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ARID1B, a member of the human SWI/SNF chromatin remodeling complex, exhibits tumour-suppressor activities in pancreatic cancer cell lines

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Background: The human ATP-dependent SWItch/sucrose nonfermentable (SWI/SNF) complex functions as a primary chromatin remodeler during ontogeny, as well as in adult life. Several components of the complex have been suggested to function as important regulators of tumorigenesis in various cancers. In the current study, we have characterised a possible tumour suppressor role for the largest subunit of the complex, namely the AT-rich interaction domain 1B (ARID1B).

Methods: We performed Azacytidine and Trichostatin A treatments, followed by bisulphite sequencing to determine the possible DNA methylation-induced transcription repression of the gene in pancreatic cancer (PaCa) cell lines. Functional characterisation of effect of ARID1B ectopic expression in MiaPaCa2 PaCa cell line, which harboured *ARID1B* homozygous deletion, was carried out. Finally, we evaluated ARID1B protein expression in pancreatic tumour samples using immunohistochemistry on a tissue microarray.

Results: *ARID1B* was transcriptionally repressed due to promoter hypermethylation, and ectopic expression severely compromised the ability of MiaPaCa2 cells to form colonies in liquid culture and soft agar. In addition, ARID1B exhibited significantly reduced/loss of expression in PaCa tissue, especially in samples from advanced-stage tumours, when compared with normal pancreas.

Conclusion: The results therefore suggest a possible tumour-suppressor function for *ARID1B* in PaCa, thus adding to the growing list of SWI/SNF components with a similar function. Given the urgent need to design efficient targeted therapies for PaCa, our study assumes significance.

Pancreatic cancer (PaCa) continues to be a devastating disease despite recent improvements in the understanding of its biology. Pancreatic tumours are frequently associated with post-resection recurrence and are refractory to available treatment options (Buchler *et al*, 1991). Most patients harbour distant metastasis at the time of presentation, and therefore not eligible for resection; the 5-year survival rate for such patients is close to nil. Identification of PaCa oncogenes exhibiting recurrent activation including *KRAS* and *AKT2*, and of tumour suppressor genes (TSGs) exhibiting recurrent inactivation including *TP53*, *CDKN2A*

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and *SMAD4* has improved our understanding of pancreatic tumours (Bardeesy and DePinho, 2002). Recent advances in the field of cancer genomics have revealed interesting insights into the complex biology of the disease and are expected to hasten the development of efficient targeted therapies. Given the ease of targeting, researchers worldwide have focused their efforts on characterisation of PaCa oncogenes; the study of PaCa TSGs has been neglected to a significant extent. Of note, recent studies have indicated that a majority of driver mutations in all cancers occur in TSGs rather than oncogenes (Bozic *et al*, 2010).

Epigenetic modifications are cardinal regulators of eukaryotic gene expression, especially with respect to embryonic development and oncogenic transformation. The human SWItch/sucrose nonfermentable (SWI/SNF) ATP-dependent chromatin-remodelling complex, components of which are orthologs of the *Drosophila* trithorax family (Kennison, 1995; Schuettengruber *et al*, 2007), includes either of the two ATPase-containing subunits, namely BRG1 and BRM. The largest subunit named BAF250 or AT-rich interaction domain 1 (ARID1) exists in two mutually exclusive isoforms viz BAF250a (ARID1A) and BAF250b (ARID1B) (Wang *et al*, 1996). Based on presence or absence of these subunits, four distinct complexes can be envisaged. In addition, another variant (the PBAF complex) has been reported, which harbours *BRG1* in association with BAF180 and BAF200 (Yan *et al*, 2005). Mutation/deletion-induced inactivation of BRG1 and, to a lesser extent, of *BRM* has been described in many tumour types (Weissman and Knudsen, 2009). Recently, inactivation of SNF5 (a core component of the complex), resulting in alleviation of repression of Polycomb group proteins such as EZH2, was shown to be an important driver event in malignant rhabdoid tumours (Wilson *et al*, 2010). In addition, tumour-suppressor roles for BAF155 and BAF57 (core components of the complex) have also been validated (Weissman and Knudsen, 2009).

