Generation and characterisation of a mouse model of IDH-mutant astrocytomas

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Declaration

I, Raquel Fonseca hereby declare that this dissertation is the result of my own work except where specifically indicated in the acknowledgements and in the text of the thesis.

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2022
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Impact statement

The generation of mouse models corresponding to established and well-characterised human cancers is key to the development and optimisation of therapies, such as drug delivery and test of drug responses.

**Scientific context:** Historically, such preclinical models aimed at the generation of a neoplasm that mimics the morphological appearances of human tumours, owing to the fact that little was known about driver mutations and combinations of mutations characterising specific tumour types. This approach has fundamentally changed, as extensive genomic and epigenetic profiling of human cancers have identified molecularly stratified tumour types, which subsequently require a corresponding preclinical model. For the example in IDH mutant astrocytomas, the combination of mutations that molecularly defined these tumours (i.e. (i) neomorphic IDH mutation, leading to the production of a so-called onco-metabolite, (ii) loss-of-function-mutation in the ATRX gene, (iii) loss-of-function-mutation of the p53 gene and (iv) optionally an CDKN2A/B homozygous deletion in high-grade astrocytomas.

**Impact of this model system on the scientific community:** This model unifies the mutational spectrum of IDH-mutant astrocytomas, and at the same time provides a rapid, highly efficient and reproducible intrinsic tumour model. It is a highly suitable platform for testing delivery of drugs by experimental preclinical methods, such as nucleic acids encapsulated into nanoparticles (polymerosomes). Such experiments are currently underway and underpinned the utility of such models. The model also provides an important platform for comparative analysis of methylome and genome, to establish molecular parallels to human IDH-mutant astrocytomas, once a computational pipeline is available to match epigenetic information obtained from arise on human and murine tumours.

Whilst being far from a perfect mimic of the human counterpart, it provides an important steppingstone for the scientific community, and to attain further knowledge for the generation of future model systems.

**Impact on capacity building of early-stage researchers:** Development of this model was a key component of the programme developed by the Aipband consortium, (“An Integrated Platform for Developing Brain Cancer Diagnostic Techniques”), funded by
the EU Horizon 2020 scheme, comprising programs in neuroscience, engineering (including big data science), healthcare and economics. Aim of the programme was to (i) identify novel blood biomarkers from patients with gliomas, (ii) design multiplex biosensors, (iii) develop a big data-empowered intelligent data management infrastructure, and (iv) develop cloud-based diagnostic systems. This was complemented by carefully arranged into local training courses, network-wide events, secondments, personalised career development plans, with strong involvement of the private sector, to maximize the abilities of early-stage researchers (ESR) in creative & innovative thinking, knowledge transformation, and encourage a business-orientated mind-set and entrepreneurship.
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Abstract

IDH-mutant astrocytomas affect adults in their 20s-40s with an incidence of approximately 5 individuals per million annually, i.e., at least 300 new tumours are diagnosed, yearly, in the UK\(^1\). These tumours carry mutations in the genes encoding isocitrate dehydrogenase enzyme 1 or 2 (IDH1/2), leading to the accumulation of a new metabolite, 2-hydroxyglutarate (2HG), which affects cell metabolism and causes global DNA hypermethylation, thought to drive neoplastic transformation\(^2\). The impact on these individuals’ lives is significant, and there is still uncertainty about prognosis of individual tumours. The generation of Idh-mutant mouse models has failed to recapitulate the formation of gliomas due to unwanted effects during embryonic development\(^3\). ATRX is a chromatin regulator which plays an important epigenetic role, depositing histones at heterochromatin and telomeric DNA\(^4\). Its loss is associated with genomic and telomeric instability and has been identified as a defining molecular determinant for a large subset of IDH-mutant low-grade gliomas\(^5,6\), but its role in tumour initiation and progression is still unclear\(^7,8\). We have generated a mouse model that recapitulates features of glioma, expressing human IDH\(^{R132H}\), the most common form in astrocytomas\(^9\). Using this model, we use CRISPR/Cas9 technology to introduce additional genetic alterations, resulting in tumours expressing IDH\(^{R132H}\), p53, ATRX and CDKN2A mutations, i.e., the same mutational profile described in human patients. Moreover, we were able to generate distinct tumour groups with a correspondent mutational signature and compare these groups to evaluate the impact of such profiles in survival and methylation.

Overall, despite expressing different mutations in tumour suppressor genes which should impact the tumour behavioural, these tumours did not show significant differences between the distinct groups, probably due to the overriding impact of the mitogen PDGF\(_B\), which seemed to mask all the nuances that could be cause by the different combination of genetic mutations in each group.

Ultimately, this mouse model represents a robust, effective, and rapid method for induction of tumours with a genetic profile that recapitulates the human counterparts, with strong potential for the study of candidate drugs, drug delivery approaches or new therapeutic strategies.
Chapter I: Introduction

1.1 Glioma: Terminology, classification, and pathogenesis

Glioma is an umbrella term, comprising around 30% of all brain tumours. Gliomas are the most common type of brain tumours in adults and account for 80% of all malignant brain tumours. The term glioma encompasses different types of tumours, the most common ones being glioblastoma, astrocytoma, oligodendroglioma, or ependymoma, which all vary in their presumed cell of origin, symptoms, aggressiveness, malignancy, and route of treatment.

The symptoms of the disease can vary depending on the type of tumours, as well as its size, location, and rate of growth. Patients most commonly present with headaches, confusion, memory loss, irritability, personality changes, and seizures.

As for most primary brain tumours, the pathogenesis of gliomas is unknown, but there are numerous studies over the last two decades that provide evidence and postulate that these tumours originate from neural stem/progenitor cells in the central nervous system, and are initiated and propagated by the activation of oncogenic pathways that render these cells neoplastic.

Currently, therapeutic options are limited and largely like efficacy. Most commonly, surgical resection aims at the removal of large parts of the tumour, and is usually followed by radiation and chemotherapy, using temozolomide (TMZ). TMZ is an alkylating agent that induces the methylation of purine bases of DNA (O6-guanine; N7-guanine and N3-adenine). O6-methylguanine incorrectly pairs with thymine and triggers the mismatch repair system leading to a double-strand break of the genome and consequent arrest of the cell cycle and induction of apoptosis. The therapeutic advantage of TMZ is that it can be administered orally.

1.2 Epidemiology

The incidence of gliomas varies significantly by histopathological type, gender, race, ethnicity, and geographic location. In adults, the most common type of glioma is the IDH-wildtype glioblastoma (subsequently in short, glioblastoma), which is estimated to occur at an incidence of 3.7 per 100,000 people in most industrialized countries. The highest incidence of glioblastoma is among 75–84-year-old people, whilst IDH-mutant oligodendrogliomas and astrocytomas are encountered most commonly in people aged 35–44 years.
The annual incidence of gliomas in the paediatric population (0–14 years) in the United States (2007–2011) was 2.8 per 100,000 persons. Among children, these tumours are most common in those aged 1–4 years, where incidence is 3.3 per 100,000. Incidence among those aged <1, 5–9, and 10–14 years is 2.3, 2.8, and 2.4 per 100,000, respectively. Pilocytic astrocytoma is the most common type of paediatric glioma, with an annual incidence of 0.9 per 100,000.\textsuperscript{16}

Geographically, brain tumour incidence is similar in Europe, (annual age-standardized rate of 5.5 per 100,000 persons), North America (5.3 per 100,000 persons), Northern Africa (5.0 per 100,000 persons), Western Asia (5.2 per 100,000 persons), and Australia/New Zealand (5.3 per 100,000 persons). The reported incidence is very low in sub-Saharan Africa (0.8 per 100,000 persons), South-Central Asia (1.8 per 100,000 persons), and Oceania (0.5 per 100,000 persons).\textsuperscript{17}, most likely due to a lack of access to diagnostic facilities.

In the United Kingdom, gliomas represent 1.5% of all cancer cases and there were over 4500 new cases reported in 2017, an incidence rate that has been increasing since the early 1990s. The incidence rates by sex are similar, with 51% of the brain, CNS and intracranial tumours cases in females, and 49% in males. The highest incidence of gliomas is in older people, with an average a quarter of new cases (23%) in people over 75 years old, and the highest rates are in 80 to 89 (female) and 80 to 84 (male) years of age. In contrast to most cancer types, gliomas also occur frequently at younger ages and are the second most common cancer type in patients aged 0 to 14 years old.\textsuperscript{18}, it is important to recognise that the glioma types are different between younger and older populations.

1.3 Glioma classification and grading

The traditional diagnostic approach to brain tumours has been based on the histological examination of tumour specimens by pathologists, classifying diffuse gliomas as astrocytoma, oligoastrocytoma or oligodendroglioma, according to morphological features such as nuclear size, shape, mitotic activity, and cell density. Each subtype was further characterised by its grade of malignancy (World Health Organisation [WHO] grades I-IV).\textsuperscript{19} More recently, the discovery of how mutations in specific genes affect glioma pathogenesis have led to a biomarker-driven classification. In 2016, the WHO (World Health Organization) published an update on the classification of central nervous system tumours, which incorporates molecular criteria to create an integrated classification system of tumour types and grades according to the molecular alterations of the specimens, and this was further refined in the 2021 WHO classification of central nervous system tumours.
The majority of diffuse gliomas are classified according to the molecular profile (Figure 1). IDH-wildtype Glioblastomas are the most aggressive type. They often exhibit epidermal growth factor receptor (EGFR) amplification and/or telomerase reverse transcriptase (TERT) promoter mutation. Oligodendrogliomas are defined by a combined IDH mutation and 1p/19q codeletion and they also carry a TERT promoter mutation. Oligodendrogliomas often are associated with a relatively favourable outcome. Astrocytomas are defined by IDH mutation, TP53 mutation and ATRX loss of function mutation. Chromosomal arms 1p and 19q are, by definition, intact in IDH-mutant astrocytomas. Their prognosis can vary from very favourable to relatively poor, depending on molecular features such as copy number variation and the morphological criteria of mitotic activity and necrosis. Although it is not formally defined by the WHO 2016 update, there is evidence that IDH-mutant astrocytomas with CDKN2A/B homozygous deletion progress very quickly, most akin to IDH-mutant glioblastoma20-22. Importantly, this nomenclature from the 2016 WHO classification of CNS tumours has now been superseded in the 2021 CNS WHO classification, which is reflected in Figure 1.

Figure 1 Classification of diffuse gliomas in adults according to histologic and molecular features. The previous classification based exclusively on histology has been complemented by a classification that combines both histology and molecular characteristics of each tumour. The IDH mutation is now an essential diagnostic biomarker, with its status separating IDH-wildtype from IDH-mutant gliomas. IDH-wildtype glioblastoma usually presented histologically with high-grade features but can in rare occasions also show histological features of diffuse lower grade astrocytoma. Moleculally, glioblastoma have some or all of the following features, chromosome 7 gain, chromosome 10 loss, TERT promoter mutation, are IDH-wildtype, and are graded as CNS WHO grade 4. IDH-mutant tumours are further divided into astrocytoma, IDH-mutant, of the WHO grades 2, 3, and 4, and are, in addition to an IDH mutation, characterised by p53 deletion and ATRX loss; or oligodendroglioma, IDH-mutant and 1p/19q co-deleted. Oligodendrogliomas typically carry in addition to the IDH mutation and the 1p/19q codeletion, a TERT promoter mutation. Oligodendrogliomas are graded CNS WHO 2 or 3.
Moreover, the discovery of further mutations in high-grade gliomas defined additional types of glioblastoma, for example, the histone H3 K27M midline glioma, the histone H3 G34R/V high-grade glioma, and BRAF V600E-mutant gliomas. The IDH-wildtype glioblastoma is molecularly defined by chromosome 7 gain, chromosome 10 loss, and by frequent EGFR amplification and TERT promoter mutations.

1.3.1 IDH-mutant astrocytomas

IDH-mutant astrocytomas are graded CNS WHO grade 2, 3, or 4. The CNS WHO grade 2 IDH-mutant astrocytoma was previously named “diffuse astrocytoma”, the CNS WHO grade 3 IDH-mutant astrocytoma previously “anaplastic Astrocytoma”, and the CNS WHO grade 4 IDH-mutant astrocytoma was previously termed “IDH-mutant glioblastoma”, and historically (until the publication of the 2016 update of the WHO classification of brain tumours) the IDH-mutant glioblastoma was referred to as “secondary glioblastoma”, to reflect the often observed progression from low-grade precursors. IDH-mutant astrocytomas affect approximately 2-3 individuals per million annually, i.e., at least 150 new tumours are diagnosed, every year, in the UK. IDH-mutant astrocytomas mainly arise in young adults in their 20-40’s and are much less common in people over 55. Despite having a relatively high five year-survival rate, IDH-mutant astrocytomas of low grade usually progress and recur as more aggressive tumours with poorer prognosis. During this progression, they often acquire CDKN2A/B deletions.

IDH1 and IDH2 mutations have emerged as driver mutations in diffuse gliomas, and determination of the IDH status by immunohistochemistry (IHC), supplemented by sequencing has now become a standard in the diagnosis of these tumours. Later, it has been recognised (and defined accordingly) that IDH mutations occur in astrocytomas and oligodendrogliomas. The molecular distinction is the presence of 1p/19q chromosome co-deletion, TERT promoter mutation, and often a loss of the trimethylation signal of histone H3 K27 (H3 K27me3) in oligodendrogliomas, whilst IDH-mutant astrocytomas are characterized by p53 deletion and a mutation in the ATRX (Alpha-Thalassemia/Mental Retardation Syndrome, X-Linked) gene, often resulting in a loss of protein expression. ATRX and TERT mutations are mutually exclusive. The most aggressive cases show a CDKN2A/B deletion, which then mandates the diagnosis of IDH-mutant astrocytoma, CNS WHO grade 4.
Diffuse astrocytomas grow as infiltrative masses with blurred anatomical boundaries, which makes them often difficult to resect. Whilst lower grade tumours have no or low mitotic activity, this increases for higher grades, along with the presence of necrosis and/or microvascular proliferation.

1.3.2 Isocitrate Dehydrogenase (IDH)

IDH1 and IDH2 are NADP⁺-dependent isocitrate dehydrogenases, enzymes that convert isocitrate to α-ketoglutarate (α-KG) (Figure 3). IDH1 R132H is the most common IDH mutation in glioma (89% of IDH1 and IDH2 mutations combined), and other common IDH1 mutations are R132S (3.9%), R132G (1.5%) and R132C (1.3%). IDH2 mutations are less frequent in IDH-mutant gliomas (approximately 5% of all IDH-mutant tumours) and R172K (2.7%), R172M (0.8%) and R172W (0.7%) were identified. More recently, 32 specimens of a multi-institutional study were identified as mismatch repair-deficient IDH-mutant astrocytomas (PMMRDIA), a novel epigenetic group of high-grade gliomas found in children, adolescents, and young adults. Another group of IDH-mutant astrocytomas, occurring in the infratentorial compartment (cerebellum) was also identified as a new subtype, distinct from the supratentorial counterparts with a predominance of non-canonical IDH-mutation variants, which are rare in supratentorial tumours. IDH1 and IDH2 mutations are mutually exclusive and alter only one allele, in a dominant fashion. These residues are in the active site of the enzyme and are critical for binding isocitrate, thus, the presence of these mutations abolishes the ability of wildtype IDH1 or IDH2 to convert isocitrate to α-KG and initiates a new NADPH-dependent pathway that catalyses the conversion of α-KG to (D)-2-hydroxyglutarate (D2HG), an oncogenic metabolite that affects cell metabolism. Mutant IDH proteins lower the bioavailability of α-KG while increasing levels of D2HG, which competitively inhibits α-KG-dependent dioxygenases, such as histone lysine demethylase 4 (KDM4, also called JMJD2A), 5-methylcytosine hydroxylase 2 (TET2), fat mass and obesity-associated protein (FTO), and prolyl hydroxylases (PHDs).
Figure 2 Frequency of the mutations on the IDH gene in human patients. IDH1R132H is the most common mutation, present in 89% of all IDH-mutant cases. Other, less common IDH1 mutations found in humans are R132S (3%), R132G (1%) and R132C (1%). Mutations in IDH2 are less frequent and make up for around 5% of all IDH-mutant tumours. R172K (3%), R172M (1%) and R172W (1%) are the types of mutations described on this gene.

Histone methylation is a mechanism for chromatin remodelling to regulate gene expression. KDM4 contributes to the demethylation of histone H3 at residue lysine-9 and lysine-36 (H3K9 and H3K36, respectively) and its inhibition by D2HG results in the accumulation of triple methylation of H3K9 (H3K9me3). However, no significant increase in H3K36 methylation levels is observed in IDH-mutant cells compared to their wildtype counterparts. In addition, IDH mutation was reported to cause upregulation of H3K27 trimethylation (H3K27me3). H3K27 is the substrate of histone lysine demethylase 6 (KDM6, also called JMJD3), while there is no direct evidence of inhibition of KDM6 by D2HG. Increased H3K9me3 blocks differentiation of 3T3-L1 adipocytes.

Moreover, accumulation of D2HG inhibits genomic base 5-methylcytosine (5mC) demethylase TET2, leading to DNA hypermethylation, which is called glioma CpG island methylator phenotype (G-CIMP). Nearly all IDH-mutant gliomas show O-6-methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation. Promoter methylation reduces MGMT expression, which sensitizes glioma cells to temozolomide.
Recently, a study found that increased levels of D2HG in IDH-mutant cancer cells can block FTO enzyme activity (FTO, Fat mass and obesity-associated protein, also known as alpha-ketoglutarate-dependent dioxygenase), leading to global N⁶-methyladenosine (m⁶A) RNA hypermethylation. In leukaemia cells, Myc oncogene and CEBPA (CCAAT Enhancer Binding Protein Alpha) transcripts with increased m⁶A methylation tend to degrade, resulting in reduced tumour growth. Prolyl hydroxylases (PHDs), another class of α-KG-dependent dioxygenases, are inhibited by D2HG overexpression, protecting HIF1a from degradation and impairing collagen maturation. HIF1a promotes angiogenesis while impairment of collagen maturation by D2HG during development causes haemorrhage. Overall, the accumulation of D2HG results in DNA hypermethylation at different levels, which impairs gene expression. A third isoform, IDH3, is an NAD⁺-dependent isocitrate dehydrogenase and has not been found to be mutated in cancer.

The diagnostic value of these mutations extends to many other cancer types, namely certain types of leukaemia and chondroid tumours. Interestingly, IDH mutations are associated with better prognosis and longer survival in glioma patients, whereas in AML the presence of these mutations represents a poor clinical outcome. This suggests that the biological role of IDH mutations is tissue-dependent and may be further influenced by other mutations.

**Figure 3 Mechanisms of IDH mutation.** In a normal situation, IDH catalyses the conversion of isocitrate to α-KG, as part of the Krebs cycle, with the production of NADPH in the process. When an IDH mutation is present, this conversion does not occur, and a different metabolite is produced instead – D2HG, in a reaction that produces NAD+ leading to DNA hypermethylation.
1.3.3  Alpha-thalassemia/mental retardation syndrome X-linked (ATRX)

The ATRX gene was discovered nearly 30 years ago through a study assessing patients with the x-linked mental retardation syndrome (ATRX syndrome) presenting with α-thalassemia, severe psychomotor impairments, and patterns of facial dysmorphism\textsuperscript{54}.

The ATRX protein has two isoforms (180 and 280 kDa) and is highly enriched by GC-rich and repetitive sequences. The C-terminus harbours the helicase/ATPase domain, classifying ATRX as part of the SNF2 (SWI/SNF2) family of chromatin-remodelling proteins, discovered on yeast by two independent screenings aimed at identifying mutations in genes that affect the mating-type switching (SWI) and sucrose fermentation (Sucrose Non-Fermenting - SNF) pathways\textsuperscript{55}. At the N-terminus of the ATRX protein lies the ATRX–DNMT3–DNMT3L (ADD) domain, receiving its name from having cysteine-rich motifs with similar features to the DNMT3 proteins involved in DNA methylation. The ADD domain comprises a GATA-like zinc finger, a plant homeodomain (PHD)-like zinc finger, and a C-terminal α-helix\textsuperscript{56} (Figure 4).

![ATRX structure and mutations heat map](image)

**Figure 4 ATRX structure and mutations heat map.** Heat map of mutations found in glioma tumours using data from the Catalogue of Somatic Mutations in Cancer (COSMIC). Only mutant ATRX gliomas with DNA sequence data were included and mutation frequency per 10 amino acid stretch was analysed. The domains of ATRX are depicted at the top of the figure and are assigned according to previous reports. Mutation frequency throughout the protein is illustrated in a heat map that is aligned with the protein diagram. NLS1 and NLS2: nuclear localization signals; TD1 and TD2: targeting domains to PML bodies, P-Box: SNF2 conserved element involved in transcriptional regulation; Q-box: the stretch of glutamine residues which is a potential protein interaction domain. Adapted from Haase et al (2008)\textsuperscript{6}
ATRX and DAXX form a chromatin re-modelling complex that loads the histone variant H3.3 into telomeric and other repetitive heterochromatic regions, crucial for maintaining chromosome stability (Figure 5). In addition, ATRX itself is recruited to telomeres after the detection of replication stress, where it is thought to assist in stress relief via its intrinsic helicase activity\(^5\). Loss of ATRX expression is mutually exclusive with TERT promoter mutations (and thus in oligodendrogliomas also with 1p/19q codeletion)\(^5\). The TERT gene encodes a telomerase catalytic subunit that deposits nucleotides to telomeres, to help maintain their length. The promoter region is the most important regulatory element of this gene and point mutations here usually result in increased levels of telomerase activity, leading to the infinite capacity of division by the cells - a known characteristic of cancer cells\(^5\).

An alternative pathway for this mechanism is known as alternative telomere lengthening (ALT) and is often associated with ATRX mutations\(^5\). ATRX deposits H3.3 at telomeres, maintaining the DNA B-form and allowing the normal progression of the replication fork. However, when

**Figure 5: Role of ATRX in maintaining chromosome stability by deposing H3.3 at telomeres.** Aberrant transcription of DNA repetitive regions such as telomeres can compromise genome integrity. ATRX localizes this highly repetitive and methylated regions and, when a histone is lost during cell cycle, it recruits the H3.3 chaperone DAXX, which deposits this histone variant to the place where the canonical histone was lost. Finally, DAXX is then able to interact with a methyl transferase to mediate direct modification of K9me3 on H3.3, guaranteeing that these regions are not inadequately transcribed. This mechanism is particularly important outside of S-phase, when canonical histones are not available.
ATRX is lost, DNA forms G-quadruplexes (G4, nucleic acid secondary structures rich in Guanine) at the end of telomeres which prevents telomere elongation by stalling the replication fork and leads to HR-mediated fork restart which induces ALT.\textsuperscript{60}

ATRX mutations have been reported in at least 15 types of human cancer, including neuroblastoma, osteosarcoma, and pancreatic neuroendocrine tumours.\textsuperscript{61} In the context of brain tumours, ATRX loss can also occur in conjunction with histone H3F3A G34R or G34V mutations (nearly always), and in a proportion of histone H3F3 K27M mutant gliomas, which occur in the midline of the central nervous system, i.e., involving the spinal cord, brainstem, or thalamus. Brain tumours with these mutations occur predominantly in children and young adults.\textsuperscript{22} Further tumours that have frequent ATRX mutations are the anaplastic astrocytoma with piloid features.\textsuperscript{62}

Due to the relatively widespread presence of ATRX mutations in a range of brain tumours (and in fact, in many other tumours type as well), it is essential to identify a corresponding driver mutation. In the context of the tumour types in this study, it is the IDH mutation. In most cases, the ATRX mutation can be identified by immunostaining for the ATRX protein, leading to a loss of nuclear expression. However, importantly, a retained expression of the protein does not necessarily exclude an ATRX gene mutation: a proportion of IDH-mutant astrocytomas has retained protein expression in tumour cell nuclei, but sequencing analysis with next-generation sequencing technology uncovers ATRX mutations in these tumours.\textsuperscript{63}

### 1.3.4 The tumour suppressor gene TP53

The tumour suppressor gene TP53 encodes the transcription factor p53, which is activated in response to cancer-associated stress signals, such as DNA damage and oncoprotein activation. Cellular levels of p53 are low under physiological conditions, due to rapid ubiquitin-dependent degradation by the E3 ubiquitin ligase MDM2. In the event of cellular stress, p53-MDM2 interaction is disrupted, either by post-translational modifications of p53 and/or MDM2 or by an interaction between p14\textsuperscript{ARF and MDM2, which enables p53 accumulation and activation (Figure 6). Once activated, p53 can elicit several different cellular responses, including growth arrest, senescence and apoptosis.\textsuperscript{64}

The key role of p53 in tumour suppression is dramatically illustrated by the prevalence of TP53 mutations in cancer: it is estimated that 50% of all human tumours carry a TP53 mutation.\textsuperscript{65} Most cases in Glioblastoma are missense mutations in the DNA binding domain, leading to
high expression of a gain of function oncogenic variants of the p53 protein. The mutational status of TP53 is associated with Glioblastoma progression, with p53 inactivation being correlated with a more invasive, less apoptotic, more proliferative and stem-like phenotype.\(^{66}\)

**Figure 6: p53-MDM2 pathway.** Under normal conditions, cellular p53 levels are kept low by complexing with MDM2 with consequent ubiquitin signalling, leading to degradation. In the event of DNA damage, p14 will interact with MDM2, leaving p53 free to target p21, causing cyclin-CDK complex disruption and failing to phosphorylate pRB. Hypophosphorylated pRB can then interact with E2F, causing cell cycle arrest.

### 1.3.5 Cyclin-dependent kinase inhibitors CDKN2A/B

Mutations in the CDKN2A/B genes are frequent events in cancer, including in brain tumours such as IDH-wildtype glioblastoma, IDH-mutant astrocytoma, CNS WHO grade 4, pleomorphic xanthoastrocytoma, and in other tumours such as colon cancer\(^{67}\), lung cancer\(^ {68}\), melanoma\(^ {69,70}\), ovarian cancer\(^ {71}\), bladder cancer\(^ {72}\) and sarcoma\(^ {73}\).
The **CDKN2A** gene is located within the frequently deleted chromosomal region 9 of p21 and contains two introns and three exons.

**CDKN2A** encodes two proteins, p16\(^{INK4a}\) and p14\(^{ARF}\) (or ARF, for alternate reading frame), which are transcribed from alternative first exons and common downstream exons: exon 1α encodes p16\(^{INK4a}\), whereas p14\(^{ARF}\) is encoded from exon 1β. As a result, they are translated from different reading frames and therefore possess completely different amino acid sequences\(^74\).

p16\(^{INK4a}\) is a cyclin-dependent kinase inhibitor that functions as a negative regulator of the cell cycle. It binds to Cyclin-dependent kinase 4/6 (CDK4/6), inhibiting CDK4/6-cyclin D1 complex formation and consequent hyper-phosphorylation of RB1, which remains free to bind to the transcription factor E2F, arresting cell cycle progression\(^75\). The same family of cyclin–CDK inhibitor proteins (CDIs) also includes the p15\(^{INK4b}\) – encoded by **CDKN2B** that interacts with CDK4/6 in the same way\(^76\).

As for the alternate open reading frame protein (p14\(^{ARF}\)), it interacts with the p53 regulatory protein, MDM2, blocking its ability to interact productively with p53, resulting in cell cycle arrest and apoptosis (Figure 7)\(^65\).

The **CDKN2B** gene is adjacent to **CDKN2A** and with similar characteristics: it is composed of two exons, but has two alternative exons 1, which transcribe into two alternative proteins – p10, which acts on the p53 pathway, by disturbing the p53-MDM2 complex; and p15, that contributes to cell cycle arrest by impairing cyclin-kinase complex formation, similarly to p16\(^77\).

Overall, both tumour suppressor genes are recruited to suppress the inadequate proliferation of defective cells, thus mutations in both or any of these genes result in uncontrolled proliferation of these cells, leading to tumourigenesis\(^78\).

In glioma, homozygous deletion of **CDKN2A/B** in IDH-mutant astrocytomas is significantly associated with poor clinical outcome\(^79\). A review about the prognostic significance of **CDKN2A** homozygous deletion concluded that this is a predictor of significantly shorter overall survival (OS) and progression-free survival (PFS) in both IDH-mutant lower-grade glioma and IDH-mutant Glioblastoma\(^80\).

In fact, many recent publications emphasise how inadequate the current grading system – based on histological features such as necrosis and microvascular proliferation - has become, after the introduction of **IDH** status as a primary diagnostic marker; and **CDKN2A** is pointed as a strong prognostic marker that should be included as a criterion in future grading systems for **IDH**-mutant astrocytomas\(^81,82,83\).
According to the newest recommendations for grading criteria, a malignant IDH-mutant astrocytoma exhibiting CDKN2A/B homozygous deletion or occurrence of microvascular proliferation or necrosis, or a combination of these features might be considered grade IV.\(^{84,85}\)

The role of CDKN2B gene in tumour development has not been well studied, possibly because the number of CDKN2A-independent CDKN2B mutations in human cancers is low. However, studies have shown that the inactivation of both genes was necessary for RB phosphorylation and to encompass cellular senescence. Thus, it is likely that these genes can compensate for each other, adding the importance of including CDKN2B deletion in our model, especially in the absence of CDKN2A expression\(^70\).

