Whole-genome sequencing identifies genetic alterations in pediatric low-grade gliomas

Jinghui Zhang1, Gang Wu1, Claudia P Miller2, Ruth G Tatevossian3, James D Dalton3, Bo Tang3, Wilda Orisme3, Chandanamali Punchihewa3, Matthew Parker1, Ibrahim Qaddoumi3, Fredrick A Boop5, Charles Lu6, Cyriac Kandoth6, Li Ding7, Ryan Lee3, Robert Huether1, Xiang Chen1, Erin Hedlund1, Panduka Nagahawatte1, Michael Rusch1, Kristy Boggs8, Jinjun Cheng3, Jared Becksfort1, Jing Ma3, Guangchun Song2, Yongjin Li1, Lei Wei3, Jianmin Wang9, Sheila Shurtleff3, John Easton8, David Zhao1, Robert S Fulton6, Lucinda L Fulton6, David J Dooling6, Bhavin Vadodaria8, Heather L Mulder8, Kerri Ochoa6, Charles G Mullighan3, Amar Gajjar4, Richard Kriwacki10, Denise Sheer11, Richard J Gilbertson2, Elaine R Mardis6, Richard K Wilson6, James R Downing3, Suzanne J Baker2 & David W Ellison3 for the St. Jude Children’s Research Hospital–Washington University Pediatric Cancer Genome Project

The most common pediatric brain tumors are low-grade gliomas (LGGs). We used whole-genome sequencing to identify multiple new genetic alterations involving BRAF, RAF1, FGFR1, MYB, MYBL1 and genes with histone-related functions, including H3F3A and ATRX, in 39 LGGs and low-grade glioneuronal tumors (LGGNTs). Only a single non-silent somatic alteration was detected in 24 of 39 (62%) tumors. Intragenic duplications of the portion of FGFR1 encoding the tyrosine kinase domain (TKD) and rearrangements of MYB were recurrent and mutually exclusive in 53% of grade II diffuse LGGs. Transplantation of Tpr53-null neonatal astrocytes expressing FGFR1 with the duplication involving the TKD into the brains of nude mice generated high-grade astrocytomas with short latency and 100% penetrance. FGFR1 with the duplication induced FGFR1 autophosphorylation and upregulation of the MAPK/ERK and PI3K pathways, which could be blocked by specific inhibitors. Focusing on the therapeutically challenging diffuse LGGs, our study of 151 tumors has discovered genetic alterations and potential therapeutic targets across the entire range of pediatric LGGs and LGGNTs.

LGGs arise most frequently in children and young adults and are the most common pediatric central nervous system (CNS) neoplasms. Although LGGs grow slowly, those that cannot be surgically resected cause considerable morbidity and premature death. Current adjuvant therapies with irradiation and pharmaceuticals extend survival but contribute to morbidity; thus, there is an urgent need for targeted therapeutics in patients with inoperable disease.

Studies of pediatric LGGs and related LGGNTs have implicated abnormalities of the extracellular signal–regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway in oncogenesis, but detailed knowledge of driver mutations in these diverse tumors is lacking. Up to 15% of children with the hereditary tumor syndrome neurofibromatosis type 1 (NF1) develop pilocytic astrocytoma, the most common type of LGG. Neurofibromin 1, the protein product of the NF1 tumor suppressor gene, is a negative regulator of RAS in the MAPK/ERK pathway. NF1–related LGGs account for less than 15% of pediatric LGGs; however, almost all sporadic cerebellar pilocytic astrocytomas show MAPK/ERK pathway activation in association with KIAA1549-BRAF fusion gene, which results in the expression of BRAF protein that lacks its autoinhibitory domain and is constitutively active. Other mechanisms activating the MAPK/ERK pathway in LGGs are comparatively rare and include RAF1 fusion genes and BRAFV600E or KRAS mutations, although the BRAFV600E mutation is present in approximately 15% of LGGNTs. Although nearly all World Health Organization (WHO) grade I LGGs from the intracranial posterior fossa harbor one of the above mutations, they occur less frequently in supratentorial grade I LGGs and rarely in diffuse grade II tumors. Notably, the genetics of inoperable diseases,
which cause considerable morbidity and mortality in children, particularly midline supratentorial and diffusely infiltrating LGGs, remain poorly characterized.

In this study, we sequenced the whole genomes of 39 pediatric LGGs and LGGNTs, along with their matching normal DNA samples, identifying multiple new genetic abnormalities. The principal new findings—duplication of the region in FGFR1 (fibroblast growth factor receptor 1) encoding the TKD and rearrangements of MYB or MYBL1—occur most frequently in diffuse cerebral LGGs.

Figure 1 Clinicopathological characteristics and genetic alterations in tumors from series 1 examined by whole-genome sequencing or RNA-seq.

(a) Summary of clinicopathological characteristics (tumor site, pathology, age at surgery, sex) and key genetic alterations identified in 56 LGGs and LGGNTs by whole-genome sequencing (n=38) or RNA-seq (n=44). Very few structural variations or SNVs are found across the whole-genome sequencing tumor series. Numbers in colored cells refer to gene fusions listed at the right. DNET, dysembryoplastic neuroepithelial tumor. (b) Selected Circos plots summarizing aspects of whole-genome sequencing data, including a solitary KIAA1549-BRAF fusion (SJLGG001), MYB rearrangement (SJLGG005), duplication in FGFR1 affecting the TKD (SJLGG006) and NF1 mutation (SJLGG022). The genomic profiles of two oligodendrogial tumors, SJLGG006 and SJLGG034, show some key differences between this type of tumor in children and in adults, the latter having IDH1 and CIC mutations along with deletion of both 1p and 19q. SJLGG039 exemplifies five LGGs with complex rearrangements.

RESULTS
The genomic landscape of LGGs and LGGNTs
The study cohort (Supplementary Fig. 1) consisted of 151 tumors from 149 affected individuals in 3 series: (i) tumors analyzed by whole-genome sequencing (n=39) and/or transcriptome sequencing (RNA-seq; n=46), (ii) diverse LGGs and LGGNTs for evaluating the frequency and clinicopathological associations of all mutated genes discovered through whole-genome sequencing or RNA-seq (n=84), and (iii) non-cerebellar LGGs and LGGNTs without matching germline
samples included to increase the representation of those tumors for which genetic abnormalities are largely unknown (n = 22).

