

From the Department of Medical Biochemistry and Biophysics  
Karolinska Institutet, Stockholm, Sweden

**Functional investigation of prognostic biomarkers and  
therapeutic targets in glioma brain tumors**

**Kaveh M Goudarzi**



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**FUNCTIONAL INVESTIGATION OF PROGNOSTIC BIOMARKERS AND  
THERAPEUTIC TARGETS IN GLIOMA BRAIN TUMORS**

**THESIS FOR DOCTORAL DEGREE (Ph.D.)**

By

**Kaveh M. Goudarzi**

*Principal Supervisor:*

Docent Mikael Lindström  
Karolinska Institutet  
Department of Medical Biochemistry  
and Biophysics  
Division of Genome Biology

*Opponent:*

Professor Cecilia Williams  
Royal Institute of Technology  
Department of Protein Science  
& Karolinska Institutet  
Department of Biosciences and Nutrition

*Co-supervisors:*

Professor Monica Nistér  
Karolinska Institutet  
Department of Oncology-Pathology

*Examination Board:*

Professor Eckardt Treuter  
Karolinska Institutet  
Department of Biosciences and Nutrition

Assistant Professor Daniel Hägerstrand  
Karolinska Institutet  
Department of Oncology-Pathology

Professor Galina Selivanova  
Karolinska Institutet  
Department of Microbiology, Tumor and Cell  
Biology

Professor Jiri Bartek  
Karolinska Institutet  
Department of Medical Biochemistry  
and Biophysics  
Division of Genome Biology

Docent Sven Nelander  
Uppsala University  
Department of Immunology, Genetics and  
Pathology



*Dedicated to my beloved family*

## **ABSTRACT**

Gliomas are known to be the most prevalent primary tumors of the central nervous system, of which glioblastoma is the most aggressive type with a median survival less than 2 years and no available cure. Studies in this thesis investigated the significance of two potentially important proteins in glioma biology, the transcription factor PROX1 and the histone chaperone NPM1, and the interplay of the therapeutic targets p53 and mTOR.

Study I combined dataset mining and experimental studies to elucidate the function of PROX1 in glioblastoma. *PROX1* mRNA was significantly lower in tumors with a mesenchymal signature, and its modulation in cultures derived from a heterogeneous glioblastoma tumor, transitioned the cells between non-mesenchymal and mesenchymal gene expression-based profiles. Concomitant with this were changes in proliferation rate, cell cycle proteins and stem cell markers. Further, the results revealed PROX1 regulation by SOX2, a connection that could be controlled by a CDK2 small molecule inhibitor. The findings from this study highlight the value of PROX1 as a prognostic tool and the functional role of PROX1 in glioma tumor development.

In study II, immunohistochemistry analysis confirmed a significant overexpression of NPM1 in human glioblastoma samples. Moreover, the subcellular localization of NPM1 was assessed in glioma cell lines. NPM1 displayed a cloudy nuclear staining compared with the more pronounced nucleolar staining observed in normal cells. Moreover, NPM1 depletion altered the nucleolar structure, but had no major effect on cell viability. Interestingly, enforced expression of NPM1 reduced apoptosis in histone H1.5 depleted cells, suggesting that NPM1 acts in a pro-survival manner in cells.

In study III, the p53 response to drug-induced nucleolar stress and simultaneous inhibition of mTOR pathway by the use of various natural and synthetic compounds was analyzed. mTOR inhibition interfered with the p53 pathway stress response, as assessed by the impairment of p53 stabilization and reduced mRNA and protein levels of its downstream target p21. This provides information that may help improve combinatorial drug treatment regimens for cancer patients.

Collectively, these studies provide novel insights into the biology of glioma brain tumors useful for patient diagnosis and more effective personalized therapies.

**Keywords:** cancer, glioma, glioblastoma, nucleophosmin, PROX1, p53 pathway, mTOR pathway, nucleolar stress

## LIST OF SCIENTIFIC PAPERS

**Kaveh M Goudarzi**, Jaime A Espinoza, Min Guo, Jiri Bartek, Monica Nistér, Mikael S Lindström, Daniel Hägerstrand

**Decreased PROX1 levels transition glioblastoma cells into a mesenchymal gene expression subtype** *Submitted Manuscript*.

Karl Holmberg Olausson, Tamador Elsir, **Kaveh M Goudarzi**, Monica Nistér, Mikael S Lindström

**NPM1 histone chaperone is upregulated in glioblastoma to promote cell survival and maintain nucleolar shape** *Sci Rep. 5, 16495* (2015)

**Kaveh M Goudarzi**, Monica Nistér, Mikael S Lindström

**MTOR inhibitors blunt the p53 response to nucleolar stress by regulating RPL11 and MDM2 levels** *Cancer Biol Ther. 15, 1499-514* (2014)

## RELATED PUBLICATION NOT INCLUDED IN THIS THESIS

**Kaveh M Goudarzi**, Mikael S Lindström

**Role of ribosomal protein mutations in tumor development (Review)** *Int J Oncol. 48, 1313-24* (2016)

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## LIST OF ABBREVIATIONS

CNS	Central Nervous System
CCLE	Cancer Cell Line Encyclopedia
CSC	cancer stem cell
DRB	adenosine analogue 5,6-dichloro- $\beta$ -D-ribofuranosyl-benzimidazole
EMT	epithelial-to-mesenchymal transition
GC	guanine-cytosine
GSC	glioma stem cell
GSEA	gene set enrichment analysis
HGCC	Human Glioblastoma Cell Culture
IDH1/2	isocitrate dehydrogenase 1 or 2
MDM2/HDM2	mouse/human double minute 2 homolog
MGMT	O(6)-methylguanine-DNA methyltransferase
mTOR	mammalian target of rapamycin
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PROX1	Prospero Homeobox 1
PMT	proneural-to-mesenchymal transition
RP	ribosomal protein
RT-PCR	reverse transcription polymerase chain reaction
SVZ	subventricular zone
TCGA	The Cancer Genome Atlas
TERT	telomerase reverse transcriptase
5'TOP	5'terminal oligopyrimidine tract
UBF	upstream binding factor
UV	ultraviolet
WHO	World Health Organization



# 1 INTRODUCTION

## 1.1 WHAT IS CANCER?

Cancer is a generic term given to a group of related disorders, characterized by an aberrant mass of tissue growing excessively in an uncoordinated manner, and capacity to invade and impair adjacent tissue as well as different sites (Robbins & Cotran Pathologic Basis of Disease). According to the WHO, cancer accounts for the highest rate of death and morbidity in Europe (similar to the U.S.) after the cardiovascular diseases. Around 70% of cancer deaths take place in low- and middle-income countries, and it accounted for 8.8 million deaths in year 2015 on a global scale (<http://www.who.int/cancer/en/>). It was reported that cancer burden has continued to grow with 6.6% increased mortality between 2000 and 2015 in the European region, while countries such as Sweden, Switzerland and the United Kingdom have succeeded to reduce the mortality, showing that it is possible. Importantly, strategies for prevention, early detection, improved diagnosis and treatment could effectively reduce the mortality associated with cancer, as highlighted by the WHO (<http://www.who.int/cancer/en/>).

Cancer cells divide abnormally, can infiltrate adjoining tissues and metastasize to other organs through the blood and lymphatic systems. Traditionally, cancers have been classified by their tissue type: Carcinomas are the most common malignancies and originate from the epithelial cells forming the skin or lining of the internal organs; sarcomas derive from cells of mesenchymal origin in the connective or supportive tissue such as bone, muscle, blood vessels or lymphoid tissues; leukemia could derive from blood-forming tissue like the bone marrow; lymphoma and multiple myeloma start in the cells of the immune system lymphocytes and plasma cells, respectively; melanomas originate from melanocytes of skin or eye, and cancers of the CNS arise from the cells of brain and spinal cord.

It is widely believed that cancer cells display several abnormal properties including chronic proliferation signaling, bypassing growth suppressors, resisting apoptosis, enabling replicative immortality, activating angiogenesis, inducing invasion and metastasis (Hanahan and Weinberg, 2011). Additionally, reprogramming of energy metabolism and evading the immune system are considered in recent years among the cancer hallmarks (Hanahan and Weinberg, 2011). Indeed, a better knowledge about the capabilities of cancer cells will help the development of novel pathogenesis-based therapeutic strategies.

## 1.2 ON THE ROAD TO CANCER

Before cancer occurs, normal cells undergo multistep genetic, epigenetic and morphological alterations, which result in the formation of pre-malignant lesions. Hyperplasia is a pathology term describing an excessive cell proliferation while the cells still appear normal under the microscope, and dysplasia is referred to the stage when transformed cells look abnormal and the tissue disorganized, prior to the growth of a fully malignant lesion. Accumulating evidence suggests that tumorigenesis begins long before formation of clinically detectable lesions through “field cancerization” that refers to the evolution of normal somatic cells into cancer-primed cell populations, possessing the required underlying mutations and phenotypic predispositions such as chromosomal instability and mutation of TP53 gene (Curtius et al., 2018). Of note, these events can be initiated through exposure to mutagens and/or age-related mutations (Curtius et al., 2018). Additionally, chronic inflammation is viewed as a predisposition factor for the onset of cancer (Curtius et al., 2018). Therefore, it should potentially be possible to detect a “cancerized field” early on before the pro-tumorigenic mutations lead to selection and expansion of mutation carrying cells in surrounding ageing or inflamed but otherwise healthy tissue microenvironment (Curtius et al., 2018), which in turn could result in increased lifespan through preventive measurements.

The cancer stem cell (CSC) hypothesis has remained work in progress, and postulates an intra-tumoral hierarchy, where a small fraction of cancer cells exhibits higher self-renewal capacity and pluripotency that is required for tumorigenesis (Reya et al., 2001; Stiles and Rowitch, 2008; Tan et al., 2006). This “stemness”, combined with an enhanced capacity for invasion and notorious resistance to therapy bestows a deadly triad that links such cell population to poor outcome for the cancer patient.

### 1.3 GLIOMA BRAIN TUMORS

Malignant CNS tumors represent the deadliest cancers considering the years of potential life lost post diagnosis, with estimated mean of ~20 years (Rouse et al., 2016). Gliomas are the most common type of primary intracranial tumors, which comprise about 80 percent of all malignant brain tumors and have largely unknown etiology (Goodenberger and Jenkins, 2012; Ostrom et al., 2014; Reifenberger et al., 2017). While gliomas can occur anywhere in the CNS they generally arise from the glial tissue in the brain (Ostrom et al., 2014).

These complex ecosystems of different cell types were previously classified merely based on histological criteria and degree of anaplasia from slow-growing principally benign grade-I lesions usually associated with a favorable prognosis to grade-IV tumors, also known as glioblastomas, which represent the most aggressive primary brain tumor in adults (Furnari et al., 2007). The international classification of CNS tumors by WHO was recently revised to incorporate molecular biomarkers such as *IDH1/2* status, *MGMT*-promoter methylation, and 1p/19q co-deletion identified by genome-wide molecular profiling efforts with neuropathological diagnosis based on histological features, *i.e.* the WHO grade and type (Reifenberger et al., 2017). Therefore, the revision aims to implement molecular diagnosis and thereby precision medicine in neurooncology by defining distinct tumor entities as precisely as possible leading to improved patient outcomes (Reifenberger et al., 2017).

#### 1.3.1 Glioma WHO grade-IV (glioblastoma)

Glioblastomas are the most devastating and common gliomas (~45% of all gliomas), which to date have remained associated with extremely aggressive clinical courses and only 0.05% to 4.7% of patients surviving 5 years following diagnosis (Ostrom et al., 2014).

In effect, glioblastoma classification includes a spectrum of biologically distinct tumors having different age of onset, tumor location and prognosis. Microscopically, these tumors are often presented with nuclear atypia and cellular pleomorphism, high mitotic activity, as well as pseudopalisading necrotic areas and microvascular proliferation that distinguish them from the gliomas of lower grades (Hambarzumyan and Bergers, 2015). In particular, the high degree of histopathological diversity and inter- and inpatient tumor heterogeneity poses several obstacles for understanding the disease, and improved diagnosis and treatment regimens.

A major obstacle in glioblastoma treatment is the diffuse tumor infiltration/invasion into the surrounding healthy tissue in characteristically distinctive patterns known as Scherer's structures, resembling threads of a spider's web, which enables them to escape complete surgical resection and chemo- and radiation therapy (Cuddapah et al., 2014). Despite a challenge posed by the brain's limited extracellular space, glioma cells are thought to gain mobility and collectively use existing brain structures such as nerve tracts and blood vessels to invade nearby tissue. Additionally, the conditions of pro-tumoral inflammation and/or EMT in the extracellular matrix can make way for cancer cells, facilitating tumor invasion (Cuddapah et al., 2014; Noroxxe et al., 2016).

At present, the standard of care for most glioblastomas can extend patients' lives by about a year (15-17 months median survival in contemporary clinical trials), and tumors eventually recur after multimodal treatments (Furnari et al., 2007; Stupp et al., 2009). The treatment for newly diagnosed cases include maximal resection followed by radiotherapy and/or 6 cycles of adjuvant chemotherapy with Temozolomide (Stupp et al., 2005). Notably, the common risks for detrimental impact on neurological and cognitive functioning of the patients should be considered, which could hinder complete surgical removal of the tumor.

However, molecular neuropathology has shown promise for better diagnosis of the tumors, and incremental improvements in survival are anticipated with the therapeutics in development.

The advent of large-scale genomic and epigenomic studies has provided deeper insights into molecular pathology of gliomas and identified novel targets for therapy such as the mutant *IDH1* enzyme (Brennan et al., 2013b; Parsons et al., 2008; Sturm et al., 2014; TCGA, 2008; Verhaak et al., 2010). Based on DNA and RNA profiling of bulk tumors and identified aberrations in *EGFR*, *NF1*, *PDGFRA* and *IDH1*, glioblastomas were clustered into four main subtypes: proneural (PN), neural (N), classical (CL), and mesenchymal (MES) (Brennan et al., 2013a; TCGA, 2008; Verhaak et al., 2010). The glioblastoma-associated genetic changes validated in these studies distinguish three core signaling axes: (I) p53 pathway, (II) receptor tyrosine kinase (RTK)/phosphatidylinositide 3-kinase (PI3K)/Ras pathway, and (III) retinoblastoma (Rb) signaling. Interestingly, the profiling based glioblastoma subtypes also bear resemblance to gene expression profiles of normal brain cells; for a review regarding glioma genetics and biology please see (Alcantara Llaguno and Parada, 2016).

Furthermore, general advancements in tumor biology and especially in the area of tumor microenvironment research have led to the development of novel targeted therapies (Bielen et al., 2011; Dresemann, 2005), including cutting-edge immune-based treatments such as dendritic cell vaccines, which are currently undergoing evaluation and/or optimization for glioblastoma. Dendritic cells are the most robust antigen presenting cells, which could be engineered for enhanced recognition of tumor cells by patients' immune system and activate a long-lasting response for their elimination. Another immune-based approach advancing to late-stage clinical trials for glioblastoma patients exploits checkpoint inhibitor drugs, developed to block tumor cells blunting of the immune response. Finally, identification of novel technologies and better prognostic biomarkers linked to tumor growth and metabolism for the assessment of treatments is focus of intense investigation.

### **1.3.2 Glioma biology – stem cells in focus**

Glioma biology is complex, and gliomagenesis involves processes that recapitulate CNS development with dynamic regulation and plasticity in cell populations within the heterogeneous tumor tissue. In other words, defined neurodevelopmental programs in normal stem and progenitor cells of the brain could re-emerge in “glioma stem cells” (GSCs) during tumorigenesis. It is thought that the activation of stem cell regulatory programs accounts for maintenance of the tumor under stress conditions, and likely enhances tumor growth, resistance to therapy, invasion, and angiogenesis (Bao et al., 2006; Singh et al., 2004a; Singh et al., 2003). Thus, the GSCs are important in glioma research and they are increasingly viewed as legitimate therapeutic targets (Altaner, 2008; Hambardzumyan et al., 2008).