**Figure 7 CDKN2A/B genes and pathways.** CDKN2A/B are tumour suppressor genes that promote cell cycle arrest in the event of damage, at different levels. P14ARF/p10 interact with MDM2, resulting in increased levels of p53, promoting cell cycle arrest. p16/p15ARF act directly on cyclin-CDK complexes of the RB pathway.
1.3.6 Other pathways commonly dysregulated in IDH-mutant astrocytoma

The retinoblastoma gene (RB) was originally identified as the tumour suppressor gene mutated in a rare childhood cancer called retinoblastoma\(^8^6\). Subsequent studies showed that RB functions in a pathway that is often functionally inactivated in a large majority of human cancers\(^8^7-^8^9\). Phosphorylation plays a key role in regulating the activities of the RB protein. The RB protein contains numerous phosphorylation sites that are phosphorylated by Cyclin D/CDK4, Cyclin E/CDK2, and Cyclin A/CDK2 kinases during cell cycle progression\(^9^0\).

Briefly, hypophosphorylated RB binds to the E2F transcription factor family, an important DNA damage checkpoint before G1 progression of the cell cycle. Generally speaking, hypophosphorylated RB is active in the inhibition of cell proliferation and tumour suppression while the hyperphosphorylated RB is inactive\(^9^1\).

Although RB mutations are common in some primary brain tumours, that is not the case for diffuse astrocytomas. However, the RB pathway is indirectly affected by other genes with mutations associated with these tumours – p15 (CDKN2B) and p16 (CDKN2A) act on cyclin/CDK complexes, impairing RB phosphorylation; p10 (CDKN2B), p14 (CDKN2A) and p53 activate p21, a CDK inhibitor; all resulting in damages of the RB pathway, at different levels, and ultimately, leading to cell cycle arrest (Figure 8).
Figure 8: Rb pathway (a) Different sites of pRB are phosphorylated by different complexes of a cyclin and a cyclin-dependent kinase, resulting in cell cycle progression (Cyclin D/CDK4, Cyclin E/CDK2, and Cyclin A/CDK2). (b) Disruption of any of these complexes results in hypo-phosphorylation of RB, which can then bind to E2F transcription factor family, resulting in cell cycle arrest.

Furthermore, a recurrent local copy number gain in the telomeric region of chromosomal arm 12p was reported as a feature of IDH-mutant astrocytomas with gemistocytic histology - a small group of approximately 5% of IDH-mutant diffuse astrocytomas characterized by the presence of gemistocytic cells. Gemistocytic astrocytes (gemistocytes, from the Greek “gemis-tos” which means “filled up”) appear as swollen with plump processes and massive accumulation of the intermediate filament glial fibrillary acidic protein (GFAP) and are more aggressive, thus more prone to progress to a higher grade. Although the size of the 12p arm regions varied between cases, cyclin D2 (CCND2) seems to be consistently affected. A higher abundance of cyclin D2 may disrupt the cell cycle by preventing the transition from phases G1 to S, resulting in lower mitotic activity despite higher pleomorphism and a higher number of multinucleated cells which explains the morphology of said tumours.
1.4 The cell of origin of gliomas

A cell of origin is the physiological progenitor from which neoplastic cells of a given type of cancer develop. Its identification can give important insight into tumour heterogeneity - different tumour subtypes may arise from distinct cells within the tissue that serves as the cell of origin - and consequently, give crucial information to dictate tumour aetiology and design new therapeutic strategies\textsuperscript{94}.

Neural cells in the adult central nervous system (CNS) are broadly classified into neurons, astrocytes, oligodendrocyte precursor cells (OPCs), and oligodendrocytes. In addition to these lineage-committed progenitor and mature cells, the brain has several compartments hosting specialized stem cells, termed adult neural stem cells (NSC), which are a self-renewing subpopulation of astroglial cells that have the capacity to differentiate into all types of neural cells\textsuperscript{95} (Figure 9).

\textbf{Figure 9 Neural cell in the adult neural system}. Neural stem cells are multipotent, self-renewing cells that differentiate into progenitor cells – oligodendrocyte progenitor cells (OPCs), astrocyte progenitor cells (APCs) or Neural progenitor cells, also called neuroblasts (that migrate through the Rostral Migratory Stream: RMS) – which further differentiate into mature oligodendrocytes, astrocytes, and neurons, respectively.
NSCs were first described in 1992 as cells that could be isolated from the CNS of adult and embryonic mice and propagated in the presence of epidermal growth factor to give rise to large spheres of cells - neurospheres, which could be generated from a single cell and subsequently dissociated to produce a new neurosphere that also contained neurons and glia. Thus, these neurosphere-producing cells had the properties of a stem cell, i.e., capable of self-renewal and pluripotent.

NSC are more abundant in developing brains, having a crucial role in the formation of the nervous system; however, NSC can also be found in the adult brain, most abundantly in two neurogenic niches – the sub ventricular zone and the dentate gyrus of the hippocampus.

The SVZ, lining the lateral ventricles, is the largest neurogenic niche in the adult mammalian brain and is composed of a wall of ependymal cells followed by three other types of cells - NSC, fast proliferating precursors and neuroblasts. NSC occasionally give rise to multipotent intermediate progenitors, which further divide to generate neuroblasts. In rodents, these neuroblasts migrate along the rostral migratory stream (RMS) to the olfactory bulb, where they finally differentiate into mature neurons (Figure 10).

**Figure 10 Subventricular zone (SVZ).** Located at the lateral ventricle, the SVZ is composed of a wall of ependymal cells (brown), NSC (in blue), transient amplifying cells (TAC, dark pink) and neuroblasts (NB, light pink). Neuroblasts migrate through the Rostral Migratory Stream (RMS) to the olfactory bulb where they differentiate into mature neurons.

In the human SVZ, some differences have been reported, mostly in the anatomical organization and the behaviour of the NSC: (i) the human SVZ features a gap between the ependymal wall and the neurogenic astrocytes, which is missing in rodents; (ii) the human SVZ is organized by layers – ependymal layer, the gap- or hypocellular layer, the astrocytic ribbon (containing the astrocyte-like NSC and neuroblast) and the transitional zone (which mainly contains myelinated axons and oligodendrocytes); (iii) the multipotent transit-amplifying cells are missing
(only NSC and neuroblasts can be found in human SVZ) and finally, (iv) the existence of migration of neural progenitor cells along the RMS is debatable or as least reduced (Figure 11).97.

Figure 11 Cell organization of the human SVZ. The SVZ in humans is organized in four layers: I) the Ependymal layer, composed of multiciliated ependymal cells (brown); II) the Hypocellular gap, composed of expansions of ependymal and astrocyte-like NSC (blue); III) Astrocytic ribbon, where astrocyte-like NSCs and some neuroblasts (pink) are located; and IV) Transitional zone to the parenchyma, rich in myelin axons (purple) and oligodendrocytes (green). Notable differences from the rodent SVZ (described in Figure 10).

NSCs exhibit remarkable self-renewal potential and differentiation plasticity to differentiate into multiple types of neural cells, in culture, which makes them the most likely candidate for a cell of origin of gliomas.98,99 Interestingly, these cells exhibit many markers also expressed by cancer stem cells (CSC) (or in this context, glioma initiating cell, GIC) isolated from human gliomas, such as Nestin, GFAP and SOX2. In fact, cultured CSCs are able to form renewable NSC-like neurospheres and genetically modified mouse or human NSC can form gliomas after implantation into recipient (host) mice100-103. Furthermore, delivery of viral vectors to the SVZ, to introduce oncogene overexpression and/or tumour suppressor genes inactivation has been proved as an efficient strategy to generate mouse models of glioma104-107. Also, lineage-tracing experiments using genetically engineered mouse models (GEMMs) reported that NSCs can transform into high-grade gliomas after the loss of TP53, NF1 and/or PTEN108-110. Finally, human glioblastomas are frequently located next to the SVZ, further supporting this theory that they are originated by NSC111. In addition to neuroblasts (also called neuronal progenitor cells)112, neural stem cells could also differentiate into glial progenitor cells including OPCs113 and astrocyte progenitor cells (APCs).
OPCs are a life-long slowly dividing cell type in adult brains\textsuperscript{114} which constitute the most proliferating pool in the brain, hence OPCs are also a strong candidate as the cell of origin of gliomas\textsuperscript{12}. They show self-renewal capacity both in vivo and in vitro and share cellular markers with most human gliomas (NG2, Olig2, PDGFRa)\textsuperscript{115-120}. Overexpression of an oncogene (EGFR) under a non-stem cell marker promoter induced tumours recapitulating human oligodendroglioma\textsuperscript{121,122}. Importantly, this study described oligodendrogliomas as ‘EGFR amplified, TP53 deleted and CDKN2A-deleted tumours, which are now diagnosed as glioblastomas. In fact, EGFR amplification is almost exclusively found in human Glioblastoma samples, and non-existent in oligodendroglioma samples\textsuperscript{22}. Thus, although the tumours formed by this mouse model might resemble oligodendrogliomas, histologically, these more likely represented forms of developing glioblastomas. Also, a PDGFr-expressing B-type stem cells subpopulation in the adult mouse SVZ can act as primary progenitor of neurons and oligodendrocytes and increased PDGFRB signalling in these cells stimulates proliferation and block differentiation, causing tumour-like growths resembling astrocytomas\textsuperscript{123}. PDGFR overexpression alone and combined with TP53 and NF1 deactivation transformed murine OPCs into lower grade oligodendrogliomas and high-grade gliomas\textsuperscript{124-127}. Again, since these studies were carried out before the discovery of the IDH mutation as a defining diagnostic marker, these samples possibly resembled a morphological phenotype of oligodendroglioma but represented in fact other tumour types with clear cell morphology. Finally, fate-mapping experiments showed that OPCs can be transformed into malignant glioma by introducing NF1 and TP53 mutations at an early or adult stage\textsuperscript{128-130}.

In conclusion, the cell of origin of gliomas is still a matter of considerable debate. However, it is reasonable to assume that different types of precursor or progenitor cells of the CNS can initiate tumorigenesis and that the features of each tumour may depend on the characteristics of the cells of origin (which includes location as well as developmental stage) in combination with the somatic mutations accumulated over time.

1.5 Mouse models of glioma

Mouse models provide a powerful tool for the study of tumour biology and can be useful for testing new therapies. Amongst many model organisms, mice share many anatomical, cellular, and molecular characteristics with humans, known to have critical properties and functions in cancer. Importantly, around 80% of mouse genes have a human orthologue, thus providing po-
tentially experimentally tractable model systems\textsuperscript{131}. Moreover, the possibility to experimentally control parameters such as diet, temperature, and light cycles; manipulating genetic background and generating large numbers of genetically similar (syngeneic) individuals in both experimental and control groups allow the study of variables in a highly controlled way.

In the first published experiments describing the generation of brain tumours, gliomas were induced by chemicals such as methylcholanthrene (in the 1920s-30s)\textsuperscript{132}, or with viruses exhibiting oncogenic potential such as Rous Sarcoma Virus (in the 1960s-70s)\textsuperscript{132}. Genetic mutations induced by this approach target the genome randomly and the effects are not easily predictable and are therefore rarely used nowadays.

Transgenic mice were first reported by Brinster et al (1984) and opened a new era for neuro-oncology study. In this study, fertilized eggs were microinjected with plasmids containing the Simian Virus 40 (SV40) early region genes - that encode for both large T antigen and small T antigens - under a metallothionein promotor/enhancer, that regulate cellular proliferation. SV40 binds and inactivates pRB and p53, resulting in accelerated cell growth (as described in paragraph 1.4.5), ultimately leading to malignant transformation. These mice developed brain tumours from choroid plexus (choroid plexus tumours)\textsuperscript{134}. This method was improved later by using tissue-specific promoters to overexpress genes with oncogenic potential, aiming at the expression in cell types that were thought to represent the likely origin of brain tumours, for example, expressing SV40 under the control of GFAP promoters leading to the formation of astrocytomas\textsuperscript{135}.

In the mid-1990s, tumour suppressor inactivation further advanced the way of generating tumour models\textsuperscript{136-138}. Targeted mutation with functional inactivation (colloquially often termed “Knock out”) of these tumour suppressor genes was achieved using homologous recombination to replace critical exons with alterative sequences\textsuperscript{131}. However, a considerable pitfall of these models was the essential function of many tumour suppressor genes in embryonic development and that the genetic inactivation in the germline was often associated with embryonic lethality, as, for example with the tumour suppressor genes Rb\textsuperscript{139} or PTEN\textsuperscript{140}.

A technological refinement that could address these biological questions allowed for an inducible gene targeting in mice: the LoxP-Cre system. In this approach, the gene of interest (one or multiple exons) is engineered to carry a recognition sequence (LoxP) on both sides (i.e., 5’ and 3’) of the region to be excised (or inverted, depending on the orientation of the LoxP sites - see Figure 12). The recombination is accomplished by the action of the enzyme Cre recombinase,
which forms and excises a loop between the two LoxP sites. The result is the removal (or inversion) of the sequence flanked by the two sites (Figure 12). Several approaches can be used for Cre Recombinase expression: (i) Cre transgene is expressed under the control of a cell- or tissue-specific promoter (e.g. GFAP, Nestin, Sox2); (ii) Tamoxifen-inducible Cre transgene or (iii) topical delivery of Cre, using, for example, adenovirus.

**Figure 12 Cre-Lox system.** This system works by flanking the gene of interest by recognition sites (loxP), which induces recombination upon Cre exposure. The orientation of the loxP sites will determine the recombination events – if facing the same direction, the sequence between two loxP will be deleted, whereas if these sites are facing each other, exposure to Cre will cause the inversion of the whole sequence between them.

More recently, CRISPR/Cas9 technology has provided a faster, cost-effective, and more efficient way of producing genetically modified mice. This system uses a single guide RNA (sgRNA) complementary to the target sequence of the genome, followed by a protospacer-adjacent motif (PAM). This short recognition site (~20bp) recruits a nuclease Cas9 that will bind and cleave the genome at that locus. Once the double-stranded breaks occur, the natural mechanisms of DNA repair are activated, resulting in short insertions or deletions that generate loss-of-function mutations (Figure 13). Until now, three main types of Cas9 variants have been developed that differ in their mechanisms of action. The first system to be adapted for the mouse was the wild-type Cas9 protein from the type II CRISPR system of *Streptococcus pyogenes*.

In addition to CRISPR and the Cre-lox system, other approaches to the development of mouse models of intrinsic brain tumours, predominantly gliomas, include the RCAS-Tva system, transposase systems (PiggyBac and sleeping beauty) and in utero electroporation.
Figure 13 CRISPR/Cas9 for genome engineering. CRISPR uses a guide RNA specifically designed to target the gene of interest, recruiting a nuclease to the spot that will bind and cleave the genome activating the mechanisms of DNA repair that will introduce random mutations in the sequence, resulting in gene silencing.

Numerous mouse models of high-grade astrocytic glioma (often referred to as Astrocytoma or Glioblastoma) have been developed over the years. A number of these mouse models involve the introduction of oncogenic mutations in the germline or specific cell subpopulations in the brain\textsuperscript{157}; transplantation models, that either allograft previously manipulated cell lines\textsuperscript{158} or xenograft human cancer cell lines\textsuperscript{159} into the mouse brain; transgenic expression of a viral oncogene\textsuperscript{160} and conditional transgenic expression of oncogenes/tumour suppressor genes in specific cells/tissues\textsuperscript{161}. These include overexpression of active forms of Ras, Akt, PDGF and BRAF as well as GFAP-Cre-mediated inactivation of Nf1, p53, Rb and PTEN in neural progenitor and neural stem/progenitor cells of the subventricular zone (SVZ)\textsuperscript{104,108,150,151,158,162-165}.

Most of the glioma models have been established so far in mice, however, also other species have been used to study the mechanism of carcinogenesis including brain tumours, such as drosophila\textsuperscript{166} or zebrafish\textsuperscript{167,168} systems. Importantly, none of these models faithfully recapitu-
lates the genetic profile of IDH-mutant astrocytomas, i.e., combining IDH, p53, ATRX and optionally also the CDKN2A mutations directly in vivo. Therefore, one of the key objectives of this work is to create a preclinical model that most closely recapitulates the genetic constellation of mutations in human IDH-mutant astrocytomas.

1.5.1 \textit{Idh1}^{R132H}/\textit{p53}^{-/-}/\textit{Pten}^{-/-} mouse model

\textit{Bardella et al} (2016) found that adult mice expressing the \textit{Idh} mutation of arginine 132 into histidine (henceforth designated \textit{Idh1}^{R132H}) in the brain SVZ develop features of gliomagenesis, including the increased number of neural stem cells (NSCs) and their progeny with the gene expression profile of the \textit{Idh1}^{R132H} SVZ closely overlapping those of human gliomas\textsuperscript{169}. \textit{Holmen et al} have designed a model that combines \textit{Idh} mutation, \textit{Cdkn2a deletion, Atrx, and Pten} loss but was unsuccessful to establish tumours without \textit{Pten} deletion, which does not resemble the human cases of astrocytoma reported\textsuperscript{47}. Furthermore, a key feature of human astrocytomas is the triad of \textit{IDH}, \textit{ATRX}, and \textit{p53} mutation which has not been recreated in their model. Instead, \textit{Pten} mutations are often associated with \textit{IDH}-wildtype glioblastomas\textsuperscript{170}.

By a combination of the gene-editing tools for transgenic mice, our group has previously generated tumours by injecting neonatal \textit{Idh1}^{R132H}/\textit{p53}^{loxP}/\textit{Pt}^{loxP} mice with a retrovirus expressing the mitogen PDGFB and Cre Recombinase. Injections were made at P1 into the left ventricle and tumours formed 20-40 days after injection with over 90% incidence.

Our results showed that the \textit{Idh} mutation delays self-renewal and proliferation of neural stem/progenitor cells \textit{in vitro} and \textit{in vivo}. Interestingly, this is in discrepancy with the previous report where the presence of mutant \textit{Idh1}^{R132H} in stem/progenitor cells led to an expansion of the progenitor pool. It is possible that our study more accurately tracked the fate of recombined, \textit{Idh}^{R132H} cells with a mutation-specific antibody, whilst the previous study examined only indirect evidence of recombination\textsuperscript{171}.

Moreover, we also derived naive NSCs from mice containing a combination of floxed sites and performed \textit{in vitro} Adeno-Cre mediated recombination and confirmed that re-implantation generated tumours that are comparable to primary tumours\textsuperscript{172,173}.

1.5.2 Pre-clinical models of low-grade glioma

IDH mutant astrocytomas and IDH mutant oligodendrogliomas can present as Low-grade gliomas, and then correspond to CNS WHO grade 2. However, both forms also exist as higher grades, i.e., the oligodendrogliomas also as CNS WHO grade 3, and the Astrocytoma also as
CNS WHO grade 3 and grade 4. In particular, the higher-grade forms recur and lead to disability and premature death, although they don’t have a prognosis as poor as that of an IDH-wildtype glioblastoma. Therefore, modelling of these lower grade gliomas is an unmet scientific and preclinical need, and currently there is a lack of modern systems that specifically recapitulate the molecular features and clinical presentation of great to astrocytomas and oligodendrogliomas. This includes the difficulty in recapitulating the effects of the neomorphic IDH mutations (Table 1). Efforts have been made by several research groups to develop in vivo and in vitro models that would reveal invaluable tools to investigate this malignancy, test new drugs and validate therapeutical candidates. Table 1 captures the key existing models developed to date.

**Table 1: Pre-clinical models of low-grade gliomas**

<table>
<thead>
<tr>
<th>Model</th>
<th>Method of tumour induction</th>
<th>Genes Involved</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Ethyl-nitrosourea (ENU)-induced gliomas in pregnant rats</td>
<td>BRAF, TP53, PDG-FRA, CDKN2A, EGFR (no IDH mutation) *</td>
<td>174, 175, 1, 76, 177, 178</td>
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<tr>
<td>GEMM</td>
<td>Expression of mutant IDH1 in mouse SVZ</td>
<td>IDH</td>
<td>179, 180, 1, 71</td>
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<tr>
<td>GEMM</td>
<td>Sleeping Beauty transposase system</td>
<td>IDH, TP53, and ATRX</td>
<td>181, 47</td>
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<tr>
<td>GEMM</td>
<td>RCAS/TVA model</td>
<td>IDH, CDKN2A, ATRX, PTEN, and PDGFA</td>
<td>182, 187</td>
</tr>
<tr>
<td>GEMM</td>
<td>RCAS/TVA model</td>
<td>IDH, PDGF, and TP53</td>
<td>182, 187</td>
</tr>
<tr>
<td>Mouse allograft</td>
<td>PDGFB overexpressing mouse NSC into mouse brain</td>
<td>PDGFB</td>
<td>183, 184</td>
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<tr>
<td>Patient derived cell line</td>
<td>BT142: stable cell line from patient with grade 3 IDH-mutant astrocytoma</td>
<td>IDH1/2</td>
<td>185, 186</td>
</tr>
<tr>
<td>Patient derived cell line</td>
<td>TS603: IDH-mutant line generated from grade 3 IDH-mutant oligodendroglioma (1p/19q co-deletion)</td>
<td>IDH, 1p/19q codeletion, TERT</td>
<td>185, 186</td>
</tr>
<tr>
<td>Patient derived organoid</td>
<td>Organoid created from LGG tissue</td>
<td>IDH, TP53, CIC, NOTCH1, NOTCH2</td>
<td>185, 186</td>
</tr>
<tr>
<td>Genetically modified human neurospheres</td>
<td>hESCs with lentiviral modification</td>
<td>IDH, TP53, and ATRX</td>
<td>187, 188</td>
</tr>
<tr>
<td>iPSC</td>
<td>human LGG iPSC</td>
<td>IDH</td>
<td>188, 190, 1, 91</td>
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<tr>
<td>PDX</td>
<td>Various LGG orthotopic xenografts</td>
<td>IDH, FUBPI, and CIC</td>
<td>189, 190, 1, 91</td>
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<tr>
<td>In silico</td>
<td>Mathematical model of LGG response to combinations of chemotherapy</td>
<td>Not applicable</td>
<td>192, 193</td>
</tr>
</tbody>
</table>

**Abbreviations:**
**GEMM**: genetically engineered mouse model; **LGG**: Low-grade glioma; **hESC**: human embryonic stem cells; **NSC**: Neural stem cells; **iPSC**: induced pluripotent stem cells; **PDX**: patient derived xenografts

*These rat models preceded the discovery of the IDH mutation. However, these neoplasms were described as low-grade gliomas, and therefore were included in this table.

### 1.6 Experimental outline

Based on the previous models developed in our group, my project has been focusing on creating a new mouse model for astrocytoma that recreates the genetic profile of *IDH-mutant* astrocytomas, i.e., *ATRX* loss, *p53* mutation and potentially *CDKN2A/B* homozygous deletion.

Initially, *Idh*1<sup>loxP(R132H)</sup> and *p53*<sup>loxP</sup> animals have been bred to generate *Idh*1<sup>R132H+/+</sup>;*p53*<sup>loxP/loxP</sup> descendants that will then be bred with a constitutively Cas9-expressing mouse *(Gt(ROSA)26Sor<sup>tm1.1(CAG-cas9*, -EGFP)Fezh</sup>/J, Jackson Laboratories Stock No: 024858) generating *Idh*1<sup>R132H+/+</sup>;*p53*<sup>loxP/loxP</sup>; *Cas9<sup>+/+</sup> mice, which will be used to conduct this study (Figure 14 A, B).

Neonatal mice are co-injected with two plasmids, followed by electroporation to help incorporate DNA into the SVZ cells: (i) a plasmid expressing Cre recombinase and two sgRNAs, targeting signature genes mutated in astrocytoma (*ATRX* and *CDKN2A*) (Figure 14 E) and a plasmid conditionally expressing PDGFB and Firefly Luciferase (Fluc), in the presence of Cre (Figure 14 F); or (ii) a single plasmid expressing Cre Recombinase, one, two or three sgRNAs targeting *ATRX, CDKN2A* and/or *CDKN2B*, PDGFB and Firefly Luciferase (Figure 14 G, H). This approach shall result in a cleaner version of the model, as exclusively the cells infected by the Cre-expressing plasmid will combine all the mutations we wish to introduce in the model *(Idh*1<sup>R132H/p53<sup>-/-</sup>/Atrx<sup>-/-</sup>/Cdkn2<sup>a</sup><sup>-/-</sup>/Cdkn2<sup>d</sup><sup>-/-</sup>). Moreover, only mutated cells will express PDGFB, hence, accelerated proliferation, which should give rise to tumours in a short period; and the expression of luciferase will also be an advantageous tool for tumour monitoring and for better complying with 3R (Figure 14 C, D).
Figure 14: Constructs designed for this project: (A) p53loxP animals have floxed TP53 gene, causing its deletion upon Cre exposure. (B) Idh1loxP(R132H) mice have a conditional point mutation (R132H) in idh exon 3 which replaces the idhWT gene upon Cre expression (inversion of the idhWT cassette followed by deletion of the idhWT cassette, using different loxP sites). (C) CRISPR/Cas9 construct expressing one sgRNA, used for making lentivirus for single deletion of Atrx, Cdkn2a and Cdkn2b, in vitro, or in vivo, by co-injection with a plasmid expressing PDGFB and Cre Recombinase (D). (E) CRISPR/Cas9 construct expressing two sgRNA (targeting Atrx and Cdkn2a) and Cre Recombinase. All cells infected by this plasmid will express idh, p53, Atrx and Cdkn2a mutations. In vivo, this plasmid can be co-injected with a construct with floxed PDGFB and luciferase cassettes (F). PDGFB will boost the proliferation of mutated cells, and luciferase expression will be used for in vivo motorization of the injected animals. (G-I) are constructs that express the sgRNAs (one, two or three), Cre Recombinase, PDGFB and Luciferase all in one plasmid. Different combinations of these plasmids were designed to assess the most efficient strategy for the model.

At the same time, we will be establishing NSC from these mice and infecting them with lentivirus targeting ATRX and CDKN2A in vitro, followed by recombination by Adeno-Cre to then allograft them into NOD/SCID mice, which we expect to result in tumour formation (Figure 14 C, D).

Tumours generated from both models will then be compared with healthy counterparts by RNA sequencing, and with human tumour samples from our Biobank, for studying tumour progression and looking for possible new biomarkers. After the identification of these potential biomarkers, functional studies will be carried out.
2 Chapter II: Materials and Methods

2.1 Animal Management

All procedures performed on mice were according to Institutional and UK Home Office guidelines (Project license PA79953C0). The ARRIVE guidelines were followed as part of the institutional policy and the licensing of the experiments. All mice were kept at the Biological Service Facility, UCL. Mice were housed in laminar-filtered airflow cages under pathogen-free conditions at constant temperature (22 °C ± 2 °C), with relative humidity (55% ± 5%), 12-h dark/light cycles and ad libitum access to food and water.

2.2 Transgenic mice and genotyping

The knock-in mice p53loxP/loxP and Idh1loxP(R132H) were used in this study, and R26RloxP/loxP was used as a reporter in all mice. Cas9 mouse (Gt(ROSA)26Sor1.1(CAG-cas9*,-EGFP)Feh/J) was bought from Jackson Laboratories. A combination of conditional gene mutations was generated by intercrossing of single gene conditioned knock-in mice. Idh1loxP(R132H)/+ knock-in mice were a kind gift from Prof. Andreas von Deimling (DKFZ). An inverted exon 3 of Idh1 bearing GA to AT mutation at a dedicated position was inserted into the third intron of wild-type Idh1. After adeno-Cre mediated recombination, the wild-type exon 3 is replaced with mutant exon 3 resulting Idh1R132H mutant allele. DNA was extracted from mouse ear notches and genotyping was performed using Phire II polymerase (Thermofisher) all primers are shown in Error! Reference source not found.. PCR cycle conditions for each gene are described in table 2 was carried out under the following conditions: 94° C for 2 min; 37 cycles of 94° C for 30 s, 58° C for 60 s; 72° C for 10 min.
2.3 Stereotaxic injections and electroporation

Intra-cranial/intraventricular injections into neonatal mice were performed on postnatal day (P) 0 – P4. Pups were anesthetised with isoflurane (Isoflurane-Vet, Merial Animal Health Ltd) during injections and maintained on a heating pad at approximately 25º C during the whole procedure. A 10 µl 26-gauge Hamilton syringe (outer diameter 0.474mm and inner diameter 0.127mm, 51 mm needle length) was mounted, then cleaned and rinsed with 70% EtOH and PBS. Prior to injections, virus stock solution was mixed with polybrene (final concentration 8 µg/ml) to facilitate cell infection. Injections were targeted at 0.5 – 1 mm left of the sagittal suture (approximately on the level with the centre of the left eye). Once the needle had visibly penetrated the skull, the syringe was inserted a further 2 mm and 3-5 µl of virus/plasmid was injected into the left ventricle. After 5 seconds the needle was removed slowly, and the animals were set on a heated mat with the litter mates until awake from anaesthesia. The whole litter was then reunited with the progenitors and kept under supervision until acknowledged by the mother and returned to their nest. A secondary check-up was carried out around four hours later to ensure all animals had fully recovered from the procedure.

