Histone-mutant interneuron progenitors co-opt *PDGFRA* for gliomagenesis

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ABSTRACT

Histone H3.3 glycine 34 to arginine/valine (G34R/V) mutations occur in deadly hemispheric high-grade gliomas. These mutations show exquisite regional and temporal specificity, which suggests a developmental context permissive to their effects. Here we present the molecular landscape of G34R/V tumours (n=86) and show that 50% bear activating mutations in *PDGFRA*, with strong selection pressure for *PDGFRA*-mutant clones at recurrence. We show that, while they are considered gliomas, G34R/V tumours arise in foetal interneuron progenitors derived from the ventral forebrain, which express *GSX2* and *DLX* homeobox transcription factors. Further neuronal differentiation is impaired in these progenitors by G34R/V. High expression and frequent mutations of *PDGFRA* in G34R/V HGGs are facilitated in this lineage-of-origin, where *PDGFRA* forms a chromatin loop with the adjacent *GSX2*, hijacking its active chromatin conformation. At the single-cell level, G34R/V tumours harbour dual neuronal/astroglial cell identities and lack oligodendroglial programs, that are actively repressed by *GSX2/DLX*-mediated cell-fate specification. G34R/V CRISPR-removal does not impact tumorigenicity suggesting this mutation becomes dispensable, while *PDGFRA* mutations are potent oncogenic drivers. Collectively, our results suggest that G34R/V gliomas arise in foetal inhibitory interneuron progenitors where they stall differentiation and co-opt *PDGFRA* via inappropriate expression and activating mutations to promote oncogenicity and gliogenesis.

INTRODUCTION

High-grade gliomas (HGGs) are deadly primary brain tumours and a leading cause of mortality in children and young adults. These tumours frequently harbour somatic mutations in genes encoding histone 3 (H3) variants or affecting epigenetic modifiers with remarkable neuroanatomical and age specificity[1-5]. HGGs in adolescents and young adults (12 - 35 years old), primarily occur in cerebral hemispheric lobes and bear heterozygous mutations in the noncanonical H3.3 variant leading to glycine 34 to arginine or valine (G34R/V) amino-acid substitutions[3-6], or in *Isocitrate Dehydrogenase 1 or 2* (*IDH1/2*)[7], or more rarely in *SET Domain Containing 2* (*SETD2*)[8]. Despite accounting for more than 30% of hemispheric HGGs, G34R/V HGGs are understudied and likely underestimated because of misdiagnosis due to their unique histopathological heterogeneity, with dual neuronal-glial compartments present at variable degrees. Indeed, close to 30% of central nervous system primitive neuroectodermal tumours (CNS-PNETs), a now-obsolete entity of mixed high-grade neuronal tumours, have been shown to be G34R/V-mutant HGGs[9,10]. At the molecular level, on the other hand, G34R/V HGGs show unifying features: they almost invariably carry mutations in *ATRX* and *TP53*[3-5,8,11,12], lack immunoreactivity for the oligodendroglial marker OLIG2[2], and cluster distinctly from other glioma entities based on DNA methylation[2,4,10]. In contrast to K27M or K36M amino acid substitutions which can affect canonical H3.1/2 or H3.3 variants, G34R/V occur exclusively on the non-canonical H3.3 variant. G34R/V mutants have been suggested to act *in cis*, with the bulky amino acid replacement preventing post-translational modification of the neighbouring H3.3K36 residue[13] and/or its recognition by specific readers[14]. Limited information exists on oncogenic or developmental pathways in these tumours. Compiling comprehensive cohorts to study the molecular landscape of this entity has been challenging as this age group spans paediatric and adult care, in addition to a high rate of misdiagnosis. To address this knowledge gap, we assembled the largest cohort to date of primary G34R/V HGG tumours, comprehensively profiled them at the genomic, epigenomic, and transcriptomic (bulk and single-cell) levels and developed *in vitro* and *in vivo* models. We delineate the unique molecular characteristics of G34R/V tumours, define their developmental origins, characterize inter- and intra-tumour heterogeneity, and uncover specific vulnerabilities that may be amenable to targeted therapies in this deadly cancer type.

RESULTS

High-frequency mutations in Platelet Derived Growth Factor Receptor alpha (PDGFRA) in G34R/V HGGs

To define the genetic landscape of G34R/V HGGs, we assembled a multi-institutional cohort of G34R (67 at diagnosis, 4 recurrences) and G34V tumours (10 at diagnosis, 5 recurrences), the largest reported for this entity to date. We compared G34R/V mutation profiles to midline K27M HGGs and to the other hemispheric HGG subgroups occurring in children or adults younger than 50 years old, namely *IDH1*- and/or *SETD2*-mutant HGGs or tumours wild-type for these mutations (**Fig. 1A, Table S1**). In addition to the known genetic alterations in *TP53* and *ATRX* (95% and 84% respectively), we observed a previously unappreciated high frequency of mutations in *PDGFRA* (43/86) specifically in G34R/V HGGs. These mutations were present in 48% of G34R and 50% of G34V tumours at diagnosis. Notably, in three of the four available G34R/V primary/recurrence pairs, the *PDGFRA* mutation was acquired at relapse. Altogether, co-occurrence of G34R/V & *PDGFRA* mutations was 7- to 8-fold higher than in all other HGG subgroups, where the highest observed frequency of $PDGFRA^{MUT}$ was 7% in this cohort (*p = 2.8E-55, chi-square test*). No other recurrent mutations (frequency > 10%) were identified beyond *PDGFRA* (**Ext. Fig. 1**). In contrast to K27M HGGs[1-3,5], *PDGFRA* was not frequently amplified in G34R/V tumours (6/47, 13%), a frequency similar to what we observed in other hemispheric HGG subgroups, including *IDH1*-mutant and IDH1/H3-WT (*p = 0.64, chi-square test*).

