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Novel mutations target distinct subgroups of medulloblastoma

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Summary

Medulloblastoma is a malignant childhood brain tumour comprising four discrete subgroups. To identify mutations that drive medulloblastoma we sequenced the entire genomes of 37 tumours and matched normal blood. One hundred and thirty-six genes harbouring somatic mutations in this discovery set were sequenced in an additional 56 medulloblastomas. Recurrent mutations were detected in 41 genes not yet implicated in medulloblastoma: several target distinct components of the epigenetic machinery in different disease subgroups, e.g., regulators of H3K27 and H3K4 trimethylation in subgroup-3 and 4 (e.g., *KDM6A* and *ZMYM3*), and CTNNB1-associated chromatin remodellers in WNT-subgroup tumours (e.g., *SMARCA4* and *CREBBP*). Modelling of mutations in mouse lower rhombic lip progenitors that generate WNT-subgroup tumours, identified genes that maintain this cell lineage (*DDX3X*) as well as mutated genes that initiate (*CDH1*) or cooperate (*PIK3CA*) in tumourigenesis. These data provide important new insights into the pathogenesis of medulloblastoma subgroups and highlight targets for therapeutic development.

Medulloblastoma is the most common malignant childhood brain tumor[1]. The disease includes four subgroups (Sonic Hedgehog (SHH)-subgroup, WNT-subgroup, subgroup-3 and subgroup-4) defined primarily by gene expression profiling that display differences in karyotype, histology and prognosis[2]. Studies of genetically engineered mice show these tumours arise from different cell types: SHH-subgroup medulloblastomas develop from committed cerebellar granule neuron progenitors (GNPs) in *Ptch1*$^{+/-}$ mice[3,4]; WNT-

subgroup tumours are generated by lower rhombic lip progenitors (LRLPs) in *Blbp-Cre* ; *Ctnnb1*$^{+/lox(Ex3)}$; *Tp53*$^{flx/flx}$ mice[5]; while subgroup-3 medulloblastomas likely arise from an undefined class of cerebellar progenitors[6]. The identification of medulloblastoma subgroups has not changed clinical practice. All patients currently receive the same combination of surgery, radiation and chemotherapy. This aggressive treatment fails to cure two thirds of patients with subgroup-3 disease, and probably over-treats children with WNT-subgroup medulloblastoma who invariably survive with long term cognitive and endocrine side effects[2,7]. Drugs targeting the genetic alterations that drive each medulloblastoma subgroup could prove more effective and less toxic, but the identity of these alterations remains largely unknown.

The genomic landscape of medulloblastoma

To identify genetic alterations that drive medulloblastoma, we performed whole genome sequencing (WGS) of DNA from 37 tumours and matched normal blood (discovery cohort). Tumours were subgrouped by gene expression (WNT-subgroup, n=5; SHH-subgroup, n=5; subgroup-3, n=6; subgroup-4, n=19; 'unclassified' [profiles not available], n=2. Figure 1; Supplementary Figures 1-3 and Supplementary Table 1). Validation of all putative somatic alterations including single nucleotide variations (SNVs), insertion/deletions (indels) and structural variations (SVs) identified by CREST[8], was conducted for 12 tumours using custom capture arrays and Illumina-based DNA sequencing (Supplementary Table 2). Putative coding alterations and SVs were validated in the remaining 25 'discovery cohort' cases by polymerase chain reaction and Sanger-based sequencing. Mutation frequency was determined in a separate 'validation cohort' of 56 medulloblastomas (WNT-subgroup, n=6; SHH-subgroup, n=8; subgroup-3, n=11; subgroup-4, n=19; unclassified, n=12; Figure 1, Supplementary Table 1).

WGS of the 'discovery cohort' detected 22,887 validated or high-quality somatic sequence mutations (SNVs and indels), 536 validated or curated SVs, and 5,802 copy number variations (CNVs, 92% concordant with 6.0 SNP mapping arrays; Supplementary Tables 3-6, Supplementary Figures 4-7). In all but five tumours with the highest mutation rates, >50% of SNVs were C>T/G>A transitions (Supplementary Figure 8). The mean missense:silent mutation ratio was 3.6:1 and 40% of all missense mutations were predicted to be deleterious, suggesting a selective pressure for SNVs that impact protein coding (Supplementary Table 5). Global patterns of total SNVs and amplifications varied significantly among medulloblastoma subgroups, even when corrected for age and sex, supporting the notion that these tumours are distinct pathological entities (Figure 1, Supplementary Figure 6). Custom capture-based analysis of the allele frequency of all somatic mutations in 12 medulloblastomas allowed us to predict the ancestry of certain genetic alterations, suggesting that aneuploidy precedes widespread sequence mutation in medulloblastomas with highly mutated genomes (Supplementary Figures 9-11).

