Recurrent internal tandem duplications of BCOR in clear cell sarcoma of the kidney

Angshumoy Roy1,2,3,4,5, Vijetha Kumar1,2, Barry Zorman4, Erica Fang1,2, Katherine M. Haines6, HarshaVardhan Doddapaneni6,7, Oliver A. Hampton6,7, Simon White7, Abhishek A. Bavle4, Nimesh R. Patel1,2, Karen W. Eldin1,2, M. John Hicks1,2,3,4,5, Dinesh Rakheja8,9,10, Patrick J. Leavey10, Stephen X. Skapek10, James F. Amatruda10, Jed G. Nuchtern3,5,11,12, Murali M. Chintagumpala3,4,5, David A. Wheeler6,7, Sharon E. Plon3,4,5,6,7, Pavel Sumazin3,4 & D. Williams Parsons3,4,5,6,7

The X-linked BCL-6 co-repressor (BCOR) gene encodes a key constituent of a variant polycomb repressive complex (PRC) that is mutated or translocated in human cancers. Here we report on the identification of somatic internal tandem duplications (ITDs) clustering in the C terminus of BCOR in 23 of 27 (85%) pediatric clear cell sarcomas of the kidney (CCSK) from two independent cohorts. We profile CCSK tumours using a combination of whole-exome, transcriptome and targeted sequencing. Identical ITD mutations are found in primary and relapsed tumour pairs but not in adjacent normal kidney or blood. Mutant BCOR transcripts and proteins are markedly upregulated in ITD-positive tumours. Transcriptome analysis of ITD-positive CCSKs reveals enrichment for PRC2-regulated genes and similarity to undifferentiated sarcomas harbouring BCOR–CCNB3 fusions. The discovery of recurrent BCOR ITDs defines a major oncogenic event in this childhood sarcoma with significant implications for diagnostic and therapeutic approaches to this tumour.
CSK is a high-risk childhood cancer that comprises 2–5% of primary renal tumours diagnosed in children1–3. Although first distinguished as a clinicopathologic entity from the more common Wilms tumour in the 1970s (ref. 3), it remains a biologically and clinically ill-defined neoplasm1–4. Children with CSK are predominantly young and male (median age 3 years; male to female ratio of >2:1), and the tumour is notable for late recurrences and metastases to bone and brain1,2,5. Although current intensive treatment regimens have resulted in improved outcomes for children with CSK, survival for patients with relapsed tumours remains poor1,2.

Histologically, CSKs are characterized by a diversity of morphological patterns that can confound accurate diagnosis in up to a quarter of cases2–5. Genetic studies to date have been generally unrevealing, with a t(10;17)(q22;p13) translocation resulting in YWHAE–NUTM2 gene family fusions in a minority (12%) of cases as the only recurrent somatic aberration reported6. Unlike Wilms tumour, CSK is not associated with familial cancer predisposition syndromes, suggesting that the genetic drivers for these tumours remain to be discovered4,5.

Here using whole-transcriptome sequencing (RNA-seq) and whole-exome sequencing (WES), we report on the identification of highly recurrent internal tandem duplications (ITDs) in the X-linked BCL-6 co-repressor (BCOR) gene that is mutated in various human cancers7–10. These genomic data provide insight into the biological processes perturbed in BCOR-mutated CSKs and reveal unexpected similarities between CSKs and soft-tissue sarcomas harbouring BCOR–CCNB3 fusions.

Results
Identification of recurrent somatic ITDs in BCOR. To identify recurrent genomic aberrations in CSK, we performed whole-transcriptome paired-end sequencing (RNA-seq) of three fresh-frozen tumour samples (cases 347T, 383T and 385T) and WES of one of the tumours and its matched peripheral blood sample as part of a clinical genomics study11 (Supplementary Table 1 and Supplementary Data 1). No recurrent in-frame fusions, fusions targeting known cancer genes or YWHAE–NUTM2 fusions were identified by RNA-seq (Supplementary Data 2). However, WES of tumour 347T identified a putative stop-loss variant within the terminal coding exon 15 of the BCOR gene on Xp11.4 that was absent in the matched germline reads (Supplementary Fig. 1a and Supplementary Table 2).

On closer inspection, WES tumour sequencing reads harbouring the variant BCOR allele were found to have adjacent soft-clipping (Supplementary Fig. 1a). Notably, analysis of the aligned RNA-seq reads from all three tumours revealed similar soft-clipped subsequences (Supplementary Fig. 1b). Since soft clipping by mapping algorithms may be indicative of reads spanning genomic breakpoints of structural variations2–5, we analysed the clipped BCOR sequences using the Basic Local Alignment Search Tool13 and discovered in-frame ITDs within exon 15 of BCOR in all three cases (Table 1). Local realignment of discordant mate pairs showed a distinct focal increase in read coverage corresponding to the ITDs (Fig. 1a and Supplementary Fig. 2), which were subsequently confirmed by targeted PCR and sequencing (Fig. 1b,c).