Tumor series 1 included a discovery set of 39 paired tumor-germline samples analyzed by whole-genome sequencing at an average of 45x haploid coverage (Supplementary Fig. 2 and Supplementary Table 1). All somatic structural variations and sequence mutations (single-nucleotide variations (SNVs) and small insertions or deletions (indels)) in RefSeq exons were validated by orthogonal sequencing methods. Exhaustive validation of all somatic structural variations and all somatic sequence mutations in non-repetitive regions of the reference human genome was undertaken for 16 tumors using custom capture arrays. The background mutation rate ascertained from validated SNVs in these tumors ranged from $5.7 \times 10^{-9}$ to $8.7 \times 10^{-8}$ mutations per base (Supplementary Table 2 and Supplementary Note). Seven tumors from the whole-genome sequencing series were also analyzed by high-coverage exome sequencing (245x average coverage), which showed that whole-genome sequencing was able to detect >85% of somatic coding variants, including subclonal mutations in these tumors (Supplementary Table 3 and Supplementary Note).

Notably, the median number of non-silent somatic sequence mutations and structural variations per tumor in the whole-genome sequencing (discovery) series was one, suggesting that few genetic alterations are required for oncogenesis (Fig. 1 and Supplementary Fig. 3). Despite this low lesion burden, we found multiple recurrent abnormalities in different histopathological subtypes, including KIAA1549-BRAF fusions in pilocytic astrocytomas, frequent BRAFV600E mutations in pleomorphic xanthoastrocytomas (PXAs) and rearrangements and amplification of MYB in diffuse gliomas, as well as intragenic duplications of the region of FGFR1 encoding the TKD, all of which recurred at a frequency of more than 6% when sought across the cohort of 151 tumors (Figs. 1–4 and Supplementary Table 4). Other validated whole-genome sequencing coding alterations, structural variations and sequence mutations (SNVs and small indels) occurred at a frequency of less than 4% across the study cohort (Figs. 2–4 and Supplementary Tables 4–8). However, among these were NF1 and FGFR1 sequence mutations, epismes-associated FGFR1-TACC1 and FGFR3-TACC3 gene fusions, a rearrangement of MYBL1, an H3F3A mutation (encoding a p.Lys27Met substitution) in three supratentorial diffuse astrocytomas and three new gene fusions involving BRAF or RAF1: FXR1-BRAF, BRAF-MACFI1 and QKI-RAF1. When considering sequence mutations alone, the only genes with a mutation rate
significant higher than the background rate were BRAF, NFI, H3F3A and FGFR1 (Supplementary Table 9).

Only 4 of 39 tumors (10%) in the whole-genome sequencing series lacked a MYB or MYB1 rearrangement, FGFR1 alteration, or aberration in a gene in the NFI-RAS-RAF pathway. One of these, SJLGG0034, was an oligodendroglioma from a subject aged 15 years that showed genetic aberrations characteristic of adult-type disease: an IDH1 mutation and deletion of both chromosomes 1p and 19q (Fig. 1). This tumor also had the highest number of sequence mutations in the whole-genome sequencing series, with six non-silent mutations in five genes: IDH1 (p.Arg132His), CIC (p.Val676fs and p.Ser726Arg), CHD2 (p.Asp1722Val), STYK1 (p.Pro101Leu) and BA13 (splice-site mutation affecting p.Ile869). No other tumor in the entire study cohort harbored IDH1 or IDH2 mutations.

Key abnormalities in the other three tumors in this subset of the whole-genome sequencing series were an ETV6-NTRK3 fusion associated with CDKN2A deletion, an H3F3A mutation and a rearrangement of WHSC1 (Supplementary Table 4). H3F3A, WHSC1 and three other genes found to have mutations in series tumors—ATRX, EP300 and CHD2—have histone-related functions. Despite the paucity of somatic lesions in most tumors, multiple structural variations, probably resulting from a single complex rearrangement event, were detected in five cerebral tumors, SJLGG039, SJLGG038, SJLGG033, SJLGG035 and SJLGG005, with 19, 13, 5, 4 and 3 structural variations, respectively. For SJLGG039, 18 of 19 structural variations were interconnected interchromosomal variations (Fig. 1b). Each structural variation breakpoint corresponded to the end of a (low-level) 0.12-copy gain (1.8 Mb–3.3 Mb) that was scattered across chromosomes 1, 3, 4, 10, 11, 12, 16 and 22. This alteration suggests a copy number gain of 1 in approximately 25% of cells in the tissue sample, whereas the pattern of structural variations and copy number variations (CNVs) suggests that focal amplifications are remnants of low-level chromosomal gains followed by loss of DNA in a complex rearrangement termed ‘chromothripsis’. Whole-genome sequencing identified fusion of ST6GAL1 (exon 2) to the first coding exon (exon 4) of WHSC1, and this was validated by RNA-seq, Sanger sequencing and interphase FISH (iFISH) (Supplementary Fig. 4). More details of the structural variations in SJLGG038, SJLGG033, SJLGG035 and SJLGG005 are provided in Supplementary Figures 5–8 and the Supplementary Note.

Across the tumor cohort, we identified two tumors with a germline NFI1 mutation (Supplementary Table 8). The first, a series 1 tumor (SJLGG022), had a germline splice-site mutation affecting Arg2214 coded by exon 43, as well as a somatic 4-bp frameshift mutation affecting Thr2263. No additional somatic mutations were detected in this cerebellar pilocytic astrocytoma. The second, SJLGG001225, showed a germline nonsense mutation encoding p.Trp571*. Loss of wild-type NFI1 was due to somatically acquired loss of heterozygosity (LOH) at this locus. This was first suggested in the sequence chromatogram by an increase in the mutant allele fraction from 50% in the germ line to 80% in the tumor (Supplementary Fig. 9). LOH was apparently caused by somatic copy number loss of chromosome 17 (Supplementary Fig. 10). Both tumors with an NFI1 germline mutation lost the second wild-type allele, one by acquiring a frameshift mutation, the other by LOH.

Whole-genome sequencing and SNP array data showed the presence of aneuploidy in a subset (11%) of LGGs and LGGNTs, but most tumors had very few somatic copy number alterations (Supplementary Table 10). Paired copy number analysis using whole-genome sequencing identified subclonal gain or loss across multiple chromosomes in three tumors: SJLGG008, SJLGG039 and SJLGG042 (Supplementary Fig. 11).

