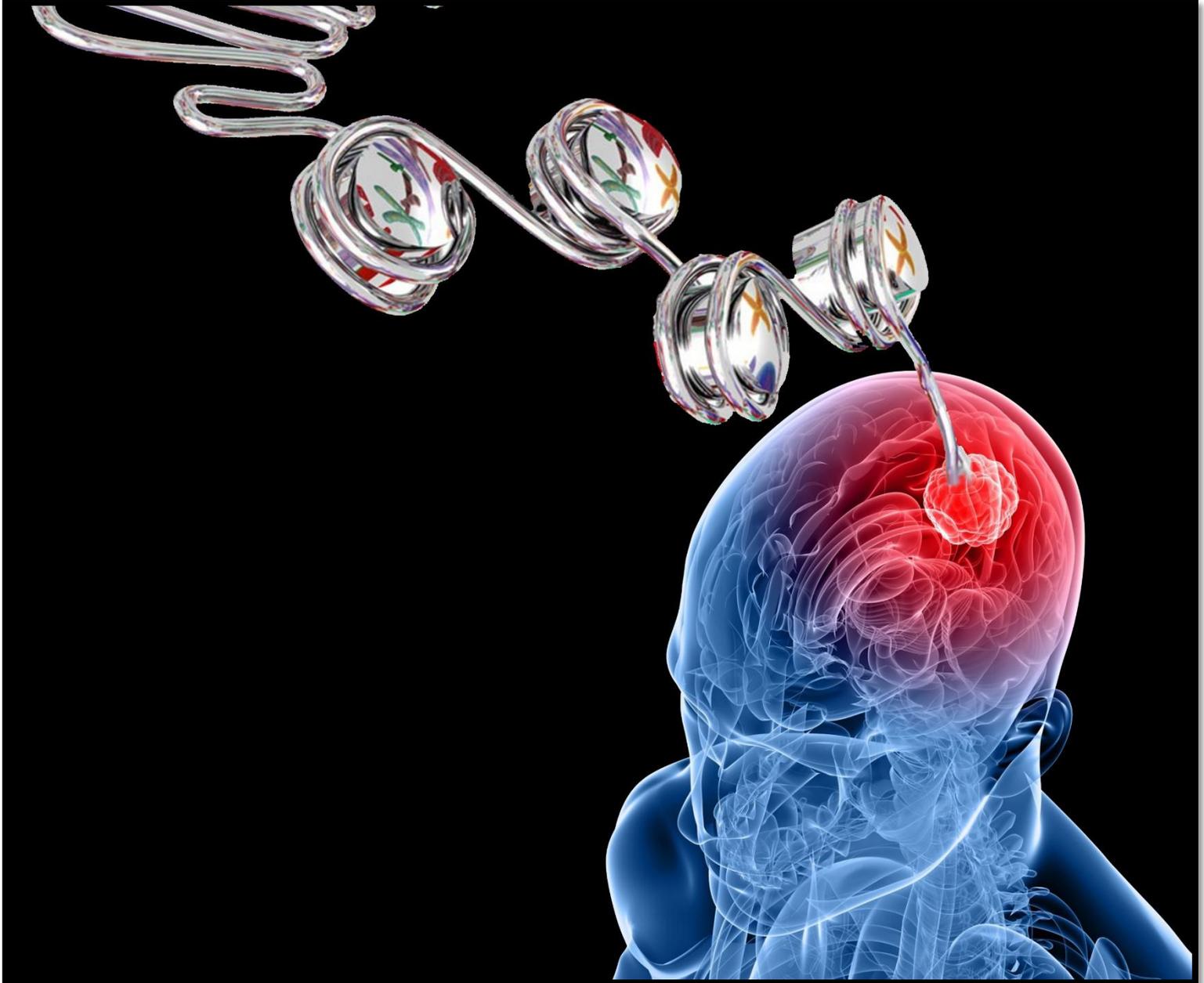




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The role of Polycomb groups proteins in Glioblastoma Multiforme



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Abstract

Glioblastoma multiforme (GBM) is the most malignant and common brain tumor in adult patients. GBM is characterised by intra and inter tumor heterogeneity thus making it very difficult to treat. The subpopulation of chemoresistant GBM cancer stem cells (GSCs) results in a high recurrence rate and poor prognosis despite decades of research and advances in treatment. Current treatment methods have reached its clinical and the request for a more personalised approach with targeted therapy arises. Polycomb group (PcG) proteins, are interesting candidates as novel therapeutic, prognostic and predictive markers in GBM. PcGs form a vital link between the epigenetic plasticity of GBM and the biology of cancer stem cells (CSCs), being essential epigenetic regulators. PcG proteins EZH2, BMI1 and CBX7 have been shown to possess oncogenic or tumor suppressor functions in different tumors including GBM. CBX7, a member of Polycomb Repressive Complex 1 (PRC1), is a rather new topic of interest in GBM research and is implicated to play a role in GBM progression and GSCs biology. The *CBX7* gene is downregulated in more than 80% of all GBM patients due to promoter hypermethylation. Loss of CBX7 results in disrupted regulatory control of among others; E-cadherin, cyclin E, osteopontin and YAP/TAZ transcriptional targets like CTGF. This all contributes to the aggressive and invasive nature of GBM. Recent studies suggest a potential role for CBX7 in the maintenance and renewal of GSCs via INK4a and ARF. It has already been established that BMI1 the CSCs self-renewal properties can largely be explained through its effects on INK4a/ARF. The influence of CBX7 on INK4a/ARF is very similar to the influence of BMI1 thus suggesting it might be very interesting to pursue CBX7 as possible regulator of GSCs via its proposed effects on INK4a and ARF. To date, not much is known about how CBX7 expression and function is regulated but a few studies from the last couple of years, concerning microRNAs (miRNAs), have shown remarkable mechanisms by which for example miR-9 and miR-18a are able to control CBX7 protein levels. MiRNAs might even hold therapeutic potential in the treatment of GBM by targeting CBX7. Currently, a number of strategies are being investigated where, via the use of Gold Nanoparticles (GNPs), miRNAs are able to target specific CSCs within brain tumors. More research is needed to determine the exact role of CBX7 in GBM, its influence on GSCs and its interaction with miRNAs. Given time, miRNAs which target PcG proteins might present a valid personalised treatment option for GBM patients.

Introduction

Glioblastoma Multiforme accounts for 46,6% of all malignant central nervous system (CNS) tumors and is therefore the most common of all malignant CNS tumors in adults. GBM is a highly aggressive and invasive brain tumor associated with one of the poorest 5-year survival rates among all human cancers. (Lee, Ryu, Won, & Kwon, 2015) (Krex et al., 2007) Most patients suffering from GBM, especially the elderly, have an overall survival of 12 months and even in the most favourable cases, most patients die within 24 months after diagnosis. (Stupp et al., 2005) (Louis et al., 2007) The current standard treatment for GBM is an aggressive combination therapy where maximal safe surgical resection is followed by extensive concurrent chemoradiation (CTRT) with Temozolomide. This radical first-line treatment is followed by 6 more months of chemotherapy. (Stupp et al., 2005) (Cihoric et al., 2017) Unfortunately, conventional chemotherapy in GBM has its limits due to poor blood-brain barrier (BBB) penetration (Lee et al., 2015) Despite improvements in intensive multimodal treatment strategies and diagnostics over the past decades, prognosis improvement is slim, treatment possibilities are very limited and there is a high change of recurrence. (Bhargava, Patil, Mahalingam, & Somasundaram, 2016)