Targeted DNA sequencing of BCOR exon 15 in a validation cohort of 11 additional CSKs (Supplementary Table 1) revealed in-frame ITDs in 8 additional tumours (Table 1 and Fig. 1b,d), resulting in an overall mutation frequency of 11/14 (78%), including tumours from 7 of 9 males and 4 of 5 females. Sequencing of cloned ITD alleles identified 5 distinct ITD types with overlapping genomic breakpoints within exon 15 of BCOR, ranging in size from 87 to 114 bp (Table 1). The ITDs are predicted to involve amino acids 1,701–1,755 within the C-terminal PUFD (polycomb-group RING finger homologue (PCGF) Ub-like fold discriminator)14 domain of the protein (Fig. 1d). In one case, the ITD in BCOR was interrupted by a 3-bp insertion (Table 1), as has been observed for ITDs in the FLT3 tyrosine kinase15.

All BCOR ITDs were confirmed to be absent from patient-matched peripheral blood and/or adjacent normal kidney samples, when available (Fig. 1b and Supplementary Table 1). Testing of two metastatic relapsed lesions revealed identical ITDs as in the primary tumour (Fig. 1b). In males, who are hemizygous at the BCOR locus, the wild-type allele was virtually undetectable (Fig. 1b), suggesting that ITD acquisition is an early event in CSK tumorigenesis. BCOR ITDs were not found in a cohort of other childhood renal tumours (18 Wilms tumours and 9 congenital mesoblastic nephromas) and soft-tissue sarcomas (n = 10). Analysis of a non-overlapping cohort of 13 CSKs subjected to transcriptome sequencing as part of the National Cancer Institute’s Therapeutically Applicable Research to Generate Effective Treatments (TARGET) initiative (http://ocg.cancer.gov/programs/target) identified ITDs within exon 15 of BCOR in 12 of 13 cases (Supplementary Data 3), including one additional ITD type (type VI), which were verified by realigning the RNA-seq reads to ITD-specific modified reference transcriptomes (Methods and Supplementary Fig. 3).

### Table 1 | BCOR internal tandem duplications identified in CSK patients.

| Sample | Patient age (years) | Patient sex | ITD length (bp) | ITD genotype | Nucleotide (gDNA)* | Nucleotide (cDNA) | Amino acid (protein) |
|--------|---------------------|-------------|-----------------|---------------|-------------------|----------------|---------------------|
| 347T   | 0.9                 | M           | 96              | Type I        | chrX:9,911,364–9,911,459 | c.5171_5266dup | p.L1724_W1755dup |
| 383T   | 1.1                 | M           | 96              | Type I        | chrX:9,911,364–9,911,459 | c.5171_5266dup | p.L1724_W1755dup |
| 385T   | 1.9                 | M           | 87              | Type IV       | chrX:9,911,418–9,911,492 | c.5138_5225dup | p.L1701_L1737dup |
| 380T   | 1.2                 | M           | 93              | Type II       | chrX:9,911,374–9,911,466 | c.5164_5256dup | p.E1722_D1752dup |
| 382T   | 1.8                 | M           | 90              | Type III      | chrX:9,911,405–9,911,494 | c.5136_5225dup | p.D1712_V1741dup |
| 384T   | 2.2                 | M           | 96              | Type I        | chrX:9,911,364–9,911,459 | c.5164_5256dup | p.E1722_D1752dup |
| 474T   | 0.6                 | M           | 93              | Type II       | chrX:9,911,374–9,911,466 | c.5164_5256dup | p.E1722_D1752dup |
| 499T   | 3.5                 | F           | 96              | Type I        | chrX:9,911,364–9,911,459 | c.5171_5266dup | p.L1724_W1755dup |
| 501T   | 1.6                 | F           | 93              | Type II       | chrX:9,911,374–9,911,466 | c.5164_5256dup | p.E1722_D1752dup |
| 504T   | 2.7                 | F           | 114             | Type V        | chrX:9,911,418–9,911,531 | c.5099_5212dup | p.G1738E & p.V1701_L1737dup |
| 624T   | 2.9                 | F           | 96              | Type I        | chrX:9,911,364–9,911,459 | c.5171_5266dup | p.L1724_W1755dup |

*Genomic coordinates as per NCBI build 37/hg19, negative strand.

*cDNA, complementary DNA; gDNA, genomic DNA.
and by local realignment of discordant mate-pair mapping to the \textit{BCOR} transcript (Supplementary Fig. 2). In total, therefore, \textit{BCOR} ITDs were identified in 23/27 (85\%) of CCSKs analysed.

