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# Aneurysmal bone cyst variant translocations upregulate *USP6* transcription by promoter swapping with the *ZNF9*, *COL1A1*, *TRAP150*, and *OMD* genes

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**Aneurysmal bone cysts (ABC) are locally aggressive bone tumors that often feature chromosome 17p13 rearrangements. One of the ABC 17p13 rearrangements – t(16;17)(q22;p13) – was recently shown to create a *CDH11-USP6* fusion in which the *USP6/TRE17* oncogene is overexpressed through juxtaposition with the *CDH11* promoter. Herein, we characterize four different ABC translocations involving 17p13, and we show that each is associated with a novel *USP6* fusion oncogene. Specifically, we demonstrate that t(1;17), t(3;17), t(9;17), and t(17;17) result in *USP6* fusions with *TRAP150 (thyroid receptor-associated protein 150)*, *ZNF9* (*ZiNc Finger 9*), *Osteomodulin*, *and COL1A1* (Collagen *1A1*), respectively. The oncogenic mechanism in these fusion genes is akin to *CDH11-USP6*, with the *USP6* coding sequences juxtaposed to the promoter regions in each of the four novel translocation partners. The novel fusion partners appear well suited to drive *USP6* transcription in the bone/mesenchymal context: osteomodulin is expressed strongly in osteoblastic lineages, and the *COL1A1* promoter has an oncogenic role in the mesenchymal cancer dermatofibrosarcoma protuberans. In summary, these studies show that *USP6* oncogenic activation results from heterogeneous genomic mechanisms involving *USP6* transcriptional upregulation by juxtaposition with ectopic promoters.**
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## Introduction

Aneurysmal bone cyst (ABC) is a locally aggressive bone lesion that occurs predominantly during the first two decades of life (Vergel De Dios *et al*., 1992; Rosenberg *et al*., 2002). The clonal nature of ABC is evidenced by various rearrangements of chromosome band 17p13, with the most frequent of such rearrangement being translocation t(16;17)(q22;p13) (Panoutsakopoulos *et al*., 1999; Dal Cin *et al*., 2000; Winnepenninckx *et al*., 2001; Wyatt-Ashmead *et al*., 2001; Althof *et al*., 2004; Oliveira *et al*., 2004a). Recently, we demonstrated that the t(16;17) creates a novel fusion oncogene in which the promoter of *CDH11* (Cadherin 11, or osteoblast cadherin) is juxtaposed to the entire coding sequence of *USP6* (ubiquitin-specific protease 6). *USP6* has an extremely restricted range of expression in normal cells, and the *CDH11-USP6* fusion oncogene results in unscheduled *USP6* expression in the ABC progenitor cells (Oliveira *et al*., 2004a). CDH11 is a member of the family of cadherin proteins involved in calcium-dependent cell–cell adhesion (Okazaki *et al*., 1994; Nollet *et al*., 2000) and is highly expressed in osteoblast lineage cells (Okazaki *et al*., 1994). *CDH11* contributes only regulatory sequences to the *CDH11-USP6* oncogene (Oliveira *et al*., 2004a), indicating that *CDH11* provides a highly active promoter which upregulates *USP6* transcription but without altering the USP6 protein structure.

*USP6* – also known as *TRE2* or *TRE17* – was first identified as a potential oncogene based on its transforming properties in NIH-3T3 transfection studies (Nakamura *et al*., 1988; Nakamura *et al*., 1992). USP6 contains an N-terminal TBC domain and a C-terminal ubiquitin protease domain (Paulding *et al*., 2003), and participates in Cdc42/Rac1 Rho GTPase signaling, with effects on cell adhesion and actin remodeling (Masuda-Robens *et al*., 2003).

In the present study, we determined whether variant 17p13 rearrangements in ABC are associated with alternate mechanisms of *USP6* transcriptional upregulation. We also evaluated *USP6* expression in a spectrum of tumor types in order to determine whether high levels of *USP6* expression are restricted to ABC with 17p13 cytogenetic aberrations.

## Results

### *t(1;17) fuses the* TRAP150 *promoter to the* USP6 *coding sequence*

A 7-year-old male presented with a 7 cm ABC in the right distal tibia (Table 1). Cytogenetic analysis revealed a balanced 17p13 translocation: t(1;17)(p34.1–34.3;p13). Fluorescence *in situ* hybridization (FISH) analysis confirmed the presence of *USP6* rearrangement and excluded cryptic *CDH11* locus rearrangement. 5′RACE PCR reactions identified a chimeric product composed of the noncoding exon 1 of the chromosome 1p34.3 gene *TRAP150* (*thyroid receptor-associated protein 150*) fused to a splicing variant of *USP6* exon 1 (Genbank Accession no. AY624559) (Figure 1). FISH analysis for *TRAP150* confirmed rearrangement of this gene in the t(1;17) (Figure 2a).

