Epigenetic activation of a RAS/MYC axis in H3.3K27M-driven cancer

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Histone H3 lysine 27 (H3K27M) mutations represent the canonical oncohistone, occurring frequently in midline gliomas but also identified in haematopoietic malignancies and carcinomas. H3K27M functions, at least in part, through widespread changes in H3K27 trimethylation but its role in tumour initiation remains obscure. To address this, we created a transgenic mouse expressing H3.3K27M in diverse progenitor cell populations. H3.3K27M expression drives tumorigenesis in multiple tissues, which is further enhanced by Trp53 deletion. We find that H3.3K27M epigenetically activates a transcriptome, enriched for PRC2 and SOX10 targets, that overrides developmental and tissue specificity and is conserved between H3.3K27M-mutant mouse and human tumours. A key feature of the H3K27M transcriptome is activation of a RAS/MYC axis, which we find can be targeted therapeutically in isogenic and primary DIPG cell lines with H3.3K27M mutations, providing an explanation for the common co-occurrence of alterations in these pathways in human H3.3K27M-driven cancer. Taken together, these results show how H3.3K27M-driven transcriptome remodelling promotes tumorigenesis and will be critical for targeting cancers with these mutations.
Diffuse Intrinsic Pontine Glioma (DIPG) is a devastating paediatric high-grade glioma (HGG) that diffusely penetrates the brainstem and is the leading cause of paediatric brain tumour death. Surgery is not possible, radiation is largely palliative, and conventional chemotherapy and targeted agents have proven ineffective. Around 80% of DIPGs harbour lysine 27-methionine (K27M) substitutions in histones H3.3 (H3F3A) and H3.1 (HIST1H3B/C)-3. More recently H3K27 mutations have also been found in a range of other tumours including haematopoietic malignancies and carcinomas-8. Thus, H3K27M is assumed to be an oncohistone, although the precise nature of its oncogenic function remains obscure.

H3K27 is a key hub for transcriptional regulation: acetylation (H3K27ac) is associated with active enhancers while trimethylation (H3K27me3) represses transcription-9. H3K27M has increased affinity for, and inhibits the activity of, Enhancer of zeste homologue 2 (EZH2), the catalytic subunit of Polycomb repressive complex 2 (PRC2) that deposits H3K27me3, leading to widespread H3K27me3 loss. However, H3K27M also leads to H3K27me3 gains in some areas, suggesting that aberrant gene expression and repression may both be important in DIPG. Despite clonal analysis indicating that H3K27M is the tumour-initiating mutation in DIPGs, in vivo models of H3K27M-driven cancer, which to date have focused on brain tumours, have failed to generate tumours without additional oncogenic drivers. The endogenous H3.3a locus was modified to allow H3.3K27M expression in Nestin-positive cells, where neuronal H3.3K27M expression decreased the latency of gliomas driven by Trp53 loss and constitutively active Pdgfra17. In experiments using RCAS-TVA retroviral delivery, neuronal targeting of Nestin-positive brainstem progenitors with H3.3K27M accelerated tumours driven by Pdgfb overexpression and Trp53 loss. Similarly, H3.3K27M decreased the latency of glioma formation in in utero electroporation experiments introducing H3.3K27M alongside Trp53 loss. Pdgfb overexpression, or WT or constitutively active (D842V) Pdgfb overexpression19,20. None of these models yielded tumours where the sole exogenous mutation was H3.3K27M. Further, to produce tumours, H3.3K27M was introduced alongside other strong oncogenic drivers making it difficult to parse out the direct role of the mutant histone in oncogenesis. Overall, these models suggest that H3K27M cooperates with p53 dysfunction and constitutive RAS/MAPK activation, but is not itself sufficient to initiate tumorigenesis, at least in the cells and developmental time points tested thus far. This cooperation with p53 and the RAS/MAPK pathway is supported by the frequent co-occurrence of PDGFRα and TP53 alterations with H3K27M in human DIPGs, although in human tumours H3K27M is clearly the initiating event21,22. Thus, how H3.3K27M alters the cell state to permit or initiate tumorigenesis and the basis of its cooperation with additional mutations remains elusive. A clearer understanding of these mechanisms is critical to developing appropriate therapies for this devastating disease.

Here we used a mouse model expressing H3.3K27M from the Fabp7 promoter in both neural and non-neural precursor cells to investigate H3K27M-driven cancer. H3.3K27M alone was sufficient to drive development of lymphomas and carcinomas, while Trp53 loss was required for high grade gliomas to form. H3.3K27M imposed a common transcriptome, mirroring early gliomagenesis, which was shared between H3K27M-driven mouse tumours from all sites, H3.3K27M mouse embryonic brainstem and human DIPGs. A main component of the H3.3K27M transcriptome was an activated RAS/MYC axis which could be targeted therapeutically. H3K27M-mutant tumours acquired secondary mutations to reinforce this activation, suggesting a model where early epigenetic pathway activation by H3K27M is later reinforced by pathway activating genetic mutations and explaining the co-occurrence of these mutations in human DIPG.

Results

H3.3K27M increases cancer incidence and leads to early death. CD-1 mice, expressing FLAG/HA-tagged H3.3K27M (H3F3a-K27M) under the control of the Fabp7 promoter, were engineered by microinjection, with colonies established from 3 founders (Supplementary Fig. S1a–c). Fabp7 promoter-directed H3.3K27M expression was compatible with viable embryonic and postnatal development; adult mice had similar phenotype and litter size to CD1 wild-type mice, although E12.5 and E14.5 H3.3K27M-positive embryos occasionally developed signs of central nervous system haemorrhage (Supplementary Fig. S1d). At the gene expression level, Fabp7 expression is highest in brain during embryogenesis, but is expressed in adult brain as well as in other embryonic and adult tissues including liver, intestine, heart, lung and bone (Supplementary Fig. S1e). Correspondingly, we saw that H3.3K27M expression was highest in brain, yet readily detectable in other tissues (Supplementary Fig. S1f, g)23–26. Importantly, the H3.3K27M-FLAG/HA transgene was not overexpressed versus endogenous H3.3 in the mouse samples or when compared to H3K27M levels in human DIPG (Supplementary Fig. S1h, i).