Our earlier studies based on array-based comparative genomic hybridisation (aCGH) carried out on PaCa cell lines (Bashyam *et al*, 2005) and xenografts (Kwei *et al*, 2008) led to the identification of several localised amplifications and deletions in specific chromosomal regions. Characterisation of two recurrent amplifications at 18q11.2 and 7q21 led to the assignment of an oncogenic function to GATA6 (Kwei *et al*, 2008) and SMURF1 (Kwei *et al*, 2011), respectively, in PaCa. More importantly, we identified several localised homozygous deletions validated by multiplex PCR, including one located at 6q25.3 that harboured a single annotated gene viz *ARID1B* (Bashyam *et al*, 2005). In the current study, we propose a tumour-suppressor function for *ARID1B* in PaCa based on functional studies, methylation analysis of promoter CpG islands and expression analysis in pancreas tumour samples.

MATERIALS AND METHODS

PaCa cell line propagation and manipulation. PaCa cell lines were procured from the ATCC, USA; propagation and DNA/RNA isolations were performed as described earlier (Bashyam *et al*, 2005). For Azacytidine and Trichostatin A (TSA) (Sigma, St. Louis, MO, USA) treatments, 0.2 million cells were seeded in 60-mm dishes and exposed (amounts (in μM) indicated in Figures 1A and B) for 24 h, followed by a fresh exposure for 24 h. Quantitative reverse transcription PCR was performed to evaluate *ARID1B* transcript levels relative to *GAPDH* as described in Supplementary Methods S1.

Analysis of ARID1B CpG methylation. Putative CpG island was identified upstream of the *ARID1B* start codon using MethPrimer (www.urogene.org/methprimer/index1.html) and CPGPLOT (www.ebi.ac.uk/Tools/emboss/cpgplot) with default parameters. PCR primer pairs specific to modified DNA sequence and flanking the putative *ARID1B* CpG-rich sequence were designed using MethPrimer such that each amplicon size was <400 bp (Figure 1C top panel and Supplementary Methods S1). All primers (except 3PR) did not include CpG dinucleotide (sequences are listed in Supplementary Methods S1). Bisulphite modification of genomic DNA isolated from cell lines was performed using EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA), as per manufacturer's instructions. PCR products were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA), as per standard protocol. Recombinant plasmids were sequenced using the $3130 \times$l genetic analyser (ABI Inc, Foster city, CA, USA), as per the manufacturer's instructions. A conversion frequency of $>$ 98% was confirmed in each recombinant plasmid.

Ectopic expression of ARID1B and functional assays. *ARID1B* cDNA construct (KIAA1235), a kind gift from the Kazusa DNA research institute (Chiba, Japan), encodes a partially truncated 1485 amino acid protein (devoid of the N-terminal 805 amino acids). Previous studies have validated functional activity of the ARID1B protein expressed from this construct (Inoue *et al*, 2002, 2011). *ARID1B* cDNA was cloned into the pcDNA3.1HisC vector as described in Supplementary Methods S1. MiaPaCa2 cells were transfected with the ARID1B-pcDNA3.1HisC recombinant plasmid construct in 60-mm culture dishes using lipofectamine 2000 (Invitrogen), as per manufacturer's protocol; pcDNA31.HisC vector was transfected separately as a control. Transfected cells were passaged into 100-mm dishes in presence of 100 μg ml^{-1} Neomycin (G418) to obtain a single cell spread. Neomycin-resistant colonies appeared after 20–21 days of selection and were isolated by localised trypsinization method, grown separately on 96-well plates and further expanded in 6-well plates. Expression of the Neomycin gene in recombinant clones was confirmed by RT–PCR (data not shown). *ARID1B* expression was confirmed using RT–PCR in the ARID1B clones (but not in the vector clones), as per strategy outlined in Supplementary Methods S1. In separate transfection experiments, colonies (obtained from both *ARID1B* and vector transfectants of MiaPaCa2 and PANC1 cells) were pooled independently following 21 days of selection under Neomycin to perform a colony-formation assay (Supplementary Methods S1). Other functional assays were performed as described in Supplementary Methods S1.

