Protein arginine methyltransferase 6-dependent gene expression and splicing: association with breast cancer outcomes

Dennis H Dowhan^{1*}, Matthew J Harrison^{1*,†}, Natalie A Eriksson^{1*}, Peter Bailey^{2*}, Michael A Pearen¹, Peter J Fuller³, John W Funder³, Evan R Simpson³, Peter J Leedman⁴, Wayne D Tilley⁵, Melissa A Brown², Christine L Clarke⁶ and George E O Muscat¹

¹Institute for Molecular Bioscience and ²School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Queensland 4072, Australia

³Prince Henry's Institute for Medical Research, Clayton, Victoria 3168, Australia

⁴Laboratory for Cancer Medicine, Western Australian Institute for Medical Research and University of Western Australia Centre for Medical Research, Royal Perth Hospital, Perth, Western Australia 6000, Australia

⁵Dame Roma Mitchell Cancer Research Laboratories, School of Medicine, Hanson Institute, The University of Adelaide, Adelaide, South Australia 5000, Australia

⁶University of Sydney Western Clinical School, Westmead Institute for Cancer Research, Westmead Millennium Institute, Westmead, New South Wales 2145, Australia

(Correspondence should be addressed to D H Dowhan; Email: d.dowhan@uq.edu.au)

*(D H Dowhan, M J Harrison, N A Eriksson and P Bailey are co-first authors)

†(M J Harrison is now at School of Biomedical Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia)

Abstract

Protein arginine methyltransferase-6 (PRMT6) regulates steroid-dependent transcription and alternative splicing and is implicated in endocrine system development and function, cell death, cell cycle, gene expression and cancer. Despite its role in these processes, little is known about its function and cellular targets in breast cancer. To identify novel gene targets regulated by PRMT6 in breast cancer cells, we used a combination of small interfering RNA and exon-specific microarray profiling *in vitro* coupled to *in vivo* validation in normal breast and primary human breast tumours. This approach, which allows the examination of genome-wide changes in individual exon usage and total transcript levels, demonstrated that *PRMT6* knockdown significantly affected i) the transcription of 159 genes and ii) alternate splicing of 449 genes. The *PRMT6*-dependent transcriptional and alternative splicing targets identified *in vitro* were validated in human breast tumours. Using the list of genes differentially expressed between normal and *PRMT6* knockdown cells, we generated a *PRMT6*-dependent gene expression signature that provides an indication of PRMT6 dysfunction in breast cancer cells. Interrogation of several well-studied breast cancer microarray expression datasets with the *PRMT6* gene expression signature demonstrated that PRMT6 dysfunction is associated with better overall relapse-free and distant metastasis-free survival in the oestrogen receptor (ER (ESR1)) breast cancer subgroup. These results suggest that dysregulation of *PRMT6*-dependent transcription and alternative splicing may be involved in breast cancer pathophysiology and the molecular consequences identifying a unique and informative biomarker profile.

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Introduction

Protein arginine methylation is a common post-translational modification that was first described over 40 years ago (Paik & Kim 1967). It is catalysed by a family of 11 human protein arginine

methyltransferases (PRMTs) and results in the transfer of either one or two methyl groups from the methyl donor *S*-adenosyl-L-methionine to guanidino nitrogen atoms in arginine residues of the target protein. This alters the function and activity of the protein and

impacts on a diverse range of cellular processes including differentiation, DNA repair, transcription, RNA processing, signal transduction, cell localisation and apoptosis (Bedford & Richard 2005). As would be expected of proteins that regulate such diverse processes, their aberrant expression and activity have been linked to a number of human diseases, including cancer (Aletta & Hu 2008).

PRMTs regulate transcription by functioning as co-activators for a range of transcription factors, including steroid/nuclear receptors, p53, YY1 and NF- κ B; E2F1; MEF2C and β -catenin (Bedford & Clarke 2009, Lee & Stallcup 2009). We have recently shown that PRMT6 can also co-activate steroid hormone receptors (SHRs), including the ER α , progesterone receptor and glucocorticoid receptor (GR (NR3C1)), in a methylation-dependent manner (Harrison *et al.* 2010). This appears to contrast with the studies demonstrating that PRMT6 can repress transcription by methylating histone 3 at arginine 2 (H3R2), which prevents the mixed lineage leukaemia methyltransferase complex binding to and methylating histone 3 at lysine 4 (H3K4; Guccione *et al.* 2007, Hyllus *et al.* 2007, Iberg *et al.* 2008). Therefore, whether PRMT6 functions as a transcriptional enhancer or repressor in relation to gene expression remains controversial.

PRMTs also regulate alternate splicing in a methylation-dependent manner. Co-activator-associated arginine methyltransferase 1 (CARM1) can methylate a number of proteins involved in pre-mRNA processing and promotes skipping of both a splicing reporter and the endogenous *CD44* gene (Cheng *et al.* 2007). PRMT6, which can methylate the splicing factors RDA288 and heterogeneous nuclear ribonucleoprotein D-like (Cheng *et al.* 2007), promotes exon skipping of vascular endothelial growth factor (*VEGF* (*VEGFA*)) and spleen tyrosine kinase (*SYK*) genes in MCF-7 and T-47D breast cancer cells (Harrison *et al.* 2010).

Initial studies have identified several PRMT proteins as having a key role in SHR-mediated prostate cancer, breast cancer, gastric cancer, lymphoma and leukaemia (Teyssier *et al.* 2010). Many proteins involved in RNA splicing/processing are methylated and/or associated in complexes with PRMTs. The distinct possibility arises that PRMTs are able to directly methylate and regulate proteins involved in RNA processing events and alternative splicing. Exon profiling in the framework of transcriptomic analysis is an emerging area in disease diagnosis and prognosis (Dutertre *et al.* 2010). The fact that certain PRMTs are associated with RNA

processing and gene expression makes them a clear choice to examine their ability to regulate alternative splicing and transcription in breast cancer.

Materials and methods

Cell culture and transfection

MCF-7 cells were maintained in DMEM nutrient mixture F-12 plus 10% FBS. T-47D cells were maintained in RPMI-1640 medium plus 10% FBS, 5 μ g/ml insulin, 10 mM HEPES and 1 mM sodium pyruvate. Both breast cancer cells lines were maintained at 37 °C and 5% CO₂. For small interfering RNA (siRNA) experiments in MCF-7 and/or T-47D cell lines, cells were transfected for 48 h with PRMT6-specific siRNA or control siRNA duplexes at a final concentration of 10 nM using RNAiMAX (Invitrogen, Victoria, Australia), with six biological replicates for each treatment regime, as described previously (Harrison *et al.* 2010). siRNA sequences were PRMT6-siRNA-1, sense 5'-CGGGACCAGCUGUACUACGTT-3', PRMT6-siRNA-1, antisense 5'-CGUAGUACAGCUGGUCCCGTT-3', PRMT6-siRNA-2, sense 5'-GCACUUGUAUUUCCGUAUATT-3', PRMT6-siRNA-2, antisense 5'-UAUACGGAAUA-CAAGUGCTT-3'. A siRNA control, Silencer Select negative control #1 (Ambion, Texas, USA), was used as a negative control (control siRNA), which has a proprietary unreleased sequence.

