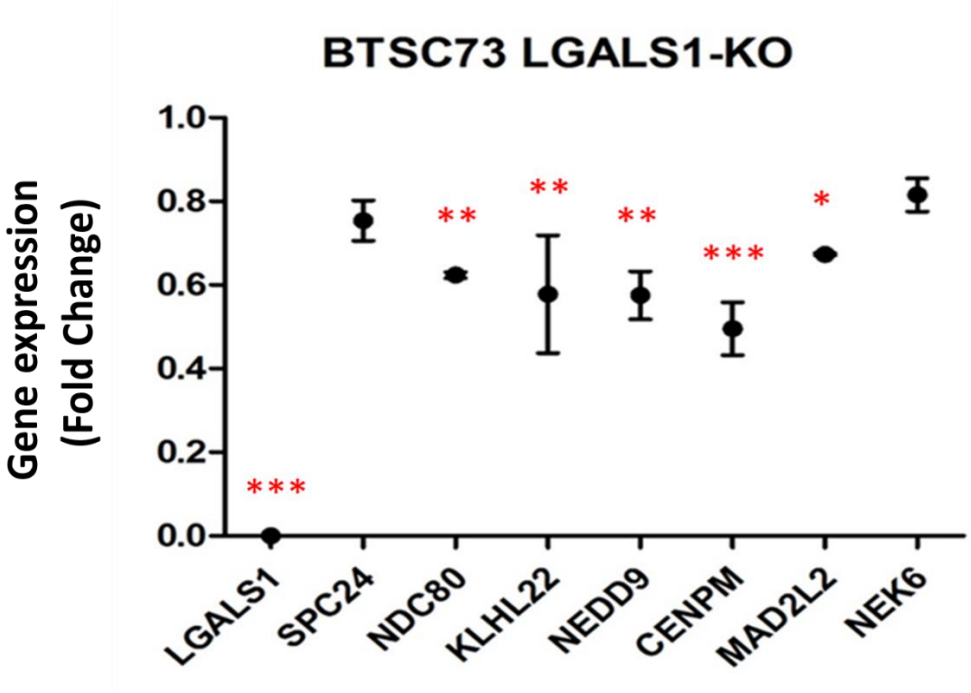


Figure 19. Validation of RNA-seq data by RT-qPCR. *LGALS1* KO is correlated with reduced expression of genes that are involved in the mitotic spindle regulations in BTSC73. In all samples, gene expression is normalized to the housekeeping gene GUSB. (n = 3 biological replicates). Two-tailed t-test. Gene expression results were analyzed relative to the levels found in CTL cells, set at 1. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with CTL cells.

