JOHN WILEY & SONS, LTD., THE ATRIUM, SOUTHERN GATE, CHICHESTER P019 8SQ, UK

*****PROOF OF YOUR ARTICLE ATTACHED, PLEASE READ CAREFULLY*****

After receipt of your corrections your article will be published initially within the online version of the journal.

PLEASE AIM TO RETURN YOUR CORRECTIONS WITHIN 48 HOURS OF RECEIPT OF YOUR PROOF, THIS WILL ENSURE THAT THERE ARE NO UNNECESSARY DELAYS IN THE PUBLICATION OF YOUR ARTICLE

- ❑ **READ PROOFS CAREFULLY**

ONCE PUBLISHED ONLINE OR IN PRINT IT IS NOT POSSIBLE TO MAKE ANY FURTHER CORRECTIONS TO YOUR ARTICLE

  - This will be your only chance to correct your proof
  - Please note that the volume and page numbers shown on the proofs are for position only

- ❑ **ANSWER ALL QUERIES ON PROOFS** (Queries are attached as the last page of your proof.)

  - List all corrections and send back via e-mail to the production contact as detailed in the covering e-mail, or mark all corrections directly on the proofs and send the scanned copy via e-mail. Please do not send corrections by fax or post

- ❑ **CHECK FIGURES AND TABLES CAREFULLY**

  - Check size, numbering, and orientation of figures
  - All images in the PDF are downsampled (reduced to lower resolution and file size) to facilitate Internet delivery. These images will appear at higher resolution and sharpness in the printed article
  - Review figure legends to ensure that they are complete
  - Check all tables. Review layout, title, and footnotes

- ❑ **COMPLETE COPYRIGHT TRANSFER AGREEMENT (CTA) if you have not already signed one**

  - Please send a scanned signed copy with your proofs by e-mail. **Your article cannot be published unless we have received the signed CTA**

- ❑ **OFFPRINTS**

  - Free access to the final PDF offprint or your article will be available via Author Services only. Please therefore sign up for Author Services if you would like to access your article PDF offprint and enjoy the many other benefits the service offers.

Additional reprint and journal issue purchases

- Should you wish to purchase additional copies of your article, please click on the link and follow the instructions provided: http://offprint.cosprinters.com/cos/bw/
- Corresponding authors are invited to inform their co-authors of the reprint options available.
- Please note that regardless of the form in which they are acquired, reprints should not be resold, nor further disseminated in electronic form, nor deployed in part or in whole in any marketing, promotional or educational contexts without authorization from Wiley. Permissions requests should be directed to mailto: permissionsuk@wiley.com
- For information about 'Pay-Per-View and Article Select' click on the following link: http://olabout.wiley.com/WileyCDA/Section/id-404512.html

WILEY AUTHOR DISCOUNT CLUB

We would like to show our appreciation to you, a highly valued contributor to Wiley's publications, by offering a **unique 25% discount** off the published price of any of our books*.

All you need to do is apply for the **Wiley Author Discount Card** by completing the attached form and returning it to us at the following address:

The Database Group (Author Club)
John Wiley & Sons Ltd
The Atrium
Southern Gate
Chichester
PO19 8SQ
UK

Alternatively, you can **register online** at www.wileyeurope.com/go/authordiscount
Please pass on details of this offer to any co-authors or fellow contributors.

After registering you will receive your Wiley Author Discount Card with a special promotion code, which you will need to quote whenever you order books direct from us.

The quickest way to order your books from us is via our European website at:

http://www.wileyeurope.com

Key benefits to using the site and ordering online include:
- Real-time SECURE on-line ordering
- Easy catalogue browsing
- Dedicated Author resource centre
- Opportunity to sign up for subject-orientated e-mail alerts

Alternatively, you can order direct through Customer Services at:
cs-books@wiley.co.uk, or call +44 (0)1243 843294, fax +44 (0)1243 843303

So take advantage of this great offer and return your completed form today.

Yours sincerely,

Verity Leaver
Group Marketing Manager
author@wiley.co.uk

*TERMS AND CONDITIONS

This offer is exclusive to Wiley Authors, Editors, Contributors and Editorial Board Members in acquiring books for their personal use. There must be no resale through any channel. The offer is subject to stock availability and cannot be applied retrospectively. This entitlement cannot be used in conjunction with any other special offer. Wiley reserves the right to amend the terms of the offer at any time.

REGISTRATION FORM
For Wiley Author Club Discount Card

To enjoy your 25% discount, tell us your areas of interest and you will receive relevant catalogues or leaflets from which to select your books. Please indicate your specific subject areas below.

Accounting [ ]
- Public [ ]
- Corporate [ ]

Chemistry [ ]
- Analytical [ ]
- Industrial/Safety [ ]
- Organic [ ]
- Inorganic [ ]
- Polymer [ ]
- Spectroscopy [ ]

Encyclopedia/Reference [ ]
- Business/Finance [ ]
- Life Sciences [ ]
- Medical Sciences [ ]
- Physical Sciences [ ]
- Technology [ ]

Earth & Environmental Science [ ]

Hospitality [ ]

Genetics [ ]
- Bioinformatics/ Computational Biology [ ]
- Proteomics [ ]
- Genomics [ ]
- Gene Mapping [ ]
- Clinical Genetics [ ]

Medical Science [ ]
- Cardiovascular [ ]
- Diabetes [ ]
- Endocrinology [ ]
- Imaging [ ]
- Obstetrics/Gynaecology [ ]
- Oncology [ ]
- Pharmacology [ ]
- Psychiatry [ ]

Non-Profit [ ]

Architecture [ ]

Business/Management [ ]

Computer Science [ ]
- Database/Data Warehouse [ ]
- Internet Business [ ]
- Networking [ ]
- Programming/Software Development [ ]
- Object Technology [ ]

Engineering [ ]
- Civil [ ]
- Communications Technology [ ]
- Electronic [ ]
- Environmental [ ]
- Industrial [ ]
- Mechanical [ ]

Finance/Investing [ ]
- Economics [ ]
- Institutional [ ]
- Personal Finance [ ]

Life Science [ ]

Landscape Architecture [ ]

Mathematics [ ]
Statistics [ ]

Manufacturing [ ]

Materials Science [ ]

Psychology [ ]
- Clinical [ ]
- Forensic [ ]
- Social & Personality [ ]
- Health & Sport [ ]
- Cognitive [ ]
- Organizational [ ]
- Developmental & Special Ed [ ]
- Child Welfare [ ]
- Self-Help [ ]

Physics/Physical Science [ ]

Please complete the next page /

I confirm that I am (*delete where not applicable):

a **Wiley** Book Author/Editor/Contributor* of the following book(s):
ISBN:
ISBN:

a **Wiley** Journal Editor/Contributor/Editorial Board Member* of the following journal(s):

SIGNATURE: …… Date: ……

PLEASE COMPLETE THE FOLLOWING DETAILS IN BLOCK CAPITALS:

TITLE: (e.g. Mr, Mrs, Dr) …… FULL NAME: ……

JOB TITLE (or Occupation): ……

DEPARTMENT: ……

COMPANY/INSTITUTION: ……

ADDRESS: ……

……

TOWN/CITY: ……

COUNTY/STATE: ……

COUNTRY: ……

POSTCODE/ZIP CODE: ……

DAYTIME TEL: ……

FAX: ……

E-MAIL: ……

YOUR PERSONAL DATA
We, John Wiley & Sons Ltd, will use the information you have provided to fulfil your request. In addition, we would like to:

1. Use your information to keep you informed by post of titles and offers of interest to you and available from us or other Wiley Group companies worldwide, and may supply your details to members of the Wiley Group for this purpose.
   [ ] Please tick the box if you do **NOT** wish to receive this information

2. Share your information with other carefully selected companies so that they may contact you by post with details of titles and offers that may be of interest to you.
   [ ] Please tick the box if you do **NOT** wish to receive this information.

E-MAIL ALERTING SERVICE
We also offer an alerting service to our author base via e-mail, with regular special offers and competitions. If you **DO** wish to receive these, please opt in by ticking the box [ ].

If, at any time, you wish to stop receiving information, please contact the Database Group (databasegroup@wiley.co.uk) at John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, PO19 8SQ, UK.

TERMS & CONDITIONS
This offer is exclusive to Wiley Authors, Editors, Contributors and Editorial Board Members in acquiring books for their personal use. There should be no resale through any channel. The offer is subject to stock availability and may not be applied retrospectively. This entitlement cannot be used in conjunction with any other special offer. Wiley reserves the right to vary the terms of the offer at any time.

PLEASE RETURN THIS FORM TO:
Database Group (Author Club), John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, PO19 8SQ, UK author@wiley.co.uk
Fax: +44 (0)1243 770154

AUTHOR QUERY FORM

Dear Author,
During the preparation of your manuscript for publication, the questions listed below have arisen. Please attend to these matters and return this form with your proof.
Many thanks for your assistance.