H3.3K27M mice had 62% reduced survival (all-cause mortality) compared to CD1 controls at two years (p = 5.5 × 10^-5; Fig. 1a, Supplementary Data 2), and were 67% more likely to die from cancer (p = 0.005; Fig. 1b). They had a 2.7-fold increase in both lymphoma (p = 3.1 × 10^-4) and carcinoma (p = 9.6 × 10^-4) compared with controls (Fig. 1b, Supplementary Data 2)28–30. H3.3K27M lymphomas most frequently involved the mediastinum (45%) and were disseminated in 36% of mice, with immune-phenotyping showing them to be T-cell lymphomas (B220^-/CD3^+. Supplementary Fig. S2a). Carcinomas were usually adenocarcinoma and primarily involved the lung (Fig. 1c), with significantly increased incidence in H3.3K27M mice compared with CD1 controls (p = 0.02). Both lymphomas and carcinomas expressed H3.3K27M and FABP7, indicating that they were driven by transgene expression rather than forming spontaneously (Supplementary Fig. S2b). Together, this indicates that H3.3K27M expression drives a significantly increased incidence of lymphoma (p = 4.2 × 10^-4) and carcinoma (p = 0.001) in mice. This is the first evidence that H3.3K27M alone can cause cancer and that it functions as a pan-cancer driver.

H3.3K27M induces gliomas and increases lymphomagenesis in Trp53-deficient mice. As H3.3K27M and TP53 alterations are highly co-associated in human gliomas, we crossed H3.3K27M mice with Trp53^+/− deficient animals. Morphologic and immunophenotypic high grade gliomas (HGG) developed at low penetrance in both H3.3K27M/Trp53^+/− and H3.3K27M/Trp53^−/− mice, but not in H3.3K27M, Trp53^+/− or Trp53^−/− mice (Fig. 1b, c and Supplementary Fig. S2c, Supplementary Data 2). Most had leptomeningeal dissemination, with tumour masses involving the cerebellum, cerebral hemispheres and/or base of the third ventricle. A similar proportion (~70%) of mouse HGG and human DIPG overexpressed OLIG2 (Supplementary Fig. S2c, d).

H3.3K27M/Trp53^−/− mice developed more lymphomas (56%) than H3.3K27M (42%) or Trp53^−/− (32%) animals, and had a 2-fold reduced median survival compared with Trp53^−/− mice (p < 0.0001; Fig. 1b, d, Supplementary Data 2)30,31. All tumours in H3.3K27M mice, regardless of tissue of origin, maintained H3.3K27M expression (Supplementary Fig. S2b). Overall, H3.3K27M mice have increased cancer incidence with decreased latency, with gliomas and lymphomas both increased by Trp53 loss.
H3.3K27M-driven mouse and human tumours have similar genomic features. We performed whole exome sequencing (WES) on 10 tumours from 8 H3.3K27M, H3.3K27M/Trp53+/− or H3.3K27M/Trp53−/− mice to investigate the evolution of H3.3K27M-driven tumorigenesis. We compared the genetic features of the H3K27M mutant mouse tumours with those of human brain and non-brain tumours. The mutation frequency was similar between mouse and human tumours (Fig. 1e, Supplementary Data 3). The burden of copy number variations (CNV) and the fraction of the genome affected by CNVs was also similar between mouse tumours and human H3.3K27M DIPG (Fig. 1f, g and Supplementary Fig. S2e; Supplementary Data 3).
Further analysis showed that SNVs and CNVs were acquired in known driver genes of human cancer (including DIPG). These included \( Kras \) (HGG, carcinoma), \( Nf1 \) (HGG), \( Pten \) (carcinoma), \( Myc \) (HGG, carcinoma) and \( Notch \) (lymphoma) (Fig. 1h). Interestingly, somatic \( Trp53 \) alterations were acquired in 2 tumours, highlighting the cooperative importance of p53 dysfunction in H3.K27M-driven tumorigenesis. KP29 (H3.K27M\(^{-/-}\)) lung carcinoma lost the copy of chromosome 11 (Supplementary Fig. S2e) carrying the wild-type \( Trp53 \) allele, resulting in a \( Trp53^{-/-} \) tumour, while K1327 (\( Trp53^{+/-} \)) lung carcinoma acquired an R62Q mutation in the p53 transactivation domain, which is critical for apoptotic signalling (Supplementary Fig. S2f)\(^{33,35} \). The latter was detected by Sanger sequencing but was a sub-clonal, presumably later-occurring, variant. Overall, H3.K27M mouse tumours show similar mutation patterns to human DIPG, with frequent mutations in the RAS/MAPK/PI3K pathway (50%) and MYC (20%; Fig. 1h).

H3.K27M rapidly disrupts transcriptional networks to establish a glioma-like, proliferative phenotype in mid-development with SOX10. In order to investigate the earliest events resulting from H3.K27M expression we examined brainstem tissue from E14.5 mouse embryos, ~5 days after H3.K27M expression is induced from the \( Fabp7 \) promoter. We further generated isogenic cell lines expressing H3.K27M or empty vector (EV) using the human oligodendrocyte precursor cell (OPC) line MO3.13, as OPCs are a proposed cell of origin for DIPG\(^{34} \).