Within G34R/V HGGs, 36/43 (80%) of *PDGFRA* mutations occurred in the 3rd-4th extracellular Ig-like domains (**Fig. 1B)**. In contrast, we rarely observed the D842V/Y mutations (n = 2) located in the autoinhibition site of *PDGFRA*, which are prevalent and well-characterized in gastrointestinal stromal tumours[15]. Most extracellular mutations, which seem exclusive to brain tumours and particularly paediatric gliomas[16] (**Table S2**), are poorly characterized. We thus performed *in silico* modelling of two mutations, the cysteine 235 to tyrosine/phenylalanine (C235Y/F), the most prevalent in our cohort (n = 14), and lysine 385 to methionine (K385M). These results suggest that these mutations promote ligand-independent dimerization of PDGFRA (**Ext. Fig. 2A-C**), similar to the neomorphic Y288C mutant[17] that renders this receptor tyrosine kinase (RTK) constitutively active.

Transcriptional and epigenetic programs in G34R/V gliomas indicate a ventral interneuron progenitor origin

The high co-occurrence of PDGFRA mutations with G34R/V suggests a G34R/V-context dependency, likely linked to development given the spatiotemporal pattern of these tumours, as was recently demonstrated for several related entities[18,19]. Thus, to define the cellular lineage at the origin of G34R/V HGGs, we assessed enrichment of cell type specific signatures derived from single-cell RNA-seq (scRNA-seq) atlases spanning mouse and human forebrain development[18,20,21], using gene set enrichment analysis (GSEA). Compared to other HGG entities, transcriptomes of G34R/V gliomas showed strong enrichment of radial glia, neuronal progenitor and inhibitory interneuron gene programs, and were depleted of excitatory neuron and oligodendroglial signatures (**Fig. 2A, Ext. Fig. 3A, Table S3**). Enrichment of astrocyte gene programs in G34R/V was similar to other HGGs, but stronger than hemispheric non-glioma entities such as the CNS-PNET high-grade neuroepithelial tumour with BCOR alteration (HGNET-BCOR)[22] (**Ext. Fig. 3B)**.

Notably, G34R/V tumours showed enrichment of foetal, but not postnatal, interneuron signatures. Inhibitory interneurons are generated during foetal brain development, in transient progenitor domains of the ventral embryonal telencephalon termed ganglionic eminences[23,24]. In contrast, excitatory neurons are born dorsally in the neocortex (**Fig. 2B**). A network of transcription factors (TFs) patterns these domains to specify the fate of their progeny. In the medial ganglionic eminence (MGE), *NKX2.1*+ progenitors give rise to both interneurons and oligodendrocytes. *GSX2* is expressed throughout the ganglionic eminences, but most highly in the lateral and caudal ganglionic eminences (LGE/CGE), where *GSX2* acts upstream of *DLX1/2*. All three factors (*GSX2, DLX1/2*) are required for interneuron specification and repression of oligodendroglial fate[25,26]. Accordingly, G34R/V HGGs significantly upregulated *GSX2* (**Ext. Fig. 3C**), which is normally expressed in radial glia and interneuron progenitors, consistent with its role maintaining neuronal progenitors in an immature state[27]. They also upregulated the human radial glia marker *MOXD1*[28,29], interneuron lineage-associated transcription factors including *DLX1/2*, and interneuron markers *GAD2, SCGN*, *NPY*, and *CALB2*[24,30]. In contrast, G34R/V HGGs lacked expression of TFs specifying dorsal excitatory neurons such as *EOMES* and *NEUROD2*. This transcriptional profile was unique to G34R/V tumours; expression of forebrain interneuron TFs and gene signatures was absent not only in midline K27M tumours, but also in other hemispheric HGGs.

Epigenomic profiles of G34R/V tumours were also consistent with an interneuron progenitor program. *GSX2* and *DLX1/2* displayed an active chromatin conformation, enriched for the activating H3K27ac mark and lacking the repressive H3K27me3 mark (**Fig. 2C, Table S4)**. On the other hand, the set of genes enriched for H3K27me3 included genes related to neuronal synapse, axon and ion-channel associated functions (e.g. *NTRK2, NXPH1, CHRNA4*) (**Ext. Fig. 3D**). The oligodendrocyte lineage factor *OLIG2*, previously shown to be silenced through DNA methylation in this entity[2], completely lacked both H3K27me3 and H3K27ac marks in G34R/V gliomas. Furthermore, G34R/V HGGs lack activation of a core module of transcription factors necessary for oligodendrocyte specification (**Ext. Fig. 3E**).

Finally, to verify their resemblance to immature neurons, we extracted the GSEA leading-edge genes driving G34R/V-enriched and depleted expression signatures, as well as genes displaying differential H3K27ac/me3 enrichment, and profiled their expression along a normal interneuron differentiation trajectory (**Fig. 2D**). G34R/V-upregulated genes were primarily expressed in interneuron progenitors. Conversely, genes down-regulated and H3K27me3-enriched in G34R/V peaked in expression late in normal interneuron maturation. Altogether, the transcriptomic and epigenomic profiles of G34R/V HGGs suggest that they arise within ventrally-derived progenitors committed to interneuron differentiation, and that they are restricted from progressing to a mature neuronal state.

G34R/V mutations promote H3K27me3 retention at genic promoters and silencing of mature neuronal genes

H3K27me3 patterns in G34R/V gliomas may either reflect their developmental origin or derive from G34R/V-mediated epigenetic dysregulation. H3.3 G34R/V substitutions were previously shown to impair the catalytic deposition of H3K36me3 by SETD2[13,31], a mark coupled to active transcription and known to directly antagonize H3K27 methylation[32]. Using liquid-chromatography/mass spectrometry (LC/MS) of histones extracted from patient-derived cell lines, we observed H3K36me3 loss coupled to reciprocal gain of H3K27me3 specifically on H3.3G34R/V-mutant histone peptides (**Ext. Fig. 4A**), confirming the *cis*-effect of these mutations. At the genome-wide level, chromatin immunoprecipitation (ChIP-Seq) showed H3K27me3 deposition to be significantly enriched at a large number of genic promoters (n = 4,383) in G34R/V HGGs compared to non-G34R/V cortical HGGs. This gain in H3K27me3

contrasted with the relatively balanced changes observed in H3K27ac deposition where only ~100 promoters showed differential enrichment in G34R/V (**Ext. Fig. 4B-C**).