Construction of tissue microarray (TMA) and immunohistochemistry (IHC). A PaCa TMA was constructed using the Minicore tissue arrayer (Alphelys, Plaisir, France) as described previously (Ginestier *et al*, 2002). A total of 52 sample pairs (tumour and matched normal) in the form of formalin-fixed paraffin-embedded blocks representing pancreatic tumours were collected from the Nizam's Institute of Medical Sciences, Hyderabad, following approval from the hospital ethics committee. The samples included well, moderate and poorly differentiated ductal adenocarcinoma; specialized adenocarcinoma including cyst-adenocarcinoma, mucinous and papillary adenocarcinoma and clear cell carcinoma; and neuroendocrine tumours. The array was constructed with at least two representative 1-mm cores from each tumour and normal sample. IHC was performed on 4-μm sections on an automated immunostainer (Ventana Benchmark XT, Tucson, AZ, USA) using ARID1B (clone 2F2, Novus Biologicals, Littleton, CO, USA; dilution of 1 : 200) and p53 (clone DO-1, EMD Millipore Calbiochem, Billerica, MA, USA; dilution of 1 : 100) antibodies separately, as per manufacturer's instructions. For ARID1B, nuclear staining intensity (absent, weak, moderate and strong; 0–3 scale) and fractional epithelium staining (absent, up to 25, 50, 75 and 100%; 0–4 scale) were evaluated by the pathologist and summated for a final staining score. For p53, $>$20% positivity was considered as nuclear stabilisation.

