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SHORT COMMUNICATION

Proliferation-associated Brn-3b transcription factor can activate cyclin D1 expression in neuroblastoma and breast cancer cells

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Brn-3b transcription factor enhances proliferation of neuroblastoma (NB) and breast cancer cell lines *in vitro* and increases the rate and size of *in vivo* tumour growth, whereas reducing Brn-3b slows growth, both *in vitro* and *in vivo*. Brn-3b is elevated in >65% of breast cancer biopsies, and here we demonstrate that Brn-3b is also elevated in NB tumours. We show a significant correlation between Brn-3b and cyclin D1 (CD1) in breast cancers and NB tumours and cell lines. Brn-3b directly transactivates the CD1 promoter in co-transfection experiments, whereas electrophoretic mobility shift assay and chromatin immunoprecipitation assays demonstrate that Brn-3b protein binds to an octamer sequence located in the proximal CD1 promoter. Site-directed mutagenesis of this sequence resulted in loss of transactivation of the CD1 promoter by Brn-3b. Thus, Brn-3b may act to alter growth properties of breast cancer and NB cells by enhancing CD1 expression in these cells.
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Introduction

Brn-3b transcription factor was originally isolated from neuroblastoma (NB)-derived ND7 cell line on the basis of homology within the conserved Pit-Oct-Unc (POU) domain of the related Brn-3a protein (Lillycrop *et al*., 1992). *Brn-3b* is expressed in neurons of the developing and adult nervous system (Xiang *et al*., 1993; Turner *et al*., 1994; Xiang, 1998) but also in non-neuronal tissues including breast epithelium (Budhram-Mahadeo *et al*., 2001; Budhram-Mahadeo and Latchman, 2006). Brn-3b proteins have complex functions that depend on which cell types express them. For instance, in the retina, Brn-3b is expressed in the ventricular zone and in retinal ganglion cells (RGC) (Xiang *et al*., 1993, 1996) and is essential for normal retinal development since *Brn-3b* 'knock-out' (KO) mice are blind (Erkman *et al*., 1996; Gan *et al*., 1996). This results from inappropriate differentiation/axonal outgrowth and death within the RGC layer and ventricular zone during late embryogenesis and post-natally (Xiang, 1998).

In contrast to its effects in the retina, elevated expression of Brn-3b in cancer cells is strongly associated with increased proliferation and altered growth. *Brn-3b* expression in proliferating NB cell lines is decreased if cells undergo differentiation by serum withdrawal or upon stimulation with retinoic acid (RA) (Budhram-Mahadeo *et al*., 1995; Smith and Latchman, 1996). Furthermore, forced overexpression of Brn-3b protein in breast cancer and NB cells increases proliferation *in vitro* and enhances *in vivo* tumour growth in xenograft models, whereas reducing Brn-3b (by antisense) decreases proliferation *in vitro* and result in smaller, slower growing tumours *in vivo* (Dennis *et al*., 2001; Irshad *et al*., 2004). Brn-3b also confers resistance to growth inhibitory stimulus, RA, and increases migratory potential of NB cells (Irshad *et al*., 2004). Brn-3b is elevated in >65% of breast cancers (Budhram-Mahadeo *et al*., 1999), but its expression in primary NBs has not so far been studied.

As a transcription factor, Brn-3b protein regulates the expression of critical genes that control different processes; for example, in neuronal cells, Brn-3b repress genes associated with differentiation, namely, α-*internexin*, *SNAP*25 and *synaptophysin* (Latchman, 1998a), whereas in breast cancer cells, it represses *BRCA1* associated with cell cycle arrest (Budhram-Mahadeo *et al*., 1999). In contrast, Brn-3b activates heat shock protein 27 (*Hsp27*), associated with invasiveness and drug resistance in breast cancers (Lee *et al*., 2005) and the cyclin-dependent kinase, *CDK4*, required for cell cycle progression (Samady *et al*., 2004).