Novel copy number variations, structural alterations and heritable mutations are rare in medulloblastoma

The repertoire of focally amplified or deleted genes appears to be very limited in medulloblastoma. We detected expected[2] gains of *MYC*, *MYCN* and *OTX2* in subgroup-3 and 4, but no novel recurrent amplifications (Figure 1, Supplementary Figure 12, Supplementary Table 7). In keeping with recent reports[9], high-level amplification of *MYCN* in subgroup-3 sample #16 (sample numbering as Figure 1) was generated by chromothripsis; although chromothripsis was observed infrequently (n=2/37 of 'discovery cohort'; Supplementary Figure 13).

Focal homo- or heterozygous deletion of genes previously implicated in medulloblastoma were also detected (e.g., *PTCH1*, *PTEN*, Figure 1)[10,11] but novel recurrent focal deletions were rare. Three subgroup-4 tumours (#11-13) and one unclassified tumour, deleted *DDX31*, *AK8* and *TSC1* at 9q34.14 in concert with *OTX2* amplification, suggesting these alterations are cooperative ($P<0.0005$, Fisher's exact). The breakpoint in this deletion occurs in *DDX31* and two samples contained a missense mutation (subgroup-4 #15) and complex rearrangement (unidentified case SJMB026) in this gene, suggesting *DDX31* is the target of these alterations (Supplementary Figure 14).

Over 50% of SVs detected by WGS broke the coding region of at least one gene, but less than 2% (n=6/314, excluding two tumours with excessive SVs) encode potential in-frame fusion proteins (Supplementary Figure 15); none affect the same gene or signal pathway. Therefore, fusion proteins are likely to be an uncommon transforming mechanism in medulloblastoma.

Although germline mutations in *TP53*, *PTCH1*, *APC*, and *CREBBP*, predispose to medulloblastoma[11-14], only 23 mutations previously associated with cancer were detected in 'discovery cohort' germlines: only one of these - in a known case of Turcot's syndrome - was accompanied with a somatic mutation (germline APC Y935*/somatic deletion: WNT-subgroup #11, Supplementary Table 8). Thus, inherited forms of medulloblastoma appear to be rare in our cohort.

Novel recurrent sequence mutations target distinct medulloblastoma subgroups

Since SVs and CNVs are unlikely to drive most medulloblastomas, we looked to see if recurrent (>2 samples), somatic SNVs and/or indels might target discrete genes and pathways. This analysis identified 49 genes, across all 93 tumors, that were targeted by non-silent, recurrent, somatic mutations: 84% (n=41/49) are not yet implicated in medulloblastoma (Supplementary Tables 9 and 10). Several of these congregated in disease subgroups and converged on specific cell pathways (Figure 1; Supplementary Figure 8 and Table 11).

Writing, reading and erasing of histone methylation is deregulated in subgroup-3 and 4 medulloblastomas

The H3K27 trimethyl mark (H3K27me3) represses lineage specific genes in stem cells (Supplementary Figure 8)[15]. H3K27me3 is written by the polycomb repressive complex 2 (PRC2) that includes the methylase EZH2[16,17], and erased during differentiation by the demethylase KDM6A[18]. As H3K27me3 is erased, chromatin remodelers recruited to H3K4me3 promote differentiation e.g., CHD7[19,20]. This process is tightly controlled during development and deregulated in cancers: *EZH2* is mutated in lymphomas[21], and upregulated in breast[22] and prostate[23] cancer; while biallelic inactivation of *KDM6A* (Xp11.2) or *KDM6A* and its paralog *UTY* (Yq11), occurs in adult female and male cancers, respectively[24].