H3.K27M has an inhibitory effect on PRC2, and its expression in E14.5 midbrain/hindbrain (MB/HB) or MO3.13 cells reduced H3.K27M\(_{m3}\) compared to controls (Fig. 2a, b and Supplementary Fig. S3a). This was accompanied by an increase in the proliferative markers PCNA and phospho-H3.S10 in H3.K27M mice compared with WT (Fig. 2a). A similar increase in proliferation was seen in the MO3.13 cells compared with empty vector (EV) and H3.3WT-expressing control cells (Fig. 2c). In a scratch assay, while EV and H3.3WT cells failed to close a wound after 24 h, H3.K27M-expressing MO3.13 cells had fully closed the wound, further suggesting that H3.K27M confers protumorigenic behaviour on MO3.13 cells (Supplementary Fig. S3b).

To better understand the acute transcriptional effects of H3.K27M expression and the proliferative E14.5 phenotype, we performed RNA sequencing (RNA-Seq), identifying 992 up- and 50 down-regulated genes differentially expressed between H3.K27M and WT MB/HB at E14.5 (Supplementary Fig. S3c, Supplementary Data 1). H3.K27M expression level strongly correlated with the degree of gene upregulation (Pearson’s correlation 0.999, \( p \approx 0.001 \); Supplementary Fig. S3d, e). In keeping with the known inhibitory effect of H3.K27M on PRC2, gene set enrichment analysis (GSEA) revealed significantly increased expression of known PRC2 target genes (Fig. 2d), including the PRC2-repressed \( Hox \) gene family that has important functions in brain development (Supplementary Fig. S3f, g)\(^{35,36} \). By chromatin-immunoprecipitation (ChIP) we found that the \( Hoxa3, Hoxb4 \) and \( Hoxc8 \) promoters had reduced H3.K27M\(_{m3}\) in H3.K27M MB/HB compared with WT (Supplementary Fig. S3h) suggesting that H3.K27M upregulates them via PRC2 inhibition. H3.K27M expression in MO3.13 cells also resulted in \( Hoxb4 \) upregulation (Supplementary Fig. S3i).

Fewer than 10% of the significantly upregulated genes were direct PRC2 targets as defined in MSigDB\(^{37,38} \) indicating that, despite the importance of PRC2 in H3.K27M function, pathways beyond PRC2 are also relevant to the transforming effects of H3.K27M (Figs. 3a, 5a and Supplementary Fig. S4a). Among the upregulated expressed genes at E14.5, nearly 20% were associated with OPcs/oligodendrocyte differentiation, <10% with astrocyte differentiation and very few with neuronal differentiation or NSCs (Fig. 2e and Supplementary Fig. S4b)\(^{39} \). Promoters of neuronal differentiation were downregulated, while several inhibitors of neurogenesis were upregulated (Fig. 2e and Supplementary Fig. S4c)\(^{39} \). Oligodendrogenic markers (OLIG1, OLIG2, O4) were increased at both E14.5 and E16.5, neuronal markers (TUJ1, DCBN) were decreased at E14.5 and the astrocyte marker GFAP was unchanged (Supplementary Fig. S4d), suggesting that H3.K27M affects cell specification.

To evaluate the potential of H3.K27M to regulate oligodendrocyte transcriptional programmes, we differentiated MO3.13 OPCs into oligodendrocytes (OL) using serum starvation and phorbol 12-myristate 13-acetate (PMA)\(^{40} \), as monitored by decreasing NG2 expression (Fig. 2f, g). S100 calcium binding protein beta (S100-\( \beta \)), myelin basic protein (MBP) and \( 2′,3′ \)-cyclic nucleotide 3'-phosphodiesterase (CNP), which are upregulated during OPC differentiation into immature/early OL\(^{41,42} \), were induced by H3.K27M expression and maintained after treatment with differentiation media for 4 days (Fig. 2g, h). SOX2, a marker of undifferentiated and cancer stem cells that is increasingly implicated in numerous oncogenic processes\(^{43} \), was also induced in H3.K27M, but not in E14.5 or E16.5, neuronal markers (TUJ1, DCBN) were decreased at E14.5 and the astrocyte marker GFAP was unchanged (Supplementary Fig. S4d), suggesting that H3.K27M affects cell specification.
differentiation programme to promote early/mid OL gene expression but not terminal differentiation, instead continuing to drive proliferation and induce characteristics of undifferentiated cells.

While loss of H3K27me3 is permissive for gene transcription, this loss alone is not enough; transcriptional upregulation is also dependent on the presence of transcription factors. We combined differential gene expression, transcription factor binding sites and protein-protein interactions to identify potential master regulators of the H3K27M phenotype. We found that the transcription factor SOX10 directly regulates ~30% of genes upregulated by H3.3K27M in E14.5 MB/HB, 3.4x more than are direct PRC2-targets (Fig. 3a). SOX10 influences lineage commitment in the developing brain to promote oligodendrogenesis45, and we noted
that it was upregulated in H3.3K27M MB/HB at E14.5 (Figs. 2e, 3b and Supplementary Fig. S4b, e) as well as H3.3K27M-expressing MO3.13 cells and human H3.3K27M-mutant DIPG (Fig. 3c and Supplementary Fig. S3i), suggesting it may cooperate with H3.3K27M. The genes regulated by SOX10 play key roles in many important biological processes that may be important for mediating the oncogenic function of H3.3K27M (Fig. 3d). A reduction in H3K27me3 at the Sox10 promoter in H3.3K27M E14.5 MB/HB and throughout the promoter and gene body of H3.3K27M-expressing NSCs46 (Fig. 3e and Supplementary Fig. S4i) suggested that H3.3K27M epigenetically mediates SOX10 upregulation through H3K27me3 loss. Furthermore, H3.3K27M-expressing NSCs exhibited significant loss of H3K27me3 in the promoters of Sox10 target genes (Fig. 3f)46. Overall, the majority of genes upregulated in the early response to H3.3K27M are not canonical PRC2 target genes. This data suggests that SOX10 may be at least partially responsible for mediating this early response in the MB/HB by promoting oligodendrogenic expansion at the expense of neuronal differentiation.

