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Distinct *H3F3A* and *H3F3B* driver mutations define chondroblastoma and giant cell tumor of bone

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It is recognized that some mutated cancer genes contribute to the development of many cancer types, whereas others are cancer type specific. For genes that are mutated in multiple cancer classes, mutations are usually similar in the different affected cancer types. Here, however, we report exquisite tumor type specificity for different histone H3.3 driver alterations. In 73 of 77 cases of chondroblastoma (95%), we found p.Lys36Met alterations predominantly encoded in *H3F3B*, which is one of two genes for histone H3.3. In contrast, in 92% (49/53) of giant cell tumors of bone, we found histone H3.3 alterations exclusively in *H3F3A*, leading to p.Gly34Trp or, in one case, p.Gly34Leu alterations. The mutations were restricted to the stromal cell population and were not detected in osteoclasts or their precursors. In the context of previously reported *H3F3A* mutations encoding p.Lys27Met and p.Gly34Arg or p.Gly34Val alterations in childhood brain tumors, a remarkable picture of tumor type specificity for histone H3.3 driver alterations emerges, indicating that histone H3.3 residues, mutations and genes have distinct functions.

Chondroblastoma is a cartilaginous tumor presenting in childhood to early adulthood that arises in active growth plates, most commonly in long bones[1]. Although locally destructive and prone to recurrence, it is considered a benign tumor that rarely metastasizes[1]. The biology of chondroblastoma is not understood, including its relation to other tumors located in the epiphysis, such as giant cell tumors of bone.

Using massively parallel sequencing, we sequenced the complete genomes of six chondroblastomas and normal DNA samples from the same individuals (**Supplementary Table 1**). Overall, we identified a relatively low somatic mutation burden, comprising between 542 and 668 substitutions (0.2 substitutions/Mb) and 15 to 25 indels (0.006 indels/Mb) per genome (**Table 1** and **Supplementary Table 2**). The predominant substitution class was C-to-T transitions, which showed enrichment at CpG dinucleotides. Copy number and rearrangement analysis showed that tumors were diploid overall, with a paucity of structural changes (**Table 1** and **Supplementary Fig. 1**).

A striking observation was that all six chondroblastomas harbored recurrent somatic mutations in one of two genes, *H3F3A* or *H3F3B*, which encode the replication-independent histone H3.3. *H3F3A* and *H3F3B* reside on chromosomes 1 and 17, respectively. They have different exonic and intronic DNA sequences, but both encode histone H3.3 proteins of identical amino acid sequence[2,3]. Cancer driver mutations have previously been identified in *H3F3A* but not in *H3F3B*. All six chondroblastomas carried p.Lys36Met histone H3.3 alterations, five of which were encoded in *H3F3B* and one of which was encoded in *H3F3A*. No other putative driver variants were identified. We extended the investigation of *H3F3A* and *H3F3B* to an additional 71 chondroblastomas and, in total, found histone H3.3 alterations in 73 of 77 (95%). All 73 mutations resulted in a p.Lys36Met alteration, with 68 presenting in *H3F3B* and 5 presenting in *H3F3A* (**Fig. 1** and **Table 2**).

We screened seven additional types of bone and cartilage tumors for the presence of *H3F3A* and *H3F3B* mutations (**Table 2**). In giant cell tumor of bone, we found mutations in 49 of 53 cases (92%). All mutations were in *H3F3A* and caused changes at glycine 34, with 48 encoding p.Gly34Trp alterations and one encoding a p.Gly34Leu alteration (**Fig. 1** and **Table 2**).