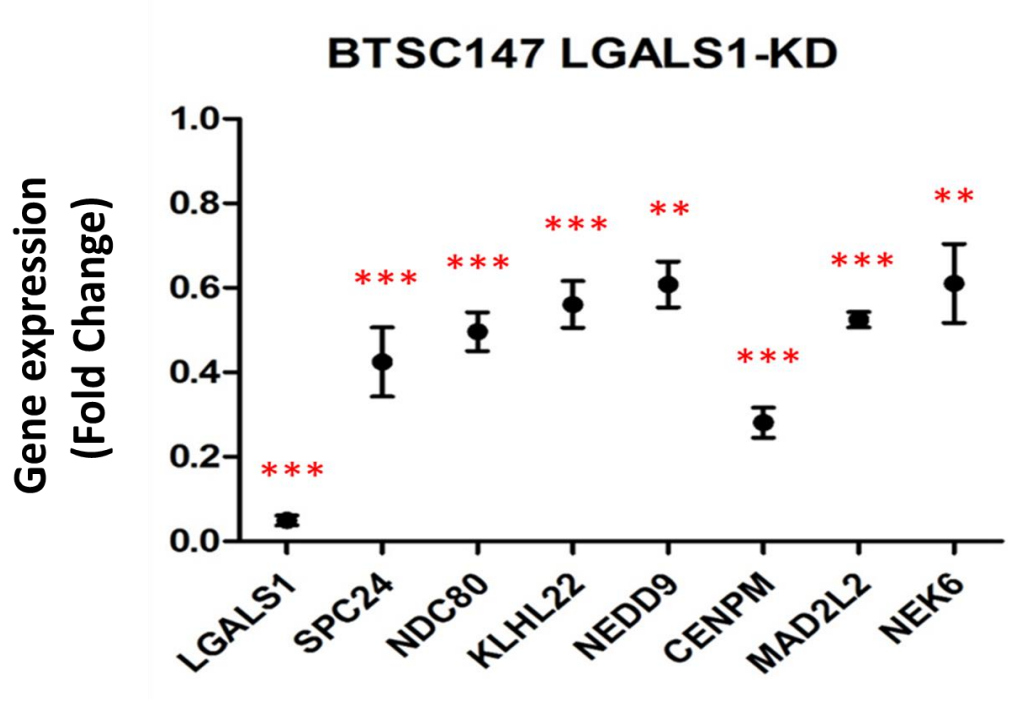


Figure 20. Validation of RNA-seq data by RT-qPCR. *LGALS1* KD is correlated with reduced expression of genes that are involved in the mitotic spindle regulation in BTSC147. In all samples, gene expression is normalized to the housekeeping gene GUSB. n = 3 biological replicates. Two-tailed t-test. Gene expression results were analyzed relative to the levels found in CTL cells, set at 1. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with CTL cells.

Deregulation of the genes in recruitment of NuMA to mitotic centrosomes pathway led to the hypothesis that galectin1 is involved in the modulation of the mitotic spindle assembly, chromosome segregation and/or cytokinesis. To address this hypothesis, I first asked if galectin1 is localized with mitotic spindle components. I performed IF staining using antibodies to galectin1 and α-tubulin, which is a mitotic spindle component, in BTSC73. Our results showed that galectin1 is partially colocalized with α-tubulin (Figure

21). Since galectin1 is expressed in all regions inside the cell, it is difficult to conclusively state whether galectin1 is colocalizing and interacting with the components of the mitotic spindle. Therefore, in future studies we propose to perform a proximity ligation assay (PLA) to confirm that galectin1 interacts with mitotic spindle components such as α-tubulin and pericentrin.

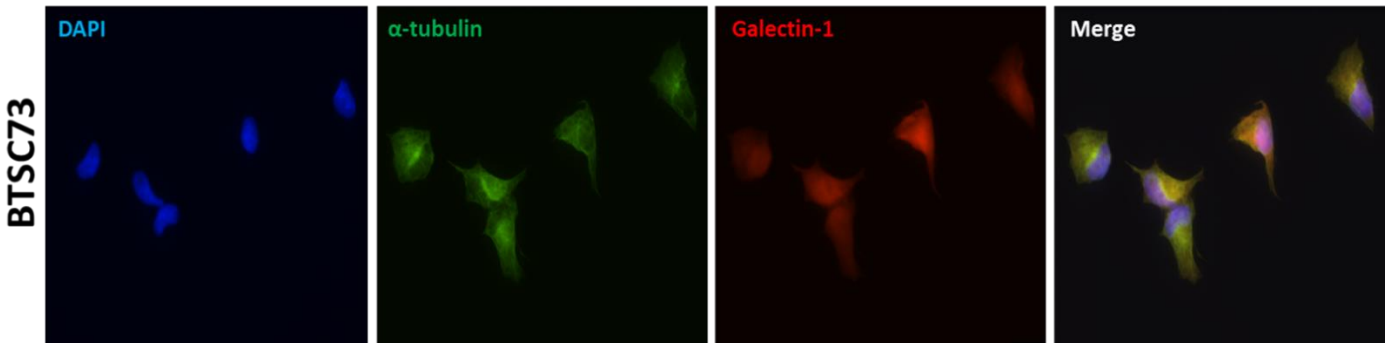


Figure 21. Galectin1 partial colocalization with α-tubulin. Co-immunofluorescence staining of galectin1 with α-tubulin (a mitotic spindle component) in BTSC73. Colocalization of galectin1 with α-tubulin can be observed. Scale bar = 50 µm.

