Putative tumour suppressor gene necdin is hypermethylated and mutated in human cancer

L E De Faveri[1], C D Hurst[1], F M Platt[1], C F Taylor[2], J-A Roulson[3], M Sanchez-Carbayo[4], M A Knowles[1] and E J Chapman*,[1]

[1]Section of Experimental Oncology, Cancer Research UK Centre, Leeds Institute of Molecular Medicine, St James's University Hospital, Beckett Street, Leeds LS97TF, UK; [2]CRUK Cancer Centre Genomics Facility, St James's University Hospital, Beckett Street, Leeds LS97TF, UK; [3]Department of Pathology and Tumour Biology, St James's University Hospital, Beckett Street, Leeds LS97TF, UK and [4]Tumour Markers Group, Spanish National Cancer Research Center, Madrid, Spain

Background: Necdin (NDN) expression is downregulated in telomerase-immortalised normal human urothelial cells. Telomerase-immortalised normal human urothelial cells have no detected genetic alterations. Accordingly, many of the genes whose expression is altered following immortalisation are those for which epigenetic silencing is reported.

Methods: NDN expression was examined in normal tissues and tumour cell lines by quantitative real-time PCR and immunoblotting. Immunohistochemistry was performed on urothelial carcinoma (UC). Urothelial carcinoma and UC cell lines were subject to HumanMethylation27 BeadChip Array-based methylation analyses. Mutation screening was performed. The functional significance of NDN expression was investigated using retroviral-mediated downregulation or overexpression.

Results: NDN protein was widely expressed in normal tissues. Loss of expression was observed in 38 out of 44 (86%) of UC cell lines and 19 out of 25 (76%) of non-UC cell lines. Loss of NDN protein was found in the majority of primary UC. Oncomine analysis demonstrated downregulation of expression in multiple tumour types. In UC, tumour-specific hypermethylation of NDN and key CpG sites where hypermethylation correlated with reduced expression were identified. Six novel mutations, including some of predicted functional significance, were identified in colorectal and ovarian cancer cell lines. Functional studies showed that NDN could suppress colony formation at low cell density and affect anchorage-independent growth and anoikis in vitro.

Conclusion: NDN is a novel tumour suppressor candidate that is downregulated and hypermethylated or mutated in cancer.

Expression of telomerase is detected in the majority of human tumours and allows cells to avoid replicative senescence by maintaining telomere length. Telomere-independent effects are also described (Smith *et al*, 2003). Expression array analysis identified genes that are consistently altered in telomerase-immortalised NHUC (TERT-NHUC; Chapman *et al*, 2006) compared with isogenic mortal counterparts (Chapman *et al*, 2008). During these studies we identified Necdin (*NDN*) as a candidate tumour suppressor gene.

NDN maps to 15q11, a maternally imprinted region implicated in Prader–Willi syndrome (PWS). Necdin is a multifunctional protein. Due to its association with PWS, interest has focused on its role in neuronal development and differentiation (Kuwajima *et al*,

2006). However, it also has functions that could be of importance in suppression of tumorigenesis (Chapman and Knowles, 2009). *In vitro* studies demonstrate the role of NDN in suppression of colony formation and haematopoietic stem cell quiescence (Liu *et al*, 2009). Anti-angiogenic effects have also been described (Moon *et al*, 2005).

For the first time, we have examined expression of NDN protein in a range of normal human tissues and tumour cell lines. Novel mutations in *NDN* were identified. Methylation of *NDN* has been examined in tumour cell lines and primary urothelial carcinoma (UC).

Annually, in the UK, more than 10 000 people are diagnosed with UC. Ninety per cent of cases are transitional cell carcinoma,

derived from the cells lining the bladder, the remainder of cases are squamous cell carcinoma or adenocarcinoma. Rarely, sarcoma or small-cell carcinoma are observed.

Integration of expression and hypermethylation data has allowed identification of key CpG sites involved in transcriptional silencing. Oncomine expression microarray data were examined to determine the expression of NDN transcript in multiple tumour types compared with normal tissue. The functional significance of NDN expression was examined *in vitro* using retroviral-mediated transduction. We present the first evidence to support our hypothesis that *NDN* is a potential tumour suppressor gene with a role in multiple tumour types (Chapman and Knowles, 2009).

MATERIALS AND METHODS

Immunohistochemistry (IHC). Necdin IHC was optimised using anti-mouse NDN (AB9372, Millipore, Watford, UK). A total of 1×10^7 NHUCs (NDN positive), TERT-NHUC-pFBhy (NDN low/negative) and TERT-NHUC pFBhy-NDN (ectopic overexpression) were pelleted and 5 ml of 4% formalin/PBS were added overnight. Formalin was replaced with 70% ethanol. Pellets were embedded into 3% agarose, formalin fixed and paraffin embedded. Additional controls were brain, bladder, ureter or UC with known NDN transcript level. Deparaffinised and rehydrated sections were treated with 3% hydrogen peroxide. Antigen retrieval was by pressure cooking with citric acid buffer pH 6 for 2 min, Avidin Biotin blocking kit, catalysed signal amplification system (Dako, Ely, UK), followed by counterstaining with haematoxylin and eosin was performed.

Immunohistochemistry was performed on normal tissue microarray (TMA) II (Provitro, Berlin, Germany) or sections from Leeds Multidisciplinary Research Tissue Bank. Necdin expression was classified as strong (3), moderate (2) weak (1) or absent (0). Immunohistochemistry was performed using Menapath X-cell Plus polymer HRP detection kit (Menarini diagnostics, Wokingham, UK). A preliminary panel contained 72 paraffin-embedded UC from patients at St James's University Hospital, U.K. Tumour samples were obtained with informed consent and the approval of the Local Research Ethics Committee and graded and staged according to the 1973 World Health Organization recommendations and T.N.M classification.

Tissue microarrays contained triplicate cores from 94 tumours from patients at Hospital Guadalajara and Hospital Central de Asturias, Spain. Three observers scored and discrepancies were resolved to give a single score for each core or section. For TMAs, where triplicate cores from a tumour had differing scores, if staining for a potential tumour suppressor gene, the lowest scoring core was recorded. Conversely, the highest scoring core was recorded for potential oncogenes. This principal was also applied if whole sections showed non-homogenous staining.

Cell culture. In all, 40 of 45 bladder cell lines are previously described (Yeager *et al*, 1998; Sarkar *et al*, 2000), LUCC6, LUCC7 and LUCC8 were derived at LIMM from G3pTa, G3pT2/3 and G2pTa UC, respectively. HCV29 was derived from non-malignant transitional epithelium from a patient who had received radiation therapy for UC (Bean *et al*, 1974) and SVHUC are *in vitro* transformed urothelial cells (Christian *et al*, 1987). Culture media used were as follows: Keratinocyte growth medium kit 2 (C-20111, PromoCell, Heidelberg, Germany) with 0.09 mM CaCl$_2$, 3 mM glycine and 1% FCS (LUCC6 and LUCC8). LUCC7 was grown as described (Sarkar *et al*, 2000) plus 30 ng ml^{-1} cholera toxin (Sigma-Aldrich, Dorset, UK). NHUC, TERT-NHUC and LUCC cell lines (excluding LUCC2) were cultured on Primaria surfaces (BD Biosciences, Oxford, UK). Twenty-five non-UC cell lines and culture media are described in Supplementary Table 1. MMG1

primary acral melanoma cell line was from Professor Akifumi Yamamoto (Saitama Medical University International Medical Centre). SMYM-PRGP melanoma cells were from Dr Hiroshi Murata (Shinshu University School of Medicine, Japan; Murata *et al*, 2007).