2.4 Electroporation

When no viral encapsulation was preceded, plasmid DNA was directly injected into the left SVZ. The procedure took place as described before – after anaesthetized by inhalation of isoflurane in a heated chamber, 1-3 µl of plasmid DNA was injected into new-born SVZ using a
10 μL 26-gauge Hamilton syringe and up to five electric pulses of 90 Volts, 50 ms length, with 1 s intervals were applied to the pups allowing the delivery of the plasmids into NSCs. Mice were then allowed to recover from the procedure in a heated environment and reunited with the mother under close supervision and monitoring.

2.5 Neurosphere derivation, maintenance, and passaging

Neuroospheres were derived from the SVZ of mice of postnatal age P5-P10. Briefly, mice were culled humanely by asphyxiation in CO2. SVZ was dissected within 30 min after the mice were confirmed dead. The SVZ was isolated and minced with a sterile razor and then dissociated using the Worthington Papain tissue dissociation kit (Cat No. LK003150) as per the manufacturer's protocol. Dissociated tissue was passed through a 0.45 μm filter before plating onto an uncoated 10 cm dish. After 5 – 7 days NSCs form large floating spheroid structures while differentiated cells are dead or adherent. To acquire monolayer cell culture, neurospheres were dissociated with Accumax (Stemcell Technologies, #07921) and seeded on a poly-lysine and laminin double-coated plate. All cells were passaged every 4 days.

2.6 Tissue Culture

HEK 293T, Platinum E, SVZ isolated mouse NSCs and neurosphere derived mouse tumours are described in this project. Mouse NSCs and mouse cancer cells were maintained on poly-lysine (Sigma) and laminin (Sigma) coated plates. All cells were maintained at 37° C, 5% CO2, 90% humidity and in appropriate media (NSC: DMEM/F12 (LONZA) containing 20 ng/ml EGF (Pepro-Tech), 20 ng/ml FGF (PeproTech), 2 % B27 (Invitrogen) and 1 % Penicillin/Streptomycin (P/S, Invitrogen); HEK293T: DMEM supplemented with 10 % FBS and 5 % P/S) Medium was changed every 4 days unless stated otherwise. Passaging was performed when cells were around 90 % confluent and were detached using Accutase solution® (Sigma, A6964).

2.7 Adeno-Cre recombination

The Cre-expressing adenovirus was produced using HEK293A cells. For infection, 2.2x10⁶ cells were seeded as a monolayer in a 10 cm petri dish and infected with 2 μl of Cre-expressing adenovirus (10¹⁰ IFU/ml), the next day. After 16 h, the medium was replaced with fresh medium. Recombination was confirmed by genotyping.
2.8 Plasmids and cloning

The LentiGuide-Puro-sgATRX (Addgene # 52963, Appendix 7.1) and LentiGuide-Hygro-sgCDKN2A (Addgene # 99375, Appendix 7.2) sgRNA expression vectors were made by digesting the parental vectors with Fast Digest Esp3I (Fermentas) for 30 min at 37°C and gel-band purifying the resulting linearized vectors for subsequent ligations. sgRNA sequences were designed using the CHOPCHOP sgRNA designer tool (https://chopchop.cbu.uib.no/). Sense and anti-sense oligonucleotides corresponding to the desired sgRNA were purchased by Thermo Fisher, mixed at equimolar ratios, and annealed by PKN (Thermo) at 37°C for 30 min, 95°C for 5 min, followed by a ramp down to 4°C by 0.1°C/sec. The annealed oligonucleotides were then diluted at 1:50 and ligated into the Esp3I digested lentiviral vectors by incubation with Quick Ligase (NEB) for 30 min at RT. The ligation mixture was transformed into SbtI3 maximum competent cells. Ampicillin-resistant colonies were validated by DNA sequencing. px333-sgATRX-sgCDKN2A-Cre was a kind gift from Prof. David Kirsch (Duke University) and was cloned using a similar approach, using the restriction sites for BbsI and Bsal (NEB). The following sgRNA oligos were used: ATRX sense (3’ CACCGTGGCCGTAAAAAGTCTGGGG5’), antisense (3’ AAACCCCCAGAACTTTTACGGCCAC 5’); CDKN2A sense (3’ CACCGCGCTGCGTCGTGCACCGGG 5’), antisense (3’ AAACCCCCAGACACGGCAGCCG 5’). Finally, CMV-PDGRFB-Fluc (VB190627-1017ykt, Appendix 7.13), pLV-U6>sgATRX-U6>sgCDKN2A-U6>sgCDKN2B:CMV>Cre:T2A:hPDGFB:P2A:Luciferase (VB200210-1021zhd, Appendix 7.14), pLV-U6>ATRX-CMV>Cre:T2A:hPDGFB:P2A:Luciferase (VB200211-1250kfw, Appendix 7.7) and pLV-U6>sgATRX-U6>sgCDKN2A-CMV>Cre (VB210923-1307ned, Appendix 7.10), plasmids were customized and bought from Vector Builder.

2.9 Lentiviral production, titration, and infection

Lentiviruses were made by Lipofectamine 2000-based co-transfection of HEK293T cells with the respective lentiviral expression vectors and the packaging plasmids psPAX2 (Addgene # 12260, Appendix 7.4) and pCMV (Addgene # 8454, Appendix 7.2) in a ratio of 4:3:1. Virus-containing supernatant was collected at 48 and 72 hours after transfection, pooled together (15 mL total per 10 cm tissue culture dish), passed through a 0.45 µm filter, aliquoted, and frozen at -80 °C until use. 0.3x10⁶ cells were seeded in double-coated 6 well plates in 2 mL of media. The following day, 1 mL of lentivirus with 5 µg/mL polybrene was added to the respective well and the plates were then centrifuged at 2000 rpm for 2 hours at RT. 12-16 hours later the virus was removed, and cells were expanded to 10 cm plates and grown for 72 hours before being
placed under drug selection. Cells were selected by growth in puromycin (1 μg/mL), blasticidin (5 μg/mL) or hygromycin (400 μg/mL) for 4 days.

For in vivo applications, lentivirus was concentrated after filtration, using Retro X concentrator overnight, then span and resuspended in 1/100th of the initial volume in PBS and finally aliquoted and frozen until use. For titration, Confetti cell lines were transduced by increasing levels of lentivirus, and their fluorescent expression was observed after 72 h in an inverted confocal microscope.

2.10 Retrovirus production, titration, and infection

The PDGFB-IRES-Cre retroviral construct was kindly provided by Prof. Peter Canoll (Columbia University Medical Centre, USA, Appendix 7.17). The modifications to this construct were designed by us and outsourced for manufacturing by Vector Builder. The use of PDGFB-IRES-CRE retrovirus to generate gliomas has been described previously \(^\text{193,194}\). Retrovirus was amplified in Platinum E cells. Briefly, transfection mixtures containing plasmid DNA, Lipofectamine® and OptiMem medium were added dropwise to a ~60% confluent 10 cm plate of platinum E cells and incubated overnight at 37°C. The transfection medium was replaced with fresh culture medium and cells were incubated for a further 24 h at 37°C. After 24 h, the culture medium was extracted into a syringe, passed through a 0.45 μl filter and mixed with Retro X concentrator (Clontech, PT5063-2) at a 1:3 (concentrator:medium). This mix was incubated for 16 – 20 h at 4°C and the centrifuged at 1500 g and 4°C for 1 h and 30 mins. The supernatant was extracted, and the pellet was re-suspended in OptiMem. For titration, 0.1 x 10⁶ unrecombined NSCs were seeded into coated wells of a 24-well plate and cultured for 24 h. After 24 h, to each well, a mixture containing 5 μl of retrovirus, polybrene (final concentration 8 μg/ml) and 1 ml of culture medium was added. Control wells received culture medium with polybrene and no virus. Cells were incubated in this mixture for 24 h then washed with PBS. A regular medium was added, and cells were cultured for a further 48 h. After 48 h, each test well was washed with PBS then cells were fixed within the well for 10 mins at room temperature in 4 % PFA. Cells were then incubated in 1 μg/ml Hoechst 33342 (Invitrogen, H3570) for 20 mins. Finally, cells were washed in PBS and then imaged on an inverted Zeiss lsm 710 confocal microscope.

2.11 Adeno-associated virus (AAV) production and titration

HEK293A cells were plated using DMEM media containing FBS without penicillin and streptomycin on a 15 cm tissue-culture dish, at 40 % confluency. The following day, the cells were
transfected using Lipofectamine 2000 with the AAV vector along with AAV2 and AAV-pHelper packaging vectors (Cell Biolabs) at a ratio of 1:1:1. The following day, fresh DMEM media with 10% FBS was added. 48 hours later, the cells and media were collected and subjected to 4 freeze-thaw cycles by alternating between an ethanol dry ice bath and 37°C. After the last thawing, cells were treated with sodium deoxycholate and benzonase to degrade nucleic acids and lipids, respectively. Cell debris was removed by centrifugation and the supernatant was collected and passed through a 0.45 μm filter. Purification was made by HPLC and collected vectors were kept in a dialysis cassette overnight and finally spun in size-selective columns (Amico Ultra 15; Merck), aliquotted, and frozen at -80°C until use. Titration was done by measuring GFP expression levels by qPCR.

2.12 Adenovirus production

The adenovirus expressing Cre Recombinase was amplified using HEK ad293 cells (Cell Biolabs, AD-100). Briefly, approximately 5 x 10⁶ HEK293A cells were seeded in a 10cm plate and cultured in a cell incubator (37°C, 5 % CO²) overnight. Adenovirus from stock (3 – 5 μl) was directly added to HEK293A cells for infection. Adenovirus was harvested after 3-day incubation. To harvest the virus, cells were directly scraped out of the plate using a scraper (Corning, 3010). The cell suspension was transferred into a 50 ml Falcon tube and centrifuged at 2000 rpm for 5 min. The supernatant was discarded, and the cell pellet was then re-suspended in 1 ml PBS. The cell suspension was then transferred into a 15 ml Falcon tube. To release the virus, the cell suspension was frozen and thawed 3 times. The tube was briefly vortexed between each freeze-thaw cycle. Ultimately, the tube was centrifuged at 1200 rpm for 5 min and the supernatant containing virus was passed through a 0.45 μm filter, which was pre-hydrated with PBS. The adenovirus was stored at -80°C until use.

2.13 Histology and IHC

Whole-brain tissue was fixed overnight in BSF for 12-24h. The following day brains were washed in PBS and processed in an automated tissue processor (Leica ASP300). The protocol involves Formalin, Ethanol (70%, 90%, 100%), Xylene and paraffin wax incubations overnight. The next day embedding was carried out using Embedding station (Leica EG1150H), where paraffin wax was dispensed into a metal mould where the processed tissue was placed with heated forceps, with the surface of interest face down; the mould was then placed on a small cold plate to set slightly while the tissue was held in place and gently pressed to give a flat cutting surface. The tissue cassette with sample ID tag was placed on top and the mould transferred to a larger cold plate to allow wax block to set. Once set, the wax blocks were removed.
from the mould and excess wax trimmed off. 5 µm sections were cut from the paraffin blocks using a microtome (Leica, RM2235). Sections were floated out on a 42°C water bath and picked up on Polysine Adhesion Slides (Thermo Scientific, #10219280). Slides were air dried for at least 30mins and then placed in a 60°C oven for at least one hour before staining. IHC was performed on an automatic immunostaining machine (Roche Ventana Discovery immunostainer) following manufacturer’s guidelines (Antibodies, see Table 2). Tissue sections were digitally scanned on a Hamamatsu Nanozoomer S360 whole slide scanner at 40x magnification.

**Table 3 Antibodies used for IHC**

<table>
<thead>
<tr>
<th>Antibody (target)</th>
<th>Host Species</th>
<th>Dilution</th>
<th>Reference</th>
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<tbody>
<tr>
<td>IDH1&lt;sup&gt;R132H&lt;/sup&gt;</td>
<td>Rabbit</td>
<td>1:20</td>
<td>Atlas HPA001906</td>
</tr>
<tr>
<td>GFP</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Abcam ab290</td>
</tr>
<tr>
<td>P16</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Abcam ab54210</td>
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<td>P15</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Thermo Fisher PAS-99491</td>
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<td>ATRX</td>
<td>Rabbit</td>
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<td>Atlas HPA001906</td>
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<td>Rabbit</td>
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<td>Rabbit</td>
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<tr>
<td>Secondary Rabbit</td>
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<td>1:200</td>
<td>Abcam, ab207995</td>
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2.14 Haematoxylin & Eosin (H&E) Staining

H&E staining was performed on a Leica ST5020 auto staining robot using the following protocol: FFPE sections were first dewaxed with two rounds of xylene (2 min each time), followed by rehydration with two times of absolute alcohol (2 min each time), 70% alcohol for 2 min and water for 2 min. These rehydrated sections were then stained with Harris haematoxylin (Leica, 3801560E) for 5 min. Prior to Eosin staining (1%, 3 min), slides were washed with water for 1 min, 1% of acid alcohol for 14 sec, and 1-minute washing to remove acid alcohol residues. Finally, slides were dehydrated with 70% alcohol, absolute alcohol and xylene as mentioned above.

2.15 Immunofluorescent labelling of cultured cells

After selection, infected NSCs were seeded in a 24-well plate double-coated, in cover slips, at 0.1x106 cells/well and grown for 24 h. The following day, cells were washed in PBS and fixed in 4% PFA for 15 min at RT. Follows a permeabilization step with PBS-Tw (0.2% Tween) for 10 min and 1 h blocking in 10% goat serum. Cells were then incubated with primary antibody at 4°C, overnight. The next day, cells are washed in PBS and incubated in the appropriate secondary antibody, protected from the light, for 1 h at RT. Cells are washed with PBS-Tw, then PBS and finally PBS with Hoechst (1:15000) for 15 min and then mounted in slides for imaging in a Zeiss lsm 710 confocal microscope.

Table 4 List of antibodies used for immunocytochemistry

<table>
<thead>
<tr>
<th>Antibody (target)</th>
<th>Host Species</th>
<th>Dilution</th>
<th>Reference</th>
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<td>IDH1R132H</td>
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<td>P16</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Abcam ab54210</td>
</tr>
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<td>P15</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Thermo Fisher PA5-99491</td>
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<td>Rabbit</td>
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### 2.16 Genomic DNA extraction

Genomic DNA from ear biopsies extraction was described before (paragraph 2.2, page 37). DNA from cell pallets was extracted using GeneJET Genomic DNA Purification Kit (Thermo Scientific, K0721) according to the manufacturer’s protocol. DNA from FFPE tissue (e.g., human glioma tissue) was extracted with the Maxwell 16 FFPE plus LEV DNA Purification Kit (Promega, AS1135). Briefly, tissue from 5-10 unstained sections was scraped out using a blade and incubated with 180 μl of incubation buffer and 20 μl of Proteinase K at 70°C overnight. On the next day, 400 μl of lysis buffer was added to each sample, which was then transferred to LEV DNA Cartridge provided in the kit. The Cartridge was loaded to Maxwell 16 MDx Instrument (Promega, AS3000) to extract DNA automatically according to the manufacturer’s protocol.

### 2.17 RNA extraction and qPCR

NSCs were cultured in 10cm plates to confluency, then washed in cold PBS. Cells were scraped using a cell scrapper, then pelleted and used for RNA extraction using Qiagen RNeasy Micro Kit (Qiagen, #74004), following the manufacturer’s instructions. For gene expression, cDNA libraries were prepared using SuperScript™ VILO™ cDNA Synthesis Kit (ThermoFisher #11754050), using 14μL of RNA per sample, and incubated at 25°C for 10 min, followed by one hour at 42°C and 85°C for 5 min to terminate the reaction. Eluted RNA was diluted in 60μL of RNAse-free water, and quantitative PCR was carried out using Taqman probes and SYBR Green methods, on different occasions. The former used Taqman Genotyping Master Mix (ThermoFisher # 4371357) and inventoried TaqMan™ Gene Expression Assay (FAM) (ThermoFisher #4351368) for Atrx, Cdkn2a and Cdkn2b. The latter used the SYBR Green kit (Thermo Scientific, K0221). In both cases, gene expression was normalized for the housekeeping gene Gapdh. All qPCR experiments were performed on HT7900 instrument or QuantStudio 5 (Applied Biosystem) with the following programme: 50°C for 2min; 40 cycles of 95°C for 15 sec, 60°C for 1min; and a dissociation stage by increasing the temperature from 60°C to 95°C at a ramp rate of 2%. All probes used are listed on Table 4.
Table 5 List of TaqMan gene expression assays for quantitative PCR. All probes were obtained from Thermofisher Scientific (Catalogue number 4331142)

<table>
<thead>
<tr>
<th>Target</th>
<th>Probe</th>
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<td>FAM-MGB</td>
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2.18 Sample preparation for Mouse Methylation Beadchip array

DNA extracted as described on paragraph 2.16 were then submitted to a clean-up protocol, using Agencourt AMPure XP beads (Beckman Coulter, # A63880). Briefly, 2500ng of DNA in 50μL are mixed in a low-binding 0.2 mL Eppendorf with 50 μL of beads and mixed in a Hula mixer for 5 min. Tubes are then placed in a magnetic rack for 2 min for separation of the beads. All supernatant is then removed and replaced with fresh 70% ethanol (this step should be done twice). Finally, all ethanol is removed and the caps are left off for 30 s, to allow for the ethanol to dry. The tubes are then removed from the rack and 40 μl of Nuclease-free water is added to each tube, flicking it to mix the beads. After 15 min of incubation at RT, tubes are replaced on the magnetic rack for 2 min and finally the supernatant is collected to a clean 0.2 mL tube.

DNA QC is performed using a Tapestation and Nanodrop, and 500 ng of each sample are used for the next steps.

Bisulphite conversion was performed using Zymo Lightning BSC deep well Kit (Zymo, # D5033-E), following manufacturer instructions: 130 μl of Lightning Conversion Reagent was added to 20 μl of each DNA sample in a Conversion plate, then transferred to a thermal cycle to perform the following steps: 98°C for 8 minutes; 54°C for 60 minutes. Then 600 μl of M-Binding Buffer was added to the wells of a Zymo-Spin™ I-96 Binding Plate mounted on a Collection Plate, where the samples from the Conversion Plate were added and centrifuged at ≥ 3,000 x g (5,000 x g max.) for 5 minutes. The flow-through is discarded and washed with 400 μl of M-Wash Buffer three times. The place the Zymo-Spin™ I-96 Binding Plate was then eluted in 15 μl of Elution buffer, onto an Elution Plate.

To ensure the best quality of DNA, samples then undergo to a restore protocol (Illumina Infinium HD FFPE Restore Protocol, #WG-901-2004): each sample was incubated with 4 μl of 0.1 NaOH, at RT, for 10 min; then mixed with Amp Mix Restore Reagent and Primer Pre Restore
Reagent (PPR) in a pre-restore plate, incubating at 37°C for 1 hour. 7 volumes (560 μL) of Zymo DNA Binding Buffer were then added to each volume of DNA, mixed and transferred to the wells of the ZymoSpin I-96 Plate mounted on a Collection Plate. After washes, 13 μl of Elution Restore Buffer Reagent is added directly to the column matrix of each well, incubated at RT for 5 min, centrifuged for elution. 10 μl of eluted DNA solution generated was then incubated at 95°C for 2 min, then immediately transferred to ice for 5 min. 5 μl of Convert Master Mix Reagent was then added to each well and incubated at 37°C for 1 hour, then mixed with 7 volumes of Zymo DNA binding buffer to each volume of DNA in the plate. Follows two washes steps with Zymo Wash Buffer and then DNA is eluted in 10 μl of dH2O which is immediately used for microarray processing.

Infinium HD Methylation Assay is processed according to manufacturer’s instructions but using a higher volume of start material (8 μl).

### 2.19 DNA Dissociation of neoplasms to establish cell lines

Fresh brains were collected in dissection media (DMEM, Lonza) and transported on ice to a dissection microscope. If no sign of tumour were perceptible macroscopically, the brain would be placed on a petri dish and cut coronally at the injection site. This would usually expose the tumour, which was then dissected and minced with a sterile razor blade. Minced tissue was then pipetted into Accumax (Sigma, A7089) and incubated at 37°C until homogenised (no longer than 1 hour). The homogenised solution would then be filtered through a 0.45 μm filter and added into 15 mL of cell culture media - DMEM/F12 (LONZA) containing 20 ng/ml EGF (PeproTech), 20 ng/ml FGF (PeproTech), 2% B27 (Invitrogen) and 1% Penicillin/Streptomycin (P/S, Invitrogen). After 10 days, neurospheres have usually formed, and lines could be maintained by normal cell culture routines.

### 2.20 IncuCyte analysis of proliferation

The IncuCyte ZOOM system (Essen Bioscience), which can image monolayer cells automatically and analyse cell confluence, was used in this study to quantify cell proliferation rate. To compare the proliferation rate of cells with different genetic backgrounds and/or under different treatment, 1500 cells were seeded per well of a 96-well plate (Nunc, 167008), and scanned in the IncuCyte for 10 days (12h between scans). Cell confluence was then assessed using ZOOM software.
2.21 D2HG enzymatic assay

D-2-Hydroxyglutarate levels were measured using D2HG Colorimetric Assay Kit (Abcam, ab211070) according to the manufacturer’s protocols. Briefly, 1x10^7 cells were rapidly homogenised with 100µL of ice-cold D2HG Assay Buffer, for 10 minutes, on ice, followed by 5-minute centrifugation (10000 x g, at 4°C). Up to 45µL of each sample were then added into three wells of a 96 well clear plate (Nunc, 167008) and mixed with 50µL of the reaction mix, containing D2GH assay buffer, D2HG enzyme and D2HG substrate mix. One of the samples (spiked sample) was used as an internal control by adding 5 µL of 1mM D2HG standard, to correct for any sample interference. The plate was then incubated at 37°C for one hour, then measured absorbance at 450 nm, using a FLUOstar plate reader. DHGH amount was calculated based on the following equation:

\[
\text{D2HG amount (nmol)} = \left( \frac{\text{OD sample (corrected)}}{\text{OD (spiked sample)}} \right) - \left( \frac{\text{OD sample}}{5} \right) \times 5
\]

The D2HG concentration in the Sample is:

\[
C = \frac{X}{V} \times D = \frac{\text{nmol}}{\mu L} = \frac{\text{mmol}}{L} \text{ or } \text{mM}
\]

Where: 
X = Amount of D2HG from the calculation above (nmol)

V = Sample volume added into reaction well (µL)

D = Sample Dilution Factor

5 = Amount spiked in Sample well (5 nmol)

2.22 Western Blot

Cells growing in 10 cm plates at 80% confluency were centrifuged at 400 g for 3 min at 4°C and the media was removed by gentle aspiration. The cell pellet was then washed once in 1 mL of ice-cold PBS, transferred to a 1.5-mL Eppendorf tube, and centrifuged at 400 g for 3 min at 4°C. The PBS was carefully aspirated, and the cell pellet was lysed in RIPA lysis buffer (Thermo, 89900) supplemented with a protease inhibitor cocktail (Complete, Roche Applied Science, 11836153001) and phosphatase inhibitors (PhosSTOP, Sigma, 04906837001). Whole-cell extracts were quantified using the Bradford Protein Assay and 20 µg of protein per sample was boiled in 2x Laemmli Sample Buffer (Bio-rad, 161073EDU), resolved by SDS-PAGE (NuPAGE 4-12% Bis-Tris Gel, Invitrogen, NP0321BOX), transferred onto nitrocellulose membranes, blocked in 5% BSA in Tris-Buffered Saline with 0.1% Tween 20 (TBS-T) for 1 h, and probed with the indicated primary antibodies overnight at 4°C. Membranes were then washed three times in TBS-T, probed with the indicated horseradish peroxidase-conjugated (HRP) secondary antibodies
for 1 h at room temperature, and washed three times in TBS-T. Bound antibodies were detected with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amer-sham ECL Prime Western Blotting Detection Kit, RPN2232). All antibodies used are listed in Table 6.

Table 6 Primary and Secondary antibodies used for Western Blot.

<table>
<thead>
<tr>
<th>Antibody (target)</th>
<th>Host Species</th>
<th>Dilution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH1R132H</td>
<td>Mouse</td>
<td>1:500</td>
<td>Dianova DIA-H09</td>
</tr>
<tr>
<td>P16</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Abcam ab54210</td>
</tr>
<tr>
<td>ATRX</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Atlas HPA001906</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Rabbit</td>
<td>1:10000</td>
<td>Cell Signalling 9661</td>
</tr>
<tr>
<td>Secondary Mouse IgG</td>
<td>Goat</td>
<td>1:10000</td>
<td>Sigma, A9917</td>
</tr>
<tr>
<td>Secondary Rabbit IgG</td>
<td>Goat</td>
<td>1:10000</td>
<td>Sigma, A6667</td>
</tr>
</tbody>
</table>

2.23 Allografts

NSC lines were established from Idh/p53 new-born mice and infected with lentivirus expressing sgATRX, as described before (Paragraphs 2.5, 2.9). After confirming the genotype by PCR and Immunocytochemistry, cells were collected from a 10 cm dish into a 15 mL Falcon tube. The dish was washed with PBS to ensure all cells were collected. The falcon tube was spun, and the supernatant was removed. Pellet was re-suspended in the determined volume of PBS to ensure there was enough for 20 µl per mouse + ~10% spare (1x10⁶ cells in 20 µL/ mice). Cells were then transported in a 1.5 ml Eppendorf to the animal facility on ice.

NOD-SCID female mice were placed into the isoflurane chamber to be anaesthetised. Once unconscious, one by one the mice were attached to the stereotactic frame and 20 µL of cells were loaded into the needle. A small incision was then made on the head to expose the brain and 10 µL of cells were slowly injected into the ventricle (bregma: 1.5 mm lateral and 2 mm deep) with a 25-µl Hamilton syringe (Hamilton model 1702 RN, point style 2) with a 22 G needle (Hamilton, 80230) attached. The incision was closed with a single suture and then the remaining 10 µL was injected and, finally, the needle was carefully removed from the brain. The mouse was then removed from the stereotactic frame and injected with 200 µl of Carprofen, subcutaneously, in the back of the neck.
3 Establishing mouse neural stem cell lines with a genetic profile of human IDH-mutant astrocytoma

3.1 Introduction

This part of the project aims at generating cell lines that recapitulate the genetic profile of human IDH-mutant astrocytomas. The starting material comprised neural stem cell lines with an inducible IDH1 R132H mutation and an inducible p53 gene deletion. Based on these lines, we aim for the functional “knockout” of the tumour suppressor genes, ATRX, CDKN2A, and CDKN2A/B, which are mutated in human IDH-mutant astrocytomas, but also to further investigate how these cell lines compared to controls, for example by examining basic biological functions such as cell growth, apoptosis, or migration. Furthermore, such cell lines allow subsequent in vitro and in vivo studies, such as gene expression analysis, epigenetic profiling, or drug testing, work that shall be carried out alongside tissue obtained from the mouse model that we propose to develop. In this part of the project, we tested different guide RNAs for each of the genes, but also different delivery systems, selection, and read-out techniques to validate the decreased expression of each gene.

Neural stem cell lines established from new-born $ldh1^{R132H/+};p53^{loxP/loxP};Cas9^{+/+}$ mice, were kept in culture and were used for all the experiments described in this chapter.

Firstly, we aimed to target the Atrx gene. Several guide RNAs were designed and tested using different viral systems but, ultimately, Atrx deletion was successful 72h after lentiviral infection of the most efficient guide and validated by IHC. Cdkn2a and Cdkn2b deletion were validated in the same fashion.

