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Dedicated to my beloved family

ABSTRACT

The majority of malignant brain tumors in adults are astrocytic gliomas. These are classified into four malignancy grades according to the World Health Organization (WHO) criteria, including grade I pilocytic astrocytoma, grade II astrocytoma, grade III anaplastic astrocytoma and grade IV glioblastoma. Understanding the underlying molecular defects of these tumors gives us the possibility to design new effective therapies.

PROX1 is a transcription factor that has an important role in the developmental process of various organs during embryogenesis, including the central nervous system. In paper I we examined a group of 56 paraffin embedded astrocytic brain tumors for their expression of PROX1. We found that the number of PROX1+ cells was correlated to high tumor grade. We concluded that PROX1 maybe used as a diagnostic tool distinguishing between grade III, IV tumors and grade II astrocytomas. This finding led us to investigate PROX1's prognostic and predictive value in grade II tumors, because these tumors have a highly unpredictable course of progression to a more malignant grade, which hinders the clinicians' choice of treatment. In paper II we studied the expression levels of PROX1 in 128 grade II astrocytic brain tumors and found that it is a highly dependable, predictive factor for short term survival in patients with astrocytic gliomas but not for oligodendrogliomas. We propose that in the future PROX1 maybe used in the clinical routine as a biomarker to help in prediction of prognosis for astrocytic brain tumors. In order to understand why PROX1 is up regulated in these tumors, we determined its expression in different established glioma cell lines, to serve as a tool for the functional studies of the protein (paper III).

A hallmark of glioblastoma is mutations in the p53 tumor suppressor gene. p53 is also frequently mutated in other tumors including those from lung, breast and skin. In paper IV we used a mouse skin model, p53QS-val135/QS-val135 (p53QS), to examine the importance of p53 transcriptional regulatory function in tumor suppression. This model contains a double mutation in the N-terminus of p53 abrogating the transactivation domain and a modification at amino acid 135 partially affecting DNA binding. Ras oncogene-induced senescence was lost in both p53QS and p53-/- keratinocytes. Likewise, p53QS, similar to p53-/-, cooperated with v-rasHa to enhance malignant conversion. The tumors arising in p53QS keratinocytes displayed strong nuclear p53 expression, thus the p53QS-val135 allele was maintained during tumor formation. While p53-/- keratinocytes displayed diminished response to TGF-beta, p53QS and p53wt keratinocytes responded equivalently, indicating that the requirement of p53 for maximizing TGF-beta-mediated growth regulation is independent of its transactivation domain and that TGF-beta-mediated growth regulation is not required for p53 mediated tumor suppression in the skin.

LIST OF PUBLICATIONS

I. **Tamador Elsir**, Anna Eriksson, Abiel Orrego, Mikael S. Lindström, Monica Nistér.
Expression of PROX1 is a common feature of high grade malignant astrocytic gliomas. J. Neuropathol. Exp. Neurol., 2010, 69:129-138

II. **Tamador Elsir***, Mingqi Qu*, Shala G Berntsson, Abiel Orrego, Tommie Olofsson, Mikael S. Lindström, Monica Nistér, Dan Ribom, Anja Smits.
PROX1 is a predictor of survival for astrocytic gliomas but not for oligodendrogliomas grade II. Submitted

III. **Tamador Elsir**, Susanne Öhlin, Jian Zhao, Susanne Heller, Monica Nistér, Mikael S. Lindström.
Differential expression of transcription factor PROX1 in human glioma cell lines. Manuscript

IV. Roshini Ponnamperuma, Kathryn King*, **Tamador Elsir***, Adam Glick, Geoffrey Wahl, Monica Nistér, Wendy Weinberg.
The transcriptional regulatory function of p53 is essential for suppression of mouse skin carcinogenesis and can be dissociated from effects on TGF-beta-mediated growth regulation. J. Pathol., 2009, 219:263-274

*Shared authorship

LIST OF ABBREVIATIONS

ABCG2	ATP-binding cassette sub-family G member 2
AML	Acute myeloid leukemia
ARF	Alternative reading frame
CNS	Central nervous system
CSC	Cancer stem cell
DBD	DNA binding domain
EGFR	Epidermal growth factor receptor
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GMC	Ganglion mother cell
IDH	Isocitrate dehydrogenase
LEC	Lymphatic endothelial cell
LGG	Low grade gliomas
LOH	Loss of heterozygosity
LRH1	Liver receptor homologue 1
MAP2	Microtubule- associated protein 2
MDM2	Murine double minute 2
MGMT	O^6–methylguanine-DNA-methyltransferase
NeuN	Neuronal specific nuclear protein
NB	Neuroblast
NLS	Nuclear localization signal
NR BOX	Nuclear receptor box
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PIP BOX	PCNA interacting protein motif box
PRD	Proline rich domain
PTEN	Phosphatase and tensin homologue
RB	Retinoblastoma protein
SCID	Severe combined immunodeficiency
SV40	Simian Virus 40
SVZ	Subventricular zone
TAD	Transactivation domain
TGF-ß	Transforming growth factor beta

TIC	Tumor initiating cell
UV	Ultraviolet
WHO	World Health Organization

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1. INTRODUCTION

1.1 Basic cancer biology

Humans have been confused by the causes and development of cancer since ancient times. Thoughts and theories changed throughout the years until the biological revolution of the twentieth century. It began with the discovery of the DNA double helix by Watson and Crick in the early 1950s, which lead to the understanding of the genetic code, consequently some cancer mysteries were unraveled. Now it became clear that most types of cancers instead of invading the body from the outside in fact arise from the normal tissue in which the cancer was first detected. Scientists were also able to pinpoint the specific genetic mutations (changes) that lead to the formation of cancer. Then came the discovery of tumor suppressor genes and oncogenes in the 1970s and 80s. Tumor suppressors have the ability to slow down cell division, repair damaged DNA or force the cell into apoptosis (programmed cell death), and are therefore often found inactive or lost in cancers. Proto-oncogenes are growth promoting genes which can control cell division and differentiation and are activated or mutated to form oncogenes in different cancers [187].

In present times, scientists have learnt to deal with each different type of cancer in an individual manner, after realizing that each cancer type behaves in a unique way. Each cancer type requires certain treatment and different tumors respond to treatment at different rates. Nevertheless, it's agreed upon that most cancers acquire the same abilities in order to develop into a malignant growth, known as the hallmarks of cancer. These are; self sufficiency in growth signals, avoiding apoptosis, sustained angiogenesis, unlimited replication potential, the ability to invade surrounding tissue and metastasis and the insensitivity to antigrowth signals [1]. There have been several attempts to find a cure for cancer using biological techniques that force the cancer cell to die and resist anti-apoptotic signals. Although there are many examples of successful cancer therapies, unfortunately, there is still a major problem with drug resistance and certain types of cancer can still not be cured.

1.2 Tumors of the central nervous system

There are many types of brain tumors with a wide range of biological aggressiveness. They are classified according to histopathological criteria that have been set by the World Health Organization (WHO). Some of the tumors are difficult to treat and the median survival time for patients diagnosed with such tumors is short. Much research has been performed in order to achieve a better understanding of the molecular mechanisms underlying the development of such tumors but unfortunately prognosis is still bad for patients with aggressive tumors.

Gliomas are the most common primary brain tumors of the Central Nervous System (CNS) mainly affecting adults. They arise from glial cells, which include astrocytes, oligodendrocytes, ependymal cells and microglia, normally functioning as the support cells of the CNS. Astrocytes are five times more abundant than neurons and have many specific roles in the normal CNS including primary roles in synaptic transmission and information processing [2]. In the case of injury reactive astrocytes have a key role in sealing an injured blood brain barrier [2].

1.2.1 Histological classification of gliomas

According to the guidelines of the WHO classification, gliomas are graded into four grades [188]. Criteria such as pleomorphism, frequency of mitosis, regional zones of necrosis and endothelial cell proliferation are taken into consideration. Malignancy ranges from grade I to IV, grade IV being the most malignant [188]. In a clinical setting, this malignancy scale is a key factor influencing the choice of therapies.

Grade I Pilocytic astrocytomas are relatively slow growing and are most frequent in children and young adults. These masses normally stay benign but there are a few examples of them undergoing malignant transformation. A tandem duplication at chromosome 7q34 was found to be highly specific to these pilocytic astrocytomas and not other types of astrocytomas [3]. This tandem duplication leads to the production of a novel fusion gene BRAF, which in turn can transform NIH3T3 cells [3].

Grade II gliomas, also known as diffuse astrocytomas are well differentiated slow growing gliomas. They have a high tendency for malignant transformation into grade III and, ultimately, grade IV glioblastoma [4, 5]. Grade II tumors represent 10-15% of all astrocytic brain tumors

with a higher incidence in young adults between ages 30-40, 10% of these grade II gliomas have the ability to target those under the age of 20. The growth fraction for these tumors, when measured according to the presence of the proliferation marker Ki67 is usually less than 4%.

Astrocytomas can further be subdivided into fibrillary, protoplasmic or gemistocytic astrocytomas [6]. Fibrillary astrocytomas are the most frequent and can be defined by their consistent immunoreactivity to the astrocytic marker glial fibrillary acidic protein (GFAP). The nucleus is often elongated, hyperchromatic and irregular [7]. Protoplastic astrocytomas are rare and express low levels of GFAP and posses uniformly round to oval nuclei. Gemistocytic astrocytomas have a unique appearance with a population of gemistocytes constituting around 35% of all the tumor cells. These gemistocytes have plump, glassy eosinophilic cell bodies, with abundant glial filaments in their cytoplasm. They have nuclei that are usually eccentric, with distinct nucleoli and densely clumped chromatin.

Oligodendrogliomas display distinct histology with a homogenous population of cells with round nuclei and perinuclear halos as they appear on paraffin sections giving the “honey comb” appearance [7]. Oligodendrogliomas have lately received a lot of attention since they are successfully treated with chemotherapy [8]. 80% of oligodendrogliomas have a deletion of chromosomal arms 1p and 19q which is associated with longer patient survival and also longer and better response to chemotherapy [9]. Oligoastrocytomas as their name suggests are a mix, combining features from both oligodendroglial and astrocytic tumors. They can either be biphasic where you can distinguish a clear separation between the two entities, oligodendroglial and astrocytic, however this is a less common variant. The more common variant is where the oligodendroglial and astrocytic tumor cells are closely mixed. There is also a favorable outcome for those with 1p/19q deletions in oligoastrocytomas [10].

Grade III gliomas also known as anaplastic astrocytomas are diffusely infiltrating, malignant astrocytomas with increased cellularity, nuclear atypia and with higher Ki67 immunoreactivity compared to grade II astrocytomas. These tumors tend to progress to glioblastoma (grade IV) so from a clinical, morphological and genetic point of view these tumors represent an intermediate stage between the diffuse astrocytomas and glioblastomas.
Grade IV gliomas also known as glioblastomas, are histopathologically characterized by their nuclear atypia, cellular pleomorphism, mitotic activity, microvascular proliferation, vascular thrombosis and necrosis.

1.3 Glioblastomas

Glioblastoma multiforme (GBM) is the most common primary brain tumor accounting for approximately 12-15 % of all intracranial tumors and is also the most malignant, graded by the WHO as a grade IV brain tumor. Despite all the efforts in research, treatment for patients with GBM has not changed much in recent years and the disease is still associated with a fatal outcome whereby the median survival is one year [4]. Glioblastomas are highly invasive which makes their complete resection very difficult. Radiotherapy and chemotherapy only help to prolong half of the patients lives by an extra year. The yearly incidence of GBMs is around 2-3/ 100000 [11]. GBM is a heterogeneous tumor type both on the genetic and histological level, affecting mainly adults between 45-75 years, with old age being the strongest negative prognostic factor for these tumors [11]. The main treatment is by combining surgical removal, radiation and chemotherapy (temozolomide). Regardless these tumors remain to be highly resistant to therapy [12, 13].

1.3.1 Primary and secondary glioblastomas

Two different types of glioblastoma have been described, primary and secondary, defined by their clinical characteristics. When the tumors progress from lower grade gliomas i.e. the patient had been previously diagnosed with an astrocytic tumor of a lower grade, they are called secondary glioblastomas but when they appear as *de novo* tumors they are called primary glioblastoma. Histologically, primary and secondary glioblastomas are similar, but there are some important differences at the genetic level. Distinguishing between the two entities is of considerable importance as the primary glioblastomas are the most common and often associated with a shorter survival time.