Query References	Query	Remarks
Q1	Please confirm that given names (red) and surnames/family names (green) have been identified correctly	
Q2	Please check if link to ORCID is correct.	
Q3	CD45 (2B11+PD7/26; Dako) –please specify the dilution.	
Q4	Abcam –please supply town, country and, if USA, state.	
Q5	Promega –please supply town, country and, if USA, state.	
Q6	Qiagen –previously, you referred to Qiagen, Hilden, Germany. Later you refer to Qiagen, Courtabouef, France. Please specify which one this is.	
Q7	Bioline –please supply town, country and, if USA, state.	
Q8	Qiagen –Qiagen, Courtabouef, France or Qiagen, Hilden, Germany? Please specify.	
Q9	Applied Biosystems –please supply town, country and, if USA, state.	
Q10	Sigma-Aldrich –please supply town, country and, if USA, state.	
Q11	Life Technologies –please supply town, country and, if USA, state.	
Q12	The Images/text in the figure(s) (FIG-2, FIG-4, FIG-5) are not clear/sharp, although the file is supplied with required resolution. It will be good if you could modify the text to appear clearly with a font size of 6pt and resupply the figures. The replacement figures must not be sent by email. Please upload them to FTP site: https://ftp.laserwords.com Login name: guest210 Password: s17m425t	
Q13	Ref. 10. Please supply volume number and page range. Ref. 12. Aviv Regev –not clear what the meaning of this name is at this position in the reference. Also, please supply volume number and page range, if now available. Ref. 16. Please supply volume number and page range, if now available.	

Funding Info Query Form

Please confirm that the funding sponsor list below was correctly extracted from your article: that it includes all funders and that the text has been matched to the correct FundRef Registry organization names. If no FundRef Registry organization name has been identified, it may be that the funder was not found in the FundRef registry, or there are multiple funders matched in the FundRef registry. If a name was not found in the FundRef registry, it may not be the canonical name form, it may be a program name rather than an organization name, or it may be an organization not yet included in FundRef Registry. If you know of another name form or a parent organization name for a "not found" item on this list below, please share that information.

Funding Agency	**FundRef Organization Name**
Institut National de la Santé et de la Recherche Médicale	Institut National de la Santé et de la Recherche Médicale
Italian Ministry of Health (Ricerca Corrente RC L2029, IRCCS Galeazzi Orthopaedic Institute)	"Not Found"
Royal Orthopaedic Hospital NHS Foundation trust, Birmingham, England	"Not Found"
Kuwait Medical Genetics Centre (KMGC), Ministry of Health, Kuwait	"Not Found"
Department of Biomedical, Surgical and Dental Sciences (University of Milan, grant number 15-63017000-700)	"Not Found"
Centre Léon Bérard, Lyon, France	"Not Found"

Journal of Pathology
J Pathol 2018; **0:** 000–000
Published online in Wiley Online Library
(wileyonlinelibrary.com) DOI: 10.1002/path.5071

ORIGINAL PAPER

Genetic analyses of undifferentiated small round cell sarcoma identifies a novel sarcoma subtype with a recurrent *CRTC1–SS18* gene fusion

Abdullah Alholle[1†], Marie Karanian[2,3†], Anna T Brini[4‡], Mark R Morris[5‡], Vinodh Kannappan[5], Stefania Niada[4], Angela Niblett[6], Dominique Ranchère-Vince[2], Daniel Pissaloux[2,3], Christophe Delfour[7], Aurelie Maran Gonzalez[8], Cristina R Antonescu[9], Vaiyapuri Sumathi[6§*], Franck Tirode[3,10§*] and Farida Latif[1§*]

[1] *Institute of Cancer and Genomic Sciences, University of Birmingham, Birmingham, UK*
[2] *Department of Biopathology, Centre Léon Bérard, Lyon, France*
[3] *University of Lyon, Université Claude Bernard Lyon 1, CNRS 5286, INSERM U1052, Cancer Research Centre of Lyon, Lyon, France*
[4] *IRCCS Galeazzi, Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy*
[5] *Research Institute in Healthcare Science, Faculty of Science and Engineering, University of Wolverhampton, Wolverhampton, UK*
[6] *Department of Musculoskeletal Pathology, The Royal Orthopaedic Hospital, Robert Aitken Institute of Clinical Research, University of Birmingham, Birmingham, UK*
[7] *Anatomy and Pathological Cytology Department, Centre Hospitalo-Universitaire Gui de Chauliac, Montpellier, France*
[8] *Anatomy and Pathology Department, Montpellier Cancer Institute, Montpellier, France*
[9] *Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY, USA*
[10] *Department of Translational Research and Innovation, Centre Léon Bérard, Lyon, France*

**Correspondence to: V Sumathi, Department of Musculoskeletal Pathology, The Royal Orthopaedic Hospital, Robert Aitken Institute of Clinical Research, Vincent Drive, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK. E-mail: vaiyapuri.sumathi@nhs.net; Or F Tirode, Cancer Research Centre of Lyon, INSERM U1052, Centre Léon Bérard, 28 Rue Laënnec, 69373 Lyon Cedex 08, France. E-mail: franck.tirode@lyon.unicancer.fr;*
Or F Latif, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK. E-mail: f.latif@bham.ac.uk

†Equal contributions.
‡Equal contributions.
§Equal contributions.

Abstract

In recent years, undifferentiated small round cell sarcomas (USRCSs) have been divided into a variety of new, rare, sarcoma subtypes, including the group of Ewing-like sarcomas, which have the morphological appearance of Ewing sarcomas, but carry *CIC–DUX4*, *BCOR–CCNB3* and other gene fusions different from the classic *EWSR1–ETS* gene fusion. Using high-throughput RNA-sequencing (RNA-seq) analyses, we identified a novel recurrent gene fusion, *CRTC1–SS18*, in two cases of USRCS that lacked any known translocation. RNA-seq results were confirmed by reverse transcription polymerase chain reaction, long-range polymerase chain reaction, and fluorescence *in situ* hybridization. *In vitro*, we showed that the cells expressing the gene fusion were morphologically distinct and had enhanced oncogenic potential as compared with control cells. Expression profile comparisons with tumours of other sarcoma subtypes demonstrated that both cases clustered close to *EWSR1–CREB1*-positive tumours. Moreover, these analyses indicated enhanced *NTRK1* expression in *CRTC1–SS18*-positive tumours. We conclude that the novel gene fusion identified in this study adds a new subtype to the USRCSs with unique gene signatures, and may be of therapeutic relevance.

Keywords: undifferentiated small round cell sarcoma; Ewing sarcoma; RNA-seq; gene fusion

Received 20 September 2017; Revised 2 February 2018; Accepted 8 March 2018

No conflicts of interest were declared.

Introduction

Diagnosing small round blue cell tumours on biopsy has been challenging because of their lack of specific features in small specimens. Among undifferentiated small round cell sarcomas (USRCSs), Ewing-like sarcoma (ELS) shares some of the morphological features of Ewing sarcoma (ES), but lacks the classic *EWSR1–ETS* gene fusion [1,2]. ESs are mainly characterized by chromosomal translocations at chromosome 22q12 that fuse *EWSR1* with one of the *ETS* gene family of transcription factors, such as *FLI1* or *ERG*, in 90–95% of ES cases. The classic ES gene fusion protein acts as an oncoprotein, and plays an essential role in tumourigenesis and

proliferation of ES cells [2]. Recent studies have identified recurrent gene fusions in ELS, namely *CIC–DUX4* and *BCOR–CCNB3* [3–6]. The identification of these gene fusions suggests that other, as yet to be identified, gene fusions could be associated with this type of tumour. Furthermore, novel gene fusions in ELS have been reported recently in case reports, including *CIC–FOXO4*, *BCOR–MAML3*, and *ZC3H7B–BCOR* [7–9]. Identifying these genetically defined entities may contribute towards understanding the pathogenesis and the behaviour of these tumours.

By applying RNA sequencing (RNA-seq) technology to investigate USRCS, we discovered a novel *CRTC1–SS18* gene fusion in two samples from two different cancer centres. Combining both samples, we were able to find similarities at the clinical, pathological, and molecular levels. Moreover, by cloning the fusion gene we were able to demonstrate its oncogenic properties, adding the *CRTC1–SS18* fusion gene to the increasing number of described oncogenes.

Materials and methods

One patient sample (case 1) was obtained from the Royal Orthopaedic Hospital NHS Foundation Trust Tumour Bank (with permission; REC 12/EM/0048). The other patient sample (case 2) was obtained from resection material sent to the Centre Léon Bérard for molecular diagnosis. Both samples were acquired with informed consent from the patient and or/next of kin, and ethical approval from institutional and local research committee boards. Patient samples were anonymized and used in accordance with the principles expressed in the Declaration of Helsinki.