Exon microarray data analysis

Labelling and hybridisation of the Affymetrix Exon 1.0 ST Arrays were performed by the Ramaciotti Centre for Gene Function Analysis (UNSW, Sydney, Australia) following the recommended Affymetrix protocols. Exon array data were analysed using XRAY excel array expression analysis software (Biotique Systems, Inc., Nevada, USA). Normalized data and raw gene expression files have been submitted to the NCBI Gene Expression Omnibus (GEO; Barrett *et al.* 2005), accession number: GSE36542.

Input files were normalised with full quantile normalisation to remove variation produced amongst samples during array labelling, hybridisation and scanning. Principal component analysis was performed using GeneSpring GX version 11 (Agilent, Victoria, Australia) on each sample to confirm similarity between samples with the same treatment. Principal component analysis scores were used to form a three-dimensional plot of each sample.

Probe sets then underwent filtering (to ensure that only high quality probe sets were included in the

analysis), GC content-based probe screening, log transformation and background correction using GC-matched probes. Individual probe sets that are not significantly expressed above background can produce false positives for alternative splicing and so were excluded from tests of alternative exon usage. Probe sets are ranked as Core, Extended or Full depending on their reliability. Only Core probe sets, which are derived from high-quality genomic features like RefSDefault (www.ncbi.nlm.nih.gov) or Ensembl (www.ensembl.org), were used in the analysis. The expression score for each probe set was then derived via Tukey's median-polish and Mixed Model, and Nested ANOVA was then used to identify changes in gene expression or alternative splicing between groups. Alternative splicing results were corrected using the Benjamini and Hochberg False Discovery Rate to remove false positives. Genes were considered to be differentially expressed or alternatively spliced when $P < 0.01$ (Supplementary Tables 2 and 3).

To determine splice events within significantly alternatively spliced genes, probe sets showing 'non-parallel' lines when average group expression is plotted over the probe sets were identified. Probe sets displaying 'non-parallel' expression were assigned to a particular exon using X:map (<http://xmap.picr.man.ac.uk/>). Changes in exon-specific splicing were then compared to known alternative splicing events using X:map (built using the Ensembl database (www.ensembl.org)) and FAST DB (<http://www.fastdb.com/fastdb2/frame.html>), built by combining databases within www.ensembl.org, <http://genome.ucsc.edu> and www.ncbi.nlm.nih.gov.

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was extracted from cells using Trizol Reagent (Invitrogen) and purified using RNeasy RNA columns, with on-column DNase treatment (Qiagen, Victoria, Australia). RNA was normalised using u.v. spectrophotometry and agarose gel electrophoresis. RNA integrity was determined using RNA 6000 Nano chips (Agilent) on a 2100 BioAnalyser (Agilent) according to the manufacturer's instructions. cDNA was synthesized from 600 ng of total RNA using TaqMan reverse transcription reagents, according to the manufacturer's instructions (Applied Biosystems, Victoria, Australia).

Gene expression levels were determined by 384-well quantitative real-time PCR (Q-RT-PCR) on a 7900HT Fast RT-PCR System (Applied

Biosystems) as described previously (Harrison *et al.* 2010). RT-PCR was performed using Assay on Demand TaqMan primer/probes (Applied Biosystems) or Sybr green; PRMT6 (Hs00250803_s1) CARM1 (Hs00406354_m1), PRMT1 (Hs01587651_g1), NCOA4 (Hs00428331_g1), FKBP4 (Hs00427038_g1), HIPK3 (Hs00178628_m1), ARL6IP1 (Hs00760013_s1), YWHAE (Hs00356749_g1), MINA (Hs00262155_m1), PTEN (Hs02621230_s1), IGFBP3 (Hs00181211_m1), THEM4 (Hs00940013_g1), BRCA2 and CDKN1A interacting protein α (BCCIP α ; Hs01567098_m1) and BCCIP β (Hs01555220_m1). All expression levels were normalised to *18S* RNA or *RLP0* expression levels using TaqMan primer/probe sets (Applied Biosystems). Target cDNA levels were analysed by Q-RT-PCR in 10 μ l reactions using TaqMan PCR master mix (Applied Biosystems), TaqMan probe/primer sets and cDNA (5% of the starting 600 ng of RNA). PCR was initiated at 95 °C for 10 min to activate Amplitaq Gold DNA polymerase followed by 45 cycles of 95 °C for 15 s and 60 °C for a 1 min two-step thermal cycling. Relative changes in gene expression were calculated using the $\Delta\Delta C_t$ method. For each experiment, six replicate wells of cells were used for each experimental condition and all experiments were repeated twice.

Alternative splicing validation of ninein (GSK3B interacting protein) (NIN), catenin (cadherin-associated protein)-delta 1 (CTNND1) and thioesterase superfamily member 4 (THEM4) was performed by Q-RT-PCR on a 7900HT Fast RT-PCR System (Applied Biosystems) in a 10 μ l reaction using SYBR-green master mix (Applied Biosystems) using manufacturer's instructions. Primers, which were designed to specifically amplify the alternatively spliced isoforms, were NIN inclusion – forward 5'-GAGTCACTATTTTAGTTAAGC-3', NIN inclusion – reverse 5'-AGATTCATATCTCA-GAAGTTC-3', NIN skipping – forward 5'-GAACTA-GAGCAGTTTCACCAG-3', NIN skipping – reverse 5'-CAGATTCATATCTTCCCTCCT-3', CTNND1 inclusion – forward 5'-ACACCCTTGATGCA-GGACGAG-3', CTNND1 inclusion – reverse 5'-AATCTTCTGCATGGAGGGGTA-3', CTNND1 skipping – forward 5'-ACAACCTATCCACAC-CAAATG-3', CTNND1 skipping – reverse 5'-AATCTTCTGCATCAAGGGTGT-3', THEM4 inclusion – forward 5'-CATTTTCTTGCTGGTAAATG-3', THEM4 inclusion – reverse 5'-GTTGGTCAGGCTG-GTCTTGAA-3' and THEM4 skipping – forward 5'-ACAAGGACCTAAGACTGCTCT-3', THEM4 skipping – reverse 5'-AAGCTTTGGGTCAAGAAA-ATG-3'. RT-PCR was initiated at 95 °C for 10 min to activate Amplitaq Gold DNA polymerase followed

by 45 cycles of 95 °C for 15 s and 56 °C for a 1 min two-step thermal cycling. Relative changes in gene expression were calculated using the $\Delta\Delta C_t$ method. For each experiment, six replicate wells of cells were used for each experimental condition and all experiments were repeated twice.

