

Two Somatic Biallelic Lesions Within and Near *SMAD4* in a Human Breast Cancer Cell Line

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Loss of chromosome arm 18q is a common event in human pancreatic, colon, and breast cancers and is often interpreted as representing loss of one or more tumor-suppressor genes. In this article, we describe two novel biallelic deletions at chromosome band 18q21.1 in a recently characterized human breast cancer cell line, HCC-1428. One lesion deletes a fragment of approximately 300 kb between *SMAD4* and *DCC* that encodes no known genes. The second lesion is an in-frame *SMAD4* deletion (amino acids 49–51) that affects the level of *SMAD4* protein but not the *SMAD4* message. This change accelerates 26S proteasome-mediated degradation of both endogenous and exogenous mutant *SMAD4*. Examination of normal DNA from the same patient demonstrated that both lesions are somatic and associated with loss of both normal alleles. These data support the concept that two independent tumor-suppressor loci exist at chromosome segment 18q21.1, one at *SMAD4* and the other potentially at an enhancer of *DCC* or an unrelated novel gene. © 2005 Wiley-Liss, Inc.

INTRODUCTION

Breast cancer was responsible for the deaths of an estimated 40,200 women in 2003 just in the United States (Ries et al., 2003). Unfortunately, many of the genetic causes of this devastating disease remain unknown. Cancer geneticists have long labored to discover the tumor suppressors and oncogenes altered in breast carcinoma. One approach to identifying novel tumor suppressors has been to characterize biallelic deletions (also known as homozygous deletions) in the genomes of cancer cell lines and xenografts. This strategy proved indispensable for the positional cloning of tumor-suppressor genes including *RBI* (Lee et al., 1987), *CDKN2A* (Kamb et al., 1994), *SMAD4* (Hahn et al., 1996b), and *PTEN* (Li et al., 1997). With the human genome project now complete, biallelic deletions should prove easier to map and candidate tumor-suppressor genes easier to identify.

Representational difference analysis (RDA) allows an investigator to perform a genomewide, unbiased screen for biallelic deletions (Lisitsyn and Wigler, 1993). RDA, a PCR-based subtraction-hybridization technique, was integral to the identification of the biallelic deletions utilized to clone the candidate tumor suppressors *PTEN* (Li

et al., 1997), *LRP1B* (Liu et al., 2000), and *BRCA2* (Schutte et al., 1995).

This study employed RDA in a screen for biallelic deletions in the genome of the recently isolated breast cancer cell line HCC-1428. RDA uncovered one novel biallelic deletion in the HCC-1428 genome, on the 18q21.1 chromosome segment. We were able to demonstrate that the biallelic deletion does not alter the open-reading frames (ORFs) of the *SMAD4* or *DCC* candidate tumor-suppressor genes, also on chromosome segment 18q21.1. We mapped the extent of the deletion, determined its length as 260–330 kbp, and localized it as being between the *SMAD4* and *DCC* loci. Sequence analysis of the *SMAD4* gene in the HCC-1428 cell line identified only an allele with a

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novel in-frame and protein destabilizing mutation whose predicted effect was to delete amino acids 49–51 of the MH1 domain. The existence of a second biallelic deletion of approximately 300 kilobase pairs (kbp) between the *SMAD4* and *DCC* genes suggests the potential to select for loss of an additional tumor-suppressor locus resident on chromosome segment 18q21.1.

MATERIALS AND METHODS

Cell Lines

Human breast carcinoma cell lines MDA-MB-468, MDA-MB-415, MDA-MB-474, MDA-MB-453, MCF7, and T-47D, human colorectal carcinoma cell line SW-480, human glioblastoma cell line U-87, and the African green monkey kidney, SV40-transformed COS-1 cell line were acquired from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured according to the distributor's instructions. Human mammary epithelial cells (HMEC) were purchased from Clonetics (San Diego, CA) and cultured per the distributor's instructions. The Gazdar laboratory isolated breast carcinoma cell lines HCC-1428, HCC-1428 BL, HCC-1937, and HCC-1143; they are now available from the ATCC (Gazdar et al., 1998).

Representational Difference Analysis

Representational difference analysis (RDA) was performed essentially as described by Lisitsyn and Wigler (1993). Specifically, the HCC-1428 genome served as the "driver genome," or template for the driver; the HCC-1428 BL genome as the "tester genome," or template for the tester. *Bgl*II restriction endonuclease (New England Biolabs, Beverly, MA) was employed to construct the driver and the tester. Our RDA methodology did differ from the original protocols in the ratio of driver to tester that was used for subtraction-hybridization. To set up the first-round subtraction-hybridization reaction, driver and tester were mixed in an 80:1 ratio (40 μ g driver:500 ng tester). For the second-round subtraction-hybridization, an 800:1 ratio was employed (40 μ g driver:50 ng tester). In the third-round subtraction-hybridization, driver and tester were mixed in a 400,000:1 ratio (40 μ g driver:100 pg tester).

Screening Products of Representational Difference Analysis of the HCC-1428 Genome for Deleted Sequences

RDA products were cloned into the pZeroII vector (Invitrogen, Carlsbad, CA) using the

*Bam*HI site, electroporated into DH10B *E. coli*. (Invitrogen), and 800 colonies were selected for sequencing. We performed all sequencing in this study on the ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). In a PCR screen for deleted fragments, the HCC-1428 BL genome was used as the template for a positive control reaction.

Polymerase Chain Reaction

All polymerase chain reactions (PCR) in this study were performed under the following conditions unless otherwise indicated: 10 μ L volume with 16.6 mM $(\text{NH})_2 \text{SO}_4$, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl_2 , 10 mM 2-mercaptoethanol, 0.25 mM dNTPs, 6% DMSO, 350 ng of each primer, and 0.5 U of Platinum Taq (GibcoBRL, Carlsbad, CA). PCR reactions were performed on 10 ng of genomic DNA. All PCR reactions were carried out on a Hybaid Omnigene Thermocycler (Thermo Electron Corporation, Waltham, MA). PCR primers used in this study are available on request.

Bacterial Artificial Chromosome Isolation and End Sequencing

The sequence-tagged site (STS) SHGC-7133 was used to PCR-screen the Human Bacterial Artificial Chromosome Release II Library (Genome Systems, St. Louis, MO). We used a NucleoBond Plasmid Kit (Clontech, Palo Alto, CA) to isolate bacterial artificial chromosomes (BACs) 494J9 and 471M4. SP6 and T7 primers were used to sequence BAC ends.