**Expression of \textit{BCOR} mRNA and protein in tumours.** Targeted reverse transcription–PCR (RT–PCR) of an intron-spanning segment of the \textit{BCOR} transcript (exons 14 and 15; Supplementary Table 3) confirmed expression of the mutant allele in all ITD-positive tumours tested (\(n = 4\)), including two from female patients, suggesting that the mutant allele had not been silenced through X-inactivation (Fig. 2a). Transcript abundance estimation of RNA-seq data from 6 ITD-positive CCSKs using Cufflinks\textsuperscript{16} showed strong upregulation of \textit{BCOR} as compared with 11 Wilms tumours, 31 assorted sarcomas and 1 ITD-negative CCSK (Fig. 2b).

**Figure 1 | Recurrent somatic ITDs in the \textit{BCOR} gene in CCSKs.** (a) View of aligned whole-transcriptome sequencing reads from a single ITD-positive CCSK (347T) demonstrating a marked focal increase in read coverage corresponding to the ITD in exon 15 of \textit{BCOR} on Xp11.4. Only unpaired reads of discordant mate pairs were used for local realignment. A representation of the ITD within the \textit{BCOR} gene is shown beneath. The parental segment (P) that is duplicated is depicted in green and the tandem duplicated segment (ITD) is shown in red. (b) Targeted PCR and gel electrophoresis of \textit{BCOR} exon 15 in samples from four representative male and two female subjects showed the expected wild-type products (288 bp) in the peripheral blood (C) and adjacent normal kidney (N) tissues, and larger products corresponding to the ITDs (87–114 bp) in the primary CCSK tumours (T) and relapsed metastatic tumours (R). Nearly undetectable levels of the wild-type products were observed in tumour samples from males; in females, both ITD-positive and wild-type products were evident. (c) Sanger sequence trace from case 347T showing the immediate sequence context surrounding the proximal genomic breakpoint in \textit{BCOR} exon 15 (hg19 coordinates, negative strand). The wild-type genomic sequence around the breakpoint including the termination codon and 3′-UTR are shown above and the parental and duplicated segments of the ITD are below. The proximal breakpoint at the second base of the stop codon (TGA) alters it to a TTA (leucine). (d) Schematic of predicted \textit{BCOR} protein sequences from ITD-positive CCSKs demonstrating the clustering of all ITDs within the C-terminal PUF domain. ITD types I–V were numbered based on genomic breakpoints and ITD sequence (Table 1). The \textit{BCOR} wild-type protein sequence (amino acids (aa) 1,701–1,755) is shown on top with the predicted protein sequence of each ITD-positive case below. Parental segments that have been duplicated are shown in green and the ITDs in red. Novel junctional amino acids (bold black font, underlined) were introduced by the ITDs in cases 385T and 504T. A stretch of 14 residues (aa 1,724–1,737) is common to every ITD type. ANK, ankyrin repeats; BBD, BCL6-binding domain.
fractions of mutant and wild-type BCOR transcripts in ITD-positive tumours by remapping unaligned RNA-seq reads to individual tumour-specific synthetic reference transcriptomes, revealing that 96–100% of BCOR expression was contributed by mutant transcripts (data not shown). Notably, the four undifferentiated sarcomas (UDS) harbouring BCOR–CCNB3 fusions\(^{18}\) tested were also shown to have high BCOR expression (Fig. 2b).

These findings were corroborated on the protein level by immunoblotting BCOR in five ITD-positive cases and three adjacent normal kidney specimens. An antibody to the full-length protein confirmed upregulated BCOR expression in the ITD-positive CCSKs compared with normal kidney (Fig. 2c). Similarly, immunohistochemistry showed strong and diffuse nuclear staining in all CCSKs tested (n = 6) but not in Wilms tumours (Fig. 2d–f) or congenital mesoblastic nephromas (data not shown).

**Transcriptome analysis of CCSKs.** Unsupervised hierarchical clustering using RNA-seq data revealed similarities between the transcriptomes of ITD-positive CCSKs and BCOR–CCNB3 fusion-positive UDS and suggested that RNA-expression programs in these tumours are distinct from those of Wilms tumours, other sarcomas or the single ITD-negative CCSK tested (Fig. 3a). When the CCSKs from both our study cohort and the TARGET consortium cohort were analysed separately by unsupervised clustering, the ITD-negative CCSKs appeared to be distinct from ITD-positive tumours in both studies (Supplementary Fig. 4). Genes that were found to be differentially expressed between ITD-positive CCSKs and Wilms tumours (Supplementary Data 4) were utilized for gene-set enrichment analysis (GSEA) using MSigDB-curated gene sets\(^{19}\). The 10 most significantly enriched gene sets identified were related to PRC2 targets or associated with the trimethylated histone H3 on Lys27 (H3K27me3) mark (Fig. 3b,c and Supplementary Table 4) (FWER \(P < 0.001\)). Significant enrichment of the Hedgehog signalling pathway (FWER \(P < 0.001\)) and downstream targets CCND1 and PDGFR\(a\) was also found in CCSKs (Supplementary Figs 5 and 6), confirming previous reports\(^{20}\).