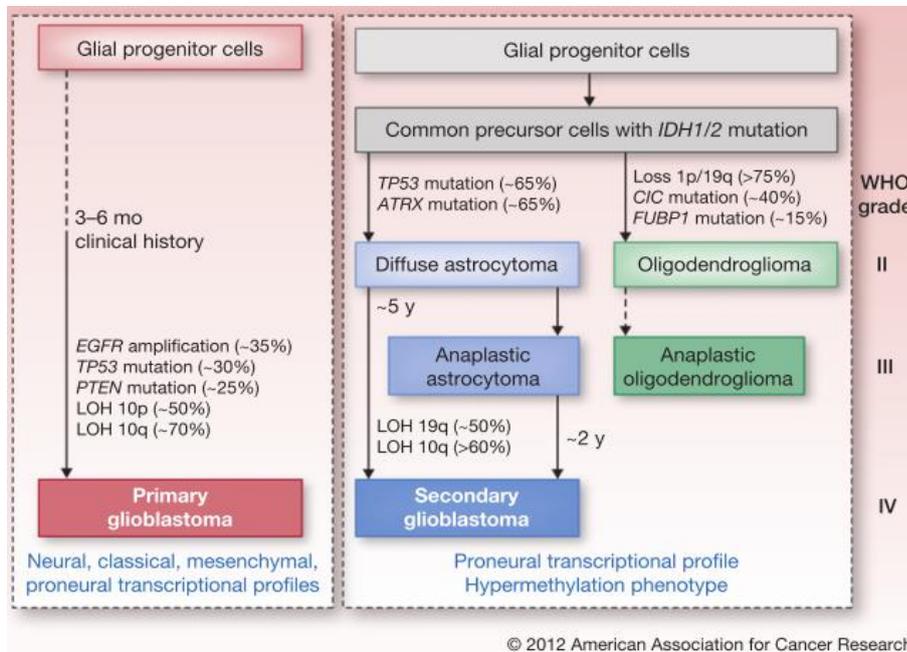
During the past decade, vast improvements has been made in uncovering the genetics and epigenetics of GBM. Especially epigenetic abnormalities need to be understood more extensively as epigenetic modifications are very common in GBM. (Nawaz et al., 2016) The complexity of GBM, due to heterogeneity between patients and within tumors make an overall applicable and effective treatment method implausible. Despite new techniques the clinical limits in terms of efficacy and side effects have been reached. Where tumorigenesis and chemo resistance are promoted by multiple mechanisms and factors, a more classified and targeted approach might prove to be more effective. (Polivka et al., 2017) The request arises for classification of GBM patients based on genetic, epigenetic and translational profiles. This profiling data could be beneficial for early detection of GBM, predicting patient outcome, the elucidation of predictive biomarkers and novel targeted therapeutic strategies. (Lee et al., 2015) (Nawaz et al., 2016)

Cancer stem cells (CSCs) arise to be exciting new therapy targets because of their cellular properties that lend the tumor its ability to overcome current treatment with chemoradiation. (Tannishtha, Morrison, Clarke, & Weissman, 2001) CSCs are believed to be of significant influence on high recurrence rates in GBM patients. Conventional therapies mostly target the cells with limited proliferative potential. (Howard et al., 2017) CSCs fall through the cracks and allow them to remain viable after therapy and regenerate the tumor. Targeting the CSCs would unable the tumor to maintain its structure and growth. (Chen et al., 2012) CSC-directed treatment methods might prove more effective than conventional methods, it could reduce recurrence and possibly even improve overall survival. (Sundar, Hsieh, Manjila, Lathia, & Sloan, 2014)

Due to their role in CSC biology and tumor progression, polycomb group (PcG) proteins are obvious candidates as novel therapeutic, prognostic and predictive markers in GBM. (Crea, Paolicchi, Marquez, & Danesi, 2012) The aim of this thesis is to elaborate on the complexity of GBM and show the exciting progress of elucidating potential new targets within PcGs as diagnostic, prognostic and predictive biomarkers of GBM. Moreover, this thesis will touch upon the subject of potential therapeutic implications for clinical practice in GBM. The main question being: How are polycomb group proteins involved in the progression and maintenance of glioblastoma multiforme?

Chapter 1 Glioblastoma Multiforme

GBM amounts to 15 % of all primary CNS tumors and is part of a specific class of primary CNS tumors; Gliomas. Gliomas form the largest group of primary CNS tumors. Gliomas arise from glial cells such as astrocytes, oligodendrocytes, and ependymal cells. (Ostrom et al., 2016) (Ostrom et al., 2013) GBM is a World Health Organisation (WHO) grade IV glioma, the highest grade glioma. The different WHO grades are determined by histopathological examination. A few examples of classifying characteristics are: nuclear atypia, mitotic activity, cellular pleomorphism, vascular thrombosis, microvascular proliferation and necrosis. (Szopa, Burley, Kramer-Marek, & Kaspera, 2017) GBM can be divided into primary and secondary GBM. Most of GBM cases, 90%, are considered primary GBMs because of the specific ‘de novo’ development from glial cells by multistep tumorigenesis. (Fig.1) Primary GBM arises mostly in elderly patients whereas secondary GBMs are most found in younger patients (< 45 years). Secondary GBMs develop from a pre-existing glioma of WHO grade II or III. (Lee et al., 2015)(Urbanska, Sokolowska, Szmidt, & Sysa, 2014).



Secondary GBMs typically show a lower degree of necrosis, are mostly located in the frontal lobe, and have a better prognosis compared to primary GBMs. Primary and secondary GBM are histologically almost indistinguishable but their genetic and epigenetic profiles are very different.

Where secondary GBM display IDH1 mutations, those mutations are mostly absent in primary GBM. These kind of variations in

Figure 1. The genetic pathways forming primary and secondary GBM. Major mutations contributing to tumorigenesis are also displayed. Figure from (Ohgaki & Kleihues, 2013)

profiles make for diagnostic markers to distinguish secondary GBM from primary GBM. (Ohgaki & Kleihues, 2013)

Molecular signature; genetic aberrations

To date, histopathological analysis of GBMs form the basis for diagnosis and the course of treatment. Classification and sub-classification of GBMs has proven to be a challenge. Recent implementation of sequencing and profiling technologies provided a more complete analysis of the molecular abnormalities which underlie gliomagenesis. (Li et al., 2013) This analysis also confirms the highly anaplastic nature and the morphological heterogeneity of GBM. (Zhang, Zhang, Cao, Cheng, & Zhang, 2012) The two clinical forms of GBM both have their own distinct molecular signature. To give a few

examples; primary and secondary GBMs often present amplification/mutations in a distinct set of genes. (Szopa et al., 2017) Table 1 displays genetic aberrations which often occur in GBM.

Genetic aberrations	Occurrence Primary GBM	Occurrence Secondary GBM
Amplification/mutation of the <i>EGFR</i> gene	36-60%	8%
<i>PTEN</i> mutation	25%	4%
<i>CDKN2A</i> -p16INK4a deletion	31-78%	8%
<i>TP53</i> mutations	28%	65%
<i>MGMT</i> promoter methylation	36%	75%
<i>IDH1</i> mutations	5%	75%

Table 1: Examples of the occurrence of genetic irregularities in primary versus secondary GBM
Note: data from (Szopa et al., 2017)

Intra-tumoral heterogeneity

Recent studies, analysed expression signatures of GBM single cells which displayed an intra-tumoral heterogeneity of expression subclasses inside a single tumor. GBM can be divided into 4 different molecular subgroups, the Verhaak Classification groups, which are based on the different expression profiles looking at signature genes within each group. Verhaak et al. used The Cancer Genome Atlas (TCGA) data to correlate gene expression-based GBM subtypes with alterations in DNA sequences and copy numbers. They established a classification of 4 subgroups of GBM; Proneural, Neural, Classical, and Mesenchymal.