In two population-based studies, the frequency of primary and secondary GBMs was found to be approximately 95% primary and 5% secondary GBMs [11, 14]. Primary GBMs are more common in older patients (mean age 62 years) whereas secondary GBM frequently occurs in middle-aged individuals (mean age 45 years), which may explain the longer survival.
On a genetic level primary glioblastomas are characterized by deletion or mutation of INK4a, EGFR amplification, PTEN mutation and loss of heterozygosity [15] of 10q. Recently TP53 mutations have been described in approximately 28% of primary glioblastoma cases. Secondary glioblastomas on the other hand usually harbor TP53 mutations [11, 16]. Dysregulation of

EGFR, INK4a and PTEN can also be found but at much lower frequency than in the primary GBM. Secondary GBMs share the high frequency of 10q LOH with the primary GBMs (Figure 1).

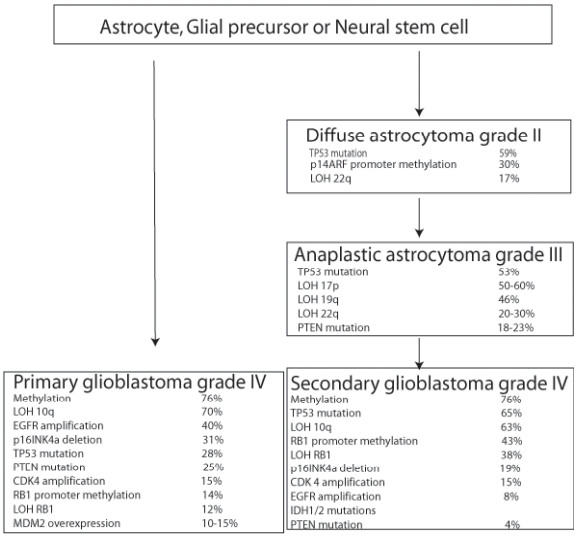

Figure 1. The most common genetic and epigenetic alterations in primary and secondary glioblastomas. Modified from Furnari et al 2007 [17] .

1.3.2 Molecular subclasses of high grade glioma

Recent studies have challenged the classical, histological means of glioma classification and put forth the possibility of a new, more objective classification, based on molecular expression profiling [18]. One such project aiming to classify high grade gliomas according to their molecular signature is The Cancer Genome Atlas (TCGA) project [19]. The strength of this project is the use of a large number of glioblastoma samples (n=206), and a multi-dimensional analysis, including gene expression, DNA copy numbers analysis and methylation aberrations. This work provided a number of new insights, including novel information on already known genes like TP53 and frequent mutations in novel players like PIK3R1 (phosphatidylinositol-3-kinase regulatory subunit gene).

In a separate study a sub-classification of high grade gliomas into three main classes, proneural proliferative (prolif) and mesenchymal (Mes) was suggested [20] [21]. This classification was based on large scale gene expression profiling, revealing a prognostic value for these subgroups independent of all known prognostic factors, including the WHO histological classification. By selecting markers that are specific for each subclass, they were able to examine both normal human tissues and a large cohort of high grade gliomas. Biologically they found that the expression profile of these three groups was similar to that of three biological processes. Thus, the PN group was similar to the expression profile of neurogenesis, Prolif to that of proliferation and Mes to angiogenic processes. They found that PN subtype defines a younger patient group (around 40 years) with a longer survival and has intact PTEN and EGFR loci but has a uniquely activated Notch pathway. Both Prolif and Mes subtypes represent an older patient group (50 years), with shorter survival, PTEN loss and Akt activation.

1.3.3 Growth factors and growth factor receptors in brain tumors

The first growth factor discovered in the brain was the epidermal growth factor (EGF), for its ability to provoke premature eye opening in new born mice [22]. Soon after, its mitogenic properties were recognized and its receptor was identified [23, 24]. EGFR is now known to be an oncogene, frequently amplified, overexpressed or mutated in glioblastomas [25, 26]. EGFR is a receptor that binds both EGF and transforming growth factor alpha (TGFα) which structurally resemble each other. It has been shown that TGFα /EGFR can induce tumors *in vivo* when in a nude mouse model and that these tumors maybe treated with EGFR tyrosine kinase inhibitors [27]. There have been clinical trials for GBMs using two different tyrosine kinase inhibitors, gefitinib and erlotinib [28, 29]. Unfortunately proof for significant clinical benefit is still poor when the inhibitors are administered as monotherapies but there are efforts to produce effective therapy in combination with other agents.

Platelet derived growth factor (PDGF) and its receptor are expressed in the human brain and are involved in its normal development [30, 31]. These proteins were also found to be overexpressed in brain tumors [32]. In secondary glioblastomas PDGFRα overexpression is common [33-35]. PDGFB, when injected into newborn mice brains as a retrovirus leads to the formation of brain tumors [36, 37]. Transgenic expression of PDGFB in mouse brain astrocytes did not show a phenotype unless in a p53 null background [38]. Some clinical trials have been

performed using a PDGFR inhibitor Imatinib for glioblastoma treatment. In combination with hydroxyurea this tyrosine kinase inhibitor showed an antitumor effect [39, 40].

Overexpression of both PDGF and EGF in mice, presented the opportunity to mimic the human *in vivo* malignancy situation [41]. This presented the possibility to study the development of cancer and understand more about its progression. There are examples of other brain tumor mouse models which involve introducing mutations to tumor suppressor genes. Such a model was described whereby introducing heterozygous distruptions to the p53, Nf1, and Pten tumor suppressor genes lead to the development of high-grade astrocytomas [42, 43]. Most recently, it has been shown that inactivation of these same tumor suppressor genes (p53, Nf1, Pten) in neural stem/progenitor cells is sufficient to induce astrocytoma formation [44].

1.3.4 Glioblastoma treatment

Over the last 30-40 years there have been hundreds of clinical therapy trials with only small levels of success. Glioblastomas remain notoriously resistant to treatment even after complete surgical resection, external beam radiation therapy and maximum tolerated doses for chemotherapy with agents like temozolomide or nitrosourea [13]. There are a few factors that make glioblastomas difficult to treat. Firstly, difficulty in drug delivery due to the blood brain barrier and the high tumor interstitial pressure. Secondly, the extreme heterogeneity of these tumors makes it difficult for any single therapy to target all the different genotypic and phenotypic differences in the cells. Cancer stem cells are increasingly an important part of the puzzle, where these cells with their stem cell-like properties harbor resistance mechanisms [45]. The fourth factor is glioblastomas' invasive characteristics where it can spread to the surrounding brain tissue, the brain stem and spinal cord, and reside behind an intact blood brain barrier. Finally these tumors have the ability to collect abundant DNA repair machinery that interferes with the effectiveness of chemotherapy and radiotherapy [13].

1.3.5 Established prognostic factors for gliomas

In general, unfavorable prognostic factors for patients with low grade gliomas are age over 40, a tumor over 6 cm in diameter, tumor crossing the midline, astrocytic tumor type and presence of neurological deficit before surgery [46]. Favorable factors include mutations of the IDH1 and

IDH2 genes in the case of both astrocytomas and oligodendrogliomas of both low grade and high grade. The IDH1 gene encodes cytosolic $NADP^+$- dependant isocitrate dehydrogenase and IDH2 gene encodes mitochondrial $NADP^+$- isocitrate dehydrogenase. These enzymes normally convert isocitrate to α-ketoglutarate while $NADP^+$ is reduced to NADPH. Mutations of these genes in gliomas are localized to a single amino acid residue, R132 for IDH1 and R172 for IDH2 and cause a decrease in the normal enzymatic activity of the endcoded protein [47-49]. It was found that the mutation in IDH1 caused some conformational changes in the protein resulting from the change of the amino acid arginine, leading to the ability of the enzyme to convert α-ketoglutarate to R(-)- hydroxyglutarate (2HG). 2HG accumulation has been associated with tumors [48, 50]. IDH1 and IDH2 gene mutations were analyzed in 1010 diffuse gliomas. This included a group of astrocytomas of grade II and III, oligodendrogliomas of both grade II and III, and also a group of grade III oligoastrocytomas. It was found that IDH1 mutations were most frequently occurring in astrocytomas while IDH2 mutations were most frequent in oligodendrogliomas [51]. It has also been shown that mutations of these genes are characteristic of secondary glioblastoma and not of the primary form.

O^6–methylguanine-DNA-methyltransferase (MGMT) promoter methylation is also a prognostic indicator of favorable outcome for glioblastoma patients treated with alkylating agent chemotherapy, like temozolomide. Patients with a methylated MGMT gene promoter treated with temozolomide were found to benefit much more than patients with no methylation [52]. This is due to the fact that in the absence of MGMT in the cell, O^6–alkylguanine accumulates in the DNA, which subsequently causes mismatch repair of the DNA, in the form of incorrect pairing with thymidin, leading to eventual DNA damage and cell death [53, 54](Table 1).

Table 1. Prognostic factors of both favorable and poor outcome in glioma patients

Prognostic factors of poor outcome	**Tumor histology**
Old age	All gliomas
Tumor crossing the midline	All gliomas
Astrocyte histology	All gliomas
Tumor size	All gliomas
Poor Karnofski performance status	All gliomas
Contrast enhancement	All gliomas
Prognostic factors of favorable outcome	**Tumor histology**
LOH 1p/19q	Oligodendrogliomas,Oligoastrocytomas
IDH1 mutation	Astrocytomas, Oligodendrogliomas
IDH2 mutation	Astrocytomas, Oligodendrogliomas
MGMT promoter methylation	Glioblastomas

1.3.6 Cancer Biomarkers

Cancer biomarkers are biological markers that assess the differences between stages of diagnosis and prognosis. They may indicate whether the patient maybe resistant to a specific treatment, if they have good or bad prognosis (examples are the above mentioned prognostic markers) and can also be very useful in diagnosing the type of cancer or malignancy grade of the tumors. Some biomarkers may serve as a good target for the tumor therapy [55, 56]. An example of this is the overexpressed PDGFR in brain tumors and the efforts to target this biomarker through PDGFR inhibitor (Imatinib)[57]. Over the years there have been great efforts to discover biomarkers for different cancer types and there has been success in some cases. With the new advances in technology the number of cancer biomarkers will continue to increase.

1.4 Cancer Stem Cells

Cancer stem cells (CSC), also known as the tumor initiating cells (TIC) have attracted intense interest since their discovery. They are defined as a population of cancer cells that has similar properties as normal stem cells i.e. have the capacity for self renewal indefinitely and for generating different types of progeny through differentiation. These CSCs upon transplantation, even at a high dilution, have the ability to generate tumors that resemble the parental tumor.

The first CSCs were described by John Dick's lab in 1994 from acute myeloid leukemia [20]. A small subset (0.01-1%) of $CD34^{+}/CD38^{-}$ cells was identified. These rare cells were found to have unlimited proliferative capacity and were found able to induce leukemia in severe combined immune deficient mice (SCID) [58].

During the last years CSCs have been isolated from many types of solid tumors including breast cancer, colorectal cancer, prostate cancer, pancreatic cancer, medulloblastomas and glioblastomas [59-63]. However, the molecular characteristics of CSCs are still poorly understood in most cases.

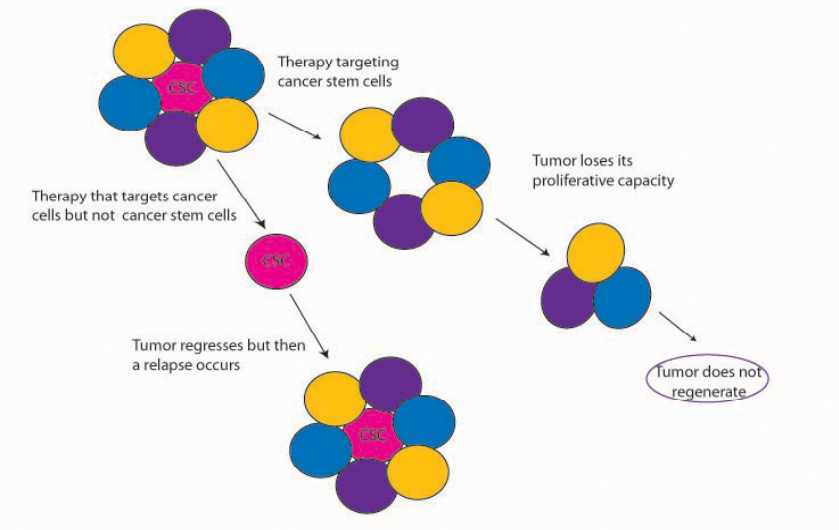

FIGURE 2. Therapy targeting the stem cell population maybe the means to tumor. eradication. Conventional therapies aim to shrink the tumor mass. Cancer stem cells are therapy resistant subsequently this might lead to regeneration of the tumor.

Modified from Reya et al. 2001 [64].