All of the BCOR ITDs identified in this study target the C-terminal PUFD domain through which BCOR physically interacts with PRC1\(\text{ref. (14) within a variant PRC1 (PRC1.1/BCOR complex)}\)^\(^{21,22}\). This complex also includes KDM2B and the E3-ubiquitin ligase RNF2 that directly monoubiquitylates lysine-119 of histone H2A (H2AK119ub1; refs 23,24). Given the observed enrichment of PRC2 targets from our GSEA analysis and the fact that PRC1-dependent H2AK119ub1 is a recruitment mark for PRC2 at distinct genomic loci\(^{25,26}\), we investigated the PRC2 target set in more detail. Although PRC2 target genes were both upregulated (n = 125) and downregulated (n = 70; P < 0.05; t-test, one-tailed, unequal variance) in CCSKs (Fig. 3c and Supplementary Data 5) relative to Wilms tumours, analysis using GSEA and the Database for Annotation, Visualization and Integrated Discovery (DAVID)\(^{27,28}\) toolset also revealed a highly significant enrichment for homeobox proteins (FWER \(P < 0.001\); Supplementary Fig. 7) and upregulation of distinct classes of homeobox proteins, which are the canonical targets of polycomb-mediated repression.

**Discussion**

In conclusion, the discovery of highly recurrent BCOR ITDs in CCSK highlights the power of unbiased next-generation sequencing (NGS) to identify genetic drivers of tumorigenesis. ITDs are an unusual class of genetic alterations, previously reported as oncogenic gain-of-function mutations, most
regions bound to H3K27me3, SUZ12 and EED in human H9 ES cells. As a deviation from the mean in Wilms tumours in units of s.d. The PRC2 target gene set (BENPORATH_PRC2_TARGETS) includes genes with promoter genes are upregulated in CCSKs relative to Wilms tumours (\( \text{ITD}^-\)).

Expression profiling revealed widespread upregulation of PRC2 targets in these tumours, suggesting disruption of polycomb regulation (Fig. 3c) as a potential pathogenic mechanism in CCSK. Expression profiling revealed ITD-positive CCSKs to share significant similarity with \( \text{BCOR-CCNB3} \) fusion-positive UDS, a soft tissue and bone tumour type not previously considered related to CCSK but now shown to have in common somatic alterations affecting the C-terminal PUFD domain of BCOR. The fact that both CCSKs and \( \text{BCOR-CCNB3} \) fusion-positive sarcomas are defined by mutations in an X-linked gene may underlie the known male predominance of these two tumour types. Further studies are necessary to understand the effects of the \( \text{BCOR} \) ITD in cell and animal models and uncover the underlying consequences of this genetic alteration on PRC function and the mechanism of CCSK oncogenesis.

Methods

Patient enrolment and study design. Informed consent was obtained for enrolment of patient 347 on the NHGRI and NCI-funded Clinical Sequencing Exploratory Research programme BASIC3 study at Baylor College of Medicine (BCM) and Texas Children’s Hospital (TCH) under a BCM Institutional Review

Figure 3 | Transcriptome profiling of renal tumours and soft-tissue sarcomas. (a) Unsupervised hierarchical clustering revealed ITD-positive CCSKs (\( n = 6 \)) to cluster separately from Wilms tumours (\( n = 11 \)), the lone ITD-negative CCSK (case 381) and other soft-tissue sarcomas (STS; \( n = 31 \)). The transcriptomes of \( \text{BCOR-CCNB3} \) fusion-positive UDS (\( n = 4 \)) cluster together and are closely related to those of the ITD-positive CCSKs. The heatmap reflects the variance in gene expression relative to the mean across all samples (\( n = 53 \)) with upregulated genes in yellow and downregulated genes in blue. (b) GSEA of CCSKs revealed significant upregulation of PRC2 targets. Enrichment score computed by the GSEA algorithm (y axis) is plotted against the PRC2 target genes (x axis) rank ordered by the degree of differential expression in ITD + CCSKs compared with Wilms tumours. High-ranking PRC2 target genes are upregulated in CCSKs relative to Wilms tumours (c) Heatmap showing expression values of PRC2 targets in individual ITD-positive CCSK cases (targets) includes genes with promoter regions bound to H3K27me3, SUZ12 and EED in human H9 ES cells. \( p \), nominal \( p \)-value; FDR, false discovery rate; FWER, family-wise error rate.