Immunohistochemistry

Case 1

For immunohistochemistry, 2-µm-thick sections were cut, and antigens were retrieved in an epitope retrieval solution of pH 8 (RE7116; Novocastra, Newcastle upon Tyne, UK) at 68 °C for 17 h in a stirred water bath. The antibody clones, dilutions and sources were as follows: anti-CD99 (12E7, 1:25; Dako, Ely, UK), anti-vimentin (V9, 1:100; Novocastra), anti-CD31 (JC70, 1:100; Dako), anti-CD34 (QBend10, 1:50; Dako), anti-cytokeratin AE1/AE3 (1:100; Dako), anti-CD45 (2B11 + PD7/26; Dako) •, anti-cytokeratin MNF116 (1:50; Dako), anti-desmin (D33, 1:100; Dako), anti-α-smooth muscle actin (SMA) (1A4, 1:200; Dako), anti-epithelial membrane antigen (EMA) (E29, 1:100; Dako), anti-HMB45 (1:200; Dako), anti-S100 (NCL-L-S100p, 1:1000; Novocastra), anti-Wilms tumour 1 (WT1) (C-19, 1:500; Santa Cruz, Insight Biotechnology Limited, Wembley, UK), anti-TLE1 (M-101, 1:50; Santa Cruz), anti-ERG (Erg-1/2/3 C-1, 1:50; Santa Cruz), anti-INI1 [1:25; BD Transduction Laboratories (BD Biosciences), Becton Dickinson UK, Oxford, UK], anti-BCOR (C-10, 1:50; Santa Cruz), anti-ETV4 (16, 1:50; Santa Cruz), and anti-Ki67 (MIB1, 1:200; Dako).

Case 2

Sections were cut at a thickness of 4 µm from formalin-fixed paraffin-embedded (FFPE) tissue, and immunostained with a VentanaBenchmark XT automatic stainer (Ventana, Tuscon, AZ, USA). Signals were revealed with the ultraView Universal Dab Detection kit (Ventana). The following antibodies were used: anti-CD99 (12E7; Dako), anti-EMA (E29, 1:50; Dako), anti-desmin (D33, 1:80; Dako), anti-cytokeratin AE1/AE3 (AE1/AE3, 1:50; Dako), anti-caldesmon (h-CD, 1:100; Dako), anti-myogenin (F5D, 1:100; Dako), anti-S100 (Z0311, 1:800; Dako), anti-CD34 (QBend-10, 1:25; Dako), anti-INI1 (25, 1:50; BD Transduction Laboratories), anti-BCOR (C-10, 1:50; Santa Cruz), and anti-ETV4 (16, 1:50; Santa Cruz). Immunohistochemistry for neurotrophic receptor tyrosine kinase 1 (NTRK1) was performed with a 32-min incubation with the anti-NTRK1 antibody (ab76291, clot EP1058Y, dilution 1:200; Abcam •) on a Ventana ULTRA machine with Cell Conditioning Solution 1 pretreatment for 64 min.

Fluorescence *in situ* hybridization (FISH) analyses

FISH analyses were performed on FFPE tissue sections with the ZytoLight SS18 Dual Color Break Apart Probe (#Z-2097-200; Zytovision, Bremerhaven, Germany) by assessment of at least 100 non-overlapping intact nuclei by two independent operators. The positive threshold for calling the FISH assay positive was 15%.

Array-comparative genomic hybridization (aCGH) analyses

Genomic DNA was extracted from FFPE tissue with a QIAamp DNA micro kit (Qiagen, Hilden, Germany). Genomic DNA and human reference DNA (Promega •) were labelled with cyanine 5 and cyanine 3, respectively, by use of the Genomic DNA High-Throughput *ULS* Labeling Kit (Agilent Technologies, Santa Clara, CA, USA), and co-hybridized onto a 4x180K Sureprint G3 Human CGH microarray (Agilent Technologies), according to the manufacturer's recommendations. Data were analysed with Agilent Genomic Workbench software v7.0 or with Cytogenomics software (v2.9.2.4; Agilent), and expressed according to the human reference genome hg19 (GRCh37; Genome Reference Consortium Human Reference 37). The identification of aberrant copy number segments was based on the ADM-2 segmentation algorithm with a threshold of 6.0.

Fresh frozen tissue RNA-seq

Total RNA was extracted from fresh frozen tissue (case 1) with a Qiagen RNeasy Mini kit (Qiagen •) according to the manufacturer's protocol. RNA quality and

quantity were measured with an Agilent 2100 bioanalyzer (Agilent Technologies). An RNA sample was sent to Oxford Gene Technology (Begbroke, UK) for performance of RNA-seq with the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA). In brief, cDNA libraries were prepared from 1 µg of total RNA with the Illumina TruSeq RNA Sample Prep Kit v2. All sequencing was paired-end (100 bp) and performed over 100 cycles, and the read files (Fastq) were generated from the sequencing platform via the manufacturer's software. Mapping and alignment were processed with the Tuxedo suit. The human sequence genome (hg19) was used as a reference and aligned to the sequence reads. FusionCatcher software was used to identify gene fusions from RNA-seq data [10]. RNA-seq data have been deposited (SRA accession: SRP131744).

FFPE RNA sequencing

RNA was extracted from FFPE tissue sections (case 1 and case 2) with Trizol reagent (Thermo Fisher Scientific, Courtaboeuf, France), and subsequently extracted with phenol/chloroform. The RNase-free DNase Set (Qiagen, Courtaboeuf, France) was used to remove DNA. The DNase was eliminated by a further Trizol extraction. All RNAs were quantified by spectrophotometry (NanoDrop; Thermo Fisher Scientific), and quality was controlled (DV200 value cutoff of >13%) by use of a TapeStation with Hs RNA ScreenTape (Agilent Technologies). One hundred nanograms of total RNA was used to prepare a library with a TruSeq RNA Access Library Prep Kit (Illumina). Fourteen libraries were pooled at 4 nM with 1% PhiX as an internal control. Sequencing was performed (75 cycles, paired end) with a NextSeq 500/550 High Output V2 kit and an Illumina NextSeq 500 (Illumina). Alignments were performed with the STAR algorithm [11] against the GRCh38 reference genome, and fusion gene assessments were made with STAR-Fusion [12], FusionCatcher [10] and FusionMap [13] tools. Expression profiles were extracted from fastq files with Kallisto [14], and transformed as log2(TPM + 2) prior to quantile normalization with the Limma package v 3.32.2 performed in the R environment v3.4.1 [15]. Only genes with a coding sequence annotation (based on Ensembl GRCh38p5 annotation) and with a maximum expression value across all samples of >2 were considered for the clustering analysis, which was performed by the use of Ward's distance on the 10% most variant genes based on their interquartile range. RNA-seq data have been deposited (SRA accession: SRP131744).

Reverse transcription polymerase chain reaction (RT-PCR) and Sanger sequencing

cDNA was generated from total RNA with SuperScript III (Invitrogen) and random primers (Promega). The RT-PCR reactions were performed withy 2.5 µl of 10X buffer, 2.5 µl of dNTPs (2.5 mM), 5 µl of 5X GC-rich solution, 1 µl of forward primer (20 pmol), 1 µl of reverse primer (20 pmol), and 0.1 µl of Fast Start DNA polymerase (Roche, Burgess Hill, UK). Primer sets used for polymerase chain reaction (PCR) amplification were CRTC1-F (TCGAACAATCCGCGGAAATT) and SS18-R (GTGCTGGTAAAAGAGACTGCA), and PCR products were visualized with 2% (w/v) agarose gel (Bioline •). The PCR products were extracted from the gel and purified with a QIAquick Gel Extraction Kit (Qiagen •). A BigDye Terminator V3.1 kit (Applied Biosystems •) was used for the cycle sequencing reaction, and PCR products of *CRTC1–SS18* gene fusions were directly Sanger sequenced with an ABI 3730 DNA analyser (Applied Biosystems).

Long-range PCR (LR-PCR)

LR-PCR was carried out with PrimeSTAR GXL DNA polymerase (Takara Bio, Shiga, Japan). Each LR-PCR reaction was set up with 50 ng of DNA, 1 × 5X PrimeSTAR GXL buffer, 200 µM each dNTP, 0.2 µM forward primer, 0.2 µM reverse primer, and 1.25 U of PrimeSTAR GXL DNA polymerase enzyme, made up to a final volume of 50 µl with sterilized distilled water. The PCR was carried out with the following conditions: 30 cycles of 10 s at 98 °C and 10 min at 60 °C. The LR-PCR primers used in this study are listed in supplementary material, Table S1. The size of the PCR product from the gene fusion was unknown; therefore, genome walking through both genes and a rough estimation of the product size was carried out. The genome walking covered the exonic and intronic regions of both genes involved in the fusion. LR-PCR was performed on the genomic DNA of the tumour sample. One forward primer (F3) was anchored on exon 1 of *CRTC1*, and different reverse primers spanning ~2.5 kbp of intron 1 were used to amplify this region and to identify the breakpoint. The samples were electrophoresed on 0.9% agarose gels to determine the size of the PCR product. After confirming the breakpoint of *CRTC1–SS18* gene fusion at the genomic level, LR-PCR was performed on both the tumour sample and the corresponding normal tissue to confirm that this fusion was somatic. The PCR product was then extracted from the gel and sequenced.