Across the study cohort of 151 tumors (Figs. 2–4 and Supplementary Table 4), KIAA1549-BRAF fusions were detected in 70 pilocytic astrocytomas, 2 pilomyxoid astrocytomas (PMAs)
Figure 5  FGFR1 aberrations in LGGs and LGGNTs. (a) Wild-type (WT) FGFR1 and FGFR1 with TKD duplication. With TKD duplication, two full-length TKDs (TKD-1 and TKD-2) are separated by a linker between the end (TKD-1) and beginning (TKD-2) of the autoinhibited, activation-competent kinase domain spanning amino acids 459–766. Linker length varies between 74 and 104 amino acids. (b) Normalized (tumor minus germline) whole-genome sequencing read coverage of FGFR1 in sample SJLGG008, with a 5-kb duplication encompassing exons 10–18. (c) Protein blot showing autophosphorylation of TKD-duplicated FGFR1 (Dp006, Dp008, Dp044) in 293T cells (pFGFR1). FGFR1 autophosphorylation is associated with activation of the MAPK/ERK pathway, as indicated by increased phosphorylation of ERK1 and ERK2 (pERK1/2). Introducing a kinase-dead (KD) alteration (p.Asp623Ala) into either TKD-1 (Dp006KD-prox) or TKD-2 (Dp006KD-dist) of Dp006 abrogates MAPK/ERK pathway upregulation, but an active proximal TKD will still autophosphorylate FGFR1, whereas one with an active distal TKD will not. (d) FGFR1-TACC1 fusion protein detected in SJLGG018 preserving the TKD. (e) Normalized (tumor minus germline) whole-genome sequencing read coverage plot (top) and structural variation connection plot (bottom) for the FGFR1-TACC1 locus suggesting formation of an episome. IG, immunoglobulin-like domain; TM, transmembrane domain; PA, pilocytic astrocytoma; OA, oligoastrocytoma; DA, diffuse astrocytoma; O, oligodendroglioma; DNET, dysembryoplastic neuroepithelial tumor; TACC, TACC domain.
and a single brainstem ganglioglioma and were present in 59%, 90% and 80% of pilocytic astrocytomas and PMAs in the supratentorial, posterior fossa and spinal anatomical compartments, respectively. BRAFV600E mutations were detected in a high proportion of PXAs (70%) and at lower frequencies in diffuse astrocytomas (23%), gangliogliomas (33%) and pilocytic astrocytomas (6%). Abnormalities of genes encoding proteins influencing the MAPK/ERK pathway were detected in almost all (95%) pilocytic astrocytomas and PMAs and in 82% of all LGGs and LGGNTs in the study. Among LGGs characterized by diffuse infiltration of adjacent brain, grade II gliomas and angiocentric gliomas, aberrations of MYB or MYBL1 and of FGFR1 or FGFR3 (duplication of the TKD-encoding region, missense mutation or fusion to TACC1 or TACC3, respectively) were detected in 68%. Of the remaining tumors, one oligodendroglioma was characterized by alterations typical of adult-type disease (SJLGG034), three contained an H3F3A mutation (encoding p.Lys27Met), four others harbored a BRAFV600E mutation, and one had a FAM131B-BRAF fusion. Only 9.9% of LGGs and LGGNTs, all outside of series 1 and mostly cerebral in location, had no detectable genetic alteration.

**FGFR1 alterations in LGGs and LGGNTs**

Whole-genome sequencing identified an intragenic duplication of the entire FGFR1 region encoding the TKD in 2 of 39 tumors. This duplication encompassed exons 10–18 to produce an in-frame fusion separated by a linker element of variable length (Fig. 5). Altogether, across the study cohort, there were 13 tumors with this structural variation,
Figure 7 Activation of MAPK/ERK and PI3K pathways in LGGs and LGGNTs with FGFR1, MYB and BRAF abnormalities. (a,b) Multiplex immunoassay phosphorylation levels for three MAPK/ERK (MEK, ERK, JUN) (a) and three PI3K (AKT, p70-S6K, NF-κB) (b) pathway components are given relatively to levels in normal brain for tumors with either FGFR1 duplication (n = 11), MYB rearrangement (n = 6) or KIAA1549-BRAF fusion (n = 18). Error bars, s.e.m. Gene expression profiling showed no significant elevation in the levels of corresponding mRNA transcripts, with the exception of JUN. (c) Protein blots showing upregulation of the ERK (pERK1/2) and PI3K (pAKT and pS6) pathways in three groups of LGGs characterized by MYB (AKT inhibits GSK-3β), FGFR1 (FGFR1 dup) or KIAA1549-BRAF (pGSK-3β) by phosphorylation, pGSK-3β. (d) Diagrams of MAPK/ERK (left) and PI3K (right) pathway components downstream of FGFR1, showing the targets of pharmaceuticals used to inhibit the actions of TKD-duplicated FGFR1 (Fig. 8).

4 of which were identified as primary tumors and 2 as recurrent tumors. First and second surgeries were 17 and 19 months apart, and no anaplastic progression was found in either recurrent tumor. Genomic profiles of the paired primary and relapse tumor samples analyzed by whole-genome sequencing (SJLGG006_D and SJLGG006_R, respectively) were identical; alongside the FGFR1 duplication, there were no tier 1 SNVs, one tier 2 SNV and six tier 3 SNVs.

All but 3 of the 11 primary tumors with FGFR1 duplication affecting the TKD were diffuse (grade II) gliomas, and all but 1 were located in the cerebral cortex (Figs. 2–5). Although relatively infrequent across the entire cohort of LGGs and LGGNTs (7.4%), FGFR1 duplication involving the TKD was present in 24% of grade II diffuse cerebral gliomas. The entire tumor cohort was screened by iFISH for FGFR1 amplification or rearrangement, and none was found. However, missense mutations in FGFR1 affecting the TKD were detected in three LGGs (Supplementary Table 4).