The presence of CSCs has raised an ongoing debate discussing the cell of origin for these cells, meaning a specification of the initial cell that receives the first oncogenic change. Possibilities are that CSCs come from a normal stem cell (inherited the stem cell phenotype), a restricted progenitor or a differentiated cell that is transformed and has acquired the ability to self renew [65]. There are some distinct similarities between tissue stem cells and cancer stem cells, in support of the first theory (CSCs originate from tissue stem cells). Both types of cells govern their growth and self renewal using the same biological processes and pathways including Notch, Wnt and hedgehog pathways [66]. Another important connection is that some of the genes primarily targeted for oncogenic transformation are those in control of normal tissue stem cell regulation and self renewal including p53, Bmi-1, Pten, Sox2, p19ARF and Nf1. p53 is mutated in around 50% of all human cancers and its role in tissue stem cell self renewal was recently described [67], revealing that p53 suppresses the self renewal of adult neural stem cells demonstrating yet another level of control in cells. p53 upregulation can lead to senescence of induced pluripotent stem cells [21] [68]. PTEN is also a known tumor suppressor and a negative regulator of neural stem cells through cell cycle control [69]. Bmi-1 is part of the polycomb family of proteins who regulate self renewal through forming large protein complexes that repress transcription by regulating chromatin structure [15]. It is proposed that Bmi-1 may play a unique role in maintenance of adult neural stem cells, unrelated to chromatin structure, and that is by repressing the $p16^{Ink4a}$ senescence pathway [66, 70].

Sox2 is a transcription factor that has an important role in neural stem cell maintenance by allowing them to proliferate and inhibiting their differentiation [71]. In normal fetal brains Sox2 expression can be found in the subventricular zone (SVZ) an area known to harbor neural stem cells and Sox2 is highly co-expressed with GFAP in this area [72]. Sox2 is overexpressed in all glial tumors of astroglial, ependymal and oligodendroglial components including glioblastomas [72]. It has also been shown that by introducing Sox2, together with other transcription factors such as Oct3, c-Myc and Nanog into terminally differentiated somatic cells, the cells were reprogrammed to generate induced pluripotent stem cells, iPS cells [73, 74].
More efforts are concentrating on the understanding of CSCs in hope to answer some questions; are they responsible for cancer metastasis and recurrence? Can we create therapies sufficient to eliminate these cells and consequently the tumor? (Figure 2)

1.4.1 Brain tumor stem cells

Scientists have tried to use their knowledge of neural stem cells to better understand brain cancer stem cells. Neural stem cell research has been of great interest in the neurodegenerative disease field and may lead to major insights into some complicated conditions such as Multiple Sclerosis and Parkinson's disease [75].

Most normal neural stem cells reside in the sub ventricular zone (SVZ) and the sub granular zone (SGZ) in the dentate gyrus of the hippocampus [76]. Normal brain stem cells have been characterized as expressing markers, including, nestin [77], Musashi-1 [78] and CD133 [79], as well as by their capacity to generate both glial and neuronal cell lineages [79]. CD133, also known as prominin-1, is expressed in both the cytoplasm and nucleus of the neural stem cells and has a function in the development of the CNS [79-81].
Due to the expression pattern of CD133 in the neural stem cells scientists used it as a tool to identify and isolate cancer stem cells. Successful attempts were reported in a number of studies, demonstrating that the $CD133^+$ cells were able to initiate and sustain tumors after transplantation into mouse brains. The ability to culture cells in the form of neurospheres, introduced the possibility to select for cells that have the stem cell characteristics, having a high capacity for proliferation, self-renewal and differentiation [82-84]. Ionizing radiation is commonly used for treatment for GBMs, but gliomas are generally quite radiation-resistant. It has been suggested by Bao et al. that it is the $CD133^+$ cancer stem cells that can survive ionizing radiation through activation of the DNA damage checkpoint response and an increased DNA repair capacity [85].

A contradicting finding was presented later in 2008, showing that $CD133^-$ cells also give rise to tumors upon transplantation [86]. In this study they also suggested that CD133 expression is not essential for tumor initiation but more likely tumor progression. It is possible to select for cancer stem cells by sorting for a side population of cells that have the ability to exclude the nuclear dye Hoechst 33342 [87, 88]. These cells manage this process by active transporters of the ABC family such as ABCG2 [89]. This is of great interest since it is these same transporter proteins that have the ability to exclude chemotherapeutic agents, thus making the cells therapy-resistant. Later on it was found that these tumor cells share this characteristic with both normal stem cells and progenitor cells [89].

1.5 The PROX1 gene

1.5.1 Neural stem cells in Drosophila and the role of prospero.

A report in 1991 [90] showed that prospero (named pros after the magician in Shakespeare's "The Tempest"), had a key role in determining the cell fate in the Drosophila CNS. Today the embryonic development of the Drosophila CNS is well described [91]. Drosophila neurons arise by the division of the mother cell called the neuroblast (NB) into two daughter cells, one of which retains the stem cell like characteristics of the NB and another smaller cell called the ganglion mother cell (GMC), which will terminally differentiate into neurons [90, 92, 93]. Prospero asymmetrically localizes into the GMC and takes part in its differentiation by activating neuronal differentiating genes, repressing neuroblast-specific genes and repressing cell cycle genes (Figure 3).

In the absence of prospero, both daughter cells act in a stem cell-like manner, expressing self renewal markers, proliferating and failing to differentiate [94-97]. The absence of prospero also leads to accumulation of cells forming brain tumor like masses, as neuroblasts division gives rise to daughter cells that have the ability to self renew [98]. Prospero has a key role in regulating stem cell self renewal [98, 99]. Berger *et al* showed that prospero can be regulated by the cell cycle protein cyclin E, whereby when cyclin E is lost there is an even distribution of prospero resulting in the differentiation of all neuroblasts into glial cells [100].

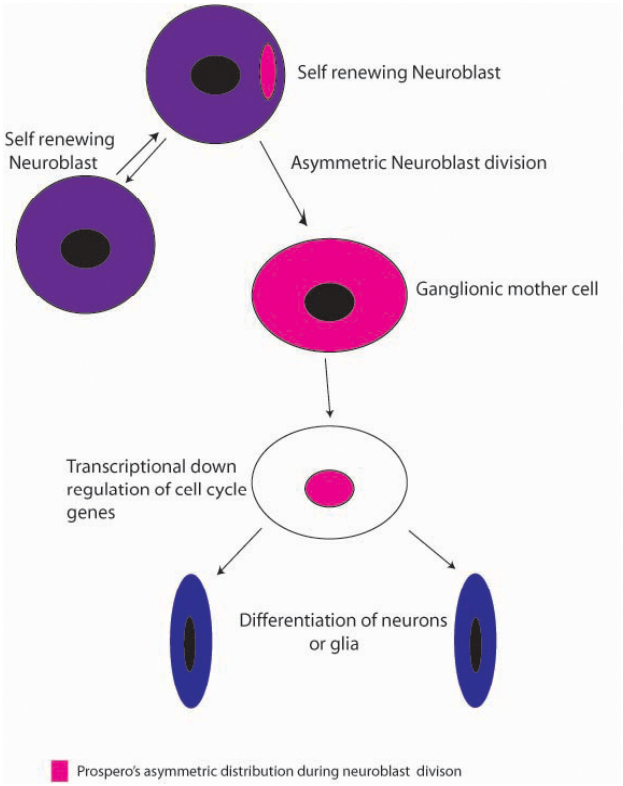

FIGURE 3. Prospero localization during asymmetric division of the drosophila neuroblast
Prospero is asymmetrically localized to the GMC where it enters the nucleus and transcriptionally regulates genes, leading to the terminal differentiation of the cells.
Modified from Myster and Duonio 2000 [93].

1.5.2 Transcription factors

Transcription factors are proteins that contain a DNA binding domain, transcription activation or transcription repression domains, which they use to regulate the expression of other genes. They execute this by binding either enhancer or promoter regions of DNA related to the genes they intend on regulating. This transcriptional regulation may initiate up-regulation (increase the expression) or downregulation (decrease the expression) of the gene in question. Transcription factors may be able to function alone through direct binding of the DNA or they may need to

form complexes with other proteins. This important function of transcription factors gives them a key role in many biological processes such as embryonic development and cell cycle regulation. Both PROX1 and P53 are transcription factors and will be discussed in the following chapters.

1.5.3 Identification and structure of the PROX1 gene

PROX1 is a member of the homeodomain protein family with an atypical homeodomain. This family is characterized by a 180 base pair DNA sequence, approximately 60 amino acids (homeodomain) which can bind DNA, consequently enabling PROX1 to transcriptionally regulate other genes. The homeodomain of PROX1 is described as atypical because it does not share any sequence homology with other homeodomains, nevertheless it creates the helix-loop-helix-turn-helix fold (three helix bundle motif) structure which is a major characteristic of other homeodomains [101].

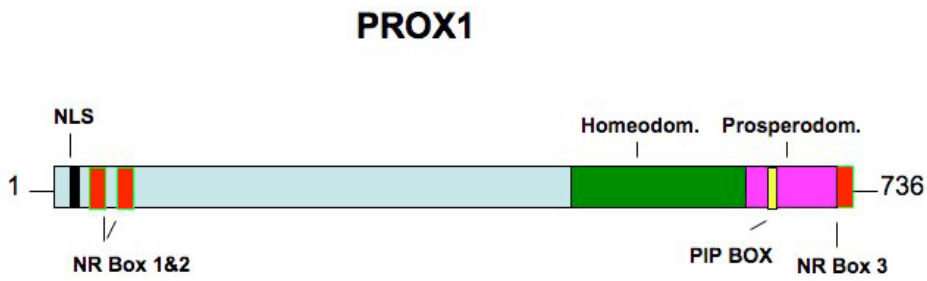

FIGURE 4. PROX1 domain structure

PROX1 contains a homeodomain, a prospero domain, a nuclear localization signal (NLS), three nuclear receptor boxes (NR BOX) and a PCNA interacting protein motif [102] [102].

Mouse *Prox1* was initially identified by homology with Drosophila prospero [103], followed by the identification of the human *PROX1* by homology with the chicken *Prox1*[104]. Sequence comparison of the human *PROX1* homeodomain revealed a 65% homology with the Drosophila

prospero and a 94% homology with the chicken and mouse *Prox1* genes. This high level of conservation of gene sequence between different vertebrates, suggests a similar role for the gene in these species.

The human *PROX1* gene is localized on chromosome 1q32.2-q32.3 and is composed of 5 exons and 4 introns. *PROX1* has a cDNA length of 2924 base pairs with a deduced number of 736 amino acids and a calculated molecular mass of 82.3 kDa [104]. Both the homeodomain and the prospero domain of the protein are located at the C-terminus and are critical for the proteins DNA binding ability, they are highly conserved between species including Drosophila. The PROX1 protein also contains a nuclear localization signal (NLS) and three nuclear receptor boxes (NR Box 1-3) [105, 106](Figure 4).
There have been multiple *Prox1* transcripts previously described, one of 8 kb present in most tissues during development and another of approximately 2 kb present in the eye lens [106].

1.5.4 Vertebrate PROX1

Prox1 has an important role in the development of a number of organs, as *Prox1* null mice die before birth (mid gestation) due to multiple developmental defects [107].
In 1993, the expression pattern of Prox1 in the fetal murine CNS was described [103]. They showed that its expression is restricted to the early neuronal precursors of the SVZ and suggested a role for Prox1 in neuronal differentiation. In the developing mammalian CNS, Prox1 was ascribed a role similar to that of Drosophila prospero in the early steps of differentiation of neural stem cells [108]. A study revealed the specific role for Prox1 in neurogenesis, and found that it is expressed in a transient specialized population of cells at the progenitor stage and that in the absence of Prox1 these cells do not exit the cell cycle and complete neural differentiation [109].

Prox1 expression in the developing eye lens and retina also determines its essential role for the normal development of the eye. In the absence of Prox1 there was abnormal cellular proliferation and down regulated expression of the cell cycle inhibitors $p27^{kip1}$ and $p57^{kip2}$, aberrant expression of E-cadherin and inappropriate apoptosis, affecting the normal development of the eye lens. Prox1 also has a transcriptional role in the lens, activating the βB1-

crystallin genes in the developing chicken lens. βB1-crystallin is one of the crystallins (alpha, beta, gamma) that constitute the majority of proteins forming the lens [104, 110, 111].

Wigle and Oliver [112] identified the crucial role for Prox1 in lymphatic system development, and ever since studies have continued to show the importance of Prox1 and have characterized it as one of the most prominent lymphatic markers to date [113, 114]. The lymphatic system and the blood vascular system both arise from a common source but then separate during development. It has been shown that any malfunction in the process of separation can lead to some dangerous abnormalities such as accumulation of fluid in the peritoneal cavity, arteriovenous malformations and edema [115]. Over expression of Prox1 in blood vascular endothelial cells up-regulates lymphatic-specific endothelial cells genes and represses blood vasculature-specific genes [116].