Melt curve analysis was performed to ensure that only one product was amplified in the Q-RT-PCRs. To determine the specificity of the amplified product, PCR fragments amplified from MCF-7 cell cDNA using the above primer sets were sub-cloned into pCR2.1 using the TOPO-TA cloning kit (Invitrogen) and sequenced. We also verified that the Q-RT-PCR assay produces a linear standard curve by plotting the log of the starting quantity of the template against the C_t value after serially diluting the pCR2.1-cloned PCR products and analysing them by Q-RT-PCR using the appropriate primers. We also verified that primer sets designed to detect inclusion do not detect the pCR2.1-cloned skipping spliced product, and primer sets designed to detect skipping do not detect the pCR2.1-cloned PCR product including the variable exon in Q-RT-PCR assays. Statistical analysis of RT-PCR data was performed using either one- or two-tailed Student's *t*-tests as appropriate.

Human breast tissue samples

Data and de-identified breast tissue samples (either fresh frozen tissue or purified total RNA) were obtained from the tissue banks listed in the Acknowledgements section. The breast tissue samples used were from individual cases of primary invasive ductal carcinoma (IDC) or normal breast samples, with no known history of breast disease, were collected following breast reduction mastoplasty or from women who had volunteered normal breast biopsy tissue. This study was approved by the human research ethics committees of all participating institutions.

Q-RT-PCR of human breast tissue samples

Commercial micro-fluidic cards, the TaqMan Low-Density Arrays (TLDA, Applied Biosystems) and Q-RT-PCR using 384-well 7900HT Fast RT-PCR System were used to profile gene expression for breast tissue samples. The geNorm software imbedded within the ABI/intergenomics StatMiner V4.1 software package was used to compute least expression variation and select the most appropriate, stable and robust combination of internal control genes with which to normalise the expression data (against the mean of the most stable controls). TLDA were used according to the manufacturer's instructions and analysed as described previously

(Myers *et al.* 2009, Raichur *et al.* 2010). The TaqMan Gene Expression Assays used as controls were MRPL19 (Hs00608519_m1), PGK1 (Hs99999906_m1), PPIA (Hs99999904_m1), TFRC (Hs99999911_m1) and UBC (Hs00824723_m1) median. The TaqMan Gene Expression Assays used were AKR1C2;AKR1C1 (Hs00413886_m1), SLIRP (Hs00364015_m1), EDF1 (Hs00610152_m1), FKBP4 (Hs00427038_g1), HIPK3 (Hs00178628_m1), NCOA4 (Hs00428328_m1), NQO1 (Hs00168547_m1), PA2G4 (Hs00854538_g1), PRKCD (Hs00178914_m1), PTEN (Hs00829813_s1) and TRIP13 (Hs00188500_m1). For each sample, 1.5 µg of total RNA was reverse transcribed using random hexamers with SuperScript III reverse transcriptase (Invitrogen) in a total volume of 45 µl. A volume of DNase/RNase-free water and an equal volume of TaqMan universal master mix was added to each TLDA fill reservoir. Each GCT sample was run once whilst the cell lines were run three times. Four reservoirs per sample were filled. The TLDA includes all gene assays and endogenous controls in triplicate. After sealing the plate, it was run on an ABI 7900HT Real-Time instrument.

Examination of alternative splicing in breast tissue samples was done by 384-well Q-RT-PCR as described earlier, using IPO8 (Hs00183533_m1) and TBP (Hs00427620_m1) median as the controls for that specific cohort of samples. TaqMan primer/probes (Applied Biosystems) used were BCCIP α (Hs01567098_m1), BCCIP β (Hs01555220_m1), NINEIN-inclusion – forward: 5'-CACGTGCAGTGATGCAGC-3', NINEIN-inclusion – reverse: 5'-CTTCCTGAGATCCATTTAATGTCC-3', NINEIN-inclusion-Probe: 5'-6-FAM-TGAACCTTCTGAGATATGAAT-MGB-3', NINEIN-skipping – forward: 5'-GAGCTCTTGGAAGACCAAA-3', NINEIN-skipping – reverse: 5'-CTTCCTGAGATCCATTTAATGTCC-3', NINEIN-skipping-Probe: 5'-6-FAM-CAGGAGGGAAGATATGAATC-MGB-3', THEM4-inclusion – forward: 5'-ACACCTACTGAATGGATTCAAGACT-3', THEM4-inclusion – reverse: 5'-AGGATCCGCCTGCCTTG-3', THEM4-inclusion-Probe: 5'-6-FAM-CATTTTCTTGCTGTTA-MGB-3', THEM4-skipping – forward: 5'-ACGTACACCTAC-TGAATGGATT-3', THEM4-skipping – reverse: 5'-CTGGGCCTGTGACATTTGTTTC-3', THEM4-skipping-Probe: 5'-6-FAM-CATTTTCTTGACCAAAAGC-MGB-3'. TaqMan primer/probes (Applied Biosystems) for SYK have been described previously (Harrison *et al.* 2010).

The TLDA and Q-RT-PCR of breast tissue samples were analysed using the relative quantification method of C_t and the normalised C_t values (ΔC_t) of the

replicates between the control/calibrator and the target sample (normal breast) were compared as described previously (Myers *et al.* 2009, Raichur *et al.* 2010).

Analysis of breast cancer microarray datasets

Statistical analysis was performed in R (<http://www.r-project.org/>). Gene expression datasets were retrieved from Bioconductor (<http://bioconductor.org>). The gene signature used comprised the 159 genes that were differentially expressed (i.e. up- and down-regulated) in a significant manner relative to controls (≥ 1.3 -fold, $P < 0.01$) as identified in exon array analysis (Supplementary Table 2, see section on supplementary data given at the end of this article). Genes listed in Supplementary Table 2 that did not have a fold change ≥ 1.3 but did have a probability of differential expression $P < 0.01$ (highlighted in grey) were not included in the signature. Experimental data packages examined include *BreastCancerUPP* (Miller *et al.* 2011), *BreastCancerNKI* (van de Vijver *et al.* 2002) and *BreastCancerTRANSBIG* (Desmedt *et al.* 2008). PRMT6 signature scores were computed using the sig.score function from the *Genefu* package (Haibe-Kains *et al.* 2010a). This function computes a signature score from a gene list (aka gene signature), i.e. a signed average as previously published (Sotiriou *et al.* 2006, Haibe-Kains 2009). Briefly, positive or high signature scores are indicative of PRMT6 dysfunction (and by inference low PRMT6 expression levels), whereas negative or low signature scores are indicative of normal PRMT6 function (and by inference high PRMT6 levels). Survival analysis was performed using the *SurvComp* package (Haibe-Kains *et al.* 2008, 2010b). PRMT6 signature scores were split according to their 33% and 66% quantiles. Upper and lower quantiles were used for survival analysis. We considered relapse-free survival (RFS) as the survival endpoint. When RFS was not available, we used distant metastasis-free survival (DMFS) data. All survival data were censored at 10 years. Survival curves were based on Kaplan–Meier estimates.