Cell line identity was verified by short-tandem repeat DNA typing using Powerplex 16 kit (Promega, Southampton, UK). Profiles were compared with publically available data (ATCC, DSMZ). Where no reference profile was available it was confirmed that the profile did not match any cell line in the DSMZ database. All cell lines are routinely tested with PCR Mycoplasma Test Kit III (PromoKine, Heidelberg, Germany). NHUC and TERT-NHUC were derived as described (Chapman *et al*, 2006).

Quantitative real-time PCR (QRT-PCR). RNA was extracted from cell lines using RNeasy mini kit (Qiagen, Crawley, UK) or from frozen sections using Picopure RNA isolation kit (Arcturus Bioscience, Mountain View, CA, USA). cDNA was synthesised and expression of NDN was quantified using TaqMan QRT-PCR (assay Hs00267349_s1) and SDHA (Hs00417200_m1) (Applied Biosystems, Warrington, UK). Reference samples were pooled NHUC cDNA (for UC) or normal human adult universal cDNA (Source Bioscience LifeSciences, Nottingham, UK) for non-UC cell lines.

Western blot. A measure of 15 µg total protein was separated in 12% polyacrylamide gels. Antibodies used were anti-Necdin (07–565, Millipore) and anti-α-tubulin (MCA77G, AbD Serotec, Oxford, UK). DSH1 and RT4 did not express α-tubulin; therefore, blots were re-probed with anti-actin (Santa Cruz, Santa Cruz, CA, USA; data not shown).

Methylation analysis. Sequenom massARRAY EpitTYPER assay was used to screen NDN for CpG hypermethylation in TERT-NHUC and isogenic NHUC. Amplicons were designed using the EpiDesigner programme (http://www.EpiDesigner.com; Supplementary Table 2). Assay was performed at Sequenom (Hamburg, Germany) using standard protocols described in the MassARRAY EpiTYPER Application Guide (http://www.sequenom.com).

Methylation detection in UC cell lines and primary tumours was performed using Illumina HumanMethylation27 BeadChip arrays by Cambridge Genomic Services (University of Cambridge, Cambridge, UK) according to Illumina's Infinium II Methylation protocol. DNA was extracted from 42 fresh frozen TaG2 UC with minimal genomic alteration (<2% genome altered by aCGH; Platt *et al*, manuscript in preparation) and 45 bladder cell lines using QIAamp DNA kit (Qiagen). Five hundred nanograms of DNA was bisulphite modified using EZ DNA methylation kit (Zymo, Irvine, CA, USA) and applied to the chip. Gene annotation was performed using the Mar 2006 (NCBI/26/hg18) assembly at the UCSC database (http://genome.ucsc.edu/). The array returns a β-value for each probe, which is representative of the average level of methylation, where 0 is no methylation and 1 is 100% methylation. Necdin is a maternally imprinted gene, therefore, control samples exhibit a β-value of approximately 0.5. Hypermethylation at a probe site was defined as a β-value $> +20\%$ of the average value obtained from the control normal samples on that array. Urothelial carcinoma cell lines were compared with cultured NHUC and UC to uncultured normal urothelium.

Gene expression array profiling. Whole-genome expression profiling (Hurst *et al.*, manuscript in preparation) was performed using GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix, High Wycombe, UK). A measure of 5 µg of total RNA was labelled using the WT-Ovation Pico Target Prep v1.0 system (NuGen Technologies, Leek, The Netherlands) and hybridised according to the array manufacturer's instructions. Arrays were scanned and CEL files imported into Partek Genomics Suite 6.5. Data were normalised and probe intensity measures were

generated using the Robust Microarray Analysis algorithm (Irizarry *et al*, 2003).

Sequencing of *NDN*. Ten microlitre of PCR reactions contained: 10 ng DNA, 4 pmol of each primer, HotStarTaq mastermix (Qiagen) and 10% DMSO. For melanoma cell lines, 10 ng BSA was added. Cycling conditions were: 15 min at 95 °C then 36 cycles of 30 s at 95 °C, 30 s at 57 °C, 1 min at 72 °C and a final cycle at 72 °C for 10 min. The single exon of *NDN* was amplified in two fragments using primers: 1 F 5′-GAAGAGCTCCTGGACGCA GA-3′ and 1 R 5′-TCAGCGCCACCCTGTCCAGC-3′, 2 F 5′-TCA TCCTCGCCCGGGTGTTCG-3′ and 2 R 5′-GTGAGGGTCAGA AACCATTCA-3′. Unincorporated primers and deoxynucleotides were removed using shrimp alkaline phosphatase and exonuclease I. Sequencing reactions were carried out using the PCR primers and a BigDye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were compared with NCBI reference sequence NG_009380.1. Mutation nomenclature uses A of ATG as nucleotide 1.

Retroviral transduction. MGC/IMAGE Clone 1697/3347128 (Geneservice, Cambridge, UK) was sub-cloned as an *Eco*R1-*Xho*1 fragment into pFB Hygro (adapted from pFB neo from Agilent, Cheshire, UK). Oligos targeting NDN (Supplementary Table 3) or a non-silencing control oligo (Douglas *et al*, 2008) were cloned into pRetroSuper-puro (Tomlinson *et al*, 2007). Constructs were transfected into PhoenixA (ATCC) using Mirus TransIT-293 (Cambridge Bioscience, Cambridge, UK). After 48 h, medium was harvested and filtered through a 0.4-μm filter. Supernatant containing 8 μg ml^{-1} of polybrene (Sigma-Aldrich) was added to sub-confluent target cells for 6 h. After 48 h, cells were transferred into selection medium.

Phenotypic assays. For growth curves, cells were plated at 3.1×10^3 cells per cm^2. To assess anchorage-independent growth, 4.2×10^3 cells per cm^2 were plated in 0.3% Noble agar (Sigma-Aldrich) in medium onto a base of 0.6% agar. Cells were fed weekly with 0.3% agar/medium. After 21 days, colonies were stained and colonies > 200 μm diameter were counted in five unselected fields. To assess colony formation at low density, 55 cells per cm^2 were plated. After 7 days (TERT-NHUC) or 14 days (UM-UC3 and TCCSUP), cells were stained with methylene blue. Total colonies > 30 cells were counted. For determination of resistance to anoikis, cells were seeded on six-well ultra low attachment plates (Corning, Amsterdam, The Netherlands) at 2.6×10^4 cells per cm^2. After 0, 24 and 48 h, viable cells were quantified using Guava ViaCount and Guava EasyCyte Plus Flow Cytometry System (Millipore).