Gene	Proneural (n = 37)	Neural (n = 19)	Classical (n = 22)	Mesenchymal (n = 38)	Total No. of Mutations
<i>TP53</i>	20 (54%)	4 (21%)	0 (0%)	12 (32%)	36
<i>PTEN</i>	6 (16%)	4 (21%)	5 (23%)	12 (32%)	27
<i>NF1</i>	2 (5%)	3 (16%)	1 (5%)	14 (37%)	20
<i>EGFR</i>	6 (16%)	5 (26%)	7 (32%)	2 (5%)	20
<i>IDH1</i>	11 (30%)^a	1 (5%)	0 (0%)	0 (0%)	12
<i>PIK3R1</i>	7 (19%)	2 (11%)	1 (5%)	0 (0%)	10
<i>RB1</i>	1 (3%)	1 (5%)	0 (0%)	5 (13%)	7
<i>ERBB2</i>	2 (5%)	3 (16%)	1 (5%)	1 (3%)	7
<i>EGFRvIII</i>	1 (3%)	0 (0%)	5 (23%)	1 (3%)	7
<i>PIK3CA</i>	3 (8%)	1 (5%)	1 (5%)	1 (3%)	6
<i>PDGFRA</i>	4 (11%)	0 (0%)	0 (0%)	0 (0%)	4

Table 2: Distribution of Frequently Mutated Genes across GBM Subtypes. Significance of the difference in number of events between subtypes and remainder of the subtypes was determined using a two-sided Fisher's exact test, corrected for multiple testing using a Familywise Error Rate. Bold type indicates *p* values significant. ^a = *p* value significant at 0.01 level. Table and legend from (Verhaak et al., 2010)

This classification might have prognostic and predictive relevance since every subgroup has a characteristic copy number profile, a different response to therapy and varies in survival. These differences should be taken into account in patient-specific therapy design. (Verhaak et al., 2010) Sottoriva et al. showed that 6 out of 10 cases, fragments from the tumor classified into at least 2 GBM subgroups. (Sottoriva et al., 2013)

In addition, GBM is thought to contain a small population of self-renewing CSCs. These GBM cancer stem cells (GSCs) are able to self-renew and can differentiate into multiple phenotypes; neuronal, macroglial, and mixed neuronal/astroglial. (Farias-Eisner et al., 2012) Both intratumoral heterogeneity and the existence of these CSCs may explain the complications in validating oncological biomarkers. This may even contribute to incorrect patient selection for targeted therapy, treatment failure, drug resistance and the high recurrence rate of GBM. (Sottoriva et al., 2013) (Sundar et al., 2014)

Cancer stem cell hypothesis

The first evidence for CSCs originated in 1994 and came from an observation in acute myeloid leukaemia. Over the years the evidence for CSCs accumulated and was found in a variety of solid tumours (human and mouse studies). The evidence was provided through transplantation studies using prospectively isolated tumour cells. For example, Xu et al. has shown that 4,1% of a GBM cell population used in their studies accounted for CSCs using a Hoechst 33342 dye exclusion assay. The CSC hypothesis (fig.2) suggests that a population of stem-like cells exists within a tumor which is responsible for tumor initiation, propagation and maintenance. (Visvader & Lindeman, 2008) (Sundar et al., 2014) (Xu, Zhang, Tian, & Wu, 2013)

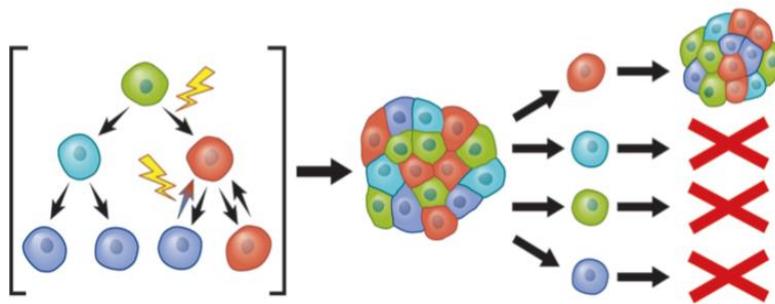


Figure 2. The CSC model, a small population of CSCs; capable of self-renewal. This population is in control of tumor initiation, propagation and maintenance. Figure from (Sundar et al., 2014)

The CSCs origin is still being questioned. The similarity in gene-expression profiles comparing GSCs and Neural stem cells (NSCs) suggests the concept that CSCs might be malignant variants of NSCs. (Jackson & Alvarez-Buylla, 2008) Next to being originated from progenitor NSCs, evidence also suggests CSCs acquired their stem-like properties

developing from more differentiated cells in the lineage that dedifferentiated. (Visvader & Lindeman, 2008) Subsequently the stochastic model proposes that tumor growth is determined by the clonal evolution of accumulated genetic mutations and that many clones possess comparable levels of tumorigenicity. These 2 models however are not mutually exclusive, instead they should be seen as a complementary drive of tumorigenesis. (Tannishtha et al., 2001)(Sundar et al., 2014)

Chapter 2 GBM cancer stem cells and polycomb proteins

Biomarkers of GSCs

Targeting GSCs is extensively complicated and years of studies haven't been able to single out a marker which definitively identifies and/or defines GSCs. Several biomarkers concerning GBM have been studied over the years. CD133 is the best-studied GSC biomarker. In addition, CD15 was also convincingly shown in various GSCs to be a marker of tumor initiation. Regardless of compelling evidence outlining the crucial correlation with GSCs, CD133 is not a universal biomarker for the identification of GSCs. Even CD133 and/or CD15 negative GSCs are capable of establishing a tumor and even some GBMs do not contain a single CD133 or CD15 positive GSC. (Wang et al., 2008)(Yan et al., 2011)(Sundar et al., 2014) (Nakano, 2015)

The search for new therapeutic, prognostic and predictive markers in GBM continues and a large amount of pre-clinical studies suggest PcGs to be exciting candidates with high potential. PcGs are found to play a critical role in the self-renewal, metastasizing and therapy resistance characteristics of CSCs. (Richly, Aloia, & Di Croce, 2011) PcGs form a vital link between epigenetic plasticity and the biology of CSCs, being essential epigenetic regulators (Crea et al., 2012)