Next, plasmids were designed that could target up to all the three tumour suppressor genes simultaneously, which were also validated 72h after lentiviral infection of $ldh1^{R132H/+};p53^{loxP/loxP};Cas9^{+/+}$ NSC, by IHC. However sufficient as a validation of the plasmids to be used in vivo, this approach could not be explored to establish triple-KO cell lines desired to progress further studies because these plasmids did not allow the selection of the infected cells. For this reason, we developed a parallel strategy in which we combined a guide RNA with a fluorophore, resulting in cells with a tumour suppressor gene deletion and capable of expressing a fluorescent signal. By infecting a population of NSC with these viruses, we could then select the cells based on the colour they expressed, which would allow us to establish different lines combining different mutations so that further studies could be carried out.
3.2 Results
3.2.1 ATRX deletion in human astrocytoma cell lines

ATRX is an important biomarker for glioma, and we will focus our efforts in generating cell lines and tumours with ATRX deletion, that would resemble the human counterparts. To confirm that ATRX can be detected in tissue, by IHC, and in cell lines, by IF; and that it’s loss can be confirmed by these methods, we used tumour samples obtained from the tumour tissue bank in the Division of Neuropathology at the National Hospital of Neurology and Neurosurgery (NHNN) with either lost or retained ATRX expression. We used FFPE sections of tumours with retained or lost expression of ATRX, comprising high-grade (A, C) and low-grade tumours (B, D) (Figure 15). Formalin-fixed and paraffin-embedded (FFPE) sections (A) and (B) showed retained expression of ATRX in tumour cells, whereas the sample shown in (C) and IDH-mutant high-grade astrocytoma and (D), and IDH-mutant low-grade astrocytoma, showed loss of ATRX expression in tumour cell nuclei and retained ATRX expression in non-neoplastic cells, such as endothelial cell nuclei and resident astrocytes or neurones. The right panel shows cell lines established from tumour samples that were cultured, fixed, and stained for ATRX. (E) and (F) are IDH-wildtype glioblastoma cell lines that express ATRX in tumour cell nuclei, whilst (G) and (H) were established from IDH-mutant astrocytomas and show loss of nuclear ATRX expression.

These results confirm the presence of ATRX expression in high-grade tumours and correspondent cell lines as well as the loss of expression in low grade tumours and cell lines and set a tone for what is the one of the aims of this project, i.e., to recreate these features in mouse cell lines and tumours.

<table>
<thead>
<tr>
<th>Formalin-fixed, paraffin embedded tissue</th>
<th>PFA Fixed cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>B</td>
<td>F</td>
</tr>
<tr>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>D</td>
<td>H</td>
</tr>
</tbody>
</table>

Figure 15 ATRX staining in human brain tumours. The left panel shows formalin-fixed, paraffin embedded (FFPE) sections of high grade (A, C) and low grade (B, D) tumour samples. (A) and (B) have retained
expression of ATRX whereas (C) and (D) show loss of ATRX expression in tumour cell nuclei, whilst ATRX is still retained in non-neoplastic cells, such as nuclei of endothelial cells or resident astrocytes. Accordingly, the right panel shows paraformaldehyde (PFA)-fixed cells of IDH-wildtype glioblastoma, (E) and (F), with retained ATRX expression. (G) and (H) are cell lines established from IDH-mutant astrocytomas and show loss of ATRX expression.

3.2.2 Generation of IDHR132H/P53/Cas9 cell lines

To test and validate the CRISPR KO of the signature biomarkers of IDH mutant astrocytomas, we started by establishing NSC lines from transgenic mice with floxed p53 and IDH genes, as described in paragraph 1.6. The transgenic mice were initially crossed with a genetically engineered mouse line that constitutively expresses Cas9 (locating the Cas9 expression cassette into the ROSA26 locus; The Jackson Laboratory, 026179), resulting in Idh1\textsuperscript{R132H/+}; p53\textsuperscript{loxP/loxP}; Cas9\textsuperscript{+/-}-flox-STOP cassette which prevents the expression of the downstream bicistronic sequences (Cas9 and EGFP). Although under the control of a CAG promoter, widespread expression of Cas9 and EGFP is prevented by the STOP cassette. After exposure to Cre recombinase, expression of cas9 and EGFP is observed, i.e., EGFP can be used as a reporter of recombination.

To generate cell lines conditionally expressing Cas9, IDHR132H and p53 deletion, new-born mice were culled and the SVZ dissected, where most NSC are located. These cells lines were cultured in appropriate growth conditions to maintain their stemminess and exposure to Cre recombinase would induce recombination, resulting in a IDH\textsuperscript{mut}/p53\textsuperscript{del}/Cas9 line that was used to validate all the in work, \textit{in vitro}, before proceeding to \textit{in vivo} approaches.

3.2.3 Validation of the inactivation of ATRX expression \textit{in vitro}

ATRX deletion in experimental cell lines was achieved by CRISPR/Cas9 technology and was first validated in vitro, targeting NSC from the SVZ of new-born animals, which are believed to be the tumour initiating cells in glioma. To obtain the most effective deletion we screened the 13 best-scoring sgRNAs from different guide RNA online design tools (Figure 16). Details on all the guides used are shown in Table 6. NSC were established from Idh1\textsuperscript{R132H/+}; p53\textsuperscript{loxP/loxP}; Cas9\textsuperscript{+/-} mice and infected with lentivirus expressing the sgRNAs (A). The efficiency of the guide RNA in reducing ATRX expression, determined by staining for ATRX, varied considerably between the different guide sgRNA candidates, ranging from no effect to a loss of expression. Figure 16B shows all the guides tested, organized in decreasing order of efficacy. The most efficient sgRNA was selected and used for further experiments. After recombination with Adeno-Cre, trans-
duced lines were selected by resistance to puromycin (4-5 days), and NSC genotype was accessed by immunocytochemistry (C) and qPCR (D), confirming ATRX loss. IDH\textsuperscript{R132H} mutation, and p53 deletion.

These results show the complete knockout of ATRX, in vitro, after antibiotic selection, as well as the expression of IDH R132H mutation, in NSC, after transduction with lentivirus expressing sgRNA and Adenovirus expressing Cre recombinase, validating the ATRXsg1 for an efficient ATRX KO.

![Figure 16 ATRX deletion in IDHR132H/P53-/- NSC.](image)

Figure 16 ATRX deletion in IDHR132H/P53-/- NSC. (A) NSC were extracted from new-born Cas9\textsuperscript{het}, Idh\textsuperscript{R132HloxP}; p53\textsuperscript{loxP} mice and infected with lentivirus expressing sgATRX and adeno-Cre, to induce recombination, resulting in a Cas9\textsuperscript{het}; Idh\textsuperscript{R132H}; p53\textsuperscript{del}; Atrx\textsuperscript{del} cell line. (B) The top scoring guides from different online design tools were screened for the most efficient deletion – NSC were infected with lentivirus expressing each guide, then fixed and stained for ATRX. sgRNAs are organized in decreasing order of deletion efficiency. ATRXsg1 had the best deletion efficiency and was used for further studies. (C) NSC genotype was confirmed by immunocytochemistry – both control and ATRX deletion cells show IDH\textsuperscript{R132H} mutation, and only ATRX deletion revealed loss of ATRX expression - ; and qPCR (D).
### Table 7 Top-scoring guide RNAs tested for ATRX deletion in vitro

<table>
<thead>
<tr>
<th>Design tool</th>
<th>Sequence</th>
<th>exon</th>
<th>efficacy</th>
<th>PAM</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
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<td>CACCGGTGGCGGTAAAGTTCTGGG</td>
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<td>ACGGCCCATTAAGGTTCAAGAGG</td>
<td>14</td>
<td>0.61</td>
<td>AGG</td>
<td>CACCGACGGCGCATTAAGGTTCAAG</td>
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<tr>
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<tr>
<td></td>
<td>CTTTCGACCAAGTGGCGAGG</td>
<td>9</td>
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<td>AGG</td>
<td>CACCGCCTTTGCAAGGTCGGGC</td>
<td>AAAACCACAAACCTTGGGGG</td>
</tr>
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<td>AGG</td>
<td>CACCGTGAAACCCTACACTCTCGG</td>
<td>AAAACCAGGAGGTTTCTGGG</td>
</tr>
</tbody>
</table>

### 3.2.4 Cdkn2a deletion in mouse NSC

The latest update on CNS tumour classification has included CDKN2A as a marker of high-grade, independently of the classical histological features, such as necrosis and macrovascular proliferation. High-grade IDH-mutant astrocytomas often report ATRX and Cdkn2a deletions, which should be represented in this mouse model. In the same line of the experimental work done for ATRX deletion, we aimed delete CDKN2A expression, and started by validating this KO, in vitro. NSC established from Idh1R132H/+; p53<sup>loxP/loxP</sup>; Cas9<sup>+</sup> mice were infected with lentivirus expressing sgRNA targeting the exon 2 of the Cdkn2a gene (5' GCGCTGCGTCGTGCACGGGGCGG3'). Immunocytochemical staining for p16 revealed complete abolishment of p16 expression (Figure 17). As previously stated, both proteins expressed by this gene differ only on the first exon, so by targeting the second exon we should be able to knock out both genes with a single guide.
NSC transfected by the lentivirus expressing sgCDKN2A and AdenoCre loss expression of p16, suggesting a successful deletion of Cdkn2a. These results encouraged us to proceed for in vivo work.

![Diagram](image)

**Figure 17 Cdkn2a deletion in IdhR132H/p53-/- NSC.** (a) NSC were extracted from new-born Cas9<sup>het</sup>;Idh<sup>R132HloxP</sup>;p53<sup>loxP</sup> mice and (b) infected with lentivirus expressing sgCdkn2a and Adeno-Cre to induce recombination, resulting in a Cas9<sup>het</sup>;Idh<sup>R132Hdel</sup>;p53<sup>del</sup>;Cdkn2a<sup>del</sup> cell line.

### 3.2.5 Effects of Atrx and Cdkn2a deletion on NSC proliferation

ATRX and CDKN2A have been described as having opposite effects on tumour proliferation and survival in humans. ATRX role in tumour progression is still unclear, but some groups have associated the ATRX mutation with slower progression and longer survival. On the other hand, CDKN2A is a well-established mutation in common mutation in many high-grade gliomas and other tumour types. In the context of IDH mutant astrocytomas it mandates grading as CNS WHO grade 4, according to the 2021 WHO classification of CNS tumours. To assess the effect of ATRX and CDKN2A mutations independently, NSC were transfected with lentivirally expressed sgRNAs targeting ATRX and CDKN2A, respectively, and after selection with an antibiotic and validating the knock-out by IHC, as demonstrated previously, 7500 cells were seeded in a 12-well plate, and imaged using an in vivo-cell detection device (Essen IncuCyte), every 12h for 12 days (Figure 18). Results show that cells all cells acquire growth advantage after Cre Recombination (i.e., when expressing IDH and p53 mutations) compared with naïve cells. Cells
transduced with the virus targeting CDKN2A seem to increase proliferation rates, but sgATRX decreased the multiplication speed of the cells.

These results agree with the latest WHO classification, in that CDKN2A deletion is associated with rapidly progressing, high-grade tumours.

Figure 18 Effect of ATRX and CDKN2A deletion in NSC proliferation. Cre-mediated recombination, with loss of p53 function and expression of mutant IDH (R132H) increases in the proliferation of NSC. After CRISPR-Cas9-mediated deletion of ATRX, proliferation decreased, whilst proliferation increased in cell lines treated with sgRNAs targeting (inactivating) CDKN2A.

3.2.6 Validation of Atrx, Cdkn2a and Cdkn2b deletion in mouse NSC

The individual KO of ATRX and CDKN2a was achieved by transduction of NSC with a lentivirus expressing a guide targeting each gene. However, this approach had limitation in that only one gene could be targeted at time. To achieve the KO of several genes at the same time, we designed plasmids expressing one, two or three sgRNAs, in different combinations, to screen for the best approach to achieve a triple KO in NSC, i.e., Atrx, Cdkn2a and Cdkn2b.

NSC established from Idh1^{R132H/+};p53^{loxP/loxP};Cas9^{+/−} mice were infected with lentivirus expressing different constructs (Figure 19 A): (numbers in the list correspond to the numbers in the figure).

1. Control (Empty vector);
3. pLV-U6>sgATRX-U6>sgCDKN2A-CMV>Cre
4. **pLV-U6>sgATRX-CMV>Cre:T2A:hPDGFB:P2A:Luciferase.**

72h after transfection, cells were plated on coverslips, fixed with PFA and stained for ATRX, p16, p15 and IDH\textsuperscript{R132H}. (B) ATRX was expressed in nuclei of control cells and decreased by 40-50% when transfected with any one of the plasmids. P16 expression also decreased 60-80% after infection with plasmids that express the validated sgCDKN2A (#2, #3) compared to control (#1). Finally, the expression of mutant IDH\textsuperscript{R132H} was quantified to assess Cre recombination efficiency. None of the control cells expressed the IDH mutation, but all the other cells (#2, #3, #4) expressed IDH\textsuperscript{R132H}, at various levels. P15 (CDKN2B) could not be determined successfully at this point.

These results suggested that Atrx and Cdkn2a could be mutually KO using this approach, however, it should be noted that the caveat of these plasmids is the absence of a selection marker, i.e., the overall effect on protein expression is dependent on the transfection efficiency.

**Figure 19** Atrx and Cdkn2a deletion in IDH\textsuperscript{R132H}/P53\textsuperscript{−/−}/Cas9\textsuperscript{+/−} NSC. (A) NSC were extracted from newborn Cas9het;IdhR132H loxP;p53loxp mice and infected with lentivirus expressing 1: Control (Empty vector); 2: LV-ATRX-CDKN2A/Cre-PDGFB-Luc; 3: LV-ATRX-CDKN2A-Cre and 4: LV-ATRX-Cre-PDGFB-Luc. (B) ATRX expression decreased in cells infected by plasmids 2, 3 (~40%) and 4 (50%), compared with controls in (1). p16 expression decreased in cells infected by plasmid 2 (80%) and 3 (50%). None of the control cells (1) expressed IdhR132H but all cells infected by the plasmids 2, 3 and 4 expressed the mutant Idh, confirming Cre Recombination (50-90%).
3.2.7  *Ex vivo, in vitro* lineage tracing approach to identify combinations of mutations

The delivery and transfection efficiency of the lentivirus vectors *in vitro* was very high, however, the absence of an antibiotic resistance construct in these plasmids precluded isolation and enrichment of cells by multiple, and consecutive antibiotic selection rounds. As a result, it was not possible with this strategy to establish cell lines with Atrx, Cdkn2a and Cdkn2b deletions, or combinations of double and/or triple deletions. To circumvent this limitation, each sgRNA was combined with a fluorophore, encoded in a lentiviral plasmid. This should allow us to sort the cells expressing specific fluorophores, and then use these cell lines for further studies. NSC established from *Idh1*^{R132H/+};*p53*^{loxP/loxP};*Cas9+/-* mice were infected with lentivirus expressing different constructs, combining a guide sequence targeting a tumour suppressor gene and a cassette expressing a fluorophore (Figure 20):

A: pLV-U6>sgATRX-Ef1>mOrange;

B: pLV-U6>sgCDKN2A-Ef1>BFP2;

C: pLV-U6>sgCDKN2B-Ef1>eGFP.

72h after infection, fluorophore-expressing cells were confirmed by flow cytometry and cells were plated in coverslips, fixed with PFA and stained for ATRX, p16 and GFP. (#A) Cells infected with plasmid A showed mOrange expression and ATRX expression decreased, comparing with naïve controls; (#B) transduction with plasmid B resulted in cells expressing the blue fluorophore BPF2 and decreased IHC staining for p16 for these cells; (#C) after infection with plasmid C, an increased GFP expression was detected by flow cytometry and IHC staining. P15 expression was not tested at this point.

![Figure 20 Atrx, Cdkn2a and Cdkn2b deletion in IDH^{R132H}/P53^{+/-}Cas9^{+/-}: NSC combined with fluorophores](image)

Figure 20 Atrx, Cdkn2a and Cdkn2b deletion in IDH^{R132H}/P53^{+/-}Cas9^{+/-}: NSC combined with fluorophores: cells transfected with plasmid expressing a guide targeting (A) ATRX and a cassette expressing
mOrange were confirmed by flow cytometry for expression of the orange fluorophore (middle panel) and IHC for decrease of ATRX expression (bottom panel); (B) Cdkn2a and BFP2 cassette expressed the blue fluorophore by flow cytometry and showed decreased p16 expression, tested by IHC; (C) Cdkn2B and eGFP reported cells expressing GFP both by flow cytometry and IHC.

After titration of the virus concentration, all 3 plasmids were combined and simultaneously transfected into NSCs. One week after transfection, cells were sorted for different combinations of single, double, and triple--positives, depending on the number of fluorophores expressed, resulting in eight different lines, with different combinations of mutations (Figure 21).

Figure 20 shows a pilot experiment to confirm the fluorophore expression by transduced cells, detected by flow cytometry and a preliminary immunofluorescence (IF) shows the protein expression before and after transfection (pre-sorting). These populations were then sorted by colour combination before re-testing protein expression by IF. However, these cells did not recover from FACS sorting and we were unable to test the different population for ATRX, CDKN2A or CDKN2B KO, nor use these lines for further experiments.

Figure 21 Establishment of different cell lines combining various combinations of mutations. IDH$^{R132H}$/P53-/-/Cas9$^{L1}$ NSC were transfected with a combination of three lentiviruses combining a guide targeting a tumour suppressor gene and a fluorophore. Cells were then sorted according to the number of fluorophores expressed, resulting in 8 different lines combining different mutations.

### 3.3 Discussion

The updated WHO 2016 classification of tumours of the central nervous system for the first time combined molecular markers and histological features to provide an “integrated diagnosis.” Following the discovery of IDH mutations as driver of astrocytoma pathogenesis, multiple mouse models were created to recapitulate the pathogenicity of mutant IDH1$^{R132H}$, but none of these models faithfully recapitulated tumour initiation or growth, without the introduction of additional driver mutations. Here, we aimed to develop a model that combines CRISPR/Cas9 and Cre-Recombinase technologies to create a model with such genetic profile in
neural stem cells (NSC) in vitro, and in mouse brains in vivo. Mixed background (FVB and C57BL/6NJ) mice were genetically modified by introducing an IDH$^{R132H}$ expression cassette flanked with loxP sites (“floxed”), whilst parts of the p53 expression cassette were also floxed. Neural stem cells (NSC) were derived from new-born mice, and we established cell lines by inducing recombination by infection of these cultured cells with adenovirus expressing Cre-recombinase. As a result, such a cell line expresses mutant IDH1 (R132H) and has a partially deleted p53 locus with loss of function of the p53 oncoprotein$^{171}$.

The application of the CRISPR/Cas9 system in genetic engineering requires two components, the single guide RNA (sgRNA) and the Cas9 nuclease$^{197}$. Cas9 nuclease recognizes the protospacer adjacent motif (PAM) in the targeted region, which is adjacent to sgRNA, and creates double-strand breaks (Figure 13). Double-strand breaks are rapidly repaired by either non-homologous end-joining (NHEJ) or homology-directed repair. NHEJ-mediated DNA repair often creates short deletions, occasionally large deletions, insertions, and point mutations$^{198}$. Despite its popularity, in vivo gene editing approaches have been limited by problems with Cas9 delivery due to its large size (over 4kb for Streptococcus pyogenes, SpCas9), limited by the viral packaging capacity$^{199}$. In this project, we overcame this issue by breeding Cas9 expressing mice, which would obviate the need for viral delivery of this protein. Finally, the additional mutations were achieved by selecting sgRNAs targeting each gene mentioned before. ATRX is a large gene, and therefore we screened the best-scoring sgRNAs from different design tools and chose those with the best performance (Figure 16 B). Guides were cloned into a lentiviral plasmid and transduced into NSC, followed by antibiotic selection. After recovery, IHC and qPCR revealed an excellent KO efficiency. Cdkn2a and Cdkn2b deletion were achieved using commercially available sgRNAs, previously validated in cell lines. It is important to note that the cell lines that were transduced with lentivirus in order to inactivate/silence Atrx, Cdkn2a and Cdkn2b, were selected by resistance to antibiotics (the viral plasmids and coded for the corresponding resistance cassette). Following the selection, a population was harvested which was considered as sufficient to demonstrate inactivation/silencing of the driver genes ATRX, Cdkn2a and Cdkn2a. However, it also was thought that this approach may have caused an impact on to the functional assays that we performed with these engineered cell lines. As it is likely that more than one clone survived the antibiotic selection, it is likely that the potentially heterogenous population undergoes a shift during further propagation with a single dominant clone emerging.

The next step was to design a single plasmid expressing all sgRNAs and Cre to generate $^{ldh1^{R132H/+}};^{p53^{del}};^{Atrx^{del}};^{Cdkn2a^{del}};^{Cdkn2b^{del}}$ cell lines (in short IDH-p53-ATRX-CDKN2A-
CDKN2B). Considering that the large size of the plasmid LV-ATRX-CDKN2A/B-Cre-PDGFB-Luc could affect its transduction efficiency, we initially designed separate plasmids with different combination of elements and screened for gene knock-out after cell transduction. We achieved encouraging results by this approach, i.e., each plasmid performed as expected, but the most significant limitation of this approach was the absence of a reporter or antibiotic resistance elements that would allow us to isolate the transduced (infected) cells and generate lines specifically with all of the following mutations, \textit{Idh1^{R132H/+};p53^{del};Atrx^{del};Cdkn2a^{del};Cdkn2b^{del}}. Another limitation in assessing the efficacy of knockdown of CDKN2B was the lack of suitable markers to assess their knockdown or ablation on murine histological sections of brain tumours. p15 was tested at this time, but these proteins are not equally expressed by all cells. In fact, unlike ATRX, which is expressed by normal cells, p15 expression levels in normal tissue are very low, and this explains why the comparison of protein expression between naive and engineered cells is not ideal to assess the gene status of this protein. The human protein atlas suggests that although CDKN2B is expressed by cells of the CNS (neurones, astrocytes, and oligodendrocytes), protein expression cannot be detected in these cells. Therefore, instead of using immunohistochemical detection of nuclear protein, we aimed to determine expression by qPCR and copy number variation assays CNV (chapter 5).

We attempted to generate all the relevant combinations of mutations in NSC by cloning each sgRNA in a plasmid, whereby each plasmid expresses a different fluorescent protein, and then perform fluorescent activated cell sorting (FACS) for single, double, and triple positives. Although fluorescent cells seem to have reduced ATRX and p16 expression, these cells were not viable after sorting, and therefore we could not proceed with this approach. This coincided with another experimental limitation, in that IDH-mutant cells generally grow relatively slowly and cannot be passaged for longer than 14 days, due to a competitive advantage of an initially very small proportion of IDH-wildtype cells, which however gradually outgrew the IDH-mutant population in culture. Therefore, due to this limitation, we limited our efforts to further characterise these genetically modified cell lines and use the opportunity to perform in vivo studies.
4 Introducing mutations of IDH-mutant astrocytoma into mice: IDH1 (R132H), p53, ATRX and CDKN2A/B

4.1 Introduction

The main goal of this project was to establish a new mouse model that faithfully recapitulated the genetic profile described in human patients of IDH-astrocytoma, according to the new WHO 2016 classification.

To achieve this goal, we combined different gene editing techniques that would allow us to combine several genetic characteristics of IDH-mutant tumours in a single, novel mouse model.

Mouse models are currently the most valuable and common approach for disease studying, due to the genetic and physiological similarities between the species. Besides the obvious advantages of these animals, related with ease of maintenance and breeding, the development of methods for the creation of transgenic, deletion, and knock-in mice provided added impetus and powerful tools for mouse research, leading to a dramatic increase in the use of mice as model organisms.

The animals used in these experiments were bred to express Cas9, a protease capable of cleaving DNA, which can be leaded by a guide RNA to a specific site of the genome, targeting the gene of interest\(^{200}\). Moreover, these animals were engineered with loxP sites surrounding the IDH and p53 genes, which causes these genes to flip when exposed to Cre Recombinase\(^{201}\). Ultimately, combining CRISPR/Cas9 and Cre/LoxP gene editing techniques, we should be able to combine some of the mutations described in human patients of IDH-mutant astrocytoma in a single mouse model – IDH\(^{R132H}\), p53 deletion, ATRX and CDKN2A/B mutations.

4.2 Results

4.2.1 Allografting NSC into NOD-SCID mice did not result in tumorigenesis

On a first attempt to generate tumours, we have induced Idh, p53, and Atrx mutations in a Cas9-expressing cell line, established from NSC from the SVZ of new-born mice. After recombination, transduction and recovery, approximately \(1 \times 10^6\) \(\text{Idh}^{R132H};\text{p53}^{\text{del}};\text{Cas9}^{\text{het}};\text{Atrx}^{\text{del}}\) NSC were allograft into the left ventricle of adult female NOD-SCID mice (Figure 22a,b). Approximately 6 months after injection, mice were culled due to hunched posture, but slices of the brain stained with H&E revealed no tumours were initiated by the cells injected (c).
This suggested that allografting engineered NSC into the SVZ of NOD-SCID animals would not result in tumour growth, most likely due to the low proliferation rates of these cells, as well as possible low allograft success.

![Diagram](image_url)

**Figure 22 NSC allografts in NOD SCID mice.** (A) Mice were anesthetized with isoflurane and attached to the stereotactic frame. (B) A small incision was then made on the to expose the skull. The injection needle was aimed at the indicated position, and then advanced through the coronal suture. 1x10^6 cells, re-suspended in 20μl of PBS were slowly injected into the left caudoputamen (coordinates of the injection site: 1.5 mm lateral from bregma and 2 mm deep starting from the skull surface). (C) After six months, a proportion of animals started showing a hunched posture and were culled, but H&E staining of brain slices did not show any tumour formation.

4.2.2 **Adeno-associated virus (AAV) delivery of sgRNAs failed to knock out ATRX in vivo**

After achieving promising results in vitro, we used a similar strategy to achieve ATRX KO in the SVZ of new-born mice. To achieve ATRX deletion in the mouse SVZ, and based on our previous model that generates tumours by injecting retrovirus expressing PDGFB and Cre recombinase in new-born transgenic mice (discussed in the previous chapter), we injected new-born mice carrying the conditional alleles *Idh1^R132H/+;p53^[loxP/loxP];Cas9^+/−*, with AAV expressing GFP and the best-scoring gRNA targeting ATRX (Figure 16b, ATRXsg1), together with retrovirus expressing Cre Recombinase (resulting in *Idh1^R132H* expression and p53 deletion) and PDGFB – a mitogen that is intended to accelerate cell proliferation, resulting in tumours in ~25-40 days. Mice developed signs of intracranial pressure after approximately 4 weeks, and autopsy showed development of large intrinsic tumours. However, these tumours showed no (Figure 23) no ATRX de-
letion (b, c). To further understand the absence of gene edition, we investigated GFP expression, as the AAV backbone also had an EGFP cassette (Figure 14c). GFP stained cell morphology (d) suggested that the AAV most likely infected astrocytes rather than NSC (e), with no staining in the SVZ (f).

These results suggested that AAV is unlikely to successfully transduce NSC and other types of viral plasmids should be tested.

**Figure 23: AAV failed to edit ATRX in the murine SVZ.** Mice injected with PDGFB-IRES-Cre and AAV expressing sgATRX formed tumours after 30 days (A) but ATRX deletion could not be confirmed (B, C). The pale areas within the tumour in A correspond to areas of necrosis, consistent with the overall high-grade appearances of these tumours. GFP staining (D) suggested that the cells targeted by the virus either were astrocytes, due to their morphology (E), whilst cells of the subventricular zone did not express GFP (F). It is however possible that a population of neural stem/progenitor cells during development was targeted and eventually differentiated into astrocytes still harbouring the GFP expression vector.
4.2.3 Lentiviral delivery of sgRNA resulted in a regional knock out of Atrx

As part of our explorative strategy, and given the encouraging results obtained in vitro, using lentiviral delivery of sgRNAs to delete Atrx and Cdkn2a, we injected new-born mice of the genotype Idh1<sup>R132H/+;p53<sup>loxP/loxP;Cas9<sup>+</sup></sup>/− with concentrated lentivirus targeting Atrx, mixed with PDGFB-IRES-Cre retrovirus in a 4:1 ratio. Four weeks later, animals were culled and showed large intrinsic tumours with morphological characteristics of high-grade gliomas, morphologically similar to those as described previously. Immunohistochemical staining for ATRX protein shows that the majority of the tumour expresses nuclear Atrx, but several small patches showed loss of ATRX expression (Figure 24). The limited ATRX deletion can be explained by a competitive advantage of cells that were infected by the PDGFB-IRES-Cre virus only. A possible strategy to overcome this limitation would be the generation of a single plasmid containing all sgRNAs, Cre and PDGFB, to avoid the possibility of having distinct populations of cells - infected with either both, one, and none of the plasmids. On the other hand, such approach would require a larger construct, which may have a negative effect on its efficiency. For this reason, we have designed different constructs and trialled them, individually and combined, to find best strategy for achieving homogeneous tumours combining all mutations. (Figure 14 E-I).

Figure 24 Lentiviral delivery of sgATRX to mice SVZ resulted in regional Atrx deletion. (A) 30 days after intraventricular injection of the retrovirus PDGFB-IRES-Cre and lentivirus expressing the guide targeting Atrx, tumours were formed and showed deletion of ATRX in some (B) but not all (C) regions of the tumour.