RESULTS

Necdin is expressed in normal human tissues but downregulated in tumour cell lines. For the first time, we demonstrated that NDN protein was expressed in multiple tissue types (Figure 1A and Supplementary Table 4). In most tissues examined expression was cytoplasmic. Expression ranged from absent (0) to strong (3; Supplementary Figure 1). Cultured NHUCs expressed NDN transcript and protein. Of 25 tumour cell lines, 22 tumour cell lines exhibited reduced expression of transcript (relative to universal cDNA). Of the five cell lines showing the smallest amount of transcript downregulation (U266, 1847, SHYSY5Y, LNCaP and MelJuso), only 1847 cell line demonstrated strong NDN protein expression.

Melanoma cell lines (MMG1, SMYM-PRGP and SKMel5) showed expression greater than universal cDNA (Figure 1B). Expression of NDN protein was detected in MMG1 and SMYM-PRGP but not SKMel5 (Figure 1C). MMG1 and ovarian cell line 1847 showed an additional smaller band reacting with the NDN

antibody at approximately 33 kDa. An alternatively spliced isoform, NDN variant bAug10 (NCBI), is described, consisting of 299aa, with a predicted protein mass of 33.8 kDa.

Quantitative real-time PCR was previously performed on UC cell lines (Chapman *et al*, 2008). Here we performed western blotting on a more extensive panel. Loss of NDN protein expression was seen in 38 out of 44 (86%). Necdin protein was detected only in 253J, CAL-29, 97–24, HT1197, LUCC6 and LUCC8 (Figure 2A).

Necdin transcript and protein expression is reduced in UC. Downregulation of NDN expression compared with NHUC was detected in 35 out of 58 (60%) of UC and NDN transcript levels were not significantly related to stage or grade (Chapman *et al*, 2008). Here, we stained a panel of UC by IHC. Necdin was scored as 0 to 3. A score of 0 or 1 was considered to be reduced expression (loss) relative to normal urothelium and 2 or 3 as positive. Of 72 (61%) tumours, 44 tumours showed loss of NDN expression. Overall, the frequency of loss of NDN expression was higher in the TMA (86%) than our initial panel.

Necdin transcript is reduced in multiple types of primary tumours. Oncomine analysis (http://www.oncomine.org) demonstrated downregulation of NDN transcript in multiple tumour types including bladder (Sanchez-Carbayo *et al*, 2006), colorectal (Sabates-Bellver *et al*, 2007; Skrzypczak *et al*, 2010), ovarian, oesophageal (Kim *et al*, 2010) and breast (Richardson *et al*, 2006) compared with the normal tissue (Figure 3). Of note, premalignant tissues (Barrett's oesophagus, vulva intraepithelial neoplasia) also show downregulation of NDN transcript.

***NDN* is hypermethylated in TERT-NHUC, UC cell lines and UC.** Sequenom Epityper MassARRAY methylation analyses of TERT-NHUC showed differential CpG methylation, validating *NDN* as a potential epigenetic target (Figure 4A). Infinium HumanMethylation27 BeadChip array analysis was performed on bladder cell lines. Hypermethylation at cg13828758 and cg12532169 best correlated with transcript and protein expression (Table 1) and showed an inverse correlation. Of the 23 out of 45 (51%) cell lines with hypermethylation of cg13828758 and 24 out of 45 (53%) cell lines with hypermethylation of cg12532169 (Figure 4B), only LUCC6 expressed NDN protein. LUCC6 has trisomy 15 (unpublished data). As *NDN* is a maternally imprinted gene, it is feasible that LUCC6 may have duplicated the silenced allele giving a hypermethylated result, yet still contain an unmethylated paternal allele from which the protein is expressed. 253J (with high NDN expression at the transcript and protein level) had the lowest level of hypermethylation at these probe sites. Methylation array analysis of 40 TaG2 UC, detected hypermethylation at cg13828758 in 8 out of 40 (20%) and at cg12532169 in 22 out of 40 (55%; Figure 4C).

***NDN* sequence variants were detected in tumour cell lines.** Sequence variants were identified in 2 of 10 colorectal carcinoma cell lines. c.187 G > A (p.D63N), c.659 G > A (p.R220Q) and c.195 C > T (p.G65G) in DLD-1 and c.626 C > T (p.A209V) and c.774 C > T (p.Y258Y) in HCT116. An additional variant c.488 T > C (p.M163T) was identified in one of five ovarian cell lines examined (1847) (Supplementary Figure 2). 1847 expressed NDN protein, whereas DLD-1 was NDN negative. Missense SNP rs114629863 C/T (p.P88L) was detected in 96-1. Of note, 96-1 lacks NDN transcript or protein expression, yet does not exhibit *NDN* hypermethylation. Synonymous SNP rs2192206 C/T was detected in 25 out of 77 (32%) of tumour cell lines. As matched normal DNA was not available for the cell lines, we are unable to confirm the somatic origin of these variations.

Necdin promotes anoikis and represses colony formation and anchorage-independent growth. Necdin was stably overexpressed
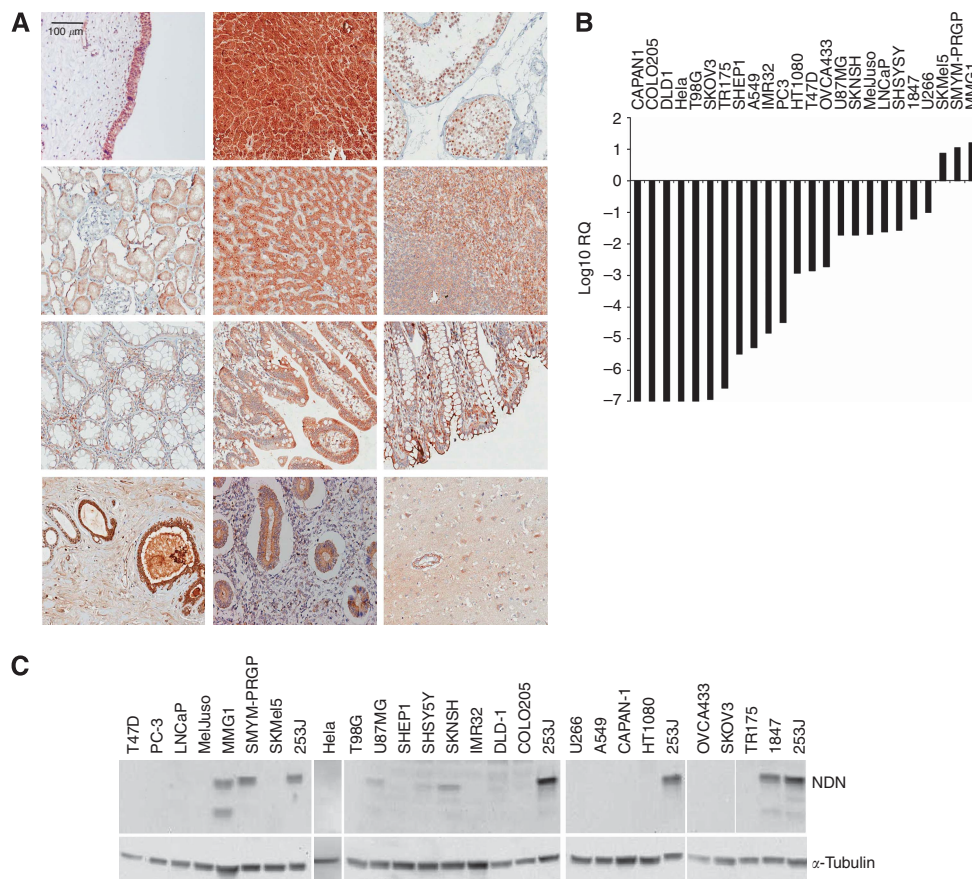
Figure 1. (**A**) Expression of necdin was detected by immunohistochemistry in normal human tissues. Top left to bottom right: bladder, pancreas, testis, kidney, liver, spleen, colon, small intestine, rectum, breast, endometrium, brain. Images were captured and scanned using Aperio Scanscope CS system (Aperio) and viewed using ImageScope. (**B**) Necdin transcript expression detected by quantitative real-time PCR in a tumour cell line panel. CAPAN1 to T98G had no detectable NDN transcript (in the presence of detectable endogenous control). These are given an arbitrary Log10[RQ] of − 7. Expression is normalised to Universal cDNA, which therefore has a Log10 [RQ] of 0. (**C**) Despite, widespread expression in normal tissues, expression of necdin protein was detected by immunoblotting in only a minority of tumour cell lines. 253J (UC cell line) is a positive control.
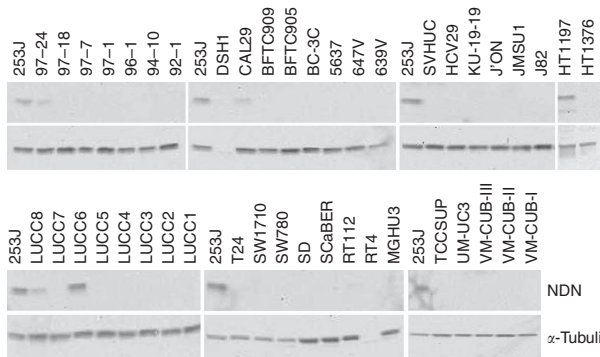
Figure 2. Expression of necdin protein by immunoblotting was only detected in 6 of 44 UC cell lines.