Hypergeometric distribution analyses revealed selective mutation of histone modifiers in subgroup-3 and 4 medulloblastomas (Supplementary Table 11). Six subgroup-4, one subgroup-3, and one unclassified medulloblastoma contained novel inactivating mutations in *KDM6A* (Figures 1 and 2; Supplementary Figures 8 and 16). The single female with a *KDM6A* splice site mutation deleted the second allele that escapes X inactivation[25] (subgroup-4 #15), and 57% (n=4/7) of *KDM6A*-mutant male medulloblastomas deleted chromosome Y, compared with only 6% (n=3/51) of male, *KDM6A* wild-type tumours ($P<0.005$, Fisher's exact; Figure 1). Thus, a two-hit model of *KDM6A–UTY* tumour suppression appears to operate in subgroup-4 medulloblastomas. Notably, mutations in six other KDM family members (*KDM1A*, *KDM3A*, *KDM4C*, *KDM5A*, *KDM5B* and *KDM7A*) were detected exclusively in subgroup-3 and 4 tumours, implicating broad disruption of lysine demethylation in these medulloblastomas (Figure 1, Supplementary Table 11; Supplementary Figure 16).

Subgroup-3 and 4 medulloblastomas also gained and overexpressed *EZH2* (7q35-34) that writes H3K27me3, and contained novel inactivating mutations in effectors and regulators of the H3K4me3 mark[26] (Figure 2a; Supplementary Figure 8). Gain of 7q was significantly enriched among subgroup-3 and 4 medulloblastomas ($P<0.005$ Fisher's exact) and correlated directly with EZH2 expression. Indeed, *EZH2* was the 8th most significantly overexpressed gene on chromosome 7 among subgroup-3 and 4 medulloblastomas that gained chromosome 7q relative to those with diploid chromosome 7 ($P<0.005$, Bonferroni correction). Nonsense and frameshift mutations were detected in *CHD7* in four subgroup-3 and 4 tumours. *ZMYM3* (Xq13.1) that participates in a protein complex with KDM1A to regulate gene expression at the H3K4me3 mark[27] was targeted by novel frameshift, nonsense and missense mutations in three male subgroup-4 medulloblastomas. All three tumours with mutations in *ZMYM3* also mutated *KDM6A* (subgroup-4 #19, 20) or *KDM1A* (subgroup-4 #21) suggesting these alterations are cooperative. Remarkably, *KDM6A*, *CHD7* and *ZMYM3* mutations were confined to subgroup-3 and 4, and clustered in samples with sub-median EZH2 expression levels (Figure 2a; $P<0.05$, Fisher's exact). These data suggest that subgroup-3 and 4 medulloblastomas retain a stem-like epigenetic state by aberrantly writing (EZH2 upregulation) or preserving (*KDM6A-UTY* inactivation) H3K27me3, or disrupting H3K4me3 associated transcription (*CHD7* and *ZMYM3* inactivation). Indeed, human and mouse subgroup-3 and 4 medulloblastomas contained significantly more

H3K27me3 than did WNT or SHH-subgroup tumours (Figure 2b). Thus, gain of *EZH2* and loss of *KDM6A* likely maintains H3K27me3 in subgroup-3 and 4 medulloblastomas.

Finally, we looked to see if the differential expression of H3K27me3 among medulloblastoma subgroups reflects ancestral chromatin marking in the progenitors that generate these tumours (Figure 2b). Relatively low levels of H3K27me3 were detected in LRLPs and committed GNPs that generate WNT and SHH-subgroup medulloblastomas respectively[3-5], potentially explaining why mutations that preserve this epigenetic mark are absent from these tumours. We recently showed that subgroup-3 medulloblastomas arise from a rare fraction of cerebellar progenitors[6]. We are currently investigating if these progenitors are found among the H3K27me^3 positive cells seen in the external germinal layer (Figure 2b).

Novel mutations in WNT-subgroup medulloblastomas target CTNNB1-associated chromatin remodelers and regulators of LRLPs