Cancer stem cells were initially identified in brain tumors by their expression of the neural stem cell surface marker CD133 (Singh et al., 2003), and while high expression of genes such as *SOX2*, *NESTIN*, *OCT4*, *KLF4*, *NOTCH1*, and *GFAP* has been repeatedly associated with stem/progenitor cells in the glioblastoma literature, none of these markers is able to define the GSCs alone. Recently, glioma stemness was linked to a core set of four master transcription factors: *SALL1*, *POU3F2* (*OCT7*), *OLIG2* and *SOX2*, that were proposed to contribute to tumor progression and underlie therapeutic resistance (Suva et al., 2014).

Of note, the effects of EMT programs on stemness features, cell proliferation and survival has linked it to tumorigenesis (Brabletz et al., 2018). During EMT, polarized epithelial cells progressively change morphology and biochemical characteristics to acquire a more

mesenchymal phenotype. As crucial mediators of cellular plasticity supporting tumor progression, the EMT-activating transcription factors (EMT-TFs) have been associated with poor clinical outcome in many cancer types of both epithelial and non-epithelial origins (Brabletz et al., 2018). A common source of confusion regarding the EMT term in cancer biology arises from the frequent expression of EMT-TFs in non-epithelial tumors including gliomas (Brabletz et al., 2018). EMT is not a uniform biological process defined by one single pathway, and the EMT-TFs such as SNAIL, SLUG, TWIST and ZEB families have other non-redundant and tissue-specific roles (Brabletz et al., 2018). Nevertheless, the divergent tumorigenic events involved in EMT result in more similar phenotypic endpoints and a generic gene expression signature was recently proposed to score EMT status amongst clinical samples (Tan et al., 2014). Using this generic signature for EMT or proneural-to-mesenchymal transition (PMT) – a term increasingly recognized in the glioblastoma literature, it was established that glioblastoma tumors (and cell lines) were primarily mesenchymal, and that patients with proneural tumors displayed better overall survival (Jiang et al., 2017; Patel et al., 2014).

Although not fully elucidated, EMT/PMT in glioblastoma is generally associated with a hypoxic tumor microenvironment, inflammation, enrichment of macrophages/microglia, and activation of signaling pathways such as SNAIL, NF1, and NF-kB (Iwadate, 2016). Intriguingly, a study from Moustakas laboratory revealed that Snail overexpression in glioblastoma cells increased tumor invasiveness in a mouse xenograft model, while it depleted the gliomasphere formation capacity *in vitro* and pluripotency and tumor growth *in vivo* (Savary et al., 2013). Another recent study established that immunological changes in a microenvironment enriched with macrophage/microglia induce proneural to mesenchymal transition in glioblastoma cells, as previously reported through NF-kB activation (Bhat et al., 2013) – but it also revealed that genetic inactivation of *NF1* drives chemo-attraction of tumor-associated macrophages/microglia, pointing to an interplay between tumor cells and their microenvironment during EMT (Wang et al., 2017). Finally, a mesenchymal shift could be triggered by radiation exposure *in vivo* (Halliday et al., 2014) and since mesenchymally transitioned cells are more radio-resistant (Bhat et al., 2013) it could impact the effectiveness of radiotherapy (Halliday et al., 2014).

Collectively, the GSCs are thought to arise from adult neural stem cells or multipotent neural progenitor cells that persist in proliferative niches in the human CNS found for instance in the SVZ, or alternatively, from differentiated lineages such as astrocytes (Stopschinski et al., 2013) or oligodendrocyte progenitors. Experimentally, these cells can

be modeled to grow as spheres *in vitro* (similar to neural stem cells), and have been inferred the capacity to initiate tumors and recapitulate histology of the initial tumor in animal models (Chen et al., 2012; Singh et al., 2003; Singh et al., 2004b). Another report revealed that glioblastoma single cell transcriptomes (Patel et al., 2014) correlate with those of the normal outer radial glia, *i.e.* outer subventricular zone glia niche of neural stem cells of the neocortex (Pollen et al., 2015). Specifically, the transcriptional signatures of the outer radial glia cells were enriched in cells from primary glioblastomas, pointing to common sets of genes that control self-renewal (and migration) in the healthy developing brain and tumor tissues (Pollen et al., 2015). More recently, a landmark study proposed that intratumoral heterogeneity in glioblastoma is the expected outcome of fate decisions made by GSCs and their progeny, independent of an evolving mutational signature (Lan et al., 2017). The authors studied clonal evolution by following serial xenotransplantation of barcoded glioblastoma cells to map their individual fate, and found a hierarchy defined by proliferative activity, in which slow-growing stem-like cells evolve into progenitor cells with enhanced proliferation rate and capacity for self-maintenance, that in turn bring about more quiescent cells (Lan et al., 2017).

### 1.3.3 Molecular biomarkers of glioma – an ongoing challenge

As noted earlier, determination of a number of molecular biomarkers such as *IDH1/2* mutations, 1p/19q co-deletion, *MGMT* promoter methylation, and *EGFRvIII* amplification could be useful in routine clinical practice, in accordance with the recently revised WHO guidelines. Of note, a preceding practical framework classified gliomas into five groups based on *IDH* mutations, 1p/19q co-deletion and *TERT* promoter mutations, and suggested incorporation of *TP53*, *EGFR*, or *PTEN* among other alterations for the refinement of glioma diagnosis (Eckel-Passow et al., 2015).

An important advancement in the recent classification of glioma entities is related to the *IDH* mutation status found among low- and intermediate-grade gliomas (mostly WHO grade II and III) (Parsons et al., 2008; Yan et al., 2009). *IDH1* and *IDH2* are key enzymes involved in cellular energy metabolism. *IDH1* mutations were identified in a large fraction of young patients and in most patients with secondary glioblastomas, associated with significantly better overall survival (Parsons et al., 2008). In brief, *IDH* mutation is tightly associated with an aberrant DNA and histone methylation profile that leads to widespread hypermethylation of CpG islands, termed ‘glioma-CpG island methylator phenotype’ (G-CIMP) (Noushmehr et al., 2010).

Of note, methylation of the *MGMT* promoter has a prognostic value in anaplastic grade III tumors, and can predict whether patients with *IDH*-wild-type glioblastoma would benefit from alkylating chemotherapy with Temozolomide (Stupp et al., 2014). Furthermore, co-deletion or loss of heterozygosity on chromosomes 1p/19q could be used for the diagnosis of oligodendroglioma entity, generally associated with both *MGMT* promoter methylation and *IDH* mutation, and distinguished from tumors of astrocytic lineages for example by an assessment of their wild type *TP53* status (Stupp et al., 2014). Additionally, the 1p/19q co-deletion has predictive value for chemotherapeutic response in oligodendrogliomas. Table 1 summarizes the expected survival of gliomas; adapted from Stupp et al., ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up (Stupp et al., 2014).

**Table 1.** Expected survival of glioma (Stupp et al., 2014)

<b>Grade and cell type</b>	<b>Median survival</b>
Grade II	
Astrocytoma	7–10 years
Oligodendroglioma <sup>a</sup>	>10–15 years
Grade III	
Anaplastic astrocytoma	3.5 years
Anaplastic oligodendroglioma <sup>a</sup>	>10 years
Grade IV	
Glioblastoma	15 months, 2-year survival 27%
MGMT	
Methylated	23 months, 2-year survival: 49%
Unmethylated	13 months, 2-year survival: 12%

<sup>a</sup> With LOH 1p/19q. MGMT, methyl-guanine methyl transferase

It is anticipated that novel techniques for the analysis of DNA methylation, copy number variations and mutational profiling by next generation sequencing (NGS) will lead to defining and developing better biomarkers, which pave the way for improved patient selection and treatment response in the era of precision medicine in neuro-oncology.

Prospero-Related Homeobox 1 (PROX1) has emerged in recent years as a key factor in developmental biology and implicated in several cancers including gliomas, which is the main focus of this thesis work.

#### **1.4.1 PROX1 in development**

*PROX1* is localized on chromosome 1q32.2-q32.3, and encodes a transcription factor comprised of two domains, an “atypical” homeodomain and a conserved Prospero domain (Zinovieva et al., 1996), each contributing to its specific binding to DNA, for a review about PROX1 see (Elsir et al., 2012). The N-terminal part of this 83kDa protein harbors a nuclear localization signal and two nuclear receptor boxes (NR-boxes) (Elsir et al., 2012). The homeobox protein Prospero in *Drosophila* mediates transcriptional repression of stem cell genes and activation of differentiation genes, thereby counteracting tumorigenesis (Betschinger et al., 2006; Doe et al., 1991; Freeman and Doe, 2001; Hirata et al., 1995; Knoblich et al., 1995; Li and Vaessin, 2000). Specifically, Prospero is required in the ganglion mother cells (GMC) to carry out three main functions: to activate neuronal differentiation genes in the larval brain, repress genes specific for neuroblasts, and repress the cell cycle genes (Doe et al., 1991; Freeman and Doe, 2001; Hirata et al., 1995; Knoblich et al., 1995; Li and Vaessin, 2000). In mouse, *Prox1* has been found essential in several aspects of morphogenesis and embryonic development of different organs including lymphatic, hepatocyte, pancreatic, heart, lens, and the central nervous system (CNS) (Elsir et al., 2012; Risebro et al., 2009; Seth et al., 2014; Sosa-Pineda et al., 2000; Wigle et al., 1999; Wigle and Oliver, 1999). Also, *Prox1* contributes to physiological control of stem and progenitor cells in the developing and adult brain (Elsir et al., 2012). Accumulating evidence from developmental studies has indicated the importance of WNT signaling in NSC self-renewal and proliferation (Clevers et al., 2014; Kalani et al., 2008), and *Prox1* is a downstream target for the canonical Wnt signaling in adult hippocampal neurogenesis (Karalay et al., 2011). *Prox1* was also reported to suppress *Notch1* expression, controlling the balance between self-renewal of neural progenitor cells and neuronal differentiation (Kaltezioti et al., 2010).

PROX1 has been reported as part of important transcription factor complexes/networks in several independent studies (Elsir et al., 2012). Accumulating evidence points to *Prox1* involvement in regulation of energy homeostasis, metabolism and circadian rhythms in the liver where it forms complexes with HDAC3, BMAL1, ERR $\alpha$  and PGC-

1 $\alpha$  (Charest-Marcotte et al., 2010; Dufour et al., 2011; Steffensen et al., 2004). Recent studies revealed that an HDAC3-PROX1 corepressor module acts on HNF4 $\alpha$  to control hepatic triglycerides (Armour et al., 2017; Kwon et al., 2016). Further, Prox1 was found to co-regulate the rhythmic control of metabolic outputs and the metabolic gene networks by interacting with ERR $\alpha$ , and PGC-1 $\alpha$ , through binding to the promoters of metabolic genes and blocking ERR $\alpha$ /PGC-1 $\alpha$  complex activity (Charest-Marcotte et al., 2010; Dufour et al., 2011). PGC1- $\alpha$  is a known regulator of mitochondria biogenesis and expressed at high levels in tissues with high metabolic demands (e.g. heart, skeletal muscle, kidney and brain) (Austin and St-Pierre, 2012). Of particular interest, PGC-1 $\alpha$  is also expressed in the developing brain and involved in metabolism and/or survival of GABAergic neurons (pertaining to or affecting the neurotransmitter GABA) (Cowell et al., 2007). Intriguingly, preliminary results from gene expression and immunoblotting analysis of U-343 glioblastoma cell cultures performed in our laboratory implicates PROX1 in regulation of PPARGC1A/PGC1 $\alpha$  levels, connected with corresponding changes in the mitochondrial morphology (Goudarzi KM et al., unpublished data), which merits further investigation. Finally, PROX1 is also involved in glucose metabolism and large-scale meta-analyses of genome-wide association studies confirmed that the rs340874 single-nucleotide polymorphism in *PROX1* is linked to fasting glycemia as well as type 2 diabetes mellitus (Kretowski et al., 2015; Lecompte et al., 2013).

#### 1.4.2 PROX1 in cancer

PROX1 has been shown in several instances to be involved in cancer development (Elsir et al., 2012). Depending on the context, PROX1 has both been suggested to have tumor suppressive capacity and be involved in tumor progression. PROX1 was associated with suppression of neuroblastoma cells, possibly by inhibition of Cdc25A and induction of p27-Kip1 to counteract Cyclin E1 overexpression (Foskolou et al., 2013). However, PROX1 enhanced colorectal cancer progression, and it may promote cell proliferation (Petrova et al., 2008). The same report indicated *PROX1* as a Wnt-beta-catenin-TCF/LEF target in colon adenomas, contributing to the transition from an early to dysplastic stage (Petrova et al., 2008). Connected to this, Prox1 was found to regulate the number of stem cells via cell proliferation to promote expansion of the stem cell population in intestinal adenomas and colorectal cancer after activation of the Wnt pathway, and that the effects were mediated by Annexin A1 and Filamin A (Wiener et al., 2014).

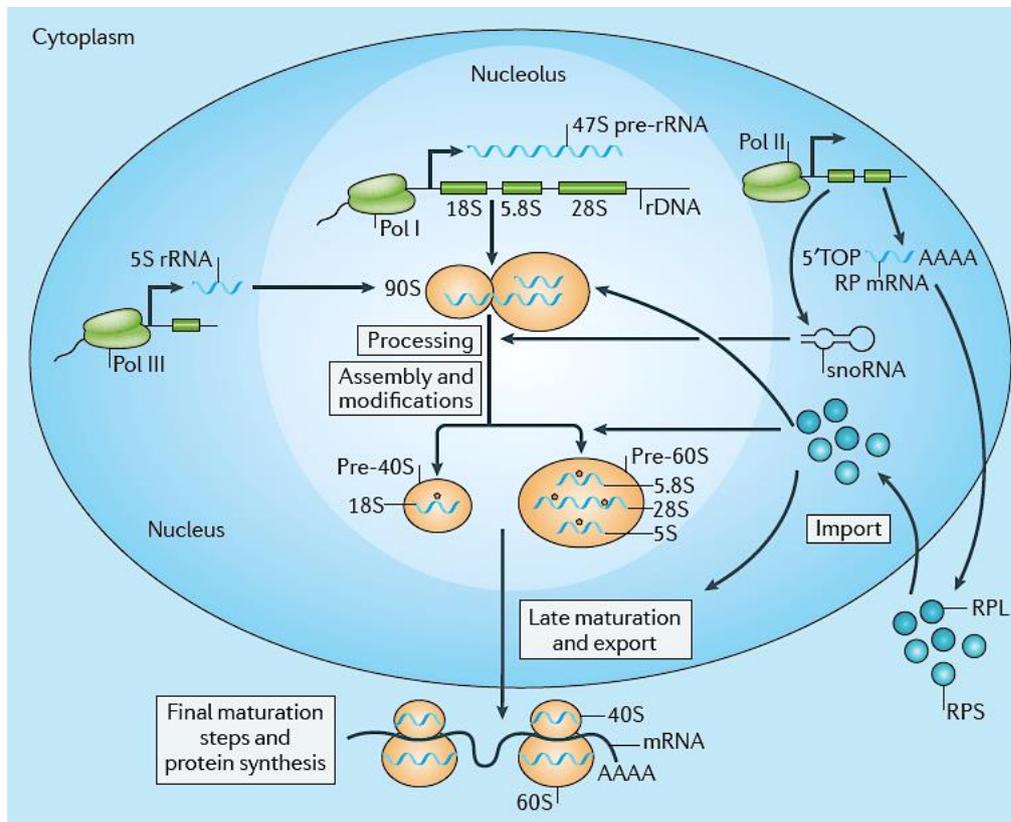
Hypermethylation of *PROX1* has been reported to occur in breast cancer and lymphomas and in brain metastases of breast cancer (Laerm et al., 2007) (Nagai et al., 2003; Versmold et al., 2007). Moreover, mutations of *PROX1* and loss of heterozygosity have been reported in a few cancers (Laerm et al., 2007) (Nagai et al., 2003; Takahashi et al., 2006; Versmold et al., 2007; Yoshimoto et al., 2007).