notably in the receptor tyrosine kinases FLT3 and \( \text{Kit} \)\(^{15,29,30} \). As demonstrated in the current study, this rarity may be due in part to the use of computational mapping algorithms that discard discordant mate pairs in NGS data. Systematic application of comprehensive ITD-detection algorithms to tumour genome data will be necessary to more rigorously evaluate the prevalence of this class of genetic alterations. The discovery of \( \text{BCOR} \) ITDs in the vast majority of CCSKs, but not in Wilms tumours, offers the potential for a molecular diagnostic test for these cancers. Recently, Ueno-Yokohata et al.\(^{31} \) published a study describing the identification of recurrent \( \text{BCOR} \) ITDs in CCSKs using conventional RT–PCR to investigate the correlation between \( \text{BCOR} \) promoter CpG hypomethylation and the observed high expression of \( \text{BCOR} \) in CCSKs. The observed frequency and types of \( \text{BCOR} \) ITDs is very similar to the findings reported in this article. In both studies, CCSK tumours harbouring \( \text{BCOR} \) ITDs exhibit high expression of \( \text{BCOR} \) mutant transcripts and protein. Understanding the mechanism by which \( \text{BCOR} \) expression is upregulated in these tumours will require further studies, including assessing the activity of the PRC1.1/BCOR complex in the context of mutant \( \text{BCOR} \). Although \( \text{BCOR} \) co-purifies with KDM2B\(^{22} \), it is unknown if that involves direct interaction with KDM2B or other members of the PRC1.1 complex; regardless, it is intriguing to speculate that the ITDs disrupt the structure and/or function of the complex leading to derepression at target promoters, including that of \( \text{BCOR} \) itself, which is a target bound by KDM2B in mouse embryonal cells\(^{32} \).

Consistent with possible disruption of the PRC1.1/BCOR complex in CCSKs, transcriptome profiling revealed widespread upregulation of PRC2 targets in these tumours, suggesting disruption of polycomb regulation (Fig. 3c) as a potential pathogenic mechanism in CCSK. Expression profiling revealed ITD-positive CCSKs to share significant similarity with \( \text{BCOR-CCNB3} \) fusion-positive UDS, a soft tissue and bone tumour type not previously considered related to CCSK but now shown to have in common somatic alterations affecting the C-terminal PUFD domain of BCOR. The fact that both CCSKs and \( \text{BCOR-CCNB3} \) fusion-positive sarcomas are defined by mutations in an X-linked gene may underlie the known male predominance of these two tumour types. Further studies are necessary to understand the effects of the \( \text{BCOR} \) ITD in cell and animal models and uncover the underlying consequences of this genetic alteration on PRC function and the mechanism of CCSK oncogenesis.
Discordant mate-pair mapping. FASTQ files were aligned to the RefSeq RNA transcriptome using Bowtie2. Discordant mate pairs, where one mate mapped to BCOR and the other was unmapped, were extracted and a pileup plot was produced with Integrative Genomics Viewer (IGV). Unmapped mates were remapped to the genome using BLAT to reveal the junction regions of the BCOR ITD.

BCOR ITD analysis from TARGET project transcriptome data. FASTQ files from the TARGET project CCSK cases (Supplementary Data 3) were first aligned to the RefSeq RNA transcriptome using Bowtie2. The FASTQ files of samples showing evidence of BCOR ITDs (insertions in exon 15 of BCOR) were remapped to a modified transcriptome with both BCOR ITC and wild-type BCOR transcripts using RSEM as described above. Reads spanning the ITDs are displayed in Supplementary Fig. 3. Discordant read mapping, as described above, was also applied to verify ITD peaks (Supplementary Fig. 2).

Hierarchical clustering. Unsupervised hierarchical clustering was implemented in R (ref. 39) using the hclust procedure with average (Unweighted Pair Group Method with Arithmetic Mean) agglomeration and (1—Spearmann correlation) distance. The dendrogram and heatmap were graphed with heatmap.2 (ggplot package: http://cran.r-project.org/web/packages/ggplot/index.html). For the clustering of 6 ITD-positive CCSK cases, 1 ITD-negative CCSK, 4 BCOR–CCNB3 fusion-positive sarcomas, 11 Wilms tumours and 31 other sarcomas, mRNA expression FPKM values for 12,775 genes were included after filtering for HGNC protein-coding genes with average expression above 1.5 FPKM and coefficient of variation (CV) above 0.3 over the set of all 53 tumours. The enrichment of gene expression in TARGET cohort CCSKs (http://target.nci.nih.gov/datasetMatrix/). Briefly, mRNA expression FPKM values for 3,355 genes were included after filtering for HGNC protein-coding genes with average expression above 1.5 FPKM and CV above 0.3 over the set of all 13 tumours.

Gene-set enrichment analysis. GSEA calculations were performed with the GSEA programme (v. 2.0.14) (ref. 19). The Broad Molecular Signatures Database (MsigDB v5.0) set c2 (curated gene sets) was used. For each tumour type comprising 9 RNA-seq libraries including 2,384,317 FPKM values for 3,355 genes were included after filtering for HGNC protein-coding genes with average expression above 1.5 FPKM and CV above 0.3 over the set of all tumours in the comparison. In addition, ribosomal protein I family (RPL) and ribosomal protein S family (RPS) ribosomal genes were removed. For ITD-positive CCSKs (6 cases) compared with Wilms tumours (11 cases), the GSEA analysis involved 4,538 gene sets and the expression of 11,513 genes. The GSEA programme was run with 10,000 randomized gene sets for statistical significance estimation, and the default signal-to-noise metric between the two phenotypes was used to rank all genes.