CDK4 protein controls G_1 transition in cells as they progress through the cell cycle (Arnold *et al*., 1991) by associating with regulatory partners such as cyclin D1 (CD1) (also referered to as PRAD1, CCND1) (Motokura *et al*., 1991) to phosphorylate specific substrates

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such as retinoblastoma protein (Israels and Israels, 2000). However, whereas CDK4 is inhibited by its association with inhibitory protein such as $p21^{cip1/waf1}$, the regulatory cyclin subunits are regulated at the level of expression and by protein stability as cells progress through the cell cycle (Guo *et al*., 2005). Therefore, factors that control expression of *CD1* will play an important role in cell cycle progression, particularly through the G_1 phase.

Like *Brn-3b*, *CD1* is critical for normal retinal development (Fantl *et al*., 1995; Sicinski *et al*., 1995) since *CD1* KO mice suffer loss of retinal cells with resultant blindness (Sicinski *et al*., 1995). More importantly, a 2.5-fold decrease in *CD1* is seen in retinas of Brn-3b KO mutants compared with wild-type (WT) littermates in microarray analysis (Mu *et al*., 2001). Similar to *Brn-3b*, *CD1* also increases proliferation of breast cancer cells (Zwijsen *et al*., 1996) and is elevated in many breast cancers (Barnes and Gillett, 1998; Budhram-Mahadeo *et al*., 1999). Furthermore, transgenic mice that are engineered to overexpress CD1 protein in the breast, develop tumours in the mammary gland (Wang *et al*., 1994).

In this study, we show correlation between *Brn-3b* with *CD1* in breast tumours and NBs and demonstrate that Brn-3b proteins could directly transactivate *CD1* promoter.

Results and discussion

Brn-3b mRNA is expressed at high levels in biopsies taken from patients with NB tumours

Because Brn-3b could significantly enhance growth and migratory potential, while conferring resistance to growth inhibitory stimulus in NB cell lines (Irshad *et al*., 2004), we tested whether *Brn-3b* was elevated in NB tumour biopsies. Quantitative reverse-transcription –PCR (qRT–PCR) was used to measure total *Brn-3b* mRNA from cDNA (prepared from RNA obtained from NB biopsies). The related *Brn-3a* POU transcription factor (associated with differentiation in NB cell lines) was also measured. Values were adjusted using housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and differences were expressed as fold changes in *Brn-3b* or *Brn-3a*, compared to levels found in control (human SHSY5Y cells – set at 1). Figure 1a shows high levels of *Brn-3b* mRNA in many tumour samples, with ~75% showing elevated *Brn-3b* (over 100-fold higher) compared with levels found in control. In contrast, majority of these tumour samples had very low levels of the mRNA encoding the related but antagonistic *Brn-3a*. This opposite expression of