or downregulated by shRNA. Ectopic expression did not affect the rate of proliferation or saturation density of NHUC, TERT-NHUC or UM-UC3 (data not shown). Knockdown of NDN expression in 253J and LUCC6 was confirmed and did not affect the rate of proliferation nor colony formation on plastic at low plating density. However, colony formation at low density was repressed by ectopic expression of NDN in TERT-NHUC and UM-UC3 (Figure 5A). Ectopic expression of NDN in UM-UC3 inhibited anchorage-independent growth (Figure 5B) and promoted anoikis.

On average, UM-UC3 cells transduced to re-express NDN, exhibited a 16% decrease in viable cells after 24 h culture relative to empty-vector transduced cells on non-adherent plates (ratio t-test, $P = 0.05$; Figure 5B).

DISCUSSION

NDN first came to our attention as a gene downregulated following telomerase-mediated immortalisation of NHUC (Chapman *et al*, 2008). It was downregulated after expression of both hTERT and the non-telomere lengthening variant, hTERT-HA (Counter *et al*, 1998). Downregulation of NDN is therefore related to telomerase activity rather than telomere-dependent effects. Unlike hTERT, hTERT-HA does not immortalise NHUC, indicating that down-regulation of NDN is not sufficient for, or a product of immortalisation.

In contrast to the restricted expression of NDN described in the mouse (Maruyama *et al*, 1991), NDN transcript is detected in various human tissues (Jay *et al*, 1997), although protein expression had not been previously examined. For the first time, we have shown that NDN is expressed at the protein level in a range of human tissues including bladder, brain, colon and liver. These preliminary studies were limited by the low number of specimens from each normal tissues examined. Further investigation involving multiple normal tissue specimens will be required.
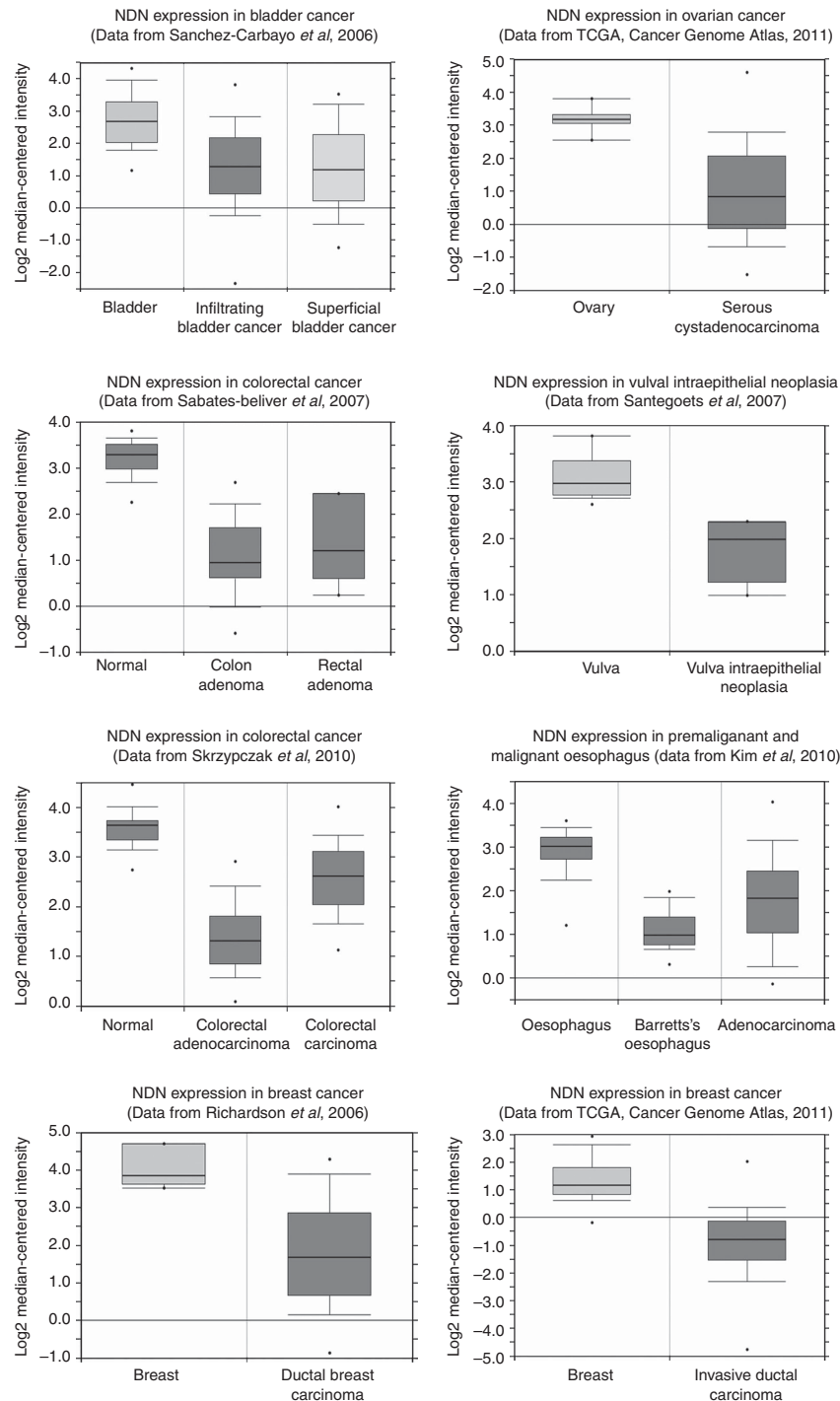
Figure 3. Oncomine analysis of deposited expression microarray data shows downregulation of NDN transcript in multiple tumour types compared with normal tissue.