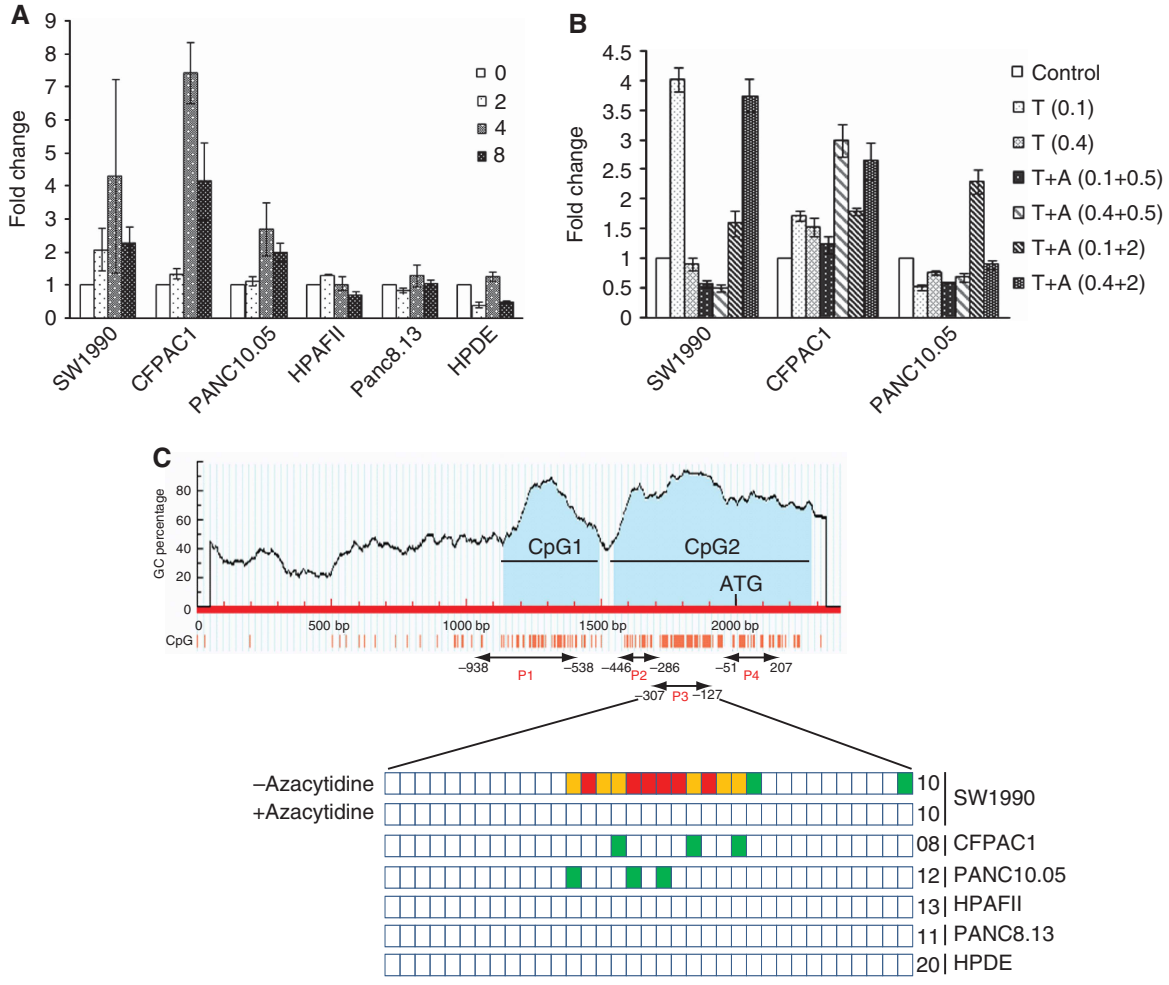

Figure 1. Evaluation of CpG methylation-induced transcriptional repression of *ARID1B*. Panels (**A**) and (**B**) show effect of Azacytidine and TSA, respectively, on several PaCa cell lines; fold increase in *ARID1B* transcript level in each cell line is plotted separately in response to varying concentrations (in μM indicated) of Azacytidine and TSA. The transcript level in absence of treatment in each cell line is normalised to 1.0. Error bars represent s.e.m.; each Azacytidine/TSA experiment was performed at least thrice. A, Azacytidine; T, Trichostatin (**A**). Panel (**C**) (top) shows position of the four *ARID1B* primer pairs used for PCR amplification of putative *ARID1B* promoter CpG island. Nucleotide position of the 5′ and 3′ ends of the four amplicons with respect to the translation initiation codon (ATG) are indicated. The bottom panel depicts the result of bisulphite sequencing-based evaluation of methylation status for the P3 region. Each row of boxes represents result for one cell line; each box represents one CpG dinucleotide and per cent of cytosine methylation is denoted by a colour code (white, <10%; green, 10–33%; orange, 34–66%; red, >66%). The total number of clones analysed for each cell line is given at the end of each row of boxes. Methylation status in SW1990 was also evaluated, following Azacytidine (8 μM) treatment.

RESULTS

ARID1B exhibits promoter CpG methylation-induced transcriptional repression in PaCa cell lines. TSGs are inactivated in tumour cells through several distinct mechanism(s), not the least being mutation and methylation-induced transcription silencing. We screened genomic DNA isolated from 10 PaCa cell lines for presence of mutations in the coding region, promoter, UTRs and splice consensus sequences by PCR-DNA sequencing and did not detect a single mutation (data not shown). In addition, the same *ARID1B* transcript isoform was detected in PaCa cell lines and pancreatic normal tissue (data not shown). Azacytidine, frequently used to determine genes transcriptionally repressed through methylated CpGs in the promoter, caused a significant elevation in *ARID1B* transcript levels in SW1990, PANC10.05 and CFPAC1, but not in PANC8.13, HPAFII and PANC1, commensurate with lower (SW1990, PANC10.05 and CFPAC1; Supplementary Figure S1) and higher (PANC8.13, HPAFII and PANC1; Supplementary Figure S1) *ARID1B* transcript levels (Figure 1A). In addition, Azacytidine had no significant effect on *ARID1B* transcript level in the nontumorigenic human pancreatic ductal epithelial (HPDE) cells (Figure 1A) known to exhibit adequate expression of SWI/SNF components, including ARID1B (Ouyang *et al*, 2000; Shain *et al*, 2012). Elevation in *ARID1B* transcript levels was evident in SW1990, PANC10.05 and CFPAC1 when subjected to TSA treatment as well (Figure 1B).

Next, we identified CpG-rich sequences in the putative promoter region of *ARID1B*, spanning about 2 kb upstream of the translation initiation codon (Figure 1C top panel). Bisulphite sequencing using primer pairs specific for the CpG-rich regions (Figure 1C and Supplementary Methods S1) revealed significant methylation of CpGs located in the P3 region (present immediately upstream to the *ARID1B* translation initiation codon; Figure 1C top panel), particularly in SW1990. Further, the methylation (in SW1990) was completely alleviated upon Azacytidine treatment (Figure 1C, bottom panel), suggesting that methylation was responsible for *ARID1B* transcription silencing. Moderately significant methylation in P2 and P4 regions was also observed (Supplementary Figure S2), though no significant methylation was