4.2.4 Combination of lentiviral and retroviral delivery of Cre Recombinase, sgRNAs and PDGFB did not result in tumorigenesis in new-born mice

To address and improve the only partial expression of sgRNA, i.e., a regional ATRX knock out only, probably caused by cells targeted by the retrovirus PDGFB-IRES-CRE being sufficient to
initiate tumorigenesis, and subsequently outgrowing Atrx-deficient cells, we designed a new set of plasmids. We separated sgRNA and Cre recombinase in one lentiviral vector and have floxed PDGFB in a second vector (retrovirus). The combination of both viruses, injected into the SVZ of Idh1<sup>R132H/+; p53loxP/loxP; Cas9<sup>+</sup> mice failed to generate tumours, even after 245 days. Different lentiviral plasmids were tested for this approach, and all failed to initiate tumorigenesis - lentiviral plasmids from Addgene containing (i) Cre Recombinase and an sgRNA targeting Cdkn2a (LV-Cdkn2A-Cre, Addgene #89644), (ii) Cre Recombinase, Cas9 and a cloning site for an sgRNA, in which we cloned the guide against Atrx that we had previously validated (LV-Cas9-ATRX-Cre, Addgene #82415) (Figure 25, Figure 26). Additionally, we obtained a plasmid expressing Cre Recombinase and cloning sites for two sgRNAs, where we cloned the validated sgRNAs targeting ATRX and CDKN2A (LV-ATRX-CDKN2A-Cre). None of these approaches were successful in initiating tumorigenesis (Table 7).

![Diagram](https://example.com/diagram.png)

**Figure 25** Different strategies for delivering Cre Recombinase, PDGFB and sgRNAs to the new-born mice brain were tested. (a) Combinations of various lentiviral and retroviral plasmids expressing floxed luciferase and PDGFB were tested. (1) a commercially available plasmid expressing Cdkn2a and Cre; (2) a commercially available lentiviral plasmid expressing Cas9, Cre Recombinase and a locus for cloning a guide RNA, where we cloned our validated guide targeting Atrx; (3). (b) A single lentiviral plasmid approach using (4) a plasmid combining guides against Atrx, Cdkn2a and Cdkn2b, Cre recombinase and PDGFB; (5) the same plasmid but with a unique guide RNA, targeting Atrx, and finally (6) the same without luciferase cassette.
### Table 8: Animals injected with each combination of plasmids and their outcome.

At least 15 mice underwent each of the strategies described above and let to live for over 210 days, or until showing neurological symptoms. None of the animals developed symptoms nor had tumours from the histology analysis. All plasmid details can be found in the Appendix of this thesis.

<table>
<thead>
<tr>
<th>#</th>
<th>Plasmid</th>
<th>Abbreviation</th>
<th>Reference</th>
<th>N&lt;sub&gt;tumours&lt;/sub&gt; / N&lt;sub&gt;Injected&lt;/sub&gt;</th>
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<td>246</td>
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<tr>
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<td>0/43</td>
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<tr>
<td>6</td>
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<td>264</td>
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Figure 26 Survival curve for animals injected with lentiviruses expressing commercially available plasmids expressing Cre Recombinase and sgRNA targeting Atrx and Cdkn2a. (1) 23 mice were injected with Addgene’s LV-CDKN2A-Cre and observed for 210 days; (2) 15 animals were injected with LV-Cas9-ATRX-Cre intraventricularly, and observed for 215 days and (3) we cloned sgRNAs in a lentiviral plasmid (LV-ATRX-CDKN2A-Cre), produced lentivirus and injected 44 mice which were observed during 264 days. None of these animals showed neurological symptoms, and after culling, histological analysis did not any reports of neurogenesis.

4.2.5 Simultaneous delivery of Cre Recombinase and sgRNAs in a single lentiviral plasmid did not generate tumours

The regional deletion of ATRX detected using a combination of lentivirus and retrovirus, together with the fact that lentivirus infects dividing and non-dividing cells, and given the promising results of in vitro experiments (Figure 19), we proceeded to inject new-born Idh1R132H/+; p53loxP/loxP;Cas9+/− with concentrated lentivirus expressing Cre Recombinase, PDGFB and a combination of three sgRNAs, targeting ATRX, CDKN2A and CDKN2B, as described before (Figure 19 A2). Modifications of this vector (Figure 19 A3, A4), injected into new-born mice, did not result in formation of subventricular perforations or expansive neoplasms, and all animals were terminated at 246 days. None of the mice in this cohort showed histological evidence of a brain tumour (Table 7).
Different combinations of lentiviruses combining Cre Recombinase, sgRNAs and PDGFB did not result in tumour formation. Three different combinations of plasmids were designed and, after successful validation in vitro, were tested in animals. (4) 61 mice were injected intraventricularly with LV-ATRX-CDKN2A/B-Cre-PDGFB-Luc, and observed for 246 days; (5) 43 new-born animals received LV-ATRX-Cre-PDGFB-Luc and were kept in observation for 246 days, and (6) LV-ATRX-Cre-PDGFB was injected into the SVZ of 42 rodents, followed by daily check-ups for 264 days. None of these animals developed neurological symptoms, and post mortem histological studies did not show any sign of tumour initiation.

4.2.6 Injection of naked plasmids, followed by electroporation, does not deliver plasmid DNA to the SVZ of new-born mice

Electroporation has been described as an effective method for plasmid DNA delivery into the mouse brain without the need to encapsulate the plasmid in a viral vector. It uses electrical fields to transiently destabilize the membrane allowing the entry of normally impermeable macromolecules into the cytoplasm. This approach potentially removes the necessity for viral encapsulation, and therefore can reduce the size of plasmid DNA that needs to be delivered. This is thought to increase success rate, i.e., its transduction efficacy. We attempted to deliver a plasmid expressing a fluorophore (LeGO-Venus, Addgene #27350, Appendix 7.7), followed by electrical pulses that should guide the DNA towards the SVZ, where it would infect NSC. Animals were culled at days 1, 3, 7 and 10 after injection, but no fluorescent signal was detected by microscopic examination. Given this outcome, and the relatively complex setup of the experimentation devices, this approach was not pursued further.

4.2.7 Retroviral delivery of Cre Recombinase, PDGFB and sgRNAs in a single plasmid resulted in tumorigenesis

Despite the proven capacity of inducing mutations in vitro, lentiviruses did not generate tumours in the experiments performed here. Therefore, we reverted to the use of retroviruses,
an approach that we had applied successfully in previous studies. We used the retroviral plasmid expressing PDGFB-IRES-CRE as a backbone to clone the guide RNAs targeting Atrx, Cdkn2a and Cdkn2b, previously validated in vitro (Chapter 3), and used it for the production of retrovirus, and injected it into new-born Idh1R132H/+;p53loxP/loxP;Cas9loxP/loxP, as described previously (RV-ATRX-CDKN2A/B-Cre, Table 7, #7, Appendix 7.5). This approach generated tumours in 77% of the animals (n=95/123), from 25 days after injection.

Coronal sections of areas containing brain tumours were prepared and immunoassayed to detect expression of mutant IDH1 (R132H), and loss of expression of ATRX. Many of the brains (95 out of 123) showed development of large intrinsic neoplasms, with a morphology characteristic of diffuse gliomas with high grade features such as frequent mitotic figures (yellow arrow), microvascular proliferation (green arrow), and necrosis (blue arrow). The location of the tumours was predominantly in the left hemisphere, as expected from the injection site into the left hemisphere, and caused significant displacement of CNS structures, such as hippocampus, and there was almost always a significant midline shift (Figure 28).

Figure 28 Injecting RV-ATRX-CDKN2A/B-Cre retrovirus into the SVZ of new-born transgenic mice resulted in tumorigenesis in nearly 80% of the animals injected. Tumours developed mostly on the left hemisphere, where the virus was injected, and showed characteristics of high-grade gliomas, such as mitotic figures, necrosis, and macrovascular proliferation.

4.2.8 An alternative retrovirus was less effective initiating tumorigenesis

The 2021 WHO classification of IDH-mutant astrocytomas classifies astrocytoma grade 2 as tumours reporting a mutant IDH, plus ATRX loss and p53 mutation. Grade 3 tumours should show additional chromosomal copy number changes and, finally, grade 4 IDH-mutant astrocy-
tomases are characterized by additional CDKN2A/B heterozygous deletion (Figure 1). We attempted to recreate the most accurate models of IDH-mutant astrocytomas, by engineering said mutations into the SVZ of transgenic mice. Another retroviral plasmid, expressing ATRX, CRE and PDGFB (RV-ATRX-Cre-PDGFB, Table 7, #9, Appendix) was also trialled and successfully resulted in tumorigenesis. Although generating tumours in some of the cases, this plasmid was significantly less efficient than RV-ATRX-CDKN2A/B-Cre-PDGFB, i.e., delivery of this plasmid resulted in tumours in only 32% of the animals injected. However, ATRX was deleted in 70% of these tumours, an only 4 did not show total deletion (9/13 tumours had total ATRX deletion) (Figure 29). Interestingly, most of these animals (88%) did not show any neurological symptoms, until being culled, 60-120 days after injections.

The lower efficacy of this approach compared to the one described in the previous paragraph lead not to pursuit this over and focus on the approach that resulted in the best results, i.e., the most efficient and predictable retrovirus.
Animals injected with RV-ATRX-Cre-PDGFB. This construct had a low efficiency, with only 20% of the injected animals developing tumours. However, 69% of these tumours showed total ATRX deletion. Although some animals developed neurological symptoms (8%), the majority did not show any signs of disease, even 120 days after injection. The scale bar corresponds to 2mm on the upper panel, and 100um on the lower panel.
4.2.9 PDGFB is necessary for tumorigenesis

To establish if the presence of PDGFB alone is capable of transforming stem or progenitor cells, we performed control experiments where we intracerebrally injected non—transgenic control mice with a retrovirus expressing PDGFB-IRES-CRE. Although none of these animals (n=6) demonstrated neurological symptoms during an observation time of up to 80 days, histology analysis of these brains revealed that two of these animals had developed brain tumours. We also tested if the presence of PDGFB is required for the transformation of stem or progenitor cells in Idh1<sup>R132H/+</sup>- p53<sup>loxP/loxP;</sup>Cas9<sup>+/−</sup> animals. To this end, we generated vector modified from that described in section 4.2.7, but without PDGFB (RV-ATRX-CDKN2A/B-Cre, Table 7, #8, Appendix 7.10). These animals (n=22) were observed for up to 160 days, never developed neurological symptoms, and autopsy with histological examination of the brains showed no anatomical abnormalities, and in particular, no brain tumours.

We conclude that PDGFB is necessary for tumorigenesis and its presence, alone, can be sufficient to initiate tumour growth.
4.2.10 Brain tumours display an heterogenous expression of Idh\textsuperscript{R132H} and ATRX

Next, we proceeded to analyse the effects of the retrovirus containing the construct RV-ATRX-CDKN2A/B-Cre-PDGFB (Table 7, #7, Appendix 7.5) in Idh\textsuperscript{1R132H/+};p53\textsuperscript{loxP/loxP};Cas9\textsuperscript{loxP/lox} mice. 125 mice were injected at age P0-P1. These mice developed neurological symptoms, specifically hunched posture, and external signs of hydrocephalus (bulked head) and were culled by exposure to CO\textsubscript{2}. The brain was harvested, cut into thick coronal slices, and processed into paraffin wax, as described before (paragraph 2.13).

In animals expressing conditional alleles for mutant IDH1, immunostaining for mutant IDH1 (R132H) showed widespread, consistent expression of the mutant protein. However, we identified variability in the efficacy of gene editing with the ATRX sgRNA (Figure 1), (A) with some areas retaining ATRX expression (i.e., gene editing was not effective), (B) whilst other areas showing distinct loss of ATRX expression (successful gene editing). (C) Some tumours showed mixed populations of positive and negative ATRX-expressing cells and others, yet (D) showed some areas with regional ATRX deletion next to areas where ATRX was expressed by every cell.

These results shed light into a caveat of this model, in which the ATRX KO cannot be predicted every time and must be evaluated, individually, after the animal is culled and tumour is dissected.
Figure 30 Tumour histology revealed a heterogeneous expression of ATRX. (A) Some samples had no apparent ATRX knockdown; (B) others had a clear ATRX knockdown whilst expressing mutant IDH1 (R132H). (C) Others revealed a mixed population of positive and negative cells for ATRX; (D) shows areas with ATRX knockdown (red arrow) and others without (blue arrow). The scale bar corresponds to 250µm.

4.2.11 Cell lines established from fresh tumours show phenotypes corresponding to those in the tumour tissue

In addition to the histological characterisation of the cell populations in the brain tumours generated by injection of RV-ATRX-CDKN2A/B-Cre-PDGFB (Table 7 #7, Appendix 7.5), cells were derived from in vivo generate tumours for further characterisation in vitro. This was of particular importance after observations that the ATRX KO was not heterogeneous throughout ever
tumour generated by our approach, as described in the previous paragraph. Brains were harvested fresh, and tumours were identified under a dissection microscope, extracted, and dissociated for propagation in vitro (as described on paragraph 2.18). After propagation (3-5 passages), cells were stained for the expression of nuclear ATRX protein and visualised/detected by immunofluorescence. Assessment by fluorescent microscopy confirmed a heterogenous population of ATRX positive (retained) and ATRX negative (“knock out”) tumour cells. Subsequently, this was further validated by flow cytometry analysis, which shows, corresponding to tissue sections, a population of ATRX positive and negative cells in most instance. Figure 31 shows examples of tissue histology and flow cytometry analysis of the corresponding tumour cell lines. Cells not expressing ATRX were gated (P3) from assay controls, for the purpose of showing the extent of gene editing and consequential loss of function in these cell cultures, i.e., P3 represents the fraction of cells that lost ATRX expression. Figure 31 A, B shows histology of an ATRX-retained tumour, and accordingly, Figure 31 C and D, flow cytometry of these cells showed that only approximately 20% of all cells lost expression of ATRX (P3). Figure 31 E,F show a tumour where almost every cell has lost expression of ATRX, with (G,H) the corresponding cell line showing almost 80% of cells with no ATRX signal (P3). Although most of the cell lines followed this trend, there were a few instances where no matching profile was established between histology (Figure 31 I, J) and cell line (Figure 31 K, L). Such discrepancy can be explained by intra-tumoral heterogeneity, as the histological sections were obtained from a different area than the samples derived fresh cell isolation and propagation.

These results showed a positive correlation between the profile of the cell lines established from the tumours and the histology of the remaining fixed tissue fixed tissue, in most of the cases.
Figure 31 Tumour tissue and corresponding flow cytometry of tumour-derived cultured cells. Cell cultures (up to 5 passages) were established from tumours and profiled by flow cytometry for ATRX expression. The majority of dissected brain tumours show a corresponding expression of ATRX in the histological section and the flow cytometry analysis (n=9/12) (A-H), but occasionally there were discrepancies, as shown in (I-L). (A,B) Histology shows a mixed population with ATRX retained expression in the majority cells and only a minority of cells showing loss of expression, corresponding to (C,D) 22% of cells not expressing ATRX shown by flow cytometry. (E,F) Tumour cells with lost ATRX expression. This aligns well with the corresponding cultured cells which show by (G,H) flow cytometry 66% loss of expression. (I,J) histology showing only rare cells with loss of ATRX expression, however the corresponding cultured cell population shows approximately 50% loss of expression (K,L). The scale bar corresponds to 1mm on the first images of each row, and 50um on the second set of histology images.
4.2.12 Brain tumours show similarities to human astrocytomas

The model system, using a combination of Cre-mediated activation of IDH1\textsuperscript{R132H} and inactivation of parts of the p53 gene, and sgRNA-mediated knock out of ATRX generates brain tumours that resemble morphologically and genetically human IDH-mutant astrocytomas with high-grade features, i.e., corresponding to CNS WHO grade 3 and 4. Specifically, these tumours express mutant IDH1 protein, and have mutations in p53 and ATRX. Despite these compelling similarities, there are however a few caveats. (i) the activation of IDH1\textsuperscript{R132H} and the inactivation of ATRX is not always achieved in all tumour cells. These tumours were selected and profiled, to assess to what extent they may still resemble the human counterparts. ATRX and IDH1\textsuperscript{R132H} status were assessed by IHC (Figure 32). We selected tumours with (M-P) complete Atrx loss, in the context of an Idh R132H mutation or (E-H) an IDH-wildtype background; (I-L) no ATRX deletion, in IDH-mutant or (A-D) in IDH-wildtype background.

<table>
<thead>
<tr>
<th>H&amp;E</th>
<th>IDH\textsuperscript{R132H}</th>
<th>ATRX</th>
<th>GFP</th>
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<td>A</td>
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Figure 32 The mouse model generated tumour with different genetic combinations. We were able to identify 4 different groups depending on IDH and ATRX status: (A-D) IDH wildtype, ATRX loss, (E-H) IDH-mutant, ATRX loss; (I-L) IDH-mutant, ATRX loss and (M-P) IDH wildtype, ATRX retained. The scale bar corresponds to 100µm.
4.2.13 Tumours with ATRX loss showed matched CDKN2A loss

The clinical importance of CDKN2A homozygous deletion, in the context of IDH-mutant astrocytomas was first highlighted in a large, multicentre study\textsuperscript{79}, followed by diagnostic recommendations\textsuperscript{81} and is now formalised in the 2021 CNS WHO classification\textsuperscript{205}. This evidence indicates that CDKN2A/B is an important prognostic marker (specifically in the context of IDH-mutant astrocytomas), with impact on survival in patients: the presence of the homozygous deletion usually results in faster progression and worse outcome. Therefore, a key experimental objective was the creation of a construct to model the human disease. The retrovirus used for this mouse model expressed a construct with a sgRNA targeting CDKN2A, previously validated, \textit{in vitro} (Paragraph 3.2.3), with a similar promoter (U6). Western blot analysis for protein expression was performed and determined that ATRX and p16 protein expression were similar in all tumours tested, i.e., all tumours showing loss of ATRX expression, also showed Cdkn2a deletion, and p16 was expressed in all tumours in which Atrx was intact, i.e., showed nuclear expression in corresponding tumours. This was further confirmed at mRNA level, using RNA extracted from FFPE slides from tumours with ATRX deleted or retained. The expression of ATRX and CDKN2A in these samples was assessed by qPCR, showing that all samples that reported ATRX loss (Figure 33B), also have loss CDKN2A expression (Figure 33 C).

These results conclude that the mutual deletion of ATRX and CDKN2A was successful, \textit{in vivo}. 
All tumours with ATRX deletion also showed CDKN2A deletion. (A) Cells established from all four groups of tumours were tested for IDH<sup>R132H</sup>, ATRX and p16 protein expression by Western blot, showing that ATRX and p16 expression is matched in every case. RNA extracted from tumours from these groups was used to assess the expression of these genes using qPCR. (B) ATRX loss tumours showed lower ATRX expression, compared with ATRX retained samples (p=0.003); (C) and the same samples also showed lower CDKN2A, when compared with the ATRX retained samples group (p=0.0035).

<table>
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<tr>
<th>Tumour profile</th>
<th>IDH&lt;sup&gt;R132H&lt;/sup&gt;</th>
<th>ATRX KO</th>
<th>ATRX</th>
<th>p16</th>
<th>Tubulin</th>
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<td>IDHR132H</td>
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<td>Tubulin</td>
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**Figure 33**
4.2.14 Successful homozygous deletion of CDKN2B in vitro but not in vivo

CDKN2A/B homozygous deletion is a prognostic (as well as diagnostic, in terms of grading) marker in IDH-mutant astrocytomas, i.e., the presence of a CDKN2A/B deletion mandates grading as CNS WHO grade 4 IDH-mutant astrocytoma. CDKN2A deletions in human cancers (including IDH-mutant astrocytomas) are commonly associated with CDKN2B deletion, and therefore we aimed to model this in murine tumours. To this end, we used commercially available sgRNAs targeting these genes and included them into the plasmid. CDKN2A deletion was successful in tumours that also showed ATRX deletion, as shown by Western blot and qPCR (see paragraph 4.2.13 “Tumours with ATRX loss showed matched CDKN2A loss”, page 79). According to the Human Protein Atlas, CDKN2B is expressed in the brain, but protein expression cannot be detected on this tissue. We also attempted to detect the deletion of CDKN2B in vitro by IHC, but realised that there was only a weak signal, with a low signal-to-noise ratio, and also in the brain, expression was very limited, compared to the expression seen in tumour cell lines (Figure 34A). Figure 34 B shows that RNA extracted from tumour cell lines showed varied expression of CDKN2B, which also did not match CDKN2A or ATRX expression in the same lines, suggesting that CDKN2B gene targeting construct was unsuccessful and did not result in a successful deletion. Figure 34C shows the amplification plot of a qPCR from RNA extracted from FFPE tumour tissue of ATRX retained and ATRX deleted tumours and of normal brain, where the red arrow points at the curves resulting from the normal brain samples. The signal for these samples was detected at cycles 28-32, i.e., significantly higher than the tumour samples, indicating that CDKN2B expression in normal brain was significantly lower (i.e., requiring many more PCR cycles to create a signal) than tumour samples. C\textsubscript{T} values also show no differences of CDKN2B expression between ATRX retained and ATRX deleted tumours, suggesting that the attempted CDKN2B deletion using CRIPSR/Cas9 was not successful.

These results suggest that, despite our efforts and the promising results in vitro, in vivo deletion of CDKN2B was no successful.
Figure 34 CDKN2B expression does not match ATRX or CDKN2A expression in cell lines derived from mouse tumours. Cell lines established from tumours with retained ATRX (n=1) or with ATRX loss (n=3) were established and investigated for CDKN2B status. (A) Immunocytochemical staining of p15 protein in tumour cell lines yielded inconclusive results. Normal NSC showed already a low expression of p15 compared with tumour cell lines, with high non-specific labelling (low signal-to-noise ratio) making results difficult to interpret. (B) mRNA expression of these genes was tested by qPCR. Results show that although ATRX and CDKN2A reported similar expression across different lines, CDKN2B expression varied between different lines, suggesting that CDKN2B deletion was not successful. (C) the amplification plot of a quantitative PCR from FFPE tissue from normal brain (n=1), ATRX loss (n=9) and ATRX retained (n=7) tumours revealed that higher cycle number were necessary for CDKN2B probe to generate signal for the normal brain samples (red arrow) compared with all the tumour samples. (D) shows that no differences between both tumour groups, regarding CDKN2B expression, suggesting that CDKN2B deletion was not successful.
4.2.15 Tumour proliferation and apoptosis

As previously described, the mouse model generated with our experimental approach resulted in tumours with four distinct genetic profiles. Despite representing a limitation to the model for investigating drug efficacies, or validating biomarker candidates, this model allowed us to identify and to compare four different groups of tumours, and to investigate the impact of the ATRX deletion in tumour initiation, progression, and apoptosis. Representative tumours for each group previously described were analysed histologically for proliferation (ki67 antigen, and phosphorylated histone H3 (pH3), as well as the apoptosis marker cleaved Caspase 3. There were no significant differences in proliferation nor apoptosis among the four groups (Figure 35).

Figure 35 Tumour proliferation and apoptosis. Tumours from each group (1-4) were identified per its mutational profile (N=4). FFPE samples were stained for the cell cycle marker ki67 and the apoptosis marker Caspase 3. There were no differences in proliferation or apoptosis between the four groups. The scale bar corresponds to 100μm.
4.2.16 Expression of glial lineage markers in experimental brain tumours

The main characteristic of that makes astrocytomas difficult to resect is their diffusely infiltrative growth pattern with an apparently seamless transition towards the normal brain. This may represent an obstacle when defining which cells are neoplastic and which ones are normal and clearly define the region of the tumours, however with a mutant protein detectable with imitation specific antibody (Ab H07, directed against IDH1 R132H), this difficulty can easily be overcome. However, the more detailed characterisation of neural and glial standards and differentiation markers could be influenced/modulated by the presence of mutant IDH1 protein or deleted ATRX protein. Oligodendrocyte transcription factor (Olig 2)\textsuperscript{116} and Sex-determining region Y-box 2 (Sox 2)\textsuperscript{206} are expressed in most diffuse gliomas, and indeed, tumours generated in our model expressed these two glial lineage markers (Figure 36A-H). Microtubule-Associated Protein 2 (MAP 2) is a protein that stabilizes microtubules in the dendrites of postmitotic neurons, and it is also expressed in many glial tumours\textsuperscript{207}, but the tumours generated here expressed no MAP2, (I-L). Finally, Glial Fibrillary Acidic Protein (GFAP), an intermediate filament that provides mechanical support to cells, and detected in astrocytes and some types of neural stem cells (amongst other types of cells), is a marker of astrocytoma (as well as other gliomas) both in clinical and experimental settings, used to determine glial differentiation\textsuperscript{208}. In our study, only), IDH wildtype samples did not express GFAP (D,H), whilst the IDH-mutant tumours expressed this protein (M,N).
Figure 36 Glial marker expression in the experimental tumours. Representative tumours from each group were stained for well-described glial markers: (A-D) SOX2 and (E-H) Olig2 were expressed by all tumour genotypes, whilst (I-L) MAP2 was negative. (M, N, O, P) The differences in GFAP expression are most likely not related to the different IDH status, but more likely explained by a different degree of tumour cell infiltration and overgrowth of underlying CNS tissue, which contains also reactive astrocytes. (L, P). The scale bar corresponds to 100µm.
4.2.17 IDH-mutant tumour cell lines showed increased levels of D2HG

The production of the oncometabolite D2HG is a well-described and characterised effect caused by the mutation in the IDH1 gene (codon 132) and the IDH2 gene (codon 172), as described in paragraph 1.3.1, page 15. To confirm that the IDH mutation induced in these tumours was resulting in the same the human counterparts, D2HG levels were determined in cell lines established from several tumours (n=4) (Abcam, ab211070). In this assay, D2HG is oxidized to a α-ketoglutarate in the presence of the IDH-mutant enzyme, whilst the intermediate reduces a probe to a coloured product with absorbance at 450 nm.

As previously shown, IDH-mutant tumours generated in this model showed production of high levels of D2HG whilst not seen in IDH wildtype tumours.

![D2HG levels](image)

**Figure 37** IDH-mutant, but not IDH-wildtype tumour cells produce the oncometabolite D2HG. Cells established from tumour cell lines expressing IDH-mutant (IDH Mut) or IDH wildtype (IDH WT) (n=4) were measured by an enzymatic assay measuring D2HG. IDH-mutant tumour cell lines showed increased levels of D1HG, compared with IDH-wildtype samples.
4.2.18 ATRX deletion is not associated with tumour growth and survival

The role of ATRX in tumour growth or prognosis is still debatable. A total of 123 Idh1R132H/p53loxP/loxP;Cas9loxP/loxP animals were injected with RV-ATRX-CDKN2A/B-Cre-PDGFB (Figure 38, Table 7, #7, Appendix 7.5). Of these, 20 never developed any neurological symptoms and were terminated after 200 days, by exposure to CO₂. Brains were analysed histologically and showed no evidence of tumour formation, or any other abnormalities, in particular in the region of the subventricular zone. The remaining 113 mice developed neurological symptoms, such as hunched posture and domed head, and histological examination confirmed intrinsic tumours with features of high-grade gliomas in 95 mice, whilst 10 developed symptoms but no tumours were detected histologically, but other finding explaining neurological signs, such as hydrocephalus, were found. When comparing the survival data from each group of animals, the loss of ATRX did not appear influence survival of these animals, both in the context of Idh wildtype (A) or Idh-mutant (B) tumour genotype. In fact, survival was not different between groups, even when comparing IDH wildtype and Idh-mutant, regardless of the ATRX status (C), or vice versa, i.e., ATRX retained vs. ATRX loss (D).

Our results suggest that the deletion of ATRX did not have a significant impact in animal survival.

Figure 38 Survival analysis of the impact of the different combinations of mutations. (A) ATRX loss did not have an impact on the survival of IDH-wildtype animals, (B) nor IDH-mut. (C) When evaluating the survival data of IDH-wildtype vs IDH-mut animals, without considering the ATRX status, there were no differences between groups. (D) ATRX loss did not affect survival time, even when the IDH mutation status was not considered.
4.2.19 Distinct methylation profiles were revealed between the tumour samples and normal brain

Although the exact role of the oncometabolite produced by the mutant IDH enzyme, catalysed from α-ketoglutarate, is still unclear, it is believed that said D2HG accumulation leads to hypermethylation of the DNA. To establish if the introduction of an IDH mutation and an ATRX deletion in our model system had similar epigenetic effects as observed in human tumours, we performed Illumina mouse methylation arrays on the 4 genotypes of tumours generated here. DNA was extracted from 6 samples of each group and analysed on an Illumina Mouse Methylation Beadchip (Figure 39). (A) Overall, all tumour samples showed a distinct methylation profile, compared to normal brain that was used as control. (B) however, the global DNA methylation of all brain tumours was reduced compared to brain control samples, and (C) clustering using the optimal probes grouped all samples from each arm together (except for 1 outlier). Comparison of individual tumour genotypes instead shows no statistically significant differences, i.e., the distinct clusters in the tSNE plot that can be observed could be explained by overfitting. (D).