To better understand how H3.3K27M and SOX10 interact we depleted SOX10 expression in MO3.13 cells (Supplementary Fig. S4g), and repeated the 4-day differentiation assay (Fig. 2f). SI100-β and MBP are both SOX10 target genes42. H3.3K27M did not induce expression of these genes after SOX10 knockdown, confirming the importance of SOX10 in executing the H3.3K27M transcriptome (Fig. 3g). As expected, all differentiation conditions decreased cell proliferation (p < 0.0001; Fig. 3h and Supplementary Fig. S4i). Strikingly, SOX10 depletion reversed the growth advantage conferred by H3.3K27M in regular media and, when combined with differentiation media, H3.3K27M cells grew substantially more slowly than EV and H3.3WT controls (p < 0.0001 when normalising by EV condition to control for the dominating media effect; Fig. 3h and Supplementary Fig. S4h). Moreover, SOX10 depletion had a synthetic lethal effect with H3.3K27M expression, particularly under differentiation conditions, while EV and H3.3WT cell viability was unaffected (p < 0.0001; Fig. 3i and Supplementary Fig. 4i). Collectively these data suggest that H3K27M co-operates with SOX10 in the regulation of transcription programmes involved in cell fate, proliferation, and survival of OL cells consistent with our findings in H3K27M brains, mouse HGGs, and human DIPGs. In keeping with this, the H3.3K27M/SOX10 signature was highly upregulated in both H3.3K27M-mutant mouse HGG and human DIPG (Fig. 3i). Furthermore, 20-30% of differentially expressed genes in the E14.5, mouse HGG and human DIPG datasets are SOX10 target genes, suggesting that SOX10 activity is maintained throughout tumorigenesis.

H3.3K27M drives a core transcriptome in different cellular contexts. To understand the effect of H3.3K27M in different tissues we compared RNA-Seq of HGG, lymphoma and brain from H3.3K27M mice with adult wild-type mouse brain, spleen, thymus and bone marrow48, as well as human DIPG and normal brain. Single-sample GSEA (ssGSEA) was used to analyse relative pathway activity across different samples. T-stochastic neighbour embedding (t-SNE) models and hierarchical clustering revealed that, while brain samples clustered separately from bone marrow, spleen and thymus, reflecting expected inter-tissue transcriptional differences, H3.3K27M-driven tumours clustered together, distinctly from their tissues of origin (Fig. 4f and Supplementary Fig. S5a). Similarly, mouse H3.3K27M HGG and lymphomas grouped with human H3.3K27M DIPG, while control H3.3K27M adult mouse brain samples grouped with normal human brain (Fig. 4g). This suggests that H3.3K27M establishes a transcriptome that is conserved across tumour types and overrides tissue-specific expression patterns. Gene sets or transcriptional pathways commonly regulated across all 3 datasets included proliferative (RAS signalling, cell cycle, MYC), extracellular remodelling (epithelial-mesenchymal transition, adhesion) and metabolic (glycolysis, oxidative phosphorylation) terms. All 3 datasets showed strong enrichment of the mesenchymal subtype and strong repression of neural/proneural glioblastoma subtype signatures (Fig. 4e)47. These data suggest that H3.3K27M rapidly establishes a transcriptome in the brainstem that recapitulates features of gliomagenesis.

To further investigate the similarities between the early effects of H3.3K27M on the brainstem and gliomagenesis we compared the transcriptomes of H3.3K27M mutant E14.5 brainstem with H3.3K27M/Tp53+/- mouse HGGs and human DIPGs. The differentially expressed genes and pathways in all 3 datasets were strikingly similar (all two-way overlaps p ≤ 10^-7; Fig. 4a, b). 167 commonly regulated genes were enriched in extracellular remodelling pathways, consistent with the highly invasive nature of human DIPG (Fig. 4c), and 52 were SOX10 target genes, underscoring the importance of SOX10 activity in establishing the early transcriptional response to H3.3K27M that is maintained throughout tumorigenesis (p = 10^-56; Fig. 4d). The 53 pathways commonly regulated across all 3 datasets included proliferative (RAS signalling, cell cycle, MYC), extracellular remodelling (epithelial-mesenchymal transition, adhesion) and metabolic (glycolysis, oxidative phosphorylation) terms. All 3 datasets showed strong enrichment of the mesenchymal subtype and strong repression of neural/proneural glioblastoma subtype signatures (Fig. 4e). These data suggest that H3.3K27M rapidly establishes a transcriptome in the brainstem which recapitulates features of gliomagenesis.
developmental stage, 30–40% of the RAS/MYC targets and ECM genes differentially expressed in each of H3.3K27M E14.5 MB/HB, mouse HGG and human DIPG are SOX10 target genes (Supplementary Fig. S5c).

Recent data has suggested repression of CDKN2A may be important in H3.3K27M-mediated tumorigenesis, as this gene retains its promoter H3K27me3 in the presence of the oncohistone, and the expression level of p16 regulates the proliferative capacity of H3.3K27M-mutant cells\textsuperscript{18,46}. We examined Cdkn2a promoter H3K27me3 in E14.5 MB/HB, finding that H3.3K27M reduced Cdkn2a methylation at this developmental time point (Supplementary Fig. S5d). There was not an accompanying expression change in Cdkn2a in RNA-Seq data and, in our mouse HGG and human DIPG datasets, it was SOX10-regulated E14.5 DEG
upregulated (Supplementary Fig. S5e, f), suggesting that p16 repression is not a key feature of tumorigenesis in this mouse model.