Two structural variations identified in whole-genome sequencing data from sample SJLGG018 were predicted to form an episome that connects the 3′ UTR of FGFR1 to the intron 6 sense strand of TACC1 (Supplementary Fig. 12). The region consists of two segments with copy number gain of one and an unamplified 6-kb segment caused by loss of DNA during episome formation. Using both the split reads from RNA-seq data and RT-PCR, we confirmed an in-frame FGFR1-TACC1 fusion transcript that joins exon 18 of FGFR1 with exon 7 of TACC1. Using copy number data from SNP array analysis or RNA-seq, we were able to identify and then to validate by RT-PCR two additional tumors (SJLGG01212 and SJLGG01264) with amplification segments on FGFR1 and TACC1 and fusion of these genes. These methods also identified a single FGFR3-TACC1 fusion (SJLGG01206). Substantial activation of both MAPK/ERK and phosphatidylinositol 3-kinase (PI3K) pathways (with phosphorylated ERK1 and ERK2 at 166 times the level in normal brain and phosphorylated AKT at 58 times the level in normal brain) was shown by multiplex immunoassay for SJLGG018.

Palliative therapy (either whole-brain or focal radiation therapy) for the primary tumors was administered. All LGGs with FGFR1 amplification or rearrangement in a further five tumors: two diffuse astrocytomas, two angiocentric gliomas and one oligodendroglioma (Figs. 2 and 6 and Supplementary Figs. 6–8 and 13). No other tumor showed a potential MYB rearrangement or copy number abnormality in a further five tumors: two diffuse astrocytomas, two angiocentric gliomas and one oligodendroglioma (Figs. 2 and 6 and Supplementary Figs. 6–8 and 13).

**MYB and MYBL1 rearrangements in diffuse LGGs**

Four tumors analyzed by whole-genome sequencing, all of which were cerebellar grade II diffuse astrocytomas, harbored a new rearrangement of MYB or MYBL1. Subsequent analysis of the entire study cohort using iFISH with MYB, MYBL1 and MYBL2 probes showed a potential MYB rearrangement or copy number abnormality in a further five tumors: two diffuse astrocytomas, two angiocentric gliomas and one oligodendroglioma (Figs. 2 and 6 and Supplementary Figs. 6–8 and 13).

No other tumor showed a potential MYB or MYBL1 rearrangement. MYB amplification, manifesting as episome formation, was detected in two tumors by whole-genome sequencing, RNA-seq and iFISH. All LGGs with MYB rearrangement on which whole-genome sequencing was not performed were analyzed by RNA-seq, which detected several partner genes (Supplementary Fig. 13). All structural variations were associated to a variable extent with deletion of a 3′ UTR miRNA (miRNA)-binding site. Of two tumors with MYB amplification, one also showed a 3′ deletion, but the amplicon in the other...
extended beyond the 3′ UTR miRNA-binding sites. In all tumors with MYB rearrangement or amplification, MYB expression was increased at the protein level (Fig. 6).

MYB alterations occurred only in cerebral gliomas with infiltrative behavior: diffuse astrocytomas, oligodendrogliomas and angiocentric gliomas. Although relatively infrequent across the entire cohort of LGGs and LGGNTs (6%), MYB and MYBL1 aberrations were present in 25% of diffuse cerebral gliomas (Fig. 2 and Supplementary Table 4). No MYB, MYBL1 or MYBL2 alterations were identified by iFISH in 33 pediatric HGGs or 79 ependymomas.

Expression profiling and activation of signaling pathways
Gene expression profiling of LGGs and LGGNTs using both Affymetrix U133 Plus 2.0 arrays and RNA-seq clearly showed clustering according to genetic abnormality. Tumors also clustered according to anatomical site and pathology because of the strong associations between these variables and specific genetic abnormalities (Supplementary Fig. 14 and Supplementary Note). No pattern was noted based on sex.

Multiplex immunoassays and protein blotting showed activation of the MAPK/ERK and PI3K pathways in groups of LGGs and LGGNTs characterized by KIAA1549-BRAF fusion, duplication in FGFR1 of the region encoding the TKD or MYB alteration (Fig. 7). Components of other signaling pathways tested by multiplex immunoassay, such as the JAK-STAT pathway, showed no consistent alterations.

**TKD-duplicated FGFR1 is transforming**
Neonatal (post-natal day 2, P2) Tp53-null astrocytes transfected with a TKD-duplicated FGFR1 construct (Dp006 or Dp008) and transplanted into the brains of nude mice generated high-grade astrocytic tumors with short latency and complete penetrance (Fig. 8). Transplanted cells containing empty vector or wild-type FGFR1 constructs did not generate tumors in mice imaged 60 d after transplantation. Tumors generated with both Dp006 and Dp008 were characterized by activation of the MAPK/ERK and PI3K pathways (Fig. 8).

When transplanted into 293T cells, TKD-duplicated FGFR1 constructs, with different lengths of linker elements separating the

Figure 8 TKD-duplicated FGFR1 in neonatal astrocytes generates gliomas in vivo. (a–f) Histopathology of tumors expressing TKD-duplicated FGFR1 in mouse brain. Diffuse gliomas could have either an astrocytic (a) or oligodendroglial (b) phenotype and were focally immunopositive for GFAP (c). Tumor cells but not normal brain showed overexpression of FGFR1 (d), pErk1/2 (e) or pAkt (f). Scale bars, 100 µm. (g) Survival curves for mice transplanted with neonatal astrocytes with empty vector (MIG), wild-type FGFR1 (WT) or two variants of TKD-duplicated FGFR1 (Dp006, Dp008). All Dp006 and Dp008 mice died from the effects of a glioma, with a median survival of 23 d. (h) Protein blots of normal brain (1) or tumor cell lysates (separate mice: Dp006 (2,3) and Dp008 (4,5)). TKD-duplicated FGFR1 is associated with greater amounts of phosphorylated FGFR1 (pFGFR1, Tyr463/Tyr464), pAkt (Ser473) and pErk1/2 (Thr202/Thr204). (i) Inhibitor studies showing that the FGFR1 inhibitor PD173074 blocks downstream activation of the MAPK/ERK pathway. The MEK1 inhibitor PD0325901 does not block pErk1/2 or pAkt but does block pAkt downstream activation of the PI3K pathway. The PI3K-mTOR inhibitor BEZ235 does not block FGFR1 autophosphorylation but inhibits PI3K pathway activation.