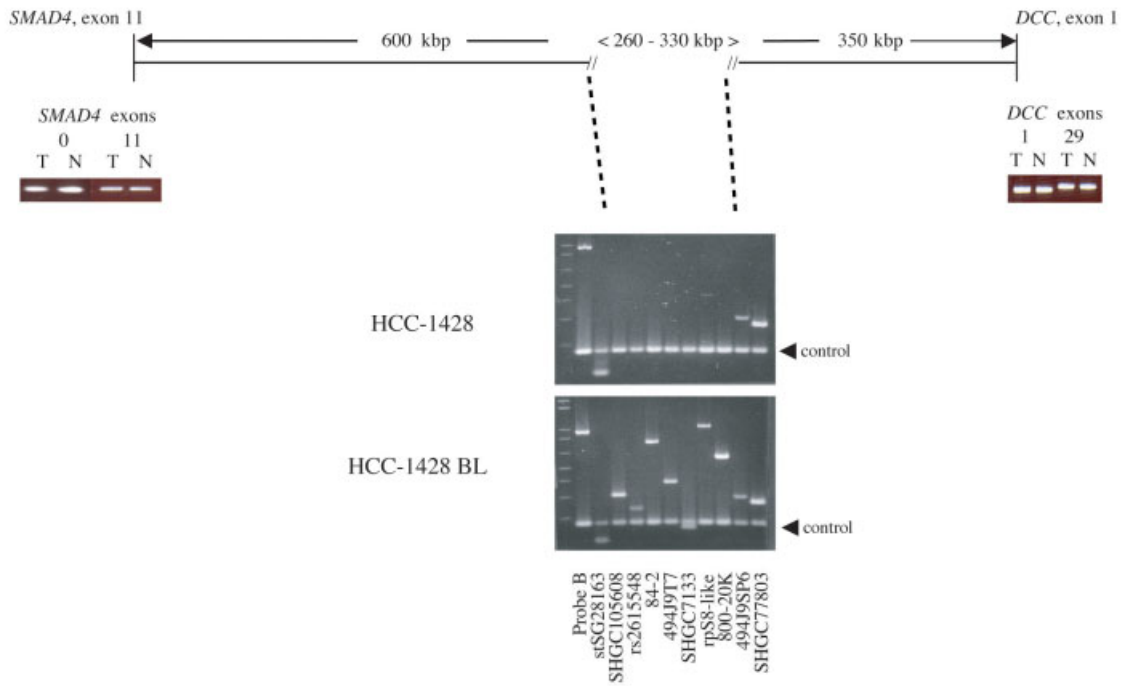
Sequence-Tagged Site Mapping

PCR conditions and primers for sequence-tagged sites used in this study are available on request as are STS coordinates on the NCBI chromosome 18 contig (accession no. NT_010966.13, October 17, 2003, version). PCR products of novel STSs probe B, 84-2, 494J9-T7, rpS8-like EST, 800-20K, and 494J9-SP6 were sequenced directly in order to confirm their identities. The control PCR fragment was from chromosome 2, NCBI accession number AC012305.7, coordinates 78,323–78,502.

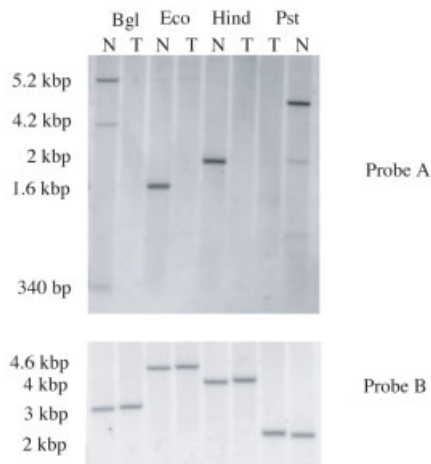
Single-Nucleotide Polymorphism Analysis

Candidate single-nucleotide polymorphisms (SNPs) in the deleted region were identified with the NCBI dbSNP database and PCR-amplified from the HCC-1428 BL genome. Sequence analysis of candidate SNP PCR products demonstrated

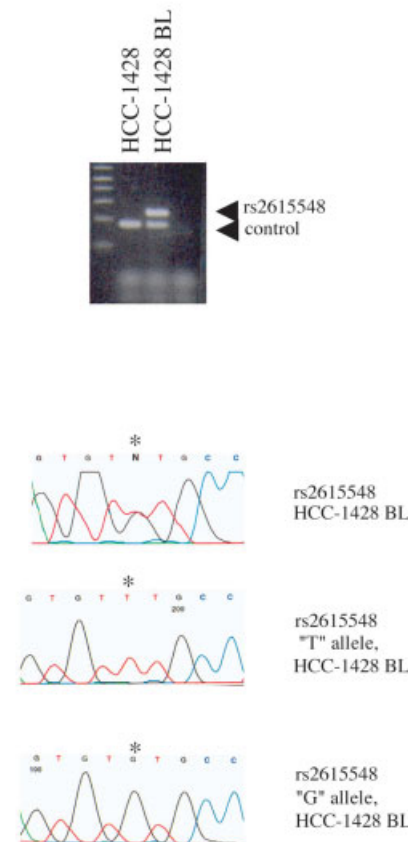
A)



B)



C)



that rs2615548 was present in two variants in the HCC-1428 BL genome. An ambiguous nucleotide (peaks at both G and T), referred to as N, was detected at the predicted rs2615548 SNP position. To confirm that both the G and T alleles were amplified from the HCC-1428 BL genome, this PCR product was cloned into the pZeroII vector, and individual clones were sequenced. The G and T alleles were detected with equal frequency. Results were confirmed with a second rs2615548 SNP PCR amplification from the HCC-1428 BL genome and subsequent product sequencing. The control PCR fragment also was from chromosome 2, NCBI accession #AC012305.7, coordinates 78,323–78,502.