Prox1 has an important role in the normal development of the liver, where it plays a key role in the migration of hepatocytes [117]. In the absence of Prox1 the liver is 70% smaller than that of the wild type due to the absence of hepatocytes from most liver lobes [118]. As in the liver, Prox1 knockout also affects the development of the pancreas both in size and morphology. Knockout mice also revealed a role for Prox1 in cell fate determination in the pancreas [119].

1.5.5 PROX1 interactions with nuclear receptor proteins

As mentioned above PROX1 contains three nuclear receptor [57] boxes, two at the N-terminus and one at the C-terminus. Nuclear receptors [57] are important regulators of genes, expression of these genes is in turn essential for a number of developmental processes. The N-terminal NR boxes 1&2 of PROX1 were found to interact with the following orphan nuclear receptors, hepatocyte nuclear factor 4 alpha (HNF4a/NR2A1) [120], liver receptor homolog1 (LRH-1/NR5A2, FTF) [121] and LRH-1 homolog steroid factor1 (SF-1/NR5A1). Both HNF4a and LRH-1 directly interact with PROX1 resulting in the repression of the human cholesterol 7alpha-hydroxylase (CYP7A1) gene. CYP7A1 is an important enzyme regulating lipid homeostasis and catalyzing the conversion of cholesterol to bile acids. SF-1 has an essential role in the development of the adrenal cortex in mammals and regulating expression of all steroidogenic enzymes as well as steroidogenic-stimulating hormones and expression of

cholesterol transporters. PROX1 interaction with SF1 leads to SF1's transcriptional repression but the formation of PROX1/SF1 complex is essential for renal development [122].

1.5.6 PROX1 interactions with other proteins

PROX1's role in the lymphatic vasculature development is crucial as mentioned previously. One of its functions is maintaining the lymphatic endothelial cells [29]. It is now clear that there is another protein involved, the nuclear hormone receptor Coup-TFII, which activates PROX1 by binding to its DNA binding region. This Coup-TFII/PROX1 complex is necessary throughout LEC progression from LEC progenitors to differentiated LEC's [123].

The proliferating cell nuclear antigen (PCNA) was also found to interact with PROX1 leading to repression of PROX1's function as an activator of the βB1-crystallin promoter in lens epithelial cells. This interaction between PCNA and PROX1 is through the interacting protein motif (PIP box) that lies between amino acids 686-693 [124, 125].

1.5.7 PROX1 tumor suppressor gene or oncogene?

In recent years there have been some studies that investigate PROX1's role in cancer and its progression. In 2003 it was found that PROX1 was affected by DNA hypermethylation in a group of hematological malignancies and lymphomas, the authors extrapolated from this that PROX1 may act as a tumor suppressor [126]. Furthermore, PROX1 was suggested to be involved in progression of pancreatic cancer, where they found low levels of PROX1 expression in the tumors, with short patient survival when compared to the normal exocrine pancreas [127].

Other studies described PROX1 as a tumor suppressor and connected its dysregulation or absence to the progression of cancer, such as in the case of hepatocellular carcinoma [128], carcinomas of the biliary system [129], sporadic breast cancer [130] and Kaposi's sarcoma [131]. A recent study shed a new light on PROX1's relationship with cancer and described that it is over expressed in colon cancer cell lines and how it promotes the progress of malignancy in colon cancer. The fact that PROX1's overexpression or knock-out effects, differ depending on the location, suggests a highly tissue specific control for PROX1's expression and function [132].

2. THE TP53 TUMOR SUPPRESSOR GENE

2.1 Discovery

In the 1970's there was a large focus on the study of tumor viruses, since it was the common assumption that they were the cause of many cancer types [187]. At the end of the 1970's several groups reported independently that Simian virus 40 (SV40 adenovirus) T- antigen co-immunoprecipitated with a protein with a molecular weight of 53,000 kDa [133, 134] that protein was consequently named p53.

After the first group of experiments published on this novel protein it was thought that p53 was an oncogene. That was due to its ability to transform cultured cells when added with the Ras oncogene [135-137]. This changed in the 1980's when these experiments could not be repeated and it was then discovered that the previous p53 clone had a mutation at codon 135 inducing its oncogenic effect [138, 139].

Following this finding came studies revealing that p53 could in contrast, inhibit transformation of cells in culture [140] and since then p53 has been considered a tumor suppressor gene.

At present we know that TP53 is one of the most commonly mutated genes in human cancer, with about 50% of studied tumors harboring the mutation. One of the most studied examples of cancer with mutated p53 is lung cancer, there is a strong link between tobacco smoke and p53 mutations. Other examples of organs where tumors may harbor a p53 mutation include the colon, breast, bladder, ovaries, prostate, head and neck (http://p53.free.fr/index.html).

2.2 p53 protein structure and functional domains

The TP53 gene is located on chromosome 17p13.1 and encodes a nuclear protein of 393 amino acid residues. It has 5 well defined functional domains (Fig 5). The transactivation domain (TA) is located in the N-terminus and responsible for p53's transcriptional activity and MDM2 binding [141, 142]. Adjacent to the TA lies the proline–rich domain (PRD), which has been described as having an important role in p53 stability, transactivation ability and induction of apoptosis [143]. In the central part of the protein is the DNA binding domain (DBD), which in accordance with its name is responsible for DNA specific sequence binding. This domain was found to be the most frequently mutated in human cancers [144]. The carboxy (C)-terminus contains the oligomerization domain, which ensures that the protein is in the correct configuration that enables it to fulfill its functions [145]. The last 30 amino acids of the protein regulate DNA binding in a negative manner, inhibiting DNA binding and transcriptional activity of p53 [146](Figure 5).

2.3 p53 regulation

Considering the important role of p53 in maintaining the stability of the mammalian genome, its expression is tightly regulated. Under normal conditions p53 is rapidly degraded in the cell through proteasomal degradation and ubiquitination that is controlled mainly by MDM2. When the cell is under stress, e g in the form of oncogene activation, DNA damage, hypoxia, or UV irradiation, p53 is activated by posttranslational modifications such as phosphorylation [147]. This activation of p53 is essential for it to function as a transcription factor that is able to induce a number of biological responses, such as apoptosis, cell cycle arrest, differentiation and senescence [147] (Figure 5). The decision of which cellular response to undergo is governed by several factors including cell type, extent of DNA damage and p53 protein levels [148] (figure 5). p53 transactivates hundreds of genes that contain a sequence specific binding site in their regulatory domain [149] and thousands of genes have been reported to respond to p53 expression [150].

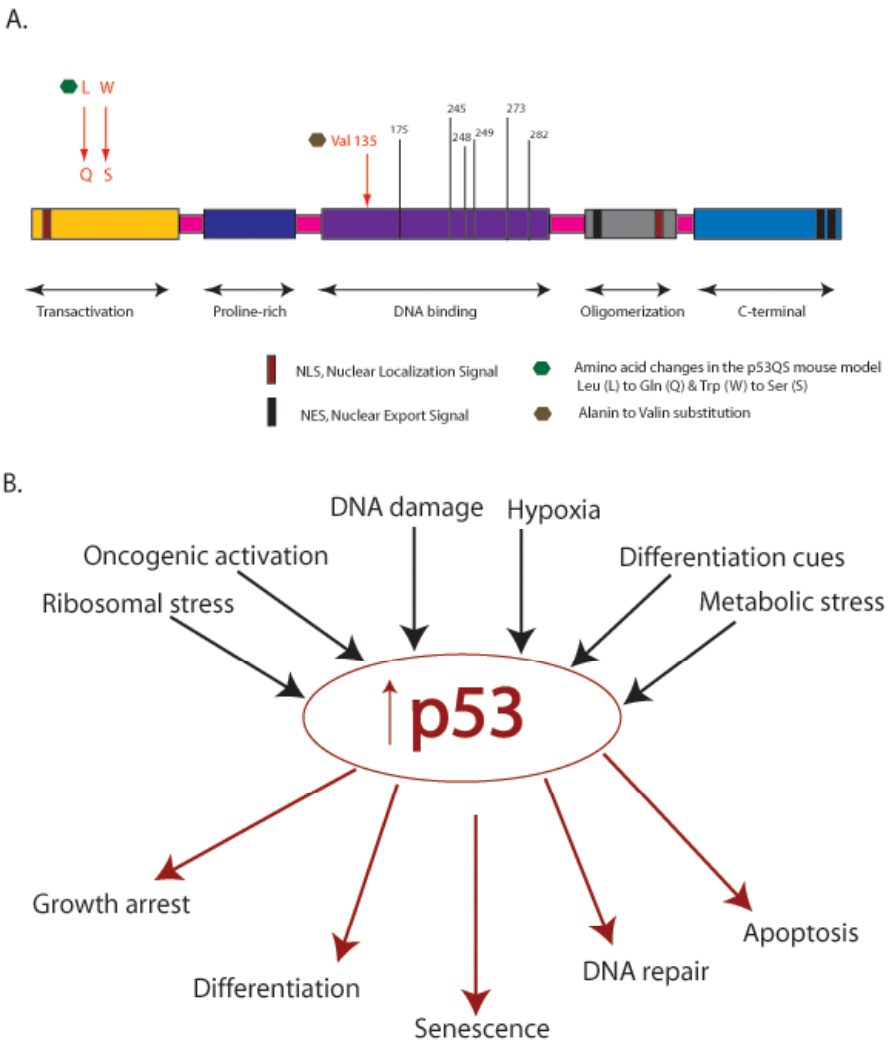

Figure 5. Schematic representation of p53's structural domains and function.

A. p53's functional domains with representation of the six common mutation residues "hot spots". B. Activation of p53 through cellular stress signals leads to its induction of various biological processes.

2.4 p53 family

Two homologues of p53 were discovered 20 years after the initial discovery of p53, namely p63 and p73 [151-153]. Both genes were found to be important during development. p63 is essential for the differentiation program of squamous epithelial cells [154] and also in the maintenance of stem cell proliferation [155]. p73 has shown an importance in neuronal development illustrated by knockout mice that show developmental defects in their CNS [156]. Despite the fact that p63 and p73 are highly conserved homologues of p53, i.e. sharing the transactivation domain, the oligomerization domain and the DNA binding domain they are rarely mutated in human cancers [157, 158]. Dysregulation of p63 and some of its isoforms have been described in several human squamous cell cancers including those of the head and neck, lung, breast, cervix, and ovaries [159, 160].

3. PRESENT INVESTIGATION

3.1 Aims of the thesis

The general aim of the thesis was to contribute to the understanding of the molecular mechanisms underlying tumor development by studying transcription factors PROX1 and p53, in the brain and skin respectively.

1. Paper 1: To investigate whether PROX1 expression can be of clinical use as a histological biomarker for distinguishing between the different malignancy grades of astrocytic brain tumors.
2. Paper 2: To evaluate the prognostic or predictive value of PROX1 for survival in patients diagnosed with grade II astrocytic tumors.
3. Paper 3: To demonstrate the expression pattern of PROX1 in different human glioma cell lines and perform some initial functional studies.
4. Paper 4: To assess whether transactivation deficient p53 confers the same malignant phenotype observed in p53-null keratinocytes when overexpressing v-ras^{HA} in these cells, thus addressing the question if p53's transactivation function is essential for tumor suppression in the skin.

4. RESULTS & DISCUSSION

4.1 Paper I: *Expression of PROX1 is a common feature of high grade malignant astrocytic gliomas*

PROX1 is a transcription factor important in the developmental pathway of various organs. It has a key role in neurogenesis, through transcriptionally regulating a group of genes (including cell cycle regulating genes) to determine the cell's fate. Previously PROX1 has been described as both a tumor suppressor gene and an oncogene. Despite the importance of PROX1 in CNS development, we are the first to investigate its role in brain tumors. In this paper we performed immunohistochemical staining on 56 human astrocytic gliomas of different grades for our analysis of PROX1 expression. Using a goat anti-human PROX1 antibody, we found a positive and statistically significant correlation between PROX1 expression and glioma tumor grade. Hence, in WHO grade IV (glioblastoma, n=15) on average 79% of the cells and in WHO grade III (anaplastic astrocytoma; n=13) 57% of the cells were strongly positive, on average 21% of cells (n=13) were clearly PROX1 positive in the grade II tumors and finally grade I astrocytomas showed significantly lower expression of PROX1, on average 1.5% of cells (n=15) were strongly positive. When comparing strongly positive cells in grade III as well as in grade IV with the grade II cases the difference was significant. Since the diagnosis and grading of astrocytomas can be challenging, PROX1 expression could be a useful diagnostic tool to distinguish high grade tumors (III, IV) from grade II tumors and so contribute to the improvement of the diagnostic accuracy.

In attempt to learn more about the PROX1 positive cells within the tumor mass, we used a double immunostaining with the proliferation markers PCNA and Ki67. $PROX1^+$ cells did not show increased proliferation compared with the average tumor cell population proliferation index. Moreover, using the double immunofluorescence technique we studied the expression of PROX1 with antibodies targeting βIII-tubulin/TuJ1, microtubule-associated protein 2 (MAP2) (both are early neuronal markers)[102, 161-163], NeuN (mature neuronal marker)[164] and GFAP (astrocytic cell marker) [165].