Necdin protein was absent from most cancer cell lines investigated but as described for other transcripts in other tumour cell lines and tumours (Stark *et al*, 2006), a direct correlation between protein and transcript levels was not observed. Post transcriptional or post-translational modifications are likely to impact on protein expression (de Sousa Abreu *et al*, 2009). The anti-NDN antibody detected an additional smaller protein in three cell lines, which may be NDN variant bAug10. The functional significance of this and whether this is a tumour-specific variant are unknown.

Our preliminary data suggest that further investigation is warranted in bladder, colorectal, ovarian and brain cell lines and

tumours. Indeed, oncomine analysis (Rhodes *et al*, 2007) demonstrates reduced expression of NDN transcript in multiple tumour types (e.g., bladder, breast, ovarian and colorectal cancer) compared with normal tissue. Downregulation of expression was also detected in two types of premalignant lesions (vulval intraepithelial neoplasia and Barrett's oesophagus), supporting our previous hypothesis that the changes in gene expression identified in our study of telomerase-immortalised cells may be early events in tumorigenesis. Telomerase activation or over-expression of its RNA components is detected in multiple premalignant lesions including those that can lead to the
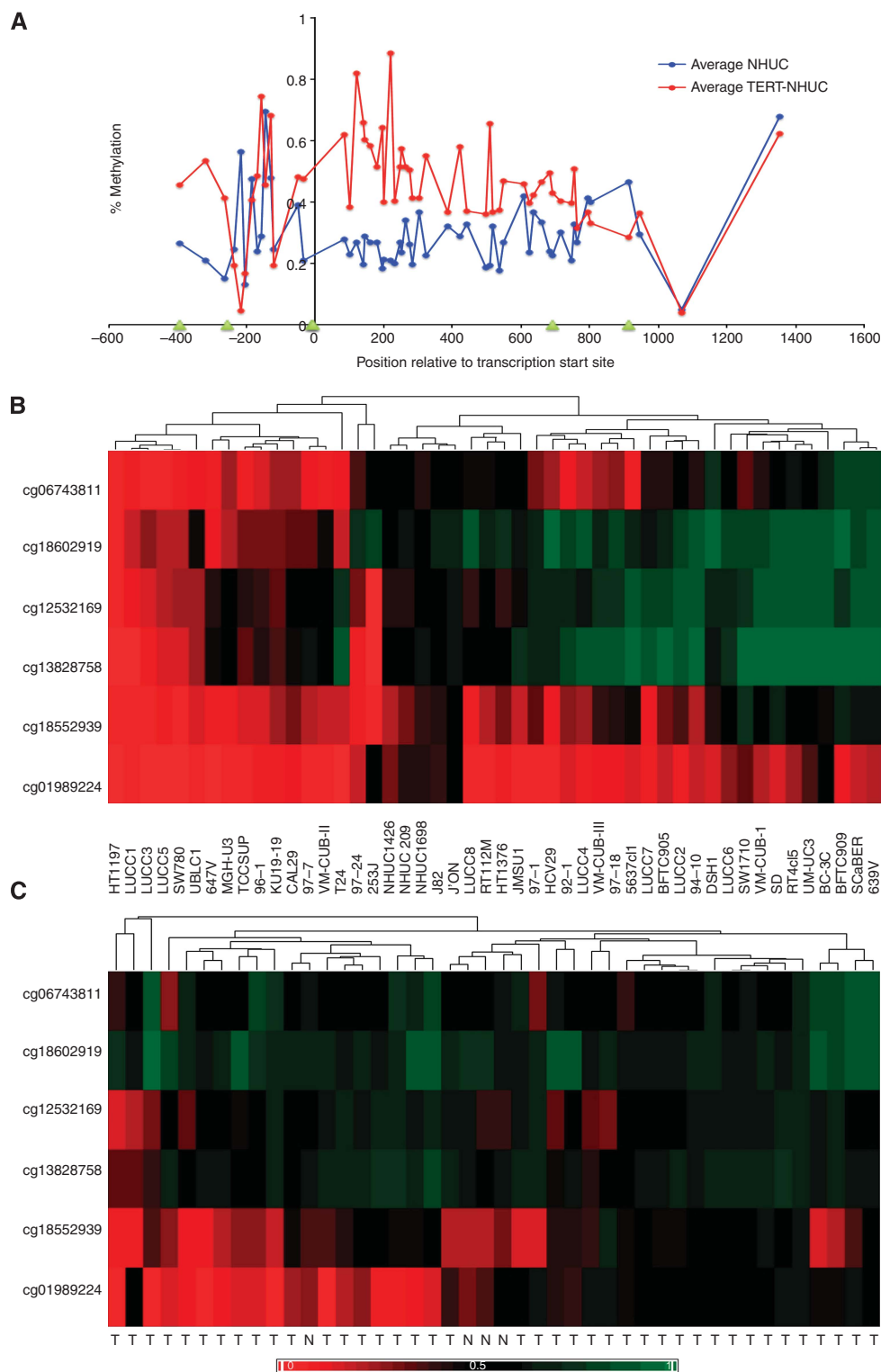
Figure 4. (**A**) Output from Sequenom MassArray Epityper analysis for of *NDN*. Chart shows the average level of methylation in NHUC and TERT-NHUC at each interrogated CpG site. Triangles represent the position of probes in the Human Methylation27 array. From left to right probes are: cg01989224, cg18552939, cg13828758, cg12532169, cg18602919 and cg06743811. (**B**) Unsupervised cluster analysis of methylation levels (β-values) detected in UC cell lines and (**C**) primary tumours by methylation array analysis. T represents tumour and N represents normal urothelium. Methylation level is shown from β-value of 0 (red) to 1 (green).

development of bladder, colon, lung, breast, oral and cervical cancer (Engelhardt *et al*, 1997; Kolquist *et al*, 1998; Leuenroth *et al*, 2005; Lantuejoul *et al*, 2007). 'Telomerase-associated' changes in gene expression such as those identified as 'signature genes' in TERT-NHUC may also occur in these premalignant lesions and may

represent one step during tumour pathogenesis. The possibility that these changes are epigenetically mediated rather than due to genetic deletion, opens up the potential for the use of demethylating or telomerase-inhibiting drugs as a tumour preventative strategy. The detection of telomerase-associated hypermethylation may also have