RAS/MAPK activation is an early response to H3.3K27M. RAS signalling was one of the most strongly activated pathways in established H3.3K27M mouse HGG and lymphoma as well as human DIPG (Fig. 4h, i and Supplementary Fig. S5b). To ask if this was an early response to H3.3K27M, we examined E14.5 MB/HB. At this time point H3.3K27M had been expressed for ~5 days and a proliferative phenotype established (Fig. 2a). Western blotting and RNA-Seq analysis of E14.5 mouse brainstem revealed that RAS/MAPK signalling was indeed already activated in an H3.3K27M associated manner (Fig. 5a–c). Pdgfra, one of the main receptors activating RAS signalling, was upregulated in H3.3K27M E14.5 MB/HB, as well as in mouse HGG and human DIPG (Fig. 5d–f and Supplementary Fig. 5h), and the promoters of Pdgfra, Hras, Kras and Nras had reduced H3K27me3 at E14.5 (Fig. 5g and Supplementary Fig. S5i). MO3.13 cells expressing H3.3K27M had increased MEK and ERK phosphorylation compared with their empty vector (EV) counterparts (Fig. 5h). H3.3K27M markedly reduced H3K27me3 in the promoters of RAS target genes in NSCs (Fig. 5i)46.

This data suggested that H3.3K27M drives epigenetic activation of the RAS/MAPK pathway by targeting multiple pathway members and its downstream target genes. To further test the dependence of the RAS/MAPK pathway activation on H3.3K27M expression, we next examined H3.3K27M-mutant primary paediatric HGG (pHGG) BT245 cells in which the H3.3K27M mutation had been reversed using CRISPR49. BT245-M27K cells showed increased H3K27me3 in the promoters of RAS target genes and significantly downregulated their expression compared with parental H3.3K27M-mutant BT245 cells (Fig. 6a, b). Similarly, depletion of H3F3A by shRNA in (H3F3A-K27M) H3.3K27M-mutant primary DIPG cells restored RAS target gene promoter H3K27me3 (Supplementary Fig. S6a)50. In H3-WT G477 pHGG cells overexpressing H3.3K27M or H3.3K27R49, K27R had no effect on H3K27me3 while K27M upregulated RAS target genes and decreased their promoter H3K27me3 (Fig. 6c and Supplementary Fig. S6b). Finally, in a panel of primary pHGG cells, H3.3K27M-mutant cells had reduced RAS target gene promoter H3K27me3 compared with H3WT cells (Fig. 6d)39. Together, this suggests that H3.3K27M-mutant tumours exhibit K27M-mediated epigenetic activation of the RAS/MAPK pathway.

Early epigenetic activation of RAS/MAPK pathway is later reinforced by genetic activation. Based on sequencing data it is well established that mutations in the RAS/MAPK pathway are frequent events in human DIPG31 suggesting activation of this pathway is an important step in DIPG tumorigenesis with clonal evolution modelling implicating these as later events15,16. Interestingly, our RNAseq data showed activation of the RAS/MAPK pathway in both mouse tumours and human DIPG regardless of whether the RAS/MAPK/P13K pathway was mutated (Fig. 6e–g, Supplementary Fig. S6c and S6d) suggesting that activation of this pathway may be universal with epigenetic/non-mutation-dependent mechanisms responsible for this activation in the non-mutant cases. Intriguingly, in another mouse model where H3.3K27M accelerated development of Trp53KO/Pdgfra-driven HGG17, the addition of H3.3K27M epigenetically activated RAS target genes compared to the H3.3WT HGG despite the pathway already being activated by constitutively active Pdgfra (Supplementary Fig. S6e, f)17 suggesting epigenetic mechanisms of pathway activation may play a role, even in mutant tumours.

The rapid induction of a RAS/MAPK signalling signature in both our mouse and cell line models following introduction of H3.3K27M as detailed above suggests that epigenetic activation of the pathway may be an early event, with mutation-dependent activation occurring later in tumour development or in a subclonal fashion. This is consistent with a model where the early epigenetic activation of RAS by H3.3K27M is later reinforced by genetic activation of the pathway.

To test if RAS/MAPK genetic alterations in the H3.3K27M mouse tumours are early or late events we investigated the clonal evolution of these tumours (Fig. 6h). For the KP29 HGG and lung carcinoma this also provided a unique opportunity to understand how H3.3K27M affects tumorigenesis of two different tissues within the same animal. These two tumours had little genetic similarity, indicating that both were primary (Fig. 1h and Supplementary Fig. S2e). RAS was activated in both tumours, with Kras alterations in the second and first clone of the HGG and lung carcinoma, respectively (Fig. 6h). The HGG additionally had a single copy NFI deletion in clone 1. The lung carcinoma also acquired a focal Mycn amplification and single-copy chromosome 11 (Trp53) loss in the first clone, and a broad single-copy loss in chromosome 4, containing Cdkn2a, in the second (Fig. 6h). Overall, as with human DIPG, most H3.3K27M-driven mouse tumours acquire additional, subclonal, oncogenic alterations beyond the truncal histone mutation and Trp53 loss. Intriguingly, these alterations converge on the RAS/MAPK/P13K pathway and MYC/MYCN, which are key features of H3.3K27M DIPG31.