Plasmid construction

The *CRTC1–SS18* expression construct was made by PCR amplification of the entire fusion construct with the cDNA generated from case 1 tumour RNA. This amplicon was subcloned into the expression vector pFlag-CMV-4 (Sigma-Aldrich •) by the use of *Eco*RI and *Xba*I restriction sites. The primers used were as follows: forward, 5′-cg g aat tcg aag atg gcg act tcg aac aat c-3′; and reverse, 5′-cg tctaga t tca ctg ctg gta att tcc ata c-3′. Plasmid constructs were verified by sequencing. Expression of this plasmid generates an N-terminal FLAG-tagged protein. The construct plasmid and associated empty vector were transfected into HEK293 cells (ATCC, Manassas, VA, USA), clones were isolated, and expression was validated by western blotting with an anti-FLAG antibody (Cat. No.

F1804, clone M2, 1:1000; Sigma-Aldrich). Clones were maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) supplemented with 1 mg/ml G418 (Life Technologies ●).

Cell migration assays

Suspensions containing 25 000 cells of a stable *CRTC1–SS18*-expressing HEK293 clone, or a clone containing the empty vector, suspended in serum-free DMEM were seeded into a 24-well format Boyden chamber cell culture insert (8-µm pore size; PET membrane) (BD Falcon, Bedford, MA, USA). The lower chamber contained DMEM, and 10% FBS as an attractant. Chambers were incubated for 16 h. Cells were fixed in methanol; cells on the upper side of the chamber were removed, and those remaining on the underside were stained with crystal violet. Migrated cells were photographed, the crystal violet was solubilized in 500 µl of 33% acetic acid, and the optical density was measured at 540 nm ($n = 20$).

Cell invasion assays

A modified migration assay was carried out in which 50 000 cells were seeded into Boyden chambers (8-µm pore size; PET membrane) (BD Falcon) precoated with 100 µl of Geltrex basement membrane matrix (Thermo Fisher Scientific). Chambers were incubated for 16 h. Invasive cells were counted by microscopy with the observer unaware of the cell type ($n = 22$).

Soft agar, anchorage-independent growth assay

Anchorage-independent growth in soft agar was assessed with the CytoSelect 96-Well Cell Transformation Assay kit (Cell Biolabs, San Diego, CA, USA), according to the manufacturer's instructions. In brief, 2500 cells per well were seeded in agar supplemented with DMEM and 10% FBS. Following incubation for 8 days, the agar was solubilized and viable cells were lysed, stained and quantified by fluorometry (excitation 492 nm, emission 520 nm; $n = 8$).

Results

Clinical presentation and pathological findings of the index case, case 1

Clinical presentation

A 35-year-old man presented to the Royal Orthopaedic Hospital with a rapidly growing lump on his right thigh. Magnetic resonance imaging revealed a heterogeneous mass within the sartorius muscle, and the findings were in keeping with a soft tissue sarcoma. A diagnosis of USRCS was made on the biopsy material of the tumour, which did not carry the chimeric gene fusions associated with ES (*EWSR1–FLI1* or *EWSR1–ERG*), mesenchymal chondrosarcoma (*EWS–NR4A3* and *TAF2N–NR4A3*), DSRCT (*EWS–WT1*), or synovial sarcoma (SS) (*SS18–SSX*). The tumour was also negative for *CIC–DUX4* and *BCOR–CCNB3*. In view of this diagnosis, the patient received four cycles of vincristine, ifosfamide, doxorubicin and etoposide. Radiologically, there was no response to chemotherapy, and the mass was excised. The patient developed bilateral lung metastases 18 months after diagnosis, and died of disease 92 months later.

Pathological findings

Grossly, an intramuscular, well-circumscribed greyish white fleshy tumour with haemorrhagic and necrotic foci measuring $7 \times 65 \times 53$ mm was observed (Figure 1A). Histologically, the tumour consisted of solid sheets and nests of small round cells surrounded by desmoplastic stroma reminiscent of desmoplastic small round cell tumour (DSRCT). The tumour cells had scant amounts of eosinophilic cytoplasm and small, irregularly shaped round nuclei with stippled chromatin. Some had prominent grooves and small nucleoli. Focal areas of necrosis and mitotic figures were identified (seven per 10 high-power fields). Rosette formation and glandular differentiation were not identified (Figure 1B,C). Immunohistochemically, the tumour cells were diffusely positive for vimentin and CD99 (Figure 1D). The tumour cells did not stain for CD31, CD34, AE1/AE3, CD45, CK(MNF116), desmin, EMA, HMB45, SMA, S100, HMB45, WT1, TLE1, ERG, ETV4, BCOR, or CCNB3. INI1 expression was retained. The Ki67 labelling index was up to 20%.

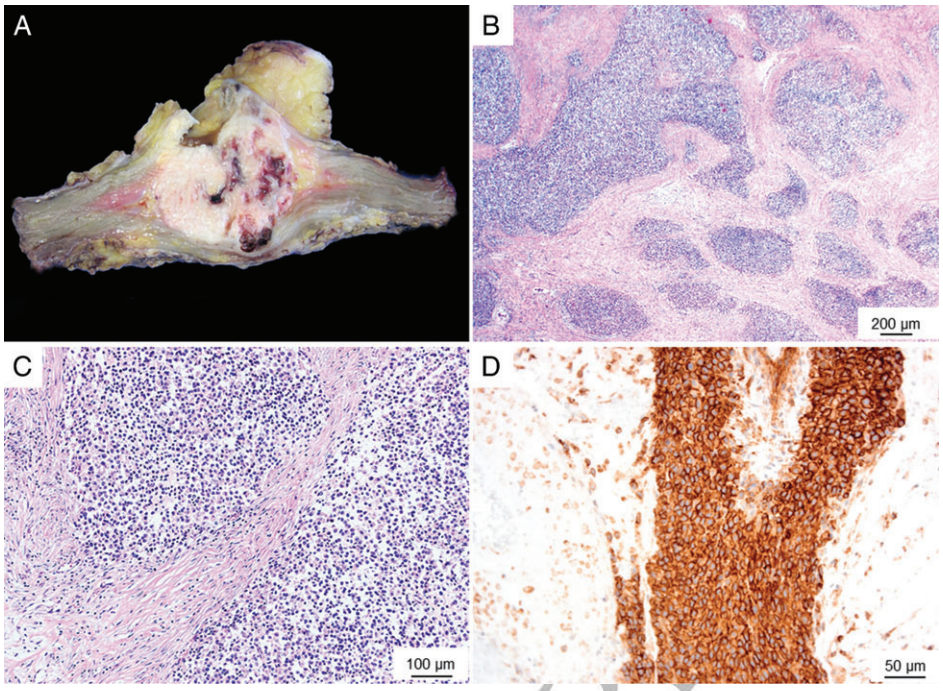
RNA-seq analysis and confirmation of gene fusion

RNA-seq analysis of the index case (case 1) revealed a novel gene fusion involving *CRTC1* and *SS18* in the tumour sample. Two alternative splicing fusion transcripts were detected that linked exon 1 of *CRTC1* with exon 2 or exon 3 of *SS18* (Figure 2A,B; supplementary material, Figure S1). A balanced translocation resulted in *SS18* being fused with exon 1 of *CRTC1*, generating an in-frame fusion protein (Figures 2A,B and 4A). The *CRTC1–SS18* fusion transcripts were confirmed by RT-PCR and Sanger sequencing (Figure 2B; supplementary material, Figure S2A). In order to map the fusion breakpoints at the genomic level, LR-PCR was carried out to reveal the genomic sequence around the breakpoints (supplementary material, Figure S2B–D and Table S1). The *CRTC1* breakpoint was found to be 8 bp from the 3′-end of exon 1 (cDNA fusion point), and the *SS18* breakpoint was 4457 bp before the 5′-end of exon 1 (Figure 2C).