Table 4

| Factor | WT | Dp006 | Dp008 |
|--------|----|-------|-------|
| Percent survival | 100 | 90 | 85 |
| Days after transplantation | 60 | 50 | 40 |

Figure 2 TKD-duplicated FGFR1 is transforming. (a) Multicolor immunofluorescence of a glioblastoma xenograft expressing TKD-duplicated FGFR1. FGFR1 expression is shown by green fluorescence; pErk1/2 by red; and Ki67 by blue. Scale bars, 200 µm. (b) Histopathology of a glioblastoma expressing TKD-duplicated FGFR1. Scale bars, 100 µm. (c) Kaplan-Meier survival curves for mice transplanted with glioblastomas expressing TKD-duplicated FGFR1. (d) Kaplan-Meier survival curves for mice transplanted with glioblastomas expressing wild-type FGFR1 or empty vector (MIG). (e) Kaplan-Meier survival curves for mice transplanted with glioblastomas expressing TKD-duplicated FGFR1 (Dp004, Dp006) and downstream activation of the MAPK/ERK pathway.
TKDs, resulted in FGFR1 autophosphorylation and activation of the MAPK/ERK pathway (Figs. 5 and 8 and Supplementary Fig. 15). FGFR1 inhibitors blocked autophosphorylation and downstream activation of the MAPK/ERK pathway, and MEK inhibitors abrogated MAPK/ERK activity (Fig. 8). When transfected into MCF7 cells, the same FGFR1 constructs resulted in FGFR1 autophosphorylation and activation of the PI3K pathway, which were both blocked by a specific FGFR1 inhibitor. A PI3K-mTOR inhibitor but not a MEK inhibitor also switched off PI3K activation.

**DISCUSSION**

LGGs encompass WHO grade I pilocytic astrocytomas, the less prevalent infiltrative WHO grade II diffuse gliomas, which have astrocytic, oligodendrogial or mixed oligoastrocytotic cytological features, and rare entities, such as the PXA and angioencephaloid glioma. In addition, LGGNTs, such as gangliogliomas, contain a glial component with LGG histopathology and are usually grouped with LGGs in therapeutic classifications (Supplementary Table 10).

Most pediatric LGGs are cerebellar pilocytic astrocytomas. These are circumscribed tumors, which are usually amenable to surgical resection and have a low recurrence rate. However, pilocytic astrocytomas at other sites, such as the brainstem or optic pathways, are less well delineated from vital structures in adjacent brain, and complete excision is usually impossible. Inoperable tumors, including diffuse grade II LGGs, grow slowly and may respond to adjuvant therapies but over time cause substantial morbidity and premature death. The molecular genetics of pediatric diffuse grade II LGGs have not been well characterized, unlike those of their adult counterparts, yet it is among these tumors that we have found the principal new recurrent abnormalities reported in this study.

Using whole-genome sequencing, we have mapped the genomic landscape of 39 pediatric LGGs and LGGNTs and shown that most of these pediatric tumors have only one somatic genetic event that affects protein coding. Furthermore, only 1 of 151 tumors (SJLGG001259_D1), with an NFI frameshift mutation, an activating FGFR1 mutation and a KRAS mutation, harbored genetic abnormalities with potentially overlapping effects on the MAPK/ERK pathway. In other tumors, NFI, RAF or RAS, FGFR1, and MYB or MYBL1 abnormalities were mutually exclusive. Whole-genome sequencing also identified several new RAF gene abnormalities, including FXR1-BRAF fusion, BRAF-MACF1 fusion and QKI-RAF1 fusion, augmenting previous accounts of other rare defects in MAPK/ERK pathway genes, such as SRGAP3-RAF1 and FAM131B-BRAF fusions.

In the present study, 24% of diffuse WHO grade II cerebral gliomas showed a previously unreported duplication of the FGFR1 region encoding the TKD, which produces FGFR1 auto phosphorylation and activation of both MAPK/ERK and PI3K pathways. Autophosphorylation of FGFR1 might arise from ligand-independent homodimerization, facilitated by an extended intracytoplasmic peptide tail and apposition of duplicated TKDs. A dual-TKD construct with truncated linker (Dp-NLK) did not stimulate the MAPK/ERK pathway, suggesting that downstream signaling is dependent on linker length as well as flexibility (Supplementary Fig. 14). Duplication of the portion of EGRF encoding the TKD has been reported in a single glioblastoma, but a similar duplication in FGFR1 has not previously been described in other CNS tumors.

Our data set includes other infrequent FGFR aberrations in LGGs and LGGNTs, including missense FGFR1 mutations and FGFR1-TACC1 and FGFR3-TACC3 fusions that are all predicted to result in constitutive FGFR signaling. Missense FGFR1 mutations have been reported as a rare event in glioblastoma and malignant melanoma and fusions with TACC genes have recently been reported at low frequency (3%) in glioblastomas. This study also reported microamplification at the FGFR3-TACC3 locus, suggesting that the FGFR3-TACC3 fusion is likely to arise from tandem duplication, as both FGFR3 and TACC3 are transcribed in the same orientation on the reference genome and are less than 50 kb apart. By contrast, FGFR1 and TACC1 are transcribed in opposite orientations and are separated by 400 kb. An epistrome structure, which was supported by the presence of two structural variations and two amplification segments in this region, makes it possible to alter the relative orientation of the two genes to generate a fusion protein containing the FGFR1 TKD and the TACC domain of TACC1. FGFR1 amplifications and other FGFR1 fusions, which are important oncogenic mechanisms in several neoplasms, were not detected in our series of LGGs and LGGNTs.

The region containing FGFR1 and TACC1 on chromosome 8 encompasses two other genes, WHSC1L1 and LETM2. The paralogous region on chromosome 4 contains, in the following order, TACC3, FGFR3, LETM1 and WHSC1. The gene order synteny was maintained in both paralogous duplications. Aside from these fusions, recurrent somatic mutations and structural variations in FGFR1 and LETM1 (p.Ser580Arg) and WHSC1 (ST6GAL1-WHSC1 fusion) were found in tumors from our study cohort, suggesting that, in the setting of minimal somatic lesions, disruption of this highly conserved multi-gene group may be important in gliomagenesis.

MYB or MYBL1 abnormalities were evident in 25% of cerebral gliomas with a diffusely infiltrative architecture, including two angiocentric gliomas. Angiocentric gliomas share some histological features with ependymoma, but we found no MYB, MYBL1 or MYBL2 alterations in a large series of ependymomas from across the neuraxis. We previously identified two structural alterations that result in MYB overexpression in pediatric diffuse cerebral gliomas: an episomal-associated amplification in MYB encompassing the transactivation domain (TAD) and a focal deletion of its negative regulatory region plus an inhibitory miRNA-binding regulatory region in its 3’ UTR. In the present study, whole-genome sequencing and RNA-seq identified new MYB and MYBL1 rearrangements that involved fusion with several different genes. Although some MYB fusion partners have reported roles in oncogenesis, all detected aberrations can produce MYB overexpression by one of the two mechanisms described above. Overall, mutually exclusive FGFR1 and MYB or MYBL1 abnormalities were present in 56% of diffuse gliomas.