Southern Blot Analysis

For Southern blot analysis, restriction-digested DNA was electrophoresed through a 0.8% agarose gel and transferred to Zeta-Probe GT membranes (Bio-Rad, Hercules, CA). Probes were labeled with ^{32}P dCTP by the random hexamer method (Feinberg and Vogelstein, 1983). Probe hybridization to membrane was carried out overnight at 68°C in 10 mL of ExpressHyb (Clontech); membranes were then washed for 15 min at 6°C in 0.3× SSC/0.1% SDS, and the results were visualized with the Storm 840 phosphoimage system (Molecular Dynamics, Sunnyvale, CA). Probe A was derived from the NCBI chromosome 18 contig, accession #NT_010966.13, coordinates 30,787,107–30,786,164. Probe B also was derived from the NT_010966.13 contig, coordinates 30,682,148–30,681,194. The cytogenetic locations of probes A and B relative to *BID-CHR18* are indicated in Figure 1A. Both probe A and probe B were synthesized by PCR and sequenced to confirm their identities.

Northern Blot Analysis

Total RNA was isolated from the indicated cell lines according to the cesium chloride/guanidium isothiocyanate method (Sambrook et al., 1989). RNA was resolved on a 1× MOPS gel by electrophoresis and transferred onto a NYTran membrane (Schleicher and Schuell BioScience, GmbH, Dassel, Germany). DNA probes were labeled with ^{32}P

dCTP by the random hexamer priming method. Blots were hybridized at 42°C in UltraHyb (Ambion, Austin, TX). The membrane was washed in 0.1× SSC/0.1% SDS at 60°C and exposed to film at –70°C using intensifying screens. After SMAD4 hybridization and exposure, the membrane was stripped by boiling for 30 sec in 0.1× SSC/0.1% SDS before being hybridized with the beta actin probe. The SMAD4 probe was constructed by *EcoRI* and *PstI* restriction digest of the SMAD4-FLAG plasmid and isolation of the 300-bp fragment at the 3' end of the *SMAD4* ORF. The beta actin probe was described previously (Wu et al., 1999).

Reverse Transcription Polymerase Chain Reaction

cDNA was synthesized from 5 µg of the whole RNA template with random primers and SuperScript II RNase H⁻ Reverse Transcriptase (GibcoBRL) according to the manufacturer's instructions. Human breast RNA was acquired from Invitrogen, human fetal brain RNA from Stratagene (La Jolla, CA). Professor V. Band, of the Radiation Oncology Department of New England Medical Center, provided RNA from the human mammary epithelial cell cultures 70N and 76N and the transformed breast cell cultures 70E6 and 76E6 (Wazer et al., 1995). PCR primers hed1R and hed3F amplify a cDNA product spanning exons 2–4 of the DCC transcript. Cyclophilin A was chosen as a housekeeper control, as a recent report indicated it is present at low copy number in both normal and cancerous breast tissue (Tricarico et al., 2002). Primers CYCA-F1 and CYCA-R1 amplify a cDNA product that includes exons 1–5. The cyclophilin A and DCC reverse transcription polymerase chain reaction (RT-PCR) products were sequenced to confirm their identities.

Western Blot Analysis, Immunoprecipitation, and Antibodies

Protein lysates (25 µg) were resolved with 4%–20% Tris-glycine gels (Invitrogen) and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). For immunoprecipitation with M2-FLAG antibody agarose beads (Sigma-Aldrich, St. Louis, MO), the cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10%

Figure 1. The HCC-1428 genome has a biallelic deletion of 260–330 kbp between tumor-suppressor genes *SMAD4* and *DCC*. (A) PCR sequence-tagged site (STS) map of the 260–330 kbp *BID-CHR18* deletion (human chromosome 18 centromere to the left, 18q telomere to the right; control PCR fragment is from chromosome 2; accession no. AC012305.7, coordinates 78,323–78,502; T, HCC-1428; N, HCC-1428 BL). (B) Southern blot analysis confirms *BID-CHR18* deletion. Probe A

spans STS 84-2. The location of probe B relative to *BID-CHR18* is indicated in panel A (Bgl, BglII; Eco, EcoRI; Hind, HindIII; Pst, PstI). (C) Sequence analysis demonstrates two alleles of single-nucleotide polymorphism rs2615548 in HCC-1428 BL genome, both deleted from the HCC-1428 genome. The control PCR fragment is identical to the panel A control.

glycerol, 1% triton, 2 mM EDTA protease inhibitor cocktail set I (CalBiochem, San Diego, CA), 1 mM Na₃VO₄, and 40 mM NaF. The beads were precipitated, resuspended in Laemmli sample buffer, and subjected to immunoblot analysis. The primary antibodies used in this study were mouse monoclonal:B-8, for Smad4 (Santa Cruz Biotechnology, Santa Cruz, CA); TU02, for tubulin (Santa Cruz Biotechnology); M2-FLAG, for FLAG (Sigma-Aldrich); JL-8, for GFP (Clontech); and Hvin1, for vinculin (Sigma-Aldrich).

Plasmids

Full-length (normal) SMAD4 and $\Delta(49-51)$ -SMAD4 cDNAs, from MCF7 and HCC-1428 cDNAs, respectively, were cloned into the C-terminal FLAG expression vector pCMVTag4A (NCBI accession no. AF073000; Stratagene). RT-PCR was performed with Pfx Platinum Polymerase (Invitrogen) according to the manufacturer's instructions. Thorough sequence analysis of Smad4-FLAG and $\Delta(49-51)$ -Smad4-FLAG confirmed their identities.

Pulse Chase Analysis

COS-1 cells at 50% confluence were transfected with either the SMAD4-FLAG or the $\Delta(49-51)$ -SMAD4-FLAG plasmids. For transfection, 5 μ g of plasmid and 60 μ L of lipofectamine (Invitrogen) per 75 cm² flask were used; each 75-cm² flask of cells represented a time point in the pulse-chase experiment. Forty-eight hours posttransfection, the cells were placed in L-methionine- and L-cystine-free DMEM and 5% dialyzed fetal bovine serum with ³⁵S-labeled L-methionine and L-cystine at 180 μ Ci/mL for 2 hr at 37°C/5% CO₂ (Amersham Biosciences, Piscataway, NJ). Chase with cold L-methionine and L-cystine was carried out for the specified periods. Sequential immunoprecipitation with M2-FLAG antibody agarose beads was performed twice before the labeled protein was resolved on a 4%–20% Tris-glycine gel according to standard protocols (Struhl, 2001). After drying, the gels were placed between 6- μ m-thick single layers of Standard Mylar (Somar International Inc., Sparks, NV), exposed for 5–7 days to the LE storage phosphorimage screen (Amersham Biosciences), and the images processed with the Storm 840 system.