Furthermore, in follow up studies we found a list of galectin1 target genes that are involved in the multinucleation and cytokinesis deficiency processes by analyzing RNA-seq data using DAVID functional annotation (Tables 9 and 10). These results support our hypothesis that galectin1 interacts with mitotic spindle proteins and plays a critical role in appropriate chromosome segregation and cytokinesis.

Table 9. Top downregulated genes in *LGALS1* KO BTSC73 that are involved in the multinucleation. (DAVID analysis).

Downregulated Genes:	**FC**	**p-value**
ANLN (Anillin actin binding protein)	0.7	0.001969788
CIT (Citron Rho-Interacting Serine/Threonine Kinase)	0.8	0.015556876
KIF20B (Kinesin Family Member 20B)	0.7	0.009912053
RNF8 (Ring Finger Protein 8)	0.7	0.000774038
CALM1 (Calmodulin 1)	0.8	0.003087402
CD2AP (CD2 associated protein)	0.6	0.000000786
CEP55 (Centrosomal protein 55)	0.6	0.000000000153

Table 10. Top upregulated genes in *LGALS1* KO BTSC73 that are involved in the multinucleation. (DAVID analysis).

Upregulated Genes:	**FC**	**p-value**
CENPV (Centromere protein V)	1.6	0.0000000000913
BIN3 (Bridging integrator 3)	1.6	0.004178021
KLHL13 (Kelch like family member 13)	1.7	4.91E-18

RCC2 (Regulator of chromosome condensation 2)	1.2	0.0000845
TTC28 (Tetratricopeptide repeat domain 28)	1.5	0.00000417

C.6. Performing Immunoprecipitation-Mass Spectrometry (IP-MS) to dissociate the mechanisms by which galectin1 regulates cell cycle, proliferation, and mitotic spindle checkpoints.

To investigate the underlying molecular mechanisms, downstream pathways, and potential binding partners of galectin1, we decided to perform IP-MS. IP-MS is a common and highly effective technique for identifying binding partners to a target protein. Finding galectin1 interactions and binding partners can assist us to better understand and characterize the function of galectin1 in cell cycle and spindle checkpoint signalling. Prior to performing IP-MS, we decided to validate the potential antibodies that can pull down galectin1 and its binding partners by using IP-WB. We used three different antibodies against galectin1((Abcam, ab108389, rabbit), (Cell Signaling Technology, #12936, rabbit) and (Cell Signaling Technology #40103, mouse)).

Next, we evaluated the presence of positive control proteins (H-Ras and HOXA5) using Western blot, but we did not observe either a galectin1 band or the presence of the positive controls after pulldown (Figure 22). Therefore, none of the commercially available antibodies that we used in this study as positive controls worked for the IP-WB. Due to limitation in finding a functional antibody, future studies should focus on generating a tagged galectin1 for identification of galectin1 binding partners.