Our comprehensive analysis of NFI, RAF or RAS, FGFR1 and MYB abnormalities across a series of LGGs and LGGNTs representative of the disease showed that nearly all LGGs and LGGNTs in the spinal and posterior fossa compartments, which are dominated by pilocytic astrocytomas, are characterized by KIAA1549-BRAF fusion genes, whereas cerebral tumors, including most diffuse gliomas, are more heterogeneous. A subset of seven LGGs (4.7%), most with a concurrent BRAF abnormality, had H3F3A mutations or abnormalities in other genes linked to histone modification, including CHD1 and CHK2. In our series, the underlying genetic causes of only 9.9% of LGGs and LGGNTs remained completely uncharacterized.

H3F3A mutations have recently been found in up to one-third of pediatric glioblastomas, the most aggressive of HGGs. Midline tumors are associated with an H3F3A mutation encoding a p.Lys27Met substitution, and this mutation is particularly prevalent in diffuse pontine gliomas. Although we detected this H3F3A mutation in only three (1.9%) of tumors in our series, this finding does indicate some overlap between the genetics of pediatric LGGs and HGGs, and it is notable that two of three diffuse grade II astrocytomas in which we found
this mutation were thalamic; the other was from the cerebral cortex. One child with a thalamic tumor has died within 2 years of diagnosis, but the others have progression-free survival beyond 10 years. None of these three tumors also contained a TP53 or ATRX mutation; instead, one contained a KRAF mutation (p.Gln61His) and another a BRAFV600E mutation. Only 1 of 33 tested HGGs (3%) had an anaplastic oligoastrocytoma that had progressed from a grade II tumor, contained a duplication in FGFR1 of the region encoding the TKD, and no MYB abnormalities were found. Any overlap between the genetics of adult HGGs and pediatric LGGs seems to be confined to rare FGFR1 missense mutations and FGFR-TACC fusions.

The histopathological features of WHO grade II diffuse gliomas occurring in children or adults appear very similar, yet their clinical behaviors and underlying genetics are distinct. Over a period of 10–15 years after surgery and despite adjuvant therapies, up to two-thirds of adult grade II gliomas progress to high-grade disease (WHO grade III or IV), heralding a poor prognosis. In contrast, childhood grade II gliomas can show relentless slow growth, but pathological progression occurs much less frequently. Our data support the hypothesis that distinct sets of genetic aberrations underlie clinicopathological differences between adult and pediatric disease. Most adult grade II gliomas (>80%) have an IDH1 or IDH2 mutation, usually encoding a p.Arg132His substitution in IDH1, which is considered to be an early transforming event. About two-thirds of adult diffuse gliomas with an astrocytic phenotype have a concurrent TP53 mutation, and >80% of grade II oligodendrogliomas show deletion of both chromosomes 1p and 19q. Progression to high-grade pathology is accompanied by the acquisition of additional genetic abnormalities, such that the range of adult diffuse gliomas from grade II to grade IV (glioblastoma) is characterized by the stepwise accumulation of specific genetic abnormalities. Rarely, adult-type grade II disease can present in childhood, and our series contained one such example, an oligodendroglioma with an IDH1 mutation encoding a p.Arg132His alteration, deletion of both 1p and 19q, and CIC mutations. There is a high concordance between IDH1 and CIC mutations in adult oligodendrogliomas, suggesting cooperation between these genes. In contrast, our data suggest that a separate set of genetic aberrations characterizes pediatric diffuse gliomas, and a single genetic aberration can be transforming in the majority of cases.

LGGs with duplication of the FGFR1 TKD or MYB overexpression show activation of the MAPK/ERK and PI3K pathways, having immunohistochemical profiles that are similar to those of pilocytic astrocytomas with KIAA1549-BRAF fusions and suggesting potential targets for therapeutic intervention. Combined activation of these pathways was found in our functional studies of TKD-duplicated FGFR1. Against a facilitative Tp53-null background in transplanted neonatal astrocytes, TKD-duplicated FGFR1 was transforming, rapidly generating high-grade astrocytic tumors that had combined activation of these signaling pathways. In vitro studies using two cell lines transfected with constructs expressing TKD-duplicated FGFR1 showed that the specific FGFR1 inhibitors PD173074 and BGJ398 and the MEK1 inhibitor PD0325901 could block FGFR1 autophosphorylation and constitutive activation of the MAPK/ERK pathway, respectively, and that upregulation of the PI3K pathway could be blocked by the specific inhibitor BEZ235. Such findings present a potential opportunity for the use of targeted therapies in the care of patients with LGGs that are unresectable and cause substantial morbidity, as they do for patients with other cancers in which FGFRs have a critical role.

Whole-genome sequencing of pediatric LGGs and LGGNTs has identified multiple previously unreported oncogenic mechanisms and facilitated discovery of a greatly extended genetic profile for pediatric diffuse (WHO grade II) gliomas. Our comprehensive analysis has also emphasized the potential therapeutic benefit of targeting upregulation of the MAPK/ERK and PI3K pathways in a disease that causes considerable morbidity and early mortality.
ONLINE METHODS
Subject cohorts and sample details. The study cohort consisted of 151 tumors from 149 affected individuals (Supplementary Fig. 1). Tissue was available at the time of diagnosis and relapse for two tumors (SJLG006_D and SJLG006_R; SJLG049_D and SJLG049_R). Archived series of 33 pediatric HGGs (WHO grade III or IV), 79 ependymomas and 11 adult anaplastic oligodendrogliomas (WHO grade III) were screened for relevant alterations. Tissue samples had been snap frozen at the time of first resection, which in all cases predated adjuvant therapy. DNA and RNA were extracted from frozen tissue and peripheral blood leukocytes\(^1\). Archived formalin-fixed paraffin-embedded (FFPE) blocks and slides were retrieved for pathology review and specific analyses.