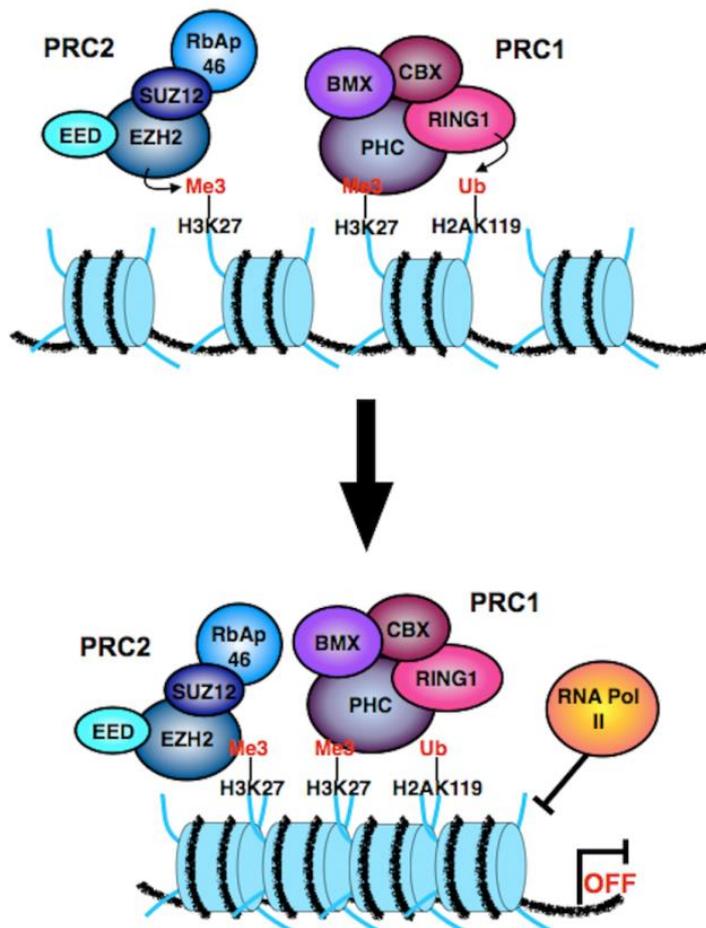


Figure 3: Epigenetic gene silencing via polycomb protein complexes. PRC2, as initiation complex, induces EZH2-mediated H3K27me3. H3K27me3 recruits the PRC1 complex which ubiquitinates H2AK119 resulting in chromatin compaction and gene silencing. Figure from (Marchesi & Bagell, 2013)

Polycomb group proteins

Epigenetic alterations mainly comprise of chemical marks on DNA and histones that jointly determine whether a gene is silenced or accessible for transcription. PcG proteins facilitate epigenetic gene modulation by two key mechanisms: DNA methylation and post-translational histone modifications. (Crea et al., 2012) In general, Polycomb group proteins are classified in two complexes: the Polycomb repressive complex 1 (PRC1) and 2 (PRC2). (fig.3) (Simon & Kingston, 2013) The PRC2 and PRC1 complexes are composed of respectively three and four subunits; gene duplication resulted in multiple paralogs for a few subunits. (Connelly, Martin, & Dykhuizen, 2016) PRC1 is a diverse complex which is comprised of RING E3 ubiquitin ligase (RING1A/B), Polycomb Group Finger (BMI-1 or MEI18), Polyhomeotic (PHC1-3) and the chromodomain-containing chromobox homolog (CBX2,4,6,7,8). (Crea et al., 2012) PRC2 is comprised of EZH1 or EZH2, EED and SuZ12. PRC2 is responsible for the trimethylation of

histone H3 lysine 27 (H3K27me3) via EZH2 catalytic subunit. H3K27me3 is identified and read by PRC1

and as a result PRC1 is recruited and chromatin-organization modifier domain (chromodomain) ubiquitinates H2A lysine 119 (H2AK119) resulting in chromatin compaction and gene silencing. (Connelly et al., 2016)(Marchesi & Bagell, 2013) (Gao et al., 2012) (Nawaz et al., 2016)

PcG protein expression levels in GBM

Obviously, the fundamental role of PcG proteins makes PcGs a therapeutic target with potentially great impact on development or malignant evolution of cancers. TCGA and other groups have done extensive genetic expression profiles studies of GBMs, revealing PcG regulatory networks have gone away in GBM. (Purow & Schiff, 2012) PcG proteins EZH2, BMI1 and CBX7 have been shown to possess

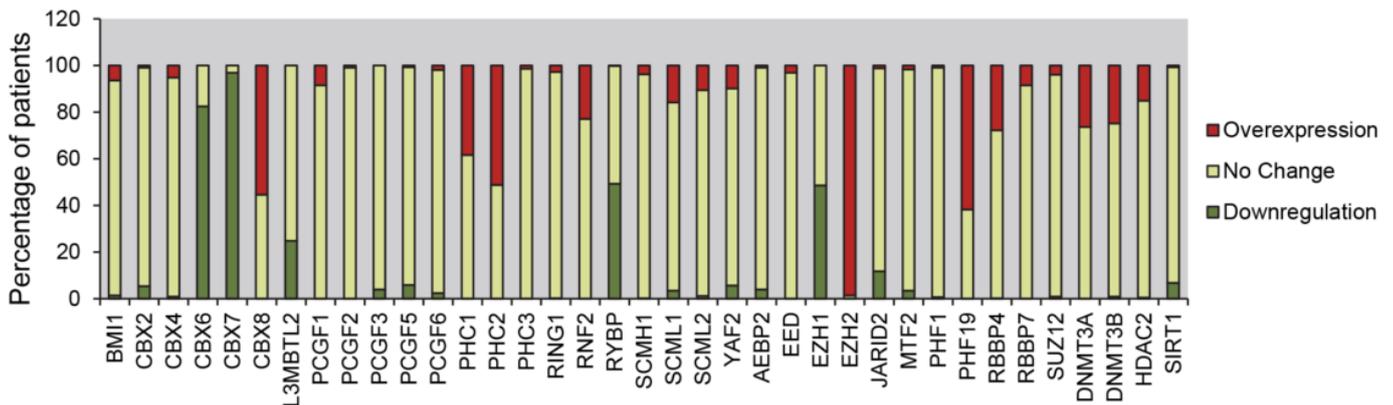


Figure 4: mRNA expression of PcG genes in GBM from TCGA. PcG gene expression in GBM patients is defined in three categories; overexpression (Tumor/Normal Ratio ≥ 2 , red); downregulation (Tumor/Normal Ratio ≤ 0.5 , green) and no change ($0.5 \leq$ Tumor/Normal Ratio ≤ 2 , yellow). The stacked bar graph depicts the percentage of each category, calculated out of total 424 patients. Figure and legend from (Li et al., 2013).

oncogenic or tumor suppressor functions in different tumors including GBMs. (Suvà et al., 2009) (Nawaz et al., 2016) As shown in figure 4, *CBX8*, *PHC2*, *EZH2* and *PHF19* PcG genes display significant overexpression profiles in in GBM patients. The most frequently downregulated PcG genes are *CBX6*, *CBX7*, *RYBP* and *EZH1*. (Li et al., 2013) In this study Li et al. also found that a widely held portion of the PcG genes are not differentially expressed in different subtypes of GBM defined by TCGA. These findings might mean that a therapeutic agent targeting PcG proteins could have a broad target range and therefore be used for multiple subtypes of GBM. Naturally, more research is needed to verify if multiple subtype agents are indeed possible.

EZH2 and *BMI1* are the most studied PcG complex proteins in cancer research, both are often overexpressed in cancers. (Mills, 2010) It has been shown, in multiple models, that these PcGs are essential in controlling proliferation and maintaining self-renewal of CSCs. (Crea et al., 2012) Recently, *Cbx6* was found to be downregulated in GBM as well as *CBX7*. These findings are really intriguing, especially because not much is known about their involvement in GBM progression. (Li et al., 2013) The mechanism which causes the *CBX6* downregulation has not yet been elucidated. What has been found is that *Cbx6* overexpression causes reduced capacity of cell growth in glioma cells.(Farias-Eisner et al., 2012)(Ma, Zhang, Sun, & Cheng, 2014) Further research in needed to find out more about the underlying mechanisms of *Cbx6* expression levels in GBM. The role of *CBX* proteins in GSCs has not yet been addressed in many studies and is an interesting new field. The next chapter will focus *CBX7*; a PcG protein which is implicated to play an important role in the progression of GBM.