### *t(3;17) fuses the* ZNF9 *promoter to the entire* USP6 *coding sequence*

A 7-year-old girl presented with an ABC in the right distal tibia measuring 5.2 cm in largest diameter (Table 1). Cytogenetic analysis revealed t(3;17)(q21;p13) as the only karyotypic abnormality. FISH analysis confirmed *USP6* locus rearrangement and excluded cryptic *CDH11* locus rearrangement. 5′RACE RACE PCR reaction identified chimeric transcripts in which the ubiquitously expressed chromosome band

**Table 1** Clinical features of ABC with alternate translocations

| *Case* | *Cytogenetics* | *Age* | *Sex* | *Location* | *Size* |
|---|---|---|---|---|---|
| 1 | t(1;17)(p34.1–34.3;p13) | 7 | M | Tibia, distal | 7 |
| 2 | t(3;17)(q21;p13) | 7 | F | Tibia, distal | 5.2 |
| 3 | t(9;17)(q22;p13) | 10 | M | Clavicle | 3 |
| 4 | t(17;17)(q12;p13) | 8 | M | Shoulder[a] | 8 |

[a]Soft tissue ABC

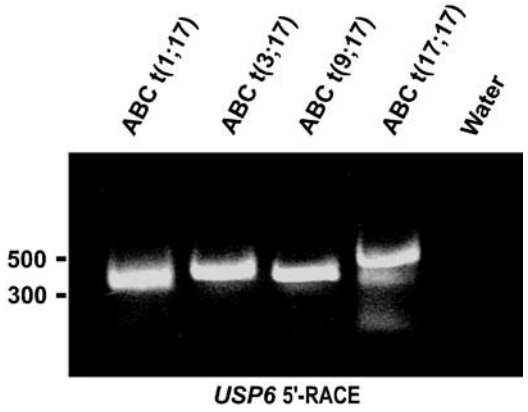


**Figure 1** *USP6* 5′ rapid amplification of cDNA ends reveals transcripts of varying sizes in each of four ABC with variant 17p13 rearrangements. Sequence analysis (see text) of each PCR product demonstrated a novel *USP6* fusion gene

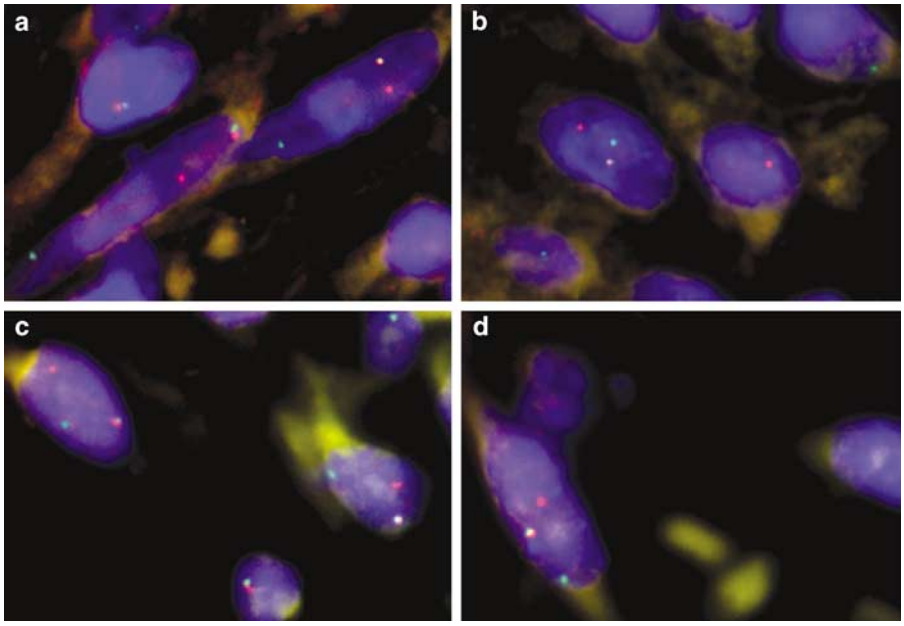


**Figure 2** Dual-color FISH demonstrates rearrangement of centromeric (green) and telomeric (red) probes flanking the *TRAP150* (**a**), *ZNF9* (**b**), *OMD* (**c**), and *COL1A1* (**d**) loci in ABC with t(1;17), t(3;17), t(9;17), and t(17;17), respectively

2q21 transcription factor gene *ZNF9* (*ZiNc Finger 9*) was fused with *USP6* (Figure 1). The fusion transcripts involved either of two splicing variants of the noncoding *ZNF9* exon 1 (Genbank Accession numbers AY624556 and AY624557) which were fused with *USP6* exon 2. *USP6* exon 2 contains the *USP6* initiation codon, and therefore the *ZNF9-USP6* fusion is predicted to result in transcriptional upregulation of the entire *USP6* coding sequence. FISH analysis for *ZNF9* rearrangement confirmed the involvement of this gene in the t(3;17) (Figure 2b). Additional FISH studies were performed to evaluate the frequency of *ZNF9-USP6* fusion in 16 ABC where previous FISH in paraffin materials had shown *USP6* rearrangement in the absence of *CDH11* rearrangement. None of these ABC had *ZNF9* rearrangement.