Whole-genome and transcriptome sequencing and analysis. Whole-genome sequencing, RNA-seq, exome sequencing and SNP or gene expression profiling by array were performed as previously described\(^{39,60}\). For both whole-genome sequencing and RNA-seq, paired-end sequencing was performed using the Illumina Genome Analyzer IIx platform with 100-bp read length.

Whole-genome sequencing mapping, coverage and quality assessment, SNP and indel detection, tier annotation for sequence mutations, prediction of deleterious effects of missense mutations and identification of LOH have been described previously\(^{25}\). Structural variations were analyzed using CREST and annotated as before\(^{60,61}\). The reference human genome assembly NCBI Build 37 was used for mapping of all samples. CNVs were identified by evaluating the difference in read depth for each tumor and matched normal tissue using a new algorithm, CONSENTING (Copy Number SEgmentation by Regression Tree In Next-Gen sequencing).

SNVs were classified into the following three tiers, as previously described\(^{60}\); tier 1: coding synonymous, nonsynonymous, splice-site and noncoding RNA variants; tier 2: conserved variants (requiring conservation score \(\geq 500\), based on either the phastConsElements28way table or the phastConsElements17way table from the UCSC Genome Browser; variants in regulatory regions were annotated by UCSC annotation (regulatory annotations included are targetScan\(S\), ORRegAnno, tbsConsSites, enhancerHubs, eponine, firstEF, LI TAF1 Valid, Poly(A), switchDitTs, encodeUvienaRnaz, laminB1 and cpgIsland-Ext)), and tier 3: variants in non-repeat–masked regions.

Tissue samples had been snap frozen at the time of first resection, which in all cases predated adjuvant therapy. DNA and RNA were extracted from frozen tissue and peripheral blood leukocytes\(^1\). Archived formalin-fixed paraffin-embedded (FFPE) blocks and slides were retrieved for pathology review and specific analyses.

High-throughput sequencing of 32 candidate genes in tumors from series 2. All coding exons of the following genes were screened in 84 LGGs and LGGNTs: ADAMTS9, ATRX, BAID, BRAF, CDK13, CHD2, CIC, DCTN1, DS3G, EP300, FGFR1, FGFR2, FGFR3, FLI1, FXR1, KRAS, LETM1, MAML2, MYB, MYBL1, NEURL4, NF1, NSMAF, PRIC285, PTK-2, QKI, RAF1, SPH1, STYK1, TFDP1, TMPRSS11D and TP53. This list includes every gene with a validated non-silent mutation present in the dominant clone (mutant allele fraction > 0.25 by whole-genome sequencing) of tumors from series 1, genes with structural variations (for example, FGFR1), genes with biology related to that of mutated genes (for example, FGFR2) and TP53.

The analysis was undertaken using PCR-based 3730 capillary sequencing at Beckman Coulter Genomics, as previously described\(^{45}\). Putative SNV's and indel variants were detected by SNPdetector25 (ref. 65). Non-silent coding variations present in tumor but absent in normal tissue were considered somatic mutations after manual review using the program consensus. To remove additional germline variations from the data set generated by sequencing tumors without matching germline samples, new non-silent mutations were compared to the 5 exomes data (National Heart, Lung, and Blood Institute Exome Sequencing Project) and to a database of germline variations identified in the Pediatric Cancer Genome Project\(^{46}\). Novel variants that passed this germline filter were manually reviewed and presented in two groups; those at a site of known somatic sequence mutation or that caused a truncation mutation were grouped with somatic mutations, and others were considered variants of unknown origin.

Analysis of the significance of mutated genes. To assess the significance of validated non-silent sequence mutations across the entire cohort, we used the Significantly Mutated Gene test\(^{57}\), which identifies genes with significantly higher mutation rates than the background mutation rate.

Experimental validation of genetic aberrations identified in whole-genome sequencing. All sequence mutations in exons (tier 1 SNVs and indels) discovered in whole-genome sequencing were validated experimentally by Sanger, 454 or MiSeq sequencing. Of the 89 high-quality tier 1 SNVs tested, 86 were validated at a rate of 96.6%. All three high-quality somatic indels were validated. All structural variations affecting coding regions were validated by Sanger sequencing.

Validation by 454 or Sanger sequencing was as previously described\(^{40}\). For MiSeq sequencing, primer pairs were designed with Primer3 to flank the genomic regions containing putative SNVs and indels. These regions were amplified using Accuprime GC-rich DNA polymerase (Life Technologies) with DNA amplified from genomic DNA used as the PCR template (Qiagen). Amplicons were barcoded and prepared for sequencing using the Nextera XT DNA Sample Prep kit (Illumina). Libraries were sequenced on MiSeq using the paired-end 150-cycle protocol, and variant analysis was performed (MiSeq Reporter). Further evaluation of SNVs and indels was performed by manual review of the BAM files using Bambino\(^{68}\).

Mutation hotspot analysis by Sanger sequencing. Mutational hotspots in BRAF, KRAS, FGFR1, IDH1, IDH2 and H3F3A were sequenced in genomic DNA from the entire series of tumors using previously published primers\(^{11,22,42}\).

Validation of structural variations. Structural variations were validated by PCR in cDNA using specific primers (Supplementary Table 1). Primer combinations yielding a structural variation–specific product were validated by direct sequencing. Additional primers were then designed for specific structural variations, covering the majority of exons within each gene partner, and these were used to screen the tumor cohort for potential structural variations, including QKI-RAF1, FXR1-BRAF, FGFR1-TACCI, ETV6-NTRK3, KIAA1549-BRAF, SRGA3-RAF1 and those within FGFR1.

Copy number analysis and expression profiling by array. Affymetrix arrays were used for the analysis of copy number alterations (SNP 6.0) and expression profiling (U133 Plus 2.0) as previously described\(^{48}\).