Chapter 3 Chromobox protein homolog 7 (CBX7)

CBX7 has been a rather new subject of an increasing number of studies over the last couple of years. CBX7 is a rather ambiguous chromobox protein, residing in the PRC1 complex, playing the dual role of tumor suppressor and oncogene in different types of cancerous malignancies. As example; there is evidence that overexpression of CBX7 leads to cellular immortalization of mouse embryonic fibroblasts (MEF) by repression of the INK4a/ARF locus in vitro and that CBX7 acts as an oncogene by producing aggressive B-cell lymphomas. (Nakano, 2015) On the other hand, CBX7 correlates with a highly malignant phenotype when strongly downregulated in GBM, thyroid carcinomas and pancreatic cancers. (Farias-Eisner et al., 2012)

Mechanisms by which the loss of CBX7 expression promotes GBM progression

Several recent findings in multiple studies suggest CBX7 is a tumor suppressor in GBM, acting as a suppressor of the proliferation and migration of glioma cells. (Nawaz et al., 2016)(Connelly et al., 2016)

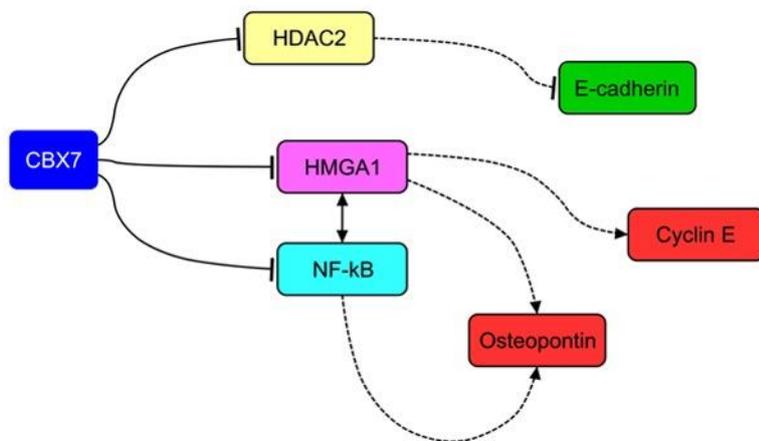


Figure 5: Mechanisms under control of CBX7. CBX7 prevents cell transformation by influencing HDAC2, HMGA1 and NF-kB levels thereby controlling E-cadherin, cyclin E and osteopontin. The downregulation of CBX7 expression in GBM results in a loss of regulatory action (shown by the dashed arrow-lines) Figure from (Pallante et al., 2015)

The precise mechanism(s) of tumor suppression is still unclear but recent reports have implicated a couple of mechanisms (fig.5):

CBX7; E-cadherin expression

CBX7 fulfils a crucial role in maintaining E-cadherin expression. E-cadherin is an essential part of cell-cell adhesion junctions thus very important in sustaining 'normal' epithelial cell morphology. The loss of E-cadherin will lead to epithelial-mesenchymal transition (EMT) which results in the mesenchymal state of GBM cells giving them their invasive nature; the ability to metastasise. (Federico

et al., 2009) CBX7 is able to control the expression of the E-cadherin gene (*CDH1*) by counteraction of the anti-transcriptional effect of histone deacetylase 2 (HDAC2). CBX7 binds to the promoter of *CDH1* and triggers acetylation of the promoter and methylation of certain sites of H3 and H4 histones by blocking HDAC2 activity. This 'histone code' results in promoting *CDH1* transcription. CBX7 downregulation results in the loss of regulatory activity on the *CDH1* promoter which leads to a mesenchymal phenotype. This phenotype correlates with the highly aggressive nature of GBM where cell migration/invasion lead to growth and metastasizing of the tumor to other parts of the brain or body. In addition, it has been found that cells undergoing EMT transform in a more de-differentiated state which enables them to be more resistant to chemotherapy. (Pallante, Forzati, Federico, Arra, & Fusco, 2015)(Pattabiraman & Weinberg, 2017)

CBX7; interaction with HMGA1

Because of the ability of CBX7 to bind to chromatin in several regions on the promoter of many different cancer-related genes, CBX7 is believed to exert control on cell cycle and cell proliferation genes. CBX7 was shown to be able to modulate cyclin E1 (*CCNE1*) expression through binding HDAC2 and forming a HDAC2/CBX7-complex bound to the *CCNE1* promoter. (Pallante et al., 2015) Cyclin E

binds CDK2 to form a complex which plays a crucial role in the G1 phase and in the G1-S phase transition of the cell cycle. (Suvà et al., 2009) The downregulation of the CBX7 gene results in a severely upregulated cyclin E1, mostly because of the antagonistic role of transcription activator High-mobility group protein A1 (HMGA1) which normally competes with CBX7 for binding to the promoter of *CCNE1*. (Forzati et al., 2012) HMGA1 removes the HDAC2/CBX7 complex from the *CCNE1* promoter to enforce its own effect on the promoter. (Forzati et al., 2012) HMGA1 overexpression and CBX7 downregulation results in a dysbalanced regulatory function in their reciprocal relationship. The altered protein levels are the result of a proposed superior regulatory mechanism leading to EMT. Pallante et al. suggested this mechanism starts with MYC and HMGA1 and eventually leads to the complete loss of E-cadherin expression via regulation of CBX7 and certain microRNAs (miRNAs). Chapter 4 will further elaborate on the role of miRNAs in GBM. (Wu et al., 2017) (Pallante et al., 2015)

CBX7; NF- κ B and Osteopontin

CBX7 is also able to repress the expression of the *SPP1* gene, which is another important gene in EMT, through counteracting HMGA1. Osteopontin, the transcriptional protein product of the *SPP1* gene, is positively regulated by HMGA1 whereas CBX7 counteracts the positive effect of HMGA1. When CBX7 is downregulated the regulation mechanism is disturbed which results in a high osteopontin expression. CBX7 loss also effects another important central positive regulator of osteopontin, the NF- κ B complex. CBX7 is a repressor of NF- κ B to regulate osteopontin levels. Once more CBX7 has an antagonistic competition with HMGA1 for the respective repression and stimulation of NF- κ B. Again, the loss of CBX7 results in high levels of osteopontin and is accompanied by a highly malignant phenotype. (Pallante et al., 2015)

Next to binding HDACs, EZH2 and CBX7 are able to physically associate with DNA methyltransferases (DNMTs). (Mohammad et al., 2009) This ability suggests a mechanism through which PcG proteins, like CBX7, are responsible for the altered DNA methylation profiles displayed in many cancer types including GBM. PcG target genes are up to 12 times more likely to be silenced by DNA methylation in cancers compared to non-PcG target genes. (Forzati et al., 2012) (Wu et al., 2017)

The role of CBX7 in migration of GBM

A recent study by Nawaz et al. specified the importance of CBX7 in the migration of GBM. Under 'Normal Brain' conditions in healthy human subject (fig. 6), CBX7 was shown to have crosstalk with the