Clinical presentation and pathological findings of case 2

Clinical presentation

RNA sequencing revealed a second case of an *CRTC1–SS18*-positive USRCS (case 2): a 42-year-old



Color

Figure 1. Macroscopy and microscopy images of case 1. (A) The macroscopic image shows a fairly well-circumscribed fleshy tumour with foci of necrosis and haemorrhage. (B) The microscopic image (low power) shows the tumour to be composed of solid sheets and nests surrounded by desmoplastic stroma. (C) The high-power view of the tumour shows small cells with scant cytoplasm. (D) Membranous staining with CD99, mimicking ES.

woman who presented to the CHU Gui de Chauliac (Montpellier, France) with a mass from the popliteal fossa, later diagnosed as an undifferentiated small cell sarcoma. The patient underwent radiotherapy and chemotherapy prior to the surgical removal of the tumour. A tumour fragment was sent to the Centre Léon Bérard's department of pathology for a second opinion and molecular diagnosis.

Pathological findings

An intramuscular mass, well circumscribed by a diffuse calcified matrix, measuring $110 \times 75 \times 70$ mm was observed. The tumour consisted of bundles and nests of cells embedded in a focally myxoid fibrous stroma (Figure 3A). There were areas of necrosis. Cytologically, the tumour was composed of oval to epithelioid cells (Figure 3B). The cells were of medium size with abundant eosinophilic cytoplasm and ovoid and vesicular nuclei with nucleoli (Figure 3C). Immunohistochemically, the tumour cells were positive for CD99 and negative for keratins, EMA, desmin, caldesmon, myogenin, S100, CD34, ETV4, and BCOR. INI1 expression was retained. NTRK1 was diffusely positive.

RNA-seq performed on the FFPE material showed an in-frame fusion between exon 1 of *CRTC1* and exon 2 of *SS18*. In this case, the translocation was unbalanced, as demonstrated by FISH (Figures 2A and 4A). It is of note that the cell morphology was similar in the tumour biopsy acquired before treatment, which also harboured the *CRTC1–SS18* fusion gene.

Genomic and transcript profiles

aCGH analyses revealed that case 1 had a diploid genome and a balanced *CRTC1–SS18* translocation, whereas case 2 had a tetraploid genome with an unbalanced *CRTC1–SS18* translocation (see Figure 4B for specific chromosomal gains and losses for each case). To enable comparison of both samples, RNA-seq of case 1 was also performed on an FFPE sample, in the same pipeline as case 2. Hierarchical clustering analysis (of RNA-seq data) demonstrated that both samples clustered together and close to the *EWSR1–CREB1*-positive tumours but not with ESs or ELSs (Figure 4C). Also, *CRCT1–SS18*-positive samples did not cluster with the recently described cutaneous melanocytomas harbouring a *CRTC1–TRIM11* fusion gene [16]. Furthermore, RNA-seq data revealed enhanced *NTRK1* expression in the two cases with the *CRTC1–SS18* gene fusion as compared with other sarcomas with known translocations such as *EWSR1–CREB1*, *BCOR–CCNB3*, *CIC–DUX4*, and *EWSR1–FLI1* (Figure 4D), and this was confirmed at the protein level by immunohistochemistry (supplementary material, Figure S3).

Functional analysis of the *CRTC1–SS18* gene fusion product

To determine whether the product of the *CRTC1–SS18* gene fusion identified had any potential oncogenic activity, the fusion gene from case 1 was cloned into a tagged mammalian expression vector, and human HEK293 clones expressing the construct were generated; a fusion protein of the predicted size

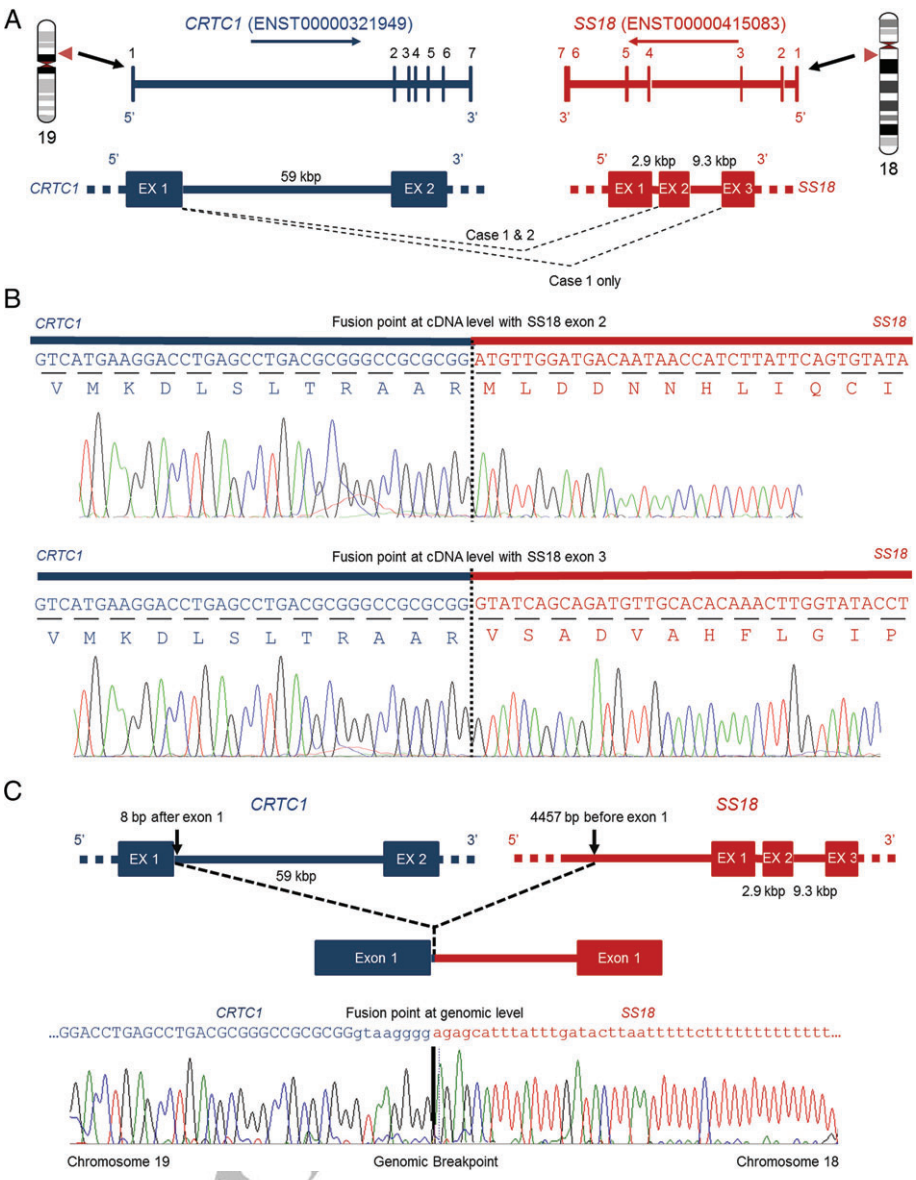
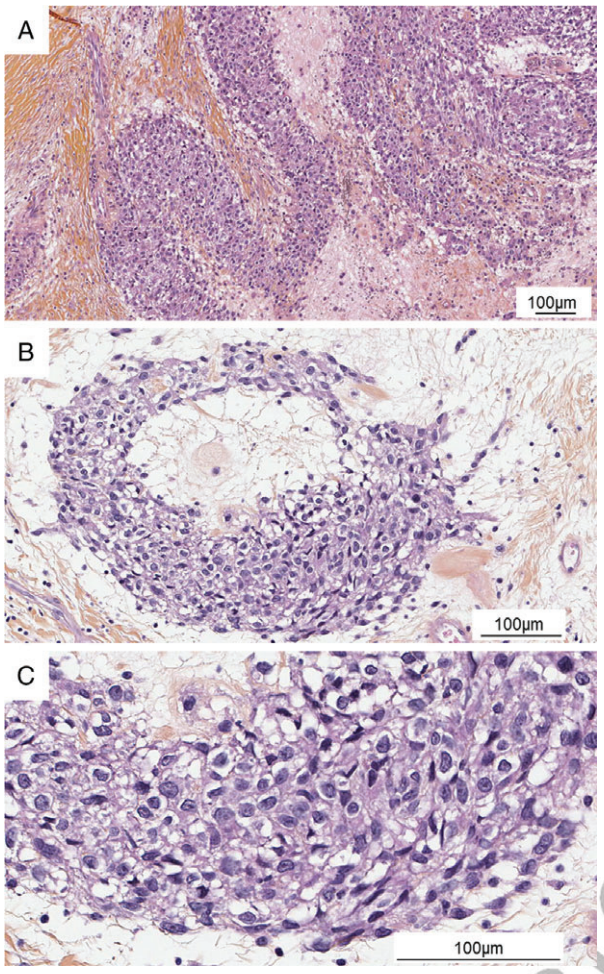


Figure 2. •RNA-seq identification of the *CRTC1–SS18* gene fusion. (A) The genomic intron–exon structure of *CRTC1* (blue) and *SS18* (red). *CRTC1*(exon 1)–*SS18*(exon 2) gene fusion was found in case 1 and case 2, whereas *CRTC1*(exon 1)–*SS18*(exon 3) alternative splicing gene fusion was found only in case 1. (B) A Sanger sequencing chromatogram of the RT-PCR product confirmed the *CRTC1*(exon 1)–*SS18*(exon 2) fusion junction and the *CRTC1*(exon 1)–*SS18*(exon 3) fusion junction in case 1. (C) Schematic of the exon–intron structure of the *CRTC1–SS18* gene fusion at the DNA level. The intergenic breakpoints for both genes are shown, and were confirmed by Sanger sequencing of the LR-PCR product in case 1.