Construction of FGFR1 duplication vectors. Full-length ORF cDNA for human wild-type FGFR1 and three FGFR1 duplication variants were amplified by RT-PCR from human brain RNA pools or LGG RNA from SJLG006_D, SJLG008_D and SJLG044_D, with forward primer FGFR1ex2-for (AACGTGGGATGTGGAGCTGGA) and reverse primer FGFR1ex18-rev (CAGTCAGCGGGCTTTAGGTGC). PCR products were cloned in PCR2.1 using a TA-cloning kit (Invitrogen) and verified by sequencing. After introducing 5’ BamHI and 3’ Xhol restriction sites by PCR, fragments encoding wild-type and duplication variants were subcloned into retroviral vector MCV-ires-EGF (MIG) digested with BamHI and Xhol to generate MIG-FGFR1-wt, MIG-FGFR1-Dp006, MIG-FGFR1-Dp008 and MIG-FGFR1-Dp044 constructs\(^{70}\). A single mutation encoding an aspartic acid to alanine substitution, p.Asp263Ala, was introduced into the wild-type FGFR1 fragment by site-directed mutagenesis to make a kinase-inactive construct (MIG-FGFR1-KD). The same kinase-dead mutation was also introduced into FGFR1 duplication variants, either at the proximal site (MIG-FGFR1-Dp006KD-prox) or at a corresponding site within the distal fragment (MIG-FGFR1-Dp006KD-dist). In addition, a dual TKD construct (NLK) was prepared with a truncated linker of 22 amino acids between the two TDKs.

**FGFR1 transfection studies.** For inhibition assays, 293T or MCF7 cells transfected with FGFR1 constructs were treated with serum-free DMEM for 12 or 18 h, respectively, and incubated with inhibitors for 3 or 2 h, respectively.
The FGFR1 inhibitors BGJ398 and PD173074, MEK inhibitor PD0325901 and PI3K-mTOR inhibitor BEZ235 were dissolved in DMSO and added to cell cultures at a concentration of 100 nM when used as single agents. For dual-agent inhibition, PD0325901 and BEZ235 were each added at a concentration of 50 nM.

Primary astrocyte cell culture and tumorigenesis. FGFR1 retroviral constructs (MIG-FGFR1-wt, MIG-FGFR1-Dp006 and MIG-FGFR1-Dp008) or control retrovirus expressing green fluorescent protein (GFP; MIG) were used to transduce -null early-passage primary mouse astrocytes (PMAs) established from 2-d-old GFAP-Cre; Trp53-/-null early-passage primary mouse astrocytes (PMAs) as previously described71–73. For tumorigenesis studies, 2 × 10^6 transduced PMAs were implanted into CD1 nude mouse brains72.

Tissue collection, immunohistochemistry and iFISH. Tumors from mice were processed and evaluated histopathologically as previously described. Immunohistochemistry using heat-mediated antigen retrieval was undertaken as previously described74. Tumors were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Sections (5 μm) were mounted on slides coated with 3-aminopropyltriethoxysilane (Aldrich). Immunohistochemistry was performed to detect the expression of FGFR1, N-terminal region of MYB (Abcam, EP769Y)22. Dual-color iFISH was undertaken as previously described74. FISH probes (Supplementary Table 11) were derived from BACs (BACPAC Resources), labeled with either Alexa Fluor 488 or Rhodamine fluorochromes and validated on normal control metaphase spreads.

Immunoblot analysis and phosphoprotein multiplex immunoassays. For immunoblotting, transfected cells were lysed and extracts clarified by centrifugation. Protein blotting was performed as previously described with antibodies to FGFR1 (2144-1, Epitomics), phosphorylated FGFR1 (Tyr653/Tyr654; AT-2031, MBL and SC-30262-R, Santa Cruz Biotechnology), p44/42 ERK (4695), phosphorylated p44/42 ERK (Thr202/Tyr204; 4370), phosphorylated AKT (7831), p70-S6 kinase and phosphorylated NF-κB p65. Protein extracts from a control cell line and a phosphatase-treated HeLa cell lysate served as positive and negative controls, respectively.

The immunoassay used antibodies to the following phosphoproteins: phosphorylated ERK1/2, phosphorylated MEK1, phosphorylated AKT, phosphorylated GSK3β and GSK3α, phosphorylated JUN, phosphorylated p70-S6 kinase and phosphorylated NF-κB p65. Protein extracts from a control cell line and a phosphatase-treated HeLa cell lysate served as positive and negative controls, respectively.

59. Zhang, J. et al. A novel retinoblastoma therapy from genomic and epigenetic analyses. Nature 481, 329–334 (2012).
60. Zhang, J. et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature 481, 157–163 (2012).
61. Wang, J. et al. CREST maps somatic structural variation in cancer genomes with base-pair resolution. Nat. Methods 8, 652–654 (2011).
62. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760 (2009).
63. McPherson, A. et al. deFuse: an algorithm for gene fusion discovery in tumor RNA-Seq data. PLoS Comput. Biol. 7, e1001138 (2011).
64. Mullighan, C.G. et al. CREBBP mutations in relapsed acute lymphoblastic leukaemia. Nature 471, 235–239 (2011).
65. Zhang, J. et al. SNPdetector: a software tool for sensitive and accurate SNP detection. PLoS Comput. Biol. 1, e53 (2005).
66. Downing, J.R. et al. The Pediatric Cancer Genome Project. Nat. Genet. 44, 619–622 (2012).
67. Dees, N.D. et al. MuSiC: identifying mutational significance in cancer genomes. Genome Res. 22, 1589–1598 (2012).
68. Edmonson, M.N. et al. Bambino: a variant detector and alignment viewer for next-generation sequencing data in the SAM/BAM format. Bioinformatics 27, 865–866 (2011).
69. Mullighan, C.G. Single nucleotide polymorphism microarray analysis of genetic alterations in cancer. Methods Mol. Biol. 730, 235–258 (2011).
70. Persons, D.A. et al. Enforced expression of the GATA-2 transcription factor blocks normal hematopoiesis. Blood 93, 488–499 (1999).
71. Bajenaru, M.L. et al. Astrocyte-specific inactivation of the neurofibromatosis 1 gene (NF1) is insufficient for astrocytoma formation. Mol. Cell Biol. 22, 5100–5113 (2002).
72. Endersby, R., Zhu, X., Hay, N., Ellison, D.W. & Baker, S.J. Nonredundant functions for Akt isoforms in astrocyte growth and gliomagenesis in an orthotopic transplantation model. J. Immunol. 160, 3135–3142 (1998).
73. Jonkers, J. et al. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat. Genet. 29, 418–425 (2001).
74. Ellison, D.W. et al. Definition of disease-risk stratification groups in childhood medulloblastoma using combined clinical, pathologic, and molecular variables. J. Clin. Oncol. 29, 1400–1407 (2011).
75. Tang, B. et al. Characterization of signal transduction through the TCR-ζ chain following T cell stimulation with analogue peptides of type II collagen 260–267. J. Immunol. 160, 3135–3142 (1998).