### *t(9;17) fuses the* OMD *promoter to the entire* USP6 *coding sequence*

A 10-year-old male presented with a 3.5 cm ABC in the right clavicle (Table 1). Cytogenetic analysis revealed the translocation t(9;17)(q22;p11–12). FISH analysis confirmed the presence of *USP6* rearrangement and excluded cryptic *CDH11* locus rearrangement. 5′RACE RACE PCR reactions identified a chimeric transcript which was composed of the noncoding exon 1 of the chromosome 9q22 gene *OMD* (*Osteomodulin*), fused to the same splicing variant of *USP6* exon 1 as described above (Genbank Accession no. AY624560) (Figure 1). FISH analysis for *OMD* confirmed rearrangement of this gene in the t(9;17) (Figure 2c).

### *t(17;17) fuses the* COL1A1 *promoter to the entire* USP6 *coding sequence*

An 8-year-old male presented with an 8 cm primary soft tissue ABC of the right shoulder (Table 1). Cytogenetic analysis revealed t(17;17)(p13;q12) as the only karyotypic abnormality. FISH analysis confirmed the presence of *USP6* rearrangement and excluded cryptic *CDH11* rearrangement. 5′RACE PCR reactions identified a chimeric transcript composed of exon 1 of the chromosome 17q21 gene *COL1A1* (Collagen *1A1*) fused to a splicing variant of *USP6* exon 1 (Genbank Accesssion no. AY624558) (Figure 1). The first 103 bp of *COL1A1* coding sequence are included in the *COL1A1-USP6* fusion transcript, but this *COL1A1*-encoded protein is a short fragment due to a stop codon in the *COL1A1* reading frame at the beginning of the *USP6*-contributed sequence. Therefore, the functional consequence of the translocation is expected to be upregulated expression of an intact USP6 protein, rather than expression of a COL1A1 fusion protein. FISH analysis for *COL1A1* rearrangement confirmed the involvement of this gene in the t(17;17) (Figure 2d). Additional FISH studies were performed to evaluate the frequency of *COL1A1-USP6* fusion in 18 ABC where previous FISH in paraffin materials had shown *USP6* rearrangement in the absence of *CDH11* rearrangement. None of these ABC had *COL1A1* rearrangement.

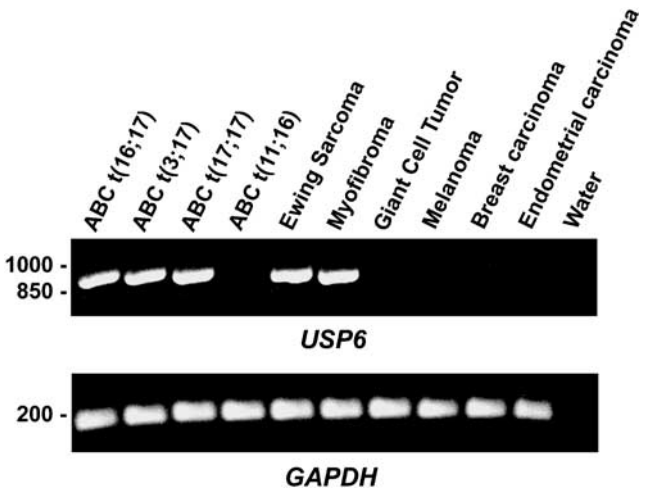


**Figure 3** *USP6* first-round RT–PCR in ABC and non-ABC tumors

### USP6 *cDNA expression profiling in ABC and non-ABC Tumors*

*USP6* cDNA expression was evaluated by RT–PCR in 13 ABCs, 12 of which had 17p13 rearrangements, and in 54 non-ABC tumors (Figure 3 and Table 2). First-round RT–PCR demonstrated *USP6* expression in 11 of the 13 ABCs (85%), and was negative only in an ABC known to lack 17p13 rearrangement and in the ABC with t(1;17)-associated *TRAP150-USP6* fusion. Second-round, nested PCR also failed to demonstrate *USP6* expression in these two ABCs, and integrity of the cDNA template was confirmed by RT–PCR amplification of the housekeeping gene *GAPDH*. In additional studies (Oliveira and Fletcher, data not shown), we determined that the *TRAP150-USP6* fusion transcript has a splicing variant which omits the sequence encoding the C-terminal end of the USP6 TBC domain, and which is the region to which the RT–PCR first-round *USP6* forward primer (*USP6* + 2726F) anneals. *USP6* expression in the t(1;17) ABC was identified readily using forward primers outside of the alternately spliced region. Notably, first-round RT–PCR demonstrated *USP6* expression in only four of 54 non-ABC tumors ($P = 0.001$), and each non-ABC with strong *USP6* expression was a neoplasm of mesenchymal derivation (Ewing sarcoma, osteoblastoma, and myofibroma). Lower levels of *USP6* expression were found in 13 non-ABC tumors after second-round, nested PCR, and were particularly evident in breast and endometrial adenocarcinoma, and in melanomas.