Table 1. NDN expression and hypermethylation at Infinium HumanMethylation27 probe (shaded)								
Cell line	Transcript	Protein	cg06743811	cg18602919	cg12532169	cg13828758	cg18552939	cg01989224
253J	+	+	+	+	–	–	–	+
HT1197	+	+	–	–	–	–	–	–
HT1376	–	–	–	+	–	–	–	–
LUCC1	–	–	–	–	–	–	–	–
LUCC2	–	–	+	+	+	+	–	–
LUCC3	–	–	–	–	–	–	–	–
LUCC4	–	–	–	+	+	+	–	–
LUCC5	–	–	–	–	–	–	–	–
LUCC6	+	+	+	+	+	+	+	–
LUCC7	–	–	–	+	+	+	–	–
LUCC8	–	+	–	+	–	–	–	–
UBLC1	–	–	–	–	–	–	–	–
SW780	–	–	–	–	–	–	–	–
97-24	+	+	–	+	–	– –	–	–
96-1	–	–	–	–	–	–	–	–
97-7	–	–	–	–	–	–	–	–
97-1	–	–	–	+	+	+	–	–
92-1	–	–	–	+	+	+	–	–
97-18	–	–	–	+	+	+	–	–
94-10	–	–	–	+	+	+	–	–
KU-19-19	–	–	–	–	–	–	–	–
TCCSUP	–	–	–	–	–	–	–	–
647V	–	–	–	–	–	–	–	–
JMSU1	–	–	–	+	–	+	–	–
RT112M	–	–	–	+	–	–	–	–
CAL29	+	+	–	–	–	–	–	–
VM-CUB- II	–	–	–	–	–	–	–	–
MGH − U3	–	–	–	–	+	–	–	–
DSH1	–	–	+	+	+	–	–	–
SW1710	–	–	–	+	+	+	+	–
VM-CUB-III	–	–	–	+	+	+	–	–
T24	–	–	–	–	+	+	–	–
BFTC909	–	–	+	+	+	+	+	–
BFTC905	–	–	–	+	+	+	–	–
5637cl1	–	–	–	+	+	+	–	–
BC-3C	–	–	–	+	+	+	–	–
SD	–	–	–	+	+	+	+	–
VM-CUB-I	–	–	–	+	+	+	+	–
UM-UC3	–	–	–	+	+	+	+	–
SCaBER	–	–	+	+	+	+	+	–
639V	–	–	+	+	+	+	+	–
RT4	–	–	+	+	+	+	+	–
J'ON	–	–	–	–	–	–	–	–
J82	–	–	–	–	–	–	–	–
HCV29	–	–	–	+	+	+	–	–

Abbreviation: NDN = necdin.

application in early disease detection or monitoring. Further studies are underway to investigate NDN expression and hypermethylation in premalignant tissues.

Our initial focus was on UC and we have shown that NDN is expressed in normal urothelium but expression is lost in the majority of UC. *NDN* is maternally imprinted and therefore
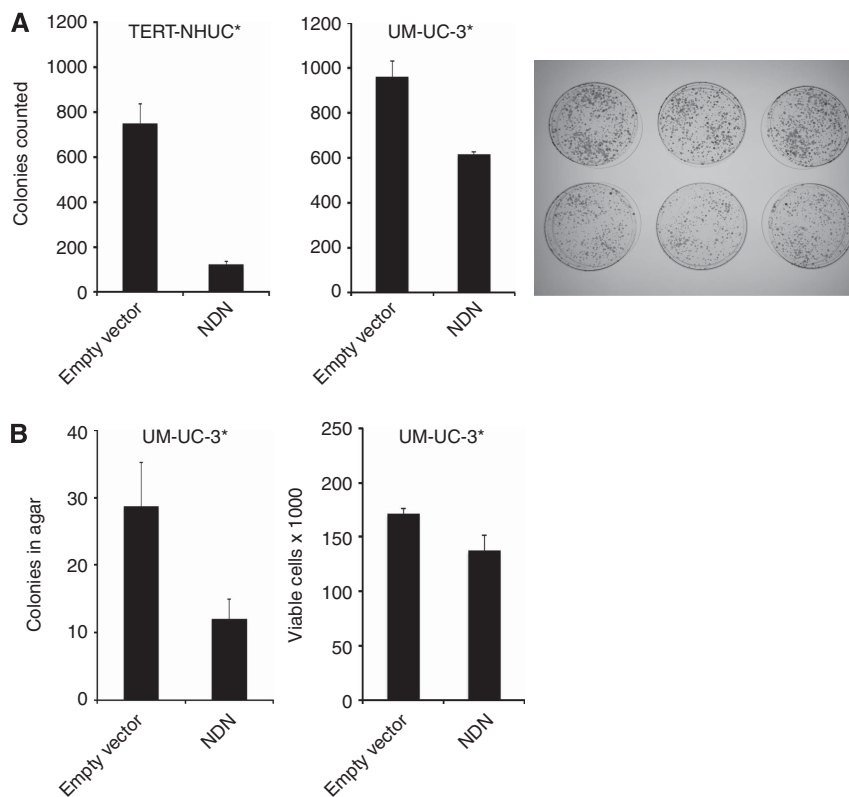
Figure 5. Phenotypic effect of NDN overexpression. (A) Colony formation at low plating density in TERT-NHUC and UM-UC3 Photo shows 9 cm diameter dishes stained with methylene blue. Top row, UM-UC3-empty vector, bottom row NDN-transduced UM-UC3. **(B)** Anchorage-independent growth of UC cell line, UM-UC3. *Indicates a statistical significant difference between empty vector and NDN-transduced cells. Data are representative of at least triplicate experiments. Error bars represent standard deviations of multiple samples.

exhibits monoallelic expression (Jay *et al*, 1997). To silence expression of the only functional copy requires only one further event. The *NDN* gene at 15q11.2 is located outside of the common region of chromosome 15 LOH in UC (Natrajan *et al*, 2003) and is predicted to be silenced by hypermethylation (Lau *et al*, 2004). Hypermethylation of *NDN* was observed in TERT-NHUC. Further, array-based methylation analyses of UC cell lines demonstrated that transcriptional silencing is closely correlated with promoter hypermethylation and two key CpG sites were identified. Hypermethylation of these key CpG sites was also detected in UC. The frequency of hypermethylation at probe cg13828758 but not cg12532169 was less in primary tumours than in UC cell lines. This may reflect tumour heterogeneity. This is the first evidence that hypermethylation of *NDN* occurs in human tumours and that key sites that correlate with transcriptional silencing are targeted. Detection of hypermethylation at an imprinted locus has the technical difficulty that methylation levels in control normal samples have β-values of approximately 0.5 (0.474 measured here for cg12532169 and 0.583 for cg13828758). Alterations in methylation status are therefore only detected in the range 0.5–1.0, half the range that could be measured in a non-imprinted gene. We chose a robust cutoff of a 20% increase in methylation level compared with control sample and this may have been over conservative in this context and led to under reporting of hypermethylation.