Proteasome Inhibitors

MG-132 (Z-Leu-Leu-Leu-al) was purchased from Sigma-Aldrich. PS-341 (also known as Bortezomib or Velcade) was a gift of Millennium Pharmaceuticals (Cambridge, MA).

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) was performed by standard methods on metaphase cells prepared from the HCC-1428 cell line. A Spectrum Orange-labeled CEP18 centromeric probe and a SpectrumGreen-labeled chromosome 18 paint probe (both obtained from Vysis, Downers Grove, IL) was used in dual-color FISH. Fluorescence signals were captured after a DAPI counterstain on an Applied Imaging Cytovision Imaging system attached to a Nikon Eclipse 600 microscope.

RESULTS

Representational Difference Analysis Identified a Novel Biallelic Deletion in the HCC-1428 Genome

The HCC-1428 RDA library provided 740 readable sequences, 62 of which (8.4%) represented sequences present in the HCC-1428 BL genome but absent in the HCC-1428 genome (Table 1). One sequence, 84-2, perfectly matched NCBI accession number AC027216.6, at chromosome band 18q2.1, coordinates 49,580–50,359. Southern blot analysis confirmed deletion of this sequence from the HCC-1428 genome (Fig. 1B).

To determine whether this novel deletion represented the somatic loss of two alleles, sequence analysis of candidate SNPs was performed. Sequence analysis of the HCC-1428 BL (paired normal) PCR products of candidate SNP rs2615548 revealed two alleles, G and T. Candidate SNP rs2615548 did not amplify from the HCC-1428 genome (Fig. 1C). The deleted locus was named biallelic deletion of chromosome 18, *BID-CHR18*.

BID-CHR18 Spans 260–330 Kilobase Pairs and Is Between Candidate Tumor-Suppressor Genes *SMAD4* and *DCC*

BLAST searches of the NCBI HTGS and NR databases mapped *BID-CHR18* to NCBI accession number AC027216.6, at chromosome band 18q21.1. PCR radiation hybrid mapping of the 84-2 fragment with the Stanford G3 Human/Hamster RH Panel also localized the deletion to 18q21.1, within 5 cM, or about 100 kbp, of STS SHGC-7133. A physical map of the deletion was constructed with publicly available STSs and the NCBI HTGS and NR databases. The centromeric end of the deletion was determined to within 40 kbp. SHGC-105608, at AC027216.6, coordinates 109,631–109,933, did not PCR-amplify from the HCC-1428 genome, whereas stSG28163, at

TABLE 1. Results of Representational Difference Analysis (RDA) of Genome of Breast Cancer Cell Line HCC-1428

Accession No. Coordinates (Gene)	Fragments Detected by RDA Process (% of total)
AF152363, 164,476-614,775 (FRA3B)	15 (2%)
HS4B958RAJ, 87,072-87,423 (EBV)	18 (2.4%)
AC027216.6, 49,960-50,298 (#84)	29 (3.9%)
All fragments not present in the HCC-1428 genome	62 (8.4%)
Fragments detected by RDA process	740 (100%)

AC027216.6, coordinates 150,505–150,627, did amplify from the HCC-1428 genome (Fig. 1A).

To map the telomeric extent of the deletion, we performed a PCR screen of a human BAC library for SHGC-7133 isolated BAC 494J9. STS 494J9 SP6, present in the HCC-1428 genome, maps to NCBI accession number AC105032.4, coordinates 22,666–22,965. Overlap of AC105032.4 with NCBI accession number AC080051.6 and a novel STS we generated from the latter, 800–20K, localized the telomeric extent of *BID-CHR18* to 27 kbp between 800–20 K and 494J9SP6 (Fig. 1A). Ultimately, sequence information from NCBI accession numbers AC027216.6, AC022701.6, and AC080051.6, as well as from the UCSC human genome server, July 2003 freeze, allowed us to conclude that *BID-CHR18* is 260–330 kbp in size and between candidate tumor suppressors *SMAD4* and *DCC* (Fig. 1A).

We performed a PCR screen of STSs SHGC-7133 and 84-2 on a panel of 40 breast cancer cell line genomes and 40 breast cancer xenograft genomes. Both SHGC-7133 and the 84-2 fragment amplified in all 80 genomes (results not shown).

Bioinformatic Analysis of *BID-CHR18* Revealed No Known Genes

BID-CHR18 is unremarkable, save for its paucity of known coding sequence. No known genes or spliced expressed sequence tags (ESTs) are contained in *BID-CHR18* as assessed by BLAST searches and analysis with the UCSC human genome browser, April 2003 freeze. *BID-CHR18* has no known microRNA genes. We performed Paracel BLAST (Paracel Inc., Pasadena, CA) of *BID-CHR18* (using accession nos. AC027216.6, AC022701.6, and AC080051.6) versus 1,138 candidate chromosome 18 exons found by trap analysis (Chen et al., 2003). This approach identified three putative exons, NCBI accession nos. BH608849, BH608723, and BH608687, within *BID-CHR18*.

However, repeated exon connection RT-PCR reactions on human fetal brain, fetal testis, and breast cDNA templates failed to amplify the spliced transcripts containing these sequences.

BID-CHR18 contains an unexceptional number of human–mouse orthologous regions. *BID-CHR18* (April 2003 UCSC freeze) was compared to the corresponding mouse region (February 2003 UCSC freeze) with the VISTA 2.0 browser. This approach revealed 51 regions of 50 bp or more that had more than 90% nucleotide identity between human and mouse. All human–mouse hits were checked for synteny and best possible matches. The similar regions covered a combined 4,998 bp, or 1.5% of the maximum possible (~330 kbp) *BID-CHR18*. This is consistent with the findings of the Mouse Genome Sequencing Consortium, which concluded that roughly 2.3% of the human genome is conserved in the mouse in small segments (≤ 50 bp) that are not coding sequences, 5' UTRs, 3' UTRs, or repeats (Waterston et al., 2002). There is synteny of the *SMAD4-(BID-CHR18)-DCC* arrangement between humans and mice. However, this also was expected because 90% of the human genome shows synteny with the mouse genome (Waterston et al., 2002).