To determine whether H3K27me3 enrichment is a direct consequence of the G34R/V oncohistone, we targeted the mutation for removal in the HSJD-GBM002 (*H3F3A*^{G34R}) cell line using CRISPR-Cas9 (**Ext. Fig. 4D**). Globally, limited differences were observed in histone marks between G34R and edited clones by ChIP-seq, with similarly modest transcriptomic changes. Expression of the *DLX* family of interneuron transcription factors was maintained in both edited and unedited lines (**Fig. 2E**), supporting the notion that specification of the interneuron transcriptional program in G34R/V gliomas reflects a developmental origin. Notably, genomic bins showing the greatest H3K36me3 loss in G34R concurrently displayed increased PRC2-SUZ12/H3K27me3 enrichment and decrease of transcriptional activity, in keeping with the presumed effect of G34R/V on these marks (**Ext. Fig. 4E-F**). We observed specific enrichment in promoter H3K27me3 in G34R/V tumours compared to other cortical HGG entities at several genes encoding brain-specific proteins implicated in neuronal function including *DPP6, ELFN2,* and *JPH4* encoding a neuron-specific junctophilin transmembrane protein (**Ext. Fig. 4E-F**). In CRISPR-edited clones, removal of the mutation reduced H3K27me3 deposition and led to concurrent gain of H3K27ac at promoters, H3K36me3 at gene bodies and increased expression of *JPH4* (**Ext. Fig. 4F**). Next, we assessed whether differentiation potential is affected by the G34R oncohistone. When edited clones were subjected to serum differentiation, we observed up-regulation of *DLX1/2,* but also *DLX5/6*, normally induced later in the course of interneuron differentiation (**Fig. 2F**). Edited clones showed enrichment of interneuron gene programs, and depletion of radial glia cell gene signatures, consistent with further progression into neuronal differentiation upon removal of the mutation (**Fig. 2G**).

To further define G34R-mediated epigenomic and transcriptomic signatures *in vivo*, we utilized the previously described *in-utero electroporation* (IUE) murine model[33]. E13.5 neocortices were electroporated with exogenous empty vector (EV) or H3.3^{G34R} in combination with *Pdgfra*^{WT}*, Atrx* shRNA, and sgRNA for *Tp53*, along with episomal vectors for Cas9 and pBase. We profiled *ex-vivo* expanded neural precursor cells derived from sorted *GFP*$^{+}$*TdTomato*$^{+}$ cells. Principal component analysis (PCA) of several histone marks and transcriptome revealed segregation of genotypes specifically with respect to H3K27me3 and H3K36me3 (**Ext. Fig. 5A**). Notably, reminiscent of H3K27me3 patterns in human G34R/V tumours and CRISPR

lines, the IUE cells also exhibited G34R-specific H3K27me3 retention at genic promoters such as *Jph4* and *Foxp2*, a transcription factor expressed in mature forebrain neurons (**Ext. Fig. 5B)**. By leveraging model systems to decouple the effect of G34 mutations from existing lineage-of-origin programs, we conclude that G34R/V oncohistones may directly impede terminal neuronal differentiation through aberrant H3K27me3 retention at specific neuronal maturation loci.

Active chromatin conformation facilitates PDGFRA co-option in G34R/V tumours

We next investigated the mechanism underlying the association of G34R/V interneuron progenitor programs with *PDGFRA*. The *PDGFRA* gene is located immediately adjacent to the interneuron transcription factor *GSX2* in mammalian genomes. In G34R/V tumours and cell lines, but not in other HGG subtypes, *PDGFRA* expression was positively correlated with *GSX2* (**Ext. Fig. 6A**). In fact, regardless of *PDGFRA* mutation status, G34R/V tumours showed significantly elevated *PDGFRA* expression compared to other HGG subgroups (*p-adj = 0.018*) (**Fig. 3A**). This transcriptional coupling observed in G34R/V tumours is likely aberrant since *PDGFRA* and *GSX2* are normally expressed in distinct cell lineages and developmental windows (**Fig. 3B, Ext. Fig. 6B-C**), suggesting that *PDGFRA* hijacks surrounding *cis* regulatory regions to promote its ectopic expression in G34R/V HGGs.

To determine whether epigenetic dysregulation underlies this transcriptional coupling, we examined *PDGFRA* adjacent *cis* elements. Consistent with expression profiles, we observed specific activation in the form of discrete H3K27ac peaks throughout a 350 kb region on chr4 encompassing *PDGFRA*, *GSX2* and *CHIC2*, that is unique to G34R/V and absent in all other HGGs including *PDGFRA*-amplified and non-amplified K27M HGGs (**Fig. 3C, Ext. Fig. 6D**). Indeed, these loci, specifically the *PDGFRA* promoter and an intragenic region within *CHIC2*, are amongst the top genomic bins showing differential H3K27ac deposition in G34R/V versus other HGGs (**Fig. 3D**). Within *CHIC2* is a VISTA validated enhancer element, *hs687,* shown to drive reporter expression in lateral ganglionic eminence of the foetal telencephalon[34], a pattern that overlaps with the nearby *GSX2*, which is detected in the ventricular zone of all three ganglionic eminences (**Fig. 3E, Ext. Fig. 6E)**.

To determine whether these H3K27ac peaks correspond to distal regulatory elements used to promote ectopic *PDGFRA* expression, we performed Hi-C chromosome conformation capture and CTCF ChIP-Seq on three G34R/V primary cell lines (**Fig. 3F**). We confirm the presence

of a topologically associated domain (TAD) encompassing *PDGFRA-RPL21P44* known to be present in non-cancerous somatic cells[35]. In addition, we uncovered a novel TAD linking *PDGFRA* and *GSX2,* anchored by CTCF in all 3 G34R/V glioma cell lines that is, in contrast, absent in iPSC-derived NPCs, neurons and glia[36] (**Fig. 3G**). Altogether, these results provide a mechanistic link for the G34R/V-context dependency of *PDGFRA* mutations, where *GSX2*-associated *cis*-regulatory elements are recruited to induce aberrant *PDGFRA* expression in G34R/V tumours, and possibly to a greater extent in *PDGFRA* mutants.