*PROX1* was previously detected by immunohistochemistry in human astrocytic brain tumors with increasing levels correlating with higher WHO grade (Elsir et al., 2010), and may therefore constitute a useful tool for the diagnosis and grading of gliomas to distinguish low-grade diffuse lesions (grade II) from high-grade (grades III–IV) malignancies. Furthermore, the authors proposed *PROX1* as a novel predictor of survival for grade II astrocytic gliomas, where high level of *PROX1* was associated with poor outcome (Elsir et al., 2012). More recently, *PROX1* was suggested as a useful pathway-specific prognostic biomarker in case of high-grade astrocytomas (Roodakker et al., 2016). While no correlation was found between *PROX1* and survival for patients with primary glioblastomas, higher presence of the protein predicted shorter survival times corresponding to the groups of 1p19q non-codeleted high-grade astrocytomas that have progressed from pre-existing low-grade tumors with an IDH mutation (Roodakker et al., 2016).

These studies raise the question of functional significance of *PROX1* expression in glioma cells, and the cellular signaling pathways it could regulate. *PROX1* may function in glioma initiation and progression by several mechanisms: (a) *PROX1* acts as a key regulator of macro-metastatic lesions in colon cancer outgrowths by promoting adaptation of tumor cells to hypoxia and nutrient deprivation (Ragusa et al., 2014) and high grade gliomas are notoriously hypoxic, hence *PROX1* could be a survival factor in human gliomas. (b) *PROX1* modulates cell proliferation and differentiation in several cellular systems, including CNS and may therefore also have such impact in glioma. A subpopulation of *Prox1*<sup>+</sup> colon cancer cells were shown to display cancer stem cell features (Wiener et al., 2014). *Prox1* depletion led to fewer stem cells and reduced cell proliferation, and decline in intestinal tumor growth via induction of Annexin A1, and reduced level of the actin-binding protein known as Filamin A. *PROX1* is therefore involved in regulating stem cells, a feature that have been shown to be important in glioblastoma cell. (c) *PROX1* regulates the migration and/or tissue invasion of certain cell types (Dadras et al., 2008; Elyada et al., 2011), and it is known that glioma cells are highly invasive and migratory.

## 1.5 RIBOSOME BIOGENESIS: AN ACHILLES' HEEL OF GLIOMA?

Cancer is characterized by a mass of cells growing and proliferating in a haphazard way (Hanahan and Weinberg, 2011), and cancer cells – with their high metabolic demands, need to couple their extensive protein production and accumulation of mass and size with the subsequent divisions to daughter cells. It is thought that bioenergetic pathways are connected to oncogenic drivers in glioma. For instance, the mTOR pathway may provide metabolic flexibility and boost survival of the glioma cells (Strickland and Stoll, 2017). A vast amount of research efforts has therefore focused on energy metabolism, protein synthesis and cell cycle control in tumor cells to exploit their vulnerabilities and open therapeutic avenues (Strickland and Stoll, 2017).



**Figure 1 | Ribosome biogenesis: the essentials.** Adapted from (Pelletier et al., 2018), with permission.

Ribosomes are central to life as the sites of protein synthesis required for proper cell growth and proliferation, and they are composed of many distinct proteins and nucleic acids. Ribosome biogenesis is elaborate, multifaceted, and a massively energy consuming process that demands the function of all three RNA polymerases and an army of non-ribosomal factors for the ribosomal RNA (rRNA) synthesis, maturation and assembly into small and large subunits in a highly coordinated manner (Pelletier et al., 2018). In brief,

the two pre-subunits pre-40S (containing 18S rRNA and 33 distinct ribosomal proteins) and pre-60S (5S, 5.8S and 28S rRNA) are assembled in the nucleolus (Figure 1) (Pelletier et al., 2018). This process is initiated by the RNA polymerase I (Pol I) transcription of 47S precursor rRNAs from ribosomal DNA (rDNA) genes, and its assembly into 90S processome together with 5S rRNA. These are subsequently exported to the cytoplasm completing their final maturation steps, forming the mature 80S ribosome, which begins the manufacturing of proteins (Figure 1) (Pelletier et al., 2018).

Recent molecular profiling studies have identified mutations in genes that encode RPs (Goudarzi and Lindstrom, 2016). Specifically, the RPL5 gene was reported to contain truncating mutation in glioblastoma and other cancer types (Lawrence et al., 2014). Moreover, a number of human disorders linked to dysregulated ribosome biogenesis and function such as Diamond-Blackfan anemia (DBA) – collectively ribosomopathies, are associated with single-copy loss of function mutations in genes encoding specific RPs, or in other cases defects in ribosome biogenesis factors (Goudarzi and Lindstrom, 2016; Mills and Green, 2017). Intriguingly, these patients are known to be at higher risk for developing cancer.

## 1.6 THE NUCLEOLUS IN A “NUTSHELL”

Nucleoli are dynamic nuclear compartments around nucleolar organizer regions (NORs), where biogenesis of ribosomes take place, and they are composed of three structural compartments: the fibrillar centers (FC) and dense fibrillar centers (DFC), where transcription and processing occur, and the granular component (GC) that is the site of pre-ribosome assembly (Figure 1) (Pelletier et al., 2018; Raska et al., 2006). As early as in the 19<sup>th</sup> century, increased number, enlarged size, and irregular morphology of nucleoli have been used to distinguish cancer cells from the normal cells in tumor tissues, and a large body of evidence has indicated nucleolar irregularities as a prognostic marker associated with tumor aggressiveness (Goudarzi and Lindstrom, 2016; Ruggero, 2012).

Apart from their main role as ribosome-producing factories, the nucleoli have emerged in recent years as the central regulatory hub for coordinating genomic organization and the maintenance of genome integrity, cell cycle control, the cellular responses to a wide variety of environmental signals and the quality control for RNA and protein production (Grummt, 2013; Lindstrom et al., 2018). As cellular stress sensors, it is believed that the nucleoli transmit genotoxic, metabolic, transcriptional and other types of stresses to the cell fate gatekeeper p53, through so called “ribosome biogenesis surveillance” pathway that involves NPM1 and other nucleolar factors (Lindstrom et al., 2007).

Given these observations, nucleolar proteins are under intensive investigation for their potential involvement in multiple types of cancer, including glioma, and nucleoli could be promising target for cancer therapeutics (Goudarzi and Lindstrom, 2016; Ruggero, 2012).

## 1.7 NPM1 IN NORMAL CELL BIOLOGY AND CANCER

### 1.7.1 NPM1 as a stress sensing nucleolar chaperone

The ubiquitously expressed NPM1 (also known as B23, NO38, or nucleophosmin) is a multifunctional non-ribosomal phosphoprotein, prominently localized in the nucleoli, and involved in normal cellular processes such as ribosome biogenesis, mRNA transport, genome stability and apoptosis (Box et al., 2016). *NPM1* is evolutionary conserved and consists of 12 exons (NPM1.1 and NPM1.3 are splice variants), encoding the 37kDa phosphoprotein Nucleophosmin 1 found in all Metazoan. The members of NPM family (NPM1-3) share common structural motifs: a core domain at the N-terminal, an acidic domain and a nuclear localization signal at the C-terminal part of the protein (Box et al., 2016).

It has been reported that NPM1 function is required for p53 stabilization in the nucleus by inhibiting p53:MDM2 interaction following UV-induced DNA damage (Colombo et al., 2002; Kurki et al., 2004). Moreover, NPM1 can directly interact with p53 to regulate its transcriptional activation induced by various stressors, and specifically was shown to induce a p53-dependent premature senescence upon its overexpression in diploid fibroblasts (Colombo et al., 2002).

### 1.7.2 NPM1 in cancer

Studies in recent years have underscored NPM1 function both as an oncogene and a tumor suppressor. Of note, reports show NPM1 mutations in leukemia and lymphoma, its overexpression in solid tumors, and rearrangements in different types of cancers (Box et al., 2016; Holmberg Olausson et al., 2015). It is believed that abundant NPM1 contributes to chromatin stability, functional rDNA transcription and ribosome biogenesis, while decreased levels result in genome instability and impairment of p53 stabilization.

NPM1 overexpression has also been reported in glioma tissue and cell lines (Chen et al., 2015). In the tumor tissues, NPM1 expression was correlated with Ki-67 index and the pathological grades, therefore it was suggested as a determinant of poor prognosis (Chen et al., 2015).

## 1.8 THE EMERGING RIBOSOMAL PROTEIN-MDM2-P53 AXIS

### 1.8.1 p53 tumor suppressor

The transcription factor p53 is a tumor suppressor that responds to a broad range of stress signals by orchestrating specific cellular responses which span from induction of a transient (cell cycle arrest) or permanent (senescence) blockade of cell proliferation to apoptosis (Biegging et al., 2014). Moreover, by integrating the activity of metabolite-sensing pathways, p53 participates in the management of cellular metabolism during nutrient and oxygen stress (Humpton and Vousden, 2016).

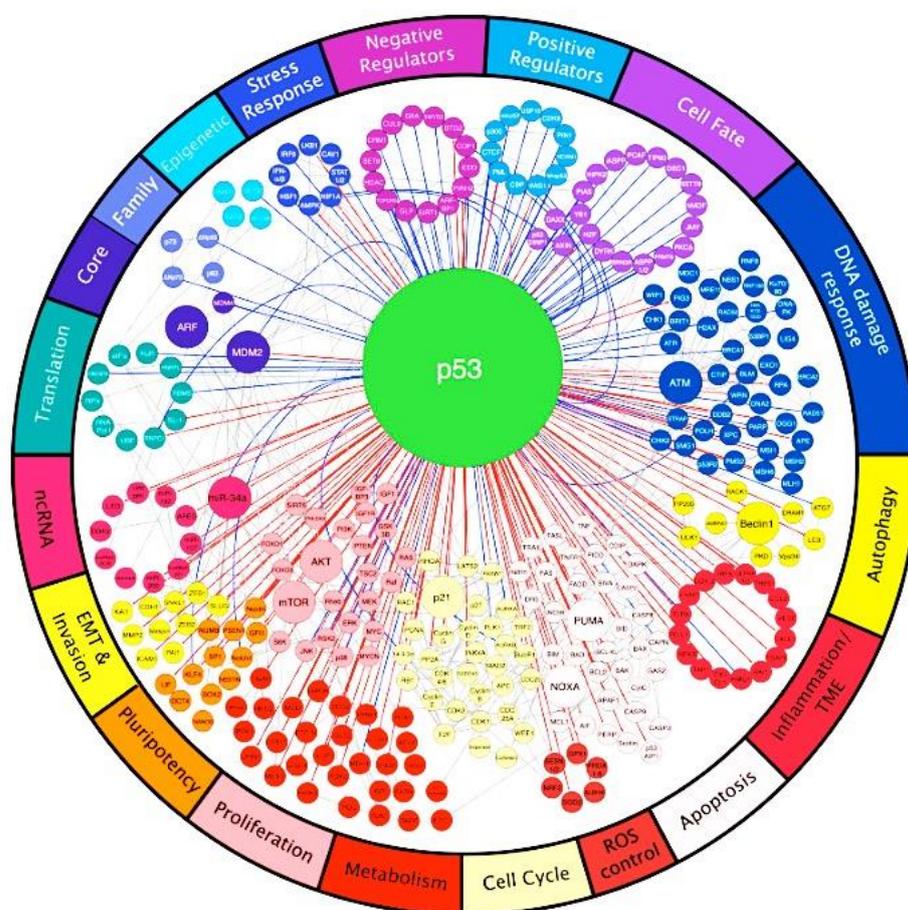


Figure 2. The p53 Network. Adapted from (Kasthuber and Lowe, 2017), with permission.

A wide variety of regulators govern the activity of p53 (Figure 2, top), which, in turn, coordinates many distinct biological processes (Figure 2, bottom); reviewed in (Kasthuber and Lowe, 2017). These cancer-associated stress stimuli include DNA damage, oxidative stress and hypoxia, as well as hyper-proliferative signals and nutrient starvation. Thus, p53 inactivation is considered a pivotal event in the tumorigenic

outgrowth, and more than half of all sporadic tumors have an inactivated p53 pathway. It is also known that individuals with a hereditary genetic condition called Li–Fraumeni syndrome (LFS) who inherit a mutant *TP53* allele are predisposed to a wide range of cancers at young age, pointing to the significance of a functional p53 protein in tumor suppression (Bieging et al., 2014).

On the other hand, a growing number of studies in recent years have underscored non-canonical functions of p53 in control of other cellular processes including stem cell maintenance, cell–cell communication within the tumor microenvironment, and cell metabolism, which could also contribute to tumor suppression (Bieging et al., 2014). In the developing and adult CNS, p53 functions are implicated in self-renewal of neural precursor cells, differentiation, and cell fate decisions. It is believed that p53 can suppress self-renewal of neural stem cells (Meletis et al., 2006), promote differentiation, and thus limit tumorigenesis by inhibiting characteristics of 'stemness' (Bieging et al., 2014). Specifically, it was revealed, through studies of (CNS)-specific *Trp53<sup>-/-</sup>Pten<sup>-/-</sup>* mice, that p53 and PTEN together restrict neural and glioma stem/progenitor cell renewal, promote differentiation and inhibit glioblastoma development (Bieging et al., 2014; Zheng et al., 2008). It was therefore proposed that p53 could inhibit tumorigenesis through suppression of stemness characteristics (Bieging et al., 2014).

Together, an elucidation of p53's physiological functions as well as p53-mediated tumor suppression can pave the way for earlier diagnosis, prognostication, and anti-cancer therapy (Bieging et al., 2014).

## 1.8.2 The mTOR pathway

Increased growth and proliferation is one of the hallmarks of cancer and is often achieved by exploitation of the mTOR pathway to globally upregulate protein synthesis and even boost the process of ribosome biogenesis itself. Therefore, it is commonly assumed that targeting such dependency in malignant cells can be an effective strategy against cancer development.

The mTOR pathway is the key integrator of intercellular signals and environmental stimuli such as stress and availability of growth factors and nutrients. Thereby, it is important in the regulation of cell cycle as a modulator of anabolic versus catabolic processes. Specifically, the crucial role of the mTOR pathway in regulating cell growth and metabolism has connected its inhibition to the protection against aging and its dysregulation to human diseases including cancer. This significance has attracted great

scientific interest in understanding mTOR signaling, as well as clinical interest in the development of mTOR inhibitors (mainly rapamycin derivatives or rapalogs), for the purpose of targeting mTOR in several solid malignancies including glioblastomas. Therefore, rapalogs such as CCL-779 (Temsirrolimus) have been tested in clinical trials, although with limited efficacy to date (Strickland and Stoll, 2017).

Of the two existing multiprotein complexes mTORC1 and mTORC2, the better-known mTOR1 is connected to mRNA translation and protein synthesis, and found upregulated in most cancers (Gentilella et al., 2015). It is believed that ribosome biogenesis and protein synthesis are induced following growth stimulating cues, through mTORC1 phosphorylation of its cellular targets RPS6 kinases (S6K1/2) at Thr389 and the protein initiation factor 4E binding proteins (4E-BP1/2/3) at Ser65 (Gentilella et al., 2015; Strickland and Stoll, 2017). Moreover, the mTORC1 complex is involved in lipid biosynthesis, as well as in regulation of glucose metabolism and mitochondrial function (Gentilella et al., 2015). However, the precise molecular mechanism of mTOR signaling is still not elucidated.