We found that $PROX1^+$ cells frequently co-expressed βIII-tubulin and MAP2 and to a lesser extent GFAP. Interestingly, the tumor cells did not express NeuN indicating that the MAP2 and βIII-tubulin positive cells were blocked in early stages of neuronal differentiation and had failed

to undergo terminal differentiation. It is not uncommon for high grade gliomas to express both astrocytic and glial markers, resembling a more immature cellular status similar to progenitor cells or stem cells. This is also supported by their consistently high expression levels of SOX2, a progenitor cell marker. It has been suggested that these cells may resemble the cells of origin (TIC) for these tumors [166].

A classic feature of human high grade malignant glioma is a large degree of heterogeneity, both intratumoral and intertumoral differences can be found. The relatively small number of tumor samples that we used to perform the double staining method on, is a limitation here due to this heterogeneous nature of the tumors. It would be of great interest to see if PROX1 does consistently mark a population of cells that proliferates slower than others in a tumor, while expressing progenitor cell markers. Stem cells are of a quiescent nature, meaning they actually proliferate at a lower rate than other dividing cells in order to not exhaust their "stemness". PROX1's marginally lower co-expression with proliferation markers Ki67 and PCNA is another factor that would support the stem cell/ progenitor cell feature.

In this paper we also show that PROX1 over expression is a special feature of the tumor cells and not the reactive non-neoplastic cells. Brain tissue that has undergone reactive change maybe difficult to distinguish from grade II astrocytomas [167], this non-cancerous change maybe due to a number of factors, including, infarction, gliosis or inflammation. We found that the expression of PROX1 in such lesions is much less than in any of the astrocytic tumor grades, excluding pilocytic astrocytomas grade I, which is consistent with the fact that grade I tumors constitute an entirely different entity from diffuse astrocytic gliomas.

From the immunohistochemical staining result it is evident that PROX1 is not expressed in any vascular structures in these brain tumors. We wanted to exclude this possibility, since PROX1 is a highly dependable lymphatic vessel marker. Although normal brain tissue does not contain lymphatics, malignant gliomas have been reported to express lymphatic markers [168].

Another point of interest is the ongoing discussion on peritumoral tissue in the brain and whether it should be considered normal or neoplastic. There are factors calling for the need to make some distinctions in the matter, including, the highly infiltrative nature of high grade gliomas, the main line of treatment being surgical removal and the ability of CSCs to give rise to a daughter tumor of the same aggressiveness, independently. This renders the situation difficult,

especially considering the resistant nature of CSCs to therapy. So how much of the adjacent brain tissue should be resected and what are the risks of a relapse? In this paper we show that unlike the "normal" tissue control (with some slight gliosis), morphologically "normal" tissue closest to the tumor mass has a relatively high expression of PROX1.

Taken together, the data we present here on PROX1 expression in high grade malignant astrocytomas versus low grade undoubtedly yields some diagnostic information. In order to use this simple immunohistochemistry technique as a routine diagnostic tool in the clinic we or others should study PROX1 expression in a large patient sample of different glioma grades, preferably, with long term clinical follow up and known outcome.

4.2 Paper II

PROX1 is a predictor of survival for astrocytic gliomas but not for oligodendrogliomas grade II

Adult low-grade gliomas (LGG) are well differentiated, diffusely infiltrative brain tumors localized mainly in the cerebral hemispheres. They affect adults with an average age of approximately 40 years at the time of diagnosis. The median survival is 5-10 years but clinical outcome varies, in some patients there is a malignant progression to a higher grade and in others it remains as a grade II tumor. However treatment is unsatisfactory and there is today no cure for LGG. There is a great need for molecular biomarkers that may serve as diagnostic and prognostic tools or even targets for therapy.

In paper I we showed that PROX1 expression increases with tumor grade and that it maybe a useful diagnostic marker for high grade astrocytic gliomas. In this study we address the prognostic value of PROX1 in a cohort of patients with grade II gliomas. A total number of 128 samples were evaluated by immunohistochemistry for their PROX1 expression. A factor of strength in this study is the use of an automated system for the staining (Ventana) where you can insure an equal distribution of the antibodies throughout the sections. This is of great importance when comparing the expression levels of a protein between different cases in a large sample size. The samples comprised a random selection of eligible paraffin embedded astrocytomas,

oligoastrocytomas and oligodendrogliomas. The number of PROX1 immunoreactive cells was counted separately by two of the authors. PROX1 expression in these tumors was classified into either positive or negative and used as a variable in multivariate survival analysis, together with established prognostic factors for this patient group. The following statistical methods were used.

Kaplan-Meier method was used to plot the survival curves and the log-rank probability test (Mantel-Cox) was used to estimate the prognostic value of the PROX-1 expression in the univariate analyses. Two survival parameters were considered: postoperative survival and overall survival. The Cox proportional hazards model calculated the impact of each of the prognostic factors defined by the EORTC studies, namely, contrast enhancement, sex, the therapeutic factors extent of resection and radiotherapy, as well as the PROX-1 expression. Due to limited number of patients in the study, the stepwise exclusion of variables was used to achieve a model with as few variables as possible in the multivariate analyses. The level for confounders to be removed from the model when adjusted for variables already in the model was set to > 0.1. The natural logarithm [96] cumulative hazard plots were made to confirm the assumption of the proportional hazard functions. In order to minimize the possibility of interaction, the significant prognostic factors in the stepwise model were also analyzed as products.

We found that patients with tumors displaying a high number of PROX1 positive cells had poor outcome, both in the univariate analysis (p=0.0151) and multivariate analysis (p= 0.0250), compared to those with low PROX1 positivity. When analyzing the separate histological subgroups, PROX1-expression was associated with shorter survival in astrocytomas and oligoastrocytomas but not in oligodendrogliomas. We concluded that PROX1 is a novel predictor of survival for grade II astrocytic gliomas.

4.3 Paper III

Differential expression of transcription factor PROX1 in human glioma cell lines

In light of the previous studies (paper I & paper II), the question that comes to mind is what is the function of PROX1 in these tumor cells? As mentioned previously PROX1 plays a key role during CNS development, so could it have the same function in the brain tumor cells? Does it have a hand in regulating the proliferation of these cells? But in order to answer any of these questions, the expression of PROX1 in glioma cell lines had to be investigated. Interestingly, we found that PROX1 expression was either absent or considerably low in most cell lines except the GFAP positive cell line U343MGa Cl2:6. To investigate the function of PROX1 in U343MGa Cl2:6 cells we carried out siRNA transfections. PROX1 could be silenced in this line using a siRNA oligo earlier found to be specific for PROX1. Hence, we could achieve at least a 50% reduction in PROX1 levels as seen in both immunoblotting and immunostaining. Silencing of PROX1 in the U343MGa Cl2:6 cell line with siRNA had a slight positive effect on cell proliferation although we did not observe any gross morphological alterations in Cl2:6 cells depleted of PROX1. We found a positive correlation between PROX1 and the transcription factor SOX2, important for maintenance of stem cells.

4.4 Paper IV

The transcriptional regulatory function of p53 is essential for suppression of mouse skin carcinogenesis and can be dissociated from effects on TGF-β-mediated growth regulation

As mentioned previously, most p53 mutations in human cancer occur in the DNA binding domain highlighting the importance of p53 specific DNA binding in tumor suppression. Most reports show that p53 transcriptional regulation is essential for it to initiate the biological processes required (as mentioned above) but there have been reports that suggest a role for p53 in different biological processes in the absence of its transcriptional regulation function. It was suggested that p53 maybe able to mediate these responses, including apoptosis, DNA repair and differentiation through transcription independent methods like direct protein to protein interactions [169-173].

The $p53^{QS}$ mouse model was designed to be able to study the p53 gene in the absence of its transcriptional regulation function. This mutant was created by introducing changes at Leucine (L) 25 and Tryptophan (W) 26, where these codons were changed to (Q) glutamine and (S) serine, hence the name QS (See figure 5). This mutant maintains p53's ability to bind DNA but renders it transcriptionally inactive. After creating this mouse model it was subsequently found that the genomic clone used to make the targeting construct contained an alanine to valine substitution at codon 135. This Val135 mutation is known to be a temperature sensitive mutation [174, 175]. Nevertheless, when examining the $p53^{QS\text{-}Val135}$ under the promoting temperature it did not gain function [176]. This mutant did not show temperature sensitivity in mouse skin keratinocytes when tested in Wendy Weinberg's lab.
A study on $p53^{QS}$ mice was reported by Johnson et al, who created the same mutant but with an intact DNA binding domain (lacking the Val 135 mutation). Surprisingly this mutant was embryonic lethal, explaining that this lethality is due to hypoxia induced activation during early embryogenesis [177].

In this paper we demonstrate that transcriptional regulation by p53 is necessary in order to function as a tumor suppressor in mouse skin carcinogenesis.
We compared the effects of the mutant p53, $p53^{QS\text{-}val135}$, which contained the above mentioned double mutations, to complete loss of p53. It has been reported that $p53^{QS\text{-}val135/QS\text{-}val135}$ mice

develop tumors more or less identical to those arising in $p53^{-/-}$ mice. We compared keratinocyte cultures derived from $p53^{QS\text{-}val135/QS\text{-}val135}$, $p53^{+/QS\text{-}val135}$, $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ mice for their ability to undergo oncogene induced senescence, a normal cellular response contributing to maintenance of normal tissue homeostasis. Primary epidermal keratinocytes isolated from the different genotypes were infected with a retrovirus encoding the v-ras^{Ha} oncogene and then grafted onto the dorsal surface of nude mice. $p53^{+/+}$ control cultures show an increased number of senescing cells following introduction of oncogenic ras. Total loss of p53 is unable to overcome this oncogene-mediated cellular senescence and $p53^{QS\text{-}val135/QS\text{-}val135}$ keratinocytes behave identically to $p53^{-/-}$ keratinocytes in this assay. We found that from $p53^{+/+}$ keratinocytes, all resulting tumours were benign papillomas, as expected. $p53^{+/QS\text{-}val135}$ grafts behaved like $p53^{+/+}$ grafts and gave rise to well-differentiated papillomas, with no predisposition towards malignant conversion, while $p53^{QS\text{-}val135/QS\text{-}val135}$ and $p53^{-/-}$ keratinocytes yielded only undifferentiated carcinomas following grafting.

We used immunohistochemistry, to confirm the presence of the p53 protein in $p53^{QS\text{-}val135/QS\text{-}val135}$ tumors, demonstrated by strong nuclear staining. We then used immunohistochemistry to examine the grafts reactivity to $p21^{waf1}$, a p53 transcriptionally regulated gene. We found that $p21^{waf1}$ positive nuclear staining is prominent in the basal layers of both $p53^{+/+}$ and $p53^{+/QS\text{-}val135}$ papillomas, while staining is absent in the $p53^{QS\text{-}val135/QS\text{-}val135}$ carcinoma. The lack of $p21^{waf1}$ expression in these $p53^{QS\text{-}val135/QS\text{-}val135}$ genotypes supports the fact that p53-mediated transcriptional regulation is compromised in homozygotes and is intact in heterozygotes. From this finding we conclude, that when the transactivation and DNA binding domains are compromised, the other domains of the p53 gene are insufficient to maintain the normal cellular senescence response to oncogenic ras. $p53^{+/QS\text{-}val135}$ keratinocytes behave similarly to $p53^{+/+}$ keratinocytes in this assay, indicating that the QS-val135 mutant p53 does not interfere with the remaining wild type protein and that it does not possess a dominant negative phenotype. These findings suggest that a functional amino terminal transactivation domain of p53 is required for the normal senescence response to activated v-ras^{Ha}.

We also report that although $p53^{QS\text{-}val135/QS\text{-}val135}$ keratinocytes behave identically to $p53^{-/-}$ keratinocytes in response to oncogenic ras induction they behave similar to the wild type p53 in response to TGF-ß. TGF-ß is known to have an anti-proliferative effect on normal epithelial cells. Loss of responsiveness to TGF-ß in epithelial cells is a feature of skin cancer development, deletion of the gene causes progression to squamous cell carcinoma [178, 179].

p53 partially controls keratinocytes' response to TGF-ß-induced growth arrest, whereby p53-null keratinocytes can only partially overcome TGF-ß-induced arrest [180]. Considering that transactivation deficiency in the $p53^{QS}$ mutant was supported by absence of $p21^{waf1}$ in $p53^{QS\text{-}val135/QS\text{-}val135}$ tumors, these findings indicate that the requirement for p53 in maximizing TGF-β-mediated growth regulation is independent of its transactivation domain and that the ability of keratinocytes to respond to TGF-β is insufficient to suppress the malignant phenotype in this model.