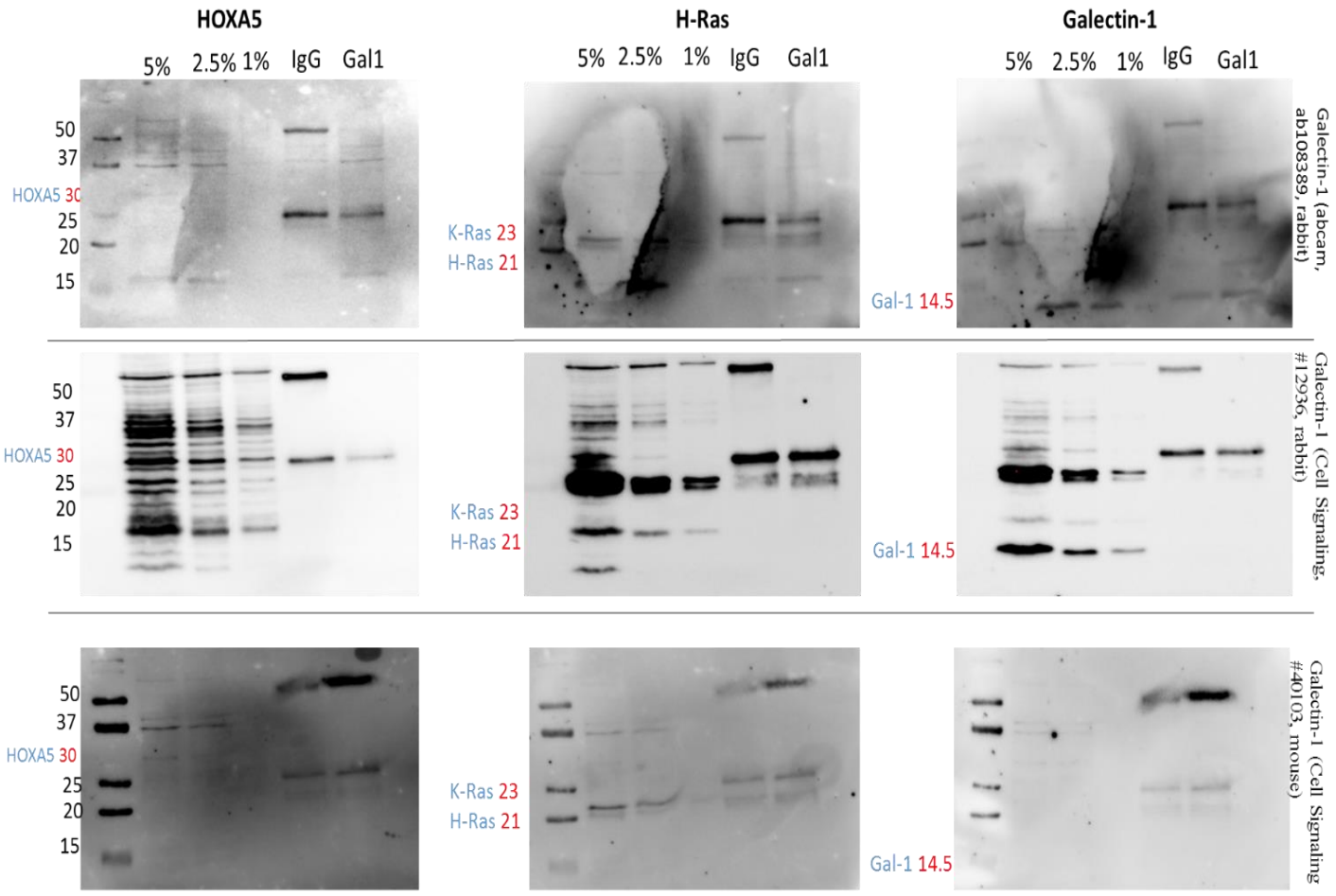


Figure 22. Immunoprecipitation (IP)/Western blot analysis of galectin1 binding proteins in BTSC73. Protein lysates of BTSC73 were subjected to IP using three different antibodies against galectin1 or IgG (negative control). 5%, 2.5%, and 1% of the cell lysates were used for the input. Western blots were probed with the galectin1, H-RAS, and HOXA5 antibodies.

D. Discussion & Future Directions

Glioblastoma is the most prevalent and aggressive primary brain tumour in adults. These tumours are composed of cells with variable differentiation and contain a rare population of self-renewing brain tumour stem cells (BTSCs) (1, 20). BTSCs evade current treatments of ionizing radiation (IR) and chemotherapy with Temozolomide (21, 22). However, the molecular mechanisms that make BTSCs resistant to therapy are not entirely clear. Glioblastoma resistance to therapy leaves the patients with a low median survival rate of 16-18 months following diagnosis (1, 7, 22). Given that BTSCs are at the core of therapeutic resistance, targeting of BTSCs is at the forefront of efforts in neuro-oncology and seems to be a promising approach for developing effective treatments.