In other bone and cartilage tumors, we found a low prevalence of histone H3.3 alterations (**Table 2**). Two osteosarcomas (2%; 2/103) had p.Gly34Arg alterations, with 1 encoded in *H3F3A* and 1 encoded in *H3F3B*. One conventional chondrosarcoma (1%; 1/75) had an *H3F3A*

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Table 1 Genome-wide somatic mutation burden of six chondroblastoma cases

Case	PD7516a	PD7517a	PD7518a	PD7520a	PD7522a	PD7523a
Substitutions	547	542	653	531	668	585
Indels	15	17	23	21	15	25
Histone H3.3 alteration	*H3F3B* p.Lys36Met	*H3F3B* p.Lys36Met	*H3F3A* p.Lys36Met	*H3F3B* p.Lys36Met	*H3F3B* p.Lys36Met	*H3F3B* p.Lys36Met

For each tumor genome, the absolute numbers of substitutions and indels are shown, along with the somatic alterations encoded in histone H3.3 genes.

mutation encoding p.Lys36Met. We identified 1 clear-cell chondrosarcoma (7%; 1/15) with an *H3F3B* mutation encoding p.Lys36Met. No histone H3.3 alterations were found in chondromyxoid fibroma ($n = 43$), chordoma ($n = 25$) or soft tissue/synovial chondroma ($n = 7$). To search for mutations of other histone H3 genes that are closely related but not identical to *H3F3A* and *H3F3B*, we analyzed the whole-exome or whole-genome sequences of 49 of 75 chondrosarcoma cases and of all 103 osteosarcoma cases included in this study. One osteosarcoma harbored a somatic variant of *HIST1H3H* of unknown significance (p.Gly12Arg). Unlike in some childhood brain tumors, we did not identify *H3F3A* or *HIST1H3H* mutations encoding p.Lys27Met, nor did we observe an association of histone H3.3 alterations with *TP53* mutations[4–9].

Giant cell tumor of bone and, to a lesser extent, chondroblastoma contain cells of the osteoclast lineage in addition to stromal cells. In giant cell tumor of bone, there has been some dispute as to which cell type is neoplastic[10]. Consequently, we separated the stromal compartment ($CD51^-CD61^-CD14^-$ cells) from osteoclasts ($CD51^+CD61^+$ cells) and osteoclast precursors ($CD14^+$ cells) in both giant cell tumors of bone and chondroblastomas harboring histone H3.3 alterations. The presence of mutations exclusively in the stromal compartment demonstrates that the neoplastic component of these tumors is represented by stromal cells and not by cells of the osteoclast lineage (**Table 3**). It is possible that histone H3.3 alterations lead directly to osteoclast recruitment, for example, by altering the expression of essential osteoclast signals such as RANK ligand or colony-stimulating factor 1.

Taken together, our findings identify histone H3.3 alterations in five classes of bone and cartilage neoplasms, demonstrating an exceptionally high prevalence of these alterations in chondroblastomas and giant cell tumors of bone and a low frequency in osteosarcomas, conventional chondrosarcomas and clear-cell chondrosarcomas. Recurrent somatic histone H3.3 alterations have previously been described in pediatric brain tumors[4–9], including in up to 60% of diffuse intrinsic pontine gliomas[8]. Otherwise, histone H3.3 alterations seem to be exceedingly rare. Among the 1.14 million coding mutations from 885,000 tumors recorded in the Catalogue of Somatic Mutations in Cancer (COSMIC)[11], there is 1 alteration at a histone H3.3 hot spot, a p.Lys36Arg variant found in a urinary tract tumor. Therefore, histone H3.3 driver alterations appear to be markedly enriched in glial tumors and in a subset of primary skeletal neoplasms.

We observed quite remarkable specificity of histone H3.3 alterations for different histogenetic tumor types, both with respect to which codon was mutated and what amino acid was newly substituted (**Fig. 1**). Histone H3.3 p.Lys36Met alterations were confined to a subset of cartilage tumors, namely chondroblastomas and chondrosarcomas, whereas glycine 34 was altered in tumors of the osteoblast lineage, namely in osteosarcomas and giant cell tumors of bone. Lysine 27, which is the principal methylation site altered in pediatric brain tumors, was not affected in bone and cartilaginous tumors. Likewise, lysine 36, which is the principal methylation site altered in chondroblastomas, was not altered in bone or brain tumors. At glycine 34, which is altered in both bone and pediatric brain tumors, changes to valine or arginine were found in brain tumors, whereas changes to tryptophan were confined to giant cell tumors of bone. Thus, there is a marked association of each tumor type with a specific histone H3.3 alteration. The most likely explanation for this phenomenon is that particular histone H3.3 alterations confer differential growth advantage to specific cell types. Although, in principle, different mutational processes in different neoplasms could account for the tumor specificity of the histone H3.3 alterations, this seems unlikely, as overall patterns of somatic substitution are similar in these tumor types.