(~57 kDa) was observed (supplementary material, Figure S4). The *CRTC1–SS18*-positive clone were morphologically distinct from control clones, showing extended pseudopodia and pronounced intracytoplasmic vacuoles (Figure 5A). We proceeded to assess the anchorage-independent growth potential of these cells (hallmark of transformation). CRTC1–SS18-expressing HEK293 cells were seeded into semisolid agar, in 96-well microtitre plates, and incubated for 8 days. The number of viable cells was then determined with a commercial fluorescence assay. Following this relatively short period of incubation, the assay indicated that there were 3.7 times the number of viable cells expressing CRTC1–SS18 as control HEK293 cells transfected with an empty plasmid. This increase in the number of viable cells was statistically significant (Figure 5B). As expression of this fusion protein appeared to increase anchorage-independent growth, we continued to assess these cells for other hallmarks of malignancy. We carried out assays to determine the migratory and invasive potential of the CRTC1–SS18-expressing cells. In a Boyden chamber assay, in which cells are encouraged to pass through 8-µm pores, significantly more HEK293 cells expressing CRTC1–SS18 migrated than control cells; this was determined both visually and with a colourimetric assay (Figure 5C). In a similar assay, in which the membranes of the chambers are coated in a basement membrane matrix to model the invasive potential of these cells, 2.6 times more CRTC1–SS18-expressing cells than control



Color

Figure 3. Microscopy images of case 2. (A) Haematoxylin and eosin (HE) (low-power objective, ×10): sheets and nests of cells in desmoplastic stroma. (B) HE (×20): nest of epithelioid cells. (C) HE (objective ×40): cells were of medium size with abundant eosinophilic cytoplasm and ovoid and vesicular nuclei with nucleoli.

cells invaded through the membrane in a 16-h period (Figure 5D).

Discussion

ELS or USRCS is a subtype of small blue round cell tumour that has a morphological appearance close to that of ES but lacks the characteristic *EWSR1–ETS* gene fusion. Recently, some of these USRCSs have been shown to carry gene fusions involving *CIC–DUX4*, *CIC–FOXO4*, *BCOR–CCNB3*, *BCOR–MAML3*, and *ZC3H7B–BCOR* [3–8]. In this study, we have identified, by RNA-seq, a novel recurrent *CRTC1–SS18* gene fusion in two USRCSs that were negative for known gene fusions in this sarcoma type. The CREB-regulated transcription coactivator 1 (*CRTC1*) gene belongs to a family of highly conserved cAMP response element-binding protein (CREB) coactivators [17,18]. *CRTC1* has already been implicated in other translocations, such as the *CRTC1–MAML2* fusion in mucoepidermoid carcinoma of salivary, bronchial and thyroid glands [19–22], and the *CRTC1–TRIM1* fusion in cutaneous melanocytomas [16]. The SS18 protein (NBAF chromatin remodelling complex subunit SS18) functions as a transcriptional coactivator, and interacts directly with members of the SWI–SNF chromatin remodelling complex [23]. The *SS18–SSX* fusion is a result of the chromosomal translocation t(X;18)(p11;q11) in almost all cases of SS, which account for approximately 10–20% of all soft tissue sarcomas [24–26].

These two cases of *CRTC1–SS18*-positive sarcoma are regarded as a distinct entity from all other ELSs, DSRCTs and poorly differentiated SSs described in the literature. Poorly differentiated SS is characterized by high cellularity, polygonal to small round cell morphology, frequent mitoses, and necrosis. These poorly differentiated SSs may be distinguished by the expression of high molecular weight cytokeratins and CD99, and having the characteristic t(X;18)(p11:2;q11:2) translocation. However, these two cases of *CRTC1–SS18*-positive sarcoma had a distinct morphology relative to that seen in poorly differentiated SS, and lacked the characteristic t(X;18)(p11:2;q11:2) translocation. Only one other ELS tumour with a *CIC–FOXO4* gene fusion has been described with desmoplastic stroma that showed immunoreactivity for CD99 and focal WT1. This tumour occurred in the neck of an elderly male. DSRCT commonly arises in the abdominal cavity in children and young adults, and is characterized histologically by nests of small round cells surrounded by desmoplastic stroma, immunohistochemically by positive staining for keratins, desmin, and WT1, and genetically by the presence of a recurrent translocation, i.e. t(11;22)(p13;q12). However, the tumours that we describe here occurred in lower limbs of adult patients, and the tumour cells were larger than cells of DSRCT, with more cytoplasm, and showed immunoreactivity for CD99 but did not carry the *EWS–WT1* gene fusion.

Morphologically, the two tumours that we present here shared features: large fibrous stroma, and small to medium cells with eosinophilic cytoplasm and vesicular nuclei. Expression profile analyses confirmed that our *CRTC1–SS18*-positive sarcomas were not related to ES or ELS, but rather to *EWSR1–CREB1*-positive tumours. It is of note that *CRTC1–TRIM11*-positive cutaneous melanocytomas were also found to resemble *EWSR1–CREB1*-positive clear cell sarcomas [16], but hierarchical clustering clearly separated both types of *CRTC1*-fused tumours. Finally, we also present here evidence, at both the RNA level and the protein level, that NTRK1 is expressed at higher levels in *CRTC1–SS18*-positive sarcomas than in other related tumours. We could not find any fusion involving *NTRK1* in these tumours, explaining its elevated level Nevertheless, NTRK1 expression may be useful as a marker for differential diagnosis, but most importantly may be used as a therapeutic target. In addition, we demonstrate that the cells expressing the *CRTC1–SS18* gene fusion