Results

siRNA depletion of PRMT6 and detection of genome-wide exon expression

MCF-7 breast cancer cells were transfected with a validated siRNA targeting PRMT6 (PRMT6-siRNA-1) or a control siRNA, as previously reported (Harrison *et al.* 2010). PRMT6 siRNA-1 specifically reduced mRNA levels of PRMT6 by $\sim 80\%$ but did not affect the levels of PRMT1 or CARM1 (Fig. 1A). Alterations

in gene transcription and alternative splicing were determined by hybridisation of labelled cDNA onto GeneChip Human Exon 1.0 ST Arrays (Affymetrix). Following full quantile normalisation, principal component analysis was performed, with exon arrays clustering into groups depending on the treatment regime (Fig. 1B). This result demonstrates that replicates of the same siRNA treatment produced a similar exon expression profile but a distinctly different profile in relation to the siRNA treatment based on clustering distance. The data were filtered by removing less reliable probes, probes with a low or high GC content, and probes that were not significantly expressed above background. The effects of the filtering are summarised in Supplementary Table 1, see section on supplementary data given at the end of this article. Of the different alternatively spliced products (referred to as transcript clusters) available for analysis following background correction, 3316 were expressed in both treatment groups.

Figure 1 PRMT6 knockdown and exon array analysis validation. (A) MCF-7 cells were transfected with control siRNA or PRMT6 siRNA-1 for 48 h. RNA was harvested and analysed for expression of *PRMT6*, *PRMT1* and *CARM1* by TaqMan Q-RT-PCR. Results were normalised to expression levels following transfection with control siRNA. Each data point represents the mean and s.d. of six transfected cultures. *** $P < 0.001$. (B) Graph of three-dimensional principal components analysis scores, with analysis performed on each sample.

Figure 2 *PRMT6* promotes and represses transcription. (A) Ingenuity analysis of exon array data following *PRMT6* knockdown, showing the top ten pathways regulated by *PRMT6* at the transcriptional level. (B) MCF-7 cells were transfected with control siRNA, *PRMT6* siRNA-1 or *PRMT6* siRNA-2 for 48 h. RNA was harvested and analysed expression of *PRMT6*, *PRMT1* and *CARM1* by TaqMan Q-RT-PCR. Results were normalised to expression levels following transfection with control siRNA. (C) T-47D cells were transfected with control siRNA or *PRMT6* siRNA-1 for 48 h. RNA was harvested and analysed to examine the expression of *PRMT6*, *PRMT1* and *CARM1* by TaqMan Q-RT-PCR. Results were normalised to expression levels following transfection with control siRNA. (D) MCF-7 cells were transfected with control siRNA, *PRMT6* siRNA-1 or *PRMT6* siRNA-2 for 48 h. RNA was harvested and analysed by TaqMan Q-RT-PCR for expression of *ARL6IP1*, *FKBP4*, *HIPK3*, *MINA*, *NCOA4*, *YWHAE*, *IGFBP3* or *PTEN*. Included are gene expression changes observed in the exon arrays (Array). Results are shown as fold change in expression compared with control siRNA values. (E) T-47D cells were transfected with control siRNA-1, *PRMT6* siRNA-1 or *PRMT6* siRNA-2 for 48 h. RNA was harvested and analysed by TaqMan Q-RT-PCR for expression of *ARL6IP1*, *FKBP4*, *HIPK3*, *MINA*, *NCOA4*, *YWHAE*, *IGFBP3* or *PTEN*. Included are gene expression changes observed in the exon arrays (Array). Results are shown as fold change in expression compared with control siRNA values. In graphs B, C, D and E, each data point represents the mean and s.d. of six transfected cultures. *** $P < 0.001$ compared to respective control. Results shown are from a single experiment, which is representative of two independent experiments.

PRMT6 influences both gene activation and repression

Analysis from genome-wide exon expression profiling of whole transcripts identified 159 genes that had a fold change ≥ 1.3 and were significantly differentially expressed compared to controls ($P < 0.01$; [Supplementary Table 2](#)). Of these 159 genes, 121 were down-regulated and 38 were up-regulated in response to PRMT6 knockdown. This demonstrated that attenuation of PRMT6 expression resulted in the activation and repression of downstream gene expression. The differentially expressed genes were analysed by Ingenuity Pathway Analysis in order to identify functional groups regulated by PRMT6. Several functional groups were found to be over-represented, including endocrine system development and function, small molecule biochemistry, cell death and cancer, indicating that PRMT6 may regulate these processes ([Fig. 2A](#)).

In order to validate the gene expression results obtained from the exon array, eight genes were chosen for re-examination by Q-RT-PCR in the ER α + MCF-7 and T-47D cell lines. These genes, taken from the list of 159 differentially expressed genes, comprised six genes with decreased expression and two with increased expression following PRMT6 knockdown. The genes were chosen for their biological significance and had relevance to either steroid hormone-dependent gene expression (FK506 binding protein 4, 59 kDa: FKBP4; homeodomain interacting protein kinase 3: HIPK3 and nuclear receptor co-activator 4: NCOA4) ([Yeh & Chang 1996](#), [Moilanen et al. 1998](#), [Riggs et al. 2003](#)) and/or cancer (NCOA4, ADP-ribosylation factor-like 6 interacting protein 1: ARL6IP1; MYC-induced nuclear antigen: MINA; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide: YWHAE; phosphatase and tensin homolog: PTEN and insulin-like growth factor binding protein 3: IGFBP3) ([Eng 2003](#), [Hu et al. 2004](#), [Teye et al. 2004](#), [Cimino et al. 2008](#), [Peng et al. 2008](#), [Guo et al. 2010](#), [Ingermann et al. 2010](#)). PRMT6 levels were decreased in MCF-7 or T-47D breast cancer cells by siRNA for 48 h. We conducted further validation, utilising two (different) non-overlapping siRNAs targeting PRMT6 (individually) to decrease the likelihood of observing non-specific off-target effects. These two siRNAs have been previously validated and do not affect PRMT1 or CARM1 RNA levels ([Fig. 2B and C](#); [Harrison et al. 2010](#)). Q-RT-PCR following knockdown of PRMT6 with both siRNAs gave similar results to the exon array, with the expression of all eight genes

approximating the fold changes observed in the exon array in both MCF-7 and T-47D cells ([Fig. 2D and E](#)).

PRMT6-dependent alternative splicing

Analysis of genome-wide exon expression profiling of individual exons identified 449 genes as being alternatively spliced compared with controls after multiple testing correction with the Benjamini and Hochberg False Discovery Rate ($P < 0.01$; [Supplementary Table 3](#)). Analysis identified that 91 of these genes were also significantly differentially expressed, demonstrating that PRMT6 can regulate both transcription and pre-mRNA processing of the same gene ([Fig. 3A](#)). The 449 differentially alternatively spliced genes identified in the array were analysed by Ingenuity Pathway Analysis to determine cellular pathways that may be regulated by PRMT6-dependent alternative splicing. Several pathways were over-represented, including cell death, cell cycle and cancer ([Fig. 3B](#)).