RepeatMasker analysis of the *BID-CHR18* demonstrated that 49% of the deleted sequence represents interspersed repeats. Specifically, *BID-CHR18* consists of 7.5% SINEs, 27.3% LINEs, 11.3% LTR elements, and 3.4% DNA transposon elements. These proportions are very similar to the average number of human genome-wide interspersed repeats: 44% combined, 13% SINE sequences, 20% LINE sequences, 8% LTR sequences, and 3% DNA transposon elements (Lander et al., 2001).

BID-CHR18 Is in the 5' Extension Intergenic Region of *DCC*

The *BID-CHR18* deletion does not extend into the *DCC* ORF of the HCC-1428 genome (Fig. 1A). At ~300 kbp, the apparently gene-barren *BID-CHR18* constitutes part of a large intergenic region upstream of *DCC*. The 350 kbp between the first exon of *DCC* and *BID-CHR18* lack any known genes or spliced ESTs, meaning that *DCC* has a 5' extension intergenic region of at least 700 kbp (distance from the 5' end to the nearest upstream gene). In contrast, a recent analysis of 5' extension intergenic regions found medians of 46 and 18 kbp for chromosomes 21 and 22, respectively (Chen et al., 2002).

RT-PCR analysis detected *DCC* transcripts in human fetal brain and adult breast cDNAs;

sequence analysis of these PCR products confirmed their identity. The *DCC* cDNA did not amplify from the HCC-1428 cDNA, nor did it amplify from nontransformed, cultured breast epithelial cells HMEC, 70N, and 76N cDNAs, possibly because of dedifferentiation of breast cells in culture (Fig. 2F). Western blot analysis of the DCC protein failed to detect it in the HCC-1428 or other breast cancer cell lines or cultures (results not shown).

The HCC-1428 18q21.1 Band Also Contains a Biallelic Deletion at *SMAD4*

Northern blot analysis demonstrated that the *SMAD4* message was not disrupted in the HCC-1428 cell line (Fig. 2D). However, sequence analysis of *SMAD4* in the HCC-1428 genome revealed only an allele with a 9-bp deletion of exon 1 ablating amino acids 49, 50, and 51 (Fig. 2C). Sequence analysis of the entire ORF, 5' UTR, and 3' UTR of the HCC-1428 *SMAD4* gene uncovered no additional mutations. The mutation was confirmed in the *SMAD4* transcript of the HCC-1428 cell line. The normal *SMAD4* exon 1 sequence did not amplify from the HCC-1428 genome, indicating that both normal copies of *SMAD4* exon 1 had been lost from the HCC-1428 genome (Fig. 2B). In addition, Western blot analysis demonstrated that endogenous mutant SMAD4 protein was almost undetectable in the HCC-1428 proteome (Fig. 2E). This *SMAD4* mutation was named, in line with the guidelines of the HUGO Mutation Database Initiative in genomic DNA, cDNA, and protein contexts, g.144_152del, c.144_152del, and p.Glu49_Lys51del, respectively (den Dunnen and Antonarakis, 2000), referred to henceforth as $\Delta(49-51)$ -SMAD4.

$\Delta(49-51)$ Mutation Enhanced SMAD4 Protein Degradation

Western blot analysis demonstrated that endogenous $\Delta(49-51)$ -SMAD4 protein was almost undetectable in the HCC-1428 proteome (Fig. 2E). To explore the effect of $\Delta(49-51)$ on the steady-state level of SMAD4 protein, we transfected normal SMAD4-FLAG and $\Delta(49-51)$ -SMAD4-FLAG constructs into COS-1 cells. Immunoprecipitation was needed to detect the mutant SMAD4-FLAG protein, which was found to have a much lower steady-state level than did normal SMAD4-FLAG (Fig. 3A). To determine whether $\Delta(49-51)$ decreased SMAD4 protein synthesis or stability, we transfected normal SMAD4-FLAG and $\Delta(49-51)$ -SMAD4-FLAG plasmids into COS-1 cells and performed pulse chase analysis. A 2-hr pulse labels

similar amounts of normal and mutant protein. In this assay, labeled SMAD4-FLAG was stable for 18 hr, but $\Delta(49-51)$ -SMAD4-FLAG had a half-life of less than 4 hr (Fig. 3B).

Proteasome Inhibition Increased the Level of Exogenous and Endogenous $\Delta(49-51)$ -SMAD4 in HCC-1428 Cells

To characterize the mechanism of the enhanced degradation of $\Delta(49-51)$, COS-1 cells transfected with normal SMAD4-FLAG and $\Delta(49-51)$ -SMAD4-FLAG were incubated with MG-132, a 26S proteasome inhibitor. A concentration of 50 μ M was needed to observe even modest stabilization at 4 hr (Fig. 3A). At this concentration, MG-132 is toxic to COS-1 cells, leading to a dramatic decrease in normal SMAD4-FLAG after more than 4 hr (not shown). However, 24-hr treatment of HCC-1428 cells with the proteasome inhibitors MG-132 (10 μ M) or PS-341 (100 nM) caused a dramatic increase in the level of endogenous $\Delta(49-51)$ -SMAD4 protein (Fig. 3C).

Chromosome Paint Analysis Demonstrated an Unbalanced Translocation of Chromosome Arm 18q in HCC-1428 Cells

FISH, using orange chromosome 18 centromeric and green chromosome 18 paint probes, was performed on HCC-1428 to determine the structure of chromosome 18. The analysis of HCC-1428 cells identified one intact chromosome 18 in all cells and an unbalanced chromosome 18 translocation, with the proximal half of 18q detected, making a net loss of one copy of distal 18q21 material (Fig. 4). Therefore, only one allele of chromosome 18 is present in the *SMAD4*-*BID*-*CHR18*-*DCC* interval.

DISCUSSION

Frequent loss of specific chromosome arms often is interpreted as representing loss of canonical, genetic two-hit tumor-suppressor genes. Potential candidate tumor-suppressor genes for human chromosome arm 18q include *SMAD4* and *DCC*. However, neither *SMAD4* nor *DCC* has a frequency of mutation of both alleles approaching that of the observed LOH and CGH in breast cancer. The rate of loss of both *SMAD4* alleles, as judged by immunohistochemistry, is 2% in sporadic breast cancer (Xie et al., 2002). Only one case of mutation of both *SMAD4* alleles has been reported previously in breast cancer (Schutte et al., 1996). A two-hit lesion of *DCC* has never been documented in breast cancer.