G34R/V HGGs are neuronal tumours devoid of oligodendroglial cells where PDGFRA mutations expand aberrant astrocytic compartments

To evaluate the effect of *PDGFRA* mutations on cellular heterogeneity of G34R/V HGGs, we profiled 6 $PDGFRA^{WT}$ and 10 $PDGFRA^{MUT}$ tumours by scRNA-seq (**Fig. 4A, Ext. Fig. 7A, Table S5)**. Projection of malignant cells to a reference developmental brain atlas[18] revealed that G34R/V tumours were predominantly comprised of neuron- and astrocyte-like cells, consistent with the reported heterogeneous histopathology. We observed a striking absence of oligodendroglial-like cells in all G34R/V tumours, in contrast to other paediatric and adult HGGs entities where they were readily detectable[37-39] and limited numbers of immune cells similar to the other gliomas (**Fig. 4B-C, Ext. Fig. 7B**). At the level of individual cells, G34R/V astrocytic and neuronal cells were highly dysplastic, with most cells displaying abnormal co-expression of both interneuron and astrocytic gene signatures and indefinite segregation between compartments (**Fig. 4C, Ext. Fig. 7C**). Across patients, G34R/V HGGs exhibited significant inter-tumour heterogeneity, with varying proportions of neuronal and astrocytic-like cells (**Fig. 4B, Ext. Fig. 7A**). Importantly, G34R/V tumours mutant for *PDGFRA* displayed an expansion of the astrocytic compartment when compared to wild type tumours (*p=0.028, wilcoxon rank sum test*), suggesting that acquisition of a *PDGFRA* mutation promotes an astrocytic state at the expense of the neuronal component.

Acquisition of PDGFRA mutations in G34R/V tumours is associated with gliomagenesis

We next examined the role of *PDGFRA* mutations in G34R/V tumour evolution from three available matched primary and recurrent tumour pairs (**Fig. 5A**, **Table S6)**. Phylogenetic trees inferred from allele frequencies revealed a remarkably high mutation burden consistent with a temozolomide treatment signature (n = 4,218) in P-1978 recurrence (G34R; $PDGFRA^{WT}$). In contrast, relatively few new mutations appeared in recurrences of patients P-1190 and P-3200 (43 and 124 mutations respectively), with *PDGFRA* being the only gene commonly mutated in

recurrences of both patients. Moreover, there was little evidence of clonal heterogeneity (**Fig. 5B)**, suggesting strong sweeping selection for the new *PDGFRA* mutations acquired. At the transcriptomic level, scRNAseq data from both patients showed an expansion of the astrocytic compartment, with concomitant decrease of the neuron-like compartment (**Fig. 5C**). To evaluate the effect of *PDGFRA* mutations on downstream MAPK/ERK or PI3K/AKT activation, we then performed phospho-ERK (pERK) and phospho-AKT (pAKT) immunohistochemistry on 2 of the primary/recurrence pairs and 2 unrelated G34R/V tumours (**Fig. 5D**, **Ext. Fig. 8B**). None of the 6 tumours stained positive for pAKT, whereas 5/6 tumours displayed pERK positivity. Notably, pERK reactivity was negative in the P-1190 primary tumour and gained intense staining at relapse concurrently with acquisition of a *PFGFRA* mutation. Together, these findings suggest that the acquisition of *PDGFRA* mutations in G34R/V activate MAPK signalling, leading to clonal selection, astrocytic expansion and potentially oncogenic addiction upon recurrence.

To delineate individual effects of *PDGFRA* and G34R/V mutations on gliomagenesis, we compared latency to tumour formation in the murine IUE and CRISPR cell line models. Consistent with known oncogenic properties of $PDGFRA^{D842V}$, immunohistochemical GFP staining showed extensive tumour growth in $Pdgfra^{D842V}$-electroporated mice in as little as one month (**Fig. 5E**). Addition of the G34R mutation did not impact latency in this model as both $H3f3a^{WT}$ and $H3f3a^{G34R}$ IUE mice showed similar tumour growth and survival (**Fig. 5F**). In contrast, GFP staining at two months upon electroporation of $Pdgfra^{WT}$ was largely negative. This finding is further reinforced in the time to tumour formation; at 12 months post-IUE, $H3f3a^{G34R};Pdgfra^{WT}$ formed tumours with ⅔ penetrance compared to $EV;Pdgfra^{WT}$ with similar latency and ⅓ penetrance. In contrast, $Pdgfra^{D842V}$ was strongly oncogenic and fully penetrant regardless of G34R presence, decreasing tumour latency for the entire cohort to 50 days. Consistent with IUE results, removal of H3.3G34V and G34R mutations in the respective patient-derived cell lines KNS-42 and HSJD-GBM002 showed limited effect on tumorigenicity in orthotopic xenograft models (**Ext. Fig. 8C-D**). These findings suggest that G34R/V mutations may be dispensable for oncogenic maintenance, and instead co-opt *PDGFRA* mutations to promote gliomagenesis.

DISCUSSION

We comprehensively profiled the molecular landscape of H3.3G34R/V HGGs in the largest multi-institutional cohort to date, which features several rare cases of matched primary and recurrence tumours. We show striking enrichment of activating *PDGFRA* mutations (50%) concurrent with elevated expression in G34R/V tumours relative to all other HGG subtypes. This frequency is likely higher in the recurrence setting, as tumours initially wild type acquired *PDGFRA* mutations with strong selective pressure for the mutant clone at recurrence. PDGFRA is an important RTK in glial development and a recurrent driver in HGGs, which show distinct mechanisms for its co-option. Indeed, *PDGFRA* amplifications are commonly observed in K27M-mutant midline gliomas (~30%)[1,3,5,40] and IDH-WT pro-neural HGGs[41]. Conversely, IDH1-mutant HGGs use a distal enhancer through DNA methylation of a proximal CTCF insulator to overexpress *PDGFRA*[35], while activating mutations, previously reported in less than11% of paediatric HGGs, are more frequently observed in hemispheric gliomas occurring in older children[16,42].