## Discussion

Primary ABC is characterized by frequent rearrangements of chromosomal band 17p13, most commonly in the form of t(16;17)(q22;p13) (Althof *et al*., 2004; Oliveira *et al*., 2004a). The t(16;17) fuses the highly

**Table 2** *USP6* cDNA expression in ABC and non-ABC tumors

| *Case* | *Tumor* | USP6 | | *GAPDH* |
|---|---|---|---|---|
| | | *1R* | *2R* | |
| 1 | ABC-01 – t(16;17) | + | | + |
| 2 | ABC-02 – t(16;17) | + | | + |
| 3 | ABC-03 – t(16;17) | + | | + |
| 4 | ABC-04 – t(16;17) | + | | + |
| 5 | ABC-05 – t(16;17) | + | | + |
| 6 | ABC-06 – t(16;17) | + | | + |
| 7 | ABC-07 – t(16;17) | + | | + |
| 8 | ABC-08 – t(16;17) | + | | + |
| 9 | ABC-09 – t(11;16) | – | – | + |
| 10 | ABC-10 – t(1;17) | – | – | + |
| 11 | ABC-11 – t(3;17) | + | | + |
| 12 | ABC-12 – t(9;17) | + | | + |
| 13 | ABC-13 – t(17;17) | + | | + |
| 14 | Osteosarcoma-01 | – | – | + |
| 15 | Osteosarcoma-02 | – | + | + |
| 16 | Ewing sarcoma-01 | + | | + |
| 17 | Ewing sarcoma-02 | – | + | + |
| 18 | Ewing sarcoma-03 | – | – | + |
| 19 | Giant cell tumor of bone-01 | – | – | + |
| 20 | Giant cell tumor of bone-02 | – | – | + |
| 21 | Giant cell tumor of bone-03 | – | – | + |
| 22 | Giant cell tumor of bone-04 | – | + | + |
| 23 | Osteoblastoma-01 | + | | + |
| 24 | Osteoblastoma-02 | + | | + |
| 25 | Myofibroma-01 | + | | + |
| 26 | Inflammatory myofibroblastic tumor | – | – | + |
| 27 | Congenital fibrosarcoma with t(12;15) | – | – | + |
| 28 | Pleomorphic rhabdomyosarcoma | – | + | + |
| 29 | Leiomyosarcoma-01 | – | – | + |
| 30 | Leiomyosarcoma-02 | – | – | + |
| 31 | Leiomyosarcoma-03 | – | – | + |
| 32 | Leiomyosarcoma-04 | – | – | + |
| 33 | GIST-01 | – | – | + |
| 34 | GIST-02 | – | – | + |
| 35 | Pleomorphic sarcoma-01 | – | – | + |
| 36 | Pleomorphic sarcoma-02 | – | + | + |
| 37 | Pleomorphic sarcoma-03 | – | – | + |
| 38 | Angiomyolipoma-01 | – | – | + |
| 39 | Angiomyolipoma-02 | – | + | + |
| 40 | Angiomyolipoma-03 | – | – | + |
| 41 | Angiomyolipoma-04 | – | – | + |
| 42 | Angiomyolipoma-05 | – | – | + |
| 43 | Chronic myelogenous leukemia K562 | – | + | + |
| 44 | Leukemia/lymphoma with t(8;13) | – | – | + |
| 45 | Follicular lymphoma-01 | – | – | + |
| 46 | Breast adenocarcinoma-01 | – | – | + |
| 47 | Breast adenocarcinoma-02 | – | – | + |
| 48 | Breast adenocarcinoma-03 | – | – | + |
| 49 | Breast adenocarcinoma-04 | – | – | + |
| 50 | Breast adenocarcinoma-05 | – | – | + |
| 51 | Breast adenocarcinoma-06 | – | + | + |
| 52 | Breast adenocarcinoma-07 | – | + | + |
| 53 | Breast adenocarcinoma-08 | – | + | + |
| 54 | Prostate adenocarcinoma-01 | – | – | + |
| 55 | Lung squamous cell carcinoma-01 | – | – | + |
| 56 | Lung squamous cell carcinoma-02 | – | – | + |
| 57 | Lung squamous cell carcinoma-03 | – | – | + |
| 58 | Endometrial adenocarcinoma-01 | – | – | + |
| 59 | Endometrial adenosquamous carcinoma-02 | – | + | + |
| 60 | Endometrial adenosquamous carcinoma-03 | – | + | + |
| 61 | Melanoma-01 | – | – | + |
| 62 | Melanoma-02 | – | + | + |
| 63 | Melanoma-03 | – | + | + |
| 64 | Melanoma-04 | – | – | + |
| 65 | Mesothelioma-01 | – | – | + |
| 66 | Mesothelioma-02 | – | – | + |
| 67 | Mesothelioma-03 | – | – | + |

active *CDH11* promoter to the entire *USP6* coding sequence, indicating that ABC pathogenesis can be explained in part by *USP6* transcription upregulation. In keeping with this evidence for a key oncogenic role in ABC, *USP6* has also been shown to possess transforming properties *in vitro* (Nakamura *et al*., 1992). We have reported previously that some ABC have variant cytogenetic mechanisms in which 17p13, but not the *CDH11* region at 16q22, is rearranged (Oliveira *et al*., 2004a). FISH and RT–PCR studies confirmed that these ABC had *USP6* rearrangements in the absence of *CDH11-USP6* fusion transcripts (Oliveira *et al*., 2004a). These observations suggested that *CDH11* promoter juxtaposition might be only one of many pathways for accomplishing *USP6* transcriptional upregulation. In the present study, we characterized the variant 17p13 cytogenetic and molecular rearrangements in four ABC, and we found that each of the variant 17p13 translocations was associated with an oncogenic *USP6* mechanism similar to that in the t(16;17)-mediated *CDH11-USP6* fusion. Namely, each of the four variant translocations resulted in a structurally similar fusion transcript in which the 5′ promoter region of a strongly expressed gene was juxtaposed to the *USP6* coding sequence.