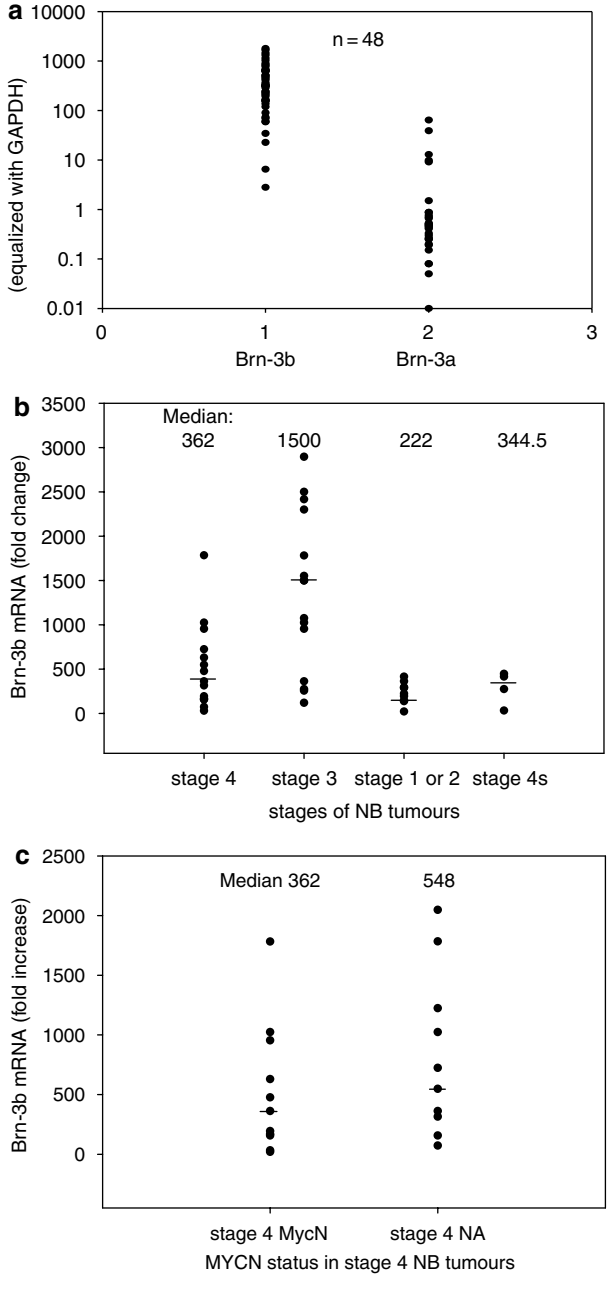


Figure 1 Brn-3b but not Brn-3a mRNA is elevated in NB tumour biopsies. (**a**) qRT–PCR was used to measure Brn-3b and Brn-3a mRNA in NB biopsies. Total Brn-3b or Brn-3a transcripts were measured using custom probe and primers (ABI assay-by-design) and QuantiTect Probe Kit (Qiagen, West Sussex, UK). Human GAPDH was quantified using TaqMan Gene Expression Assay 99999905 (Applied Biosystems, Warrington, UK). RNAs from NBs were obtained from Italian Neuroblastoma Society (Genoa, Italy) and NB biopsies were obtained from Pathology Department, Great Ormond Street Hospital (London, UK) or UKCCSG, UK. Following qRT–PCR, values were normalized to endogenous GAPDH housekeeping gene and expressed as fold induction relative to levels found in human NB cell line, SHSY5Y, known to express Brn-3a and Brn-3b (mRNA expression for each transcription factor in the cell line is arbitrarily set at 1) (Vandesompele *et al*., 2002). (**b**) Analysis of Brn-3b levels, according to NB stage (INSS), shows higher levels of Brn-3b in stage 3 and 4 tumours compared with lower stages 1 and 2 or 4s. (**c**) Correlation of Brn-3b mRNA levels, in stage 4 NB tumours with MYCN status, shows higher levels of Brn-3b in tumours with normal (non-amplified) MYCN copy numbers (stage 4 NA) compared with stage 4 tumours harbouring MycN amplification (stage 4 *MycN*). INSS, International Neuroblastoma Staging System; NA, non-amplified; NB, neuroblastoma; qRT–PCR, quantitative reverse-transcription–PCR.

Brn-3b and *Brn-3a* in NBs reflects their unique effects in NB cell lines. Thus, unlike the growth-promoting effects of *Brn-3b*, high levels of *Brn-3a* lead to differentiation of NB cells as a consequence of differential effects on specific target genes (Smith and Latchman, 1996; Latchman, 1998b). In this regard, elevated *Brn-3b* and low levels of *Brn-3a* in NB biopsies are also reflected in their opposite and antagonistic effects in NB cell lines. Taken together with previous results in NB cell lines, it is likely that elevated *Brn-3b* in NBs will play an important role in regulating the growth and behaviour of these tumours.

Further analysis of these NBs, according to tumour stages (using International Neuroblastoma Staging System (INSS)), (Brodeur, 2003) showed highest levels of *Brn-3b* in stage 3 tumours (Figure 1b) with a median of 1500 compared with less aggressive stages 1/2 (median $\sim$222) or 4 (median $\sim$344.5). Interestingly, higher stage 4 tumours appeared to express lower *Brn-3b* (median $\sim$362) than stage 3 tumours.