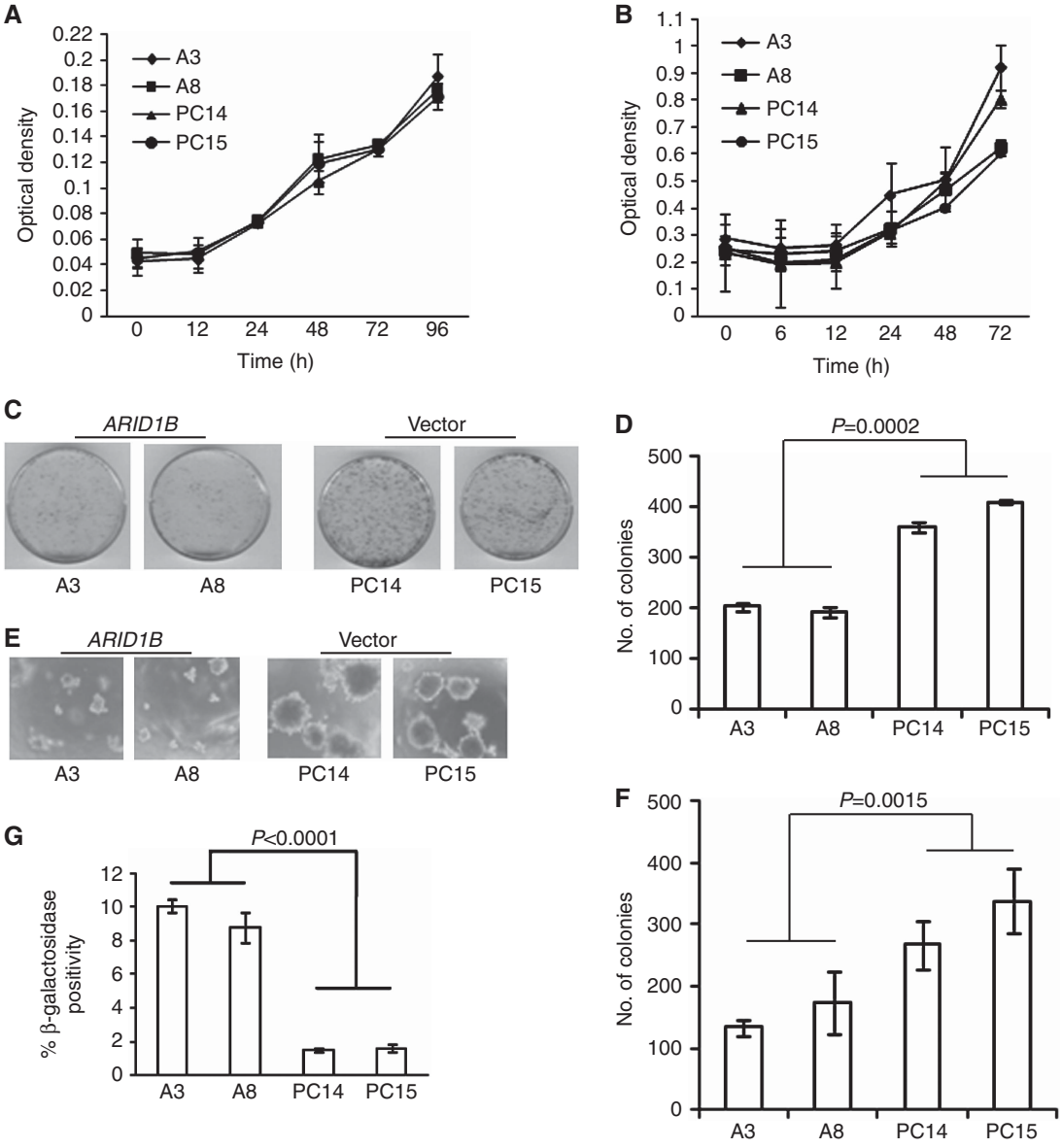

Figure 2. Functional characterisation of *ARID1B* permanent transfectants of MiaPaCa2. Panels (**A**) and (**B**) show result of MTT and crystal violet dye extraction growth assays performed for the two *ARID1B* (A3 and A8) and two vector (PC14 and PC15) clones of MiaPaCa2, respectively. Error bars represent s.e.m. of two (MTT assay) and three (crystal violet assay) independent experiments (performed in duplicate), respectively. Panels (**C**) and (**E**) show representative results of liquid and soft agar colony-formation assays, respectively. Panels (**D**) and (**F**) show graphical representation of result of liquid and soft agar colony-formation assays, respectively. Panel (**G**) shows quantitation of β-galactosidase staining in the same clones. Error bars represent s.e.m. of three independent liquid colony formation (in duplicate), soft agar and β-galactosidase staining (both in triplicate) experiments. *P*-value corresponds to unpaired Student's *t*-test.

detected in the P1 region (data not shown). In contrast, PANC8.13, HPAFII and HPDE either harboured negligible or nil methylation of the CpGs in these regions (Figure 1C and Supplementary Figure S2).