WNT-subgroup medulloblastomas contained mutations in epigenetic regulators that are different to those seen in subgroup-3 and 4 disease. CTNNB1, the principal effector of the WNT pathway, forms a transcription factor with the T cell factor/lymphoid enhancer factor (TCF/LEF)[28]. The c-terminus of CTNNB1 then recruits a series of protein complexes that remodel chromatin and promote transcription at WNT-responsive genes (Supplementary Figure 8). These include: histone acetyltransferases (e.g., CREBBP and TRRAP-TIP60 complexes)[28,29]; ATPases of the SWI/SNF family (e.g., SMARCA4)[30]; and the Mediator complex that coordinates RNA polymerase II placement (e.g., MED13)[31]. As expected, >70% (n=8/11) of WNT-medulloblastomas contained mutations that stabilise CTNNB1 (Figure 1 and Supplementary Figure 8; P<0.0001, Fishers exact)[32,33]. A single subgroup-3 case (#5) also mutated *CTNNB1*, but this mutation has not been reported in cancer, did not upregulate nuclear CTNNB1 (Figure 1) and is of unclear significance. Remarkably, six WNT-subgroup medulloblastomas mutated chromatin modifiers that are recruited to TCF/LEF WNT-responsive genes by CTNNB1 (Figure 1, Supplementary Figure 8): four WNT-subgroup tumours contained heterozygous missense mutations in the helicase domain of *SMARCA4* (P<0.002, Fisher's exact); two samples, including one with a *SMARCA4* mutation (#5), contained nonsense mutations in *CREBBP* (WNT-subgroup enrichment P<0.02, Fisher's exact); and missense mutations in *TRRAP* and *MED13* were detected in a single WNT-subgroup medulloblastoma each. Thus, in addition to stabilization of CTNNB1, the development of WNT-subgroup medulloblastoma may require disruption of chromatin remodeling at WNT-responsive genes.

A small number of WNT-subgroup medulloblastomas lack mutations in *CTNNB1* or *APC*, suggesting alternative mechanisms drive aberrant WNT-signals in these tumours. Three WNT-subgroup medulloblastomas in our series contained wild-type *CTNNB1* (#1, 10 and 11, Figure 1). Sample #11 inactivated *APC* as the sole case of Turcot's Syndrome in our study, but this tumour and sample #10 also contained novel missense mutations in *CDH1* (R63G, V329F; Figure 1 WNT-subgroup enrichment P<0.05, Fisher's exact). CDH1 sequesters CTNNB1 at the cell membrane[34], and mutations that disrupt this interaction promote WNT signaling in adult cancers[35,36]. The functional consequences of CDH1^{R63G}

and CDH1^{V329F} remain to be determined, but their restriction to WNT-subgroup tumours; mutual exclusivity with *CTNNB1* mutations; and adjacency to residues mutated in breast cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/), suggest these might promote aberrant WNT signals in medulloblastoma.

We showed previously that mutant Ctnnb1 initiates WNT-subgroup medulloblastoma by arresting the migration of LRLPs from the embryonic dorsal brainstem to the pontine grey nucleus (PGN)[5]. Therefore, to test if disruption of CDH1 might substitute for mutant CTNNB1 in medulloblastoma, we used shRNAs to knock down Cdh1 in embryonic day (E) 14.5 mouse LRLPs (Figure 3a to c). Deletion of Cdh1 expression upregulated Tcf/Lef mediated gene transcription in LRLPs and more than doubled their self-renewal capacity (Figure 3b). Furthermore, *in utero* electroporation of LRLPs with Cdh1 shRNAs impeded their migration from the dorsal brainstem to the PGN with an efficiency similar to that of mutant Ctnnb1 (Figure 3d,e; see Supplementary Methods). These data support the hypothesis that *CDH1* suppresses the formation of WNT-subgroup medulloblastoma by regulating WNT-signals in LRLPs.

WNT-subgroup medulloblastomas were also enriched for novel, recurrent somatic missense mutations in the DEAD-Box RNA helicase *DDX3X* at Xp11.3 ($P<0.0001$, Fisher's exact; Figure 1). DDX3X regulates several critical cell processes including chromosome segregation[37], cell cycle progression[38], gene transcription and translation[39]. Previously reported cancer associated mutations in *DDX3X* disrupt the ATPase activity of the protein, but seven of eight mutations identified in our series clustered in the DEAD-box domain (Supplementary Information; Supplementary Figure 8). Structural modeling predicts that these mutations interfere with nucleic acid binding, possibly altering specificity and/or affinity for RNA substrates, rather than inactivating DDX3X (Supplementary Figures 17-22). Indeed, the wild-type allele of *DDX3X* that escapes X inactivation[25] was retained by two of three *DDX3X*-mutant female medulloblastomas, and knock-down of *Ddx3x* halved the self-renewal rate of mouse LRLPs, suggesting this protein is important for the proliferation and/or maintenance of the LRLP lineage (Figure 3b).