Aggressive stage 4 NBs often have *MYCN* amplification, so *Brn-3b* levels were further analysed using *MYCN* status of the tumours. Figure 1c shows lower *Brn-3b* levels in stage 4 tumours with *MYCN* amplification compared with those with no *MYCN* amplification (NA) (Figure 1c) (mean values of 12 NB samples with *MYCN* amplification $\sim$362-fold increase compared with mean of $\sim$548 for similar numbers of NB tumours with normal copies of *MYCN*). However, because of the wide variation in Brn-3b levels in these tumours and small sample size, this is not statistically significant ($P=0.2$), and larger sample numbers will be needed to confirm these findings.

These results suggest that *Brn-3b* is significantly elevated in many NB tumours, particularly in stage 3 and 4 tumours that do not have *MYCN* amplification.

CD1 correlates with Brn-3b in cell lines and tumour samples

Since Brn-3b TF enhances cell proliferation whereas loss of Brn-3b slowed cell growth (Dennis *et al*., 2001; Irshad *et al*., 2004), we measured CD1 protein (associated with proliferation) in stably transfected NB cells that over-expressed Brn-3b (Brn-3b+) or had reduced Brn-3b protein (3b antisense) (Irshad *et al*., 2004) compared with control cells. Figures 2A(i) and (ii) show significantly higher levels of endogenous CD1 protein in Brn-3b (+) cells compared with controls (*P*-value <0.05), whereas Brn-3b antisense cells had reduced CD1 levels. This decrease in CD1 upon loss of Brn-3b was also observed in MCF7 breast cancer cells that stably expressed antisense to *Brn-3b* (Figure 2A(iii)). Thus, altering Brn-3b protein levels in NB cells or breast cancer cells were sufficient to change the growth of these cells and this was paralleled by changes in CD1 expression.

We next quantified *CD1* in tumours with known *Brn-3b* levels using RNA obtained from 48 NBs (Figure 1) and 42 (previously analysed) breast cancer biopsies (Budhram-Mahadeo *et al*., 1999; Samady *et al*., 2004; Lee *et al*., 2005). Figure 2B(i) shows good correlation of *Brn-3b* with *CD1* mRNA in $>50\%$ of NB samples ($R=0.55$), which was specific to *CD1* since there was poor correlation between *cyclin* E and *Brn-3b* in these tumours ($R=0.0002$) (Figure 2B(ii)). Similar analysis of 42 breast cancer biopsies demonstrated an even stronger correlation ($R=0.85$) between *Brn-3b* and *CD1* (Figure 2C), confirming a close association between *Brn-3b* and *CD1* in tumour samples and cell lines expressing different levels of Brn-3b protein.

Transactivation of CD1 promoter by Brn-3b

We next tested whether Brn-3b could directly regulate *CD1* expression by undertaking transient transfection studies in which *CD1* promoter reporter plasmid (CD1-848) was co-transfected with Brn-3b expression vector (in which long terminal repeat (LTR) drives expression of Brn-3b cDNA) into ND7 cells. Deletion constructs, CD1-567 or CD1-69 (Sabbah *et al*., 1999; Redeuilh *et al*., 2002) (Figure 3a) were used to map the region of *CD1* promoter required for Brn-3b transactivation. TK-renilla reporter (included in all transfections) provided an internal control for variability in transfection efficiencies.