Ectopic expression of ARID1B abrogates tumour-related characteristics of PaCa cell line. In our previous study, the 6q25.3 homozygous deletion (harbouring the *ARID1B* gene) identified in the MiaPaCa2 PaCa cell line was PCR validated using primer pairs specific for the *ARID1B* 3′-UTR region (Bashyam *et al*, 2005). Therefore, we first confirmed *ARID1B* complete deletion using PCR primers specific for exons 2, 5, 10, 12, 15, 17 and 20 (data not shown). Next, in order to determine the effect of *ARID1B* ectopic expression in MiaPaCa2 cells, we generated pcDNA3.1HisC clones of *ARID1B* (and of the vector as a control) as described in the MATERIALS AND METHODS section. We chose two vector and *ARID1B* clones each for analysis. Expression of *ARID1B* transcript in the *ARID1B* clones was confirmed using RT–PCR (Supplementary Figure S3); however, protein expression could not be detected, though three different antibodies were tested (data not shown). MTT assay (Figure 2A), crystal violet dye extraction growth assay (Figure 2B), a standard plating assay (percentage of cells adhering to the surface upon seeding; data not shown) as well as FACS-based cell cycle analysis (data not shown) did not reveal a significant difference in growth kinetics of *ARID1B* and vector clones. Similarly, the percentage of apoptotic population in the gene and vector clones was not significantly different (data not shown). However, the *ARID1B* clones exhibited significantly reduced ability to form colonies in liquid culture as compared with the vector clones (Figures 2C and D), indicating a reduced clonogenicity in case of the former. In addition, the *ARID1B* clones generated significantly