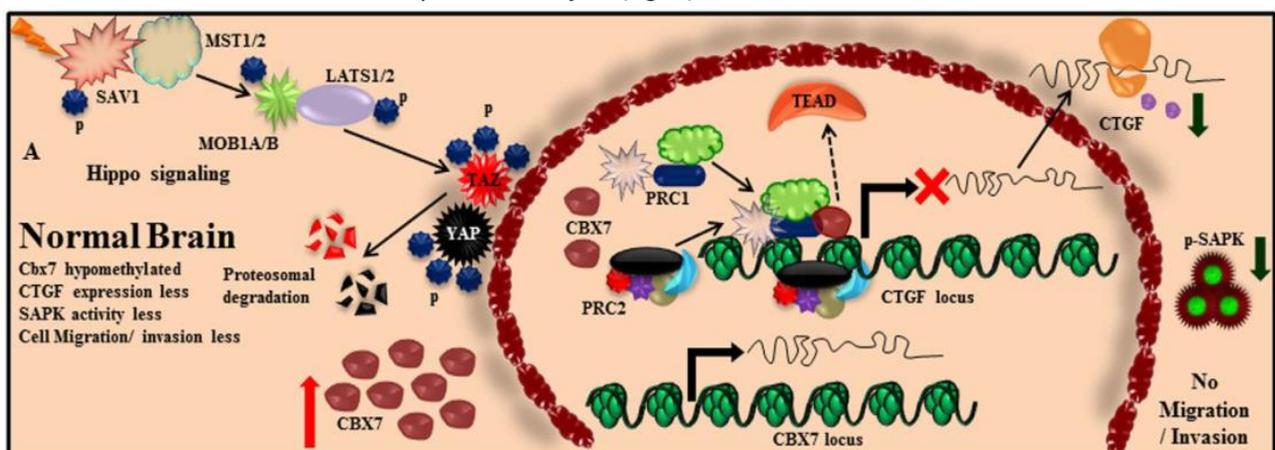


Figure 6. Mechanism for CBX7 regulation of migration under normal conditions. Under non-malignant, 'normal brain', conditions CBX7 is not hypermethylated. CBX7 levels inside the cell are in perfect balance for its proper incorporation into the PRC1 complex. Under normal conditions, CBX7 functions as a transcriptional (tumor) repressor of a wide range of oncogenes, like CTGF (target of YAP/TAZ). Figure from (Nawaz et al., 2016)

Hippo pathway (box 1); a pathway which is essential in apoptosis, cell proliferation, metastasis and invasion of cancer cells.

Box 1. Hippo signalling pathway

The Hippo pathway entails a core kinase cascade resulting in the phosphorylation of transcriptional kinases YAP/TAZ. YAP/TAZ is made in-activate by either being prohibited to enter the cell nucleus or by their enhanced proteosomal degradation. The phosphorylation is the result of an interplay of threonine kinases; mammalian STE20-like protein kinase 1 and 2 (MST1/2) and large tumour suppressor 1 and 2 (LATS1/2). Together with adaptor proteins Salvador homologue 1 (SAV1) and MOB kinase activator 1A (MOB1A) and MOB1B the threonine kinases tag YAP/TAZ creating binding sites which prevents YAP/TAZ from entering the nucleus and ultimately stimulates the degradation via the Ubiquitin Proteosomal Pathway (UPP). (Gumbiner & Kim, 2014)(Nawaz et al., 2016)

CBX7 controls the transcriptional activity of many oncogenes when being properly incorporated in the PRC1 complex. The connective tissue growth factor (*CTGF*) gene, also called *CCN2*, is one of the oncogenes whose YAP/TAZ-dependent transcription is regulated by the binding of PRC1 to the *CTGF* locus. By inhibiting YAP/TAZ-dependent transcription CBX7 prevents CTGF production and it leads to the activation of the Hippo pathway which results to proteosomal degradation of YAP/TAZ. CTGF is a secretory molecule which performs its function within several oncogenic signalling pathways in the cell membrane. (Yin et al., 2010) CTGF is known to increase phosphorylation status of five phosphoproteins including kinases like SAPK/JNK. In many human cancers the *CTGF* gene has been shown to play an important role in the initiation of metastasis and invasion. Therefore, CTGF is a negative prognostic marker in GBM. A high expression of CTGF is equivalent to low survival. (Xie et al., 2004) Under 'normal' conditions CBX7 regulates the activation of SAPK/JNK to control migration and invasion of glioma cells. (Nawaz et al., 2016)(Lin et al., 2013) Transcriptional enhancer factor (TEF) also known as TEA domain (TEAD), shown in fig. 6 & 7, is a transcription factor which is positively regulated by YAP and TAZ to facilitate growth and cell viability. In GBM (Fig. 7), CBX7 is downregulated and it has no effective incorporation in the PRC1 complex which renders the PRC1 complex dysfunctional. The loss of CBX7 will ultimately result in more migration and invasion which correlates with the aggressive and invasive nature of GBM. (Nawaz et al., 2016)

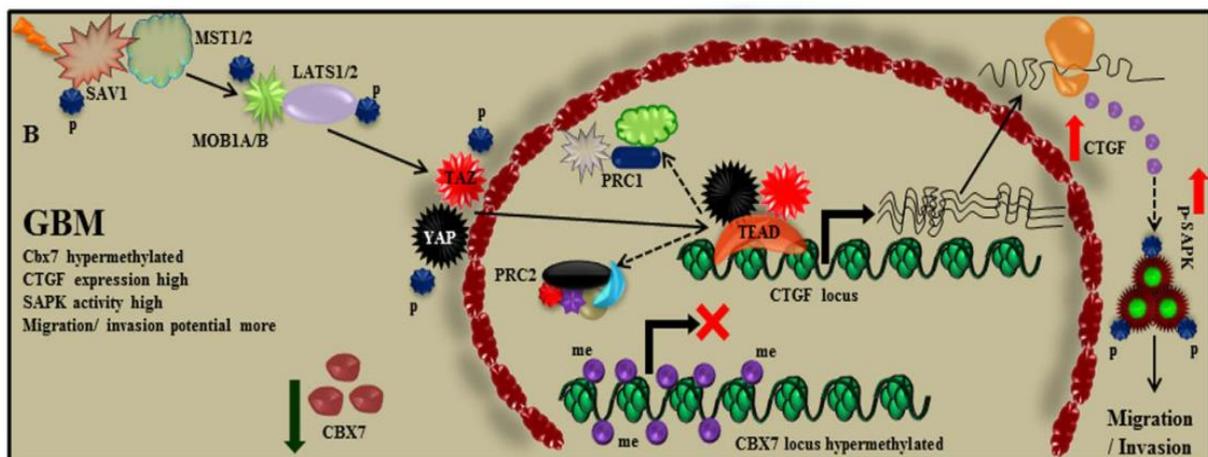


Figure 7. Mechanism for CBX7 regulation of glioma cell migration. The CBX7 locus is hypermethylated in a GBM cell which prevents a normal incorporation in the PRC1 complex. The PRC1 complex is impaired in its function to regulate YAP/TAZ transcriptional targets like CTGF. A higher CTGF expression results in activation of kinases like SAPK/JNK. Loss of CBX7 will, via this mechanism, lead to more migration/invasion. Figure from (Nawaz et al., 2016)

CBX7 and CSCs

The INK4/ARF locus is one of the firstly identified and one of the most recognised targets for CBX proteins within the PRC1 complex. CBX7 and BMI1 are both able to regulate the expression of the tumor suppressor gene *CDKN2A* which encodes for cyclin-dependent kinase inhibitors (CDKIs) isoforms like p16^{INK4a} and p15^{INK4b}, and p53 regulator ARF. INK4 proteins are able to block certain CDKs which are involved in the cell cycle. Furthermore, INK4 plays an essential role in cellular senescence, apoptosis and DNA repair. (O’Loughlen et al., 2015) ARF is able to stabilize p53 via interaction with MDM2, a protein which causes p53 to degrade. (Marchesi & Bagell, 2013) Like INK4 proteins, ARF too is able to control the G1 phase of the cell cycle. Interestingly, p16^{INK4a} is upregulated in aging organisms and it’s involved in a reduced proliferation of neural and haematopoietic stem cells. (Krishnamurthy et al., 2006)

Several recent studies have reported that EZH2 and BMI1 are both involved in the maintenance and renewal of GSCs. (Li et al., 2013) The effects of BMI1 on CSCs self-renewal properties can largely be explained through its effects on INK4a/ARF. INK4a/ARF is tightly controlled by PRCs in proliferating cells. (O’Loughlen et al., 2015) CBX7 shows many similarities with BMI1 in its ability of repressing transcription of the INK4a/ARF gene. (Gil, Bernard, & Peters, 2005) The activated pathways in CSCs have not been fully clarified but among others INK4A has been implicated in GBM (Visvader & Lindeman, 2008) As Li et al showed, BMI1 expression is not as severely disrupted in as wide a range of GBM patients as CBX7 expression (fig. 4). Therefore it might be very interesting to pursue CBX7 as possible regulator of GSCs via its proposed effects on INK4a and ARF.