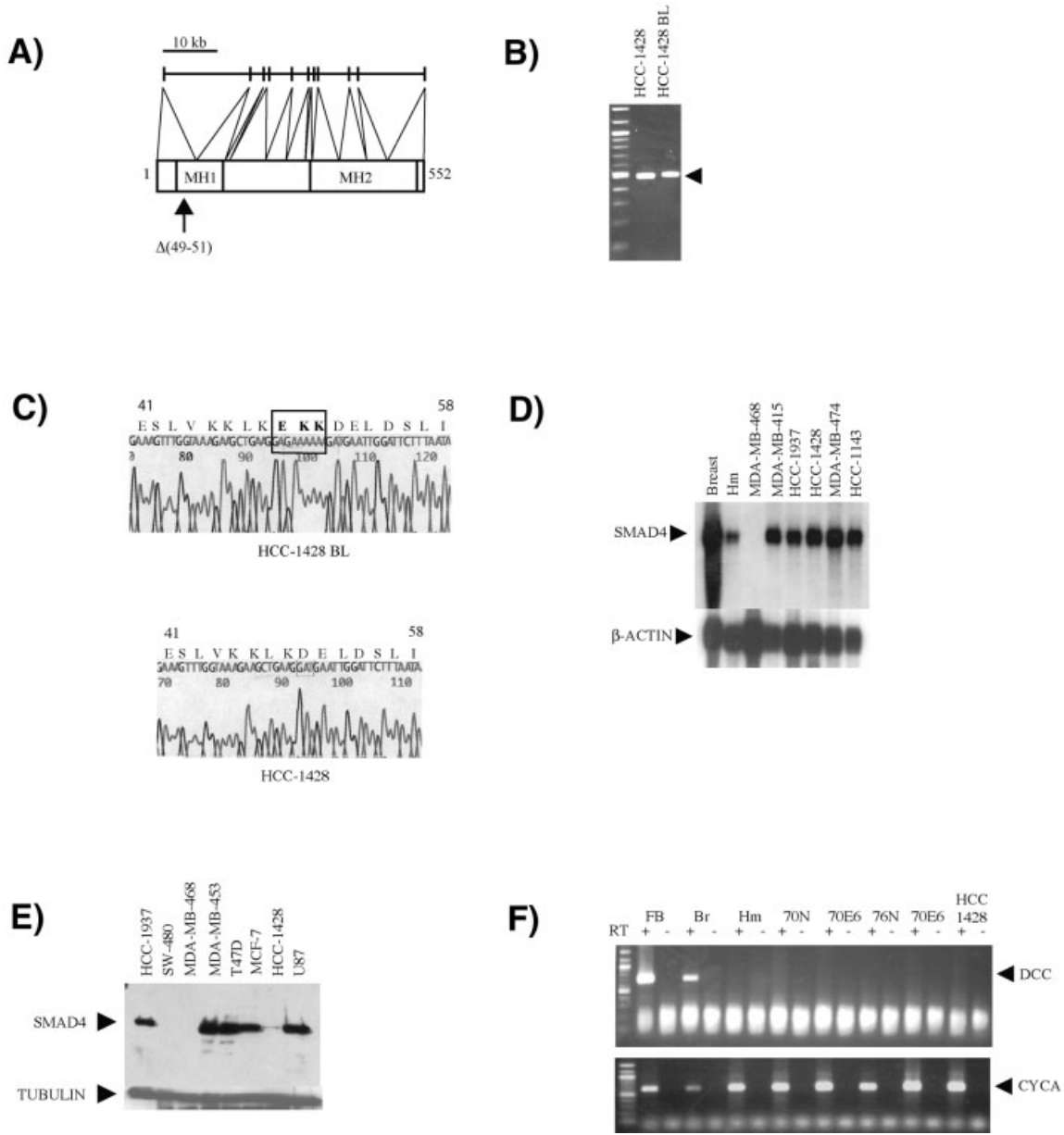


Figure 2. The HCC-1428 *SMAD4* locus contains a 9-bp deletion mutation in exon I. (A) Schematic diagram of *SMAD4* gene [above, exon/intron structure; below, position of $\Delta(49-51)$ mutation relative to domains of *SMAD4*; MH1, mad homology 1 domain, responsible for sequence-specific DNA binding; MH2, mad homology 2 domain, involved in heteromerization and transactivation (Miyaki and Kuroki, 2003); amino and carboxy terminal residues indicated as 1 and 552, respectively]. (B) PCR of *SMAD4* exon I from HCC-1428 genome amplifies only a truncated product. (C) Sequence analysis reveals a 9-bp deletion from exon I of *SMAD4* of HCC-1428 (nucleotides deleted from HCC-1428 BL genome in box, deleted amino acids in boldface).

(D) Northern blot analysis of *SMAD4* transcript demonstrates normal level in HCC-1428 cells compared to human breast tissue and other breast cancer cell lines (breast, whole RNA from human breast tissue; Hm, human mammary epithelial cell culture; MDA-MB-468, negative control). (E) Western blot analysis of human cancer cell lines shows greatly attenuated *SMAD4* protein in HCC-1428 cells (SW-480 and MDA-MB-468, negative controls). (F) RT-PCR does not amplify *DCC* cDNA from HCC-1428 or cultured human breast cells (FB, fetal brain cDNA template; Br, human breast tissue; Hm, human mammary epithelial cell culture).