Chromosomal t(1;17) resulted in fusion of the *TRAP150* to the *USP6* coding sequence. *TRAP150* encodes a 150 kDa subunit of the TRAP (thyroid receptor-associated proteins) complex and was recently cloned by Ito *et al*. (1999). TRAP150 may function as a transcriptional coactivator (Ito *et al*., 1999), and there is preliminary evidence for strong *TRAP150* expression in mesenchymal tissues, in that *TRAP150* transcripts are abundant in a human chondrosarcoma cell line (Stanford SOURCE database at http://source.stanford.edu/cgi-bin/source/sourceSearch).

The translocation t(3;17) in one ABC accomplished *USP6* overexpression through fusion with the *ZNF9* promoter. *ZNF9*, also known as cellular retroviral nucleic acid-binding protein 1 (*CNBP1*), is an ubiquitously expressed zinc-finger transcription factor involved in the regulation of the sterol metabolism (Rajavashisth *et al*., 1989). *ZNF9* expression is particularly strong in mesenchymal tissues such as cardiac muscle (Liquori *et al*., 2001). Interestingly, CCTG expansions in *ZNF9* intron 1 are found in patients with myotonic dystrophy type II (Liquori *et al*., 2001). Given that the t(3;17) breakpoint involved *ZNF9* intron 1, it is possible that a recombinational hot spot at a repetitive sequence region predisposed to formation of the *ZNF9-USP6* fusion oncogene (Kurahashi *et al*., 2000; Spiteri *et al*., 2003).

Chromosomal t(9;17) resulted in fusion of the *OMD* to the *USP6* coding sequence. Osteomodulin belongs to a family of leucine-rich repeat (LRR) proteoglycans clustered at chromosome band 9q22, which include the genes for asporin, extracellular matrix protein 2 (ECM2), and osteoglycin (Henry *et al*., 2001). Like *CDH11*, osteomodulin expression increases during osteoblastic differentiation. For example, Balint *et al*. (2003) showed induction of osteomodulin expression in

BMP-2 (bone morphogenic protein-2)-mediated osteoblast differentiation.

Translocation t(17;17) accomplished *USP6* overexpression through fusion with the *COL1A1* promoter. *COL1A1* encodes the collagen alpha-1 polypeptide chain, a major component of the collagen heterotrimeric molecule type I, which is predominantly expressed in bone, tendons, and skin. Mutations in this gene are found in osteogenesis imperfecta, Ehlers–Danlos syndrome type VIIA, and in some chondrodysplasias (Byers, 2004). In addition, polymorphisms of the Sp1 transcription factor-binding site in the promoter region of *COL1A1* are associated with increased risk for osteoporosis (Grant *et al*., 1996). Notably, *COL1A1* is a component of the *COL1A1-PDFGB* fusion oncogene created by translocation t(17;22)(q22;q13) in dermatofibrosarcoma protuberans and giant cell fibroblastoma (Simon *et al*., 1997). The *COL1A1-PDFGB* fusion is similar conceptually to the *COL1A1-USP6* described herein, in that the entire *PDFGB* gene is placed under the transcriptional control of the highly active *COL1A1* promoter, resulting in PDFGB protein overexpression and resultant activation of the PDGFRB receptor tyrosine kinase. In addition, the t(17;22) removes the *PDFGB* gene from suppressor elements upstream of the *PDFGB* breakpoint on chromosome 22 (Simon *et al*., 1997; O'Brien *et al*., 1998). In the same manner, it is possible that the *USP6* translocations in ABC accomplish *USP6* overexpression through juxtaposition with a highly active ectopic promoter (e.g. that of *COL1A1*), and also by removing the *USP6* coding sequence from the regulatory influence of its normal transcriptional control points in the *USP6* exon 1 and upstream promoter regions.

The present characterizations of four novel *USP6* fusion oncogenes have consolidated the model of transcriptional upregulation which we first established for the *CDH11-USP6* fusion in ABC (Oliveira *et al*., 2004a). Collectively, these studies show that *USP6* transcriptional upregulation is mediated by heterogeneous genomic mechanisms in ABC. The common theme in each of the 17p13 chromosomal translocations is fusion of noncoding promoter regions of highly expressed genes to the *USP6* coding sequence. Each of the five fusion partner genes (*CDH11*, *TRAP150*, *ZNF9*, *COL1A1*, and *OMD*) is highly expressed in mesenchymal lineages, and three have known roles in bone development and formation. Most ABC arise in the bone, and the expression profiles for the *USP6* fusion partners are consistent with ABC origin from a transformed progenitor cell in a mesenchymal lineage. Given that ABC have little if any mature bone, and only minor components of immature bone, the transformed progenitor cell might be prior to the point of osteoblastic commitment.