Epigenetic activation of a RAS/MYC axis in H3.3K27M-driven cancer. MYC target genes were among the most upregulated gene sets in H3.3K27M mouse HGG and lymphomas, and human DIPGs (Fig. 4h, i and Supplementary Fig. S5b). As with RAS signalling, H3.3K27M mouse HGG and human DIPG
Fig. 4 H3.3K27M drives a lineage independent tumour transcriptome. a Venn diagram comparing differentially expressed genes in H3.3K27M E14.5 MB/HB and HGG with DIPG. b Venn diagram comparing differentially expressed Hallmark and KEGG pathways in H3.3K27M E14.5 MB/HB and HGG with DIPG. c Gene ontology analysis of 167 genes commonly regulated in B. d Venn diagram showing overlap of 167 genes commonly regulated in B with SOX10 target genes upregulated in E14.5 H3.3K27M MB/HB. e Heatmap showing gene set enrichment analysis (GSEA) normalised enrichment scores (NES) of glioblastoma subtype signatures. f t-SNE of wild type tissues (bone marrow, spleen, thymus, brain) and untransformed brain, brain tumours and lymphomas from H3.3K27M mice. g t-SNE of untransformed brain, brain tumours and lymphomas from H3.3K27M mice and human H3.3K27M DIPG and normal brain. h Heatmap of select single sample GSEA (ssGSEA) hallmark enrichment scores (ES). i GSEA of RAS and MYC target genes in H3.3K27M mouse HGG. FDR: false discovery rate. Source data are provided as a Source Data file. Statistical tests: Hypergeometric (a, b, d).
Therapeutic targeting of RAS/MYC activity in H3.3K27M-driven cancer. There is currently no clinical way to target MYC, but to test whether MYC could be a therapeutic vulnerability in H3.3K27M-mutant cancer, we used Omomyc, a peptide-based dominant negative MYC inhibitor. Omomyc expression led to downregulation of HSP90, a classical MYC target gene, in MO3.13 cells (Supplementary Fig. S7i). Importantly, H3.3K27M MO3.13 cells had significantly increased cell death in response to Omomyc expression compared with control cells (Fig. 7f).

MYC is a known effector gene of RAS signalling, which targets MYC twice (Supplementary Fig. S7j): MYC-S62 phosphorylation by ERK stabilizes MYC and prolongs its activation, and AKT suppresses GSK-3-mediated MYC-T58 phosphorylation leading to MYC degradation. To test if these mechanisms were at play in the context of H3K27M we used H3.3K27M-expressing MO3.13 cells. These had increased phospho-MYC-S62 as well as activated RAS/MAPK compared with their EV counterparts, suggesting that both H3.3K27M and RAS signalling have roles in MYC activation (Figs. 5h, 7g). In support of this, RAS and MYC activation are tightly coupled in H3K27M DIPG (Fig. 7h).

We then examined the effects of AKT inhibition on phospho-MYC-T58 in MO3.13 cells. As previously reported, A443654 increased phospho-AKT-S473. Phospho-MYC-T58 was increased in both A443654-treated WT and K27M MO3.13 cells, accompanied by a 30-40% reduction in the overall amount of MYC and HSP90 (Fig. 7i). However, H3.3K27M-MO3.13 cells...
were significantly more sensitive to A443654 (IC50 29 nM vs EV IC50 72 nM, \(p < 0.0001\)) than WT MO3.13 cells, suggesting that K27M alone leads to increased dependency on MAPK-mediated MYC activation (Fig. 7j).

Thus, as a clinically viable alternative to directly targeting MYC, targeting either the MAPK or PI3K/AKT arms of the RAS cascade (Supplementary Fig. S7j) could be used. To test this as a potential DIPG therapy we investigated the effects of multiple MEK inhibitors and AKT inhibition on 5 H3K27M-mutant primary DIPG cell lines. Both MEK and AKT inhibition resulted in dose-dependent DIPG cell death with IC50s in the nM range (Fig. 7k, l, Supplementary Fig. S7k, l) supporting these as important therapeutic targets for H3K27M-driven cancer.

**Discussion**

H3K27M mutations were first identified as high-frequency drivers of DIPG, and more recently have been found in...
additional tumour types\(^2\text{-}^8\). Previous pre-clinical mouse models of K27M-driven cancer focussed on generating brain tumours and revealed a cooperative effect on p53/PDGFRα-driven mouse HGG\(^17\text{-}^19,^56\). However, the oncogenic role of H3K27M itself was unclear. To investigate this we created a mouse where H3.3K27M is expressed from the Fabp7 promoter in radial glia and astrocyte progenitors from E9.5, as well as in developing and adult non-brain tissues\(^23\text{-}^26,^56\). H3.3K27M mice developed tumours in multiple tissues, with increased frequency and decreased latency, with and without additional Trp53-deficiency. Thus, this is the first demonstration that H3.3K27M is sufficient to drive tumorigenesis in the absence of other initiating mutations, establishing its role as an oncohistone. This is in keeping with recent work where removing the K27M mutation from DIPG and HGG cells by CRISPR/Cas9 inhibited their tumorigenicity\(^48\). HGG occurred exclusively in association with Trp53 loss in our model, while H3.3K27M alone increased lymphoma and carcinoma susceptibility. Notably, the other tumour types in which H3K27M mutations have been identified in humans are haematopoietic malignancies and carcinomas\(^6\text{-}^8\), perhaps explaining the spectrum of H3K27M-related cancers we saw in our model. H3K27M-mediated disruption of PRC2 in these diseases is in keeping with frequent PRC2 deregulation in haematological neoplasms and lung carcinomas, either by mutation (Supplementary Fig. S8) or differential expression\(^27\text{-}^46\). Finally, previous studies showed an association of high levels of H3.3 and invasive phenotypes in already developed cancers\(^28\text{-}^41\). While our in vitro studies support a phenotypic effect related to the mutant histone, rather than to ectopic expression of H3, our study is limited by the lack of a mouse line expressing the wild-type histone under the Fabp7 promoter. However, transgene expression in our model was very modest compared with endogenous H3.3 and H3.3K27M in human DIPG (Supplementary Fig S1h, i), and the phenotypes we observed were related to cancer initiation rather than to metastasis, suggesting that they are likely due to the K27M mutation rather than histone overexpression.