Over the past few years, it has become clear that galectins, in particular galectin1, are involved in several aspects of cancer biology (25). Moreover, galectin1 upregulation has been reported in a wide range of cancer cell types and its upregulation is associated with poor prognosis in glioblastoma patients (29, 30). A major oncogenic protein in GB is the mutated active EGFR, termed EGFRvIII. Previous analyses by our laboratory revealed that *LGALS1* mRNA expression was highly deregulated in an EGFRvIII-dependent manner in patient-derived BTSCs (101).

In the present study, galectin1-regulated candidate target genes were profiled via performing mRNA sequencing on patient-derived BTSC73 and *LGALS1* KO BTSC73. Analysis of this dataset revealed deregulation of multiple signalling pathways including a large panel of candidate target genes belonging to G2/M transition and mitotic G2-G2/M phases which were significantly downregulated in response to *LGALS1* deletion.

Furthermore, DAVID functional annotation analysis also led to the identification of a wide range of downregulated candidate target genes involved in the modulation of cell cycle and proliferation. This panel of genes included E2F7, GSPT2, MCM5, PIM2, HIRA, SMARCA4, GTSE1, SKP2, CCND3, CDKN2D, and CDK6. Together, these data raised the hypothesis that *LGALS1* regulates cell cycle and proliferation. In further support of this hypothesis, we employed RT-qPCR and confirmed that deletion of *LGALS1* significantly downregulated the expression of some of these genes such as E2F7, GSPT2, GTSE1, MCM5, PIM2, and CDK6 in two different patient-derived BTSCs, BTSC73, and BTSC147.

Having confirmed the RNA-Seq data, we next examined if *LGALS1* regulates the proliferation of BTSCs. In order to examine the role of *LGALS1* in proliferation, we performed immunofluorescent (IF) staining of two proliferation markers, Ki67 and phospho-Histone H3 (PH3). Ki67 is a known marker of proliferation which is expressed during all active phases of the cell cycle G1, S, G2, and M, but is absent in G0 (103). Moreover, PH3 is another marker of proliferation which is mostly expressed in the late G2 and during the mitosis phase, and marks nuclei containing condensed chromatin (a mitotic marker) (104). Our results from Ki67 and PH3 immunostaining analysis revealed a significant reduction in cell proliferation in both BTSC73 and 147 upon deletion of *LGALS1*. Masamune et al., 2006, reported that galectin1 enhances proliferation of pancreatic stellate cells through regulation of NF- κB, JNK, and ERK pathways, and its effects can be blocked by treatment with thiodigalactoside which is an inhibitor of β-galactoside binding (105). Zhang et al., 2014, found that overexpression of galectin1 significantly promotes the proliferation of the epithelial ovarian cancer cells. They also observed that downregulation of galectin1 using siRNA significantly reduced the proliferation, cell growth, and invasion of these cells. They hypothesized that these significant

effects could be a result of galectin1 and H-Ras interaction leading to the activation of the ERK pathway (106). Whether galectin1 regulates cell cycle events and cell proliferation in patient derived BTSCs via converging on ERK pathway remains to be investigated in future studies.