In addition to p53-independent mTOR1 functions, a large body of evidence in recent years has pointed to its crosstalk with the p53 pathway in control of cell fate determination through a number of mechanisms.

### **1.8.3 The concept of “nucleolar stress”**

It is well established that disruption of ribosome biogenesis or nucleolar structure – also known as “ribosomal stress” or “nucleolar stress” could lead to stabilization of the p53 protein. However, mechanisms behind this p53 activation are not fully elucidated, and it is debated whether involvement of the nucleolus in p53 stabilization is indirect or direct (Deisenroth and Zhang, 2010).

The nucleoli are proposed as cellular compartments where p53 regulation occurs (Boyd et al., 2011). Specifically, this model suggests a direct involvement of the nucleolus in catalyzing p53 protein degradation via its ubiquitylation and transport, which is perturbed following nucleolar stress (Boyd et al., 2011).

Another model with substantial amount of both *in vitro* and *in vivo* experimental support is based on the re-localization of diffusible components that can disrupt the p53-MDM2 interaction.

More specifically, under normal conditions, the pre-ribosomal complex 5S RNP, consisting of the 5S rRNA RPL5, and RPL11, is incorporated into nascent 60S ribosomes in the nucleolus, and p53 is degraded through the E3 ubiquitin ligase activity of MDM2. However, following a disruption of ribosome biogenesis, the 5S RNP, also recently referred to as 'impaired ribosome biogenesis checkpoint (IRBC) complex', is redistributed from the assembly into newly synthesized 60S ribosomes in the nucleoli to the binding of MDM2, leading to p53 activation and possible suppression of tumorigenesis (Pelletier et al., 2018; Sloan et al., 2013). Therefore, a crucial consequence of ribosomal stress and 'IRBC response' could be p53-mediated cell cycle arrest and apoptosis (Mills and Green, 2017; Pelletier et al., 2018). In this model, cancer cells having higher levels of protein synthesis are more sensitive to the perturbations of ribosome biogenesis (Lindstrom et al., 2007; Mills and Green, 2017).

Many commonly used anti-cancer drugs interfere with DNA replication or block RNA pol I and RNA pol II transcription targeting dividing cells. A few compounds that induce nucleolar stress have shown some rather promising effects on glioma-derived cells with stem-like properties *in vitro*. Examples of drugs that target ribosome biogenesis include low concentrations (<10nM) of Actinomycin D, which intercalates in GC-rich region of rDNA and preferentially inhibits RNA Polymerase I activity and induces p53 through the nucleolar stress pathway (Macias et al., 2010). Other examples include cisplatin that crosslinks DNA and hijacks UBF, therefore also inhibiting RNA pol I at low concentrations, whereas 5-Fluorouracil inhibits pre-rRNA processing (Bywater et al., 2013). While Actinomycin D is not currently used in brain cancer therapy due of its systemic side effects and toxic effects on mature neurons, other compounds are in the development.

## **2 PRESENT INVESTIGATION**

### **AIM OF THE STUDY**

The overall aim of this thesis was to advance our understanding of glioma tumor biology, and provide novel molecular insights for the clinic.

The three specific aims were to:

- Mechanistically investigate the role of PROX1 and what cellular signaling pathways it regulates in glioblastoma.
- Determine the expression patterns and cellular localization of the NPM1 protein in glial tumors, and its potential role in glioma cell proliferation and apoptosis.
- Investigate if inhibitors of the mTOR pathway affect the activity of the p53 pathway in glioma cells. Specifically, how this may occur through modulation of ribosomal protein-MDM2 interaction in the setting of nucleolar stress.

## 3 RESULTS & DISCUSSION

### 3.1 STUDY I

*Decreased PROX1 levels transition glioblastoma cells into the mesenchymal glioblastoma gene expression subtype.*

#### 3.1.1 Results

Expression of *PROX1* mRNA was analyzed in a combined TCGA dataset containing low and high-grade gliomas extracted from the GlioVis portal (<http://gliovis.bioinfo.cnio.es/>). Intriguingly, the average *PROX1* mRNA expression was significantly lower in grade IV tumors than in grade II and III tumors. Upon survival analysis in the same dataset, a subgroup of cases with higher *PROX1* expression was associated with a median survival of 67.5 months that was significantly higher than the median survival of 34.9 months in the subgroup with lower *PROX1* levels. Subsequent analysis of the gene expression subtypes in grade IV gliomas associated the patient group with highest *PROX1* levels to the classical and proneural subtypes. On the other hand, the group with low *PROX1* mRNA consisted mainly of cases of mesenchymal gene expression subtype.

Further exploration was performed by Gene Set Enrichment Analysis (GSEA) in order to assess gene expression differences in a set of 45 glioblastoma cell lines in the Cancer Cell Line Encyclopedia (CCLE) (Barretina et al., 2012), identifying *PROX1*-associated gene ontology (GO) terms. It was revealed that cell cultures with high *PROX1* expression were enriched with signatures for histone methylation (H3K27Me3), PDGFRB signaling, SOX2 targets, and positive regulation of epithelial proliferation, while cultures with low *PROX1* expression were enriched for ESRRA-, HOXA9- and the Verhaak Mesenchymal glioblastoma subtype-signatures. Furthermore, an analysis of *PROX1* correlated genes, in 375 glioblastoma patients data available from TCGA, identified *MARK1* as the highest correlated ( $r=0.54$ ) gene, which encodes a serine/threonine-protein kinase involved in regulation of cell polarity and microtubule dynamics and proposed as a positive regulator of the Wnt signaling pathway (Kojima et al., 2007; Sun et al., 2001). In addition, several landmark genes including *SEMA6A* ( $r=0.50$ ), *ZEB1* ( $r=0.48$ ) and *MAP2* ( $r=0.46$ ) were found present among the top 50 correlated genes. On the other hand, in this analysis *IL15* (interleukin 15) was identified as the most anti-correlated gene ( $r=-0.40$ ), and *ESR1* (estrogen receptor (ER)-alpha) ranked among the top 20 anti-correlated genes in these samples (rank position 16,  $r=-0.35$ ).

To further evaluate PROX1 expression in glioma, protein levels were measured in a panel of glioma cultures including the ones from the U-343 system, which is a set of cell cultures that were derived from a single glioblastoma (Nister et al., 1986; Nister et al., 1987). Immunoblotting revealed that PROX1 is differentially expressed in glioma cell lines – undetected in U-343 MG while expressed at high levels in U-343 MGa. Furthermore, it was co-expressed with the glioma markers for a cancer stem cell phenotype, SOX2 and GFAP.

PROX1 is a known transcription factor. To gain insights into PROX1-regulated gene networks in glioblastoma, and investigate the functional consequences of PROX1 modulation, the U-343 cultures and newly generated stable cell lines thereof with overexpression or suppression of PROX1, were subjected to global gene expression analysis by RNA sequencing. Of note, the U-343 MG and U-343 MGa cultures were found to represent two cell phenotypes mesenchymal-like and non-mesenchymal, respectively (Nister et al., 1986; Nister et al., 1987). Interestingly, PROX1 modulation in the setting of the U-343 cultures seemed to alter these states, suggested by regulation of GFAP levels. On a larger scale, overexpression of PROX1 in the mesenchymal type glioblastoma culture U-343 MG and PROX1 suppression in the non-mesenchymal type culture U-343 MGa could transition these into the opposite type – defined by Verhaak et al. (Verhaak et al., 2010). Thus, PROX1 can regulate the transition of these transcriptional subtypes in a reversible manner in tumor cells derived from the same tumor.

Based on these results, it was found that PROX1 overexpression affected regulators of the cell cycle, and increased the rate of cell proliferation of U-343 MG cells. Furthermore, this study places PROX1 under the control of the SOX2 transcription factor, and indicates decreased levels of both SOX2 and PROX1 in response to treatment of glioblastoma cells with CDK2 small molecule inhibitor CVT-313.

Finally, experiments performed in this study identified THRAP3 as a novel interacting partner and potentially a co-regulator of PROX1 expression and/or protein stability.

### **3.1.2 Discussion**

PROX1 was previously proposed as a predictor of survival for gliomas WHO grade II, by using tumor histopathology methods (Elsir et al., 2011), but whether the observed correlation is causative has not been shown. The study herein was aimed at bringing biological insights into PROX1 expression and function in glioblastoma. The results propose utility of PROX1 expression levels as a prognostic marker in glioblastomas,

distinguishing the gene expression subtypes, and *PROX1* maintenance of a glial stemness profile and proliferation capacity with high levels of G1-cyclins.

The inter-tumor heterogeneity observed in glioblastoma patients poses a great challenge to diagnosis and therapy. Moreover, cells within a single tumor exhibit distinct phenotypes, genotypes and epigenetic states, as it was highlighted by a recently published landmark single-cell transcriptome analysis (Patel et al., 2014). The study proposed potential plasticity among glioblastoma molecular subtypes under microenvironmental influences, and underscored the significance of intratumoral heterogeneity where tumors with higher percentage of proneural cells had better clinical outcome than those with intermixture of different subtypes (Patel et al., 2014). Reflecting cancer evolutionary dynamics, such intratumoral heterogeneity and redundant signaling pathways can also provide explanations for the common failures of conventional and targeted therapies to result in long-term remissions (Sottoriva et al., 2013).

The expression analysis of TCGA data suggested that glioblastoma patients with tumors having low *PROX1* mRNA levels were enriched for mesenchymal gene expression subtype and shorter survival. Furthermore, the transcriptional analysis of *PROX1* functional effects in cell cultures, combined with co-expression analysis in the TCGA, CCLE, HGCC, and single-cell RNA sequencing data from 5 tumors (Patel et al., 2014), describes *PROX1* as a regulator of glioblastoma tumor evolution that could distinguish different cell populations in these heterogeneous tumors.

Importantly, *PROX1* was found to be a regulator of glioblastoma transcriptional profiles, as discussed earlier. According to the presented analysis of TCGA data, *PROX1* expression is increased from grade II to III, decreased in grade IV tumors, and is found significantly higher in proneural and classical glioblastomas than in those with mesenchymal profile. *PROX1* could therefore mark a transitory stage in the evolution of gliomas, potentially similar to its role in the CNS development as a determinant of the neuronal and glial cell developmental paths (Elsir et al., 2012; Torii et al., 1999). A consensus point, emerging from a wealth of molecular profiling of glioblastomas in recent years is the general recognition of the two proneural and mesenchymal subtypes. It is believed that most glioblastomas evolve from a proneural-like precursor glioma to the mesenchymal gene expression subtype; for instance, via loss of *NFI* that is a later event during tumor evolution (Ozawa et al., 2014). While detailed functions of *PROX1* in glioma tumor evolution remains to be elucidated, it appears to regulate the transition from proneural/non-mesenchymal to mesenchymal profile in glioblastoma. In early or low-

grade gliomas, one could speculate that high-PROX1 expressing cells in a tumor are derived from a cell of origin with stem like properties, which normally are committed to differentiate into oligodendrocytes (Bunk et al., 2016). In extension, glioblastomas with high PROX1 could be suspected to arise from oligodendrocyte precursor cells, which is one suggested source of glioblastomas (Lindberg et al., 2009). Given that increasing PROX1 levels in low-grade gliomas have been shown to correlate with increasing poor prognosis (Elsir et al., 2011), PROX1 could here be speculated to drive an increased stemness and proliferation phenotype during tumor cell evolution. While in glioblastomas loss of PROX1 would occur during a later stage of tumor cell evolution. As shown, increasing PROX1 levels in cells will increase stemness gene expression, proliferation and tumor formation capacity (Figure 3). Intuitively, this should coincide with a clinically less favorable situation. However, it is low PROX1 levels that correlate with shorter survival in glioblastoma. Per a tumor cell evolution model of tumors with intra-tumoral heterogeneity the mesenchymal subtype expression profile based on bulk tumor measurements could be reflected by tumors that have a higher fraction of cells that have progressed in a tumor cell evolution perspective. These different tumor cell populations raise the question of which are more relevant to target, the more progressed cells, the more stem like cells or both?

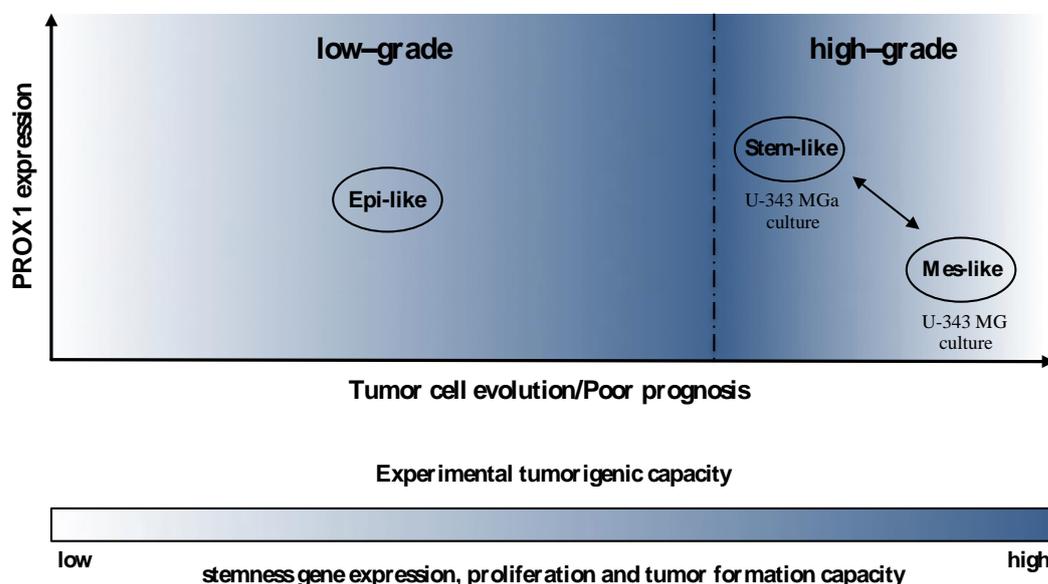


Figure 3. A hypothetical model for PROX1 in glioma tumor evolution.

How can the correlation of lower *PROX1* expression with the mesenchymal profile, and worse patient outcome be explained? Characterized by a protective p53 program, ionizing

radiation response in an *in vivo* model of glioblastoma induced apoptotic gene expression program and suppressed cell cycle progression (Halliday et al., 2014). This response was reported to alter the gene expression profile from proneural to mesenchymal. In agreement, reduced p53 level and increased rate of cell proliferation coincided following PROX1 overexpression in U-343 MG cells with mesenchymal to non-mesenchymal transition. Of note, a revised view on p53 mechanisms of tumor suppression suggests p53 inhibition of stem cell self-renewal (Meletis et al., 2006), as well as blocking reprogramming of differentiated cells into stem cells (Bieging et al., 2014). Thus, it would be of interest to further investigate PROX1 regulation of stemness gene signature in relation to decreased p53 protein levels observed in this study.