An association of mutations at specific codons with different cancer types has been reported in other cancer-relevant genes. For example, in *NRAS*, Gln61 is more frequently altered in melanoma than two other mutation hot spots, Gly12 and Gly13, which are predominantly altered in leukemias[12]. Other examples include *BRAF* and *TP53*, in which certain mutational hot spots demonstrate a bias for specific tumor types[11,13]. However, the mutual exclusivity of histone H3.3 alterations in brain tumors, giant cell tumors of bone and chondroblastomas that we report is unprecedented.

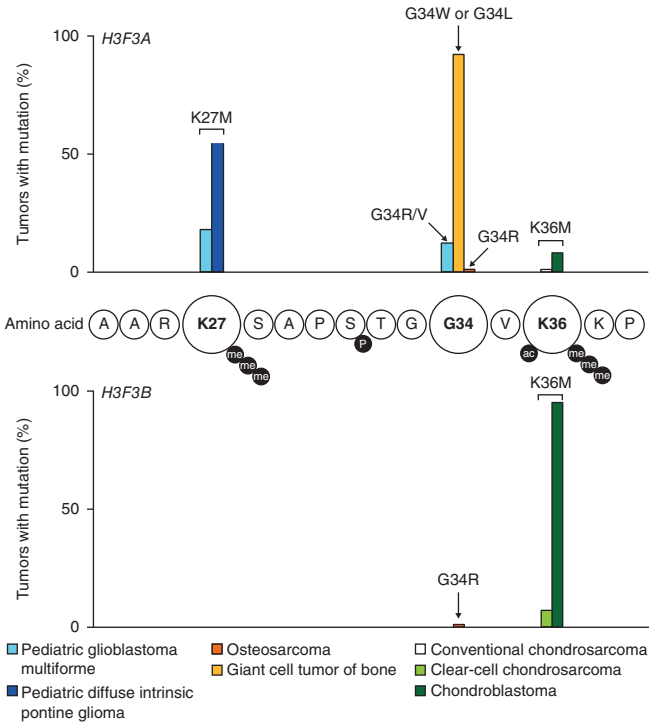

Figure 1 Prevalence and distribution of histone H3.3 somatic alterations in different tumor types. The percentage of cases in each series harboring a specific histone H3.3 alteration is indicated as follows: light blue, pediatric glioblastoma multiforme[4]; dark blue, pediatric diffuse intrinsic pontine glioma[6]; dark orange, osteosarcoma; light orange, giant cell tumor of bone; light green, clear-cell chondrosarcoma; white, conventional chondrosarcoma; dark green, chondroblastoma. Circles represent amino acids that are identical for the proteins encoded by *H3F3A* and *H3F3B*. Black circles represent post-translational modifications: me, methylation; ac, acetylation; P, phosphorylation.

Table 2 Histone H3.3 somatic alterations in bone and cartilage tumors

			H3F3A					*H3F3B*	
Tumor type	Number screened	Number mutated	Gly34Leu	Gly34Arg	Gly34Val	Gly34Trp	Lys36Met	Gly34Arg	Lys36Met
Chondroblastoma	77	73 (95%)					5		68
Giant cell tumor of bone	53	49 (92%)	1			48			
Clear-cell chondrosarcoma	15	1 (7%)							1
Osteosarcoma	103	2 (2%)		1				1	
Conventional chondrosarcoma	75	1 (1%)					1		
Chondromyxoid fibroma	43	0							
Chordoma	25	0							
Chondroma	7	0							

Prevalence of histone H3.3 alterations in eight different tumor types. p.Lys36Met and p.Gly34Trp alterations are mutually exclusive in chondroblastoma and giant cell tumor of bone ($P < 1 \times 10^{-15}$).