5. SIGNIFICANCE AND FUTURE PERSPECTIVES

Malignant gliomas are the most common form of brain tumors, are highly aggressive and invasive. They are often devastating to the patient both physically and psychologically. Despite extensive efforts, the median survival for patients with the most aggressive form is short (9-12 months) after maximum treatment. The highly infiltrative nature of theses tumors even when at a lower grade of malignancy makes it extremely difficult for successful maximum resection during surgery. Understanding the underlying molecular pathogenesis increases our possibilities for novel therapies [56]. The knowledge we acquire will bring further insight, to either improve our current therapies so that they are sufficient for treatment or establish novel clinical strategies. An example is the ongoing attempt for identification of tumor initiating cells or CSCs, which has been successful for some types of cancer. This finding promises a new cellular target for anticancer drug discovery. Identification of biological markers specific to the CSC population is a good way to identify these cells and possibly target them.
In the same light, it is of importance to discover molecular markers that assist in diagnosis and prediction of the course of disease.

Currently, neuropathologists grade gliomas based on the presence of the following criteria: increased cellular density, nuclear atypias, mitosis, vascular proliferation and necrosis [181]. Unfortunately there are cases that are difficult to assess based on these criteria and are subject to interobserver variability. These cases lead to uncertainties that subsequently compromise patient management. In an optimal clinical setting, clinicians will have the tools to give an accurate diagnosis immediately and also predict the course of progression for each tumor in a patient specific manner, especially in the case of low grade gliomas where there is a highly variable course of disease. Unfortunately at present times there is often a wait-and-see policy for grade II tumors. Clinical biomarkers are the road to successful personalized patient management. A good example is the MGMT methylation status of GBMs, providing knowledge on the patient's responsiveness to chemotherapy [182]. The emerging possibilities for treatment options, although exciting, introduce more challenges for diagnostic pathologists.

In this thesis we introduce PROX1 as a novel biomarker for malignant glioma and a prognostic marker for grade II astrocytic gliomas. Overexpression of PROX1 was consistent in all high grade tumors and its expression increased with tumor grade. Moreover, in a retrospective study of 128 patients with low grade astrocytic tumors, PROX1 was found to predict poor prognosis.

When this is substantiated in other studies, PROX1 expression analysis, using the simple immunohistochemistry technique, may be used in the routine clinical diagnosis of glioma. A high number of strongly positive PROX1 cells in a patient's tumor will primarily inform the neuropathologists of the neoplastic nature of the lesion, ruling out the possibility for a reactive condition. Secondly, it will help determine whether the tumor is grade II or of "high grade" III and IV, assisting in the choice for a suitable therapy accordingly. In the context of grade II tumors, a high number of PROX1 positive cells in a patient's sample indicates poor outcome. Consequently these low grade glioma cases could be given a more aggressive treatment due to this prior knowledge.

We will continue with the functional studies of PROX1 in order to understand PROX1's role in brain tumor progression, whether it be direct or indirect. PROX1 has been found to contribute to the progression of colon cancer to a more malignant phenotype, through the β-catenin pathway [132]. This mechanism of control is organ/ tissue specific, how it may function in brain tumor progression is not yet understood.

Many malignant glioma patients experience a quick relapse after initial remission of the tumor and a possible reason could be the elevated drug resistant nature of CSCs. It would be of great interest to investigate whether PROX1 is a marker for CSCs, considering the preliminary evidence indicating that PROX1 expression in the tumor marks a population of cells with CSC features. $PROX1^{+}$ cells are most likely progenitor cells, expressing both early neuronal markers and the glial marker GFAP, while not expressing any neuronal differentiation markers, having a relatively slow rate of proliferation and expressing SOX2. We should also consider that prospero, the Drosophila homologue of PROX1 is a key regulator of stem cell self renewal in Drosophila and that recent work on Drosophila neural stem cells (neuroblasts) has provided insights into tumor formation and stem cell biology [183-186].

Dissecting the mechanisms of function and tumor suppression of TP53 is of extreme importance. As mentioned previously, it is a key player in cell death and proliferation, and it is mutated in 50% of human cancers. In our study we show that p53 is not able to suppress a malignant phenotype in mouse skin carcinomas, in the absence of a well functioning transcriptional regulatory property, suggesting that both the DNA binding domain and the transactivation domain must be intact. We introduced a mutant $p53^{QS\text{-}val135}$, which contains a double mutation in the N-terminus abrogating transactivation activity and a modification at

amino acid 135 partially affecting DNA binding. This created a p53 mutant that acts in a similar fashion to p53 null mutants in its inability to suppress Ras oncogene-induced senescence.

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7. REFERENCES

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100(1):57-70.
2. Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. Acta Neuropathol; 119(1):7-35.
3. Jones DT, Kocialkowski S, Liu L, Pearson DM, Backlund LM, Ichimura K, et al. Tandem duplication producing a novel oncogenic BRAF fusion gene defines the majority of pilocytic astrocytomas. Cancer Res 2008; 68(21):8673-7.
4. Ohgaki H, Kleihues P. Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas. J Neuropathol Exp Neurol 2005; 64(6):479-89.
5. Vertosick FT, Jr., Selker RG, Arena VC. Survival of patients with well-differentiated astrocytomas diagnosed in the era of computed tomography. Neurosurgery 1991; 28(4):496-501.
6. Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC, et al. The WHO classification of tumors of the nervous system. J Neuropathol Exp Neurol 2002; 61(3):215-25; discussion 26-9.
7. Cavaliere R, Lopes MB, Schiff D. Low-grade gliomas: an update on pathology and therapy. Lancet Neurol 2005; 4(11):760-70.
8. Cairncross JG. Aggressive oligodendroglioma: a chemosensitive tumor. Recent Results Cancer Res 1994; 135:127-33.
9. Cairncross JG, Ueki K, Zlatescu MC, Lisle DK, Finkelstein DM, Hammond RR, et al. Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. J Natl Cancer Inst 1998; 90(19):1473-9.
10. Eoli M, Bissola L, Bruzzone MG, Pollo B, Maccagnano C, De Simone T, et al. Reclassification of oligoastrocytomas by loss of heterozygosity studies. Int J Cancer 2006; 119(1):84-90.
11. Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL, et al. Genetic pathways to glioblastoma: a population-based study. Cancer Res 2004; 64(19):6892-9.
12. Gaya A, Rees J, Greenstein A, Stebbing J. The use of temozolomide in recurrent malignant gliomas. Cancer Treat Rev 2002; 28(2):115-20.
13. Clarke J, Butowski N, Chang S. Recent advances in therapy for glioblastoma. Arch Neurol; 67(3):279-83.
14. Dropcho EJ, Soong SJ. The prognostic impact of prior low grade histology in patients with anaplastic gliomas: a case-control study. Neurology 1996; 47(3):684-90.
15. Jacobs JJ, van Lohuizen M. Polycomb repression: from cellular memory to cellular proliferation and cancer. Biochim Biophys Acta 2002; 1602(2):151-61.
16. Watanabe K, Tachibana O, Sata K, Yonekawa Y, Kleihues P, Ohgaki H. Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. Brain Pathol 1996; 6(3):217-23; discussion 23-4.
17. Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, et al. Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev 2007; 21(21):2683-710.
18. Gravendeel LA, Kouwenhoven MC, Gevaert O, de Rooi JJ, Stubbs AP, Duijm JE, et al. Intrinsic gene expression profiles of gliomas are a better predictor of survival than histology. Cancer Res 2009; 69(23):9065-72.

19. Purow B, Schiff D. Advances in the genetics of glioblastoma: are we reaching critical mass? Nat Rev Neurol 2009; 5(8):419-26.
20. Hong H, Takahashi K, Ichisaka T, Aoi T, Kanagawa O, Nakagawa M, et al. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway
Senescence impairs successful reprogramming to pluripotent stem cells
Direct cell reprogramming is a stochastic process amenable to acceleration
Cerebellar stem cells act as medulloblastoma-initiating cells in a mouse model and a neural stem cell signature characterizes a subset of human medulloblastomas. Nature 2009; 460(7259):1132-5.
21. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 2006; 9(3):157-73.
22. Koch JH, Fifis T, Bender VJ, Moss BA. Molecular species of epidermal growth factor carrying immunosuppressive activity. J Cell Biochem 1984; 25(1):45-59.
23. Grimaux M, Romain S, Remvikos Y, Martin PM, Magdelenat H. Prognostic value of epidermal growth factor receptor in node-positive breast cancer. Breast Cancer Res Treat 1989; 14(1):77-90.
24. Gusterson B, Cowley G, Smith JA, Ozanne B. Cellular localisation of human epidermal growth factor receptor. Cell Biol Int Rep 1984; 8(8):649-58.
25. Huang PH, Xu AM, White FM. Oncogenic EGFR signaling networks in glioma. Sci Signal 2009; 2(87):re6.
26. Mitsudomi T, Yatabe Y. Epidermal growth factor receptor in relation to tumor development: EGFR gene and cancer. Febs J; 277(2):301-8.
27. El-Obeid A, Hesselager G, Westermark B, Nister M. TGF-alpha-driven tumor growth is inhibited by an EGF receptor tyrosine kinase inhibitor. Biochem Biophys Res Commun 2002; 290(1):349-58.
28. Rich JN, Bigner DD. Development of novel targeted therapies in the treatment of malignant glioma. Nat Rev Drug Discov 2004; 3(5):430-46.
29. Haas-Kogan DA, Prados MD, Tihan T, Eberhard DA, Jelluma N, Arvold ND, et al. Epidermal growth factor receptor, protein kinase B/Akt, and glioma response to erlotinib. J Natl Cancer Inst 2005; 97(12):880-7.
30. Smits A, Kato M, Westermark B, Nister M, Heldin CH, Funa K. Neurotrophic activity of platelet-derived growth factor (PDGF): Rat neuronal cells possess functional PDGF beta-type receptors and respond to PDGF. Proc Natl Acad Sci U S A 1991; 88(18):8159-63.
31. Buch S, Han RN, Cabacungan J, Wang J, Yuan S, Belcastro R, et al. Changes in expression of platelet-derived growth factor and its receptors in the lungs of newborn rats exposed to air or 60% O(2). Pediatr Res 2000; 48(4):423-33.
32. D'Andrea MR, Mei JM, Tuman RW, Galemmo RA, Johnson DL. Validation of in vivo pharmacodynamic activity of a novel PDGF receptor tyrosine kinase inhibitor using immunohistochemistry and quantitative image analysis. Mol Cancer Ther 2005; 4(8):1198-204.
33. Westermark B, Heldin CH, Nister M. Platelet-derived growth factor in human glioma. Glia 1995; 15(3):257-63.
34. Hermanson M, Funa K, Hartman M, Claesson-Welsh L, Heldin CH, Westermark B, et al. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. Cancer Res 1992; 52(11):3213-9.
35. Hermanson M, Funa K, Koopmann J, Maintz D, Waha A, Westermark B, et al. Association of loss of heterozygosity on chromosome 17p with high platelet-derived growth

factor alpha receptor expression in human malignant gliomas. Cancer Res 1996; 56(1):164-71.
36. Shih AH, Dai C, Hu X, Rosenblum MK, Koutcher JA, Holland EC. Dose-dependent effects of platelet-derived growth factor-B on glial tumorigenesis. Cancer Res 2004; 64(14):4783-9.
37. Uhrbom L, Hesselager G, Nister M, Westermark B. Induction of brain tumors in mice using a recombinant platelet-derived growth factor B-chain retrovirus. Cancer Res 1998; 58(23):5275-9.
38. Hede SM, Hansson I, Afink GB, Eriksson A, Nazarenko I, Andrae J, et al. GFAP promoter driven transgenic expression of PDGFB in the mouse brain leads to glioblastoma in a Trp53 null background. Glia 2009; 57(11):1143-53.
39. Dresemann G. Imatinib and hydroxyurea in pretreated progressive glioblastoma multiforme: a patient series. Ann Oncol 2005; 16(10):1702-8.
40. Reardon DA, Egorin MJ, Quinn JA, Rich JN, Gururangan S, Vredenburgh JJ, et al. Phase II study of imatinib mesylate plus hydroxyurea in adults with recurrent glioblastoma multiforme. J Clin Oncol 2005; 23(36):9359-68.
41. Shih AH, Holland EC. Platelet-derived growth factor (PDGF) and glial tumorigenesis. Cancer Lett 2006; 232(2):139-47.
42. Kwon CH, Zhao D, Chen J, Alcantara S, Li Y, Burns DK, et al. Pten haploinsufficiency accelerates formation of high-grade astrocytomas. Cancer Res 2008; 68(9):3286-94.
43. Zhu Y, Harada T, Liu L, Lush ME, Guignard F, Harada C, et al. Inactivation of NF1 in CNS causes increased glial progenitor proliferation and optic glioma formation. Development 2005; 132(24):5577-88.
44. Alcantara Llaguno S, Chen J, Kwon CH, Jackson EL, Li Y, Burns DK, et al. Malignant astrocytomas originate from neural stem/progenitor cells in a somatic tumor suppressor mouse model. Cancer Cell 2009; 15(1):45-56.
45. Cavaliere R, Wen PY, Schiff D. Novel therapies for malignant gliomas. Neurol Clin 2007; 25(4):1141-71, x.
46. Pignatti F, van den Bent M, Curran D, Debruyne C, Sylvester R, Therasse P, et al. Prognostic factors for survival in adult patients with cerebral low-grade glioma. J Clin Oncol 2002; 20(8):2076-84.
47. Yan H, Bigner DD, Velculescu V, Parsons DW. Mutant metabolic enzymes are at the origin of gliomas. Cancer Res 2009; 69(24):9157-9.
48. Frezza C, Tennant DA, Gottlieb E. IDH1 mutations in gliomas: when an enzyme loses its grip. Cancer Cell; 17(1):7-9.
49. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, et al. IDH1 and IDH2 mutations in gliomas. N Engl J Med 2009; 360(8):765-73.
50. Aghili M, Zahedi F, Rafiee E. Hydroxyglutaric aciduria and malignant brain tumor: a case report and literature review. J Neurooncol 2009; 91(2):233-6.
51. Hartmann C, Meyer J, Balss J, Capper D, Mueller W, Christians A, et al. Type and frequency of IDH1 and IDH2 mutations are related to astrocytic and oligodendroglial differentiation and age: a study of 1,010 diffuse gliomas. Acta Neuropathol 2009; 118(4):469-74.
52. Weller M, Felsberg J, Hartmann C, Berger H, Steinbach JP, Schramm J, et al. Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the German Glioma Network. J Clin Oncol 2009; 27(34):5743-50.