Although H3.3K27M expression disrupted canonical PRC2 target genes in the E14.5 MB/HB, many other genes were also upregulated, indicating that although PRC2 is important, the mutant histone must also act through additional signalling and transcriptional pathways to drive transformation. SOX10 and its target genes were epigenetically activated by H3.3K27M and, consistent with the role of SOX10 in promoting OPC commitment\(^35,^44,^65\), we found increased expression of oligodendroglial markers at the expense of other lineages. Importantly, an OPC-like cell is hypothesised as a putative DIPG cell of origin\(^34,^55\), suggesting that SOX10 may have a key role in mediating the effects of H3.3K27M. This was confirmed in our cell culture model of OPC differentiation where H3K27M and SOX10 cooperated in activation of a mid-differentiation oligodendrogial transcription programme. Inhibition of SOX10 in this model also led to decreased proliferation and survival specifically in H3K27M cells, suggesting that H3K27M may use SOX10 as a proxy for maintaining oligodendroglial-like cells in a proliferative state. Given the frequent tissue specificity of transcription factors, it is likely that H3.3K27M will partner with other transcription factors in non-glial tissues.

H3.3K27M drove tumorigenesis in multiple tissues and, unexpectedly, the transcriptomes of H3.3K27M-driven tumours were far more similar to one another than to other WT tissues, including their tissues of origin. Remarkably, when comparing H3.3K27M mouse tissues with human DIPGs and normal brain, the mouse HGGs and lymphomas grouped with DIPG. Thus, oncohistone-mediated epigenetic changes override tissue-specific programmes to impose a core, lineage-independent H3.3K27M oncotranscriptome.

Among the most enriched pathways between H3.3K27M-driven mouse cancers and human DIPGs were target genes of RAS/MAPK and MYC. We found that, both in vitro and in vivo, these pathways were epigenetically activated in an H3.3K27M-dependent fashion, including through upregulation of PDGFRα and MYC. This is supported by recent work showing that H3.3K27M leads to in vitro activation of the RAS/MAPK cascade\(^66\) and the finding that a MYC superenhancer was among the most highly activated by the presence of H3K27M\(^67\). Together, this suggests that H3.3K27M-mediated epigenetic changes activate RAS/MAPK and MYC, with H3.3K27M epigenetically activating multiple members of the RAS/MAPK cascade as well as its downstream transcriptional targets. Notably, similar findings have been made in malignant peripheral nerve sheath tumours, where mutations of the SUZ12 component of the PRC2 complex result in transcriptional activation of RAS/MAPK target genes through chromatin landscape changes\(^68\).

Interestingly, human DIPG and H3.3K27M-driven mouse tumours both develop secondary RAS/MAPK/P13K pathway and MYC mutations. This suggests a model in which H3.3K27M initiates tumorigenesis in part through epigenetic activation of RAS and MYC and later mutational events act to lock in this activation in a portion of tumours, providing an explanation for the frequent association of PDGFRα/P13K and MYC alterations in H3.3K27M DIPG (Supplementary Fig. S9).

Importantly, we show here for the first time that H3.3K27M alone can drive tumorigenesis. In addition to offering insight into the function of PRC2 in tumour initiation, our data suggest that H3.3K27M drives cancer by extensive transcriptome remodelling, centred on epigenetic activation of a RAS/MYC axis. Targeting these pathways was synthetically lethal with H3.3K27M providing a potential therapeutic option for treating H3K27M-driven cancers.
Viability assay of empty vector (EV) or H3.3K27M MO3.13 cells 3 days post-transfection with FLAG-EV or FLAG-Omomyc plasmids. Bars show mean ± standard deviation (n = 3). **p < 0.0001, NS: not significant.

**Fig. 7** Epigenetic activation of a RAS/MYC axis in H3.3K27M-driven cancers can be targeted therapeutically. a MYC target gene single sample gene set enrichment analysis (ssGSEA) enrichment score (ES) from MYC WT (n = 25)/mutant (n = 3) H3.3K27M DIPG and normal brain (n = 20). b H3K27me3 ChIP-Seq density was profiled ± 15 kb around the transcription start site (TSS) of MYC target genes in parental, H3K27R or H3K27M G477 cells. c H3K27me3 ChIP-Seq density was profiled ± 15 kb around the TSS of RAS target genes in CRISPR-M27K versus parental BT245 pHGG cells. d Gene set enrichment analysis (GSEA) of RAS target genes in CRISPR-M27K versus parental BT245 pHGG cells. NES: Normalised Enrichment Score. FDR: false discovery rate. e H3K27me3 ChIP-Seq density was profiled ± 15 kb around the TSS of MYC target genes in normal brain, parental H3K27M and H3WT pHGG cells. f Viability assay of empty vector (EV) or H3.3K27M MO3.13 cells 3 days post-transfection with FLAG-EV or FLAG-Omomyc plasmids. Bars show mean ± standard deviation (n = 3). g Western blot of EV and H3.3K27M-expressing MO3.13 cells (results are representative of 2 independent experiments). h Scatter plot of ssGSEA ES of RAS and MYC target genes in human H3.3K27M DIPG (n = 29). r: Pearson correlation. i Western blot of EV and H3.3K27M MO3.13 cells treated or not with A443654 (50 nM) for 3 days (results are representative of 2 independent experiments). j Viability assay of EV and H3.3K27M MO3.13 cells treated or not with A443654 for 3 days. Bars show mean ± standard deviation (n = 4). k Viability assays of DIPG cells treated or not with the indicated MEK inhibitor for 3 days. Bars show mean ± standard deviation (n ≥ 5). l Viability assays of DIPG cells treated or not with A443654 inhibitor for 3 days. Bars show mean ± standard deviation (n ≥ 5). Source data are provided as a Source Data file. Statistical tests: ANOVA (a, f), t (h, j), ***p < 0.0001, NS: not significant.
Methods

Ethical approval and patient samples. The Hospital for Sick Children Animal Care Committee reviewed and approved all procedures conducted on animals, which were performed in compliance with the Animals for Research Act of Ontario and the Guidelines of the Canadian Council on Animal Care. Patient material was collected from patients presenting at The Hospital for Sick Children with a diagnosis of DIPG (median age 7.1 years, 60% male, Supplementary Data 3) and after receiving informed consent. Ethics oversight was provided by the Hospital for Sick Children Ethics Board (#1000055059).