To our knowledge, this is also the first description of somatic driver mutations presenting in different genes encoding an identical protein. The reasons for this phenomenon remain uncertain. However, the clear preferences for *H3F3A* mutations in tumors of osteoblastic lineage and glial cells and for *H3F3B* mutations in cartilaginous neoplasms indicates that the two genes are unlikely to be redundant with respect to function. One possibility is that differential gene expression may account for the selective pattern of mutation in different tumor types. We assessed the expression levels of *H3F3A* and *H3F3B* in ten cases each of giant cell tumor of bone (p.Gly34Trp mutant encoded in *H3F3A*) and chondroblastoma (p.Lys36Met mutant encoded in *H3F3B*) and did not find significant differences in gene expression between the tumor types. Our findings do not exclude the possibility that temporal differences underlie the tumor type specificity of *H3F3A* and *H3F3B*, especially as differential expression patterns of the two genes have been observed during embryonic and postnatal development in normal mouse and human tissues[14,15].

The accumulation of histone H3.3 alterations in pediatric brain tumors has sparked a number of functional investigations seeking to evaluate the effects of key regulatory sites, such as critical lysine residues that are targets of methylation. Histone H3.3 lysine-to-methionine alterations, including p.Lys9Met, p.Lys27Met and p.Lys36Met, were found to reduce methylation of these residues through inhibition of SET domain–containing enzymes[16]. Interestingly, p.Gly34Arg and p.Gly34Val substitutions decreased Lys36 methylation[16]. In addition, expression profiling of histone H3.3 p.Lys27Met mutant cell lines demonstrated genome-wide changes in gene expression that seemed to disrupt multiple cancer-related pathways[17]. Among these, upregulation of *MYCN* has been identified as a potential downstream effect of histone H3.3 alteration in a gene expression study of a mutant *H3F3A* glioma cell line encoding histone H3.3 with a p.Gly34Val alteration[18]. Collectively, these studies begin to provide mechanistic insight into histone H3.3 alterations and corroborate the genomic evidence that recurrent somatic alterations of histone H3.3 at Lys27, Gly34 and Lys36 are dominant driver events.

Our study identifies distinct histone H3.3 alterations in a variety of bone and cartilage tumors. Given the exceptionally high prevalence of mutually exclusive histone H3.3 alterations in chondroblastoma and giant cell tumor of bone, it seems likely that these tumors are defined by distinct histone H3.3 alterations, which may be of immediate diagnostic use, especially in characterizing morphologically ambiguous cases. The unprecedented tumor type specificity of histone H3.3 alterations indicates fundamental differences in the function of histone H3.3 amino acids in tumor development, which may pertain to the normal differentiation of bone and cartilage cells. It is conceivable that chondroblastomas and giant cell tumors of bone are derived from a common precursor cell, the differentiation of which is determined by the presence of specific histone H3.3 variants. A future challenge will be to investigate the functional repercussions of these mutations in appropriate biological models that control for the tissue specificity of histone H3.3 variants.

Table 3 Mutant allele fraction in different compartments of chondroblastoma and giant cell tumor of bone

	Mutant allele fraction in stromal component ($CD14^-CD51^-CD61^-$ cells)	Mutant allele fraction in mononuclear precursors ($CD14^+CD51^+CD61^+$ cells)
Case S00036864 Chondroblastoma (*H3F3B* p.Lys36Met)	10%[a]	0
Case S000297791 Giant cell tumor of bone (*H3F3A* p.Gly34Trp)	40%	0

[a]The mutant allele fraction in the stromal component of this tumor was low, as it contained a large fraction of reactive fibroblasts.