The relevance of *DCC* to tumor suppression is a topic of much controversy. *DCC* was first identified as a candidate tumor-suppressor gene by virtue of its position in a biallelic deletion in a colon tumor genome (Fearon et al., 1990). However, character-

ization of biallelic deletions of the *DCC* locus in cancer demonstrated that these lesions were always intergenic, never limited to the *DCC* gene itself. Indeed, some reported *DCC* deletions include the *BID-CHR18* locus (Hilgers et al.,

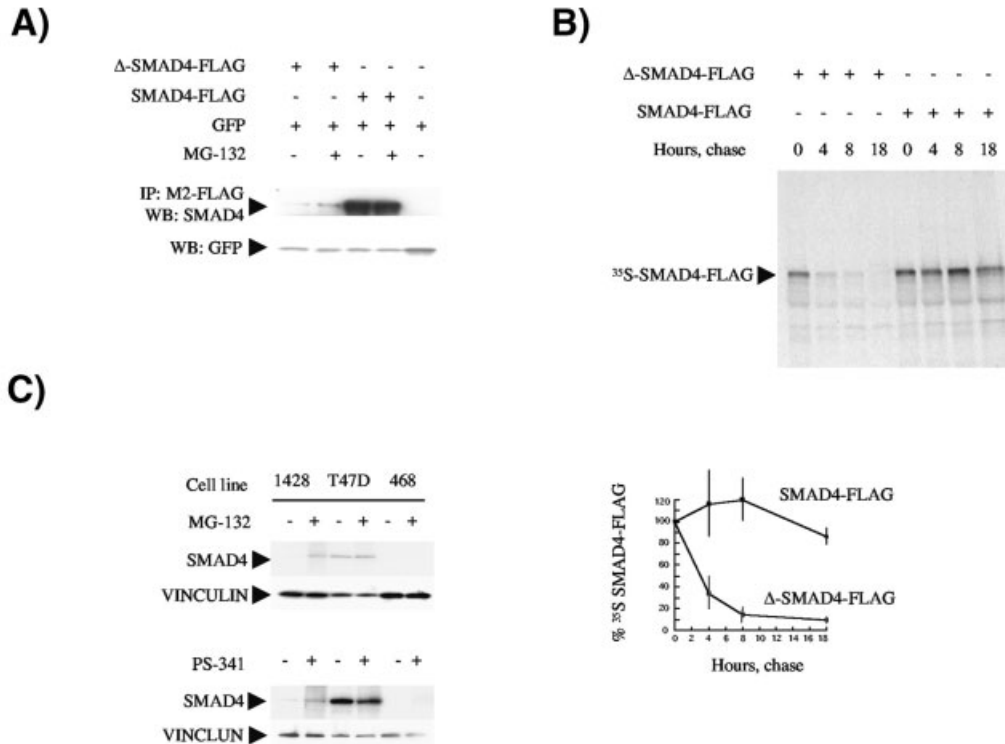


Figure 3. The $\Delta(49-51)$ mutation accelerates 26S proteasome-mediated degradation. (A) Western blot analysis showing attenuated expression of $\Delta(49-51)$ -SMAD4-FLAG versus SMAD4-FLAG in COS-1 cells [Δ -SMAD4-FLAG, $\Delta(49-51)$ -SMAD4-FLAG; MG-132, 50 μ M MG-132, 4 hr]. Experiment was performed 3 times. (B) Pulse chase analysis demonstrating accelerated degradation of $\Delta(49-51)$ -SMAD4-FLAG versus SMAD4-FLAG in COS-1 cells [above, representative pulse chase experiment; below, vertical axis; 35 S SMAD4-FLAG as percentage of

signal at 0 hr; chase, average of 3 independent experiments; vertical bars, standard error at each time point; Δ -SMAD4-FLAG, $\Delta(49-51)$ -SMAD4-FLAG]. (C) Inhibition of the 26S proteasome rescues SMAD4 protein levels in HCC-1428, T47D (unmutated SMAD4), MDA-MB-428 cells (SMAD4 biallelically deleted) (MG-132, 10 μ M MG-132, 24 hr; PS-341, PS-341, 24 hr).

2000). No convincing somatic missense, nonsense, frameshift, or in-frame deletion mutations of the *DCC* ORF have been reported in the literature (Cho et al., 1994; Miyake et al., 1994). Inherited mutation of *DCC* also has been excluded as predisposing to hereditary colon cancer (Peltomaki et al., 1991). Finally, transgenic mice heterozygous for a *DCC* knockout allele are not predisposed to colon cancer (Fazeli et al., 1997). Nonetheless, immunohistochemical studies indicated that DCC protein is absent in a subset of a variety of cancers including breast cancer (Koren et al., 2003). The presence of DCC protein has been shown to be a positive prognostic factor in stage II and stage III colorectal carcinoma (Shibata et al., 1996), and overexpression of Netrin-1, a DCC ligand, stimulates the formation of adenocarcinoma in *Apc* mutant mice (Mazelin et al., 2004).

In contrast to *DCC*, *SMAD4* has been established as a canonical two-hit tumor-suppressor gene. *SMAD4* has been shown to be biallelically deleted from a variety of pancreatic carcinoma cell line and

xenograft genomes. Many of these deletions are intragenic, eliminating only the *SMAD4* gene (Hahn et al., 1996b). Many somatic missense, nonsense, and frameshift mutations have been reported to occur in the *SMAD4* ORF in pancreatic and colon carcinoma without concurrent amplification of normal the *SMAD4* ORF sequence (Iacobuzio-Donahue et al., 2004). Inherited mutated alleles of *SMAD4* are responsible for 50% of juvenile polyposis syndrome cases (Howe et al., 1998). *SMAD4* protein has not been detected in polyps that develop in carriers of *SMAD4* mutations, indicating loss of the normal *SMAD4* allele (Woodford-Richens et al., 2001). Mice heterozygous for a *SMAD4* knockout allele are predisposed to gastric polyposis, with loss of the normal *SMAD4* allele in subsequent carcinomas (Xu et al., 2000). The *SMAD4* protein probably acts as a tumor suppressor through its signal transduction role in the TGF β pathway (Massague et al., 2000).