While BTSCs are capable of sustained self-renewal and persistent proliferation, BTSCs are shown to evade DNA damaging chemotherapy in glioblastoma tumours (19-22). Moreover, it has been reported that galectin1 plays a role in the chemoresistance of several types of cancers (93, 98). We, therefore, sought to examine the role of galectin1 in BTSC resistance to therapy. We performed limiting dilution assays in *LGALS1* KO BTSC73 and BTSC73 in the absence and presence of the chemotherapeutic agent Temozolomide (TMZ). Our results showed that deletion of LGALS1 significantly sensitized the response of BTSCs to TMZ treatment and led to a significant decrease in the number and average size of spheres in BTSC73.Therefore, these data suggest that galectin1 can modulate the chemosensitivity of BTSCs and can partially protect BTSCs against chemotherapy. Moreover, our results are consistent with findings in ovarian cancer in which galectin1 conferred resistance of epithelial ovarian cancer to the chemotherapeutic agent Cisplatin. Downregulation of galectin1 using a pool of siRNA sensitized the response of ovarian cancer cells to Cisplatin and induced apoptosis in the A2780/CP which is a Cisplatin-resistant cell line (106). Wang et al., 2017, found that downregulation of galectin1 also significantly promoted drug sensitivity of breast cancer cells to chemotherapeutic agents such as Paclitaxel (PTX) and Adriamycin (ADR). In this study, an increase in drug sensitivity could be modulated through downregulation of P-glycoprotein (P-gp) expression via inhibiting the Raf-1/AP-1 pathway (107). These studies, together with our findings in BTSCs, support a model whereby a combination of a drug that

targets galectin1 and Temozolomide treatment could be a promising avenue for overcoming therapeutic resistance in glioblastoma patients.

Another cell cycle-related pathway that was found to be significantly affected by the deletion of galectin1 in our studies was the recruitment of NuMA to mitotic centrosomes, using Reactome pathways and GSEA analysis. Nuclear mitotic apparatus protein (NuMA) is a mitotic centrosomal component that plays a crucial role in the stabilization and organization of spindle poles during mitosis (108). Further DAVID analysis showed downregulation of galectin1 target genes involved in the mitotic spindle checkpoint and chromosome segregation including, MAD2L2, CENPM, KLHL22, NEDD9, and NDC80 which were then validated by RT-qPCR in both BTSC73 and 147. In view of these findings, we asked whether galectin1 is involved in the modulation of the mitotic spindle assembly, chromosome segregation and/or cytokinesis. First, we evaluated the colocalization of galectin1 with a mitotic spindle component, α-tubulin, by performing immunofluorescent (IF) staining. Our results indicated that galectin1 is partially colocalized with α-tubulin. Altogether, these data suggest a model whereby galectin1 may regulate the mitotic spindle assembly, chromosome segregation and/or cytokinesis, future experiments using proximity ligation assay (PLA) and Co-immunoprecipitation (co-IP) of galectin1 with α-tubulin is required to validate this model.

Interestingly, by further analysis of RNA-seq data using DAVID functional annotation, we also found galectin1 target genes that are involved in the regulation of multinucleation and cytokinesis deficiency processes. These genes were ANLN, CIT, KIF20B, RNF8, CALM1, CD2AP, CEP55, CENPV, BIN3, KLHL13, RCC2, and TTC28. Suzuki et al., 2005, showed that downregulation of *ANLN* via small interfering RNA led to the appearance of multiple nuclei morphology in non–small cell lung cancers (NSCLC) which subsequently resulted in

cell death (109). Plans et al., 2008, indicated that *RNF8* is a nuclear protein that can modulate mitotic processes, such as the exit rate of cells from mitosis and cytokinesis through its ubiquitin ligase activity. They observed that deregulation of *RNF8* induced unresolved cytokinesis and aberrant mitotic figures, such as formation of multinucleated cells, and multiple mitotic spindles (110). Citron Rho-interacting serine/threonine kinase (*CIT*) is a component of the midbody in dividing cells and is required for cytokinesis (111). Moreover, elevated expression of this gene has been reported in some cancers (111). Sahin et al., 2019, identified that silencing of *CIT* using shRNA in multiple myeloma cell lines caused cytokinesis failure and multinucleation which led to significant reduction of cell proliferation in these cells (111). Furthermore, centrosomal protein 55 (CEP55) which is a microtubule-bundling protein and recruited to the midbody during cytokinesis, is necessary for the completion of cell division (112). Studies showed that either downregulation or upregulation of this gene induced cytokinesis failure and as a result increased the number of multinucleated cells (113, 114). These findings suggest a model whereby galectin1 could interact with mitotic spindle proteins and play a role in appropriate chromosome segregation and cytokinesis.