URLs. European Genome-phenome Archive (EGA), http://www.ebi.ac.uk/ega/.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Sequencing data have been deposited in the European Genome-phenome Archive (EGA), hosted by the European Bioinformatics Institute (EBI), under accession EGAD00001000646.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
S.B. and P.S.T. performed analysis of the sequence data. N. Presneau and R.H. performed the extension screen. S.S. and A.M.F. performed *in vitro* studies. P.V.L. performed copy number analysis. D.C.W. performed statistical analyses. S.L.C. performed rearrangement analysis. N. Pillay, G.G., H.D. and S.N.-Z. contributed to data analysis. S. McLaren and S. Martin coordinated sample acquisition and processing. V.G. and B.R. performed technical investigations. A.B. and J.W.T. coordinated informatics analyses. D.H., D.B., G.J., B.K., O.M., M.F.A., R.T. and A.M.F. provided samples and clinical data. P.J.C., M.R.S., P.A.F. and A.M.F. directed the research. M.R.S., S.B. and P.S.T. wrote the manuscript, with contributions from P.J.C., A.M.F. and P.A.F.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Subject samples. Informed consent was obtained from all subjects, and ethical approval was obtained from the Cambridgeshire 2 Research Ethics Service (reference 09/H0308/165). Collection and use of samples was approved by the appropriate institutional review board of each Institution.

Whole-genome sequencing. DNA was extracted from six chondroblastomas as well as matched normal tissue derived from the same individuals. Short-insert 500-bp library construction, flow cell preparation and cluster generation were performed according to the Illumina no-PCR library protocol. We performed 100-base paired-end sequencing on Illumina HiSeq 2000 analyzers, as described in the Illumina Genome Analyzer operating manual. Reads were aligned to the reference human genome (NCBI37) using the Burrows-Wheeler Aligner (BWA) with default settings[19]. Reads that were unmapped or were PCR-derived duplicates were excluded from the analysis. The average coverage of tumors was 40× and of normal tissues was 30×.

Variant detection. The CaVEMan (Cancer Variants through Expectation Maximization) algorithm was used to call single-nucleotide substitutions[20]. To call insertions and deletions, we used split-read mapping implemented as a modification of the Pindel algorithm[21]. To call rearrangements, we applied the BRASS (Breakpoint via Assembly) algorithm, which identifies rearrangements by grouping discordant read pairs that represent the same breakpoint event[22]. After processing, filters were applied to the output to improve specificity. Mutations were annotated to Ensembl version 58.

Variant validation. Variants were subjected to validation by manual inspection, and artifacts were excluded from the data set. All ambiguous variants (141 substitutions and 24 indels) as well as randomly selected indels (n = 25; 12%) and substitutions (n = 339; 8%) were experimentally validated. To this end, DNA from tumors and matched normal tissues from the same individuals was amplified by PCR (primer sequences are available upon request) targeting the variant location. Resulting PCR amplicons were sequenced on an Illumina MiSeq (read length of 150 bp) to a median depth of 7,400×. Overall specificity for the catalog of substitutions and indels was thus determined to be at least 95%. All histone H3.3 variants detected in whole genomes were separately validated by capillary sequencing, as described below.

Extension study. We screened 185 tumors by capillary sequencing (primer sequences available upon request) for the presence of *H3F3A* and *H3F3B* variants between codons 29 and 63: 70 chondroblastomas, 43 chondromyxoid fibroma, 51 giant cell tumors of bone, 14 clear-cell chondrosarcomas and 7 soft tissue/synovial chondromas. DNA from these 185 tumors was extracted from formalin-fixed, paraffin-embedded tissue as well as from fresh-frozen tissues in 22 cases, and DNA was also isolated from normal tissues from the same individuals.

Using DNA from fresh-frozen tissue, we applied massively parallel sequencing (whole-genome sequencing, whole-exome sequencing or targeted capture of histone H3.3 genes) as previously described[20,22] to screen *H3F3A* and *H3F3B* for the presence of mutations in 207 tumors: 103 osteosarcomas, 75 chondrosarcomas, 25 chordomas, 2 giant cell tumors of bone, 1 clear-cell chondrosarcoma and 1 chondroblastoma. Mutations found in histone H3.3 genes or related genes were validated by capillary sequencing or massively parallel sequencing.