Transgenic construct and mice. H3F3a mouse cDNA with C-terminal FLAG/HA tag was cloned into pCAT3-Basic, downstream from the 1.6 kb Fabp7 promoter, and the gel purified transgene introduced into ICR-CD1 mouse embryos by pro-nuclear microinjection. Of 11 positive animals, 3 male founders were chosen based on expression to develop colonies. For developmental studies littermates were used where possible, or else stage-matched CD1 control animals. B6.129S2-Tp53^In2No1/J mice were a gift from Dr. Rebecca Gladdy (Mt. Sinai Hospital, Toronto). Animals were housed at The Hospital for Sick Children animal facility which operates with a 14/10 h light/dark pattern, at 45–55% humidity and within a temperature range of 22–26°C. The Harmonizome[72]. Fabp7 RNA-Seq expression data was from the Gene Expression Database[76].

Network analysis. Transcription factor (TF) networks were scored on three metrics, similar to previous methods[77], with improvements to network weighting by gene differential expression. Differential gene expression scores were calculated as for pre-ranked GSEA. Networks of TF-DNA and TF-Protein interaction edges were constructed from MARA[79] and StringDB[80], respectively, allowing a maximal edge distance of 3 from the root TF. The network score \( N_{f,r} \) was computed by

\[
N_{f,r} = \sum_{v \in V_r} \frac{S_v}{D_{n,v} + \epsilon} + \frac{S_{v'}}{D_{n,v'} + \epsilon}
\]

where \( r \in V \) is the set of all genes (r) in the local subnetwork of transcription factor \( r \); \( S_v \) is the differential expression gene score, \( S_{v'} \) is the MARA/String relationship score; \( D_{n,v} \) and \( D_{n,v'} \) is the edge distance of gene \( v \) from \( r \) and the outer degree of the parent node in the network, respectively. Each TF network was scored as the aggregate rank of each sub-score in decreasing order, such that the higher the rank, the stronger the effect of the TF influence among differentially expressed genes.

End-point PCR and q-PCR. Total RNA was reverse transcribed using Reverse Transcription kit (Applied Biosystems). End point PCR was performed for 28 cycles unless indicated otherwise. End point PCR-validated primers were used for qPCR with Taq Universal SYBR green supermix (Bio-Rad).

Mouse exome sequencing. DNA was extracted from frozen tissue samples with DNeasy kit (Qiagen). Exome libraries were generated with the SureSelect Mouse All Exon Kit (Agilent). Paired end 125 bp Illumina HiSeq 2500 sequencing was done at The Hospital for Sick Children. We sequenced a pool of normal CD1 mice because Exome sequencing has no available CD1 reference genome or genome-wide SNP analysis. Reads were trimmed (Trimmomatic-v0.32[69]) and aligned GRCh38m with bwa-v0.7.8[60]. Indel realignment and base quality recalibration was performed with GATK-v3.6.0[81] and duplicate reads marked. Sequencing depth was 87-115× (normal) and 86-134× (tumour).

Somatic variants were called with VarScan-v2.3.5[82] and Mutect2[83], retaining those identified by both. For unmatched tumours, variants were called individually against all normals including the CD1 pool, retaining those identified with both tools against every normal and additionally discarding known variants in other mouse strains, as we could not exclude the possibility that these are also present in a particular CD1 mouse. Variants with >10x coverage in both tumour and normal samples, a minimum tumour variant allele frequency (VAF) of 0.05 and maximum normal VAF of 0.01 were annotated using Snpeff-v4.3k[84]. Variants were compared with known human cancer variants using the COSMIC-v81[85]. Clonal evolution of tumours with a matched normal was analysed with SuperFreq[86]. Copy number variants were identified using both on and off-target reads with CNVkit v0.8.6[87].

Human exome sequencing. DNA was extracted from frozen tissue samples with DNeasy kit (Qiagen). Libraries were created with Ion TargetSeq Exome 50 Mb library (ThermoFisher), sequenced on Ion Proton machines (ThermoFisher), and aligned and human gnomAD hg19 with Torrent Suite Software (ThermoFisher) at The Hospital for Sick Children. Variants were called with VarScan and annotated with Snpeff. RAS pathway and MYC mutant samples were determined by the presence of an alteration in a core RAS/MAK/PI3K pathway gene (Supplementary Data 4) or MYC, respectively. These were restricted to SNVs/indels that mutated missense, frameshift, nonsense or splice site mutations at variant-allele frequency >0.2 and with coverage >20, a copy number gain with 5+ copies, or a homozygous deletion, had to be present in the sample for any pathway gene. Wild-type samples had all pathway genes free of SNV/indels and copy number changes.

Statistical analysis. Unless otherwise stated, all p-values were calculated by two-tailed t-tests, not assuming equal variance between samples.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Raw mouse WES and RNA-seq data is available from the Gene Expression Omnibus (GEO), accession GSE120884. Human WES and RNA-seq data is available from the European Genomics Archives, accession EGAD00010064500 (ega-archive.org). Publicly available data was from GEO (https://www.ncbi.nlm.nih.gov/geo/). Human WES data and RNA-seq data is available from the European Genomics Archives, accession EGAD00010066700 (ega-archive.org).
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