In order to validate the alternative splicing array data, four genes that indicated differential exon

Figure 3 PRMT6 regulates alternative splicing. (A) Diagrammatic representation of the number of genes identified in the exon array that are regulated by PRMT6 at the levels of transcription, alternative splicing or both. (B) Ingenuity analysis of exon array data following PRMT6 knockdown, showing the top ten pathways regulated by PRMT6 at the level of alternative splicing.

alternative splicing events were chosen from the list of 449 alternatively spliced genes by their biological relevance to cancer, namely NIN, CTNND1, THEM4 and BCCIP. NIN plays a role in the cell cycle and is involved in preventing microtubule aster-organisation defects during microtubule nucleation (Abal *et al.* 2002, Dammermann & Merdes 2002, Stillwell *et al.* 2004). CTNND1 is thought to have a metastatic promoting role in breast cancer (van Roy & McCrea 2005), THEM4 is involved in apoptosis (Parcellier *et al.* 2009) and BCCIP is involved with inhibiting breast cancer cell growth (Meng *et al.* 2004). The details of the genes chosen for validation are shown in Table 1. PRMT6 knockdown coupled with exon-specific Q-RT-PCR was used to examine the alternative splicing of these genes. Results are described in the sections below.

PRMT6-dependent regulation of NIN exon skipping

Comparison of probe sets within the *NIN* gene indicates that *NIN* may be alternatively spliced following *PRMT6* knockdown. Seven probe sets (probe sets 24–30) displayed higher than expected expression following *PRMT6* knockdown, indicative of potential differential alternative splicing (Fig. 4A). The *NIN* gene is composed of 34 exons, and all 7 ‘non-linear’ probe sets map to exon 19, indicating that knockdown of *PRMT6* leads to preferential inclusion of exon 19. Two transcripts within the Ensembl database display skipping of NIN exon 19 (ENST00000389868 and ENST00000382043), and this underscores alternative splicing of exon 19 and is an identified biologically relevant RNA processing event.

Table 1 Genes analysed by Q-RT-PCR to validate differential alternative splicing observed in exon array. The *P* value (corrected) is the probability of differential alternative splicing (corrected by the Benjamini and Hochberg False Discovery Rate), and rank relates to the position of the gene amongst all genes in the array when ranked for *P* value (corrected)

Gene symbol	Gene name	<i>P</i> value (corrected)	Rank
<i>NIN</i>	NINEIN (GSK3B interacting protein)	2.1×10^{-34}	2
<i>CTNND1</i>	Catenin (cadherin-associated protein) delta 1	4.03×10^{-14}	10
<i>BCCIP</i>	BRCA2 and CDK1NA interacting protein	3.18×10^{-8}	52
<i>THEM4</i>	Thioesterase superfamily member 4	1.09×10^{-7}	60

In order to validate the regulation of NIN alternative splicing by PRMT6, we designed primers to detect inclusion and skipping of NIN exon 19 for use in Q-RT-PCR (Fig. 4B). Following reduction of PRMT6 levels by PRMT6-siRNA-1 or PRMT6-siRNA-2 in MCF-7 and T-47D breast cancer cells, we saw ~3- to 3.3-fold increase in inclusion and a ~2.3-fold decrease in skipping of NIN exon 19, leading to an increase in the inclusion: skipping ratio of approximately sevenfold (Fig. 4C, D, E and F). This is in agreement with the increase in inclusion of NIN exon 19 observed in the exon array following *PRMT6* knockdown and confirms that a cellular function of PRMT6 is to promote NIN exon 19 skipping.

PRMT6 regulates a variety of alternative splicing events

Examination of median probe set scores from the exon expression profiling indicates that PRMT6 regulates the inclusion of CTNND1 exon 20, alternative splicing of exon 3 and overall transcript levels of *THEM4*, as well as BCCIP isoform production by alternative terminal exon choice (Supplementary Figures 1A, 2A, B and 3A, see section on supplementary data given at the end of this article). The alternative splicing of specific exons within the *CTNND1*, *THEM4* and *BCCIP* genes identified from the exon array is supported by alternative transcripts present in the Ensembl databases. This indicates that these alternative splicing events are common and previously identified biologically relevant RNA processing events.

We designed Q-RT-PCR assays to detect these various alternative splicing events for CTNND1, THEM4 and BCCIP in the MCF-7 and T-47D breast cancer cell lines (Supplementary Figures 1B, 2C and 3B). Knockdown of *PRMT6* and Q-RT-PCR analysis confirmed a ~2.2-fold increase in skipping of CTNND1 exon 20, a >1.5-fold decrease in expression and preferential inclusion of THEM4 exon 3 an approximate twofold increase in the BCCIPβ and a concomitant twofold decrease in the levels of BCCIPα (Fig. 5A, B and C). These results show significant changes in the alternative splicing of specific exons within the *CTNND1*, *THEM4* and *BCCIP* genes (Supplementary Figures 1C, D, E, 2D, E, F and 3C, D and E). This indicates that one cellular function of PRMT6 is to influence a variety of selective alternative splicing events, which may have pathophysiological consequences in several cancer-related signalling pathways.

Figure 4 PRMT6 regulates alternative splicing of NIN exon 19. (A) MCF-7 cells were transfected with control siRNA or siRNA targeting PRMT6 for 48 h ($n=6$). RNA was harvested and hybridised to a Human 1.0 ST exon array, and data were analysed using XRAY software. Knockdown of PRMT6 leads to 'non-linear' expression of probe sets 24–30 (highlighted by box). Data points show the mean and s.d. of probe set scores from each treatment group. (B) Q-RT-PCR method to detect exon 19 inclusion and skipping of the *Ninein* gene. To detect inclusion of exon 19, a forward primer within exon 19 and a reverse primer spanning the exon 19/20 boundary were used. To detect skipping, a forward primer within exon 18 and a reverse primer spanning the exon 18/20 boundary were used. (C) MCF-7 cells were transfected with control siRNA (Ctrl), PRMT6 siRNA-1 (P6-1) or PRMT6 siRNA-2 (P6-2) for 48 h. RNA was harvested and assayed for *Ninein* exon 19 inclusion and skipping by Q-RT-PCR. Inclusion and skipping expression levels were normalised to levels following treatment with control siRNA. (D) The relative inclusion:skipping ratio following PRMT6 knockdown in MCF-7 cells was calculated by dividing the relative inclusion expression by the relative skipping expression detected as in (C) and normalising to the control ratio. (E) T-47D cells were transfected with control siRNA (Ctrl), PRMT6 siRNA-1 (P6-1) or PRMT6 siRNA-2 (P6-2) for 48 h and analysed as in (C). (F) The relative inclusion:skipping ratio following PRMT6 knockdown in T-47D cells was calculated by dividing the relative inclusion expression by the relative skipping expression detected as in (E) and normalising to the control ratio. In graphs C, D, E and F, each data point represents the mean and s.d. of six transfected cultures. *** $P<0.001$ compared to respective control. Results shown are from a single experiment, which is representative of two independent experiments.