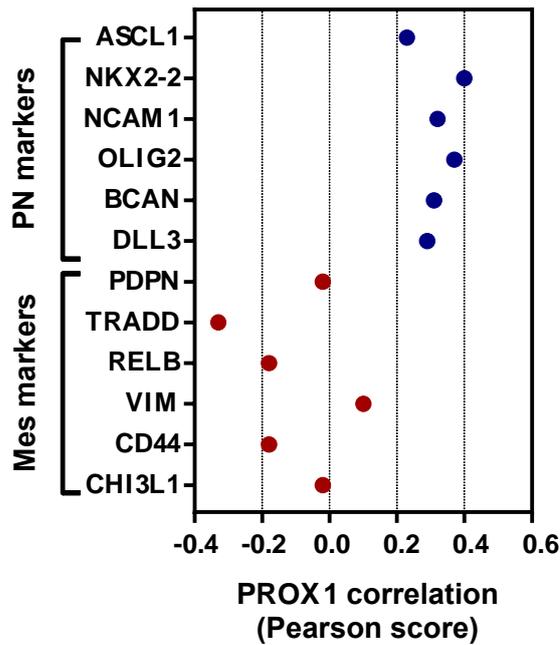
An integrated analysis of G-CIMP positive tumors associated this phenotype to *IDH1* mutations in low- and intermediate-grade gliomas, and upregulation of genes related to cell metabolism and positive regulation of macromolecules (Noushmehr et al., 2010). Furthermore, an EMT/PMT shift may be dependent on metabolism alterations, as it was recently shown (Shaul et al., 2014). Given that PROX1 could regulate metabolic processes, the expression differences observed here might reflect metabolic adjustments by the tumors as they evolve from low-grade to high-grade. In line with the Warburg's effect, it could be hypothesized that more proliferative non-mesenchymal tumors with higher *PROX1* mRNA expression preferentially convert glucose to lactate, whereas mesenchymal tumor cells could use the oxidative phosphorylation pathway in a more energy-efficient manner.

The performed analysis herein places PROX1 downstream of the pluripotency and neurodevelopmental factor SOX2, which has been suggested to be a driver of a cancer stem cell behavior, and gliomagenesis (Hagerstrand et al., 2006; Suva et al., 2014). The GSEA identified a signature for trimethylation of histone 3 on lysine 27 (H3K27me3), associated with gene silencing. The methylation of H3K27 is known to be mediated by Enhancer of Zeste homolog 2 (EZH2) (Czermin et al., 2002). *EZH2* is frequently amplified or overexpressed in a number of cancer types including glioblastoma (Lee et al., 2008), and has a canonical role in gene silencing through H3K27me3 (Vire et al., 2006). *EZH2* is strongly upregulated in Ewing embryonic tumors of undifferentiated mesenchymal origin, and blockade of *EZH2* was reported to induce expression of several hallmark epithelial and neuroectodermal differentiation genes including *SOX11* and *GFAP* in cell lines (Richter et al., 2009). In conjunction with this, SOX4, *EZH2* and HDAC3 together form a co-repressor complex that binds to the miR-31 promoter, causing

repression of miR-31 through an epigenetic mark by H3K27me3 and by histone acetylation in esophageal cancer cells (Koumangoye et al., 2015). Furthermore, PROX1 and HDAC3 interact in a co-repressor module, which co-occupy extensive genomic binding sites, revealing a metabolic signature in the mouse liver (Armour et al., 2017). Therefore, *PROX1* involvement under other glioblastoma oncogenes that functionally overlap with *SOX2* such as *EZH2* should be pursued.

We should also recall that the higher proliferative capacity of PROX1 overexpressing cells *in vitro* – also suggested recently by others (Xu et al., 2017), may not reflect the *in vivo* situation where a mixture of cells is more strictly controlled in their niche. In the tumor tissue PROX1 expressing cells may exist in a more stem-like resting state, but with potential to proliferate. In support of this notion, a previous investigation by Patel et al. observed a striking contrast between the activity of cell cycle program in the *in vitro* glioblastoma models scoring almost 100% positive for the “cell cycle module”, and the single-cell transcriptome of tumors, where it ranged from just 1.4% to 21.9% proliferating cells (Patel et al., 2014).

It was reported that the EMT status is associated with worse overall survival in glioblastomas, ovarian and gastric cancers but intriguingly no association was found in other carcinoma types investigated (Tan et al., 2014). The authors developed a generic EMT score based on cancer specific transcriptomic EMT signatures and used it to establish an EMT spectrum across various cancers, showing that glioblastomas primarily have mesenchymal gene expression pattern (Tan et al., 2014). Another recent study evaluated the expression of 12 glioblastoma signature genes (6 representative markers for each proneural and mesenchymal subtype), with the aim to develop a clinically applicable method for differentiating these transcriptional subtypes. A predominant “metagene score” was calculated by subtracting the “mesenchymal score” (as the average value of  $\Delta\Delta\text{Ct}$  of 6 markers) from the “proneural score” in glioma samples of different grades as analyzed by quantitative RT-PCR, which was able to distinguish proneural/mesenchymal, and decreased in cases of tumor recurrence and malignant transformation (Murata et al., 2015). Moreover, the mesenchymal score showed a positive correlation with the tumor grade, whereas the proneural score did not (Murata et al., 2015). Interestingly, a survey of *PROX1* correlation with these markers in TCGA data from 206 glioblastoma patients (Goudarzi KM et al., unpublished data) shown in Figure 4, suggests that *PROX1* mRNA levels alone is sufficient to distinguish between these markers, thus allowing such mRNA quantification in a feasible and clinically straightforward manner.



**Figure 4. PROX1 correlation with selected proneural /mesenchymal markers in TCGA data**

Collectively, PROX1 may constitute a promising and a clinically applicable tool to distinguish mesenchymal from non-mesenchymal tumors, and potentially assess the level of heterogeneity within tumors. Also, the significance of PROX1 in malignant transformation merits further investigation.

Future experimental inquiries should unravel additional pieces to the molecular puzzle that underlies PROX1 function, not only in gliomas and other cancers but also in healthy tissues, which in turn may help direct us towards cancer prevention, earlier diagnosis, improved prognosis, and novel avenues for therapeutic interventions.

## 3.2 STUDY II

*NPM1 histone chaperone is upregulated in glioblastoma to promote cell survival and maintain nucleolar shape.*

### 3.2.1 Results

NPM1 protein levels and subcellular localization were initially assessed in a panel of 60 gliomas (16 grade I, 16 grade II, 15 grade III, & 13 grade IV) by immunohistochemical (IHC) staining, using a previously validated monoclonal antibody (Holmberg Olausson et al., 2014). The IHC analysis revealed nucleolar staining of NPM1 in the tumors of all grades, displaying an overall increase in the signal intensity from grade I to IV astrocytomas that was significant when comparing grade I and IV tumors ( $p=0.000245$ , Chi square test). Moreover, grade IV tumors were distinguished with a clear increase in nuclear staining of NPM1 in 12 out of 13 cases. Consistently, NPM1 was detected in both nucleoli and nucleoplasm of a panel of glioblastoma cell lines tested. Moreover, IHC analysis revealed Npm1 protein in the nucleoli of both undifferentiated mice NSCs cultured as spheres and differentiated NSCs grown as monolayers, while in addition a more generalized nucleoplasmic staining was noted in the more undifferentiated cells.

To explore NPM1 function in glioma, NPM1 was depleted using siRNA, resulting in a transformed nucleolar morphology in cells, assessed by phase contrast microscopy, Acidic Toluidine Blue O and silver (AgNOR) staining assays of the nucleoli, and immunofluorescence (IF) staining of nucleolar protein fibrillar. Of note, the nucleolar re-organization in NPM1 depleted cells was not akin to structural changes induced by inhibitors of RNA polymerases I and II, Actinomycin D (5nM) and DRB (25  $\mu$ M) treatment of the cells, respectively, which were used in the analysis as controls. Moreover, the siRNA-mediated knockdown of NPM1 did not cause a major alteration in the cell cycle distribution, suggesting that most of NPM1 protein is not needed to support proliferation of the glioma cells.

We hypothesized that NPM1 depletion could sensitize cells to apoptosis and in order to test this glioma cultures were treated with siNPM1 as well as Actinomycin D. The NPM1 depletion alone did not induce evident signs of apoptosis, but decreased the survival of Actinomycin D treated cells, as indicated by increased positivity for cleaved caspase-3 protein in IF analysis.

In addition, NPM1 depletion sensitized U251MG glioma cells to concentrations of chemotherapeutic drugs Temozolomide and 5-Fluorouracil, but did not have much of an effect in the U343MGa Cl2:6 cell line.

Finally, siNPM1 treatment rendered glioma cells more sensitive to the detrimental effects of linker histone H1.5 knockdown, assessed by increased positivity for cleaved caspase-3 in IF analysis. The histone H1.5 is a key component of chromatin architecture that binds NPM1 (Holmberg Olausson et al., 2014), and this association was confirmed using a nuclear complex co-IP in the presented study II.

### 3.2.2 Discussion

Consistent with other recent reports (Chen et al., 2015; Kuo et al., 2015), high expression of NPM1 in astrocytic glioma samples (grades III-IV) was confirmed by histochemical analysis performed herein with increasing intensity in glioblastoma tissues. Of note, high NPM1 expression has been associated with poor survival in glioma patients (Chen et al., 2015; Kuo et al., 2015). Secondly, the study evaluated subcellular localization pattern of NPM1, and marked a nucleoplasmic staining in glioblastomas, in addition to the nucleolar staining, which was unitedly observed in both grade III and grade IV tumors. One interesting possible explanation for the presence of nucleoplasmic NPM1 observed in glioblastoma tissues (besides its overexpression and nucleolar spillover), consistent with the cell lines, is the change of chromatin landscape in the highest-grade tumors. Based on our observations, NPM1 should not be considered as an exclusive nucleolar marker.

NPM1 involvement in higher-order chromatin organization, and interactions with factors such as linker histone H1.5 has been previously reported (Holmberg Olausson et al., 2014; Li et al., 2012). Earlier investigations indicated H1.5 in the maintenance of condensed chromatin and found enrichment of genes involved in apoptosis following H1.5 depletion (Li et al., 2012). It was hypothesized that NPM1 could maintain the structural organization of the cell nucleoli via interaction with chromatin remodeling factors such as H1.5.

Depletion of NPM1 triggered evident nucleolar distortion. Furthermore, NPM1 depletion (by itself) sensitized glioma cell line U251MG to concentrations of Temozolomide and 5-Fluorouracil, but such pro-survival effect was not as apparent in other cell lines tested. The U251MG cells have a higher degree of aneuploidy that could be an underlying reason for higher sensitivity of these cells to drug-induced DNA damage. It should also be noted that all three glioma lines used in these experiments appear rather drug-resistant.

Therefore, it is of interest to explore these findings in a larger panel of glioma cells that also include cultures in NSC condition.

Unlike NPM1, depletion of H1.5 induced cell death in all cell lines tested. Intriguingly, the cytotoxic effect was more dramatic when the two proteins were co-depleted, which points to a pro-survival role for NPM1 connected to the maintenance of genome integrity in glioma.

Taken together, NPM1 is upregulated in glioblastoma tissues and cell lines, displaying a pattern of distribution non-exclusive to the nucleoli, and may promote viability of stressed cells during glioma pathogenesis, which merits further investigation.

### 3.3 STUDY III

*mTOR inhibitors blunt the p53 response to nucleolar stress by regulating RPL11 and MDM2 levels.*

#### 3.3.1 Results

This study investigated the p53 response to nucleolar stress concurrent with inhibition of the mTOR pathway in osteosarcoma and glioblastoma cells with wild type p53. The results revealed that both synthetic mTOR inhibitors such as Temsirolimus or PP242, and “natural” inhibitors such as Rapamycin, Resveratrol or caffeine could impair p53 stabilization induced by low dose of drug Actinomycin D (5 nM). Specifically, the protein level of p53 transcriptional target p21 was also reduced, corresponding to the p53 decline.

To assess the biological consequences of p21 inhibition as a readout for nucleolar stress and impairment of p53 activation, the cells were treated with different combinations of Rapamycin (100 nM, high concentration), Actinomycin D (5 nM, low concentration), and Nutlin-3 (10  $\mu$ M) and the drug-induced morphological effects were monitored using light microscopy. Nutlin-3 is a potent small-molecule that restores p53 activity via disruption of p53-MDM2 interaction (Vassilev et al., 2004) and is not linked with nucleolar stress. It therefore served as a control in these experiments. Visual inspection of the cell cultures and a comparison of cell viability by MTT assay suggested that combination of Rapamycin and Actinomycin D had less potent growth inhibitory effect than Rapamycin plus Nutlin-3. Further investigation revealed an increase in the percentage of cells in S-phase corresponding to Rapamycin and Actinomycin D co-treatment compared with Actinomycin D treatment alone, whereas no major difference in cell cycle distribution was observed with Rapamycin and Nutlin-3 versus Nutlin-3 only. Note that, treatment of cells with Actinomycin D and Nutlin-3 applied as single agents resulted in a cell cycle profile with accumulated cells in G1 and G2/M phases, and fewer cells in S-phase, compared to DMSO-treated control.

Rapamycin may interfere with p53 protein stabilization/activity via inhibition of mTOR pathway and a subsequent reduction in expression of RPs, specifically MDM2 binding protein RPL11 (similarly RPL5). To test this hypothesis, p53 and MDM2 protein half-life assays were coupled with the analysis of ribosomal protein binding to MDM2 using *in vivo* labeling and immunoprecipitation. We could conclude that reduced levels of RPL11 and binding of RPL11 to MDM2 may be of importance, but it appeared as this was not the only explanation. For example, we noted that Rapamycin treatment resulted in increased levels of endogenous MDM2 and an inhibition of its phosphorylation at Ser166.

### 3.3.2 Discussion

Study III assessed the utility of combinatorial anti-cancer strategies including mTOR inhibitor compounds and those that trigger nucleolar stress, and investigated the interplay of mTOR and p53 pathways in this setting. Because Rapamycin analogues or combined mTOR/PI3K inhibitors have shown promise in clinical trials for several tumor types including glioma, the experiments combined this class of drugs with small molecules that directly interfere with rDNA transcription or p53 hyper-activating drugs. Surprisingly, the pre-treatment of glioma cells with Rapamycin was found to markedly blunt p53 activation following a low dose of Actinomycin D but not when using the MDM2 inhibitor Nutlin-3. Nutlin-3 disrupts binding between p53 and MDM2 and does not require ribosomal proteins for its effects to activate p53.

The results from this study did not indicate any synergism in growth inhibitory effects of the simultaneous drug treatments. On the contrary, co-treatments with Rapamycin and Actinomycin D increased the number of BrdU<sup>+</sup> cells, coinciding with a change in cell cycle distribution towards more cells in the S phase, compared to Actinomycin D alone. In addition, induction of p21 was attenuated when cells were co-treated with the cytostatic concentration of the drugs used in the experiments. Importantly, mTOR inhibitors did not blunt the p53 response to Nutlin-3 treatment to the same extent as in the case of Actinomycin D.

Following these observations, experiments were designed to determine the mechanism(s) behind apparent mTOR-p53 pathway crosstalk based on the prevalent indirect model, where cellular localization of p53/MDM2-interacting factors could determine p53 levels. Accumulating evidence indicate that p53 is induced following impaired rRNA synthesis, disruption of rRNA modification and processing, or an imbalance of RPs. The prevalent mechanism put forward for p53 activation is through the RP-MDM2-p53 axis. In brief, excessive amount of RPs caused by an inhibition of rRNA synthesis, or availability of free ribonucleoprotein intermediates or unprocessed rRNA to bind MDM2 could lead to p53 stabilization (Deisenroth and Zhang, 2010).

An established factor as such is the essential component of 5S RNP complex RPL11, which contributes to p53 stabilization following nucleolar stress (Goudarzi and Lindstrom, 2016; Sloan et al., 2013). Notably, *de novo* synthesis of RPL11 is required for this effect. It was shown that Rapamycin suppresses mitogen and amino acid induced activation of mTOR and translation of 5' TOP mRNAs, which is mediated in some cases by S6K (Gentilella et al., 2015). Given that RPL11 is translated as a 5' TOP mRNA, it was

hypothesized that treatment of cancer cells with inhibitors of the mTOR pathway leads to reduced synthesis of RPL11, and thereby p53 destabilization in this setting. Notably, these observations may also be extended to other compounds inhibiting mTOR including caffeine and wortmannin, and may explain effects on p53, ageing and cancer.