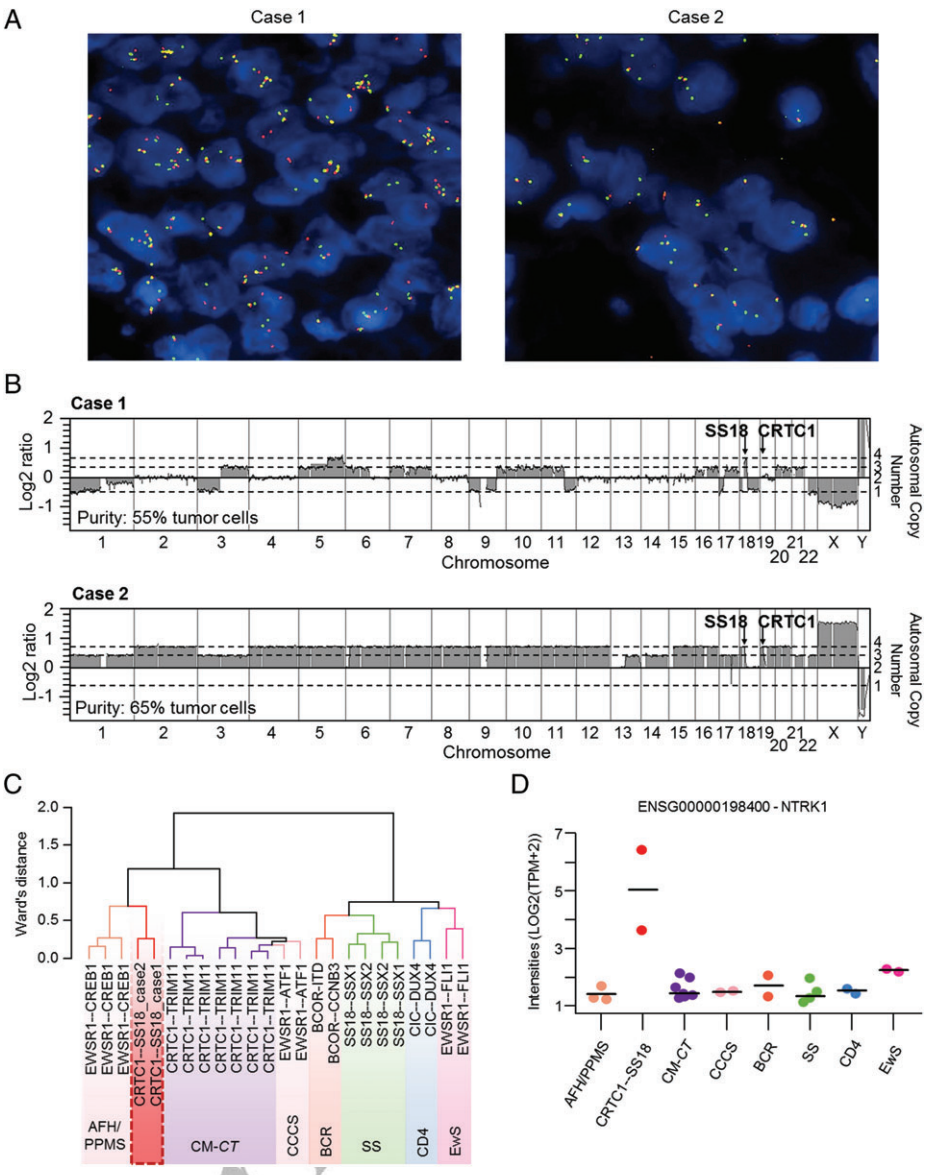
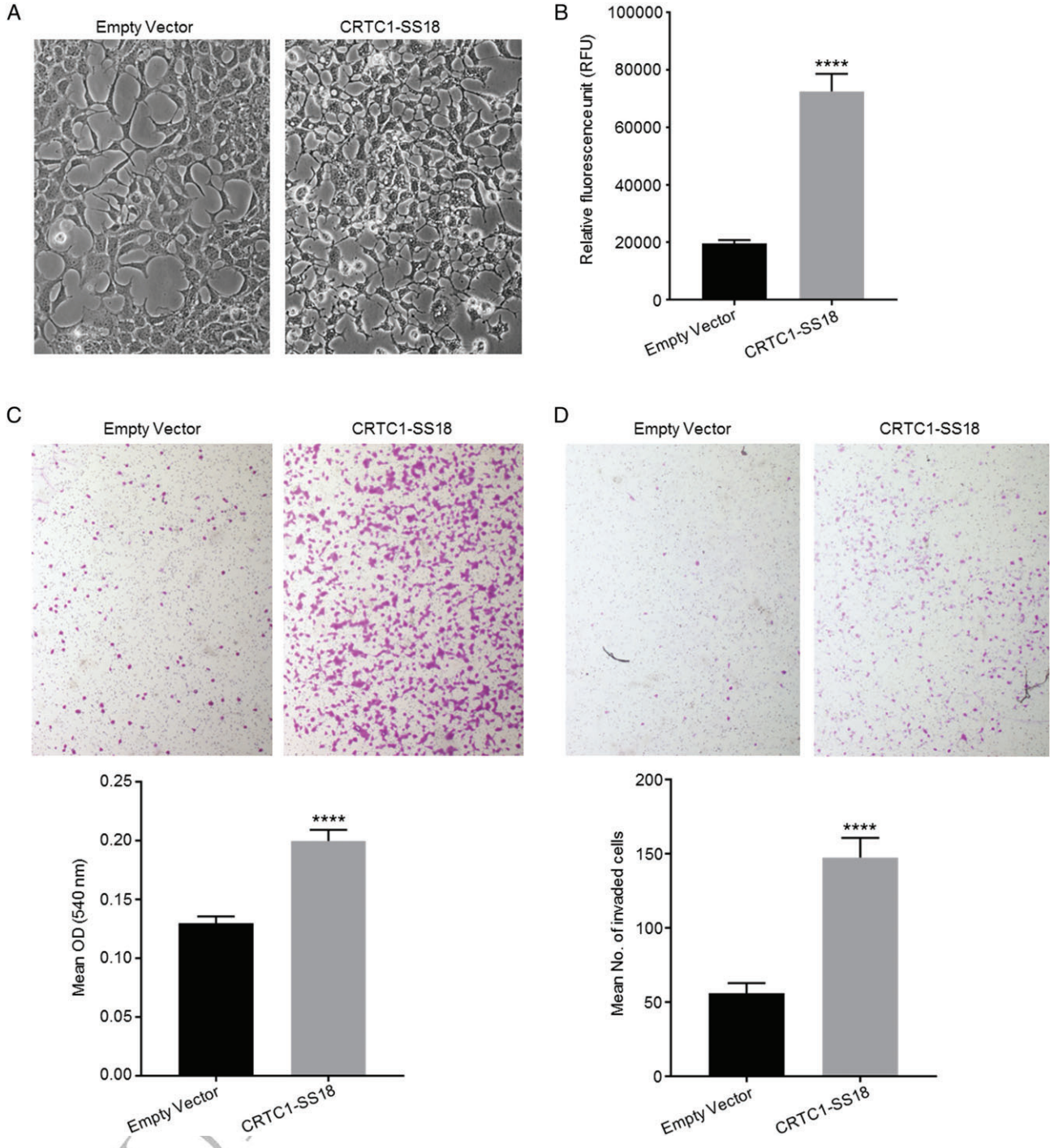


Figure 4. Genomic and transcript profiles of *CRTC1–SS18*-positive sarcomas. (A) FISH analysis with an *SS18* break-apart probe, showing multiple copies of chromosome 18 carrying *SS18*. Most cells were tetrasomic or greater for the *SS18* locus on chromosome 19. In case 1, the translocation was balanced (presence of red dots), whereas in case 2 the translocation was unbalanced (the red signal is lost while the green signal remains). (B) aCGH profiles. Case 1 had a diploid genome with the loss of chromosomes 1, 3p, and 9, and parts of 11q, 17p, 18, and 22, together with gains on chromosomes 3q, 5, 6, 7, 9, 10, 11, 16, 17, 20, and 21. The region around *SS18* on chromosome 18 was focally gained, whereas the *CRTC1* locus was normal, which is in accordance with a balanced translocation. Case 2 had a tetraploid genome with the loss of one copy of chromosomes 1, 3, 13, 14, 17, 21, and 22, and with homozygous deletion of chromosome region 17q22, impacting on *MBTD1*, *UTP18*, and *CA10*. The 5′ region of *SS18* (on the minus strand) and the 3′ region of *CRTC1* (on the plus strand) were lost, in accordance with an unbalanced translocation. (C) Hierarchical clustering of RNA-seq data placed the two *CRTC1–SS18*-positive cases close to *EWSR1–CREB1*-positive tumours. (D) *NTRK1* expression from RNA-seq data. AFH/PPMS, angiomatoid fibrous histiocytoma/primary pulmonary myxoid sarcoma; CCCS, cutaneous clear cell sarcoma; CM-*CT*, cutaneous melanocytoma with *CRTC1–TRIM11* fusion; BCR, BCOR-rearranged sarcoma; CD4, *CIC–DUX4*-positive sarcoma; EwS, Ewing sarcoma.

were morphologically distinctive from control cells and had enhanced oncogenic potential.

In summary, we have presented two cases of USRCS with a novel *CRTC1–SS18* gene fusion. It would be beneficial to screen more samples to determine the frequency of *CRTC1–SS18* gene fusion in other USRCSs. The severe clinical phenotype (lung metastasis at an early age) of case 1 (case 2 has 6 months of follow-up), the novel *CRTC1–SS18* gene fusion and the expression profile data indicate that these tumours may be classified as a new type of USRCS. Except for sporadic and unique cases, to our knowledge the two USRCSs in this study are the only cases of a sarcoma type other than SS involving *SS18* as a recurrent gene fusion partner. The discovery of this new fusion should enable better classification and study of these rare sarcomas. *CRTC1–SS18*-positive sarcoma should be considered in the differential diagnosis of USRCS, DSRCT and poorly differentiated tumours that show *SS18* split signals with FISH. Elevated levels of NTRK1



Color

Figure 5. Biological analysis of the *CRTC1–SS18* gene fusion. (A) Morphological analysis of CRTC1–SS18-expressing cells. *CRTC1–SS18* fusion-positive clones were morphologically distinct from control HEK293 cells; the cytoplasmic component was small, with pronounced vacuoles and extended, thin pseudopodia (both images taken under phase contrast at ×200 magnification). (B) Expression of the CRTC1–SS18 fusion protein in HEK293 cells significantly increased anchorage-independent growth potential. The number of viable, colony-forming cells present, following incubation in soft agar, was increased 2.1-fold in HEK293 cells expressing CRTC1–SS18 as compared with HEK293 cells transfected with a control plasmid. Viable cells were determined with a fluorometric assay. $p < 0.0001$, $t = 8.61$, degrees of freedom (d.f.) = 14. Error bars represent standard error of the mean (SEM) ($n = 8$). (C) Expression of the CRTC1–SS18 fusion protein in HEK293 cells significantly increased cell migration. The number of HEK293 cells expressing CRTC1–SS18 that migrated through 8-μm pores in a Boyden chamber assay in 16 h was significantly increased as compared with HEK293 cells transfected with a control plasmid. This was apparent by inspection by microscopy (at ×4 objective magnification) and by a colourimetric assay. $p < 0.0001$, $t = 6.2.2$, d.f. = 38. Error bars show SEM ($n = 20$). (D) Expression of the CRTC1–SS18 fusion protein in HEK293 cells significantly increased cell invasive potential. The number of HEK293 cells expressing CRTC1–SS18 that invaded through 8-μm pores coated in a basement membrane matrix in 16 h was significantly increased as compared with HEK293 cells transfected with a control plasmid. Invasive cells were counted at ×40 objective magnification; the mean number of invasive cells was 2.6 times greater for CRTC1–SS18-expressing cells than for control cells. $p < 0.0001$, $t = 6.108$, d.f. = 42. Error bars show SEM ($n = 22$). OD, optical density.

in *CRTC1–SS18*-positive sarcomas may be of therapeutic importance and amenable to treatment with tyrosine kinase inhibitors [27]. Considered all together, for the field of rare to ultra-rare sarcomas, this study offers a nice example of the need to assess samples from different cancer centres to identify recurrent fusions and to be able to characterize new sarcoma subtypes. Further collaboration between groups is therefore required to depict the whole landscape of small round cell sarcomas.