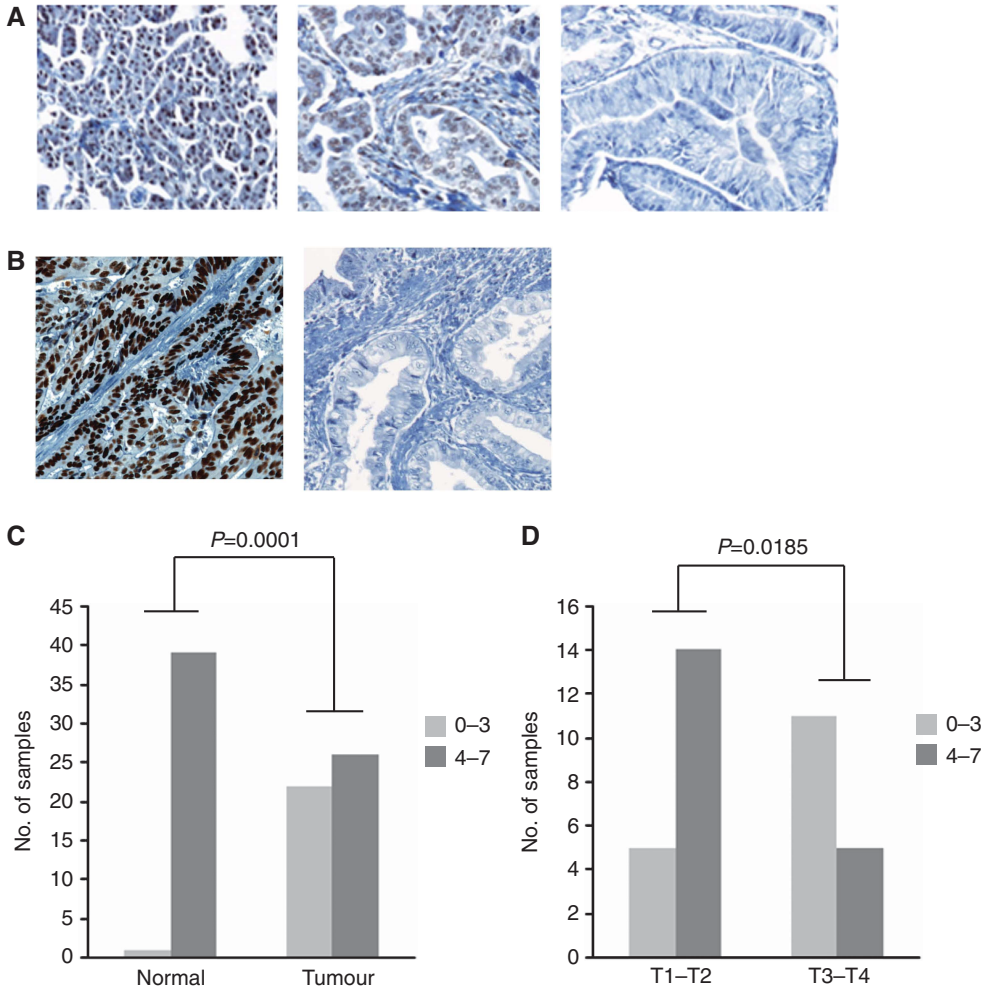

Figure 3. Analysis of ARID1B and p53 expression using a pancreatic cancer tissue microarray. Panel (**A**) shows representative results for ARID1B for normal pancreas (left, strong nuclear stain) and pancreatic tumour (middle, moderate nuclear stain and right, negative nuclear stain). Representative results for p53 IHC performed on PaCa samples are shown in panel (**B**) (left, positive; right, negative). Panel (**C**) shows graphical representation of comparative analysis of ARID1B expression in pancreatic tumour and normal samples; samples were split into two categories based on their IHC scores (0–3 representing negative to weak staining and 4–7, indicating moderate to strong staining) calculated as described in MATERIALS AND METHODS. Panel (**D**) shows graphical representation of comparative analysis of ARID1B expression in early (T1–T2) and late (T3–T4) stage pancreatic tumour samples, based on IHC scores. Fisher's exact test *P*-values are shown.

less number (and smaller size) of colonies in soft agar, indicating a reduced ability to grow in the absence of anchorage (Figures 2E and F). In order to rule out the possibility of reduced colony-formation ability of *ARID1B* clones being attributable specifically to the two selected clones and not to *ARID1B* expression, we performed liquid colony-formation assay separately using a 'pool' of *ARID1B* and vector clones obtained from an independent MiaPaCa2 transfection experiment (MATERIALS AND METHODS). The pooled *ARID1B* clones generated significantly fewer colonies compared with the pooled vector clones, whereas there was no significant difference in colonies generated from pooled vector and *ARID1B* PANC1 clones (Supplementary Figure S4), thus strengthening our premise that *ARID1B* expression was indeed responsible for reduced colony-formation ability. Given that ectopic expression of the SWI/SNF component BRG1 induces a senescence phenotype in rat mesenchymal stem cells (Napolitano *et al*, 2007) as well as in PaCa cells (Shain *et al*, 2012), we tested whether *ARID1B* expression could have a similar effect on MiaPaCa2 cells. The *ARID1B* clones exhibited significantly higher senescence-associated β-galactosidase activity when compared with the vector clones (Supplementary Figure S5 and Figure 2G).

Reduced/loss of ARID1B expression in PaCa tissues. We constructed a TMA to evaluate ARID1B and p53 expression status in PaCa tumour samples using IHC (Figures 3A and B). Interestingly, a significant proportion of tumour samples exhibited negative/reduced staining when compared with normal pancreas ($P = 0.0001$, Fisher's exact test; Figure 3C). Moreover, the loss of ARID1B expression was significantly associated with advanced-tumour stage ($P = 0.0185$, Fisher's exact test; Figure 3D), indicating perhaps it could be a late event. As a comparison, p53 exhibited nuclear stabilisation in 19 of 42 samples (representative result shown in Figure 3B) and there was no association with ARID1B status nor with tumour stage (data not shown). These results provide further evidence for a tumour-suppressor role for *ARID1B* in PaCa.

DISCUSSION

Deletion or rearrangement of the 6q25.3 region has been described in breast (Utada *et al*, 2000), cervical (Mazurenko *et al*, 2003) and

other cancers (Monoranu *et al*, 2008). In the current study, we endeavoured to characterise a homozygous deletion localised at 6q25.3, identified in the MiaPaCa2 PaCa cell line in our previous studies (Bashyam *et al*, 2005; Shain *et al*, 2012) and in other studies (Birnbaum *et al*, 2011), which included only one annotated gene viz *ARID1B*. Gene mutation is a common mode of inactivation of TSGs; however, we did not detect a single mutation in 10 PaCa cell lines tested. Azacytidine (Figure 1A) and Trichostatin A (Figure 1B) treatments, however, resulted in significant elevation in *ARID1B* transcript level in PaCa cell lines, suggesting suppression of gene expression through DNA methylation, which was confirmed by bisulphite sequencing (Figure 1C). HLTF, encoding another member of the SWI/SNF family, was earlier shown to be transcriptionally repressed following CpG methylation in lung cancer cell lines, and the repression was alleviated upon treatment with Azacytidine (Moinova *et al*, 2002).