***TP53* screen.** We screened cases with giant cell tumor of bone (47/53) for *TP53* mutations. Libraries of targeted 120-bp fragments were generated by Fluidigm, according to the manufacturer's instructions, and sequenced by Ion Torrent One-Touch version 2 and Personal Genome Machine with 316 chip, according to the manufacturer's instructions with minor modifications. Reads were analyzed by manual inspection and by SAMtools mpileup incorporated into custom software.

Detection of copy number variation. Copy number data were derived from whole-genome reads using the ASCAT (version 2.2) algorithm[23] and were validated by SNP6.0 in 5 of 6 cases. Variant zygosity was derived by determining mutation copy number, as previously described[24].

Cell separation experiments. Fresh tissue from one case of giant cell tumor of bone (radius) and one case of chondroblastoma (femur) was obtained at the time of surgery from the Royal National Orthopaedic Hospital (Stanmore, UK). Tumors were processed as previously described[25] with some modifications. After collagenase digestion for 2 h, cells were cultured for 14 d and, on reaching confluence, were passaged twice during this period to enrich for the trypsin-sensitive stromal cell population. Trypsin-resistant large multinucleated osteoclasts and a smaller population of mononuclear cells persisted in the flasks after the first treatment with trypsin, but new osteoclasts developed when cells were replated. After the second passage, cells were incubated with monoclonal mouse IgG1 antibody (23C6, ab34226, Abcam) directed against CD51 and CD61, the vitronectin receptor (VNR) expressed by osteoclasts[26,27], followed by microbeads conjugated with rat antibody to mouse IgG1 (Miltenyi Biotec). Cells were then incubated with 20 µl per 10^7 cells of microbeads conjugated with mouse monoclonal IgG2a antibody to human CD14 (Miltenyi Biotec) according to the manufacturer's recommendations for the purpose of selecting osteoclast precursors. Cells were sorted manually using a Minimax magnetic cell separator (Miltenyi Biotec) according to the manufacturer's instructions. Each fraction ($CD51^-CD61^-CD14^-$ and $CD51^+CD61^+CD14^+$ fractions from each tumor) was sorted three times. Using RT-PCR on RNA extracted from paraffin-embedded tissue (RNeasy Mini kit, Qiagen), we confirmed that cells from the stromal compartment expressed genes specific to stromal cells (*COL1A1*) and did not express markers of osteoclasts and their precursors (*CD45*, *ACP5* and *CD14*). DNA was extracted from the resulting cell populations and screened for *H3F3A* and *H3F3B* mutations by massively parallel sequencing.

Gene expression analyses. Total RNA was isolated from paraffin-embedded tissues from ten cases of giant cell tumor of bone and ten cases of chondroblastoma and was reverse transcribed using the SuperScript III First-Strand Synthesis kit (Invitrogen/Life Technologies). Gene-specific primers were designed with Primer3, and quantitative RT-PCR was performed with SsoFast EvaGreen Supermix from Bio-Rad (primer sequences are available upon request). The average of two independent analyses for each target gene and sample was calculated and was normalized to levels of the endogenous reference control gene *GAPDH*.

Statistical analysis. The significance of associations between specific histone H3.3 alterations and tumor type was tested using one-sided Fisher's exact tests.

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Corrigendum: Distinct *H3F3A* and *H3F3B* driver mutations define chondroblastoma and giant cell tumor of bone

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In the version of this article initially published, the name of author Victoria Goody was misspelled, and the following statement was omitted from the Acknowledgments: "The EuroBoNeT consortium, a European Commission–granted Network of Excellence for studying the pathology and genetics of bone tumors, also contributed to the financial support for this study." These errors have been corrected in the HTML and PDF versions of the article.