Differential expression of *PRMT6* transcriptional and alternatively spliced genes in primary breast cancer tumours

We assessed the expression of the mRNA encoding PRMT6 in a primary breast IDC cohort ($n=66$, ER+ and ER-) relative to a normal breast tissue cohort

Figure 5 PRMT6 regulates multiple aspects of alternative splicing. MCF-7 cells were transfected with control siRNA (Ctrl), PRMT6 siRNA-1 (P6-1) or PRMT6 siRNA-2 (P6-2) for 48 h and RNA was harvested. (A) The corresponding cDNA was assayed for (A) CTNND1 exon 20 inclusion and skipping, (B) THEM4 exon 3 inclusion and skipping and (C) *BCCIP α* and *BCCIP β* mRNA transcript levels by Q-RT-PCR, according to [Supplementary Figures 1, 2 and 3](#). Expression levels of specific mRNA transcripts to examine transcription, exon skipping, exon inclusion or specific mRNA isoform production were normalised to mRNA transcript levels following treatment with control siRNA. In graphs A, B and C, each data point represents the mean and s.d. of six transfected cultures. *** $P<0.001$ compared to respective control. Results shown are from a single experiment, which is representative of two independent experiments.

Figure 6 Expression of *PRMT6* and associated transcriptional targets in breast cancer. (A) The expression of *PRMT6* and (B) *PRMT6* transcriptionally regulated genes in invasive ductile carcinoma (IDC) human breast tissue samples ($n=66$) relative to normal human breast tissue ($n=50$). Data are presented as relative quantification (RQ) (log10) of genes from IDC human breast tissue samples relative to normal human breast tissue. Statistical analysis was conducted with Integromics Statminer software package, using the non-parametric Wilcoxon rank sum test. Normalisation of data was against the median of geNorm selected controls (MRPL19, PGK1, PPIA, TFRC and UBC) of least expression variation (see Materials and methods section). *** $P<0.001$.

($n=50$). We examined *PRMT6* mRNA levels by Q-RT-PCR, and observed significantly decreased *PRMT6* mRNA expression in the IDC breast cancer cohort relative to normal breast (Fig. 6A). Furthermore, the expression of the (*in vitro* identified) *PRMT6*-dependent breast cancer-related genes (Table 2) (SRA stem-loop interacting RNA binding protein: SLIRP; endothelial differentiation-related factor 1: EDF1; FKBP4, HIPK3, NCOA4, NAD(P)H dehydrogenase, quinone 1: NQO1; proliferation-associated 2G4: PA2G4; protein kinase C, delta: PRKCD; PTEN and tudor domain containing 3: TRIP13) demonstrated significant and differential expression, as observed after *PRMT6* mRNA attenuation in breast cancer cell lines (Fig. 6B). The only

Table 2 Genes identified as being transcriptionally regulated by PRMT6 and examined in human breast tissue: cancer relevance

Gene symbol	Cancer relevance	Reference
<i>AKR1C2</i> ; <i>AKR1C1</i>	Progesterone metabolism	Ji <i>et al.</i> (2004)
<i>SLIRP</i>	Nuclear receptor co-repressor	Hatchell <i>et al.</i> (2006)
<i>EDF1</i>	Sensitivity to tamoxifen resistance	Mendes-Pereira <i>et al.</i> (2012)
<i>FKBP4</i>	Breast cancer prediction marker	Yang <i>et al.</i> (2012)
<i>HIPK3</i>	Resistance to Fas receptor-mediated apoptosis	Curtin & Cotter (2004)
<i>NQO1</i>	Nitrosamine metabolism, dysregulated in nasopharyngeal carcinoma	Dodd <i>et al.</i> (2006)
<i>PA2G4</i>	Inhibition of heregulin-mediated MCF-7 breast cancer cell growth	Zhang <i>et al.</i> (2008)
<i>PRKCD</i>	Pro-proliferative factor in oestrogen-dependent breast cancer cells	McCracken <i>et al.</i> (2003)
<i>PTEN</i>	Tumour suppressor gene	Chow & Baker (2006)
<i>TRIP13</i>	Interacts with thyroid hormone receptor, lung cancer marker	Kang <i>et al.</i> (2008)

exceptions were PTEN and TRIP13, which showed a significant yet opposite expression pattern in comparison with the *in vitro* results (see Fig. 2).

We then examined the four cancer-related genes we identified and validated to be alternatively spliced upon *PRMT6* knockdown in MCF-7 and T-47D breast

cancer cell lines. Examination of the IDC breast cancer cohort (*n*=10) and normal breast tissue (*n*=12) by Q-RT-PCR revealed changes in the exon usage of three of our previously identified alternatively spliced genes in the IDC cohort relative to normal breast tissue (Fig. 7A, B and C). We found increased NIN exon 19

Figure 7 Expression of alternatively spliced transcripts in breast cancer. The expression of alternatively spliced transcripts for (A) *NIN*, (B) *THEM4*, (C) *BCCIP* and (D) *SYK* in invasive ductile carcinoma (IDC) human breast tissue samples (*n*=10) relative to normal human breast tissue (*n*=12). Data are presented as relative quantification (RQ) (log10) of genes from IDC human breast tissue samples relative to normal human breast tissue. Statistical analysis was conducted with Integromics Statminer software package using the non-parametric Wilcoxon rank sum test. Normalisation of data was against the median of IPO8 and TBP controls (see Materials and methods section). **P*<0.05, ***P*<0.01.

inclusion, decreased skipping of THEM4 exon 3, increased levels of the BCCIP β isoform and no significant change in CTNND1 exon usage (data not shown). Our previous study (Harrison *et al.* 2010) demonstrated PRMT6-dependent alternative splicing of the breast cancer tumour suppressor SYK to be regulated by PRMT6 in breast cancer cell lines. In that study, we found reduction of PRMT6 in breast cancer cells resulted in an increase in the full-length SYK transcript (SYK-L) over the shorter SYK (SYK-S) transcript, which skips exon 7. Interestingly, we found similar increase in the SYK-L mRNA transcript expression in our IDC breast cancer samples (Fig. 7D).

Expression of the PRMT6-dependent gene signature predicts disease outcome

To investigate and expand upon the clinical relevance of our findings, we evaluated a PRMT6 transcriptionally regulated gene signature in the context of clinical outcome associations in three independent publically available breast cancer datasets with available clinical outcomes (van de Vijver *et al.* 2002, Desmedt *et al.* 2008, Miller *et al.* 2011). To determine whether PRMT6-dependent gene expression is associated with disease recurrence (RFS) and/or metastasis (DMFS), we utilised a PRMT6-dependent gene expression signature based on the 159 genes that were differentially expressed in a significant manner relative to controls (≥ 1.3 -fold, $P < 0.01$; see Supplementary Table 1). PRMT6 gene expression signature scores were calculated for each patient tumour sample. The PRMT6-dependent gene expression signature is calculated as a signed average, with the sign of the PRMT6 gene expression signature score providing an indication of PRMT6 dysfunction (and by inference the level of PRMT6 expression). For example, a negative (i.e. low) PRMT6 gene expression signature score indicates that the genes comprising the PRMT6 gene expression signature and having a negative weight (i.e. the genes significantly down-regulated in the PRMT6 knockdown cells) are up-regulated relative to the PRMT6 signature genes having a positive weight (i.e. the genes significantly up-regulated in the PRMT6 knockdown cells). Accordingly, a negative or low PRMT6-dependent signature score is indicative of normal to high PRMT6 expression levels and a positive or high PRMT6-dependent signature score is indicative of PRMT6 dysfunction (i.e. low PRMT6 expression levels). In keeping with this notion, we observed a significant inverse correlation between the PRMT6 gene expression levels and PRMT6-dependent signature scores in the NKI and UPP breast cancer datasets

(Supplementary Figure 4A and B, see section on supplementary data given at the end of this article).