The high frequency of *PDGFRA* mutations unique to G34R/V HGG is likely due to their distinct developmental origin. Our data suggest G34R/V gliomas originate in interneuron progenitors found in the developing forebrain, where the oncohistone impedes further neuronal differentiation through aberrant H3K27me3 retention and altered H3K36me3 deposition, as suggested by prior biochemical analyses performed on G34R/V-mutant nucleosomes[13] (**Fig. 6**). This progenitor state likely maintains the transient, lineage-specific factor *GSX2* in an accessible chromatin conformation state, rendering the locus permissive to transcriptional exploitation by *PDGFRA.* Alternatively, the TAD observed in G34R/V may be transiently present during normal interneuron development. Regardless, this ectopic *PDGFRA* expression and subsequent acquisition of activating mutations likely promote astroglial features in the G34R/V-stalled interneuron progenitors. Notably, a *Nestin*- inducible K27M/*Tp53*^{cKO} mouse model aiming to model K27M midline gliomas led to neuronal high grade tumours, which became HGGs only upon co-expression of *PDGFRA*^{V544ins} [43], in keeping with a major role for *PDGFRA* mutations in driving gliogenesis. Specific induction of downstream MAPK signalling is probably necessary for the expansion of astrocytic lineage programs in G34R/V, as increasing ERK activation, but not AKT/PI3K pathway activation, has been shown to promote glial specification at the expense of neuronal lineages[44]. Finally, persistent high levels of *GSX2* and *DLX1/2* are likely responsible for the absence of oligodendroglial lineage programs in G34R/V HGGs, as down-regulation of these transcription factors is required for the transition from neurogenesis to oligodendrocyte formation in the ventral forebrain[25,26].

Lastly, our data suggest that *PDGFRA* mutants are robust glioma drivers in G34R/V HGGs and that, in contrast to K27M[45,46], G34R/V mutations can be dispensable for tumour maintenance. Our data show that~80% of *PDGFRA* mutations occurred in the extracellular domain, with the glioma-specific C235Y/F mutations alone accounting for 30% of all *PDGFRA* mutations in G34R/V. As recently shown for the Y288C mutant[17], these neomorphic mutations possibly have specific biochemical properties that render this RTK constitutively active, and warrant further investigations for optimal therapeutic targeting.

In sum, we unravel the specific developmental program in interneuron progenitors of the foetal ventral forebrain, which is permissive to the effects of G34R/V oncohistones. We highlight a novel mechanism where *PDGFRA* expression is ectopically promoted by the interneuron factor *GSX2*, potentially leading to the high rate of activating mutations specifically of this RTK in G34R/V. Cells which acquire a *PDGFRA* mutation undergo sweeping clonal selection, and potently activate MAPK signalling to promote gliomagenesis. Altogether, these mechanisms have important therapeutic implications as G34R/V HGGs are invariably lethal. *PDGFRA* mutations and downstream MAPK activation are potentially actionable targets, providing hope for novel therapeutic opportunities in this deadly cancer.

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Competing Interests

PB and RB receive grant funding from the Novartis Institute of Biomedical Research for an unrelated project.

Authors Contributions

C.C.L.C. and S.D. led the study design, performed functional studies, analyzed and interpreted data, and wrote the manuscript. V.L. and S.J. led the study design, performed data analysis and interpretation, and wrote the manuscript. D.H. and M. B. led bioinformatic analysis of Hi-C data and interpreted the results. A.F.A., M.Z., and C.R. performed the PDOX experiments contributed to data analysis/interpretation. D.F. facilitated patient sample acquisition, processed patient samples, and contributed to data interpretation. H.S., F.D., E.W., S.H., and T.G. performed bioinformatic analysis on exome sequencing data and interpreted the results. M.P., D. A., N.M. and P.S. generated the IUE mouse model. M.C., N.D.J., and S.M. contributed to bioinformatic analysis of the transcriptomic data and interpretation of results. J.K., S.A., S.B., and B.E. assessed and interpreted histopathology results. A.B. and A.S.H. performed experiments and data analysis/interpretation. D.M.M. and B.G. led the histone proteomics experiments and analysis. L.G.M. facilitated patient sample acquisition and contributed to manuscript preparation. N. Juretic facilitated patient sample acquisition. A.V.B. and A.M.B. performed *in silico* modelling of *PDGFRA* mutations. P.H., A.K., M.Z., A.V., L.K., K.V., J.Z., D.S., P.G.E, D.S.Z., J.R.H., D.A.K.Q., and A.G.W collected patient samples. L.G., R.B., M.D.T., P.B, D.T.W.J, and P.S. contributed to study design and data interpretation. C.L.K. and N.J. co-led and supervised all aspects of the project.

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FIGURE LEGENDS

Figure 1. PDGFRA is frequently mutated in G34R/V tumours.
A. Oncoprint showing frequent occurrence of *PDGFRA* mutations in G34R/V (n = 86), relative to K27M (n = 53), IDH1/SETD2 (n = 80), and H3/IDH1-WT (n = 28) HGG subgroups, details in Table 1. Lines linking consecutive G34R/V samples indicate primary/recurrent tumours from the same patient. **B**. Spectrum and frequency of *PDGFRA* somatic mutations identified in G34R/V HGGs. Recurrent mutations (observed in 2 or more patients) are listed. Bold face indicates mutations which were previously characterized as activating; references in Table 2.