Recent research has shown that CBX7 plays an important role in maintaining the balance between differentiation and self-renewal in hematopoietic stem cells (HSCs) and embryonic stem cells (ESCs). (Wu et al., 2017) For example, CBX7 was found to be the primary CBX protein residing in the PRC1 complex in mouse ESC. It has been discovered that CBX proteins can switch off by replacing each other in an autoregulatory loop to give the PRC1 complex its different functions like self-renewal and differentiation. When CBX7 is part of the complex it directly contributes to the repression of transcription of genes that encode for other CBX proteins like CBX2, CBX4, and CBX8 who are crucial for proper differentiation of ESCs. (O’Loughlen et al., 2015) (Ma et al., 2014) Other studies demonstrated that mouse HSCs also required CBX7 for their self-renewal. Again, *CBX2*, *CBX4*, and *CBX8* genes were repressed but upregulated upon differentiation. Fascinatingly, ectopically expressed CBX2, CBX4, or CBX8 in HSCs are capable of competing with CBX7 to be a part of the PRC1 complex which resulted in a shift of PRC1 targets thus differentiation of HSCs. This kind of interplay of CBX family proteins has to be investigated further and might differ between different types of cancer. The role of the *CBX7* gene in GBM CSCs needs to be investigated and the interplay of CBX proteins has to be taken into account. (Ma et al., 2014)

Chapter 4 Mechanisms leading the loss of CBX7

CBX7 promotor methylation

The *CBX7* gene is silenced in GBM on account of hypermethylation of its promoter. Nawaz et al. (2016) confirmed the cause of the gene silencing by bisulphite sequencing which established the hypermethylation in GBM samples (20.71%), glioma derived cell lines (39.60%) and control brain samples (5.75%). In addition, the use of a methylation inhibitor resulted in the re-expression of *CBX7* mRNA levels in three glioma cell lines. Thus, concluding the gene silencing was due to the hypermethylation pattern on the *CBX7* promoter. (Nawaz et al., 2016)

MicroRNA regulation of CBX7

To date, not much is known about how *CBX7* expression and function is regulated. A few interesting studies from the last couple of years, concerning miRNAs (Box 2), have shown remarkable mechanisms by which miRNAs are able to control *CBX7* protein levels. (Pallante et al., 2015)

Research has elucidated a few miRNAs which are believed to play a crucial role in the regulation of *CBX7*:

Box 2 MicroRNAs

MiRNAs are small non-coding RNA molecules of 19-22 base pairs which are able to bind to complementary sequence of the 3'-UTR of mRNA transcripts. MiRNAs are regulatory molecules which are able to regulate gene expression on a post-translational level. MiRNAs can, by binding mRNA, directly trigger their cleavage or inhibit their translation. (Tyagi et al., 2016) (Wu et al., 2017) (Pallante et al., 2015)

MiR-9

O'Loghlen et al. and Pallante et al. reported that the miR-9 family plays an important role in regulating *CBX7* protein levels. MiR-9 had earlier been implicated in the development of different types of brain tumors including GBM and evidently plays a key role in the proliferation of neural progenitors.

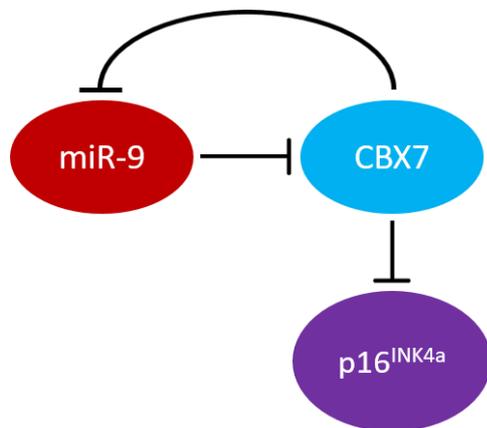


Figure 8: Negative regulatory feedback loop of miR-9 and *CBX7* influencing *p16^{INK4A}* expression.

(Pallante et al., 2015) (Malzkorn et al., 2010) (O'Loghlen et al., 2015) O'Loghlen et al. reported that *CBX7* and miR-9 regulate each other in a negative regulatory feedback loop (fig. 8). MiR-9 expression, activated by the oncogene *MYC*, results in the downregulation of *CBX7* mRNA levels. MiR-9 regulates *CBX7* expression by binding to specific sites in the 3'UTR thereafter *CBX7* regulates miR-9 expression by binding its promoter which results in repression of miR-9 transcription. This miR-9/*CBX7* feedback loop also influences *p16^{INK4a}* expression levels. *CBX7* is able to repress *p16^{INK4a}* which influences the cell cycle by controlling progression from G1 phase to S phase. This suggest that *CBX7* contributes to the fine-tuning of the initiation of *p16^{INK4a}* during replicative senescence. (O'Loghlen et al., 2015)

Although their research suggests that miR-9 directly influences the stability of *CBX7* mRNA, more research is needed to be sure that *CBX7* downregulation is not effected through the inhibition of *CBX7* translation by miR-9 or that another indirect regulation causes *CBX7* downregulation.

MiR-18a

Another recently discovered miRNA which regulates CBX7 in GBM is miR-18a. MiR-18a is an oncogenic miRNA that promotes proliferation and enables tumor progression in GBM and many other cancer types like gastric cancer, oesophageal squamous carcinoma and prostate cancer. (Luo et al., 2013) (Wu et al., 2017) Wu et al. showed that miR-18a levels correlated with WHO grade and is highly expressed in clinical GBM samples. They identified that miR-18a directly targets CBX7 mRNA which results in a downregulation of CBX7 protein levels. They found that miR-18a levels are inversely correlated with miR-18 levels which might suggest some sort of feedback mechanism which has yet to be investigated. In vitro and in vivo studies showed that miR-18a regulates the proliferation of GBM cells and controls the cell cycle by blocking the inhibiting effect of CBX7 on the expression of genes related to the cell cycle. One of their in vivo studies, MiR-18a knockdown in nude mice (Fig. 9), showed a significantly decrease in growth of intracranial tumors (using U87 glioblastoma cells). Also, the miR-18a knockdown groups had a significant longer overall survival. (Wu et al., 2017)