*USP6* has an extremely restricted range of expression in normal tissues, being detected predominantly in testis. By contrast, *USP6* expression has been shown to be ubiquitous in human cancers, although those studies were performed by Northern blotting (Nakamura *et al*., 1992), and might have been confounded by cross-hybridization of the *USP6* cDNA probe to the abundantly expressed *PRC17* and *USP32* genes with which *USP6* shares extremely high sequence homologies (Paulding *et al*., 2003). *USP6* is an unusual gene in that it derived – in the recent evolutionary past – from fusion of the *PRC17* and *USP32* genes. The PRC17 TBC-domain and USP32 ubiquitin-binding domain comprise the amino- and carboxy-terminal parts of USP6, respectively, and the *USP6* coding sequence is highly conserved with that of *PRC17* and *USP32*. Notably, these sequence homologies extend even to the *USP6* intronic and flanking genomic sequences and hence *USP6* gene FISH probes crosshybridize strongly with the *PRC17* and *USP32* loci on the long arm of chromosome 17 (Oliveira and Fletcher, unpublished data). In order to evaluate *USP6* expression in a specific manner, we therefore used an RT–PCR protocol in which the forward and reverse *USP6* primers were from regions derived from *PRC17* and *USP32*, respectively. Using this method, we were able to determine whether ABC with *USP6* fusion oncogenes featured high levels of *USP6* expression. *USP6* transcripts were identified after first-round RT–PCR in 11 of 12 ABC with *USP6* fusion genes, but in only four of 54 non-ABC tumors ($P<0.0001$, Table 2), supporting the hypothesis that *USP6* overexpression results from cytogenetic juxtaposition to an active, ectopic promoter. *USP6* expression was not detected in an ABC lacking *USP6* genomic rearrangement (Table 2, case 9), nor in an ABC with a t(1;17)-associated *TRAP150-USP6* fusion oncogene. In additional studies (Oliveira and Fletcher, data not shown), we determined that the *TRAP150-USP6* fusion transcript has a splicing variant which does not include the region – in the 3′ end of the TBC domain – to which the *USP6* forward RT–PCR primers were directed, therefore accounting for the false-negative *USP6* expression result. Intriguingly, the four non-ABC tumors with strong *USP6* expression were of mesenchymal differentiation and included one of three Ewing's sarcoma (with another Ewing's sarcoma showing weaker *USP6* expression), each of two osteoblastomas, and one myofibroma (Table 2). On the other hand, *USP6* expression was demonstrable – and at a low level – in only one of four giant cell tumors of the bone, which are neoplasms that can resemble ABC histologically, and which share with ABC an origin from mesenchymal neoplastic cells in the bone context. Demonstration of high-level *USP6* expression in Ewing's sarcoma is notable because historically the *USP6* oncogene was first identified by transfection assays using a genomic DNA library from a Ewing's sarcoma cell line (Nakamura *et al*., 1988). Likewise, the finding of high-level *USP6* expression in each of two osteoblastomas is notable, in that some osteoblastomas have components with ABC-like histology (so-called 'secondary' ABC) (Oliveira *et al*., 2004b). These findings suggest that *USP6* might have transforming activity beyond the ABC category of tumors. In future studies it will be worthwhile to perform a more comprehensive *USP6* expression profiling in mesenchymal tumors, and to evaluate potential mechanisms of *USP6* transcriptional

deregulation in tumors – such as osteoblastoma and some Ewing's sarcoma – which express *USP6* strongly but which lack 17p13 cytogenetic aberrations involving the *USP6* region. It will also be instructive to determine *USP6* expression in cherubism and brown tumors, which are histologically similar to ABC. The possibility of a genetic relationship between ABC and cherubism should be considered because some cherubism cases result from missense mutations within the ABL-binding, SH3-domain, protein: SH3BP2 (Ueki *et al*., 2001). The cherubism-associated SH3BP2 mutations can affect RAC signaling pathways (Miah *et al*., 2004) among others, whereas USP6 is a CDC42/RAC1 effector (Masuda-Robens *et al*., 2003).

In summary, our studies reveal that the heterogeneous 17p13 genomic rearrangements in ABC have a consistent functional consequence: namely, *USP6* overexpression resulting from juxtaposition to a promoter that is active in the mesenchymal/osteoblast cell context. The four novel fusion oncogenes reported here, and the *CDH11-USP6* oncogene reported previously, establish an oncogene model in which *USP6* overexpression might be sufficient for transforming activity. On the other hand, the *TRAP150-USP6* fusion transcript had a splicing variant, and we cannot exclude the possibility of other *USP6* splicing variants, or *USP6* point mutations, which could synergize with *USP6* overexpression to increase transforming activity in ABC. We also note that *USP6* might have a broader oncogenic role in mesenchymal neoplasia, warranting further study in Ewing's sarcoma, myofibroma, and osteoblastoma.