The novel deletions described in this report are true two-hit lesions. The normal paired genome

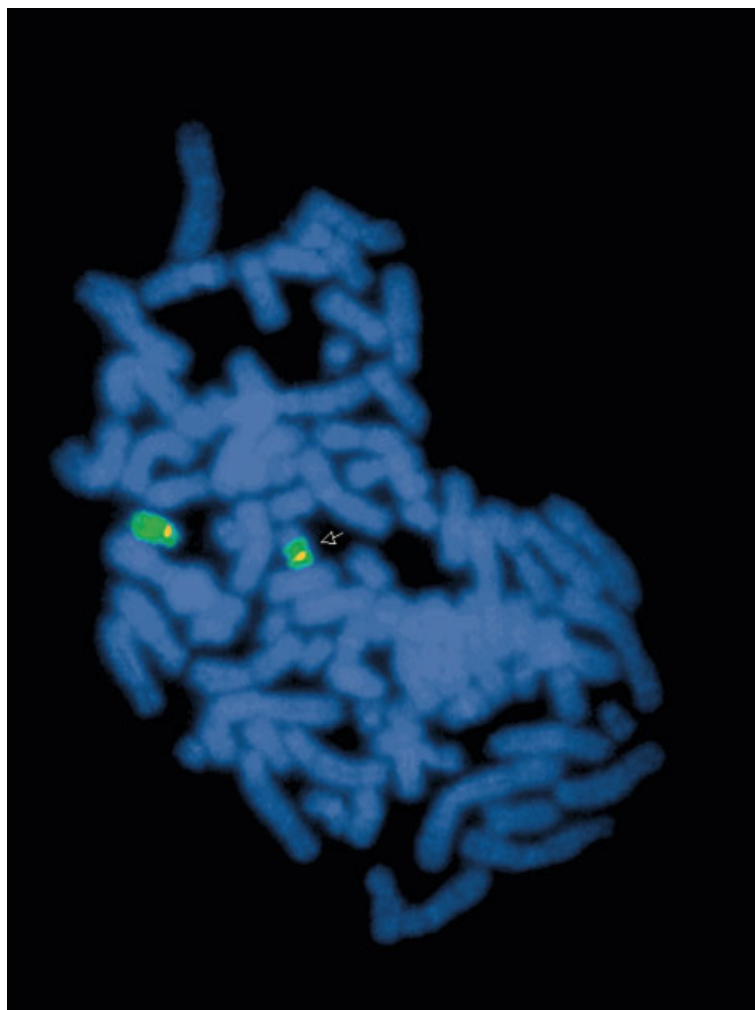


Figure 4. Identification of chromosomal deletion of distal 18q in HCC-1428. A metaphase with DAPI counterstain showing two centromeric signals of chromosome 18 (Orange) and chromosome 18 painting (Green). White arrow indicates the unbalanced chromosome 18 translocation with deletion of distal of 18q.

possesses two separate alleles at each deleted locus; both were lost en route to oncogenesis. Analysis of deletion maps from previous reports of chromosome arm 18q deletion in human cancer found complete (presumably biallelic) loss of *BID-CHR18* from other cancer cell lines and xenograft genomes. Specifically, studies of *SMAD4* (Hahn et al., 1996a; Hilgers et al., 2000) and *SMAD4* and *DCC* (Thiagalingam et al., 1996) reported *BID-CHR18* deletion from genomes of cancer cell lines and xenografts, although with the concomitant loss of the respective candidate tumor-suppressor genes. It therefore is possible that the phenotypic consequences of these large deletions are in part a result of the genetic information in *BID-CHR18*. Other studies have identified homozygous deletions centromeric to the *SMAD4-BID-CHR18-DCC* interval (Thiagalingam et al., 1996; Takei et al., 1998; Wilentz et al., 2000). Some of these deletions reduced the *SMAD4* message by affecting a non-coding exon of *SMAD4*.

The putative relationship between *DCC* expression in human breast epithelial cells with the *BID-CHR18* locus is difficult to assess. Other investigations have shown the *DCC* protein to be breast in epithelia lining ducts and acini in vivo by immunohistochemical techniques (Koren et al., 2003). In our study, the *DCC* transcript was detected in fetal brain and normal breast tissue, but not in three separate normal breast epithelial cell cultures or in HCC-1482 or other breast cancer cell lines (results not shown). *DCC* transcription appears to be down-regulated in response to cell culture, independent of malignant transformation. This phenomenon has been observed by Thompson et al. (1993), who used Northern analysis to detect *DCC* transcript in breast cancer tumors and MCF-7 xenograft cells but did not detect *DCC* transcript in MCF-7 cells in culture or in the breast cancer cell lines MDA-MB-231 and T-47D (Thompson et al., 1993). Therefore, we cannot rule out that deletion of

BID-CHR18 may have affected the expression of *DCC* in the context of the primary tumor, perhaps via elimination of a *DCC* enhancer sequence. The definitive gene content of *BID-CHR18* is difficult to assess with current resources, and it does not contain any currently known genes.

The 9-bp $\Delta(49-51)$ *SMAD4* mutation represents an additional two-hit lesion of the HCC-1428 genome. No other in-frame 3-amino-acid-deletion mutations have been reported in *SMAD4* in sporadic cancer or juvenile polyposis (JP), although one JP kindred has a 9-nucleotide deletion that eliminates amino acids 64–66 and adds a threonine (Woodford-Richens et al., 2000). The $\Delta(49-51)$ *SMAD4* mutation is in the MH1 (Mad homology 1 domain) DNA-binding domain. Based on a comparison with the crystal structure of fellow SMAD protein family member SMAD3, amino acids 49–51 are not directly involved in DNA contact; however, SMAD4 missense mutations in the vicinity reduce DNA binding in vitro (Moren et al., 2000).

Notwithstanding the DNA-binding impairment conferred by MH1 cancer-specific mutations, Moren et al. (2003) and others have convincingly demonstrated that such lesions, specifically, L43S, G65V, R100T, and P130S, all inactivate the SMAD4 protein by enhancement of polyubiquitination and 26S proteasome-mediated degradation (Xu and Attisano, 2000; Moren et al., 2003). Our own pulse chase analysis of exogenous $\Delta(49-51)$ SMAD4-FLAG protein and partial pharmacologic rescue of endogenous $\Delta(49-51)$ SMAD4 by inhibition of the 26S proteasome confirmed that this mechanism is responsible for low levels of mutant protein. Other recent studies have demonstrated that the SMAD4 MH1 domain cancer mutants R100T, G65V, and L43S have increased susceptibility to ubiquitination and proteolysis mediated by E3 ubiquitin ligase complex SCF^{skp2}. Additional investigations are needed to confirm that $\Delta(49-51)$ SMAD4 also is targeted by the SCF^{skp2} complex (Liang et al., 2004).

Partial pharmacologic rescue of endogenous $\Delta(49-51)$ -SMAD4 by inhibition of the 26S proteasome confirm that increased degradation was responsible for the low level of SMAD4 protein, not loss of any information from 18q21.1, the deleted region, or elsewhere. This finding raises the possibility that TGFB signaling attenuated by *SMAD4* mutation could be rescued in vitro or in vivo by pharmacological inhibition of the 26S proteasome.

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