Although the difference between normal brain and tumour is clear, no significant methylation patterns were seen between groups.
Figure 39 Analysis of the methylation profile of the different groups of tumours. DNA extracted from FFPE samples of each group was analysed in Illumina Mouse Beadchip. (A) Overall, all tumours showed...
lower global methylation, compared with controls. (B) The T-SNE graph shows how tumour groups cluster well together and are very distinct from control samples. (C) Heatmap showing methylation differences between each group and the controls. (D) When compared against each other (without controls), the groups cluster well, but no significant differences seem to be attributed to the mutational profile.

### 4.2.20 Tumours generated in our model system have flat DNA copy number profiles

Copy number variation (CNV) refers to an alteration (gain or loss) of a specific segment of DNA compared to a control sample. It is generally accepted that somatic CNV is highly associated with the development and progression of numerous cancers by impacting gene expression level\(^{209-211}\). To determine whether the induced mutations led to chromosomal alterations, we built CNV profiles, comparing, for each group of tumours, the number of copies of each gene to those of controls, for which we used normal whole mouse forebrain (Figure 40). Copy number profiles were obtained from all tumour samples. In most instances, the copy number profile was flat, i.e., no gains or losses of chromosomal material. A small proportion of tumours however showed gains and losses of whole chromosomes, such as chromosome 1 and 12 (A), or chromosome 5 and 11 (D) No recurrent, or consistent pattern of chromosomal abnormalities was identified across the 24 tumour samples derived from (A) IDH-wildtype/ATRX retained (n=6), (B) IDH-wildtype/ATRX loss (n=6), (C) IDH-mutant/ATRX retained (n=6), or (D) IDH-mutant/ATRX loss (n=6) tumours. The last panels show examples of the CNV of human IDH-mutant astrocytoma samples (F), with abundant chromosomal gains and losses compared to low-grade (G), which typically show flat profiles, similar to the ones generated by our mouse model samples.

Although showing clear histological characteristics of high-grade, CNV profiles of the tumours generated by our model did not resemble the profiles of patients with high-grade neoplasm, typically noisy, with several chromosomal loses and gains. Instead, the tumours generated by our group revealed flat CNV profiles.
Figure 40 Copy number variation profiles generated from samples from each tumour type (N=6). CNV profiles were generated by comparing DNA copy numbers with the sample controls for all tumours from each group: (A) IDH-wildtype, ATRX Retained, (B) IDH-wildtype, ATRX Loss, (C) IDH-mutant, ATRX Retained (D) IDH-Mutant, ATRX Loss. Unlike the copy number profile in a human IDH-mutant astrocytoma with histological high-grade features (E), the CNV profiles for experimentally induced IDH-mutant tumours are largely flat and are more akin to those seen in human low grade IDH-mutant astrocytomas (F).

4.2.21 Recombination of the p53 gene is detected in the copy number profiles of all tumours

The tumours in this model are generated by a combination of two genome engineering techniques: CRISPR/Cas9, which causes deletion of ATRX and of CDKN2A/B, and the Cre/lox system, which results in the expression of IDH (R132H) and a deletion of parts of the p53 gene. The latter is achieved by introducing LoxP sites on distinct sites of the p53 gene, flanking exons 2 and 10 (Figure 41A). Exposure to Cre recombinase deletes the genomic material between these lox sites, causing loss of function of this tumour suppressor gene\textsuperscript{212}. To confirm that we can detect
loss of genetic material corresponding to the region between exon 2 and exon 10, we identified the probes located in this region. 5 probes are located in this region, and the copy number plot (B) confirms loss of genetic material corresponding to the region spanning exons 2-10. This confirms the deletion of p53 by Cre recombination.

Figure 41 CNV profile of each probe targeting Trp53. (A) Schematic of the strategy for p53 deletion in the mouse model. LoxP sites were inserted flanking exons 2-10, resulting in the deletion of this DNA fraction, upon Cre Recombination. (B) Tumours of all genotypes (each genotype n=6) show loss of gene copies at the probes targeting the gene, corresponding to Exons 2-10, when compared with control samples, i.e., the gene deletion caused by Cre Recombination. This can be considered as an independent confirmation of the functional inactivation of the p53 gene, consistent with previous experiments using PCR primer combinations173.
4.2.22 CRISPR-induced mutations are below detection limits of the methylation array probes

The gene deletions obtained by CRISPR technology results from random INDELs introduced at the targeted loci, as result of the natural mechanism of DNA repair, after the double strand cleavage of the DNA by Cas9, which is guided to the target regions by sgRNAs. Cas9 usually binds to the PAM region and cleaves the DNA 3bp after this region. This provides us with an accurate picture of the location of these insertions and/or deletions, and therefore we could identify probes in the vicinity of this region to establish if these Mutations may have resulted in distinct, small losses or gains of genetic material. Even though we identified occasional losses or gains of genetic material specifically in these regions, the loci that were targeted by the nuclease were not covered by the probes on the array. We conclude that the genetic lesions introduced by the CRISPR Cas9 technique are too subtle to be detectable on the specific mouse methylation array due to insufficient coverage of the CpG sites.
Figure 42 CNV profiles for each probe in ATRX, CDKN2A and CDKN2B. Each panel represents the loss or gain of DNA, resulting from the signal intensity difference between each probe that aligned with these genes, for the samples of each group, compared with control. Although random chromosomal loss (red)/gain (blue) were detected, the mutations induced by CRISPR are not detectable due to the lack of probes covering the loci where Cas9 induced the DSB.

4.3 Discussion

Animal models of diseases play a key role in basic and translational research. Mice are the preferred model organism to study human biology due to their often-high degree of genetic homologies with humans, their small size, short reproduction cycle and ease of propagating them with useful litter sizes, and comparatively modest maintenance efforts. A useful experimental model is characterized by consistent and robust features and the recapitulation of either distinct, single traits, or even multiple aspects of human disease.

Three types of mouse models are commonly used for the study of CNS tumours, to investigate disease initiation, diagnostic and treatment studies: (i) xenografts, achieved by the implantation of tumour cells, derived from patients’ biopsies (patient-derived xenografts, PDX); (ii) allografts, where implanted cells have been genetically altered in vitro; or (iii) genetically engineered mouse models (GEMM) where tumorigenesis is induced in the animal by disrupting tumour suppressor genes and/or inducing oncogenes.

Each mouse model has its advantages and limitations. PDX models are widely used as preclinical cancer models and are considered better than cell culture models in recapitulating the hist-
tological features, molecular characteristics and intra-tumoral heterogeneity of human tumours. Arguably, the human disease itself (i.e., the tumour cells) can be best modelled with this approach, as it overcomes all the inter-species caveats that may occur in Genetically Engineered Mouse Models (GEMMs), such as gene homology and different drug tolerance across species. However, there are several limitations of PDX models, such as highly variable engraftment rate, absence of immune pressure and often long latency periods. The latter represents a probably the most significant problem for IDH-mutant astrocytomas of lower grades (as well as for arguably all Low-grade glial or clonal tumours), as these grow slowly, a disadvantage also in a corresponding PDX model. In particular, IDH-mutant cells are slow-growing and often challenging to maintain in culture, which may explain why xenografts of these tumours are difficult to achieve.

Our attempt to graft NSC (Neural Stem Cells), previously manipulated in vitro, to express $\text{Idh1}^{R132H};\text{p53}^{\text{del}};\text{Cas9}^{\text{het}};\text{Atrx}^{\text{del}}$ failed to generate tumours, probably due to the long latency of these tumours to develop and the low rate of engraftment success (Figure 22). Others have described that the CDKN2A/B homozygous deletions accelerate the progression of these tumours, increasing the proliferation rate, therefore, the inclusion of CDKN2A/B mutations in these cells could accelerate the growth of these tumours, increasing the likelihood of tumour development. However, the lentiviral approach limited the number of targets, and thus we were able to generate cell lines with one mutation only, as consecutive viral transductions of the cells, and selection cycles with different antibiotics had a detrimental effect on the cell lines, in that they showed a reduction of proliferation in vitro, due to the toxic effect of the selection media. An alternative attempt, combining the different sgRNAs with fluorescent proteins, should have resulted in single, double, and triple knockout cells, but these cells were not viable after sorting by flow cytometry (Figure 21). For these reasons, we decided to focus our efforts on inducing mutations in the mouse SVZ (subventricular zone) to characterise these neoplasms and compare the tumours with human counterparts.

GEMM are invaluable tools to model human diseases, including human cancers. Unlike the transplantation models using patient-derived xenografts, GEMMs can be developed in immunocompetent mice, recreating both the cell-intrinsic factors as well as tumour microenvironment, which is considered key to a “holistic” approach to studying initiation, progression, and therapeutic response of brain tumours, thus providing more accurate insight in the molecular biology of cancer as well as providing valuable platforms for biomarker and drug discovery and validation. Other advantages of GEMM include the option to introduce abnormalities in specific tissues and study tumour progression over time.
The recognition of IDH-mutant astrocytomas as a distinct biological and clinical entity sets them apart from other high-grade gliomas, most notably IDH-wildtype glioblastoma. Multiple models for IDH-wildtype glioblastoma had been generated in the past, using a broad range of genetic techniques and concepts. Instead, attempts to generate models of IDH-mutant astrocytomas had shown limitations. In particular, all models so far required additional driver mutations to generate brain tumours in an experimentally useful manner (i.e., tumour development times and tumour incidence), to be meaningful as a preclinical model. In our own previous experimental settings, we generated mice with an inducible IDH1 R132H mutation, concomitant p53 deletion, and optional additional PTEN deletion. These tumours were generated by inducing proliferation in the stem/progenitor compartment with PDGFB, a growth factor providing a strong mitogenic signal. Furthermore, IDH-mutant astrocytomas are defined by a mutation in the ATRX gene, which usually is reflected in a loss of protein expression, i.e., can be detected by immunohistochemical staining, as well as CDKN2A and CDKN2B. These additional driver mutations were achieved with the CRISPR/Cas9 system. sgRNAs targeting ATRX and CDKN2A were cloned into a viral plasmid and validated in vitro for deletion efficiency (see Chapter 3). One of the objectives was to establish if the combination of the expression of mutant IDH1, and the loss of expression of ATRX and p53 would be sufficient to elicit brain tumours, i.e., if the previously required additional expression of PDGFB is still required to trigger tumorigenesis. A further objective was to establish the role of CDKN2A/B, i.e., to investigate whether the deletion of these genes would (i) accelerate tumorigenesis and thus reduce survival, corresponding to the scenario in human IDH-mutant astrocytomas, and (ii) reduce or obviate the need for PDGFB expression, in case this is still required for eliciting IDH/ATRX/p53 mutant tumours.

Several targeting approaches have been used to generate this model. The model needed to represent a useful compromise of an effective, consistent, and robust approach for the study of therapeutic approaches, and a combination of mutations representing, as closely as possible, the genetic profile of IDH-mutant astrocytomas.

Adding an associated virus (AAV) is a very attractive method for in vivo applications, since it allows a tissue-specific delivery of DNA, using serotypes that have a better affinity for certain tissue types. It causes only a very mild immune response and infects dividing and quiescent cells in a broad range of target tissues. In addition, AAV is the preferred method for delivery of CRISPR components in vivo in many studies. Our first attempt to develop tumours in the SVZ of new-born mice combining retrovirus PDFGB-IRES-CRE and an AAV expressing a guide...
RNA targeting ATRX did not successfully initiate neoplastic transformation of stem and progenitor cells of the CNS. The AAV construct expressed green fluorescent protein (GFP), to facilitate tracing the fate of infected cells. In the brain of mice that received AAV, we detected GFP expression only in astrocytes, but not in the stem or progenitor cells, as shown in Figure 23. It is possible however that the astrocytes shown in Figure 23E represent progenies of infected stem and progenitor cells. Overall, we conclude from the experimental outcomes that AAV is not a suitable vector for the delivery of DNA constructs into stem/progenitor cells of the central nervous system to generate intrinsic neoplasms, and alternative approaches were tested and pursued further.

The next effort to deliver constructs into this subpopulation was again a combinatorial approach. We used the PDGFB-IRES-CRE construct in a retroviral vector and injected it in combination with the lentiviral plasmid that had successfully resulted in ATRX deletion in vitro (Figure 16). Animals injected with these two lentiviral vectors simultaneously developed tumours, but only a small number showed regional ATRX deletion (Figure 24). We conclude that the use of two distinct plasmids may have targeted either two separate cell populations, or alternatively, that the PDGFB-IRES-CRE retrovirus, which was previously proven to be sufficient to initiate tumorigenesis, was sufficient on its own to elicit tumours, and that cells transduced with this construct already had a growth advantage compared to the cells transduced by the lentivirus. However, the latter still showed a functional delivery in that it created small, focal losses of ATRX expression in these brain tumours (Figure 24).

To address this shortcoming, we then re-designed the vectors so that Cre Recombinase and PDGFB were expressed by separate plasmids, and, importantly, PDGFB was flanked by lox P sites, i.e. only cells infected by the lentivirus expressing Cre and sgRNAs (Figure 25a 1-3) and the retrovirus with floxed PDGFB would benefit from the proliferation elicited PDGFB and, therefore, due to this co-dependence of both constructs, a much wider and more efficient inactivation of ATRX should be expected. Moreover, to avoid different transduction efficiency between different types of viruses and the added variability of having two different plasmids, we also included lentiviruses expressing all the elements, in a single plasmid approach (Figure 25b). We anticipated that these changes could potentially influence incubation periods and tumour growth rates, and thus included a luciferase cassette to allow us to monitor these animals, post-injection. Figure 25b, #4 shows a vector expressing all three guide RNAs (targeting ATRX, CDKN2A, and CDKN2B), Cre Recombinase and PDGFB, plus luciferase, i.e., including all elements needed to initiate tumorigenesis in a single plasmid. This resulted in a large plasmid (pLV-U6>ATRX-U6>cdkn2a-U6>cdkn2b-CMV>Cre:T2A:hPDGFB:P2A:Luciferase, size 12 kb),
which could affect the virus efficiency, therefore we decided to also design and test shorter versions of the same plasmid, with a single RNA (pLV-U6>ATRX-CMV>Cre:T2A:hPDGFB:P2A:Luciferase, Figure 25b, #5 and without luciferase (pLV-U6>ATRX-CMV>Cre:T2A:hPDGFB, Figure 25b #6). Despite the successful results from in vitro experiments, for the latter group of plasmids (Figure 19), none of these combinations resulted in tumorigenesis (Figure 26, Figure 27, Table 7).

Our group previously showed the high efficiency of retrovirus to generate tumours with this model 171. Also, although lentivirus achieved satisfactory results in vitro, this delivery method had never resulted in recombination, in vivo, unless when combined with a retrovirus. Lentiviruses have been widely used in gene editing and gene therapy 236, and were presented as a superior alternative to retroviruses, mostly due to their capacity of transducing non-dividing cells, which generally should increase the transduction rate of such viruses 237. However, our model aims to target a small number of highly mitotic NSC located in the SVZ, surrounded by non-dividing cells, such as neurones, or, other unwanted target populations, such as astrocytes as shown in Figure 23.

Retroviruses share many features with lentiviruses—both their DNA is easy to manipulate, they have an ability to easily integrate into the target genome and generate stable recombinants, have a low inflammatory response, and they have a proven experimental utility, shown by us and others to initiate tumours when infecting the neurogenic population in the subventricular zone. For these reasons, we decided to clone the vector shown in Figure 25b #4 into a retroviral backbone (pMMLV-hU6>sgATRX-mU6>sgCDKN2A-h7SK>sgCDKN2B-CMV>RCAS-PDGFB/HA:IRES:Cre) and test it, in vivo, which indeed resulted in tumours, approximately 30 days after injection. Around 90% of the animals injected, intraventricularly, with RV-ATRX-CDKN2A/B-Cre retrovirus started manifesting neurological symptoms and were culled, to find diffuse tumours in around the left ventricle frontal brain.

Amongst the different cloning strategies and resulting constructs, the most effective tumour induction was accomplished with a construct comprising the (i) PDGFB expression cassette, followed by a (ii) Cre expression cassette and (iii) sgRNAs targeting ATRX, CDKN2A and CDKN2B, resulting in a tumour induction rate of 75% within up to 60 days. However, in this model we observed a degree of heterogeneity of gene delivery and this resulted in a variable loss of function (deletion) of ATRX: ATRX deletion was not seen across all cells in the tumour—some tumours showed ATRX loss in all tumour cells, others had a partial loss, which could show as a mixed populations of ATRX positive and negative cells, or regions of ATRX loss next to regions
with retained ATRX expression. Some tumours showed no ATRX deletion at all. Several factors may have contributed to this result: Cas9 causes a double strand break to the DNA, which would then be repaired by intrinsic, physiological mechanisms, introducing unpredictable indels (“Indel” being a general term that may refer to insertion, deletion, or insertion and deletion of nucleotides in genomic DNA), that are randomly introduced at the cleavage loci. These indels may result in loss of function, but, in other cases, it may not have affected gene expression if the insertion does not result in an alteration of the genetic code. Another possible reason for the unsuccessful ATRX deletion in a subpopulation of tumour cells may be the absence of cell division at the time of transduction, i.e., since retroviruses only infect cells that are actively dividing, it is expected that only a certain number of cells will be transduced at a time.

This inconsistency represents a noteworthy caveat for this mouse model, as a key requirement of a disease model is its reproducibility and robustness. On one hand, the fact that we cannot predict the outcome of the model in every single instance of an experiment, does not allow us to use this model for some glioma studies, such as patient selection for a candidate drug. On the other hand, this model resulted in different types of tumours, with varied mutational profiles, that could be grouped and compared to each other, and also to the various human glioma types. For that purpose, we excluded all the mixed population or partly deleted tumours, and divided the remaining samples into four distinct groups, depending on their mutational profile, evaluated by histological analysis (Figure 32): (A-D) IDH-wildtype (IDH WT), ATRX retained; (E-H) IDH-wildtype and ATRX Loss; (I-L) IDH-mutant and ATRX Retained and (M-P) IDH-mutant and ATRX Loss. The selection of these samples included only tumours with either ATRX loss in all cells or retained ATRX expression in the entire tumour (n= 8-10). This allowed us to characterize and compare the different tumour types between each other and infer the human counterparts that could be represented by each group.

All groups of tumours expressed the markers that are typically encountered in glial tumours, such as SOX2 and Olig2 (Figure 36) and we were able to establish cell lines representative of each group, the profiles of which were confirmed by flow cytometry (Figure 31), qPCR and Western blot (Figure 33). These profiles usually matched the histological appearances from the tissue counterparts, with some exceptions probably caused by the proliferation of a dominant population of cells in culture, over multiple passages. A possible limitation is the separation of tissue taken for cell culture and the tumour remaining in the brain for histology which potentially can result in a discrepancy due to the intra-tumoral heterogeneity and can explain the occasional examples where we observed a discrepancy between histology and in vitro data.
Introduction of the R132H IDH mutation results in reduced proliferation and longer survival \cite{169,171}. However, the mutational profile of the tumours did not affect tumour proliferation, apoptosis markers (Figure 35), or animal survival (Figure 38). This could be explained by the effect of PDGFB, a strong driver of cell proliferation, which may override more subtle effects of other genetic lesions, such as the IDH mutation or the ATRX loss. This is further underpinned by the fact that cell lines engineered in vitro, which do not express PDGFB, and show differences in proliferation, depending on the combination of mutations engineered into the cells (Figure 18).

Therefore, in order to avoid the potentially overriding effects of PDGFB, we engineered constructs that contained mutations in the p53 gene, IDH R132H, and ATRX mutations, but contained no PDGFB expression cassette Table 8, #5). However, without the proliferation-inducing function of PDGFB, no tumours developed, probably due to a very slow turnover rate of the recombined cells.

Further plasmid that was tested and successfully generated tumours with ATRX, was RV-ATRX-Cre-PDGFB, (Table 8 #9). Of 65 animals injected with this virus, containing a plasmid with a guide targeting ATRX, but not CDKN2A, only 13 mice developed tumours, which were seen at histological examination, with 69 % of these showing total ATRX deletion (Figure 29). Interestingly, these animals were often asymptomatic, even 130 days after injection, and in keeping, histology investigation revealed only small tumours around the SVZ. The low efficacy of this plasmid compared with RV-ATRX-CDKN2A/B-Cre (20 % vs 77 %) prompted us to focus on our most effective strategy. The longer latency periods for these tumours to develop could indicate that the ATRX mutation is slowing down tumour progression, which in our model would be compensated by the co-existence of the CDKN2A mutation – characteristic of high-grade tumours - resulting in no notable differences between the groups. As this remains speculative, a proof of this assumption would require further work.

CDKN2A and CDKN2B are tumour suppressor genes associated with the regulation of the cell cycle by interacting with cyclin and kinase complexes\cite{238} and the deletion of these genes is associated with poor prognosis, specifically in the context of IDH-mutant astrocytomas\cite{80,82,83,239} (of note, CDKN2A/B deletion is a feature of many cancers, including many CNS neoplasms, for example glioblastoma, IDH-wildtype, anaplastic pleomorphic xanthoastrocytoma, or High-grade astrocytoma with piloid features, but in these tumour types the CDKN2A/B status has not been shown to be of prognostic significance). In the event of DNA damage, these cyclin-dependent kinase inhibitors expressed will ultimately affect Rb phosphorylation, resulting in
cell cycle arrest (Figure 7)24. CDKN2A is a well-recognized prognostic marker of IDH-mutant astrocytomas240. In fact, CDKN2A loss mandates grading of IDH mutant astrocytomas as CNS WHO Grade 4, according to the WHO 2021 classification of CNS gliomas205. Reis et al. investigated the role of CDKN2A in survival of glioma patients240, and associated CDKN2A loss with certain molecular subtypes of astrocytoma - IDH/P53/ATRX mutated and IDH/P53 mutated, but not on IDH/ATRX mutated tumours – but it did not have a significant impact on oligodendrogliomas or oligoastrocytomas241. However, this study has used historical annotations I was performed prior to the recognition of the molecular determinants and definitions of IDH mutant astrocytoma. Hambardzumyan et al. (2009) used the RCAS/t-va technology to model adult gliomas in mice and showed that CDKN2A deletion resulted in high-grade gliomas, with shorter survival than control groups with no such deletion242. Importantly, this model also used PDGFB to induce tumorigenesis, and reported that Ntv-a mice injected with RCAS-PDGFB developed tumours with low-grade features242.

CDKN2A deletion in patients typically occurs together with CDKN2B243. We successfully deleted CDKN2A, but not CDKN2B, in our model (Figure 34). Although there is limited information in the literature about the impact of CDKN2B loss in glioma, these genes have similar functions and may compensate for each other’s loss244, indicating that loss of function of only one of the two loci may not have the predicted effect on tumour progression.

The mutant IDH protein is recognized not only as a diagnostic entity, but also an important prognostic biomarker of glioma: The IDH mutation is an essential part of the diagnosis of astrocytomas, IDH-mutant, or oligodendrogliomas, IDH-mutant and 1p/19q-codeleted. IDH-mutant gliomas are biologically distinct from IDH-wildtype gliomas; however, it is important to recognise in this concept that there are over 100 types of IDH-wildtype gliomas, i.e., this umbrella term does not specify a distinct tumour type/entity. When the IDH mutation was discovered in 2008245, it was initially used for the distinction of IDH-wildtype versus IDH-mutant glioblastomas. Subsequently, IDH-mutant glioblastomas were identified as biologically related to lower grade IDH-mutant astrocytomas. This relationship is now reflected in the 2021 CNS WHO classification which has parted from the term glioblastoma, IDH-mutant, and refers to astrocytoma, IDH-mutant, CNS WHO grades 2, 3, or 4 205. Almost 15 years after the discovery of the IDH mutation in gliomas (and subsequently in other tumour types), the mechanism of tumorigenesis is still poorly understood. It is established, that heterozygous mutation in the IDH1 or IDH2 gene (at codon 132, or 172, respectively (Figure 1) leads to the production of an oncometabolite (D2HG) which and it leads to DNA hypermethylation (Figure 3)2,246,247.
ATRX is a chromatin remodeler that deposits histone H3.3 at sites where the canonical histones have been lost, i.e., ATRX has the capacity of altering chromatin status and can affect DNA methylation status (Figure 5). Also, it has been reported that tumors with ATRX loss show widespread hypermethylation, when compared to ATRX-expressing groups. However, careful examination of the published data in this study reveal that the authors probably stratified tumors by IDH status, using the ATRX expression as proxy. Even though the published data do not allow specific scrutiny of coexistence of ATRX and IDH status, it is not surprising that the loss of ATRX is associated with a hypermethylation, as this is a feature of IDH-mutant tumors. This explains why the different mutational profiles recreated in our tumor model did not have a strong impact on animal survival or DNA methylation.

In this context, we investigated the impact of ATRX on DNA methylation, related to the IDH mutation status, i.e., modeling the molecular constellation of IDH-mutant astrocytomas. Six samples from each group were analyzed on Illumina Mouse Methylation Beadchip (Illumina #20041558) arrays.

The Infinium Mouse Methylation Beadchip is a recent “mouse version”, of the Infinium Human Mouse Methylation Beadchip (Illumina 850k EPIC), the latter being used for example for epigenetic profiling and in some circumstances also for the improvement of the accuracy of the diagnosis of CNS tumors. Others have investigated the utility of the human arrays for mouse DNA methylation studies and, although this platform is suitable for investigating the mouse methylome, the coverage is limited by the lack of probe overlap between species. The Infinium Mouse Methylation Beadchip has been tested and validated as an improved tool to study mouse DNA methylation, but given the novelty of these arrays, no further studies have been published at the time of writing this thesis.

The brain tumors generated in our study show a distinct methylation pattern, separating tumors from control tissue. When comparing the methylation profile across the 4 genotypes (IDH-mutant/ATRX loss, IDH-mutant/ATRX retained, IDH-wildtype/ATRX loss, and IDH-wildtype/ATRX retained), these appear to segregate in a t-SNE plot. However, such separation may be an effect of overfitting, which remains always a possibility if the differences are small.

To test if the experimental tumors expressing mutant IDH1, in combination with ATRX and CDKN2A loss and p53 gene deletion mimic the human epigenetic changes, we analyzed the methylome of the experimental tumors (comparing all 4 genetic combinations as above) Figure 39. All tumors show, in contrast to human IDH-mutant gliomas, a global hypomethylation, compared to CNS control tissue. Whilst the hypomethylation cannot currently be explained,
the overall limited effects that mutant IDH (and the concomitant accumulation of 2 hydroxyglutarate, which we have previously demonstrated\textsuperscript{171}), could be explained by a relatively short exposure to this onco-metabolite. Our current model (i) generates tumours in a very short period (30-60 days), which may be an exposure of insufficient duration to elicit a global hypermethylation, in particular compared to the exposure to the oncometabolite in human tumours, which is likely to span years, or even decades (Figure 43); (ii) tumours are induced in new-borns, which may represent a different microenvironment, and may target a population of stem/progenitor cells that are different from those in humans. However, also in humans, it is likely that the initiating event occurs relatively early during life, but due to the time it takes to progress into a noticeable, clinically apparent lesion, arises in young adults. Therefore, despite some experimental limitations and constraints, our current model is probably still a reasonably good representation of the pathogenic events occurring in humans. In conclusion, the advantages of this model, a rapid and efficient tumour induction, making it highly suitable for preclinical studies, may also represent a downside, in that the rapid tumour development precludes sufficient exposure time to the oncometabolite which is known to elicit global DNA hypermethylation.

The methylation array data also allowed the generation of copy number profiles (copy number variation, CNV) (Figure 40) for each tumour. All groups showed almost flat profiles, with occasional chromosomal losses or gains, which resemble the CNV of human low-grade IDH-mutant astrocytomas (Figure 40F). These similarities, i.e., flat copy number profiles in low-grade IDH-mutant astrocytomas in humans and in the current preclinical model, are not directly comparable. Histologically, our preclinical model represents a high-grade lesion with high numbers of cell divisions, presence of microvascular proliferations and necrosis. These tumours therefore are better comparable with the higher grade IDH-mutant astrocytomas which are characterised by frequent gains and losses of chromosomal material (Figure 40E). Again, this difference can be explained by the biological dynamics of experimental tumours, which are driven by PDGFB mitogenic signals, and accelerated by a p53 mutation, giving relatively little time to accumulate genomic changes (Figure 43). In fact, this method of rapid induction, resulting in flat copy number variance profiles that resemble most those of low-grade gliomas rather than the high-grade glioma characterized by CDKN2A deletion may be explained by the similar phenomenon reported in some childhood tumours, i.e., since children have a shorter life span to accumulate genetic alterations, paediatric tumours (including also high-grade intrinsic brain tumours) generally have much fewer mutations and copy number alteration than what is described in adult tumours\textsuperscript{253}. Thus, the short development time of these tumours could explain
why the CNV profiles are so different from the human counterparts of high-grade IDH-mutant astrocytomas.