It was found that depletion of RPL11 mimicked the effect of rapamycin treatment with regards to inhibition of p53 response to nucleolar stress. However, rapamycin treatment did not reduce RPL11 and p53 levels in a consistent manner in the cell lines tested. Notably, rapamycin treatment increased the endogenous level of MDM2, although phosphorylation of MDM2 at Ser-166 was inhibited. Therefore, other mechanisms should be involved that merit further research.

Taken together, these results indicate that Rapamycin among other inhibitors of mTOR pathway and ribosome biogenesis, interfered with p53 protein stabilization following nucleolar stress in osteosarcoma and glioma cell lines; a finding that could be important in the design of clinical trials involving Rapamycin-like compounds. The results also underscore the complexity of mTOR–p53 interplay in cancer, which should be further investigated.

## 4 SIGNIFICANCE AND FUTURE PERSPECTIVES

How can we treat gliomas more effectively? Conventionally, gliomas have been classified into oligodendroglioma (WHO grades II–III), oligoastrocytoma (WHO grades II–III), and astrocytoma (WHO grades I–IV). However, a lack of consensus definition for gliomas as a larger class of histologies has made it difficult to compare and evaluate results from different studies (Ostrom et al., 2014). Advancements in molecular biomarkers of glioma entities will pave the way to improved treatment approaches in neurooncology.

Specifically, glioblastomas feature distinct phenotypic, genotypic, and epigenetic states forming a complex ecosystem. Studies of molecular biomarkers are pivotal in glioblastoma research as the existing biomarkers investigated so far are not robust enough to implement in the clinic on a stand-alone basis. Moreover, of the potential biomarker genes studied in recent years, for instance via mining of databases such as TCGA, none has shown enough specificity to predict the complex course of disease progression and patient outcome. Therefore, the current view is that detailed characterization of molecular signatures in glioblastoma and a more individual therapeutic approach could provide better alternatives for the clinic and facilitate development of new therapies against glioblastoma.

Remarkably, the gut microbiota has emerged in recent years in pathogenesis of different diseases including cancer, as well as in response to therapies (Zitvogel et al., 2017). It was reported that acetate synthesized by the intestinal bacteria, is a potential oncometabolite (as opposed to butyrate) that supports the growth of human glioblastomas and brain metastases (Mashimo et al., 2014; Zitvogel et al., 2017). Moreover, with the accumulating evidence on the gastrointestinal tract and brain bidirectional communication involving immune mechanisms (Zitvogel et al., 2017), it is not farfetched to expect that similar risk factors that cause disequilibria in intestinal bacteria through our lifespan could contribute to the development of covert “cancerized fields” that eventually manifest in a specific cancer diagnosis.

In light of the aforementioned evidence on microbiota–gut–brain axis, one can anticipate that future research will bring more attention to PROX1 involvement in maintenance of stemness features as well as energy homeostasis in tissues identifying common risk factors that may connect gliomas with ‘metabolic inflammation’ and the gastrointestinal malignancies. The findings in the study I in this thesis point to PROX1 participation in diverse regulatory programs central to glioma biology. Indeed, intriguing similarities were observed between the PROX1-related molecular circuitry in glioma and the previous

reports from gastric and colorectal cancers (Laitinen et al., 2017; Ragusa et al., 2014). The RNA-seq based expression analysis of glioblastoma cultures predicted PROX1 involvement in glycolysis, inflammatory response, adipogenesis, and the regulation of NF- $\kappa$ B pathway. Meta-analyses of genome-wide association studies have associated single-nucleotide polymorphism in *PROX1* with fasting glycaemia and type2 diabetes mellitus (DIAGRAM et al., 2014; Dupuis et al., 2010), and reduced *PROX1* expression was reported to alter  $\beta$ -cell insulin secretion, thereby conferring susceptibility to type2 diabetes (Lecompte et al., 2013). Strikingly, pre-diagnostic hyperglycaemia and diabetes were linked to a lower risk of developing glioma in a recent meta-analysis (Zhao et al., 2016), and excess glucose consumption by the preclinical tumor was suggested to paradoxically account for the inverse association between blood glucose and glioma (Schwartzbaum et al., 2017). Alternatively, hyperglycaemia could induce apoptosis and inhibit proliferation of neural stem cells, possibly via activation of JNK/p38 MAPK pathways and the delay of G1-S transition in the cells (Chen et al., 2013a; Schwartzbaum et al., 2017), thereby conferring protection against glioma.

Further, NF- $\kappa$ B has emerged in recent years to integrate metabolism and inflammation with profound implications for oncogenesis (Tornatore et al., 2012), and its activation was recently associated to PROX1 in glioblastoma cells (Xu et al., 2017). Therefore, considering the aforementioned observations, it is of great interest to address PROX1 expression and function in healthy versus inflamed and tumor tissues with regards to energy homeostasis and immunity to find viable indications for cancer prevention, improved diagnosis and therapy. Moreover, since PROX1 is involved in regulation of metabolic clock gene expression (Dufour et al., 2011), and perturbed clock is linked to malignancies including gliomas (Chen et al., 2013b; Sahar and Sassone-Corsi, 2009); a possible role for PROX1 in clock-related differences between tumors and healthy cells should not be ignored.

On a related note, it was recently reported that Rapamycin upregulates triglycerides in hepatocytes by inhibiting *Prox1* expression in hepatocytes, suggesting that the mTOR pathway is involved in the regulation of triglycerides by controlling Prox1 levels (Kwon et al., 2016). Specifically, Rapamycin affects Prox1 protein but not its transcript. Rapamycin upregulated the amount of triglyceride and downregulated the expression of Prox1 in HepG2 cells by reducing protein half-life while the transcript levels remained unaffected (Kwon et al., 2016). Whether such regulation exists in the brain has not been explored, and would be of interest to investigate in the context of gliomas.

The epidemiology of glioblastomas indicates increasing incidence in adults with age, slightly higher occurrence in men, and among the most conclusive prognostic markers are age of onset, the extent of tumor resection and Karnofsky performance status (Ostrom et al., 2014; Stupp et al., 2014). Unlike other solid tumor types, glioblastomas rarely metastasizes to other organs. On the contrary, brain metastasis occur from gastrointestinal cancers, which are on the rise in Sweden (Smedby et al., 2009) and worldwide, with an underestimated frequency due to a lack of routine brain scans and overlooked asymptomatic lesions; for a systematic review of 74 reported studies with brain metastasis originated from gastrointestinal cancer see (Esmailzadeh et al., 2014). It was revealed from the data available for 2538 patients with brain metastasis that 2028 patients (79.90%) had colorectal cancer, followed by 233 patients (9.18%) with liver and 148 patients (5.83%) with gastric malignancies.

A wealth of research in recent years has underscored disruption of ribosome biogenesis or nucleolar stress in cancer pathogenesis. Current research is focused on how cancer cells maintain high efficiency and accuracy of ribosome synthesis and an important question is how ribosome biogenesis is connected to stress responses and cell cycle control. However, the mechanisms sensing nucleolar stress and how anti-proliferative p53 is activated following nucleolar stress are not fully understood, but should be resolved in the coming years. The challenges that cancer cells meet due to their high demand of ribosome manufacturing for sustained tumor growth, and their mechanisms of quality control surveillance can be exploited for the design of novel targeted therapeutics.

Despite the large amount of research efforts that has linked the mTOR pathway to cell growth and survival, there has been a limited progress for mTOR inhibitors in the clinical trials. Furthermore, it is inherently challenging to determine the function of particular ribosomes in a cell, and thus investigating the effects of the drugs on fundamental cell processes such as ribosome biogenesis and p53 activation, for example, by using biochemical analysis. Indeed, an advanced understanding of the crosstalk between mTOR and p53 pathways is required for the utility of mTOR inhibitors as therapeutic options, specifically in combinatorial strategies to overcome drug resistance and enhance efficacy. The main finding in the study III was that the natural and synthetic mTOR inhibitors could blunt the p53 response to nucleolar stress induced by chemotherapeutic agents such as Actinomycin D. First and foremost, this observation might have significance for the design of various combinatorial anti-cancer drug treatment regimens using mTOR inhibitors. In other words, the simultaneous inhibition of the p53-dependent nucleolar

pathway – likely a disadvantage in combinatorial treatments must be considered in this setting. Secondly, similar effect of physiologically relevant concentrations of natural mTOR inhibitors, for example caffeine on basal levels of p53 and or p21 in heavy coffee drinkers merits further inquiry.

This thesis explored the significance of two molecular biomarkers PROX1 and NPM1 in glioblastoma, and investigated the interplay of p53 and mTOR pathways via testing the synergism of a range of chemotherapeutic agents. Looking ahead, extensive progress is envisioned during the coming years in our understanding of glioma tumor biology. In unison, it is hoped that studies presented in this thesis will bring new perspectives on biomarkers of high-grade gliomas, provide some insight into the possible future use of mTOR inhibitors and related compounds in glioma, and contribute to future improvements in the lives of glioma patients.

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## 6 REFERENCES

- Alcantara Llaguno, S. R., and Parada, L. F. (2016). Cell of origin of glioma: biological and clinical implications. *Br J Cancer* *115*, 1445-1450.
- Altaner, C. (2008). Glioblastoma and stem cells. *Neoplasma* *55*, 369-374.
- Armour, S. M., Remsberg, J. R., Damle, M., Sidoli, S., Ho, W. Y., Li, Z., Garcia, B. A., and Lazar, M. A. (2017). An HDAC3-PROX1 corepressor module acts on HNF4alpha to control hepatic triglycerides. *Nat Commun* *8*, 549.
- Austin, S., and St-Pierre, J. (2012). PGC1alpha and mitochondrial metabolism--emerging concepts and relevance in ageing and neurodegenerative disorders. *J Cell Sci* *125*, 4963-4971.
- Bao, S., Wu, Q., Sathornsumetee, S., Hao, Y., Li, Z., Hjelmeland, A. B., Shi, Q., McLendon, R. E., Bigner, D. D., and Rich, J. N. (2006). Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Res* *66*, 7843-7848.
- Barretina, J., Caponigro, G., Stransky, N., Venkatesan, K., Margolin, A. A., Kim, S., Wilson, C. J., Lehar, J., Kryukov, G. V., Sonkin, D., *et al.* (2012). The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* *483*, 603-607.
- Betschinger, J., Mechtler, K., and Knoblich, J. A. (2006). Asymmetric segregation of the tumor suppressor brat regulates self-renewal in *Drosophila* neural stem cells. *Cell* *124*, 1241-1253.
- Bhat, K. P. L., Balasubramanian, V., Vaillant, B., Ezhilarasan, R., Hummelink, K., Hollingsworth, F., Wani, K., Heathcock, L., James, J. D., Goodman, L. D., *et al.* (2013). Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. *Cancer Cell* *24*, 331-346.
- Biegging, K. T., Mello, S. S., and Attardi, L. D. (2014). Unravelling mechanisms of p53-mediated tumour suppression. *Nat Rev Cancer* *14*, 359-370.
- Bielen, A., Perryman, L., Box, G. M., Valenti, M., de Haven Brandon, A., Martins, V., Jury, A., Popov, S., Gowan, S., Jeay, S., *et al.* (2011). Enhanced efficacy of IGF1R inhibition in pediatric glioblastoma by combinatorial targeting of PDGFRalpha/beta. *Mol Cancer Ther* *10*, 1407-1418.
- Box, J. K., Paquet, N., Adams, M. N., Boucher, D., Bolderson, E., O'Byrne, K. J., and Richard, D. J. (2016). Nucleophosmin: from structure and function to disease development. *BMC Mol Biol* *17*, 19.
- Boyd, M. T., Vlatkovic, N., and Rubbi, C. P. (2011). The nucleolus directly regulates p53 export and degradation. *J Cell Biol* *194*, 689-703.
- Brabletz, T., Kalluri, R., Nieto, M. A., and Weinberg, R. A. (2018). EMT in cancer. *Nat Rev Cancer* *18*, 128-134.
- Brennan, C. W., Verhaak, R. G., McKenna, A., Campos, B., Nounshmehr, H., Salama, S. R., Zheng, S., Chakravarty, D., Sanborn, J. Z., Berman, S. H., *et al.* (2013a). The somatic genomic landscape of glioblastoma. *Cell* *155*, 462-477.

- Brennan, C. W., Verhaak, R. G., McKenna, A., Campos, B., Nounshmehr, H., Salama, S. R., Zheng, S., Chakravarty, D., Sanborn, J. Z., Berman, S. H., *et al.* (2013b). The somatic genomic landscape of glioblastoma. *Cell* *155*, 462-477.
- Bunk, E. C., Ertaylan, G., Ortega, F., Pavlou, M. A., Gonzalez Cano, L., Stergiopoulos, A., Safaiyan, S., Vols, S., van Cann, M., Politis, P. K., *et al.* (2016). Prox1 Is Required for Oligodendrocyte Cell Identity in Adult Neural Stem Cells of the Subventricular Zone. *Stem Cells* *34*, 2115-2129.
- Bywater, M. J., Pearson, R. B., McArthur, G. A., and Hannan, R. D. (2013). Dysregulation of the basal RNA polymerase transcription apparatus in cancer. *Nat Rev Cancer* *13*, 299-314.
- Charest-Marcotte, A., Dufour, C. R., Wilson, B. J., Tremblay, A. M., Eichner, L. J., Arlow, D. H., Mootha, V. K., and Giguere, V. (2010). The homeobox protein Prox1 is a negative modulator of ERR $\alpha$ /PGC-1 $\alpha$  bioenergetic functions. *Genes Dev* *24*, 537-542.
- Chen, J., Guo, Y., Cheng, W., Chen, R., Liu, T., Chen, Z., and Tan, S. (2013a). High glucose induces apoptosis and suppresses proliferation of adult rat neural stem cells following in vitro ischemia. *BMC Neurosci* *14*, 24.
- Chen, J., Li, Y., Yu, T. S., McKay, R. M., Burns, D. K., Kernie, S. G., and Parada, L. F. (2012). A restricted cell population propagates glioblastoma growth after chemotherapy. *Nature* *488*, 522-526.
- Chen, J., Sun, J., Yang, L., Yan, Y., Shi, W., Shi, J., Huang, Q., Chen, J., and Lan, Q. (2015). Upregulation of B23 promotes tumor cell proliferation and predicts poor prognosis in glioma. *Biochem Biophys Res Commun* *466*, 124-130.
- Chen, Z., Liu, P., Li, C., Luo, Y., Chen, I., Liang, W., Chen, X., Feng, Y., Xia, H., and Wang, F. (2013b). Deregulated expression of the clock genes in gliomas. *Technol Cancer Res Treat* *12*, 91-97.
- Clevers, H., Loh, K. M., and Nusse, R. (2014). Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. *Science* *346*, 1248012.
- Colombo, E., Marine, J. C., Danovi, D., Falini, B., and Pelicci, P. G. (2002). Nucleophosmin regulates the stability and transcriptional activity of p53. *Nature cell biology* *4*, 529-533.
- Cowell, R. M., Blake, K. R., and Russell, J. W. (2007). Localization of the transcriptional coactivator PGC-1 $\alpha$  to GABAergic neurons during maturation of the rat brain. *J Comp Neurol* *502*, 1-18.
- Cuddapah, V. A., Robel, S., Watkins, S., and Sontheimer, H. (2014). A neurocentric perspective on glioma invasion. *Nat Rev Neurosci* *15*, 455-465.
- Curtius, K., Wright, N. A., and Graham, T. A. (2018). An evolutionary perspective on field cancerization. *Nat Rev Cancer* *18*, 19-32.
- Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell* *111*, 185-196.