53. Ochs K, Kaina B. Apoptosis induced by DNA damage O6-methylguanine is Bcl-2 and caspase-9/3 regulated and Fas/caspase-8 independent. Cancer Res 2000; 60(20):5815-24.
54. Stojic L, Cejka P, Jiricny J. High doses of SN1 type methylating agents activate DNA damage signaling cascades that are largely independent of mismatch repair. Cell Cycle 2005; 4(3):473-7.
55. Ross JS, Linette GP, Stec J, Clark E, Ayers M, Leschly N, et al. Breast cancer biomarkers and molecular medicine. Expert Rev Mol Diagn 2003; 3(5):573-85.
56. Sawyers CL. The cancer biomarker problem. Nature 2008; 452(7187):548-52.
57. Wen PY, Yung WK, Lamborn KR, Dahia PL, Wang Y, Peng B, et al. Phase I/II study of imatinib mesylate for recurrent malignant gliomas: North American Brain Tumor Consortium Study 99-08. Clin Cancer Res 2006; 12(16):4899-907.
58. Lapidot T, Grunberger T, Vormoor J, Estrov Z, Kollet O, Bunin N, et al. Identification of human juvenile chronic myelogenous leukemia stem cells capable of initiating the disease in primary and secondary SCID mice. Blood 1996; 88(7):2655-64.
59. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res 2003; 63(18):5821-8.
60. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. Nature 2004; 432(7015):396-401.
61. Ricci-Vitiani L, Fabrizi E, Palio E, De Maria R. Colon cancer stem cells. J Mol Med 2009; 87(11):1097-104.
62. Dontu G, Al-Hajj M, Abdallah WM, Clarke MF, Wicha MS. Stem cells in normal breast development and breast cancer. Cell Prolif 2003; 36 Suppl 1:59-72.
63. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005; 65(23):10946-51.
64. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001; 414(6859):105-11.
65. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 2008; 8(10):755-68.
66. Molofsky AV, Pardal R, Morrison SJ. Diverse mechanisms regulate stem cell self-renewal. Curr Opin Cell Biol 2004; 16(6):700-7.
67. Meletis K, Wirta V, Hede SM, Nister M, Lundeberg J, Frisen J. p53 suppresses the self-renewal of adult neural stem cells. Development 2006; 133(2):363-9.
68. Banito A, Rashid ST, Acosta JC, Li S, Pereira CF, Geti I, et al. Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev 2009; 23(18):2134-9.
69. Groszer M, Erickson R, Scripture-Adams DD, Dougherty JD, Le Belle J, Zack JA, et al. PTEN negatively regulates neural stem cell self-renewal by modulating G0-G1 cell cycle entry. Proc Natl Acad Sci U S A 2006; 103(1):111-6.
70. Wang Y, Guan Y, Wang F, Huang A, Wang S, Zhang YA. Bmi-1 regulates self-renewal, proliferation and senescence of human fetal neural stem cells in vitro. Neurosci Lett.
71. Graham V, Khudyakov J, Ellis P, Pevny L. SOX2 functions to maintain neural progenitor identity. Neuron 2003; 39(5):749-65.
72. Phi JH, Park SH, Kim SK, Paek SH, Kim JH, Lee YJ, et al. Sox2 expression in brain tumors: a reflection of the neuroglial differentiation pathway. Am J Surg Pathol 2008; 32(1):103-12.
73. Wang Y, Mah N, Prigione A, Wolfrum K, Andrade-Navarro MA, Adjaye J. A Transcriptional Roadmap to the Induction of Pluripotency in Somatic Cells. Stem Cell Rev.

74. Guo J, Li ZC, Feng YH. Expression and activation of the reprogramming transcription factors. Biochem Biophys Res Commun 2009; 390(4):1081-6.
75. Lindvall O, Kokaia Z, Martinez-Serrano A. Stem cell therapy for human neurodegenerative disorders-how to make it work. Nat Med 2004; 10 Suppl:S42-50.
76. Ma DK, Bonaguidi MA, Ming GL, Song H. Adult neural stem cells in the mammalian central nervous system. Cell Res 2009; 19(6):672-82.
77. Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. Cell 1990; 60(4):585-95.
78. Potten CS, Booth C, Tudor GL, Booth D, Brady G, Hurley P, et al. Identification of a putative intestinal stem cell and early lineage marker; musashi-1. Differentiation 2003; 71(1):28-41.
79. Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, et al. Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci U S A 2000; 97(26):14720-5.
80. Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997; 90(12):5002-12.
81. Yu Y, Flint A, Dvorin EL, Bischoff J. AC133-2, a novel isoform of human AC133 stem cell antigen. J Biol Chem 2002; 277(23):20711-6.
82. Parmar M, Skogh C, Bjorklund A, Campbell K. Regional specification of neurosphere cultures derived from subregions of the embryonic telencephalon. Mol Cell Neurosci 2002; 21(4):645-56.
83. Ahmed S. The culture of neural stem cells. J Cell Biochem 2009; 106(1):1-6.
84. Qiang L, Yang Y, Ma YJ, Chen FH, Zhang LB, Liu W, et al. Isolation and characterization of cancer stem like cells in human glioblastoma cell lines. Cancer Lett 2009; 279(1):13-21.
85. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 2006; 444(7120):756-60.
86. Wang J, Sakariassen PO, Tsinkalovsky O, Immervoll H, Boe SO, Svendsen A, et al. CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. Int J Cancer 2008; 122(4):761-8.
87. Patrawala L, Calhoun T, Schneider-Broussard R, Zhou J, Claypool K, Tang DG. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. Cancer Res 2005; 65(14):6207-19.
88. Wu C, Alman BA. Side population cells in human cancers. Cancer Lett 2008; 268(1):1-9.
89. Bleau AM, Huse JT, Holland EC. The ABCG2 resistance network of glioblastoma. Cell Cycle 2009; 8(18):2936-44.
90. Doe CQ, Chu-LaGraff Q, Wright DM, Scott MP. The prospero gene specifies cell fates in the Drosophila central nervous system. Cell 1991; 65(3):451-64.
91. Betschinger J, Knoblich JA. Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. Curr Biol 2004; 14(16):R674-85.
92. Jan YN, Jan LY. Asymmetric cell division. Nature 1998; 392(6678):775-8.
93. Myster DL, Duronio RJ. To differentiate or not to differentiate? Curr Biol 2000; 10(8):R302-4.
94. Hirata J, Nakagoshi H, Nabeshima Y, Matsuzaki F. Asymmetric segregation of the homeodomain protein Prospero during Drosophila development. Nature 1995; 377(6550):627-30.

95. Knoblich JA, Jan LY, Jan YN. Asymmetric segregation of Numb and Prospero during cell division. Nature 1995; 377(6550):624-7.
96. Bowman SK, Rolland V, Betschinger J, Kinsey KA, Emery G, Knoblich JA. The tumor suppressors Brat and Numb regulate transit-amplifying neuroblast lineages in Drosophila. Dev Cell 2008; 14(4):535-46.
97. Choksi SP, Southall TD, Bossing T, Edoff K, de Wit E, Fischer BE, et al. Prospero acts as a binary switch between self-renewal and differentiation in Drosophila neural stem cells. Dev Cell 2006; 11(6):775-89.
98. Betschinger J, Mechtler K, Knoblich JA. Asymmetric segregation of the tumor suppressor brat regulates self-renewal in Drosophila neural stem cells. Cell 2006; 124(6):1241-53.
99. Southall TD, Brand AH. Neural stem cell transcriptional networks highlight genes essential for nervous system development. Embo J 2009; 28(24):3799-807.
100. Berger C, Kannan R, Myneni S, Renner S, Shashidhara LS, Technau GM. Cell cycle independent role of Cyclin E during neural cell fate specification in Drosophila is mediated by its regulation of Prospero function. Dev Biol; 337(2):415-24.
101. Banerjee-Basu S, Landsman D, Baxevanis AD. Threading analysis of prospero-type homeodomains. In Silico Biol 1999; 1(3):163-73.
102. Laggner U, Pipp I, Budka H, Hainfellner JA, Preusser M. Immunohistochemical detection of class III beta-tubulin in primary brain tumours: variable expression in most tumour types limits utility as a differential diagnostic marker. Histopathology 2007; 50(7):949-52.
103. Oliver G, Sosa-Pineda B, Geisendorf S, Spana EP, Doe CQ, Gruss P. Prox 1, a prospero-related homeobox gene expressed during mouse development. Mech Dev 1993; 44(1):3-16.
104. Zinovieva RD, Duncan MK, Johnson TR, Torres R, Polymeropoulos MH, Tomarev SI. Structure and chromosomal localization of the human homeobox gene Prox 1. Genomics 1996; 35(3):517-22.
105. Gehring WJ, Affolter M, Burglin T. Homeodomain proteins. Annu Rev Biochem 1994; 63:487-526.
106. Tomarev SI, Zinovieva RD, Chang B, Hawes NL. Characterization of the mouse Prox1 gene. Biochem Biophys Res Commun 1998; 248(3):684-9.
107. Wigle JT, Chowdhury K, Gruss P, Oliver G. Prox1 function is crucial for mouse lens-fibre elongation. Nat Genet 1999; 21(3):318-22.
108. Torii M, Matsuzaki F, Osumi N, Kaibuchi K, Nakamura S, Casarosa S, et al. Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system. Development 1999; 126(3):443-56.
109. Misra K, Gui H, Matise MP. Prox1 regulates a transitory state for interneuron neurogenesis in the spinal cord. Dev Dyn 2008; 237(2):393-402.
110. Duncan MK, Cui W, Oh DJ, Tomarev SI. Prox1 is differentially localized during lens development. Mech Dev 2002; 112(1-2):195-8.
111. Tomarev SI, Sundin O, Banerjee-Basu S, Duncan MK, Yang JM, Piatigorsky J. Chicken homeobox gene Prox 1 related to Drosophila prospero is expressed in the developing lens and retina. Dev Dyn 1996; 206(4):354-67.
112. Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. Cell 1999; 98(6):769-78.
113. Rodriguez-Niedenfuhr M, Papoutsi M, Christ B, Nicolaides KH, von Kaisenberg CS, Tomarev SI, et al. Prox1 is a marker of ectodermal placodes, endodermal compartments, lymphatic endothelium and lymphangioblasts. Anat Embryol (Berl) 2001; 204(5):399-406.