Finally, the arrays also return copy number profiles for the probes covering the genes into which genetic modifications were induced. The method or technique by which these mutations were introduced also determined whether such genetic changes were covered by the probes on the mouse methylation array, and thus could be detected on the copy number profiles: the conditional deletion (using the Cre-lox system) of the p53 gene is achieved by a deletion of genetic material between exons 2-10. Since this genomic region is covered by several probes on the array, there is a distinct loss of DNA detected (Figure 41). In contrast, alterations to IDH (only one base pair difference between wildtype and mutant exons, after recombination) (Figure 14) is not detectable on the copy number profile, due to the small genetic lesion. Also, the ATRX, CDKN2A and CDKN2B genomic alterations cannot be identified on the copy number profile. These genes were edited using CRISPR/Cas9, i.e., Cas9 is recruited by specific sgRNAs to the target genes and induces double break strand 3 bp after the PAM sequence. This genomic lesion is subsequently corrected by DNA repair mechanisms, introducing random indels at the respective loci, resulting in gene silencing (Figure 13). These are of very small size and are not covered by the micro array probes (Figure 42).

### 4.4 Conclusion

We have chosen an approach of in situ recombination, combining a Cre-lox approach for inducing expression of mutant IDH1 and for deletion of functional domains of p53, with the CRISPR Cas9 technique to delete ATRX, CDKN2A, and CDKN2B. Our results show a dominant effect of the PDGFB, which is used to induce proliferation and expansion of the stem/progenitor population, leading to variable deletion of ATRX and CDKN2A. Yet, the model system shows many parallels to human IDH-mutant astrocytomas. Differences to the human counterparts are the global hypomethylation, and overwhelmingly flat copy number profiles, which are explained by relatively short latencies between tumour induction and development of fully grown tumours.
5 General discussion

5.1 Summary

This project aimed at establishing a mouse model of IDH-mutant astrocytoma. The key rationale to generate this mouse model was the distinct combination of genetic lesions in IDH-mutant astrocytomas. To this end, this compromise was achieved by using a retrovirus expressing PDGFB, Cre recombinase and sgRNA targeting Atrx and Cdkn2a. This generated tumours with the desired genetic profiles, which would establish the role of these mutations in tumour initiation and progression, and to provide a robust, and realistic preclinical model.

The time of the tumour initiation as well as the latency between tumour induction and development of a large, infiltrative neoplasm leading to the development of neurological signs, has been discussed earlier (Discussion, section 4.3). The time between tumour induction (postnatal day 0 or 1) and development of neurological signs, requiring killing the animal, is usually between 30-50 days, i.e., the tumour induction occurs relatively early during development, and tumour development is accelerated, compared to the presumed time it takes in humans to develop an astrocytoma. The differences in copy number changes and methylation profile between human tumours and the mouse model have been explained in the Discussion (section 4.3), and are also depicted in Figure 43. These experimental constraints explain the differences of methylation profiles and copy number changes between the mouse model and human tumours and need to be taken into consideration when using this model as preclinical system.

Instead, the experimental parameters (time of tumour induction, time to develop a tumour and tumour induction rate) are at the same time also a significant advantage of this model, rendering it as highly suitable for preclinical studies, for example to test experimental approaches to reduce tumour growth, interfere with cell cycle pathways, and test the delivery through novel methods, such as nanoparticles, viruses, or other types of vectors. The use of PDGFB for tumour initiation however needs to be considered as limitation, in that its expression in itself is already growth-promoting, by accelerating proliferation in the stem/progenitor population (Figure 26). Other models have described longer survival associated with mutant IDH1 but could not clarify the role of ATRX in tumour progression. A model was generated by inactivating TP53 and ATRX function but also this model required expression of an oncoprotein (N-ras) to elicit neoplastic growth. They found that ATRX knockdown increased tumour growth, resulting in shorter survival (reduction from 84 to 64 days by inactivating ATRX in addition to the tp53 inactivation and NRAS expression). The median reported survival of these animals was longer than that of our model (64 or 84 days compared to 40 days in our model),
even in the presence of the IDH mutation and regardless of the ATRX status. An alternative approach to generate an IDH-mutant astrocytoma model used the sleeping beauty transposon system to integrate IDH<sup>R132H</sup> into genomic DNA of neonatal mice<sup>256</sup>. In analogy to the approach used in our model system, also the transposon-mediated system used an oncogenic driver to initiate tumorigenesis, specifically NRAS G12V (and shp53, with or without sgATRX and with or without IDH1R132H). Also, this group<sup>256</sup> used an alternative model with PDGFB as driver (PDGFB/shP53/sgATRX/Ink4a/Arf<sup>−/−</sup>; with IDH-mutant or IDH-wildtype) that did not encode RAS-activating mutations, underpinning the fact that such tumours cannot be elicited without additional driver mutations or signals. In this study, IDH-mutant, ATRX knockdown tumours showed longer survival than IDH-wildtype, but also that ATRX-knockdown, in the context of an IDH-wildtype seems to decrease survival of these animals. Notably, this model used shRNA to suppress ATRX and TP53 expression, resulting in a knockdown of these genes, instead of a deletion generated in our model, by CRISPR/Cas9, which is arguably a more accurate representation of the human tumours. Furthermore, these tumours had a latency that was longer than we observed in our study (70 days for IDH-wildtype, increased to 163 days for IDH-mutant animals, compared with 40 days average survival for all animals in our model). Such an extension of survival is however not adequately interpreted in this study, as it has been achieved by simply omitting the IDH1 R132H mutation but was compared to IDH-wildtype glioblastoma in humans. This comparison can be found on many occasions in the literature, but in fact compares two completely separate tumour types, i.e., IDH-mutant tumours and an arbitrarily chosen IDH wildtype tumour (here, IDH-wildtype glioblastoma) whilst there are many more IDH wildtype entities (essentially all tumour types that have no IDH mutation), of which many have in fact a better survival than IDH-mutant astrocytomas. It is possible that the development of epigenetic differences between IDH-mutant and IDH-wildtype tumours became more apparent in the model system with longer incubation times<sup>5,256</sup>.

We had previously discussed the caveat that loss of ATRX expression can be associated with favourable survival, as this specific study has probably stratified the tumour cohort by IDH mutation, using ATRX expression as a surrogate marker for IDH mutations, or to differentiate IDH-mutant astrocytomas from IDH-mutant oligodendrogliomas<sup>248,257</sup>. Along the lines discussed above, a recent study compared the survival of patients with different grades of gliomas and, although reporting a trend for longer survival of patients with IDH and ATRX mutations, the differences were not significant<sup>258</sup>. However, this study is flawed in that it pooled multiple different types of IDH-wildtype gliomas and compares them to of IDH-mutant gliomas. Firstly, this study has, despite being conducted in 2016, still used the term oligoastrocytoma, which at the
time had already been identified as being a historical overlap between IDH-mutant astrocytomas and IDH-mutant oligodendrogliomas. Secondly, this study has also not taken into consideration the molecular stratification that should be included in the diagnosis of astrocytomas, and therefore these data intrinsically cannot show a better resolution of survival as a function of IDH mutation. Moreover, a study of the TCGA data on patients of LGG and GBM revealed that ATRX alterations had a significant impact on survival of GBM patients (p=0.029), but not LGG. This conclusion is a result of a selection bias, in that ATRX mutations were always associated with IDH mutations and with p53 mutations, i.e., correspond to IDH-mutant astrocytomas, which are, in absence of a CDKN2A/B deletion, highly enriched for low-grade astrocytomas, thus prognostically favourable tumours. If, however, IDH-mutant tumours with and without ATRX mutations are compared, this would instead include IDH-mutant oligodendrogliomas (which have no ATRX mutation, but instead a TERT promoter mutation). Therefore, in the context of an IDH mutation, the presence or absence of an ATRX mutation may not render a statistically significant difference, at least in the way this publication has analysed the data. Overall, although ATRX expression seems to have some effect on survival, its impact may depend on the combination of mutations present in the tumours, such as IDH, p53 or CDKN2A.

The fact that multiple groups have shown how IDH, ATRX and CDKN2A mutations impact the survival of patients with IDH-mutant astrocytoma, whilst we did not see significant differences between tumours with different mutational profiles, suggests that PDGFB may have a dominant effect in the pathogenesis of these tumours.

We have pursued multiple additional strategies to generate tumours, using expression vectors without the PDGFB promoter. The combined induction of mutant IDH1 in combination with deletion of ATRX and p53 resulted in acceleration of growth of neural stem cells in vitro, however, this approach did not result in formation of neoplastic lesions in vivo. Importantly, the allografting of neural stem cell lines, engineered with an IDH1, ATRX, and p53 mutation into immunosuppressed mice did not result in the formation of viable allografts either.

Our laboratory has in the past attempted to generate IDH-mutant tumours using the previously established Adeno-Cre mediated recombination. In this model, we used adenovirus-mediated delivery of cre recombinase to activate IDH1 R132H and concomitantly inactivated p53 and PTEN in young adult mice (4-6 weeks of age). This model however was fraught with a low efficacy (between 2% and 5% tumour induction rate), combined with a very long incubation time until the animals developed neurological signs indicative of tumour, of up to 300 days. In an attempt to investigate whether the similar effects on survival and methylation across
groups with different mutational profiles was, in fact, a result of the dominant effect of PDGFB expression in these tumours, we attempted to extract DNA from the tumours described in this paragraph, which combined PS3 and PTEN mutations induced by AdenoCre, thus, without PDGFB. As stated, before this model efficiency was extremely low, therefore we had a very limited number of tumours (N=6) from FFPE tissue which also was over 15 years old. DNA extracted from these blocks was of poor quality and very fragmented, and we obtained no useful signal from the methylation array of these samples, most likely due to the long period of time that these samples have been in archive.

5.2 Experimental limitations

This preclinical model for IDH-mutant astrocytoma successfully recapitulates the mutational profile and histological appearance of these tumours, but as with all experimental paradigms, also this system shows a number of limitations and discrepancies to human tumours, and a number of experimental approaches had specific limitations.

In the subsequent paragraphs, we discriminate between objectives not achieved due to experimental difficulties or shortcomings, and limitations of the experimental approach, limiting the translational equivalency of the model.

5.2.1 Limitations due to experimental difficulties

Viability of genetically engineered cells: Firstly, some of the in vitro studies were limited by the viability of the edited cell lines after several passages in culture, and this. This impacted on their analysis and attempts to enrich genetically engineered cells by flow cytometry further limited their viability, thus reducing our ability to characterise these cells with the intended assays, such as proliferation, apoptosis, or migration assays. It became apparent that this was particularly significant in cells expressing the IDH mutation, and a further confounding factor was the loss of the IDH mutation over multiple passages, a phenomenon that interestingly can occasionally be observed also in progressing IDH mutant high grade astrocytomas (Sebastian Brandner, personal communication). In fact, the difficulty of creating IDH-mutant astrocytoma cell cultures, and their successful propagation over multiple passages, has been described by others, and explains why patient derived xenograft (PDX) of IDH-mutant astrocytomas are very limited. Piaskowisky et al (2011) demonstrated that IDH-mutant glioma lines could not be maintained under standard culture conditions. Such change of genetic properties during in vitro propagation is not unique to IDH-mutant astrocytomas: A comparable observation has
been made in cultured, EGFR-amplified glioblastoma cells. Upon multiple passages, gradually tumour cells overexpressing EGFR had been eliminated by EGFR-non-overexpressing clones \(^{261}\).

**Combined deletion of CDKN2A and CDKN2B:** One of the objectives that were not achieved in this project was the difficulty in generating a CDKN2A/B combined deletion, owing to unsuccessful attempts to create a CDKN2B deletion in the addition to the successful CDKN2A deletion. This combination of mutations is a characteristic of a majority of high-grade IDH-mutant astrocytomas\(^{262}\). These tumour suppressor genes express proteins that inhibit cyclin/kinase complexes, resulting in cell cycle arrest, in the event of DNA damage, and therefore are not highly expressed in normal brain\(^{18,75,76}\). Also, the two adjacent genes regulate each other, and are thought to compensate for each other’s loss of expression, and therefore a combined deletion would have been desirable for our model. However, the low baseline expression of p15 (the gene product of CDKN2B) in the brain did not allow us to validate its deletion in cell lines, using immunohistochemical staining or Western blot analysis. In fact, quantification of mRNA expression confirmed that the CDKN2B deletion was not successful in cells and tumours transduced by RV-ATRX-CDKN2A/B-Cre. Undoubtedly, CDKN2B has an important role in tumour progression, as for example CDKN2B loss promotes progression from benign lesion to melanoma\(^{263}\), or CDKN2B expressing p15 protein has been shown to have a dominant role over CDKN2A (p14 and 16) as tumour suppressor, whereby loss of CDKN2B, but not CDKN2A, resulted in urothelial tumours in mice, when combined with oncogenic expression of HRas, suggesting that p15 is a more potent urothelial tumour suppressor, *in vivo*, than p16\(^{264}\). However, there is limited information about the isolated role of CDKN2B alone in glioma. Increased proliferation of glioma cells has been shown by p15 inhibition, via the expression of long non-coding RNA NBR2\(^{265}\), but few publications focus on CDKN2B independently of CDKN2A, specifically in the context of IDH-mutant astrocytoma. For these reasons, future work should aim for a successful deletion of CDKN2B alone or ideally, in combination with deletion of CDKN2A. More work would be needed to establish and test a wider panel of the best-scoring sgRNA, created from several different design tools, as it was done for ATRX in this study. Different sgRNAs would have to be cloned into a lentiviral plasmid and transfected into NSC, followed by antibiotic selection and, finally, the efficiency of deletion could be tested by qPCR, or even by sequencing the DNA of these transduced cells, around the locus targeted by these guides.
5.2.2 Limitations due to presumed or definite conceptual shortcomings

**Phenotypic variability of experimental tumours:** A recognised limitation of our model was the variability of genotypes and phenotypes of tumours arising from the targeted mutations. Specifically, this related to inconsistent editing of ATRX, resulting in 3 phenotypes, ATRX fully retained, ATRX lost in a subpopulation of tumour cells, and ATRX lost in all tumour cells. This variability however was used to our advantage, in that we were able to assess cellular morphology, epigenetic features and survival within the same experimental cohort. Still however, this specific feature remains a limitation of the model, as it requires expression analysis at the endpoint of experiments (i.e., on the fully developed tumour, after conclusion of possible experimental procedures). This limitation is somewhat mitigated by our standard practice (which we consider best practice) of a histological analysis of any animal reaching an experimental endpoint. There are several explanations for such heterogeneity: The deletion of ATRX and CDKN2A was achieved using CRISPR/Cas9, which induced random INDELs in the genome, usually resulting in gene loss-of-function. However, this may not be the case in every instance, as these random insertion/deletions may not always alter the translation, i.e., not resulting in gene deletion. Other explanations include variable viral integration sites, and this has been discussed in previous chapters and offers room for improvement of the present model so that it can be used as an accurate and consistent mouse model of high-grade, IDH-mutant-astrocytoma.

**Dominant effects of the PDGFB promoter driving the expression of viral constructs:** The use of PDGFB as oncogenic driver has been widely established in similar mouse models and had previously been key to the success of our Cre-lox model whereby a retroviral vector expressing PDGF and Cre recombinase in a bicistronic vector led to the simultaneous deletion of functional elements of PTEN and p53 genes, whilst at the same time driving proliferation of the recombined stem/progenitor pool. This mitogenic potential however proved a disadvantage for the current model, in which the strong mitogenic potential of PDGFB dominates the effect on stem/progenitor cells and may render the potentially more subtle effects of the IDH mutation and the ATRX mutation subordinate to PDGFB effects. The difficulties that previous, published models of IDH-mutant astrocytomas encountered, and circumvented by using similar approaches (i.e., using strong oncogenic drivers) have been discussed in the preceding chapter. Clearly, the dominant effect of PDGFB have to be considered as a compromise to the rapid induction of tumours, which is one of the key advantages of this model and is almost unmatched amongst most published intrinsic brain tumour models.
At the same time, it is debatable how such rapid tumour induction and development is a good
biological match to the much slower development of human IDH mutant tumours, even when
taking into account the different lifespans of the host organism. The different latencies from
tumour induction to tumour development between humans and experimental mice almost
certainly explain differences in mutational spectrum (copy number assays) and most likely also
methylation profile, as discussed previously, and as illustrated in Figure 43.

Figure 43 Schematic illustration of the different dynamics of tumour initiation in humans and mouse
models. In humans, damage to the DNA occurs continuously, and is usually repaired by the natural
mechanism of DNA repair. However, in some cases, these mechanisms are not effective and may result
in unrepaired mutations. IDH mutations is thought to be among the first mutations of astrocytomas,
resulting in epigenetic alterations to the cell. Over the years, the combination of environment, habits, diet,
and genetics of the individual may lead to additional mutation to occur in this cell, which eventually
becomes a tumour cell, which initially slowly, and later, upon acquisition of additional mutations, increas-
ingly rapidly divides to form a tumour. In contrary, this mouse model induces four gene mutations and
expression of the driver PDGFB within 1-3 days after birth, forming a tumour cell, that rapidly trans-
forms in a tumour over the period of 30 days. This can explain why Tumours with different mutational
profiles may not show statistically significant differences in methylation profile, copy number profile, or
survival.

5.2.3 Limitations due to biological and systemic constraints

Any model system, including the widely used murine model presents experimental shortcom-
ings, when modelling human diseases, including cancer. There are obvious differences such as
lifespan, organ physiology and size of the organism. The small body size, and the short reproduction cycle represents a major advantage, and is 1 of the key reasons why this model system is widely used to model very specific aspects of human disease. Such short lifespans (which include the necessity for rapidly induced and growing tumours) of course also limit the time available for neoplastic processes to replicate the mutational landscape, including diversity of clones, and aberrations in copy number profiles. The need for economically viable and experimentally useful, i.e., relatively fast developing tumours in mouse models also limits the utility for modelling low-grade intrinsic neoplasms such as low-grade IDH mutant astrocytomas. Consequently, all these constraints and limitations have to be considered when using such model systems for drug discovery, development and testing of efficacies, and the translation of test results of such preclinical models require significant caution.

5.3 Perspectives and future work

We successfully generated tumours that recapitulate the mutation profile of human IDH-mutant astrocytomas. Our model results in tumours with four different combinations of mutations. This could represent a way forward to investigate further how these groups correspond to the human counterparts, at transcriptome or gene expression levels; or to study the impact of candidate drugs or drug delivery, such as nanoparticle encapsulation, for cancer treatment. As discussed previously, these tumours develop relatively quickly, and therefore (are expected to) show genetic and epigenetic differences to the human counterparts. As discussed in the section on limitations of model systems (section 5.2.3, page 111), human IDH-mutant astrocytomas, in particular those with low grade histological features, develop over years, possibly even decades, which provides a different genetic and epigenetic context compared to the mouse model which has been designed and optimised for a rapid progression, to make it ideally suited as preclinical tool.

Human IDH-mutant astrocytomas, of low grade usually have a corresponding copy number profile with no significant alterations, i.e., the copy number profile derive from methylation arrays appears flat or exhibits only rare chromosomal gains and losses. IDH-mutant astrocytomas of higher grades (corresponding to CNS WHO grade 3) typically exhibit additional copy number gains and losses, with the highest grade (corresponding to CNS WHO grade 4) showing frequent alterations of CDKN2A/B.

The histological appearances of tumours generated in our model are those of high grade IDH-mutant astrocytomas. However, these tumours have a uniformly flat copy number profile, or
at most one or two chromosomal gains or losses. This contrasts with the human counterparts and is best explained by a different pathogenesis and growth dynamics.

The tumour pathogenesis of experimental model involves (i) a strong mitogenic signal that induces growth/amplification of stem/progenitor cells of the subventricular zone, and (ii) a concomitant, simultaneous mutation of the IDH, ATRX, CDKN2A and p53 gene. The combination of these events leads to a rapid growth, not requiring, facilitating, or necessitating additional genetic lesions for growth and progression. Instead, the pathogenesis of human tumours usually involves an initial driver mutation (it is thought that the mutation in the IDH1 gene and the generation of an oncometabolite) precedes other genetic events). From this initial step to the acquisition of additional lesions, such as ATRX mutation and p53 mutation, it may require extensive epigenetic changes to affect these mutations.

Further refinement of the current model would aim at alternate strategies, primarily to eliminate the necessity for the growth inducing PDGFB. Also, be the concomitant deletion of CDKN2A and CDKN2B, which has been so far accomplished only partially. This could be achieved by applying the same approach as with ATRX, i.e., to test a panel of sgRNAs, from different design tools, and assess the most efficient approach to be used in vivo.

Overall, we achieved a new, rapid, and efficient mouse model of glioma, that generates high-grade gliomas with different mutational profiles, i.e., IDH-wildtype and mutant with or without concomitant ATRX and CDKN2A loss, although the more subtle differences caused by the mutational signatures may be masked by the overriding effect of PDGFB. Despite the limitations described, these tumours are similar to the human counterparts and suitable as a preclinical model to test drug delivery methods therapeutic approaches and for the study of metabolic effects.
6 Bibliography


37 Turcan, S. et al. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. Nature 483, 479, doi:10.1038/nature10866

https://www.nature.com/articles/nature10866#supplementary-information (2012).


52 Schnittger, S. et al. &lt;em&gt;IDH1&lt;/em&gt; mutations are detected in 6.6% of 1414 AML patients and are associated with intermediate risk karyotype and unfavorable prognosis in adults younger than 60 years and unmethylated &lt;em&gt;NPM1&lt;/em&gt; status. Blood 116, 5486 (2010).


7 Appendix

7.1 lentiGuide-Puro

(Plasmid #52963)

PURPOSE
Expresses S. pyogenes CRISPR chimeric RNA element with customizable sgRNA from U6 promoter and puromycin resistance from EF-1α promoter. 3rd generation lentiviral backbone.

DEPOSITING LAB
Feng Zhang

PUBLICATION
Sanjana et al Nat Methods. 2014 Aug;11(8):783-4. doi: 10.1038/nmeth.3047. (How to cite)

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Add to Cart
2 µg of cloning grade DNA in Tris buffer

This material is available to academics and nonprofits only.

Cloning Grade DNA

52963-DNA.cg

More Information

$95

Add to Cart

BACKBONE

Vector backbone: Custom

Total vector size (bp): 10183

Vector type: Mammalian Expression, Lentiviral, CRISPR

Selectable markers: Puromycin

GROWTH IN BACTERIA

Bacterial Resistance(s): Ampicillin

Growth Temperature: 37°C

Growth Strain(s): Stbl3


Copy number: High Copy

GENE/INSERT 1

Gene/Insert name: S. pyogenes sgRNA cassette

Alt name: S. pyogenes CRISPR customizable RNA element

Species: Synthetic

Insert Size (bp): 100

Promoter: hU6

CLONING INFORMATION FOR GENE/INSERT 1

Cloning method: Restriction Enzyme

5’ cloning site: BsmBI (not destroyed)

3’ cloning site: BsmBI (not destroyed)

5’ sequencing primer: hU6-F

3’ sequencing primer: hGata4-rev (5’-ATTGTGGATGATGAACTGCC-3’)

(Common Sequencing Primers)

GENE/INSERT 2

Gene/Insert name: Puromycin resistance

Alt name: puromycin N-acetyl-transferase

Alt name: PAC

Insert Size (bp): 600

Promoter: Ef1-a

CLONING INFORMATION FOR GENE/INSERT 2
Cloning method: Restriction Enzyme
5' cloning site: BsiWI (not destroyed)
3' cloning site: MluI (not destroyed)
5' sequencing primer: EF-1a-F
3' sequencing primer: WPRE-R
(Common Sequencing Primers)

Supplemental Documents:
- GenBank file
- lentiCRISPRv2 and lentiGuide oligo cloning protocol

Articles Citing this Plasmid:
- 422 References

TERMS AND LICENSES
Academic/Nonprofit Terms:
- UBMTA

Institut Pasteur Label License for cPPT

Industry Terms:
Not Available to Industry

Trademarks:
- Zeocin® is an InvivoGen trademark.

DEPOSITOR COMMENTS
Note that this plasmid does NOT contain Cas9. It should be used in conjunction with lentiCas9-Blast (Addgene #52962) or otherwise with cell lines already expressing Cas9.

Special note from the Zhang lab: We are constantly improving our CRISPR reagents. Please check https://zlab.bio/ for the most up-to-date information.

Information for Cloning Grade DNA (Catalog # 52963-DNA.cg)

PURPOSE
Cloning grade DNA is suitable for use in PCR, cloning reactions, or transformation into E. coli. The purity and amount is not suitable for direct transfections.

Amount: 2 µg
Guaranteed Concentration: 100 ng/µl +/- 5 ng/µl
Pricing: $95 USD
Storage: DNA can be stored at 4°C (short term) or -20°C (long term).
TERMS AND LICENSES

Academic/Nonprofit Terms:

- UBMTA Institut Pasteur Label License for cPPT

Industry Terms:

- Not Available to Industry
7.2 lentiGuide-Hygro-eGFP

(Plasmid #99375)

PURPOSE
Expresses S. pyogenes CRISPR chimeric RNA element with customizable sgRNA from U6 promoter and hygromycin resistance marker with 2A eGFP from EF-1α promoter. 3rd generation lentiviral backbone.

DEPOSITING LAB
Kristen Brennand

PUBLICATION

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This material is available to academics and nonprofits only.

BACKBONE
Vector backbone: lentiGuide (plasmid #52963) (Search Vector Database)
Backbone size w/o insert (bp): 9465
Total vector size (bp): 11409
Vector type: Mammalian Expression, Lentiviral, CRISPR
Selectable markers: Hygromycin

GROWTH IN BACTERIA
Bacterial Resistance(s): Ampicillin, 100 µg/mL
Growth Temperature: 30°C
Growth Strain(s): NEB Stable
Copy number: High Copy

GENE/INSERT 1
Gene/Insert name: S. pyogenes sgRNA cassette
Species: Synthetic
Insert Size (bp): 100
Promoter: hU6

CLONING INFORMATION FOR GENE/INSERT 1
Cloning method: Restriction Enzyme
5' cloning site: BarnHI (not destroyed)
3' cloning site: BarnHI (not destroyed)
5' sequencing primer: hU6-F
3' sequencing primer: hGat4-rev (5'-ATTGTGGAATAGATCACC-3') (Common Sequencing Primers)

GENE/INSERT 2
Gene/Insert name: Hygro-P2A-eGFP
Insert Size (bp): 1844
Promoter: EF-1α

CLONING INFORMATION FOR GENE/INSERT 2
Cloning method: Gibson Cloning
5' sequencing primer: EF-1α-F 3'
sequencing primer: WPRE-R (Common Sequencing Primers)

RESOURCE INFORMATION
A portion of this plasmid was derived from a plasmid made by: Hygro Addgene plasmid #61426 (Feng Zhang lab)
These plasmids were created by your colleagues. Please acknowledge the Principal Investigator, cite the article in which the plasmids were described, and include Addgene in the Materials and Methods of your future publications.

For your Materials & Methods section:

lentiGuide-Hygro-eGFP was a gift from Kristen Brennand (Addgene plasmid # 99375; http://n2t.net/addgene:99375; RRID:Addgene_99375)

For your References section:

7.3 pCMV-VSV-G

(Plasmid #8454)

PURPOSE
(Empty Backbone) Envelope protein for producing lentiviral and MuLV retroviral particles. Use in conjunction with a packaging vector such as pCMV-dR8.2 dvpr (lentiviral) or pUMVC (MuLV retroviral).

DEPOSITING LAB
Bob Weinberg

PUBLICATION
Stewart et al RNA 2003 Apr;9(4):493-501. (How to cite)

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This material is available to academics and nonprofits only.

This item is currently unavailable outside the US without additional regulatory approval. A non-refundable shipping export licensing fee of $85 is required to cover Addgene’s additional processing costs.

This material is available to academics and nonprofits only.

BACKBONE

Vector backbone: na

(Geek)(Search Vector Database)

GENE/INSERT

Gene/Insert name: None

CLONING INFORMATION

Cloning method: Restriction Enzyme

5' sequencing primer: T7 (Common Sequencing Primers)

GROWTH IN BACTERIA

Backbone size (bp): 6363

Vector type: Mammalian Expression

Bacterial Resistance(s): Ampicillin

Growth Temperature: 37°C

Growth Strain(s): DH5alpha

Copy number: High Copy

RESOURCE INFORMATION

Supplemental Documents:

Weinberg-viral

Articles Citing this Plasmid:

501 References

TERMS AND LICENSES

Academic/Nonprofit Terms:
- UBMTA

Industry Terms:
- Not Available to Industry

Trademarks:
- Zeocin® is an InvivoGen trademark.