- Dadras, S. S., Skrzypek, A., Nguyen, L., Shin, J. W., Schulz, M. M., Arbiser, J., Mihm, M. C., and Detmar, M. (2008). Prox-1 promotes invasion of kaposiform hemangioendotheliomas. *J Invest Dermatol* 128, 2798-2806.
- Deisenroth, C., and Zhang, Y. (2010). Ribosome biogenesis surveillance: probing the ribosomal protein-Mdm2-p53 pathway. *Oncogene* 29, 4253-4260.
- DIAGRAM, C., Asian Genetic Epidemiology Network Type 2 Diabetes, C., South Asian Type 2 Diabetes, C., Mexican American Type 2 Diabetes, C., Type 2 Diabetes Genetic Exploration by Nex-generation sequencing in muylti-Ethnic Samples, C., Mahajan, A., Go, M. J., Zhang, W., Below, J. E., Gaulton, K. J., *et al.* (2014). Genome-wide trans-ancestry meta-analysis provides insight into the genetic architecture of type 2 diabetes susceptibility. *Nat Genet* 46, 234-244.
- Doe, C. Q., Chu-LaGraff, Q., Wright, D. M., and Scott, M. P. (1991). The prospero gene specifies cell fates in the Drosophila central nervous system. *Cell* 65, 451-464.
- Dresemann, G. (2005). Imatinib and hydroxyurea in pretreated progressive glioblastoma multiforme: a patient series. *Ann Oncol* 16, 1702-1708.
- Dufour, C. R., Levasseur, M. P., Pham, N. H., Eichner, L. J., Wilson, B. J., Charest-Marcotte, A., Duguay, D., Poirier-Heon, J. F., Cermakian, N., and Giguere, V. (2011). Genomic convergence among ERRalpha, PROX1, and BMAL1 in the control of metabolic clock outputs. *PLoS genetics* 7, e1002143.
- Dupuis, J., Langenberg, C., Prokopenko, I., Saxena, R., Soranzo, N., Jackson, A. U., Wheeler, E., Glazer, N. L., Bouatia-Naji, N., Gloyn, A. L., *et al.* (2010). New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk. *Nat Genet* 42, 105-116.
- Eckel-Passow, J. E., Lachance, D. H., Molinaro, A. M., Walsh, K. M., Decker, P. A., Sicotte, H., Pekmezci, M., Rice, T., Kosel, M. L., Smirnov, I. V., *et al.* (2015). Glioma Groups Based on 1p/19q, IDH, and TERT Promoter Mutations in Tumors. *N Engl J Med* 372, 2499-2508.
- Elsir, T., Eriksson, A., Orrego, A., Lindstrom, M. S., and Nister, M. (2010). Expression of PROX1 Is a common feature of high-grade malignant astrocytic gliomas. *Journal of neuropathology and experimental neurology* 69, 129-138.
- Elsir, T., Qu, M., Berntsson, S. G., Orrego, A., Olofsson, T., Lindstrom, M. S., Nister, M., von Deimling, A., Hartmann, C., Ribom, D., and Smits, A. (2011). PROX1 is a predictor of survival for gliomas WHO grade II. *Br J Cancer* 104, 1747-1754.
- Elsir, T., Smits, A., Lindstrom, M. S., and Nister, M. (2012). Transcription factor PROX1: its role in development and cancer. *Cancer metastasis reviews* 31, 793-805.
- Elyada, E., Pribluda, A., Goldstein, R. E., Morgenstern, Y., Brachya, G., Cojocar, G., Snir-Alkalay, I., Burstain, I., Haffner-Krausz, R., Jung, S., *et al.* (2011). CK1alpha ablation highlights a critical role for p53 in invasiveness control. *Nature* 470, 409-413.
- Esmailzadeh, M., Majlesara, A., Faridar, A., Hafezi, M., Hong, B., Esmailnia-Shirvani, H., Neyazi, B., Mehrabi, A., and Nakamura, M. (2014). Brain metastasis from gastrointestinal cancers: a systematic review. *International journal of clinical practice* 68, 890-899.
- Foskolou, I. P., Stellas, D., Rozani, I., Lavigne, M. D., and Politis, P. K. (2013). Prox1 suppresses the proliferation of neuroblastoma cells via a dual action in p27-Kip1 and Cdc25A. *Oncogene* 32, 947-960.

- Freeman, M. R., and Doe, C. Q. (2001). Asymmetric Prospero localization is required to generate mixed neuronal/glial lineages in the *Drosophila* CNS. *Development* *128*, 4103-4112.
- Furnari, F. B., Fenton, T., Bachoo, R. M., Mukasa, A., Stommel, J. M., Stegh, A., Hahn, W. C., Ligon, K. L., Louis, D. N., Brennan, C., *et al.* (2007). Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev* *21*, 2683-2710.
- Gentilella, A., Kozma, S. C., and Thomas, G. (2015). A liaison between mTOR signaling, ribosome biogenesis and cancer. *Biochimica et biophysica acta* *1849*, 812-820.
- Goodenberger, M. L., and Jenkins, R. B. (2012). Genetics of adult glioma. *Cancer Genet* *205*, 613-621.
- Goudarzi, K. M., and Lindstrom, M. S. (2016). Role of ribosomal protein mutations in tumor development (Review). *Int J Oncol* *48*, 1313-1324.
- Grummt, I. (2013). The nucleolus-guardian of cellular homeostasis and genome integrity. *Chromosoma* *122*, 487-497.
- Hagerstrand, D., Hesselager, G., Achterberg, S., Wickenberg Bolin, U., Kowanetz, M., Kastemar, M., Heldin, C. H., Isaksson, A., Nister, M., and Ostman, A. (2006). Characterization of an imatinib-sensitive subset of high-grade human glioma cultures. *Oncogene* *25*, 4913-4922.
- Halliday, J., Helmy, K., Pattwell, S. S., Pitter, K. L., LaPlant, Q., Ozawa, T., and Holland, E. C. (2014). In vivo radiation response of proneural glioma characterized by protective p53 transcriptional program and proneural-mesenchymal shift. *Proc Natl Acad Sci U S A* *111*, 5248-5253.
- Hambardzumyan, D., and Bergers, G. (2015). Glioblastoma: Defining Tumor Niches. *Trends Cancer* *1*, 252-265.
- Hambardzumyan, D., Squatrito, M., Carbajal, E., and Holland, E. C. (2008). Glioma formation, cancer stem cells, and akt signaling. *Stem cell reviews* *4*, 203-210.
- Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* *144*, 646-674.
- Hirata, J., Nakagoshi, H., Nabeshima, Y., and Matsuzaki, F. (1995). Asymmetric segregation of the homeodomain protein Prospero during *Drosophila* development. *Nature* *377*, 627-630.
- Holmberg Olausson, K., Elsir, T., Moazemi Goudarzi, K., Nister, M., and Lindstrom, M. S. (2015). NPM1 histone chaperone is upregulated in glioblastoma to promote cell survival and maintain nucleolar shape. *Sci Rep* *5*, 16495.
- Holmberg Olausson, K., Nister, M., and Lindstrom, M. S. (2014). Loss of nucleolar histone chaperone NPM1 triggers rearrangement of heterochromatin and synergizes with a deficiency in DNA methyltransferase DNMT3A to drive ribosomal DNA transcription. *J Biol Chem* *289*, 34601-34619.
- Humpton, T. J., and Vousden, K. H. (2016). Regulation of Cellular Metabolism and Hypoxia by p53. *Cold Spring Harb Perspect Med* *6*.
- Iwadate, Y. (2016). Epithelial-mesenchymal transition in glioblastoma progression. *Oncol Lett* *11*, 1615-1620.

- Jiang, Y., Marinescu, V. D., Xie, Y., Jarvius, M., Maturi, N. P., Haglund, C., Olofsson, S., Lindberg, N., Olofsson, T., Leijonmarck, C., *et al.* (2017). Glioblastoma Cell Malignancy and Drug Sensitivity Are Affected by the Cell of Origin. *Cell reports* 18, 977-990.
- Kalani, M. Y., Cheshier, S. H., Cord, B. J., Bababeygy, S. R., Vogel, H., Weissman, I. L., Palmer, T. D., and Nusse, R. (2008). Wnt-mediated self-renewal of neural stem/progenitor cells. *Proc Natl Acad Sci U S A* 105, 16970-16975.
- Kaltezioti, V., Kouroupi, G., Oikonomaki, M., Mantouvalou, E., Stergiopoulos, A., Charonis, A., Rohrer, H., Matsas, R., and Politis, P. K. (2010). Prox1 regulates the notch1-mediated inhibition of neurogenesis. *PLoS biology* 8, e1000565.
- Karalay, O., Doberauer, K., Vadodaria, K. C., Knobloch, M., Berti, L., Miquelajauregui, A., Schwark, M., Jagasia, R., Taketo, M. M., Tarabykin, V., *et al.* (2011). Prospero-related homeobox 1 gene (Prox1) is regulated by canonical Wnt signaling and has a stage-specific role in adult hippocampal neurogenesis. *Proc Natl Acad Sci U S A* 108, 5807-5812.
- Kastenhuber, E. R., and Lowe, S. W. (2017). Putting p53 in Context. *Cell* 170, 1062-1078.
- Knoblich, J. A., Jan, L. Y., and Jan, Y. N. (1995). Asymmetric segregation of Numb and Prospero during cell division. *Nature* 377, 624-627.
- Kojima, Y., Miyoshi, H., Clevers, H. C., Oshima, M., Aoki, M., and Taketo, M. M. (2007). Suppression of tubulin polymerization by the LKB1-microtubule-associated protein/microtubule affinity-regulating kinase signaling. *J Biol Chem* 282, 23532-23540.
- Koumangoye, R. B., Andl, T., Taubenslag, K. J., Zilberman, S. T., Taylor, C. J., Loomans, H. A., and Andl, C. D. (2015). SOX4 interacts with EZH2 and HDAC3 to suppress microRNA-31 in invasive esophageal cancer cells. *Mol Cancer* 14, 24.
- Kretowski, A., Adamska, E., Maliszewska, K., Wawrusiewicz-Kurylonek, N., Citko, A., Goscik, J., Bauer, W., Wilk, J., Golonko, A., Waszczeniuk, M., *et al.* (2015). The rs340874 PROX1 type 2 diabetes mellitus risk variant is associated with visceral fat accumulation and alterations in postprandial glucose and lipid metabolism. *Genes Nutr* 10, 4.
- Kuo, Y. H., Chen, Y. T., Tsai, H. P., Chai, C. Y., and Kwan, A. L. (2015). Nucleophosmin overexpression is associated with poor survival in astrocytoma. *APMIS* 123, 515-522.
- Kurki, S., Peltonen, K., Latonen, L., Kiviharju, T. M., Ojala, P. M., Meek, D., and Laiho, M. (2004). Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. *Cancer Cell* 5, 465-475.
- Kwon, S., Jeon, J. S., Kim, S. B., Hong, Y. K., Ahn, C., Sung, J. S., and Choi, I. (2016). Rapamycin up-regulates triglycerides in hepatocytes by down-regulating Prox1. *Lipids Health Dis* 15, 41.
- Laerm, A., Helmbold, P., Goldberg, M., Dammann, R., Holzhausen, H. J., and Ballhausen, W. G. (2007). Prospero-related homeobox 1 (PROX1) is frequently inactivated by genomic deletions and epigenetic silencing in carcinomas of the biliary system. *J Hepatol* 46, 89-97.
- Laitinen, A., Bockelman, C., Hagstrom, J., Kokkola, A., Kallio, P., and Haglund, C. (2017). High PROX1 expression in gastric cancer predicts better survival. *PLoS One* 12, e0183868.

- Lan, X., Jorg, D. J., Cavalli, F. M. G., Richards, L. M., Nguyen, L. V., Vanner, R. J., Guilhamon, P., Lee, L., Kushida, M. M., Pellacani, D., *et al.* (2017). Fate mapping of human glioblastoma reveals an invariant stem cell hierarchy. *Nature*. 549, 227-232.
- Lawrence, M. S., Stojanov, P., Mermel, C. H., Robinson, J. T., Garraway, L. A., Golub, T. R., Meyerson, M., Gabriel, S. B., Lander, E. S., and Getz, G. (2014). Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* 505, 495-501.
- Lecompte, S., Pasquetti, G., Hermant, X., Grenier-Boley, B., Gonzalez-Gross, M., De Henauw, S., Molnar, D., Stehle, P., Beghin, L., Moreno, L. A., *et al.* (2013). Genetic and molecular insights into the role of PROX1 in glucose metabolism. *Diabetes* 62, 1738-1745.
- Lee, J., Son, M. J., Woolard, K., Donin, N. M., Li, A., Cheng, C. H., Kotliarova, S., Kotliarov, Y., Walling, J., Ahn, S., *et al.* (2008). Epigenetic-mediated dysfunction of the bone morphogenetic protein pathway inhibits differentiation of glioblastoma-initiating cells. *Cancer Cell* 13, 69-80.
- Li, J. Y., Patterson, M., Mikkola, H. K., Lowry, W. E., and Kurdistani, S. K. (2012). Dynamic distribution of linker histone H1.5 in cellular differentiation. *PLoS genetics* 8, e1002879.
- Li, L., and Vaessin, H. (2000). Pan-neural Prospero terminates cell proliferation during *Drosophila* neurogenesis. *Genes Dev* 14, 147-151.
- Lindberg, N., Kastemar, M., Olofsson, T., Smits, A., and Uhrbom, L. (2009). Oligodendrocyte progenitor cells can act as cell of origin for experimental glioma. *Oncogene* 28, 2266-2275.
- Lindstrom, M. S., Deisenroth, C., and Zhang, Y. (2007). Putting a finger on growth surveillance: insight into MDM2 zinc finger-ribosomal protein interactions. *Cell cycle* 6, 434-437.
- Lindstrom, M. S., Jurada, D., Bursac, S., Orsolich, I., Bartek, J., and Volarevic, S. (2018). Nucleolus as an emerging hub in maintenance of genome stability and cancer pathogenesis. *Oncogene*. [Epub ahead of print]
- Macias, E., Jin, A., Deisenroth, C., Bhat, K., Mao, H., Lindstrom, M. S., and Zhang, Y. (2010). An ARF-independent c-MYC-activated tumor suppression pathway mediated by ribosomal protein-Mdm2 Interaction. *Cancer Cell* 18, 231-243.
- Mashimo, T., Pichumani, K., Vemireddy, V., Hatanpaa, K. J., Singh, D. K., Sirasanagandla, S., Nannepaga, S., Piccirillo, S. G., Kovacs, Z., Foong, C., *et al.* (2014). Acetate is a bioenergetic substrate for human glioblastoma and brain metastases. *Cell* 159, 1603-1614.
- Meletis, K., Wirta, V., Hede, S. M., Nister, M., Lundeberg, J., and Frisen, J. (2006). p53 suppresses the self-renewal of adult neural stem cells. *Development* 133, 363-369.
- Mills, E. W., and Green, R. (2017). Ribosomopathies: There's strength in numbers. *Science* 358.
- Murata, H., Yoshimoto, K., Hatae, R., Akagi, Y., Mizoguchi, M., Hata, N., Kuga, D., Nakamizo, A., Amano, T., Sayama, T., and Iihara, K. (2015). Detection of proneural/mesenchymal marker expression in glioblastoma: temporospatial dynamics and association with chromatin-modifying gene expression. *J Neurooncol* 125, 33-41.