Patient samples were stratified according to their PRMT6-dependent gene signature scores (high scores $\geq 66\%$ quantile; low scores $\leq 33\%$ quantile). Kaplan–Meier survival analysis for RFS or DMFS (Fig. 8A, B and C, Supplementary Figures 5 and 6, see section on supplementary data given at the end of this article) clearly demonstrated that patients falling within the ER+ subgroup and having low PRMT6 signature scores (i.e. indicative of normal PRMT6 function/normal to high PRMT6 expression levels) exhibited significantly lower ($P < 0.05$ in all cohorts analysed) probabilities of RFS and metastasis-free survival when compared to patients falling within the ER+ subgroup and having high PRMT6 signature scores (i.e. indicative of PRMT6 dysfunction/low PRMT6 expression levels). Accordingly, the PRMT6-dependent gene signature is significantly associated with clinical outcome in the ER+ subgroup and suggests the PRMT6-dependent genetic program plays a significant role in breast cancer disease progression.

Discussion

We used Affymetrix exon array in combination with RNA interference technology to investigate the global effects of PRMT6 on transcription and alternative splicing in breast cancer cells and human breast cancer. Before this study, the role of PRMT6 in regulating transcription was controversial. We previously identified PRMT6 as a SHR co-activator capable of promoting the transcription of oestrogen- and progesterone-dependent genes (Harrison *et al.* 2010). However, earlier studies demonstrated a role for PRMT6 in repressing transcription by methylating H3R2 (Guccione *et al.* 2007, Hyllus *et al.* 2007, Iberg *et al.* 2008). In order to resolve this controversy, we examined the effects of knocking down PRMT6 on global transcription in breast cancer cells. We found that PRMT6 plays a dual role and can both co-activate and co-repress transcription. This is in agreement with a recent study in lung and bladder cell lines, in which PRMT6 functioned primarily as a transcriptional enhancer but could also repress transcription of a few genes (Yoshimatsu *et al.* 2011). The ability of PRMT6 to co-activate or co-repress transcription of a given gene, as demonstrated in our knockdown studies, may be due to direct or indirect effects. Regardless of the mode of action, our study highlights the fact that the PRMT6-dependent pathways do not exclusively modulate gene expression in a positive or negative fashion. Therefore, there is no conflict between the

Figure 8 Long-term disease outcome based on *PRMT6*-dependent transcriptional signature in breast cancer. Kaplan–Meier survival analysis of patients in the NKI breast cancer cohort exhibiting high and low *PRMT6*-dependent signature scores. Analysis was performed for (A) all subgroups and (B) patients falling within the ER+ subgroup. *PRMT6*-dependent signature scores were split according to their 33% and 66% quantiles. (C) Patients falling within the ER+ subgroup ($P=3.4 \times 10^{-7}$ low vs high/intermediate). *PRMT6*-dependent signature scores were split according to their 33 and 66% quantiles.

previous studies investigating the role of *PRMT6* in gene expression, with the *PRMT6*-dependent transcriptional outcome likely to be gene dependent.

While the exact mechanism of *PRMT6*-dependent gene activation is still unknown, it has been shown that *PRMT6* can methylate histone H4R3, a target that is also methylated by *PRMT1* during oestrogen-induced gene activation (Strahl *et al.* 2001, Wagner *et al.* 2006, Hyllus *et al.* 2007). H4R3 methylation is read by tudor domain containing 3, an effector molecule that has transcriptional co-activator properties (Yang *et al.* 2010). It is possible that chromatin architecture at promoter/enhancer regions or previously laid down chromatin modifications determine whether the *PRMT6* activity enhances or inhibits transcription.

Pathway analysis of *PRMT6*-dependent transcriptionally regulated genes indicates that *PRMT6* may play a role in many cellular pathways, including those implicated in tumourigenesis. *PRMT6* has been shown to be required for the G1-S transition and proliferation of lung and bladder cancer cells and is aberrantly regulated in a number of cancers, including breast cancer (Yoshimatsu *et al.* 2011). In addition, we have previously demonstrated that *PRMT6* is required for maximal oestrogen-stimulated proliferation of MCF-7 breast cancer cells (Harrison *et al.* 2010).

We validated the results from the exon array by examining *PRMT6*-mediated changes in gene expression of eight genes, six of which have known roles in cancer. *NCOA4*, *YWHA*E, *ARL6IP1* and *MINA* are overexpressed in a number of different cancers (Hu *et al.* 2004, Teye *et al.* 2004, Cimino *et al.* 2008, Peng *et al.* 2008, Guo *et al.* 2010). The up-regulation of *YWHA*E in breast cancer is associated with disease-free and overall survival, and therefore, the down-regulation of *YWHA*E from reduced *PRMT6* levels may be unfavourable in relation to breast cancer prognosis (Cimino *et al.* 2008). Conversely, down-regulation of *PRMT6* expression and corresponding low expression of *ARL6IP1* and *MINA* may be favourable to decreased breast cancer growth.

In our study, *PTEN* and *IGFBP3* expression were increased when we knocked down *PRMT6* in the MCF-7 and T-47D cell lines. *PTEN* is a tumour suppressor gene that inhibits the PI3K pathway (Chow & Baker 2006). Loss of *PTEN* leads to the activation of a number of kinases and consequent cell cycle progression (Gera *et al.* 2004, Guertin & Sabatini 2005). *IGFBP3* has an anti-proliferative effect and induces apoptosis in breast cancer cells (Kim *et al.* 2004, 2010). This indicates that lower *PRMT6* expression may result in increased *PTEN* and *IGFBP3* expression, decreased cell cycle progression and increased breast cancer cell apoptosis.

We have demonstrated that PRMT6 has roles in steroid hormone-dependent transcription (Harrison *et al.* 2010), and pathway analysis demonstrated that PRMT6 regulates the transcription of genes involved in endocrine system development and function. HIPK3 and NCOA4 are AR co-activators (Yeh & Chang 1996, Moilanen *et al.* 1998), and FKBP4 promotes binding of hormone to the GR (Riggs *et al.* 2003). This indicates that in addition to co-activation of steroid hormone-dependent genes, PRMT6 is involved in the expression of genes that promote steroid hormone signalling. The reduction of PRMT6 in the MCF-7 and T47D ER+ cell lines results in a decrease in HIPK3 and NCOA4, two transcriptional co-activators primarily associated with androgen receptor (AR) signalling. Interestingly, AR signalling in ER+ breast cancer is thought to be associated with favourable outcomes (Castellano *et al.* 2010, Park *et al.* 2011), and the reduction of HIPK3 and NCOA4 may have a negative effect in relation to AR signalling and outcomes in ER+ breast cancer. In contrast, reduction of the full-length isoform of NCOA4 in MCF-7 cells is associated with increased breast cancer metastasis (Wu *et al.* 2011), while knockdown of *NCOA4* in MCF-7 cells results in decreased cell proliferation (Hua *et al.* 2008). The exact role *NCOA4* plays in breast cancer is not fully defined and requires further investigation.