Mutation screening was performed for the single exon of *NDN* in tumour cell lines. Perhaps not unexpectedly for an imprinted gene with such frequent hypermethylation, mutations were not observed in UC cell lines. However, mutations were identified in DLD-1 and HCT116 colon carcinoma cell lines and 1847 ovarian carcinoma cell line. The mutations occur in regions of high evolutionary conservation and we predict that they could be of

functional significance. The functional domains of NDN necessary for protein–protein interaction, nuclear matrix targeting and cell growth suppression have been identified (Taniura *et al*, 2005). The whole MAGE homology domain (amino acids 116–280) is required for interaction with p53 and could be disrupted by p.R220Q, p.M163T or p.A209V. p.R220Q and p.A209V are within the part of the domain required for cell growth suppression (amino acids 191–222). p.M163T is within domains required for nuclear matrix targeting (amino acids 144–184). No targeted mutation screen of *NDN* has previously been performed in human cancer. However, the Catalogue of Somatic Mutations in Cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/) at the time of writing, lists 43 mutations of NDN found during genome-wide mutation screens. These include 28 in lung tumours plus 5 examples in colorectal tumours. Mutations are also reported in skin squamous cell carcinoma (p.P309L), ovarian serous carcinoma (p.P41S), breast carcinoma (E269K, R142H), kidney (P34P, H161H), prostate (P88T) and endometrial cancer (G130S, R142C, R249C). This indicates that mutation screening of non-UC tumour types, particularly lung and colorectal cancers, would be worthwhile. The missense SNP rs114629863 C/T (p.P88L) was detected in a UC cell line, 96-1. This change is within the MAGE homology domain required for p53 interaction and therefore potentially could impact on protein function (Taniura *et al*, 2005). Interestingly, 96-1 is a cell line that lacks NDN transcript or protein and does not exhibit *NDN* hypermethylation. We investigated the functional significance of *NDN* silencing. Necdin is a suppressor of proliferation in post mitotic neurons (Taniura *et al*, 1998). Here, no effect of NDN on NHUC or tumour cell line proliferation was observed. Ectopic expression of NDN suppresses colony formation and growth in SAOS-2 osteosarcoma cells (Taniura *et al*, 2005). Necdin was ectopically overexpressed in cell lines that had low or minimal

endogenous levels and reduced colony formation at low density was observed in TERT-NHUC, UM-UC3 and TCCSUP. Reduced anchorage-independent growth was observed in UM-UC3. Furthermore, anoikis was promoted in UM-UC3, which may have contributed to the repression of anchorage-independent growth. In light of our current evidence supporting the idea of *NDN* as a tumour suppressor gene, future studies to investigate the effect of *NDN* on tumorigenicity *in vivo* may now be warranted. Indeed, ectopic overexpression of *NDN* in a mouse tumour cell line is reported to attenuate tumorigenicity and metastasis *in vivo*. The same study found that the gene expression signature that results from ectopic overexpression of NDN is also reported to predict prognosis in a human breast cancer cohort (Crawford *et al*, 2008). There is considerable evidence to support the hypothesis that *NDN* is a tumour suppressor gene (Chapman and Knowles, 2009).

Necdin is one of the genes inactivated in PWS. If *NDN* is proven to be a tumour suppressor gene, this raises the question of whether PWS patients have an increased cancer risk. Although it is not a recognised cancer-prone syndrome, the only specific study of cancer incidence in PWS patients reported an increased risk of myeloid leukaemia but not of other cancers (Davies *et al*, 2003). The report by Liu *et al* (2009) describing the importance of NDN in maintaining hematopoietic stem cell quiescence perhaps provides evidence to explain this observation. Prader–Willi syndrome has only been accurately diagnosed in the last 30 years since genetic testing became possible. Therefore, there are only few confirmed cases identified in middle aged or elderly patients, the population in which a general increased cancer predisposition would present (Davies *et al*, 2003). As life expectancy for well-managed PWS is now near normal, any increased cancer predisposition may be observed in the future. However, the concept of a gene whose germ line alteration results in a syndrome, which is not tumour prone, whereas a somatic alteration contributes to tumorigenesis is not unprecedented. Other examples include *FGFR2* and *FGFR3* where germ line activating mutations are implicated in craniosynostosis and chondrodysplasia syndromes but somatic mutations contribute to endometrial (Pollock *et al*, 2007) and urothelial (van Rhijn *et al*, 2002) cancers. In conclusion, we have shown that NDN is widely expressed in normal human tissue. Expression is lost in cell lines derived from a range of common tumour types. *NDN* is verified as an epigenetic target. In UC, hypermethylation appears to be the key mechanism of inactivation and this correlates with loss of expression in UC cell lines. Previously, undescribed mutations have been observed and may be the mode of inactivation in some non-UC tumours such as colorectal carcinoma. *In vitro* functional analyses indicate that NDN has roles in suppression of colony formation, anchorage-independent growth and anoikis. We propose that *NDN* is a novel candidate tumour suppressor gene with relevance to a wide range of human cancers.

REFERENCES

Bean MA, Pees H, Fogh JE, Grabstald H, Oettgen HF (1974) Cytotoxicity of lymphocytes from patients with cancer of the urinary bladder: detection by a 3-H-proline microcytotoxicity test. *Int J Cancer* **14**: 186–197.

Chapman EJ, Hurst CD, Pitt E, Chambers P, Aveyard JS, Knowles MA (2006) Expression of hTERT immortalises normal human urothelial cells without inactivation of the p16/Rb pathway. *Oncogene* **25**: 5037–5045.

Chapman EJ, Kelly G, Knowles MA (2008) Genes involved in differentiation, stem cell renewal, and tumorigenesis are modulated in telomerase-immortalized human urothelial cells. *Mol Cancer Res* **6**: 1154–1168.

Chapman EJ, Knowles MA (2009) Necdin: a multi functional protein with potential tumor suppressor role? *Mol Carcinog* **48**: 975–981.

Christian BJ, Loretz LJ, Oberley TD, Reznikoff CA (1987) Characterization of human uroepithelial cells immortalized *in vitro* by simian virus 40. *Cancer Res* **47**: 6066–6073.

Counter CM, Hahn WC, Wei W, Caddle SD, Beijersbergen RL, Lansdorp PM, Sedivy JM, Weinberg RA (1998) Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization. *Proc Natl Acad Sci USA* **95**: 14723–14728.

Crawford NP, Walker RC, Lukes L, Officewala JS, Williams RW, Hunter KW (2008) The Diasporin Pathway: a tumor progression-related transcriptional network that predicts breast cancer survival. *Clin Exp Metastasis* **25**: 357–369.

Davies HD, Leusink GL, McConnell A, Deyell M, Cassidy SB, Fick GH, Coppes MJ (2003) Myeloid leukemia in Prader-Willi syndrome. *J Pediatr* **142**: 174–178.

de Sousa Abreu R, Penalva LO, Marcotte EM, Vogel C (2009) Global signatures of protein and mRNA expression levels. *Mol Biosyst* **5**: 1512–1526.

Douglas D, Hsu JH, Hung L, Cooper A, Abdueva D, van Doorninck J, Peng G, Shimada H, Triche TJ, Lawlor ER (2008) BMI-1 promotes ewing sarcoma tumorigenicity independent of CDKN2A repression. *Cancer Res* **68**: 6507–6515.

Engelhardt M, Drullinsky P, Guillem J, Moore MA (1997) Telomerase and telomere length in the development and progression of premalignant lesions to colorectal cancer. *Clin Cancer Res* **3**: 1931–1941.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**: 249–264.

Jay P, Rougeulle C, Massacrier A, Moncla A, Mattei MG, Malzac P, Roeckel N, Taviaux S, Lefranc JL, Cau P, Berta P, Lalande M, Muscatelli F (1997) The human necdin gene, NDN, is maternally imprinted and located in the Prader-Willi syndrome chromosomal region. *Nat Genet* **17**: 357–361.