To better understand the role of DDX3X in WNT-subgroup medulloblastoma, we employed our *in utero* migration assay to assess the impact of *Ddx3x* shRNAs, mutant-*Ddx3x*^{T275M} (identified in WNT-sample #9) or mutant-*Ddx3x*^{G325E} (WNT-sample #8) on LRLPs. Remarkably, while *Ddx3x* shRNAs were expressed abundantly in E14.5 brainstem cells within 48 hours of electroporation, <0.5% of *Ddx3x*- shRNA-positive cells were present by postnatal day 1, confirming the critical importance of this gene to maintain the LRLP lineage (Figure 3d,e). In stark contrast, mice electroporated with either mutant-*Ddx3x*^{T275M} or *Ddx3x*^{G325E} consistently contained ~50% more labeled cells at postnatal day (P) 1 than did controls, although these cells migrated normally (Figure 3d,e and data not shown). Thus, mutations in *DDX3X* may contribute to WNT-subgroup medulloblastoma by increasing LRLP proliferation rather than perturbing the migration of their daughters. Notably, comparable knock-down *in utero* of *Mll2*, *Gabrg1*, and *Kdm6a* that were selectively mutated in non-WNT medulloblastomas had no apparent impact on LRLPs; supporting the value of our assay for assessing WNT-subgroup specific mutations and underscoring the importance of cell context for functional studies of genes mutated in cancer subgroups.

PIK3CA mutations promote but don't initiate WNT-subgroup medulloblastoma

Cancer-associated, activating mutations in *PIK3CA* were detected in a single case each of WNT ($PIK3CA^{Q546K}$), SHH ($PIK3CA^{H1047R}$) and subgroup-4 ($PIK3CA^{N345K}$) medulloblastoma (Figure 1; Supplementary Figure 23). Although *PIK3CA* mutations are common in adult cancers[40] and reported in medulloblastoma[41], their role in tumourigenesis remains controversial. In particular it is not known if these mutations initiate or progress cancer. To test this, we generated mice that express a conditional allele of the $Pik3ca^{E545K}$ mutation. Mice harboring $Pik3ca^{E545K}$ or $Pik3ca^{E545K}$ and $Tp53^{flx/flx}$ were bred with *Blbp-Cre* that drives efficient recombination in LRLPs[5]. *Blbp-Cre* ; $Pik3ca^{E545K}$ mice, with or without $Tp53^{flx/flx}$, survived tumour free for a median of 212 days with no evidence of aberrant LRLP migration (Figure 4a and data not shown). In stark contrast, 100% (n=11/11) of *Blbp-Cre* ; $Ctnnb1^{+/lox(Ex3)}$; $Tp53^{+/flx}$; $Pik3ca^{E545K}$ mice developed WNT-subgroup medulloblastomas by 3 months of age: only 4% (n=2/54) of *Blbp-Cre* ; $Ctnnb1^{+/lox(Ex3)}$; $Tp53^{+/flx}$ mice develop WNT-medulloblastoma by 11 months (Figure 4a,b). *Pik3ca* wild-type and mutant mouse medulloblastomas displayed similar 'classic' histologies and nuclear $Ctnnb1^{+}$, but $Pik3ca^{E545K}$ mutant tumors contained greater AKT pathway activity as measured by pS6 and p4EBP1 immunostaining. Thus mutations in *PIK3CA* likely activate the AKT pathway to progress, rather than initiate, WNT-medulloblastoma.

SHH-subgroup medulloblastomas

Four of 13 SHH-subgroup medulloblastomas contained expected biallelic inactivating alterations in *SUFU* or *PTCH1*. What drives aberrant SHH-signals in the remaining cases remains unclear. These tumours contained mutations in *MLL2*, *TP53*, and *PTEN* that have been reported previously in medulloblastoma[42]; but these mutations occur in other subgroups and are not known to activate SHH signals. Two SHH-subgroup tumours (#11 and 12) contained identical novel T48M mutations in gamma-aminobutyric acid (GABA) A receptor, gamma 1 that is predicted to be deleterious (Figure 1, Supplementary Table 9). Disruption of $GABA_A$ receptors can enhance neural stem cell proliferation[43], suggesting these mutations might deregulate the proliferation of GNPs that generate SHH-subgroup medulloblastomas.