Figure 2. Transcriptional and epigenetic programs in G34R/V gliomas indicate a ventral interneuron progenitor origin.
A. Heatmap of enrichment scores of forebrain developmental cell type signatures in G34R/V compared to IDH1, K27M, and WT HGG subgroups, by gene set enrichment analysis (GSEA). Scores are shown for all signatures significantly enriched (*adjusted p-value < 0.01*) in G34R/V vs. IDH1. **B**. Schematic of the embryonic forebrain, coronal section. LGE: lateral ganglionic eminence; MGE: medial ganglionic eminence, RGC: radial glia cell; IPC: intermediate neuronal progenitor cell. **C**. Promoter-associated H3K27ac and H3K27me3 for genes significantly differentially expressed between G34R/V and IDH1 HGG by bulk RNA-seq. Genes relevant to glioma (*OLIG2*), or with high G34R/V-enrichment of either mark (z-score > 0.9) and RNA-seq absolute log2 fold change > 3 are labelled. **D**. Trajectory inference of interneuron differentiation from mouse scRNA-seq developmental forebrain data. Top panel: density of each cell type along the inferred trajectory, pseudotime, represented by the x-axis. Bottom: heatmaps depicting the expression across pseudotime of genes enriched or depleted, respectively, in G34R/V. Expression is z-scored across pseudotime. **E**. Expression of *DLX1/2/5/6*, transcription factors in the patient-derived cell line HSJD-GBM002 in stem cell media. Green: unedited or parental clones containing G34R (n = 6); blue: edited clones with the G34R mutation removed by CRISPR (n = 6). Horizontal bar indicates the median. *p-values*: *DLX1* (0.9), *DLX2* (0.5), *DLX5* (0.04), *DLX6* (0.0008). **F**. Expression of *DLX1/2/5/6* in HSJD-GBM002 clones in differentiation media. Green: unedited or parental G34R clones (n = 2), blue: edited clones with the G34R mutation removed by CRISPR (n = 2). Horizontal bar indicates the median. *p-values*: *DLX1* (0.0001), *DLX2* (0.001), *DLX5* (< 0.0001), *DLX6* (0.04). **G**. Single-sample gene set enrichment (ssGSEA) score for RGC and interneuron gene signatures in transcriptomes of HSJD-GBM002 clones in serum.

Figure 3. Active chromatin conformation promotes *GSX2-PDGFRA* co-expression in G34R/V tumours.
A. Expression of *PDGFRA* in HGGs. *PDGFRA* is significantly upregulated in G34R/V samples compared to others (*adjusted p-value = 0.018*). **B.** *Pdgfra, Gsx2, and Dlx1/2* expression in mouse scRNA-seq developmental forebrain atlas. For each neuroectodermal cell population (tree leaves), mean expression and proportion of cells expressing the gene are indicated. OPC: oligodendrocyte progenitor cells. **C.** Composite H3K27ac ChIP-seq of primary cortical glioma G34R/V, IDH1/SETD2, and WT subgroups. Teal lines indicate H3K27ac-enriched regions that are specific to G34R/V tumours, * denotes significantly H3K27ac-

enriched in G34R/V (z-score > 0.5, *p-value < 0.05*). **D**. 5kb genomic bins ranked by H3K27ac z-score, showing the top-ranking loci with H3K27ac enrichment in G34R/V vs. non-G34 HGGs. **E.** Enhancer reporter activity of *hs687-LacZ* in coronal forebrain sections of E11.5 mouse embryos profiled by VISTA enhancer browser, and expression of nearby *Gsx2* as profiled by in-situ hybridization (ISH) in E13.5 mouse embryo by Allen Brain Atlas. LGE: lateral ganglionic eminence. MGE: medial ganglionic eminence. **F.** Hi-C heatmaps from G34R/V cell lines depicting sub-TAD structure at the *GSX2-PDGFRA* locus (black line) within the larger TAD formed by contact to the known distal insulator (dashed line). Heatmap represents the log2 ratios of observed interactions relative to expected interactions across the *GSX2-PDGFRA* locus at a 5kb resolution (red = positive, blue = negative). All observed and expected matrices were computed using HOMER accounting for both the linear distance between two given loci and the sequencing depth. Below, intersection with composite CTCF ChIP-seq from G34R/V cell lines. **G**. Hi-C heatmaps from human iPSC-derived neural progenitor cells (NPC), neurons, and glia at the *GSX2-PDGFRA* locus.

Figure 4. G34R/V tumours are devoid of oligodendrocytes, and *PDGFRA* mutant tumours exhibit expanded astrocytic compartments.

A. UMAP embedding of scRNA-seq G34R/V patient samples colour coded according to patient of origin (left, teal: $PDGFRA^{WT}$ tumours, red: $PDGFRA^{MUT}$) or consensus cell type projection (right panels). Inset pie charts depict the proportion of each cell type in each tumour. The two primary/recurrence tumour pairs are highlighted with arrowheads. **B.** Radar plots contrasting the abundance of the oligodendroglial lineage in the G34R/V tumours compared to other HGG entities including K27M, IDH1, paediatric and adult H3/IDH1-WT. Each line represents one sample, colour coded as in (A) for G34R/V tumours. Extension of the line segment represents the proportion of cells projected to each type in each tumour. Outer circle: 100%, middle circle: 50%, inner circle: 0%. **C.** Mean expression of foetal interneuron and astrocyte gene signatures in individual cells from the mouse scRNA-seq developmental forebrain atlas (left), and in cells from across G34R/V tumours (right). For tumours, cells called malignant and projected as neurons or astrocytes were included.

Figure 5. Acquisition of *PDGFRA* mutations in G34R/V tumours is associated with gliomagenesis.

A. Phylogeny of G34R/V primary and matched recurrence samples from patients P-1978, P-1190, and P-3200. Scale bar: 10 mutations. Dashed lines: potentially germline mutations. **B**. Fish plot depicting tumour clonal structure of G34R/V primary and matched recurrence samples from patients P-1978, P-1190, and P-3200. **C.** Doughnut plots of the proportion of cells projected to each type in two primary/recurrence pairs, highlighting increased proportion in astrocyte-like cells observed in the $PDGFRA^{MUT}$ recurrence (outer circle) compared to the $PDGFRA^{WT}$ primary tumour (inner circle). **D.** Immunohistochemical analysis of G34R HGGs using antibodies specific for phosphorylated ERK and phosphorylated AKT. **E.** Immunohistochemical GFP staining of coronal forebrain sections from in-utero electroporated mice. All mice received *shAtrx, sgTp53* in addition to PDGFRA WT or D842V, and H3.3 WT/G34R. **F.** Kaplan-Meier curve depicting survival of in-utero electroporated mice. All mice

received *shAtrx, sgTp53* in addition to $Pdgfra^{WT}$ or $Pdgra^{D842V}$, and empty vector/H3.3 WT/G34R.