Kim SM, Park YY, Park ES, Cho JY, Izzo JG, Zhang D, Kim SB, Lee JH, Bhutani MS, Swisher SG, Wu X, Coombes KR, Maru D, Wang KK, Buttar NS, Ajani JA, Lee JS (2010) Prognostic biomarkers for esophageal adenocarcinoma identified by analysis of tumor transcriptome. *PLoS One* **5**: e15074.

Kolquist KA, Ellisen LW, Counter CM, Meyerson M, Tan LK, Weinberg RA, Haber DA, Gerald WL (1998) Expression of TERT in early premalignant lesions and a subset of cells in normal tissues. *Nat Genet* **19**: 182–186.

Kuwajima T, Nishimura I, Yoshikawa K (2006) Necdin promotes GABAergic neuron differentiation in cooperation with Dlx homeodomain proteins. *J Neurosci* **26**: 5383–5392.

Lantuejoul S, Salon C, Soria JC, Brambilla E (2007) Telomerase expression in lung preneoplasia and neoplasia. *Int J Cancer* **120**: 1835–1841.

Lau JC, Hanel ML, Wevrick R (2004) Tissue-specific and imprinted epigenetic modifications of the human NDN gene. *Nucleic Acids Res* **32**: 3376–3382.

Leuenroth S, Riethdorf S, Erbersdobler A, Riethdorf L, Loning T, Huland H, Friedrich MG (2005) Detection of human telomerase RNA in the tumour-surrounding mucosa of bladder carcinomas as a marker for premalignant transformation. *BJU Int* **96**: 553–557.

Liu Y, Elf SE, Miyata Y, Sashida G, Huang G, Di Giandomenico S, Lee JM, Deblasio A, Menendez S, Antipin J, Reva B, Koff A, Nimer SD (2009) p53 regulates hematopoietic stem cell quiescence. *Cell Stem Cell* **4**: 37–48.

Maruyama K, Usami M, Aizawa T, Yoshikawa K (1991) A novel brain-specific mRNA encoding nuclear protein (necdin) expressed in neurally

differentiated embryonal carcinoma cells. *Biochem Biophys Res Commun* **178**: 291–296.

Moon HE, Ahn MY, Park JA, Min KJ, Kwon YW, Kim KW (2005) Negative regulation of hypoxia inducible factor-1alpha by necdin. *FEBS Lett* **579**: 3797–3801.

Murata H, Ashida A, Takata M, Yamaura M, Bastian BC, Saida T (2007) Establishment of a novel melanoma cell line SMYM-PRGP showing cytogenetic and biological characteristics of the radial growth phase of acral melanomas. *Cancer Sci* **98**: 958–963.

Natrajan R, Louhelainen J, Williams S, Laye J, Knowles MA (2003) High-resolution deletion mapping of 15q13.2-q21.1 in transitional cell carcinoma of the bladder. *Cancer Res* **63**: 7657–7662.

Pollock PM, Gartside MG, Dejeza LC, Powell MA, Mallon MA, Davies H, Mohammadi M, Futreal PA, Stratton MR, Trent JM, Goodfellow PJ (2007) Frequent activating FGFR2 mutations in endometrial carcinomas parallel germline mutations associated with craniosynostosis and skeletal dysplasia syndromes. *Oncogene* **26**: 7158–7162.

Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB, Barrette TR, Anstet MJ, Kincead-Beal C, Kulkarni P, Varambally S, Ghosh D, Chinnaiyan AM (2007) Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. *Neoplasia* **9**: 166–180.

Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, Liao X, Iglehart JD, Livingston DM, Ganesan S (2006) X chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell* **9**: 121–132.

Sabates-Bellver J, Van der Flier LG, de Palo M, Cattaneo E, Maake C, Rehrauer H, Laczko E, Kurowski MA, Bujnicki JM, Menigatti M, Luz J, Ranalli TV, Gomes V, Pastorelli A, Faggiani R, Anti M, Jiricny J, Clevers H, Marra G (2007) Transcriptome profile of human colorectal adenomas. *Mol Cancer Res* **5**: 1263–1275.

Sanchez-Carbayo M, Socci ND, Lozano J, Saint F, Cordon-Cardo C (2006) Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. *J Clin Oncol* **24**: 778–789.

Santegoets LA, Seters M, Helmerhorst TJ, Heijmans-Antonissen C, Hanifi-Moghaddam P, Ewing PC, van Ijcken WF, van der Spek PJ, van der Meijden WI Blok LJ (2007) HPV related VIN: highly proliferative and diminished responsiveness to extracellular signals. *Int J cancer* **121**(4): 759–766.

Sarkar S, Julicher KP, Burger MS, Della Valle V, Larsen CJ, Yeager TR, Grossman TB, Nickells RW, Protzel C, Jarrard DF, Reznikoff CA (2000) Different combinations of genetic/epigenetic alterations inactivate the p53 and pRb pathways in invasive human bladder cancers. *Cancer Res* **60**: 3862–3871.

Skrzypczak M, Goryca K, Rubel T, Paziewska A, Mikula M, Jarosz D, Pachlewski J, Oledzki J, Ostrowski J (2010) Modeling oncogenic signaling in colon tumors by multidirectional analyses of microarray data directed for maximization of analytical reliability. *PLoS One* **5**(10) pii: e13091.

Smith LL, Coller HA, Roberts JM (2003) Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. *Nat Cell Biol* **5**: 474–479.

Stark AM, Pfannenschmidt S, Tscheslog H, Maass N, Rosel F, Mehdorn HM, Held-Feindt J (2006) Reduced mRNA and protein expression of BCL-2 versus decreased mRNA and increased protein expression of BAX in breast cancer brain metastases: a real-time PCR and immunohistochemical evaluation. *Neurol Res* **28**: 787–793.

Taniura H, Kobayashi M, Yoshikawa K (2005) Functional domains of necdin for protein-protein interaction, nuclear matrix targeting, and cell growth suppression. *J Cell Biochem* **94**: 804–815.

Taniura H, Taniguchi N, Hara M, Yoshikawa K (1998) Necdin, a postmitotic neuron-specific growth suppressor, interacts with viral transforming proteins and cellular transcription factor E2F1. *J Biol Chem* **273**: 720–728.

Tomlinson DC, Hurst CD, Knowles MA (2007) Knockdown by shRNA identifies S249C mutant FGFR3 as a potential therapeutic target in bladder cancer. *Oncogene* **26**: 5889–5899.

van Rhijn BW, van Tilborg AA, Lurkin I, Bonaventure J, de Vries A, Thiery JP, van der Kwast TH, Zwarthoff EC, Radvanyi F (2002) Novel fibroblast growth factor receptor 3 (FGFR3) mutations in bladder cancer previously identified in non-lethal skeletal disorders. *Eur J Hum Genet* **10**: 819–824.

Yeager TR, DeVries S, Jarrard DF, Kao C, Nakada SY, Moon TD, Bruskewitz R, Stadler WM, Meisner LF, Gilchrist KW, Newton MA, Waldman FM, Reznikoff CA (1998) Overcoming cellular senescence in human cancer pathogenesis. *Genes Dev* **12**: 163–174.

Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)