Ectopic expression of SWI/SNF components into cancer cells has been shown to result in elevation of antitumor characteristics such as cell cycle arrest, apoptosis or differentiation, depending on the cancer cell type and the identity of the inactivated component (Caramel *et al*, 2008; Dunaief *et al*, 1994; Wang *et al*, 2005). Recent studies have suggested a tumour-suppressor role for components of the SWI/SNF complex in several cancers (Wilson and Roberts, 2011). SiRNA-mediated knockdown of *ARID1B* was earlier shown to cause a significant reduction of growth factor-independent growth in HPDE (Shain *et al*, 2012), further corroborating our observations. The reduced/loss of ARID1B expression in PaCa when compared with normal pancreatic tissues (Figure 3C) suggests the possible importance of the gene in human pancreatic tumours.

Previous studies on preosteoblast cell lines revealed opposing roles for ARID1A- and ARID1B-containing SWI/SNF complexes, with the former causing cell cycle arrest, whereas the latter appeared to be involved in cell cycle progression (Nagl *et al*, 2007). On the other hand, ARID1B levels increase during differentiation of embryonic stem cells (Kaeser *et al*, 2008) and ectopic expression induces increased expression of p53 and p21, followed by cell cycle arrest in HeLa cells (Inoue *et al*, 2011). It is therefore possible that ARID1B may have distinct roles depending on the cellular context. *ARID1A* appears to be a specific tumour suppressor for ovarian clear cell carcinomas (Wiegand *et al*, 2010). Interestingly, the inactivation of ARID1A in clear cell and endometrioid carcinomas also in breast cancer cell lines (Mamo *et al*, 2012) appears to be achieved predominantly through mutations, with little or no evidence of epigenetic inactivation (Wiegand *et al*, 2010). In contrast, we did not detect any ARID1B mutation in all PaCa cell lines screened. An earlier report had also revealed the absence of *ARID1B* mutations in PaCa cell lines and tumour samples (Birnbaum *et al*, 2011). The roles of ARID1A and ARID1B in different tumour types could possibly reflect differences in their tissue-specific expression.

We were unable to detect ARID1B protein expression in the permanent transfectants generated using pcDNA3.1HisC, though several clones were evaluated; the *ARID1B* transcript was however readily detected (Supplementary Figure S3). Tumour cells often exhibit reduced fitness when tumour suppressors are expressed at high levels (Kucuk *et al*, 2011). The phenotypic changes exhibited by the *ARID1B*–MiaPaCa2 clones are probably a result of low-level expression that was undetected by western blot. As chromatin modulators are expected to generate a pleiotropic effect, a small increment in expression may be expected to result in visible phenotypic changes. A previous study on MiaPaCa2 cells (which lack expression of BRM) showed undetected BRM protein expression when overexpressed, but the low expression did result in phenotypic changes (Rosson *et al*, 2005). In the current study, overexpression of ARID1B had no significant effect on growth of MiaPaCa2 cells, though the transfectants exhibited significantly reduced ability to form colonies in liquid media and soft agar (Figures 2C–F). Of interest, the tumour suppressor *ING4,* which functions through interaction with chromatin remodelling factors, elicits a reduced clonogenicity without affecting proliferation in rat fibroblast cells (Kim *et al*, 2004). Similarly, ectopic expression of BAF155 (a core component of the human SWI/SNF complex) in cancer cell lines was recently shown to reduce colony-formation ability without affecting apoptosis (DelBove *et al*, 2011).

As MiaPaCa2 cells do not express BRM (Rosson *et al*, 2005), our observations pertaining to the antioncogenic effect of ARID1B expression is expected to be a result of the action of a SWI/SNF complex harbouring BRG1 and ARID1B. Interestingly, BRG1 and ARID1B have been suggested to harbour a similar expression profile and often localise to transcriptionally active promoters (Flores-Alcantar *et al*, 2011), as against BRM and ARID1A. Given that the BRG1-containing complex is known to activate genes that participate in cell cycle arrest (Napolitano *et al*, 2007) downstream of TGF beta signalling (Xi *et al*, 2008), and that the BRG1/ARID1B SWI/SNF complex activates expression of TSGs, such as *p21* (Inoue *et al*, 2011), our results showing an increased senescence-associated β-galactosidase activity in cells expressing ARID1B (Supplementary Figure S5 and Figure 2G) assume significance. The results obtained in this study therefore propose a tumour-suppressor role for ARID1B in PaCa.

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