PRMT6 functions as a tertiary co-regulator, and from our microarray data, is able to modulate the expression of a large number of genes. In the context of breast cancer, the expression of several of the examined *PRMT6*-regulated oncogene/tumour suppressor genes indicates that reduction of PRMT6 and corresponding *PRMT6*-dependent gene changes may be involved in breast carcinogenesis.

Analysis of the exon array data identified 449 genes that are alternatively spliced in response to PRMT6. Of these genes, 121 were also regulated transcriptionally by PRMT6. Therefore, PRMT6 can have a major impact on the function of particular genes by simultaneously regulating both their expression levels and final protein composition. However, the fact that not all genes overlap in the two groups demonstrates that *PRMT6*-regulated transcription and alternative splicing are not necessarily inter-dependent processes.

Pathway analysis of the alternatively spliced genes regulated by PRMT6 indicates that PRMT6 may play roles in cancer, gene expression, cell death and cell cycle progression. Therefore, it would appear that dysregulation of PRMT6 may lead to tumourigenesis by causing aberrant alternative splicing as well as by deregulating transcription. NIN plays a key role in centrosome function, which may be regulated by

PRMT6-directed alternative splicing. PRMT6 promotes skipping of NIN exon 19, which removes a region important in the centrosomal targeting ability of NIN. Therefore, removal of this region is likely to interfere with NIN chromosomal targeting and γ -tubulin binding, resulting in mitotic abnormalities (Abal *et al.* 2002, Dammermann & Merdes 2002, Stillwell *et al.* 2004, Lin *et al.* 2006).

CTNND1 exon 20 encodes a functional leucine-rich nuclear export signal (Keirsebilck *et al.* 1998, van Hengel *et al.* 1999), and so PRMT6-directed inclusion of this exon will lead to cytoplasmic CTNND1. CTNND1 is often located in the cytoplasm but rarely in the nucleus of tumour cells, and cytoplasmic CTNND1 has been linked to a more invasive form of breast cancer (Sarrio *et al.* 2004, Shibata *et al.* 2004, Soubry *et al.* 2005). Whilst the metastatic-promoting role of cytoplasmic CTNND1 is unknown, it is possibly due to its binding to microtubules and/or activation of Rac (Franz & Ridley 2004). In addition, CTNND1 may sequester Kaiso in the cytoplasm, preventing Kaiso-regulated repression of tumour suppressor genes (van Roy & McCrea 2005), leading to a more oncogenic version of CTNND1 being expressed.

THEM4 is a mitochondrial protein that sensitises cells to apoptosis by binding to heat-shock protein 70 and promoting caspase-3 cleavage (Parcellier *et al.* 2009, Piao *et al.* 2009). Currently, nothing is known about the effect of inclusion/skipping of exon 3 on THEM4 structure and activity. Considering the importance of THEM4 in the apoptotic pathway, this PRMT6-regulated alternative splicing event is certainly worth further investigation in relation to breast cancer.

BCCIP is a BRCA2 and CDKN1A (Cip1/waf1/p21) interacting protein that plays an important role in cancer aetiology (Ono *et al.* 2000, Liu *et al.* 2001). BCCIP is alternatively spliced to form two isoforms ($-\alpha$ and $-\beta$) and *PRMT6* knockdown leads to the preferential production of BCCIP β in breast cancer cells. Whilst both BCCIP isoforms inhibit cell growth by interacting with p21, BCCIP β has a much greater effect on cell growth inhibition, particularly in breast cancer cells (Meng *et al.* 2004).

We found that *PRMT6* expression was substantially down-regulated in IDC compared with normal breast tissue. The recent study by Yoshimatsu *et al.* (2011) found PRMT6 to be significantly increased in ~33% of breast cancers. Knowing that breast cancer is not a homogeneous disease, it is not surprising that individual breast cancer tumours demonstrate dissimilar expression of *PRMT6*. Given our findings and that of Yoshimatsu *et al.*'s (2011), PRMT6 may be abnormally regulated up or down in breast cancer. Many of the

genes we identified as transcriptionally regulated after attenuation of *PRMT6* in MCF-7 and T-47D cells also demonstrated changes in expression in IDC breast cancer compared to normal breast tissue, including *SLIRP*, *EDF1*, *FKBP4*, *HIPK3*, *NCOA4*, *NQO1*, *PA2G4*, *PRKCD* and *PTEN*. Similarly, genes we have identified as alternatively spliced by *PRMT6* in our array and previous studies (*NIN*, *THEM4*, *BCCIP* and *SYK*) demonstrate changes in alternative splicing in IDC compared to normal breast tissue.

Through the identification of genes regulated by *PRMT6* at the gene expression level, we were able to investigate the significance of a *PRMT6*-dependent gene expression signature in three published breast cancer cohorts (Fig. 8A, B and C, Supplementary Figures 5 and 6). We found that low *PRMT6* expression and the corresponding high *PRMT6*-dependent gene signature correlated with increased probability of relapse-free or metastasis-free survival in ER+ breast cancer. This would indicate that the role of *PRMT6* in breast cancer is associated with ER-mediated signalling but extends beyond *SHR* transcriptional regulation.

PRMT6 can determine transcriptional gene regulation and alternative splicing decisions, including both exon retention and skipping. Therefore, *PRMT6* can be considered a master modulator of gene expression, capable of determining both transcript abundance and protein structure. Our findings indicate that *PRMT6* mRNA is aberrantly down-regulated in breast cancer and the genes we found regulated by *PRMT6* in human breast cancer cells are over-represented by genes involved in important aspects of carcinogenesis. In addition, our *PRMT6*-dependent gene signature is significantly associated with clinical outcome in ER+ breast cancer and suggests that the *PRMT6*-dependent genetic program plays a significant role in breast cancer disease progression.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-12-0100>.

Declaration of interest

DH Dowhan and George EO Muscat are co-Chief Investigators on a 2012/2013 grant submission. All other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

D H Dowhan conceived the study, performed breast tissue experimental studies, data analysis, participated the study design, coordination and manuscript preparation. M J Harrison participated the study design, performed cell line experimental studies, data analysis and manuscript preparation. N A Eriksson performed breast tissue RNA extraction, experimental studies and data analysis. P Bailey and M A Brown performed public data set mining, Kaplan–Meier survival analysis and data analysis. M A Pearen carried out PCA and Ingenuity analysis on the exon array data. P J Fuller, J W Funder, E R Simpson, P J Leedman, W D Tilley, C L Clarke and G E O Muscat provided breast tissue samples and were involved in the study, design and analysis of transcriptional profiling studies in breast cancer. P Bailey, P J Fuller, J W Funder, E R Simpson, P J Leedman, W D Tilley, M A Brown and C L Clarke assisted in manuscript preparation. G E O Muscat participated in the study design, coordination and manuscript preparation. All authors read and approved the final manuscript.

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