**Table 3** BAC clones used in FISH validations of gene rearrangement

| *Gene* | *BAC* | *BAC relationship to gene* | *BAC size (kb)* |
|---|---|---|---|
| *USP6* | CTD-2367F23 | Centromeric | 90 |
| | RP11-457I18 | Centromeric | 209 |
| | RP11-124C16 | Telomeric | 153 |
| | RP11-111I16 | Telomeric | 144 |
| | RP11-177H5 | Telomeric | 145 |
| *CDH11* | RP11-615M9 | Centromeric | 181 |
| | RP11-730A21 | Centromeric | 177 |
| | RP11-76J1 | Centromeric | 177 |
| | RP11-137A18 | Telomeric | 194 |
| | RP11-631H23 | Telomeric | 181 |
| | RP11-351A20 | Telomeric | 190 |
| *TRAP150* | RP11-1114M16 | Centromeric | 167 |
| | RP11-905I2 | Centromeric | 203 |
| | RP11-54F14 | Telomeric | 172 |
| | RP11-615M11 | Telomeric | 186 |
| *ZNF9* | RP11-1079P4 | Centromeric | 219 |
| | RP11-951F18 | Centromeric | 191 |
| | RP11-247H1 | Telomeric | 177 |
| | RP11-90I14 | Telomeric | 173 |
| *OMD* | RP11-62C3 | Centromeric | 192 |
| | RP11-148C19 | Centromeric | 167 |
| | RP11-728C20 | Telomeric | 218 |
| | RP11-19J3 | Telomeric | 168 |
| *COL1A1* | RP11-298H16 | Centromeric | 170 |
| | RP11-479I21 | Centromeric | 151 |
| | RP11-982A23 | Telomeric | 202 |
| | RP11-94C24 | Telomeric | 179 |

## Materials and methods

### *Tumor samples*

Frozen and paraffin-embedded materials from four primary ABC with alternate translocations involving the chromosome band 17p13 were studied (Table 1). The karyotypes for two of these tumors have been recently reported (Althof *et al*., 2004; Oliveira *et al*., 2004a). All cases were histologically characterized according to contemporary criteria (Rosenberg *et al*., 2002).

### *Fluorescence* in situ *hybridization (FISH)*

BAC clones flanking the *CDH11*, *USP6*, *ZNF9*, *COL1A1*, *TRAP150*, and *OMD* loci were obtained from Children's Hospital Oakland Research Institute (Oakland, CA, USA) and Research Genetics (Huntsville, AL, USA). Rearrangements of the various loci were evaluated using a split-apart approach with minicontig probes flanking both sides of each gene. The BAC addresses and insert sizes for each probe set are detailed in Table 3. DNA isolation was performed according to a previously reported protocol (Sinnett *et al*., 1998). After overnight bacterial growth, cell pellets were digested (25 mM Tris-HCL, 50 mM glucose, 10 mM EDTA, 5 mg/ml lysozyme, and 200 $\mu$g/ml RNAse), and the DNA was precipitated with 5 M potassium acetate and 100% ethanol. BAC DNA was labeled by random priming with either digoxigenin or biotin-modified nucleotides using the BioPrime® DNA Labeling System (Invitrogen, Carlsbad, CA, USA), purified by chromatography using S-200HR MicroSpin columns (Amersham Biosciences, Piscataway, NJ, USA), co-precipitated with 0.3 $\mu$g/ml glycogen, 2.5 M ammonium acetate, and two volumes of 100% ethanol, and resuspended with hybridization buffer (50% formamide, 10% dextrose sulfate, and 2 × SSC) and Cot-1 DNA (Invitrogen, Carlsbad, CA, USA).

Metaphase harvesting, slide preparation, and trypsin-Giemsa staining for cytogenetic analyses were performed as described previously (Fletcher *et al*., 1991). FISH was carried out by treating metaphase slides with 2 × SSC for 1 h at 37°C, followed by enzymatic digestion for 5 min at 37°C with Digest All-3 (Zymed, San Francisco, CA, USA). After a brief washing in 1 × PBS, the slides were treated with 10% buffered formalin for 1 min, washed in 1 × PBS, sequentially dehydrated in alcohol (70, 85, 95, and 100%), and air dried for 1 h at room temperature. Tissue sections were denaturated at 75°C for 2 min and BAC hybridization was carried out overnight in a humidified chamber at 37°C. Tissue sections were then washed in 0.5 × SSC for 5 min at 73°C and treated with CAS block (Zymed, San Francisco, CA, USA) for 10 min. Probe detection was performed using FITC-anti-digoxigenin (1 : 500) and Alexa Fluor® 594-streptavidin (1 : 500) (Molecular Probe, Eugene, OR, USA) for 30 min. Slides were then mounted in VECTASHIELD® mounting medium with 1.5 $\mu$g/ml of 4′,6-diamidino-2-phenylindole (DAPI) (Hibbard *et al*., 2000).