DEPOSITOR COMMENTS
VSVG envelope protein, for use with lentiviral and MuLV vectors. The 293T cell line can be obtained from the Weinberg lab or GenHunter

How to cite this plasmid

These plasmids were created by your colleagues. Please acknowledge the Principal Investigator, cite the article in which the plasmids were described, and include Addgene in the Materials and Methods of your future publications.

For your **Materials & Methods** section:

pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid #8454; http://n2t.net/addgene/8454; RRID:Addgene_8454)

For your **References** section:

7.4  psPAX2

(Plasmid #12260)

PURPOSE
(Empty Backbone) 2nd generation lentiviral packaging plasmid. Can be used with 2nd or 3rd generation lentiviral vectors and envelope expressing plasmid (Addgene#12259)

DEPOSITING LAB
Didier Trono

PUBLICATION
Trono Lab Packaging and Envelope Plasmids (unpublished) (How to cite)

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This material is available to academics and nonprofits only.

BACKBONE

Vector backbone: psPAX2

Gene/Insert name: None

GENE/INSERT

Cloning method: Restriction Enzyme

S' sequencing primer: See map

CLONING INFORMATION

(BamHI and EcoRI digests)

GROWTH IN BACTERIA

Backbone size (bp): 10703

Vector type: Mammalian Expression, Lentiviral; Packaging

GROWTH IN BACTERIA

Bacterial Resistance(s): Ampicillin

Growth Temperature: 37°C

Growth Strain(s): DH5alpha

Copy number: High Copy

RESOURCE INFORMATION

Supplemental Documents:  
- BamHI and EcoRI digests

Articles Citing this Plasmid:  
- 3147 References

TERMS AND LICENSES

Academic/Nonprofit Terms:  
- UBMTA

Industry Terms:  
- Not Available to Industry

Trademarks:  
- Zeocin® is an InvivoGen trademark.

DEPOSITOR COMMENTS

2nd generation packaging vector. Known to work with many of the Aebischer and Trono lab lentiviral vectors, as well as the pLKO system. Produces higher titer than pCMV-dR8.2 dvpr.
Note: This plasmid may run as a dimer. Try testing multiple colonies to select the monomer. It may be helpful to follow protocols for low copy plasmids.

Please visit the Trono lab http://tronolab.epfl.ch for cloning strategies, protocols, publications, and more. See LentiWeb http://www.lentiweb.com for discussion on cloning strategies and protocols.

How to cite this plasmid

These plasmids were created by your colleagues. Please acknowledge the Principal Investigator, cite the article in which the plasmids were described, and include Addgene in the Materials and Methods of your future publications.

For your Materials & Methods section:

psPAX2 was a gift from Didier Trono (Addgene plasmid 12260; http://n2t.net/Addgene:12260; RRID:Addgene_12260)
7.5 LentiCRISPRv2Cre
(Plasmid #82415)

PURPOSE
Lentiviral vector expressing Cre recombinase alongside Cas9 and an sgRNA cloning site

DEPOSITING LAB
David Feldser

PUBLICATION
Walter et al Cancer Res. 2017 Feb 15. pii: canres.2159.2016. doi: 10.1158/0008-5472.CAN-16-2159. (How to cite)

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BACKBONE

Vector backbone: lentiCRISPR v2 (Addgene #52961)
(Search Vector Database)

Backbone manufacturer: Feng Zhang

Total vector size (bp): 13459

Modifications to backbone: Cloned in Cre to replace Puro resistance

Vector type: Lentiviral, Cre/Lox, CRISPR

GENE/INSERT

Gene/Insert name: Cre Recombinase

Alt name: Cre

Species: P1 Phage

Insert Size (bp): 1050

Mutation: Mutated BsmBI cut site

Promoter: EFS (P2A)

GROWTH IN BACTERIA

Bacterial Resistance(s): Ampicillin

Growth Temperature: 37°C

Growth Strain(s): NEB Stable

Copy number: Unknown

Clone Information:

5' sequencing primer: TCGAGAGAATCCATCGACTTTCTGGAA-GCCAAGGGCTA
(Common Sequencing Primers)

CLONING INFORMATION

Cloning method: Gibson Cloning

RESOURCE INFORMATION

3. Articles Citing this Plasmid:

TERMS AND LICENSES

Academic/Nonprofit Terms:

- UBMTA

Institut Pasteur Label License for cPPT

Industry Terms:

- Not Available to Industry
sgRNA of choice is cloned into vector using Golden Gate assembly, with BsmBI restriction enzyme (as per resource information on Addgene plasmid #52961 by Feng Zhang).

sgRNA sequencing primer is as follows: 5'-TACGTGACGTAAAGTA

How to cite this plasmid

These plasmids were created by your colleagues. Please acknowledge the Principal Investigator, cite the article in which the plasmids were described, and include Addgene in the Materials and Methods of your future publications.

For your Materials & Methods section:

LentiCRISPRv2Cre was a gift from David Feldser (Addgene plasmid #82415; http://n2t.net/addgene:82415; RRID:Addgene_82415)

For your References section:

7.6 Lenti-sgCdkn2a/Cre

(Plasmid #89644)

**PURPOSE**
Expresses a Cdkn2a-targeting gRNA and Cre-recombinase

**DEPOSITING LAB**
Monte Winslow

**PUBLICATION**
Rogers et al Nat Methods. 2017 May 22. doi: 10.1038/nmeth.4297. ([How to cite](#))

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This material is available to academics and nonprofits only.

**BACKBONE**
Vector backbone: pLL3.3
([Search Vector Database](#))

- **Backbone size w/o insert (bp):** 7741
- **Vector type:** Mammalian Expression, Lentiviral, Cre/Lox

**GROWTH IN BACTERIA**
Bacterial Resistance(s): Ampicillin
Growth Temperature: 37°C
Growth Strain(s): NEB Stable
Copy number: High Copy

**GENE/INSERT**
Gene/Insert name: gRNA targeting Cdkn2a

| gRNA/shRNA sequence: GCGCTGCGTCGTGCACCGGG |

Species: M. musculus (mouse)
Entrez Gene: Cdkn2a (a.k.a. (also known as) ARF-INK4a, Arf, INK4A-ARF, Ink4a/Arf, MTS1, Pctr1, p16, p16(INK4a), p16INK4a, p19<ARF>, p19ARF)

**TERMS AND LICENSES**
Academic/Nonprofit Terms:
- UBMTA

Industry Terms:
- Not Available to Industry

Trademarks:
- Zeocin® is an InvivoGen trademark.

**DEPOSITOR COMMENTS**
Annotated sequence at [https://benchling.com/s/seq-OM7fqimgqUQOZa46yYg6](https://benchling.com/s/seq-OM7fqimgqUQOZa46yYg6)

To view Winslow Lab vector sequences on Benchling, please visit [https://benchling.com/winslowlab/f_/rgXCLH0mq9-winslow-lab-public-vectors-and-sequences/?sort=name](https://benchling.com/winslowlab/f_/rgXCLH0mq9-winslow-lab-public-vectors-and-sequences/?sort=name)
These plasmids were created by your colleagues. Please acknowledge the Principal Investigator, cite the article in which the plasmids were described, and include Addgene in the Materials and Methods of your future publications.

For your **Materials & Methods** section:

Lenti-sgCdkn2a/Cre was a gift from Monte Winslow (Addgene plasmid #89644; http://n2t.net/addgene/89644; RRID:Addgene_89644)

For your **References** section:

7.7 LeGO-V

(Plasmid #27350)

DEPOSITING LAB
Boris Fehse

PUBLICATION
Weber et al Mol Ther. 2008 Apr 16(4):698-706. (How to cita)

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This material is available to academics and nonprofits only.

BACKBONE

Gene/Insert name: U6 promoter, SFFV promoter, Venus

Vector backbone: LeGO

(Gene/Insert Database)

Backbone size w/o insert (bp): 6747

Vector type: Mammalian Expression, Lentiviral

GENE/INSERT

Insert Size (bp): 720

Cloning method: Restriction Enzyme

5' cloning site: BamHI (not destroyed)

3' cloning site: EcoRI (not destroyed)

5' sequencing primer: GAGCTCACAACCCCTCACTC (Common Sequencing Primers)

GROWTH IN BACTERIA

Bacterial Resistance(s): Ampicillin, 100 μg/mL

Growth Temperature: 37°C

Growth Strain(s): Stbl3

Growth Instructions: Any (like TOP10, XL10-Gold or Stbl)

Copy number: High Copy

CLONING INFORMATION

RESOURCE INFORMATION

A portion of this plasmid was derived from a plasmid made by: The Venus cDNA is from the Miyawaki lab (http://cdfs.brain.riken.jp). The lentivector backbone is a derivative of pLenti.Lox3.7 developed at the MIT (http://web.mit.edu/jacks-lab/protocols/pll37.htm).

TERMS AND LICENSES

Academic/Nonprofit Terms:
- UBMTA

Industry Terms:
- Not Available to Industry

Trademarks:
- Zeocin® is an InvivoGen trademark.

DEPOSITOR COMMENTS

HIV-1 derived third generation lentiviral vector for shRNAs. Compatible to shRNAs cloned in pSUPER (XhoI/XbaI). Use second generation (like psPAX2) or third generation (like pMD.Lg/psR + pSV Rev) systems for packaging in addition to a VSV-G expressing plasmid (or another envelope protein). Please visit the LeGO-Vector home page for more information: http://www.LentiGO-Vectors.de
These plasmids were created by your colleagues. Please acknowledge the Principal Investigator, cite the article in which the plasmids were described, and include Addgene in the Materials and Methods of your future publications.

For your **Materials & Methods** section:

LeGO-V was a gift from Boris Fehse (Addgene plasmid # 27350; http://n2t.net/addgene:27350; RRID:Addgene_27350)

For your **References** section:

Lentiviral Gene Ontology Vector, LeGO-V
Kristoffer Weber, Udo Bartsch, Carol Stocking, Boris Fehse
*Molecular Therapy* (2008); 16 4 698–706
http://www.LentiGO-Vectors.de

Important sites:

All single and double cutters:
### Vector Information

<table>
<thead>
<tr>
<th>Vector ID</th>
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<td>Ampicillin</td>
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<tr>
<td><strong>Cloning Host</strong></td>
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### Vector Map

![Vector Map](image)

### Vector Components
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<tr>
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**Note:** Components added by user are listed in **bold red** text.

**Vector Sequence**

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Validation by Restriction Enzyme Digestion

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7.9 VB200604-1242fbx

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<td>Cloning Host</td>
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**Vector Map**

![Vector Map](attachment:image)

**Vector Components**

- RSV promoter
- pUC ori
- 5' LTR-DeltaU3
- Psi
- RRE
- cPPT
- U6 promoter
- ATRX[gRNA#1]
- gRNA scaffold
- Terminator
- CMV
- Kozak
- SV40 early pA
- 3' LTR-DeltaU3
- WPRE
- Cre(ns)
- T2A
- hPDGFB[NM_002608.3]
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Note: Components added by user are listed in **bold red** text.

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Note: Components added by user are listed in **bold red** text.
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<th>DNA Fragments (bp)</th>
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7.10 VB200211-1250kfw

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**Vector Map**

![Vector Map](image)

**Vector Components**

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**Note:** Components added by user are listed in **bold red** text.

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Validation by Restriction Enzyme Digestion

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7.11 VB210316-1064vma

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<td>Vector Type</td>
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<tr>
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<tr>
<td>Antibiotic Resistance</td>
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<td>Cloning Host</td>
<td>VB UltraStable (or alternative strain)</td>
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**Vector Map**

**Vector Components**
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<tr>
<th>Name</th>
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<th>Size (bp)</th>
<th>Type</th>
<th>Description</th>
<th>Application notes</th>
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<tr>
<td>CMV enhancer</td>
<td>1-381</td>
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<td>promoter</td>
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<td>note=human cytomegalovirus (CMV) immediate early promoter</td>
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<tr>
<td>5' LTR (truncated)</td>
<td>586-761</td>
<td>176</td>
<td>LTR</td>
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**Note:** Components added by user are listed in **bold red** text.

**Vector Sequence**

1. GACATTGATT ATGACTAGT TATTAATAGT AATCAATTAC GGGTCATTA GTTCATAGCC CATATATGA GTTCCGCCTT
2. ACAATACTTA CGGTAATGG CCCGCTTGCC TGACCGCCCA ACGACCCCCG CCCATTGACG TCAATAATGA CTTATGTTC
3. CATAGTAGC CCAATAGGGA CTTTCCATTG ACGTCAATGG GTGGAGTATT TACGGTAAAC TGCCCACTTG GCAGTACATC
### Validation by Restriction Enzyme Digestion

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<th>Restriction Enzymes</th>
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<th>DNA Fragments (bp)</th>
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<td>Ascl</td>
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<td>9227</td>
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<tr>
<td>ApaLI</td>
<td>2364, 2726, 4714, 7074, 7572, 8818</td>
<td>362, 1888, 2360, 498, 1246, 2773</td>
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<td>ApaLI+Ascl</td>
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7.12 VB210923-1307ned

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<td>Vector Type</td>
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<td>Antibiotic Resistance</td>
<td>Ampicillin</td>
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<td>Cloning Host</td>
<td>VB UltraStable (or alternative strain)</td>
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**Vector Map**

![Vector Map](image-url)

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<tr>
<td>CMV enhancer</td>
<td>1-381</td>
<td>381</td>
<td>misc_feature</td>
<td>None</td>
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<td>Type</td>
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<td>Application notes</td>
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<td>Type</td>
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| AmpR       | complement     | 861       | ORF   | None                                            | codon_start=1  
gene=bla                                                  
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ote=/SeqFeature=True    
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ote=/full_name=Ampicillin resistance gene   
ote=/name=AmpR  
ote=/official_designation=Ampicillin  
ote=/uuid=233390972317459885e22af363ee0833  
ote=/vntifkey=4  
product=beta-lactamase |
| AmpR promoter | complement     | 105       | promoter | None                                      | gene=bla  
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ote=/SeqFeature=True  
ote=/level=0  
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ote=/official_designation=AmpR promoter  
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ote=/vntifkey=21 |

**Note:** Components added by user are listed in **bold red** text.

**Vector Sequence**

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  ACATAACTTA CGGTAATGG CCGGCCTGCC TGACCGCCCA AGCACCCCGG CCCATTGAGG TCAATAATAGA GCATACATC
  CATAGTAACG CCAATAGGGA CTTTCCATTG AGGTCAATGG GTGGAGTATT TACGGTAAAC TGCCCACTTG ACGTCGAGT
  ACATCAATGG GGGTGATAG CCGTTTGAIT CAGGGGATT TCCAGTCTCG CACCCATATT AGCTGTACAT GAGTTGTGTG
  TGGACAAAAT ATCACAAGGA CTTTCACAAA GTGCTGAAAC ACTCCGCCCCC ATTTGCGCAG GCGTGCTACG
  GTGGGAGGT TATATAAGTA GCTCCAAGA AAAGGCCCA CAACCCCTCA CTCGCGCCGG CATCTTCCCG
  AATCCGACCC CCGTCAGGAT CTTTGTTCTG TATAGTAGTG ATGAGAAGAC GCCTGGGTGG CAGAAGTCG
  CGGATCGCTC ACAACAGTGC TGGCTGCTCTA CTCATGGCTAT TATCGCTGGTA TATCGCTGGT
  CGTCTATTGT TATGGGAATAT CAGGGGCTCA GGGGCTGTTA GGGGCTGTTA GTGGGCTGCTG TGGGCTGCTG
  CGGGGGCAAC CCGGCTGCCA GCTGGGAGGA AATGGGTTTG GTTGGGCTCTG GTTGGGCTCTG TGGGCTGCTG
  TACGCGACAT CTCTTCAATG GGAAGAAGAC TTTTGACCCC TCTCGAGGAG CAGAAGGAGA
  TATGCGCCTG CGTCTGTACT AAGTGAATCA AATCGACGGA CCGCCGCGC TTCTGACAGC
  GACGGAGCT GAGTTTAACAG AAAGCTGG ACGCAACTT CCGCTGGGTT CGCCCTTAA
  CGTCTTGCAG CTCTGAAAAT TAGGGCCAGA TCTGGCTACG TGGGCTGCTG TGGGCTGCTG TGGGCTGCTG
  CGGATCGCTC ACAACAGTGC TGGCTGCTCTA CTCATGGCTAT TATCGCTGGTA TATCGCTGGT
  CGTCTATTGT TATGGGAATAT CAGGGGCTCA GGGGCTGTTA GGGGCTGTTA GTGGGCTGCTG TGGGCTGCTG
  CGGGGGCAAC CCGGCTGCCA GCTGGGAGGA AATGGGTTTG GTTGGGCTCTG GTTGGGCTCTG TGGGCTGCTG
  TACGCGACAT CTCTTCAATG GGAAGAAGAC TTTTGACCCC TCTCGAGGAG CAGAAGGAGA
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<tr>
<th>Restriction Enzymes</th>
<th>Cutting Sites</th>
<th>DNA Fragments (bp)</th>
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<tr>
<td>Xhol</td>
<td>2459, 3478</td>
<td>1019, 6845</td>
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<tr>
<td>Ascl</td>
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<td>Agel</td>
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<td>Nhel</td>
<td>2453, 4673</td>
<td>2220, 5644</td>
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<td>ApaLI</td>
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<td>362, 2985, 498, 1246, 2773</td>
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### Vector Map

![Vector Map](image)

### Vector Components

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<tr>
<td>Vector Size</td>
<td>7049 bp</td>
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<td>Vector Type</td>
<td>Mammalian Gene Expression MMLV Vector</td>
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<tr>
<td>Plasmid Copy Number</td>
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<tr>
<td>Antibiotic Resistance</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>Cloning Host</td>
<td>VB UltraStable (or alternative strain)</td>
</tr>
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</table>

- **AmpR** promoter
- **CMV** enhancer
- **CMV** promoter
- **5' LTR (truncated)**
- **MMLV Psi**
- **gag (truncated)**
- **ori**
- **SV40 ori**
- **SV40 promoter**
- **3' LTR (Delta-U3)**
- **Cre**
<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Size (bp)</th>
<th>Type</th>
<th>Description</th>
<th>Application notes</th>
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<tr>
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<tr>
<td>MMLV Psi</td>
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<td>None</td>
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<tr>
<td>gag (truncated)</td>
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<td>Type</td>
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<td>Application notes</td>
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| hU6          | 1674-1922   | 249       | misc_feature | None                                                                         | note=hU6  
note/=SeqFeature=True  
note/=level=0  
note=/application_notes=Pol III promoter; drives expression of small RNAs.  
note=/full_name=Human U6 promoter  
note=/official_designation=U6  
note=/uuid=0f9979e4676b4788b9441e4543b07317  
note=/vntifkey=21                                                                 |
| sgATRX       | 1924-1943   | 20        | misc_feature | None                                                                         | note=sgATRX  
note/=SeqFeature=True  
note/=level=0  
note=/name=sgATRX  
note=/official_designation=sgATRX  
note=/uuid=c6cf48af2c394b5ead006e53a6fa1dac  
note=/vntifkey=21                                                                 |
| gRNA scaffold | 1944-2019   | 76        | misc_RNA    | None                                                                         | note=gRNA scaffold  
note/=SeqFeature=True  
note/=level=0  
note=/application_notes=Facilitates interaction between gRNA and Cas9 protein to form targeting complex.  
note=/name=gRNA scaffold  
note=/official_designation=gRNA scaffold  
note=/uuid=3ed9ac2e5cc74055b29bc0949061dec8  
note=/vntifkey=53                                                                 |
| Terminator   | 2020-2025   | 6         | misc_feature | None                                                                         | note=Terminator  
note/=SeqFeature=True  
note/=level=0  
note=/application_notes=Allows transcription termination of small RNA transcribed by Pol III RNA polymerase.  
note=/name=Terminator  
note=/official_designation=Terminator  
note=/uuid=a2ca82806fa441698297d9fb7e458916  
note=/vntifkey=21                                                                 |
| CMV enhancer | 2029-2332   | 304       | enhancer    | None                                                                         | note=CMV enhancer  
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note/=level=0  
note=/name=CMV enhancer  
note=/official_designation=CMV enhancer  
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<th>Position</th>
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<th>Type</th>
<th>Description</th>
<th>Application notes</th>
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<td>ORF</td>
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<td>3' LTR (Delta-U3)</td>
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<td>4518-4847</td>
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<td>note=SV40 promoter note=/SeqFeature=True note=/level=0 note=name=SV40 promoter note=official_designation=SV40 promoter note=uuid=57b0f08bed9c4d3b88821b51b0a9ac17 note=vntifkey=21</td>
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<tr>
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<tr>
<td>Name</td>
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<td>Size (bp)</td>
<td>Type</td>
<td>Description</td>
<td>Application notes</td>
</tr>
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| ori          |          | 589       | rep_origin | None                                                                        | note=ori  
|              |          |           |        | note/_SeqFeature_=True                                                       | note/_level_=0                                                                                          |
|              |          |           |        | note=/application_notes=Facilitates plasmid replication in E. coli;         | regulates low-copy plasmid number when Rop protein is present (15-20) and medium-copy plasmid number when  |
|              |          |           |        |                                                                               | Rop is absent (100-300).                                                                              |
|              |          |           |        | /full_name=pBR322 origin of replication                                        | note=/name=ori note=/official_designation=pBR322 ori note=/uuid=e48458ed5a30458dab8769dee705600c note=/vntifkey=33 |
| AmpR         |          | 861       | ORF    | None                                                                        | codon_start=1  
|              |          |           |        | gene=bla                                                                     | note=AmpR  
|              |          |           |        | note/_SeqFeature_=True                                                       | note/_level_=0                                                                                          |
|              |          |           |        | note=/application_notes=Allows E. coli to be resistant to ampicillin.        | note=/full_name=Ampicillin resistance gene note=/name=AmpR note=/official_designation=Ampicillin          |
|              |          |           |        |                                                                               | note=/uuid=233390972317459885e22af363ee0833 note=/vntifkey=4 product=beta-lactamase                   |
| AmpR promoter|          | 105       | promoter | None                                                                        | gene=bla  
|              |          |           |        | note=AmpR promoter                                                           | note/_SeqFeature_=True  
|              |          |           |        | note/_level_=0                                                               | note/_name=AmpR promoter note=/official_designation=AmpR promoter note=/uuid=3538fe4fe522460ebaf8d4c8aca417dc note=/vntifkey=21 |

**Note:** Components added by user are listed in **bold red** text.

### Vector Sequence

1. GACATTGATT ATGACTAGT TATTAATAGT AATCAATTAC GGGGCATTA GTTCATAGCC CATATATGGA GTTCATAGCC

81 CATATACCTA CCATGGAGGA CCCGGCTGGC TGAGCCGCCA AGCAGCCCCC CCAATGAGC TCGATATGAC CTATATGTC

161 CACGTTAAGG CTTCCCTTGG AGCTCAATGG CAGCTGATTT TACGATACAT TGGCCACTTG GCGTACATC

241 AAGGCTAAGT TATGCAACTA AGGCCCTCTA TGACGTCAA GAGCGTTAAA TGCCCCGCTG TGGCATTAGT CCCAGTACAT

321 GACCTTAGG GACTTTCCGA CTTGGCAGTA CATCTACGTG TTAGTCTACG CATTACCTG GGATGTGC GGTTGCGGATT

401 ACATCAATG GCTGGCATAG CGGTCGACT CACGGGGATT TCAAGTCTC CACCCCATTT ACGCATTGAG GAGTTGCTTT
Validation by Restriction Enzyme Digestion

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<th>Restriction Enzymes</th>
<th>Cutting Sites</th>
<th>DNA Fragments (bp)</th>
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<tbody>
<tr>
<td>XhoI</td>
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<td>AciI</td>
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<td>7049</td>
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<td>7049</td>
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7.14 VB190627-1017ykt

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<td>Antibiotic Resistance</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>Cloning Host</td>
<td>VB UltraStable (or alternative strain)</td>
</tr>
</tbody>
</table>

### Vector Map

![Vector Map](image)

### Vector Components

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<thead>
<tr>
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<th>Position</th>
<th>Size (bp)</th>
<th>Type</th>
<th>Description</th>
<th>Application notes</th>
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<tbody>
<tr>
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<td>1-594</td>
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<td>Moloney murine leukemia virus 5' long terminal repeat</td>
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<tr>
<td>Name</td>
<td>Position</td>
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<td>Description</td>
<td>Application notes</td>
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<td>psi plus</td>
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<td>588</td>
<td>LTR</td>
<td>Moloney murine leukemia virus 3' long terminal repeat</td>
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<tr>
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<td>❑ complement (5683-6543)</td>
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<td>Ampicillin resistance gene</td>
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**Note:** Components added by user are listed in **bold red** text.

**Vector Sequence**

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GAATAGAAAA GTTCAGATCA AGGTCAGGAA CAGATGGAAC AGCTGAATAT GGCCCAACA GGATATCTGT GGTAAAGCAGT
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Validation by Restriction Enzyme Digestion

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<td>3049, 3628</td>
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<td>889, 5185, 6424</td>
<td>4296, 1239, 1142</td>
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<td>145, 220, 4183, 4258</td>
<td>75, 3963, 75, 2564</td>
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7.15 VB200210-1013bud

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<td><strong>Viral Genome Size</strong></td>
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**Vector Map**

**Vector Components**
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<tr>
<th>Name</th>
<th>Position</th>
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<th>Type</th>
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<th>Application notes</th>
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<td>230-410</td>
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<td>Application notes</td>
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**Note:** Components added by user are listed in **bold red** text.
Vector Sequence

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241 GTTAGACACG ATCTGACCTT GGGTACCTTC TGGCTAATCA GGGAAACCC TGATTTACCC TCATATAAGG TGCTCTGCTG
321 TGCTCTAGAT AGTCGTGTGG GCTCCTGTGTT GAAGTCTGAC TTCATACGAC CTTCTTTATG TCTTGTTGAG
401 ATCTCTAGCA GTGCGGCCGC AACAGGGACT TGAAGAGAA AGGGAAACG GAGGGACTCT CTCGACAGCA GACTCGGATT
481 GCTGAGACGC GCACGCGAAC AGGCGGAGGG CGGCAGTGTC TGGTAACGAC ACTACATCTC TCAGCAAGAG AATGCAAGA
561 GAGAGATGGG TGCGAGAGCG CTGATATTTA AAGGGGGAAG ATTAGATGCG GATGGGAGTT AATCGGGAGA GAGCGGGGAG
641 GAAAGAAAAA ATATAAATAA AACATATAAG TATGGGCAAG AAGGGGACT CAGGACTCC TCGGCTCTTA
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881 AGGAAAGAGCA AAACAAGATG AAGACACCGG CACAGCCTGA GGCCTCTGT TCTCGACACT GGGAGGAGAG ATATGAGGGA
961 CAATTGGAGA AGTAGTATTA ATAAATATAA ATAGTAAAA ATAGAGCATAT TAGGAGTAGC ACCACAGAC GCAAGAGAA
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1761 GATCCATTGC ATTAGTGAAC GGTCTGCAG GTATCTGACT GCTTTTAAAA GAAAGGAGG GAGGGGGGTC TACAGTGCA
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2001 TACAAGGCTG TTAGAGAGAT AATTGGAAAT AATTTGACTG TAAACAAAGA GATATTAGTA CAAAATACGT GAGCTGAAA
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2241 GAAATAGCAA GTAAAATAAA GCGTATGCGT TTATCAACTT GAAAGATTGG CACGGAGTGG GTCCTTTTTT CAAAGTTTGTA
2321 CAAAGACGCA GCTTATGTTA TTTAAAGTGCA CCCCTATTGT CAGTATTTCT CATTCACTACA CGATATGGCT TCGCGGTTAC
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3121 TGGCGCTGTC GGGGCGCGCA GTGCTCAGAT GCAGTAAACG GAAAGTGTGG AGAAGCGACG GTGAAAGATT TTGCGGATGAT
3201 CTCTGTATAC TCAGCAGCGG GGCTGCTGCA TAAAACATA TCCAGCAAAC TTTGGGCGCA CTAATACAGTG TTAATCAGTG
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3361 GTGACGGTGC AAAACAGGGT CAGCTGCTGC AACGGACTGA TTGCCAGAG AGTGCCTACC CAGGATGACA GATGGCGC
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Validation by Restriction Enzyme Digestion

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<td>Cloning Host</td>
<td>VB UltraStable (or alternative strain)</td>
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**Vector Map**

![Vector Map Diagram]
### Vector Components

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<th>Type</th>
<th>Description</th>
<th>Application notes</th>
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**Vector Sequence**

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7.17 PDGFB-IRES-CRE

Vector Map
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**FEATURES**

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