In addition, GSEA comparisons were run using the same parameters with the MsigDB v2 collections of gene sets supplemented with a set of 170 genes corresponding to the ‘homeobox’ protein family downloaded from the UniProtKB human proteome (UniProt proteome: UP000005640) using the following parameters: ‘family and domains’—‘protein family’—‘homeobox; ‘organism’—human; and ‘reviewed’ status as ‘yes’. All P values reported by GSEA as zero represent values lower than 10<sup>−10</sup> (1/100,000 permutations).

Targeted PCR and sequencing for ITD detection. Targeted PCR was performed on cases and controls with specific primers (BCOR-ITD_F/BCOR-ITD_R and BCOR-ITD_Intron_14_F/BCOR-ITD_3’UTR_R, Supplementary Table 3) designed to detect the ITDs, followed by agarose gel electrophoresis. To validate and map the ITDs, the ITD fragments were either directly sequenced or cloned using the TOPO-TA cloning kit (Life Technologies) followed by sequencing using BigDye Terminator v3.1 chemistry on a 3730xl DNA Analyzer (Life Technologies).

RT–PCR to examine expression of the BCOR ITD. Total RNA was isolated from available fresh-frozen tumours of two female patients (501T and 504T). Total RNA (500 ng) was used for RT–PCR with the SuperScript III First-Strand Synthesis System (Life Technologies) and specific intron-spanning primers (Supplementary Table 3) were designed on exons 14 (forward) and 15 (reverse) to target the 3’ coding sequence of the mature BCOR mRNA (NM_001123385.1). Expression of ITD in the tumours of males was also validated using the same primers.

Immunoblot assays. Total protein was extracted from fresh-frozen tissue from seven CCSK tumours and three adjacent normal kidney tissue using RIPA buffer in the presence of cOmplete, Mini, EDTA-free protease inhibitor (Roche). Protein electrophoresis and immunoblotting were performed with 30 µg of total protein on NuPAGE 3–8% Tris-Acetate gels (Life Technologies) and transferred onto a polyvinylidene difluoride membrane following standard protocols. BCOR and β-actin were detected using a mouse anti-BCOR antibody (Abcam, ab88112, 1:1,000 dilution) and a rabbit polyclonal anti-β-actin antibody (Abcam, ab8227, 1:10,000).

Somatic mutation analysis from WES data. Data analysis including alignment, variant calling and annotation was performed using the semi-automated Mercury DNA analysis pipeline to generate annotated vcf files as previously described. After merging the vcf files and the corresponding germline vcf files, the following filters were applied for calling somatic (tumour-specific) variants: variant ratio > 0.05, total tumour coverage > 50, variant coverage in tumour > 6 and variant coverage in normal < 4. Somatic variants were additionally annotated with information from the COSMIC database (Catalog of Somatic Mutations In Cancer; v59) to include frequency of variant position hits in COSMIC, nearby hits and most common hits affected. Whole-exome and transcriptome data of tumours for which patients (or their parent/legal guardian) gave consent for the deposit of their information have been deposited in dbGaP under the accession phs001026.

Library preparation for exome and transcriptome sequencing. WES was performed for patient 347 on tumour (T) and matched normal (N) genomic DNA from peripheral blood. Exome libraries for the T/N pair were generated and sequenced on a single lane of an Illumina HiSeq 2000 as previously described, yielding a mean coverage of 186 (N) and 203 (T) and a target base coverage of 97.5% at >20. Whole-transcriptome RNA sequencing (RNA-seq) for seven CCSK samples (347T, 380T, 381T, 382T, 383T, 384T and 385T) was performed using 1 μg total RNA to prepare strand-specific poly-A + RNA-seq libraries for sequencing on the Illumina platform. Library preparation details except with the following modifications are the same as described previously. Purified mRNA from total RNA was fragmented by heat at 94°C for 3–4 min. Libraries were prepared as described previously and pooled in equimolar amounts (2 libraries per pool) and sequenced on a HiSeq 2000 or 2500 to generate reads in paired-end mode (2 × 100-bp reads). On average, for these seven samples, 83.7 million paired reads (155–201 million total reads) were generated per sample. Summary sequencing statistics for the RNA-seq data are presented in Supplementary Data 1.

DNA and RNA isolation. Genomic DNA from fresh-frozen tissue (for WES libraries and PCR) or formalin-fixed paraffin-embedded (FFPE) tissue (for targeted BCOR sequencing) was isolated using the QiaAmp DNA Mini kit (Qiagen) according to the manufacturer’s protocol. Total RNA was extracted from fresh-frozen tissue (for RNA-seq libraries and RT–PCR) using the mirVana miRNA isolation kit (Life Technologies). Total RNA from FFPE tissue (for targeted BCOR sequencing) was extracted using the RecoverAll Total Nucleic Acid Isolation kit (Life Technologies).