Figure 6. Model of aberrant development in G34R/V gliomas, compared to normal development in the ventral forebrain.
Left: During normal development, radial glial cells (RGC) in the ventral forebrain give rise to oligodendrocyte precursor cells (OPC) which differentiate into oligodendrocytes, and intermediate neuronal progenitor cells (IPC) which differentiate into cortical interneurons. Right: G34R/V gliomas retain molecular features of a committed interneuron progenitor (*GSX2, DLX1/2+*) and oncohistone-mediated H3K27me3 gain may impede terminal neuronal differentiation. G34R/V tumours exhibit dual neuronal and astrocytic components. Elevated expression and oncogenic *PDGFRA* mutation may promote the abnormal astrocyte-like state. G34R/V HGGs demonstrate a TAD which brings *GSX2*-associated *cis*-regulatory elements into proximity with the *PDGFRA* promoter to induce overexpression.

EXTENDED FIGURE LEGENDS

Extended Figure 1. Recurrent somatic mutations in G34R/V HGGs.
Oncoprint depicting recurrently mutated genes (observed in > 2 patients) in G34R/V HGG, grouped by associated gene functions as determined by Gene Ontology. Columns adjacent to gene names represent the number of patients in which mutations were called, in the entire cohort or stratified by *PDGFRA* mutation status (red = $PDGFRA^{MUT}$, green = $PDGFRA^{WT}$) . Underlined genes indicate a bias towards $PDGFRA^{WT}$ G34 samples.

Extended Figure 2. Extracellular PDGFRA mutations identified in G34R/V HGGs.
Complex structure diagrams depicting extracellular PDGFRA mutations **A.** C235Y/F, **B.** Y288C, and **C.** K385M. Crystal structures of PDGFRB (PDB: 3MJG) were used to model C235 and Y288 mutations, and VGFR1 (PDB: 5T89) for K385. The C235Y/F mutations destabilize the domain, leaving C291 with the ability to form another inter- or intramolecular disulfide bond, potentially leading to dimerization and receptor activation, analogous to Y288C. In the wild-type, the positive charge of two lysine 385 residues at the receptor dimerization interface creates unfavourable repulsive charge effects. The K385M mutation may promote a small hydrophobic patch adjacent to several salt bridges to enhance dimer formation.

Extended Figure 3. G34R/V uniquely exhibits a transcriptome program specific to the ventral forebrain interneuron lineage.
A. Gene set enrichment analysis (GSEA) plots evaluating the enrichment or depletion of representative cell type signatures in G34R/V compared to IDH1 HGGs or compared to HGNET-BCOR (astrocyte signature only). NES: normalized enrichment score. **B**. Heatmap of GSEA normalized enrichment scores of forebrain astrocyte signatures in G34R/V tumours compared to HGNET-BCOR tumours. **C**. Boxplots of expression levels of the human radial glia marker *MOXD1* and markers for cortical inhibitory and excitatory neurons in G34R/V,

other HGG subgroups, and HGNET-BCOR. For genes upregulated in G34R/V, adjusted p-values for comparison of G34R/V to other entities are indicated in parentheses. **D**. Gene ontology analysis of differentially expressed genes that showed significant H3K27ac or H3K27me3 enrichment in G34R/V tumours (z-score > 0.5). **E**. Core transcription factors (inferred by H3K27ac enrichment) activated in G34R/V compared to other HGGs, non-glioma brain tumours, and normal brain. TFs from cluster 2, indicated, lack activation signal in G34R/V HGG specifically. Bolded genes indicate transcription factors annotated as necessary for oligodendrocyte differentiation (*FDR = 0.0001*) based on Gene ontology enrichment analysis.

Extended Figure 4. G34R/V promotes aberrant H3K27me3 retention at terminal neuronal genes.

A. Quantification of H3K27 and H3K36 methylation states by histone mass spectrometry on the mutant H3.3G34R or H3.3G34V histone normalized by H3.3 WT abundance in PS10-801 (left, n = 3), HSJD-GBM002 (middle, n = 1) or KNS-42 (right, n = 6) cell lines respectively. * denotes p-value < 0.05 (paired t-test). **B.** Volcano plot depicting differential H3K27me3 enrichment between G34R/V and non-G34R/V cortical HGGs at all annotated promoters. Blue data points indicate G34R/V-specific H3K27me3+ genes (*z-score > 0.5, p-value < 0.05*) involved in spontaneous synaptic transmission (*FDR = 0.0273*), calcium ion-regulated exocytosis of neurotransmitter (*FDR = 0.000269*), and regulation of GABAergic synaptic transmission (*FDR = 0.00332*). **C.** Volcano plot depicting differential H3K27ac enrichment between G34R/V and non-G34R/V cortical HGGs at all annotated promoters. Red data points indicate G34R/V-specific H3K27ac+ genes (*z-score > 0.5, p-value < 0.05*) involved in neuron fate commitment (*FDR = 0.00243*). **D.** Immunofluorescence validation of CRISPR-mediated editing of G34R in HSJD-GBM002 clones. **E.** Genome browser snapshot depicting H3K27me3 profiles in cortical tumours (above) at the *JPH4* locus. Note specific H3K27me3 enrichment at the promoter prominent in G34R/V tumours versus non-G34 HGGs. Below, profile of H3K27me3, H3K27ac, and H3K36me3 in HSJD-GBM002 CRISPR clones indicating that G34R editing is associated with decrease H3K27me3, increase of H3K27ac, and increase H3K36me3. **F**. Parallel coordinate plot depicting concurrent epigenomic changes observed in genome-wide 5kb bins. Bins were separated into quintiles based on change in H3K36me3 and tracked across the other epigenomic and transcriptomic features. Solid line = median, shaded area = 25% and 75% percentile. Note that genomic bins losing H3K36me3 (blue line) concurrently gain SUZ12 and H3K27me3, and lose H3K27ac, and expression.