- Nagai, H., Li, Y., Hatano, S., Toshihito, O., Yuge, M., Ito, E., Utsumi, M., Saito, H., and Kinoshita, T. (2003). Mutations and aberrant DNA methylation of the PROX1 gene in hematologic malignancies. *Genes Chromosomes Cancer* 38, 13-21.
- Nister, M., Heldin, C. H., and Westermark, B. (1986). Clonal variation in the production of a platelet-derived growth factor-like protein and expression of corresponding receptors in a human malignant glioma. *Cancer Res* 46, 332-340.
- Nister, M., Wedell, B., Betsholtz, C., Bywater, M., Pettersson, M., Westermark, B., and Mark, J. (1987). Evidence for progressional changes in the human malignant glioma line U-343 MGa: analysis of karyotype and expression of genes encoding the subunit chains of platelet-derived growth factor. *Cancer Res* 47, 4953-4960.
- Noroxe, D. S., Poulsen, H. S., and Lassen, U. (2016). Hallmarks of glioblastoma: a systematic review. *ESMO Open* 1, e000144.
- Noushmehr, H., Weisenberger, D. J., Diefes, K., Phillips, H. S., Pujara, K., Berman, B. P., Pan, F., Pelloski, C. E., Sulman, E. P., Bhat, K. P., *et al.* (2010). Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell* 17, 510-522.
- Ostrom, Q. T., Bauchet, L., Davis, F. G., Deltour, I., Fisher, J. L., Langer, C. E., Pekmezci, M., Schwartzbaum, J. A., Turner, M. C., Walsh, K. M., *et al.* (2014). The epidemiology of glioma in adults: a "state of the science" review. *Neuro Oncol* 16, 896-913.
- Ozawa, T., Riester, M., Cheng, Y. K., Huse, J. T., Squatrito, M., Helmy, K., Charles, N., Michor, F., and Holland, E. C. (2014). Most human non-GCIMP glioblastoma subtypes evolve from a common proneural-like precursor glioma. *Cancer Cell* 26, 288-300.
- Parsons, D. W., Jones, S., Zhang, X., Lin, J. C., Leary, R. J., Angenendt, P., Mankoo, P., Carter, H., Siu, I. M., Gallia, G. L., *et al.* (2008). An integrated genomic analysis of human glioblastoma multiforme. *Science* 321, 1807-1812.
- Patel, A. P., Tirosh, I., Trombetta, J. J., Shalek, A. K., Gillespie, S. M., Wakimoto, H., Cahill, D. P., Nahed, B. V., Curry, W. T., Martuza, R. L., *et al.* (2014). Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* 344, 1396-1401.
- Pelletier, J., Thomas, G., and Volarevic, S. (2018). Ribosome biogenesis in cancer: new players and therapeutic avenues. *Nat Rev Cancer* 18, 51-63.
- Petrova, T. V., Nykanen, A., Norrmen, C., Ivanov, K. I., Andersson, L. C., Haglund, C., Puolakkainen, P., Wempe, F., von Melchner, H., Gradwohl, G., *et al.* (2008). Transcription factor PROX1 induces colon cancer progression by promoting the transition from benign to highly dysplastic phenotype. *Cancer Cell* 13, 407-419.
- Pollen, A. A., Nowakowski, T. J., Chen, J., Retallack, H., Sandoval-Espinosa, C., Nicholas, C. R., Shuga, J., Liu, S. J., Oldham, M. C., Diaz, A., *et al.* (2015). Molecular identity of human outer radial glia during cortical development. *Cell* 163, 55-67.
- Ragusa, S., Cheng, J., Ivanov, K. I., Zangger, N., Ceteci, F., Bernier-Latmani, J., Milatos, S., Joseph, J. M., Tercier, S., Bouzourene, H., *et al.* (2014). PROX1 promotes metabolic adaptation and fuels outgrowth of Wnt(high) metastatic colon cancer cells. *Cell reports* 8, 1957-1973.
- Raska, I., Shaw, P. J., and Cmarko, D. (2006). Structure and function of the nucleolus in the spotlight. *Curr Opin Cell Biol* 18, 325-334.

- Reifenberger, G., Wirsching, H. G., Knobbe-Thomsen, C. B., and Weller, M. (2017). Advances in the molecular genetics of gliomas - implications for classification and therapy. *Nature reviews Clinical oncology* *14*, 434-452.
- Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature* *414*, 105-111.
- Richter, G. H., Plehm, S., Fasan, A., Rossler, S., Unland, R., Bennani-Baiti, I. M., Hotfilder, M., Lowel, D., von Luettichau, I., Mossbrugger, I., *et al.* (2009). EZH2 is a mediator of EWS/FLI1 driven tumor growth and metastasis blocking endothelial and neuro-ectodermal differentiation. *Proc Natl Acad Sci U S A* *106*, 5324-5329.
- Risebro, C. A., Searles, R. G., Melville, A. A., Ehler, E., Jina, N., Shah, S., Pallas, J., Hubank, M., Dillard, M., Harvey, N. L., *et al.* (2009). Prox1 maintains muscle structure and growth in the developing heart. *Development* *136*, 495-505.
- Roodakker, K. R., Elsir, T., Edqvist, P. D., Hagerstrand, D., Carlson, J., Lysiak, M., Henriksson, R., Ponten, F., Rosell, J., Soderkvist, P., *et al.* (2016). PROX1 is a novel pathway-specific prognostic biomarker for high-grade astrocytomas; results from independent glioblastoma cohorts stratified by age and IDH mutation status. *Oncotarget* *7*, 72431-72442.
- Rouse, C., Gittleman, H., Ostrom, Q. T., Kruchko, C., and Barnholtz-Sloan, J. S. (2016). Years of potential life lost for brain and CNS tumors relative to other cancers in adults in the United States, 2010. *Neuro Oncol* *18*, 70-77.
- Ruggero, D. (2012). Revisiting the nucleolus: from marker to dynamic integrator of cancer signaling. *Sci Signal* *5*, pe38.
- Sahar, S., and Sassone-Corsi, P. (2009). Metabolism and cancer: the circadian clock connection. *Nat Rev Cancer* *9*, 886-896.
- Savary, K., Caglayan, D., Caja, L., Tzavlaki, K., Bin Nayeem, S., Bergstrom, T., Jiang, Y., Uhrbom, L., Forsberg-Nilsson, K., Westermarck, B., *et al.* (2013). Snail depletes the tumorigenic potential of glioblastoma. *Oncogene* *32*, 5409-5420.
- Schwartzbaum, J., Edlinger, M., Zigmont, V., Stattin, P., Rempala, G. A., Nagel, G., Hammar, N., Ulmer, H., Foger, B., Walldius, G., *et al.* (2017). Associations between prediagnostic blood glucose levels, diabetes, and glioma. *Sci Rep* *7*, 1436.
- Seth, A., Ye, J., Yu, N., Guez, F., Bedford, D. C., Neale, G. A., Cordi, S., Brindle, P. K., Lemaigre, F. P., Kaestner, K. H., and Sosa-Pineda, B. (2014). Prox1 ablation in hepatic progenitors causes defective hepatocyte specification and increases biliary cell commitment. *Development* *141*, 538-547.
- Shaul, Y. D., Freinkman, E., Comb, W. C., Cantor, J. R., Tam, W. L., Thiru, P., Kim, D., Kanarek, N., Pacold, M. E., Chen, W. W., *et al.* (2014). Dihydropyrimidine accumulation is required for the epithelial-mesenchymal transition. *Cell* *158*, 1094-1109.
- Singh, S. K., Clarke, I. D., Hide, T., and Dirks, P. B. (2004a). Cancer stem cells in nervous system tumors. *Oncogene* *23*, 7267-7273.
- Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., and Dirks, P. B. (2003). Identification of a cancer stem cell in human brain tumors. *Cancer Res* *63*, 5821-5828.

- Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., Henkelman, R. M., Cusimano, M. D., and Dirks, P. B. (2004b). Identification of human brain tumour initiating cells. *Nature* *432*, 396-401.
- Sloan, K. E., Bohnsack, M. T., and Watkins, N. J. (2013). The 5S RNP couples p53 homeostasis to ribosome biogenesis and nucleolar stress. *Cell reports* *5*, 237-247.
- Smedby, K. E., Brandt, L., Backlund, M. L., and Blomqvist, P. (2009). Brain metastases admissions in Sweden between 1987 and 2006. *Br J Cancer* *101*, 1919-1924.
- Sosa-Pineda, B., Wigle, J. T., and Oliver, G. (2000). Hepatocyte migration during liver development requires Prox1. *Nat Genet* *25*, 254-255.
- Sottoriva, A., Spiteri, I., Piccirillo, S. G., Touloumis, A., Collins, V. P., Marioni, J. C., Curtis, C., Watts, C., and Tavaré, S. (2013). Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proc Natl Acad Sci U S A* *110*, 4009-4014.
- Steffensen, K. R., Holter, E., Bavner, A., Nilsson, M., Pelto-Huikko, M., Tomarev, S., and Treuter, E. (2004). Functional conservation of interactions between a homeodomain cofactor and a mammalian FTZ-F1 homologue. *EMBO Rep* *5*, 613-619.
- Stiles, C. D., and Rowitch, D. H. (2008). Glioma stem cells: a midterm exam. *Neuron* *58*, 832-846.
- Stopschinski, B. E., Beier, C. P., and Beier, D. (2013). Glioblastoma cancer stem cells--from concept to clinical application. *Cancer Lett* *338*, 32-40.
- Strickland, M., and Stoll, E. A. (2017). Metabolic Reprogramming in Glioma. *Front Cell Dev Biol* *5*, 43.
- Stupp, R., Brada, M., van den Bent, M. J., Tonn, J. C., Pentheroudakis, G., and Group, E. G. W. (2014). High-grade glioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* *25 Suppl 3*, iii93-101.
- Stupp, R., Hegi, M. E., Mason, W. P., van den Bent, M. J., Taphoorn, M. J., Janzer, R. C., Ludwin, S. K., Allgeier, A., Fisher, B., Belanger, K., *et al.* (2009). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *The lancet oncology* *10*, 459-466.
- Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., Belanger, K., Brandes, A. A., Marosi, C., Bogdahn, U., *et al.* (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* *352*, 987-996.
- Sturm, D., Bender, S., Jones, D. T., Lichter, P., Grill, J., Becher, O., Hawkins, C., Majewski, J., Jones, C., Costello, J. F., *et al.* (2014). Paediatric and adult glioblastoma: multiform (epi)genomic culprits emerge. *Nat Rev Cancer* *14*, 92-107.
- Sun, T. Q., Lu, B., Feng, J. J., Reinhard, C., Jan, Y. N., Fantl, W. J., and Williams, L. T. (2001). PAR-1 is a Dishevelled-associated kinase and a positive regulator of Wnt signalling. *Nature cell biology* *3*, 628-636.
- Suva, M. L., Rheinbay, E., Gillespie, S. M., Patel, A. P., Wakimoto, H., Rabkin, S. D., Riggi, N., Chi, A. S., Cahill, D. P., Nahed, B. V., *et al.* (2014). Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. *Cell* *157*, 580-594.

- Takahashi, M., Yoshimoto, T., Shimoda, M., Kono, T., Koizumi, M., Yazumi, S., Shimada, Y., Doi, R., Chiba, T., and Kubo, H. (2006). Loss of function of the candidate tumor suppressor *prox1* by RNA mutation in human cancer cells. *Neoplasia* 8, 1003-1010.
- Tan, B. T., Park, C. Y., Ailles, L. E., and Weissman, I. L. (2006). The cancer stem cell hypothesis: a work in progress. *Lab Invest* 86, 1203-1207.
- Tan, T. Z., Miow, Q. H., Miki, Y., Noda, T., Mori, S., Huang, R. Y., and Thiery, J. P. (2014). Epithelial-mesenchymal transition spectrum quantification and its efficacy in deciphering survival and drug responses of cancer patients. *EMBO Mol Med* 6, 1279-1293.
- TCGA (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 455, 1061-1068.
- Torii, M., Matsuzaki, F., Osumi, N., Kaibuchi, K., Nakamura, S., Casarosa, S., Guillemot, F., and Nakafuku, M. (1999). Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system. *Development* 126, 443-456.
- Tornatore, L., Thotakura, A. K., Bennett, J., Moretti, M., and Franzoso, G. (2012). The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. *Trends Cell Biol* 22, 557-566.
- Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., *et al.* (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 303, 844-848.
- Verhaak, R. G., Hoadley, K. A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M. D., Miller, C. R., Ding, L., Golub, T., Mesirov, J. P., *et al.* (2010). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 17, 98-110.
- Versmold, B., Felsberg, J., Mikeska, T., Ehrentraut, D., Kohler, J., Hampl, J. A., Rohn, G., Niederacher, D., Betz, B., Hellmich, M., *et al.* (2007). Epigenetic silencing of the candidate tumor suppressor gene PROX1 in sporadic breast cancer. *Int J Cancer* 121, 547-554.
- Vire, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A., Bernard, D., Vanderwinden, J. M., *et al.* (2006). The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 439, 871-874.
- Wang, Q., Hu, B., Hu, X., Kim, H., Squatrito, M., Scarpace, L., deCarvalho, A. C., Lyu, S., Li, P., Li, Y., *et al.* (2017). Tumor Evolution of Glioma-Intrinsic Gene Expression Subtypes Associates with Immunological Changes in the Microenvironment. *Cancer Cell* 32, 42-56 e46.
- Wiener, Z., Hogstrom, J., Hyvonen, V., Band, A. M., Kallio, P., Holopainen, T., Dufva, O., Haglund, C., Kruuna, O., Oliver, G., *et al.* (2014). Prox1 promotes expansion of the colorectal cancer stem cell population to fuel tumor growth and ischemia resistance. *Cell reports* 8, 1943-1956.
- Wigle, J. T., Chowdhury, K., Gruss, P., and Oliver, G. (1999). Prox1 function is crucial for mouse lens-fibre elongation. *Nat Genet* 21, 318-322.
- Wigle, J. T., and Oliver, G. (1999). Prox1 function is required for the development of the murine lymphatic system. *Cell* 98, 769-778.

- Xu, X., Wan, X., and Wei, X. (2017). PROX1 promotes human glioblastoma cell proliferation and invasion via activation of the nuclear factor-kappaB signaling pathway. *Mol Med Rep* 15, 963-968.
- Yan, H., Parsons, D. W., Jin, G., McLendon, R., Rasheed, B. A., Yuan, W., Kos, I., Batinic-Haberle, I., Jones, S., Riggins, G. J., *et al.* (2009). IDH1 and IDH2 mutations in gliomas. *N Engl J Med* 360, 765-773.
- Yoshimoto, T., Takahashi, M., Nagayama, S., Watanabe, G., Shimada, Y., Sakasi, Y., and Kubo, H. (2007). RNA mutations of prox1 detected in human esophageal cancer cells by the shifted termination assay. *Biochem Biophys Res Commun* 359, 258-262.
- Zhao, L., Zheng, Z., and Huang, P. (2016). Diabetes mellitus and the risk of glioma: a meta-analysis. *Oncotarget* 7, 4483-4489.
- Zheng, H., Ying, H., Yan, H., Kimmelman, A. C., Hiller, D. J., Chen, A. J., Perry, S. R., Tonon, G., Chu, G. C., Ding, Z., *et al.* (2008). p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. *Nature* 455, 1129-1133.
- Zinovieva, R. D., Duncan, M. K., Johnson, T. R., Torres, R., Polymeropoulos, M. H., and Tomarev, S. I. (1996). Structure and chromosomal localization of the human homeobox gene Prox 1. *Genomics* 35, 517-522.
- Zitvogel, L., Daillere, R., Roberti, M. P., Routy, B., and Kroemer, G. (2017). Anticancer effects of the microbiome and its products. *Nat Rev Microbiol* 15, 465-478.