RNA-seq analysis. RNA-seq reads were aligned using STAR v2.3.0 (ref. 34) to an index of hg19 that included GENCODE v16 gene annotation (http://www.gencodegenes.org/archive_stats.html). Alignment files were processed using Picard tools v1.54 (http://picard.sourceforge.net/), and the final BAM files indexed using SAMtools index v0.11.1 (ref. 35). RNA-seq run quality was assessed using the RNA-SeQC package using the same GENCODE 16.gtf file and hg19 reference as was used to create the index. Transcript quantification was performed using Cufflinks v2.2.0 running in quantification mode against the GENCODE v16.gtf file. FPKM (Fragments Per Kilobase of Exon Per Million Fragments Mapped) values were used for relative abundance estimation.

To estimate the ratio of BCOR ITC to wild-type BCOR from RNA-seq reads, the RefSeq RNA transcriptome (dated 4 June 2014) for protein-coding sequences was modified by replacing all BCOR transcripts with two competing BCOR sequences for alignment: one wild-type sequence containing only the last six exons of BCOR and the first 304 bp of the 3’-untranslated region (UTR), and another 304 bp transcript with the corresponding ITD bases added and the same 304 bp of 3’-UTR. All other RefSeq transcripts remained unmodified. The RNA-seq reads were aligned and quantified to this modified transcriptome with RSEM (version 1.2.17) and Bowtie2 (ref. 37).

Detection of fusion genes was performed using deFuse as previously published. Briefly, high-quality FASTQ files were subjected to analysis with default options and filters (Supplementary Data 2). Predicted read-through candidates and nominated candidates, which did not retain an open reading frame were discarded, and the remaining nominated candidates were ranked based on location of fusion breakpoints.
References
1. Gooskens, S. L. et al. Treatment and outcome of patients with relapsed clear cell sarcoma of the kidney: a combined SIOP and AIEOP study. Br. J. Cancer 111, 227–233 (2014).
2. Furtwangler, R. et al. Clear cell sarcomas of the kidney registered on International Society of Pediatric Oncology (SIOP) 93-01 and SIOP 2001 protocols: a report of the SIOP Renal Tumour Study Group. Eur. J. Cancer 49, 3489–3496 (2013).
3. Marsden, H. B. & Lawler, W. Bone-metastasizing renal tumour of childhood. Br. J. Cancer 38, 437–441 (1978).
4. Gooskens, S. L. et al. Clear cell sarcoma of the kidney: a review. Eur. J. Cancer 48, 2219–2226 (2012).
5. Argani, P. et al. Clear cell sarcoma of the kidney: a review of 351 cases from the National Wilms Tumor Study Group Pathology Center. Am. J. Surg. Pathol. 24, 4–18 (2000).
6. O’Meara, E. et al. Characterization of the chromosomal translocation t(10;17)(q22;p13) in clear cell sarcoma of kidney. J. Pathol. 227, 72–80 (2012).
7. Zhang, J. et al. A novel retinoblastoma therapy from genomic and epigenetic analyses. Genes Dev. 21, 329–334 (2011).
8. Pugh, T. J. et al. Medulloblastoma exon sequencing uncovers subtype-specific somatic mutations. Nature 488, 106–110 (2012).
9. Pierron, G. et al. A new subtype of bone sarcoma defined by BCOR-CCNB3 gene fusion. Nat. Genet. 44, 461–466 (2012).
10. Grossmann, V. et al. Whole-exome sequencing identifies somatic mutations of BCOR in acute myeloid leukemia with normal karyotype. Blood 118, 6153–6163 (2011).
11. Scollon, S. et al. Obtaining informed consent for clinical tumor and germline exome sequencing of newly diagnosed childhood cancer patients. Genome Med. 6, 69 (2014).
12. Wang, J. et al. CREST maps somatic structural variation in cancer genomes with base-pair resolution. Nat. Methods 8, 652–654 (2011).
13. Altschul, S. F. et al. Basic local alignment search tool. J. Mol. Biol. 215, 403–410 (1990).
14. Junco, S. E. et al. Structure of the polycomb group protein PGC1F in complex with BCOR reveals basis for binding selectivity of PGC1 family homologs. Structure 21, 665–671 (2013).
15. Nakao, M. et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia 10, 1911–1918 (1996).
16. Trapnell, C. et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28, 511–515 (2010).
17. Li, R. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12, 323 (2011).
18. Peters, T. L. et al. BCOR-CCNB3 fusions are frequent in undifferentiated sarcomas of male children. Mod. Pathol. 28, 575–586 (2015).
19. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl Acad. Sci. USA 102, 15545–15550 (2005).
20. Cutcliffe, C. et al. Clear cell sarcoma of the kidney: up-regulation of neural markers with activation of the sonic hedgehog and Akt pathways. Clin. Cancer Res. 11, 7986–7994 (2005).
21. Gao, Z. et al. PCGF homologs, CBX proteins, and RBYP define functionally distinct PRCI family complexes. Mol. Cell 45, 344–356 (2012).
22. Gearhart, M. D., Corcoran, C. M., Wamstad, J. A. & Bardwell, V. J. Polycomb group and SCF ubiquitin ligases are found in a novel BCOR complex that is recruited to BCOR domains. Mol. Cell Biol. 26, 6880–6889 (2006).
23. Wang, H. et al. Role of histone H2A ubiquitination in Polycomb silencing. Nature 431, 873–878 (2004).
24. de Napoles, M. et al. Polycomb group proteins Ring1A/B link ubiquitilation of histone H2A to heritable gene silencing and X inactivation. Dev. Cell 7, 663–670 (2004).
25. Kalb, R. et al. Histone H2A monoubiquitination promotes histone H3 methylation in Polycomb repression. Nat. Struct. Mol. Biol. 21, 569–571 (2014).
26. Blackledge, N. P. et al. Variant PRC1 complex-dependent H2A ubiquitination drives PRC2 recruitment and polycomb domain formation. Cell 157, 1445–1459 (2014).
27. Huang, D. W. et al. DAVID Bioinformatics Resources: expanded annotated database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Res. 35, W169–W175 (2007).
28. Dennis, J. R. et al. DAVID: database for annotation, visualization, and integrated discovery. Genome Biol. 4, P3 (2003).
29. Heinrich, M. C. et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J. Clin. Oncol. 21, 4342–4349 (2003).
30. Antonesco, F. et al. Association of KIT exon 9 mutations with nongastric primary site and aggressive behavior: KIT mutation analysis and clinical correlates of 120 gastrointestinal stromal tumors. Clin. Cancer Res. 9, 3329–3337 (2003).
31. Ueno-Yokohata, H. et al. Consistent in-frame internal tandem duplications of BCOR characterize clear cell sarcoma of the kidney. Nat. Genet. 47, 861–863 (2015).
32. He, J. et al. Kdm2b maintains murine embryonic stem cell status by recruiting PRCI complex to CpG islands of developmental genes. Nat. Cell Biol. 15, 373–384 (2013).
33. Yang, Y. et al. Clinical whole-exome sequencing for the diagnosis of mendelian disorders. N. Engl. J. Med. 369, 1502–1511 (2013).
34. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21 (2013).
35. Li, H. et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079 (2009).
36. DeLuca, D. S. et al. RNA-SeqQC: RNA-seq metrics for quality control and process optimization. Bioinformatics 28, 1530–1532 (2012).
37. Langerman, B. & Saliberg, S. L. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359 (2012).
38. McPherson, A. et al. deFuse: an algorithm for gene fusion discovery in tumor RNA-Seq data. PLoS Comput. Biol. 7, e1001138 (2011).
39. Team, R. D. C.: R: a language and environment for statistical computing. http://www.R-project.org (2008).