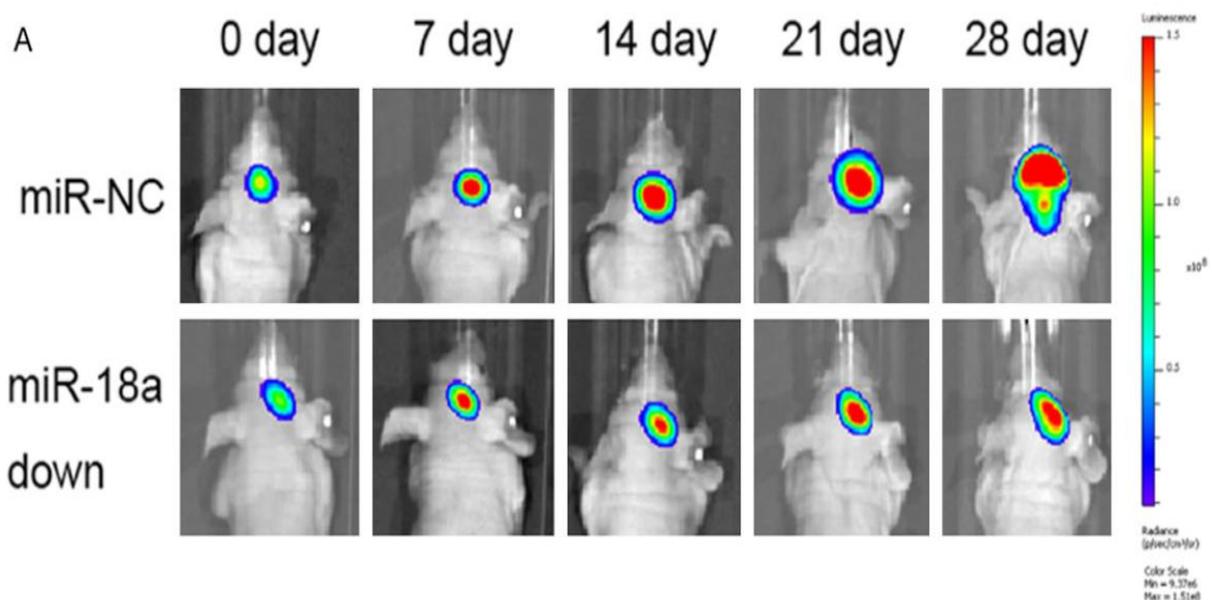


Figure 9. MiR-18a inhibition suppresses tumor proliferation in vivo. U87 cells pretreated with a lentivirus with miR18a-down or miR-NC and a lentivirus containing luciferase were implanted in the brains of nude mice, and tumor formation was assessed by bioluminescence imaging. The bioluminescent images were measured at days 7, 14, 21 and 28 after implantation. Figure and legend from (Wu et al., 2017)

The role and precise mechanisms behind the miR-9/CBX7 and miR-18a/CBX7 axes should be further investigated but might represent promising therapeutic targets in GBM treatment strategies in clinical trials.

MiRNAs as therapeutic targets

As is the case in GBM, when delivering chemotherapy a large inefficient dose is needed during the delivery process because of the blood brain barrier (BBB). In addition, chemotherapeutics have a high toxicity which damages not only cancer cells but also somatic cells. Moreover CSCs have a high chemo resistance. RNAi-based therapeutics is a very new field but holds great promise as a novel therapeutic strategy in cancer with high precision and potentially less collateral damage. Currently, clinical trials are underway using siRNAs. A number of strategies have been tried to overcome the problem of the BBB. For example, the use of Gold Nanoparticles (GNPs) to kill tumor cells has been broadly reported and this could be a method for delivering siRNAs and miRNAs to the tumor site. For example, Tyagi et al. used a polymeric nanoparticle loaded with microRNA-145 (miR-145) which could efficiently inhibit

expression of multidrug resistance gene and anti-apoptotic gene in cerebral GSCs. (Tyagi et al., 2016) (Silber et al., 2008) (Wu et al., 2017)

Further testing of miR-9 and miR-18a in pre-clinical models of GBM is needed to determine their definitive therapeutic potential for treatment of GBM by targeting CBX7.

Discussion & Conclusion

GBM remains the most common and most malignant brain tumor in adults. Extensive years of research has given us a better understanding of the biology of GBM nonetheless we are still awaiting breakthroughs resulting in effective treatment strategies. GBM is vastly heterogenous, inter as well as intra tumoral differences make targeting quite impossible. Recent genetic and epigenetic profiling, rather than histological profiling, may result in a better GBM subtype distinction in patients and a better 'fitting' treatment plan. There is still much to be unraveled and understood about the complexity of GBM to offer us more insight into the mechanisms behind the development, maintenance and progression of GBM. GBM tumorigenesis and chemoresistance are facilitated via multiple factors and it has become clear that epigenetic regulation mechanisms play an important role. Many papers have been reporting and confirming that there are small subpopulations of GSCs present within a single GBM tumor and even within a subgroup. These GSCs play an important role in recurrence, drug resistance and the resilience of GBM. Unfortunately, no single definitively marker to identify or define GSCs has been found. Research has uncovered that PcG complexes and their individual PcG proteins fulfil a crucial part in the progression of GBM. In particular, the PRC1 and PRC2 complex proteins seem to be of importance in controlling cell cycle, migration and are even implicated in their involvement in maintaining stemness in GBM CSCs. This suggest that PcG proteins are interesting subjects to investigate in relation to targeting GSCs and overall GBM treatment.

Gene expression and protein levels of PcGs are highly dysregulated in GBM. In this thesis, the CBX family was the main focus; in particular CBX7. CBX7 has been shown to play the role of tumor suppressor in GBM. This anti-oncogenic role can be validated by its ability to counteract HMGA1, HDAC2 and NF- κ B in order to prevent cell transformation. CBX7 is also responsible for the repression of gene expression of several oncogenes like CTGF. Many studies have shown that EZH2 and BMI1 play a crucial role in the maintenance and renewal of GSCs. The CBX family members, like CBX7, are presenting a new promising topic of research. CBX7 has already been implicated in their role as regulator of the balance between differentiation and self-renewal in mouse HSCs and mouse ESCs. It needs to be seen if CBX7 plays a role too in GSCs regulation. More research is required to fully comprehend the full arsenal of CBX7 activities. Most studies are reporting that the loss of CBX7 expression correlates with highly malignant forms of glioma's, becoming more and more aberrant with a higher WHO grade. The loss of CBX7 in GBM results in a great amount of dysregulation of 'normal' PRC1 function and contributes to the invasive and aggressive character of GBM. It is abundantly shown that CBX7 downregulation in human malignant neoplasias clearly correlates with a poor prognosis. More than 80% of GBM patients show a downregulation of CBX7 which makes low CBX7 a biomarker for poor diagnosis.

In addition to unravelling the mechanisms and functions of individual CBX proteins, a lot more remains to be investigated relating to the interplay among CBX and others PcG proteins and even other transcription and translation regulatory mechanisms. For example, CBX7, CBX6 and CBX8 interplay is proposed to have an important role in GBM. In mouse HSCs and mouse ESCs, CBX7 has been shown to be the primary CBX protein in PRC1 sustaining self-renewal properties and simultaneously repressing the expression of other CBX proteins which promote cell differentiation. Similar mechanisms in GBM have yet to be found but has to be taken in to account when targeting GSCs.

Multiple mechanisms have been implicated in the downregulation of CBX7. Nawaz et al. showed that hypermethylation of the CBX7 promoter was the cause of the loss of expression. MiRNAs have been implicated as one, of possibly many, causes to regulate CBX7 protein levels. MiR-9 and miR-18a are

specific miRNAs found to be associated with the loss of CBX7 in GBM. The definitive clinical therapeutic relevance of these miRNAs needs to be confirmed and the role of other miRNAs in GBM should be decrypted.

It is clear that PcG proteins are highly involved in the maintenance and progression of GBM. CBX proteins in particular are interesting because their hypothetical role in GSCs are yet to be confirmed. CBX7, but also CBX6, CBX4, CBX2 and CBX8, is greatly implicated in the tumorigenesis of GBM. The miRNA/CBX axis presents a possible engaging point for therapeutics as well as diagnostics and prognostics. More insight is needed to understand the mechanisms behind miRNAs and CBX protein interaction to even consider treatment options but with time, miRNAs which target PcG proteins might lead to promising personalised treatment options for patients suffering from GBM.

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