Extended Figure 5. Introduction of G34R in murine embryonic forebrain induces H3K27me3/H3K36me3 epigenomic reprogramming.

A. Principal component analysis (PCA) plot of H3K27me3, H3K36me3, H3K27ac, and RNA-seq at autosomal genomic regions in IUE cohort. EV: empty vector. **B.** Genome browser snapshot of ChIP-seq and RNA-seq profiles in IUE-derived neural precursor cells showing H3K27me3 profiles associated with G34R-introduction. Some genic promoters, such as *Foxp2* (right) and *Jph4* (left) show striking H3K27me3 enrichment uniquely in G34R-transduced cells.

Extended Figure 6. Hyperactive chromatin conformation promotes *GSX2-PDGFRA* co-expression in G34R/V HGGs.

A. Normalized expression of *PDGFRA* and *GSX2* in bulk RNA-seq of G34R/V tumours and cell lines (left) and other tumour entities (right). Circles: tumours, triangles: cell lines. For G34R/V tumours: open shapes: $PDGFRA^{MUT}$, closed shapes: $PDGFRA^{WT}$. Pearson correlation: G34R/V tumours (*correlation=0.69,* R^2*=0.49, p-value=0.001*), H3K27M (*correlation=0.49,* R^2*=0.24, p-value=0.09*), IDH1 (*correlation=0.12,* R^2*=0.02, p-value=0.73*), WT (*correlation=0.29,* R^2*=0.08, p-value=0.45*), HGNET-BCOR (*correlation=-0.61,* R^2*=0.37, p-value=0.27*). **B**. *PDGFRA, GSX2 and DLX1/2* expression in cell populations from scRNA-seq atlas of the developing human telencephalon. For each cell population, mean expression and proportion of cells expressing the gene are indicated. Dendrograms were constructed based on pairwise Spearman correlations of gene expression. OPC: oligodendrocyte progenitor cells. **C**. Expression of *PDGFRA* and *GSX2* in the human brain across the lifespan, using data from the BrainSpan atlas. Expression as a function of age is estimated using LOESS smoothing. "Ventral forebrain & other telencephalon" includes the medial, caudal and lateral ganglionic eminences, striatum, amygdaloid complex, and hippocampus. "Diencephalon & hindbrain" includes the thalamus, cerebellum, cerebellar cortex, and upper rhombic lip. **D.** H3K27ac profile in G34R/V tumours (n = 7), IDH1 tumours (n = 5), SETD2 tumours (n = 5), WT tumours (n = 3), and K27M tumours (n = 4). $PDGFRA^{MUT}$ samples are distinguished by darker teal. Note *PDGFRA* is amplified in one K27M sample (first yellow track). **E.** Enhancer reporter activity of *hs687-lacZ* in full mount E11.5 mouse embryos profiled by VISTA enhancer browser, and expression of nearby *Gsx2* as profiled by in-situ hybridization (ISH) in E13.5 mouse embryo by Allen Brain Atlas. CGE: caudal ganglionic eminence.

Extended Figure 7. G34 HGGs are devoid of oligodendrocyte lineage, and co-option of $PDGFRA^{MUT}$ promotes neural-to-astrocytic transition.

A. Bubble plot representing the composition of cell types in each tumour. Circle size represents the proportion of each cell type per tumour, and circle colour represents the total of malignant cells in the tumour. **B**. UMAP embedding of IDH1, K27M, H3/IDH1-WT HGG scRNA-seq datasets, colour coded by patient of origin (top) and cell type projection (bottom). Oligodendrocytes (in green) are clearly detected in all non G34R/V HGG entities. **C**. Mean expression of foetal interneuron and astrocyte gene signatures in individual cells from the normal human telencephalon. Cells of the interneuron lineage, as well as radial glial cells and astrocytes were included.

Extended Figure 8. G34R/V HGGs show intense pERK activation while G34R/V may be dispensable for tumorigenesis.

A. Digital droplet PCR (ddPCR) of *PDGFRA* amplicons specific for WT (blue) and mutant (orange) K385M and Y288C alleles in matched primary and recurrence G34V tumours of patients P-1190 and P-3200 respectively. **B.** Immunohistochemical analysis of G34R HGGs using hematoxylic & eosin (H&E), phosphorylated ERK, and phosphorylated AKT. Square outline indicates general area where the inset image is taken from. Below, inset images of pERK/pAKT under 15x magnification depicting positive pERK nuclear staining in all samples except P-1190 primary. **C.** Immunofluorescence validation of CRISPR-mediated

repair/removal of G34V in KNS-42 clones. **D.** Kaplan-Meier survival curves of KNS-42 G34V clones. Green depicts unedited or parental clones containing G34R, blue depicts edited clones.

SUPPLEMENTARY TABLES

Table S1. PDGFRA mutations in G34R/V, K27M, IDH1/SETD2, and WT HGG subgroups. (Related to Fig. 1A)

Table S2. Extended information on PDGFRA mutations found in G34R/V HGGs. (Related to Fig. 1B)

Table S3. Analysis of bulk RNA-seq profiles in G34R/V HGGs. (Related to Fig. 2 and Ext. Fig. 3)

Table S4. Analysis of H3K27me3 and H3K27ac ChIP-seq profiles in G34R/V HGGs. (Related to Fig. 2 and Ext. Fig. 4)

Table S5. Overview of scRNA-seq data for G34R/V HGGs. (Related to Fig. 4 and Ext. Fig. 7)

Table S6. G34R/V primary/recurrence mutation comparison for P-1190, P-3200, and P-1978. (Related to Fig. 5A)

Table S7. Extended information on in-house G34R/V HGG samples characterized by pathology, transcriptomics, and epigenomics.

Table S8. Extended information on G34R/V cell lines and murine IUE samples characterized by transcriptomics and epigenomics.