Discussion

We have identified several, new, recurrent, somatic mutations in specific subgroups of medulloblastoma. Alterations affecting *EZH2*, *KDM6A*, *CHD7* and *ZMYM3* appear to disrupt chromatin marking of genes in subgroup-3 and 4 tumours. Further epigenetic studies will be required to uncover the identity of these genes, but evidence suggests these may include *OTX2*, *MYC* and *MYCN*[44,45]. Since amplification of these genes was detected almost exclusively in subgroup-3 and 4 tumours that lacked mutations in *KDM6A*, *CHD7* or *ZMYM3*, it is tempting to speculate that these genetic alterations target common transforming pathways. A recent study detected recurrent mutations in three other chromatin remodelers in medulloblastoma[42]: *SMARCA4*, *MLL2* and *MLL3*, but this study did not include details of tumour subgroup. Here, we show that mutations in *SMARCA4*, *CREBBP*, *TRRAP* and *MED13* are enriched in WNT-subgroup medulloblastomas; thereby uncovering potential cooperative mutations in chromatin remodelers and their binding-partner

oncogene, CTNNB1. Thus, disruptions in the epigenetic machinery of medulloblastoma are likely to be subgroup specific and may cooperate with other oncogenic mutations. The low incidence of *MLL2* mutations detected in our study relative to Parsons et al.,[42] likely reflects differences the our study populations (see Supplementary Results).

Although medulloblastoma is more prevalent in males, especially subgroup-3 and 4 disease[46], the reason for this sex bias is unknown. One potential explanation is the location of medulloblastoma oncogenes or tumour suppressor genes on chromosome X[47]. Three of the most recurrently mutated genes detected in our study are located on chromosome X, of which two (*ZMYM3* and *KDM6A*) were observed almost exclusively in males. Mutation of these genes might explain some of the male sex-bias in medulloblastoma. The third mutated X chromosome gene, *DDX3X*, is more likely to be a WNT-medulloblastoma oncogene. Three of four female medulloblastomas carried heterozygous mutations in *DDX3X* that escapes X inactivation[25] and our functional data indicate that mutations in this gene provide a proliferative advantage to LRLPs that generate these tumours.

Our findings also have important implications for drug development. Inhibitors of the epigenetic machinery, especially those that maintain H3K27me3 e.g., EZH2 methylases, may be useful treatments of subgroup-3 and 4 disease. These tumours include the most aggressive forms of medulloblastoma for which treatment options are limited. Mutations that activate PIK3CA and DDX3X in WNT-subgroup tumors might also be targeted with novel therapeutic strategies[48,49]. Future clinical trials of drugs that target these mutant proteins must recruit the appropriate patient populations, since we show mutations display subgroup-specificity in medulloblastoma. Our accurate mouse models of WNT, SHH and subgroup-3 medulloblastoma should help considerably with future studies of the biological and therapeutic significance of the novel genetic alterations described in this study.

Methods summary

Human tumour and matched blood samples were obtained with informed consent through an institutional review board approved protocol at St Jude Children's Research Hospital. Whole genome sequencing (WGS) and analysis of WGS data were performed as previously described[50]. Details of sequence coverage, custom capture and other validation procedures are provided in Supplementary Information (Supplementary Tables 12-15). Sequence and SNP array data were deposited in dbGaP (dbGaP accession number: phs000409, SRA accession number: SRP008292). Immunohistochemistry and immunofluorescence of human and mouse tissues were performed using routine techniques and primary antibodies of the appropriate tissues as described (Supplementary Methods). Medulloblastoma mRNA and DNA profiles were generated using Affymetrix U133v2 and SNP 6.0 arrays, respectively (Supplementary Methods). Reverse transcriptase Real Time-PCR analysis of genes targeted in mouse LRLPs by shRNAs were performed as described previously[32]. LRLPs were isolated and transduced with indicated lentiviruses in stem cell cultures or targeted *in utero* with shRNAs or mutant cDNA sequences by electroporation as described (Supplementary Information)[5]. Mice harbouring a cre-inducible *Pik3ca*^{E545K} allele were generated using homologous recombination: A lox-puro-STOP-lox cassette was introduced immediately upstream of the exon containing the initiation codon, exon 9 was replaced with an exon