114. Baluk P, McDonald DM. Markers for microscopic imaging of lymphangiogenesis and angiogenesis. Ann N Y Acad Sci 2008; 1131:1-12.
115. Abtahian F, Guerriero A, Sebzda E, Lu MM, Zhou R, Mocsai A, et al. Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. Science 2003; 299(5604):247-51.
116. Makinen T, Norrmen C, Petrova TV. Molecular mechanisms of lymphatic vascular development. Cell Mol Life Sci 2007; 64(15):1915-29.
117. Sosa-Pineda B, Wigle JT, Oliver G. Hepatocyte migration during liver development requires Prox1. Nat Genet 2000; 25(3):254-5.
118. Burke Z, Oliver G. Prox1 is an early specific marker for the developing liver and pancreas in the mammalian foregut endoderm. Mech Dev 2002; 118(1-2):147-55.
119. Wang J, Kilic G, Aydin M, Burke Z, Oliver G, Sosa-Pineda B. Prox1 activity controls pancreas morphogenesis and participates in the production of "secondary transition" pancreatic endocrine cells. Dev Biol 2005; 286(1):182-94.
120. Song KH, Li T, Chiang JY. A Prospero-related homeodomain protein is a novel co-regulator of hepatocyte nuclear factor 4alpha that regulates the cholesterol 7alpha-hydroxylase gene. J Biol Chem 2006; 281(15):10081-8.
121. Steffensen KR, Holter E, Bavner A, Nilsson M, Pelto-Huikko M, Tomarev S, et al. Functional conservation of interactions between a homeodomain cofactor and a mammalian FTZ-F1 homologue. EMBO Rep 2004; 5(6):613-9.
122. Liu YW, Gao W, Teh HL, Tan JH, Chan WK. Prox1 is a novel coregulator of Ff1b and is involved in the embryonic development of the zebra fish interrenal primordium. Mol Cell Biol 2003; 23(20):7243-55.
123. Srinivasan RS, Geng X, Yang Y, Wang Y, Mukatira S, Studer M, et al. The nuclear hormone receptor Coup-TFII is required for the initiation and early maintenance of Prox1 expression in lymphatic endothelial cells. Genes Dev; 24(7):696-707.
124. Maga G, Hubscher U. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J Cell Sci 2003; 116(Pt 15):3051-60.
125. Chen X, Patel TP, Simirskii VI, Duncan MK. PCNA interacts with Prox1 and represses its transcriptional activity. Mol Vis 2008; 14:2076-86.
126. Nagai H, Li Y, Hatano S, Toshihito O, Yuge M, Ito E, et al. Mutations and aberrant DNA methylation of the PROX1 gene in hematologic malignancies. Genes Chromosomes Cancer 2003; 38(1):13-21.
127. Schneider M, Buchler P, Giese N, Giese T, Wilting J, Buchler MW, et al. Role of lymphangiogenesis and lymphangiogenic factors during pancreatic cancer progression and lymphatic spread. Int J Oncol 2006; 28(4):883-90.
128. Shimoda M, Takahashi M, Yoshimoto T, Kono T, Ikai I, Kubo H. A homeobox protein, prox1, is involved in the differentiation, proliferation, and prognosis in hepatocellular carcinoma. Clin Cancer Res 2006; 12(20 Pt 1):6005-11.
129. Laerm A, Helmbold P, Goldberg M, Dammann R, Holzhausen HJ, Ballhausen WG. Prospero-related homeobox 1 (PROX1) is frequently inactivated by genomic deletions and epigenetic silencing in carcinomas of the bilary system. J Hepatol 2007; 46(1):89-97.
130. Versmold B, Felsberg J, Mikeska T, Ehrentraut D, Kohler J, Hampl JA, et al. Epigenetic silencing of the candidate tumor suppressor gene PROX1 in sporadic breast cancer. Int J Cancer 2007; 121(3):547-54.
131. Dadras SS, Skrzypek A, Nguyen L, Shin JW, Schulz MM, Arbiser J, et al. Prox-1 Promotes Invasion of Kaposiform Hemangioendotheliomas. J Invest Dermatol 2008; 26:26.

132. Petrova TV, Nykanen A, Norrmen C, Ivanov KI, Andersson LC, Haglund C, et al. Transcription factor PROX1 induces colon cancer progression by promoting the transition from benign to highly dysplastic phenotype. Cancer Cell 2008; 13(5):407-19.
133. Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. Nature 1979; 278(5701):261-3.
134. Linzer DI, Levine AJ. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell 1979; 17(1):43-52.
135. Eliyahu D, Raz A, Gruss P, Givol D, Oren M. Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature 1984; 312(5995):646-9.
136. Jenkins JR, Rudge K, Currie GA. Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. Nature 1984; 312(5995):651-4.
137. Parada LF, Land H, Weinberg RA, Wolf D, Rotter V. Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. Nature 1984; 312(5995):649-51.
138. Eliyahu D, Goldfinger N, Pinhasi-Kimhi O, Shaulsky G, Skurnik Y, Arai N, et al. Meth A fibrosarcoma cells express two transforming mutant p53 species. Oncogene 1988; 3(3):313-21.
139. Hinds P, Finlay C, Levine AJ. Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. J Virol 1989; 63(2):739-46.
140. Finlay CA, Hinds PW, Levine AJ. The p53 proto-oncogene can act as a suppressor of transformation. Cell 1989; 57(7):1083-93.
141. Unger T, Mietz JA, Scheffner M, Yee CL, Howley PM. Functional domains of wild-type and mutant p53 proteins involved in transcriptional regulation, transdominant inhibition, and transformation suppression. Mol Cell Biol 1993; 13(9):5186-94.
142. Alarcon-Vargas D, Ronai Z. p53-Mdm2--the affair that never ends. Carcinogenesis 2002; 23(4):541-7.
143. Toledo F, Lee CJ, Krummel KA, Rodewald LW, Liu CW, Wahl GM. Mouse mutants reveal that putative protein interaction sites in the p53 proline-rich domain are dispensable for tumor suppression. Mol Cell Biol 2007; 27(4):1425-32.
144. Soussi T. p53 alterations in human cancer: more questions than answers. Oncogene 2007; 26(15):2145-56.
145. Chene P. The role of tetramerization in p53 function. Oncogene 2001; 20(21):2611-7.
146. Wolkowicz R, Rotter V. The DNA binding regulatory domain of p53: see the C. Pathol Biol (Paris) 1997; 45(10):785-96.
147. Ashcroft M, Ludwig RL, Woods DB, Copeland TD, Weber HO, MacRae EJ, et al. Phosphorylation of HDM2 by Akt. Oncogene 2002; 21(13):1955-62.
148. Chen X, Ko LJ, Jayaraman L, Prives C. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev 1996; 10(19):2438-51.
149. el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. Definition of a consensus binding site for p53. Nat Genet 1992; 1(1):45-9.
150. Wang L, Wu Q, Qiu P, Mirza A, McGuirk M, Kirschmeier P, et al. Analyses of p53 target genes in the human genome by bioinformatic and microarray approaches. J Biol Chem 2001; 276(47):43604-10.
151. Senoo M, Seki N, Ohira M, Sugano S, Watanabe M, Inuzuka S, et al. A second p53-related protein, p73L, with high homology to p73. Biochem Biophys Res Commun 1998; 248(3):603-7.

152. Osada M, Ohba M, Kawahara C, Ishioka C, Kanamaru R, Katoh I, et al. Cloning and functional analysis of human p51, which structurally and functionally resembles p53. Nat Med 1998; 4(7):839-43.
153. Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, et al. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 1998; 2(3):305-16.
154. Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 1999; 398(6729):708-13.
155. Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, et al. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 1999; 398(6729):714-8.
156. Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J, et al. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature 2000; 404(6773):99-103.
157. Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, et al. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 1997; 90(4):809-19.
158. Benard J, Douc-Rasy S, Ahomadegbe JC. TP53 family members and human cancers. Hum Mutat 2003; 21(3):182-91.
159. King KE, Weinberg WC. p63: defining roles in morphogenesis, homeostasis, and neoplasia of the epidermis. Mol Carcinog 2007; 46(8):716-24.
160. Wrone DA, Yoo S, Chipps LK, Moy RL. The expression of p63 in actinic keratoses, seborrheic keratosis, and cutaneous squamous cell carcinomas. Dermatol Surg 2004; 30(10):1299-302.
161. Blumcke I, Muller S, Buslei R, Riederer BM, Wiestler OD. Microtubule-associated protein-2 immunoreactivity: a useful tool in the differential diagnosis of low-grade neuroepithelial tumors. Acta Neuropathol 2004; 108(2):89-96.
162. Blumcke I, Becker AJ, Normann S, Hans V, Riederer BM, Krajewski S, et al. Distinct expression pattern of microtubule-associated protein-2 in human oligodendrogliomas and glial precursor cells. J Neuropathol Exp Neurol 2001; 60(10):984-93.
163. Katsetos CD, Legido A, Perentes E, Mork SJ. Class III beta-tubulin isotype: a key cytoskeletal protein at the crossroads of developmental neurobiology and tumor neuropathology. J Child Neurol 2003; 18(12):851-66; discussion 67.
164. Preusser M, Laggner U, Haberler C, Heinzl H, Budka H, Hainfellner JA. Comparative analysis of NeuN immunoreactivity in primary brain tumours: conclusions for rational use in diagnostic histopathology. Histopathology 2006; 48(4):438-44.
165. Zhu H, Dahlstrom A. Glial fibrillary acidic protein-expressing cells in the neurogenic regions in normal and injured adult brains. J Neurosci Res 2007; 85(12):2783-92.
166. Zheng H, Ying H, Yan H, Kimmelman AC, Hiller DJ, Chen AJ, et al. p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. Nature 2008; 455(7216):1129-33.
167. Schittenhelm J, Mittelbronn M, Nguyen TD, Meyermann R, Beschorner R. WT1 expression distinguishes astrocytic tumor cells from normal and reactive astrocytes. Brain Pathol 2008; 18(3):344-53.
168. Anderson JC, McFarland BC, Gladson CL. New molecular targets in angiogenic vessels of glioblastoma tumours. Expert Rev Mol Med 2008; 10(10):e23.

169. You H, Yamamoto K, Mak TW. Regulation of transactivation-independent proapoptotic activity of p53 by FOXO3a. Proc Natl Acad Sci U S A 2006; 103(24):9051-6.
170. Chylicki K, Ehinger M, Svedberg H, Gullberg U. Characterization of the molecular mechanisms for p53-mediated differentiation. Cell Growth Differ 2000; 11(11):561-71.
171. Bertrand P, Saintigny Y, Lopez BS. p53's double life: transactivation-independent repression of homologous recombination. Trends Genet 2004; 20(6):235-43.
172. Linke SP, Sengupta S, Khabie N, Jeffries BA, Buchhop S, Miska S, et al. p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination. Cancer Res 2003; 63(10):2596-605.
173. Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P, et al. p53 has a direct apoptogenic role at the mitochondria. Mol Cell 2003; 11(3):577-90.
174. Martinez J, Georgoff I, Martinez J, Levine AJ. Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. Genes Dev 1991; 5(2):151-9.
175. Michalovitz D, Halevy O, Oren M. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell 1990; 62(4):671-80.
176. Tang M, Wahl GM, Nister M. Explaining the biological activity of transactivation-deficient p53 variants. Nat Genet 2006; 38(4):395-6; author reply 6-7.
177. Johnson TM, Hammond EM, Giaccia A, Attardi LD. The p53QS transactivation-deficient mutant shows stress-specific apoptotic activity and induces embryonic lethality. Nat Genet 2005; 37(2):145-52.
178. Bachman KE, Park BH. Duel nature of TGF-beta signaling: tumor suppressor vs. tumor promoter. Curr Opin Oncol 2005; 17(1):49-54.
179. Glick AB, Lee MM, Darwiche N, Kulkarni AB, Karlsson S, Yuspa SH. Targeted deletion of the TGF-beta 1 gene causes rapid progression to squamous cell carcinoma. Genes Dev 1994; 8(20):2429-40.
180. Weinberg WC, Azzoli CG, Kadiwar N, Yuspa SH. p53 gene dosage modifies growth and malignant progression of keratinocytes expressing the v-rasHa oncogene. Cancer Res 1994; 54(21):5584-92.
181. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 2007; 114(2):97-109.
182. Weller M, Stupp R, Reifenberger G, Brandes AA, van den Bent MJ, Wick W, et al. MGMT promoter methylation in malignant gliomas: ready for personalized medicine? Nat Rev Neurol; 6(1):39-51.
183. Yu F, Kuo CT, Jan YN. Drosophila neuroblast asymmetric cell division: recent advances and implications for stem cell biology. Neuron 2006; 51(1):13-20.
184. Doe CQ. Neural stem cells: balancing self-renewal with differentiation. Development 2008; 135(9):1575-87.
185. Egger B, Chell JM, Brand AH. Insights into neural stem cell biology from flies. Philos Trans R Soc Lond B Biol Sci 2008; 363(1489):39-56.
186. Zhong W, Chia W. Neurogenesis and asymmetric cell division. Curr Opin Neurobiol 2008; 18(1):4-11.
187. Robert A.Weinberg (2007) The biology of cancer, 1st edn, Garland science
188. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK (2007) World Health Organization Classification of Tumors of the Central Nervous System, Vol 1, 4th edn, Lyon: IARCI