Acknowledgements The work was supported by the National Institutes of Health (NIH/NCI 1U01HG004685, the Cancer Prevention and Research Institute of Texas (RP120685-P1, RP120685-AC and RP120685-C1) and a Stand Up To Cancer St Baldrick’s Pediatric Dream Team Translational Research Grant (SU2CAACR-DT1113; Stand Up To Cancer is a programme of the Entertainment Industry Foundation administered by the American Association for Cancer). The results published here are in part based on data generated by the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) initiative managed by the NCI. Information about TARGET can be found at http://ocg.cancer.gov/programs/target. We acknowledge Pamela Parsons for technical assistance. The BCOR-184 antibody was a kind gift from Dr Vivian Bardwell.

Author contributions A.R. performed the next-generation sequencing analysis; V.K. and E.F. characterized the initial cases; V.K. and E.F. screened all the other validation cases and performed the expression analysis; H.D., S.W., D.A.W. and O.A.I. developed the RNA-Seq pipeline; B.Z. and P.S. performed all bioinformatics analysis; D.R., M.H., K.W.E. and A.R. performed pathology review and N.R.P. contributed to the immunohistochemistry experiments; D.R., J.F.A., P.J.L., S.X.S., J.G.N., M.M.C. and A.A.B. contributed cases and analysed the clinical files; A.R., S.E.P., P.S. and D.W.P. planned and supervised the work; all authors contributed to writing the manuscript.

Additional information Accession codes: Whole-exome and transcriptome sequence data of tumours have been deposited in dbGaP under the accession code phs001026. The TARGET project CCSK transcriptome data have been deposited in dbGaP under the accession code phs000466.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Roy, A. et al. Recurrent internal tandem duplications of BCOR in clear cell sarcoma of the kidney. Nat. Commun. 6:8891 doi: 10.1038/ncomms9891 (2015).