containing the E545K mutation. *Pik3ca*^{E545K} mice were bred with *Blbp-Cre*, *Ctnnb1*$^{lox(ex3)/lox(ex3)}$ and *Tp53*$^{flx/flx}$ mice to generate progeny of the appropriate genotype and subjected to clinical surveillance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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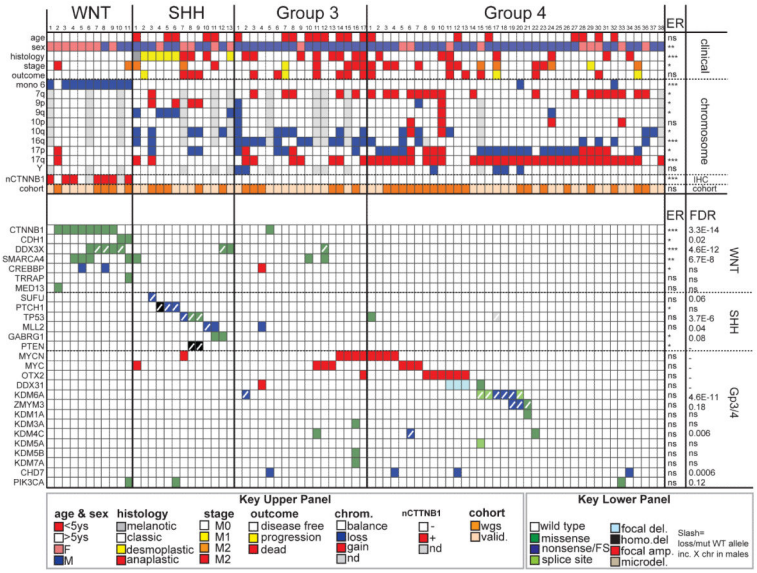

Figure 1. The genomic landscape of medulloblastoma

Top: clinical, histologic, gross chromosomal, nuclear CTNNB1 (nCTNNB1), and cohort (discovery or validation) details of 79 medulloblastomas by subgroup. Below: genetic alterations detected in 27 genes of particular interest. Color key at bottom. ANOVA (continuous) or Fisher's exact (categorical) p-value is shown right. False discovery estimates (FDR) of each mutation are shown right. ***=P<0.0005; **=P<0.005; *=P<0.05; ns=not significant.

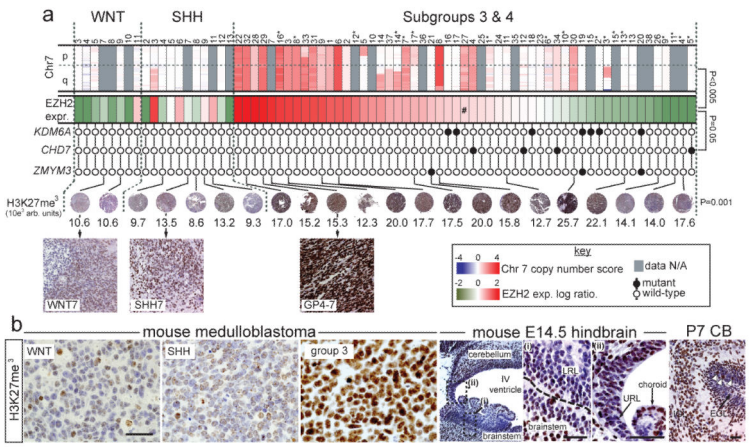

Figure 2. Deregulation of H3K27me3 in subgroup-3 and 4 human and mouse medulloblastoma

(a) Top row, SNP profiles of chromosome 7 copy number in medulloblastomas (samples as Figure 1; *=subgroup-3 cases). Second row, expression of EZH2. Subgroup-3 and 4 tumours are ordered left to right by expression level, #=median expression point (Bonferroni corrected p-value of EZH2 expression vs. chromosome 7 gain). Third row, mutation status of *KDM6A*, *CHD7* and *ZMYM3* (p-value, Fisher's exact mutations vs. EZH2 expression). Fourth row, H3K27me3 immunohistochemistry (numbers=colorimetry, p-value ANOVA). **(b)** H3K27me3 expression (right) in mouse *Blbp-Cre* ; $Ctnnb1^{+/lox(Ex3)}$; $Tp53^{flx/flx}$ (WNT), $Ptch1^{+/-}$; $Tp53^{-/-}$ (SHH) and *Myc* ; $Ink4c^{-/-}$ (group 3) medulloblastomas and (left) developing hindbrain. high power views of E14.5 (i) LRL and (ii) upper rhombic lip (URL). IGL=internal granule layer, EGL=external germinal layer. Scale bar=50μm. White arrows in P7 cerebellum pinpoint H3K27me3 cells in the EGL.