Finally, we set out to investigate how galectin1 functions to regulate cell cycle, proliferation, and resistance to chemotherapy in human BTSCs. To this end, we aimed to map galectin1 proteome and characterize its binding partner using Immunoprecipitation-Mass Spectrometry (IP-MS), endogenously. IP-MS is a common, highly accurate, and sensitive method for characterizing protein complexes and binding partners to a target protein (115, 116). Identification and mapping of galectin1 complexes help in explaining the underlying molecular mechanisms of galectin1 function.

Several galectin1 antibodies are highlighted for experimental procedures that involve IP. We, thus, tried to validate these potential antibodies in order to pull down galectin1 and its binding partners endogenously. In preliminary analysis, three different antibodies against galectin1 (Abcam, ab108389, rabbit), (Cell Signaling Technology, #12936, rabbit) and (Cell Signaling Technology #40103, mouse) were examined. Paz et al., 2001, employed Co-immunoprecipitation and Western blotting and identified that galectin1 can bind to oncogenic H-Ras in order to modulate cell transformation and Ras membrane anchorage (117). Similarly, Chung et al., 2012, reported the interaction of galectin1 and Ras proteins by performing Immunoprecipitation and Western blotting (118). Additionally, unpublished data from our lab found the interaction of galectin1 and HOXA5 in BTSCs by pulling down HOXA5 and immunoblotting galectin1 using Immunoprecipitation and Western blotting. Therefore, we decided to use H-Ras and HOXA5 as positive controls for our IP-WB. Our attempts to validate the potential galectin1 antibodies by observing either the galectin1 band or the presence of the positive controls after pulldown were not successful, suggesting that endogenous IP-MS was not a viable option due to lack of an effective antibody. Future directions of this research should consider exogenous IP with tagged protein or BioID techniques in order to map galectin1 proteomic network. BioID is our recommended approach to map galectin1 interactomes in BTSCs.

BioID (proximity-dependent biotin identification) is an efficient method to screen for candidate protein interactions in living cells. The high sensitivity of this method allows for the identification of weak and transient interactions even in insoluble and membrane-associated proteins in a wide variety of cell types and species. This technique utilizes a promiscuous biotin ligase which is fused to the protein of interest. Therefore, when this fused ligase is expressed

in cells, it can biotinylate proximal and interacting proteins which provides a history of protein interactions. These biotinylated interacting proteins can be purified and isolated by standard biotin-affinity capture and then identified with mass spectrometry. This technique has become an increasingly utilized tool to map a wide range of interactomes in living cells (119-121). Therefore, future studies with BioID can assist us to identify galectin1 interactions, and to better understand the exact molecular mechanism by which galectin1 can function in cell cycle, proliferation, spindle checkpoint signalling, and resistance to chemotherapy in human BTSCs.

Conclusion and summary:

The data that I have presented in this study suggest that galectin1 regulates brain tumour stem cells by modulating cell cycle, proliferation, and mitotic spindle assembly. The bioinformatics analysis of the mRNA-Seq data revealed a large panel of galectin1 target genes which are involved in the regulation of cell cycle, cell division, mitotic spindle assembly and chromosome segregation. These data were further confirmed by RT-qPCR of galectin1 target genes and IF staining of proliferation markers including PH3 and KI67 in two different patient-derived BTSCs, BTSC73, and BTSC147. Furthermore, I have found that deletion of galectin1 significantly sensitized the response of BTSCs to TMZ treatment, which suggests the role of galectin1 in regulating the chemosensitivity of BTSCs. Our findings in this study support a model whereby a combination of a drug targeting galectin1 and Temozolomide treatment could be a promising avenue for overcoming therapeutic resistance in glioblastoma patients. However, further studies are required to characterize galectin1 and its binding partners in BTSCs to explain the underlying molecular mechanisms of galectin1's role in cancer stem cell biology.

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