Acknowledgements

This research was funded in part by the Kuwait Medical Genetics Centre (KMGC), Ministry of Health, Kuwait, the Royal Orthopaedic Hospital NHS Foundation Trust, Birmingham, UK, the Centre Léon Bérard, Lyon, France, the Institut National de la Santé et de la Recherche Médicale, the Italian Ministry of Health (Ricerca Corrente RC L2029, IRCCS Galeazzi Orthopaedic Institute), and the Department of Biomedical, Surgical and Dental Sciences, University of Milan (grant number 15-63017000-700).

Author contributions statement

The authors contributed in the following way: AA, ATB, FT, VS, FL: designed the research and analysed the data; VS, AN, CD, AMG: provided tumour samples; VS, MK: reviewed slides and the clinical information; AA, MRM, VK, SN, CA, MK, DP: performed experiments and/or analysed data; AA, ATB, MRM, MK, FT, VS, FL: wrote the paper. All authors approved the final version.

References

1. Machado I, Navarro S, Llombart-Bosch A. Ewing sarcoma and the new emerging Ewing-like sarcomas: (CIC and BCOR-rearranged-sarcomas). A systematic review. *Histol Histopathol* 2016; **31:** 1169–1181. •
2. Antonescu C. Round cell sarcomas beyond Ewing: emerging entities. *Histopathology* 2014; **64:** 26–37.
3. Pierron G, Tirode F, Lucchesi C, *et al.* A new subtype of bone sarcoma defined by BCOR–CCNB3 gene fusion. *Nat Genet* 2012; **44:** 461–466.
4. Kawamura-Saito M, Yamazaki Y, Kaneko K, *et al.* Fusion between CIC and DUX4 up-regulates PEA3 family genes in Ewing-like sarcomas with t(4;19)(q35;q13) translocation. *Hum Mol Genet* 2006; **15:** 2125–2137.
5. Antonescu CR, Owosho AA, Zhang L, *et al.* Sarcomas with CIC-rearrangements are a distinct pathologic entity with aggressive outcome: a clinicopathologic and molecular study of 115 cases. *Am J Surg Pathol* 2017; **41:** 941–949.
6. Puls F, Niblett A, Marland G, *et al.* BCOR–CCNB3 (Ewing-like) sarcoma: a clinicopathologic analysis of 10 cases, in comparison with conventional Ewing sarcoma. *Am J Surg Pathol* 2014; **38:** 1307–1318.
7. Sugita S, Arai Y, Tonooka A, *et al.* A novel CIC–FOXO4 gene fusion in undifferentiated small round cell sarcoma: a genetically distinct variant of Ewing-like sarcoma. *Am J Surg Pathol* 2014; **38:** 1571–1576.
8. Specht K, Zhang L, Sung YS, *et al.* Novel BCOR–MAML3 and ZC3H7B–BCOR gene fusions in undifferentiated small blue round cell sarcomas. *Am J Surg Pathol* 2016; **40:** 433–442.
9. Brohl AS, Solomon DA, Chang W, *et al.* The genomic landscape of the Ewing sarcoma family of tumors reveals recurrent STAG2 mutation. *PLoS Genet* 2014; **10:** e1004475.
10. Nicorici D, Satalan M, Edgren H, *et al.* FusionCatcher – a tool for finding somatic fusion genes in paired-end RNA-sequencing data. bioRxiv 2014.
11. Dobin A, Davis CA, Schlesinger F, *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013; **29:** 15–21.
12. Haas B, Dobin A, Stransky N, *et al.* STAR-Fusion: fast and accurate fusion transcript detection from RNA-seq. Aviv Regev. *bioRxiv* 2017; https://doi.org/10.1101/120295 [Epub ahead of print].
13. Ge H, Liu K, Juan T, *et al.* FusionMap: detecting fusion genes from next-generation sequencing data at base-pair resolution. *Bioinformatics* 2011; **27:** 1922–1928.
14. Bray NL, Pimentel H, Melsted P, *et al.* Near-optimal probabilistic RNA-seq quantification. *Nat Biotechnol* 2016; **34:** 525–527.
15. R Development Core Team. *R: a Language and Environment for Statistical Computing*. R Foundation for Statistical Computing: Vienna, 2017.
16. Cellier L, Perron E, Pissaloux D, *et al.* Cutaneous melanocytoma with CRTC1–TRIM11 fusion: report of 5 cases resembling clear cell sarcoma. *Am J Surg Pathol* 2017; https://doi.org/10.1097/PAS.0000000000000996 [Epub ahead of print].
17. Conkright MD, Canettieri G, Screaton R, *et al.* TORCs: transducers of regulated CREB activity. *Mol Cell* 2003; **12:** 413–423.
18. Iourgenko V, Zhang W, Mickanin C, *et al.* Identification of a family of cAMP response element-binding protein coactivators by genome-scale functional analysis in mammalian cells. *Proc Natl Acad Sci U S A* 2003; **100:** 12147–12152.
19. Nordkvist A, Gustafsson H, Juberg-Ode M, *et al.* Recurrent rearrangements of 11q14–22 in mucoepidermoid carcinoma. *Cancer Genet Cytogenet* 1994; **74:** 77–83.
20. Tonon G, Modi S, Wu L, *et al.* t(11;19)(q21;p13) translocation in mucoepidermoid carcinoma creates a novel fusion product that disrupts a Notch signaling pathway. *Nat Genet.* 2003; **33:** 208–213.
21. Enlund F, Behboudi A, Andren Y, *et al.* Altered Notch signaling resulting from expression of a WAMTP1–MAML2 gene fusion in mucoepidermoid carcinomas and benign Warthin's tumors. *Exp Cell Res* 2004; **292:** 21–28.
22. Tirado Y, Williams MD, Hanna EY, *et al.* CRTC1/MAML2 fusion transcript in high-grade mucoepidermoid carcinomas of salivary and thyroid glands and Warthin's tumors: implications for histogenesis and biologic behavior. *Genes Chromosomes Cancer* 2007; **46:** 708–715.
23. Middeljans E, Wan X, Jansen PW, *et al.* SS18 together with animal-specific factors defines human BAF-type SWI/SNF complexes. *PLoS One* 2012; **7:** e33834.
24. Clark J, Rocques PJ, Crew AJ, *et al.* Identification of novel genes, and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. *Nat Genet* 1994; **7:** 502–508.
25. Skytting B, Nilsson G, Brodin B, *et al.* A novel fusion gene, SYT–SSX4, in synovial sarcoma. *J Natl Cancer Inst* 1999; **91:** 974–975.
26. Nielsen TO, Poulin NM, Ladanyi M. Synovial sarcoma: recent discoveries as a roadmap to new avenues for therapy. *Cancer Discov* 2015; **5:** 124–134.
27. Schram AM, Chang MT, Jonsson P, *et al.* Fusions in solid tumours: diagnostic strategies, targeted therapy, and acquired resistance. *Nat Rev Clin Oncol* 2017; **14:** 735–748.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary figure legends

Figure S1. Read coverage for *CRTC1*(Ex1)-*SS18*(Ex2) and *CRTC1-SS18*(Ex3) identified in Case 1 using RNA-seq data

Figure S2. RT-PCR validation of *CRTC1-SS18* fusions and Long-Range PCR to determine genomic break points

Figure S3. Immunostaining of NTRK1 (Case 2), showing strong positivity; scale bar = 100 μm

Figure S4. Validation of CRTC1-SS18 expression in stably-transfected HEK293 clones

Table S1. The sequence of primers used for long range PCR