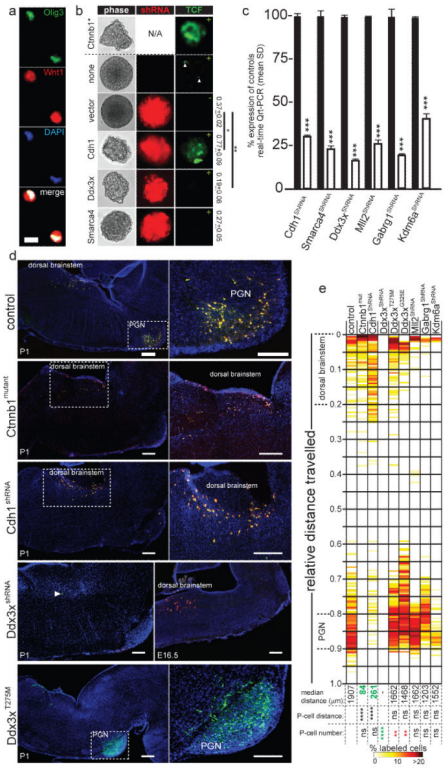

Figure 3. Genes mutated in WNT-subgroup medulloblastomas regulate LRLPs

(a) Isolated Olig3^{+}/Wnt1^{+} LRLPs were transduced in **(b)** with mutant Ctnnb1 (above hashed line) or the indicated shRNA-Red Fluorescence Protein construct (below hashed line). LRLPs were also transduced (+) or not (−) with a Tcf-Lef-enhanced green fluorescence (Tcf) reporter. Numbers right report clonal % 2′ to 3′ passage neurosphere formation (+SD). **(c)** Knock-down of genes targeted by shRNA relative to control transduced cells. **(d)** Immunofluorescence of P1 mouse hindbrains electroporated *in utero* at E14.5 with GFP (to control for equivalence of electroporation between embryos control) and the indicated construct. High-power views of indicated areas are shown right. Cells targeted by Ddx3x-shRNA are present 48 hours post electroporation but ablated by P1. Scale=200μm. **(e)** Heatmap reporting the distribution of GFP^{+}/RFP^{+} cells in eletroporated mice at P1. Median distance migrated by cells, and p-values of migration distance and cell number relative to controls is shown (****, p<0.00005; ***, p<0.0005; **, p<0.005; *, p<0.05. Red and green text reports significant increase or decrease, respectively relative to control).

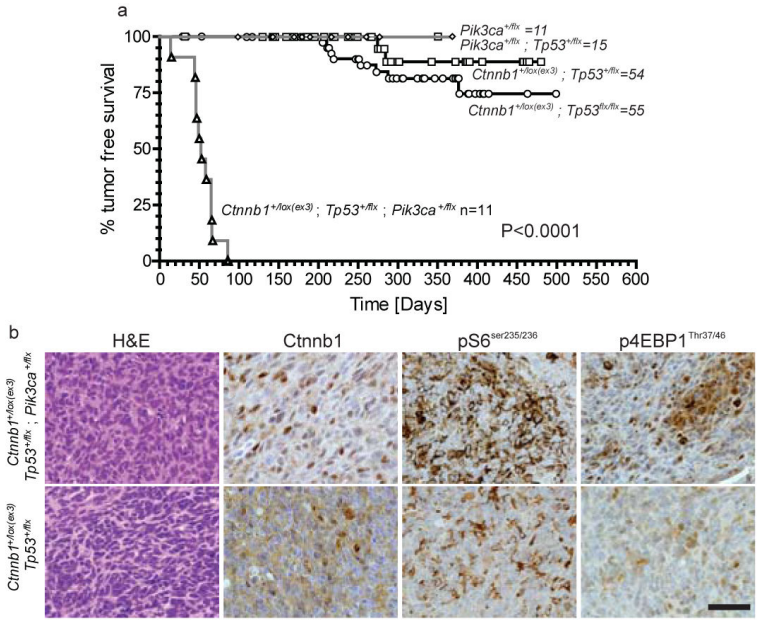

Figure 4. *Pik3ca*^{E545K} accelerates but does not initiate WNT-subgroup medulloblastoma

Tumour free survival of mice of the indicated genotype. All mice carry the *Blbp-cre* allele. Log Rank P<0.0001. **(b)** Hematoxylin and eosin and immunohistochemical stains of indicated tumors. Scale=50 μm.