For paraffin-embedded material, tissue sections were deparaffinized three times in xylene for 10 min, dehydrated twice in 100% ethanol for 2 min, and treated with 100 mM Tris and 50 mM EDTA (pH = 7.0) for 15 min at 93°C. Tissue sections were then rinsed once in 1 × PBS and proteins were digested with Digest All-3 (Zymed, San Francisco, CA, USA). Probe

**Table 4** Oligonucleotide primers for *USP6* RT–PCR evaluations

| *Gene* | *Primer* | *Sequence* |
|---|---|---|
| *USP6* | *USP6* + 1747R | CTTCCGCTCCTGTGCCTGCAAACTA |
| | *USP6* + 1781R | CTCGGTGTCCCTTGTCATACTT |
| | *USP6* + 1736R | CAGGAGCGGAAGGACATACTTA |
| | *USP6* + 2162R | GGTTCCGGAGAGTCGTCCTCACGTC |
| | *USP6* + 2726F | AGGGCCTCTACGAAGAAACTAACAAGGAAGC |
| | *USP6* + 2835F | AGACCCTCTGCAAGGGGTATAG |
| | *USP6* + 3560R | GCTGAAACCCATCAAACTTGGGAGCA |
| | *USP6* + 3644R | CCACATATGGCTTTTCATGGACTCG |
| *CDH11* | *CDH11* + 83F | GTGAATGGGACCGGGACT |
| | *CDH11* + 71F | CGCCGCTGACTTGTGAAT |
| *TRAP150* | *TRAP150* + 3F | GAGGTTCGGGCTGGTTGTTC |
| | *TRAP150* + 31F | GCTGCAGCTGCGATCTCTGT |
| *ZNF9* | *ZNF9* + 81F | CAGGCAAGGACCCTCAAAATAAACAG |
| | *ZNF9* + 104F | CAGCCTCTACCTTGCGAGCCGTCTT |
| *OMD* | *OMD* + 70F | ACCCGAGTGTTTTCCAAGAAGA |
| | *OMD* + 215F | CAAATTCATCAACCCCTGAA |
| *COL1A1* | *COL1A1*−37F | CCCAGCCACAAAGAGTCTACAT |
| | *COL1A1*−4F | AGACATGTTCAGCTTTGTGGAC |

hybridization and detection was performed as described above for metaphase preparations. FISH images were captured using a liquid cooled CCD camera (Photometrics, Tucson, AZ, USA).

### *RNA isolation, RT–PCR, and 5′ RACE PCR*

RNA was isolated from frozen tissue material from 13 ABCs and 54 non-ABC tumors after mechanical homogenization and overnight incubation in Trizol® (Invitrogen, Carlsbad, CA, USA) at 4°C. RNA reverse transcription into cDNA was performed using the GeneAmp® RNA PCR Kit (Applied Biosystems, Foster City, CA, USA) for 2 h at 42°C using random hexamers. RNA isolation from paraffin sections was performed according to a previously described protocol (Argani *et al*., 1998).

PCR reactions were performed using a Takara Ex Taq® kit with the following parameters for 35 cycles: denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1 min. The PCR primers (Table 3) were *CDH11* + 83F and *USP6* + 1781R, and nested PCR was performed – when necessary – with primers *CDH11* + 71F and *USP6* + 1736R (Table 4).

RACE cDNA synthesis and PCR was performed using the BD SMART RACE cDNA amplification kit® (BD Biosciences Clontech, Palo Alto, CA, USA). Touchdown PCR for the nested 5′-RACE reactions was performed in two PCR rounds of 35 cycles each, with the following parameters: initial denaturation at 95°C for 2 min, denaturation at 94°C for 30 s and annealing/extension at 72°C for 3 min for five cycles, denaturation at 94°C for 30 s, annealing at 70°C for 30 s, and extension at 72°C for 3 min for five cycles, and denaturation at 94°C for 30 s, annealing at 68.5°C for 30 s, and extension at 72°C for 3 min for 25 cycles, followed by a final extension at 72°C for 7 min. The primers used included universal primers provided by the manufacturer, *USP6* + 1747R and *USP6* + 2162R reverse primers. *TRAP150-USP6*, *ZNF9-USP6*, *OMD-USP6* and *COL1A1-USP6* fusion transcripts were confirmed by seminested PCR reactions using either *USP6* + 1747R or *USP6* + *1781R* and the following primers: *TRAP150* + 3F and *TRAP150* + 31F, *ZNF9* + 81F and *ZNF9* + 104F, *OMD* + 70F and *OMD* + 215F, and *COL1A1*-37F and *COL1A1*-4F (Table 4).

### USP6 *cDNA expression profiling in ABC and non-ABC tumors*

In order to amplify *USP6* specifically, we designed an RT–PCR screen in which the forward primers were within the part of *USP6* derived from *PRC17*, whereas the reverse primer was within the part derived from *USP32* (see Discussion). First round RT–PCR was performed with primers *USP6* + 2726F and *USP6* + 3644R, and nested RT–PCR was performed with primers *USP6* + 2835F and *USP6* + 3560R (Table 4). PCRs were performed for 35 cycles (1st round) and 25 cycles (nested round), according to the following parameters: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min 30 s, and a final extension for 7 min.

All PCR products were gel-purified using the QIAquick® Gel Extraction kit (Qiagen, Valencia, CA, USA) and sequenced using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

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