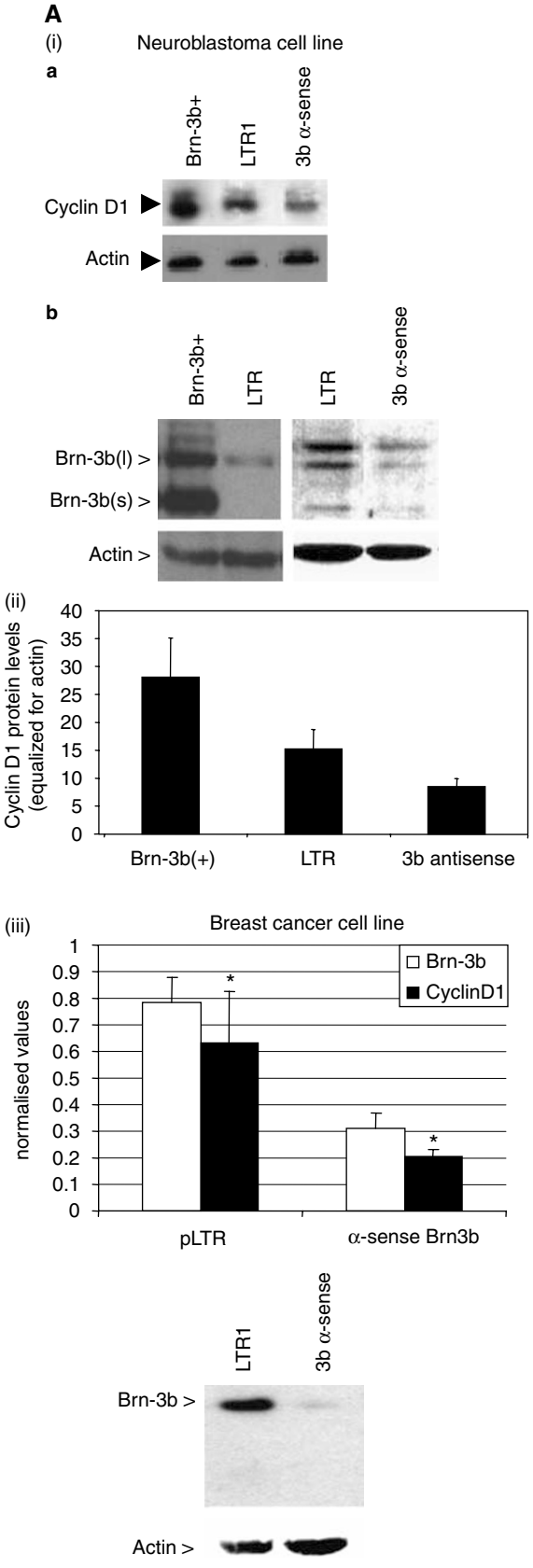
Figure 3b(i) shows that Brn-3b transactivated the longest *CD1* promoter (CD1-848) by 8- to 10-fold, compared with vector control, LTR ($P<0.005$). Similar transactivation was observed when deletion construct, CD1-573, was co-transfected with Brn-3b but the shortest promoter construct, CD1-69, was not transactivated by Brn-3b, although its basal activity was comparable to the longest promoter CD1-848 (relative luciferase assay unit (RLU) following transfection of each promoter alone) (Figure 3b(ii)). Figure 3b(iii) shows increased Brn-3b proteins in cells following transfection with Brn-3b expression vector. These results suggest that CD1-69 promoter construct contains the elements required for assembly of the intact basal transcription machinery but the regulatory elements required for Brn-3b to transactivate this promoter reside within sequence -567 and -69 of the promoter and are lost in CD1-69 construct.

We also tested whether Brn-3b could transactivate full-length *CD1* promoter, CD1-848, in MCF7 breast cancer cell lines, using similar experiments. Figure 3c shows that Brn-3b strongly transactivated the *CD1* promoter in breast cancer cells also.

The ability of Brn-3b to transactivate *CD1* promoter in NB and breast cancer cells suggests that the correlation between these two factors in tumour samples may result from direct regulation of *CD1* by Brn-3b.

Brn-3b binds directly to an octamer site in the proximal CD1 promoter

Analysis of the sequence between -567 and -69 of the *CD1* promoter for putative transcription factor binding sites, using MatInspector (http://www.genomatix.de/), revealed two A/T-rich sequences between positions -248 and -228 (Figure 4a(i)), which had homology to octamer sites recognized by these POU proteins. To test whether Brn-3b binds to this region of the *CD1*


A
(i)
Neuroblastoma cell line
a
Brn-3b+
LTR1
3b α-sense
Cyclin D1
Actin
b
Brn-3b+
LTR
LTR
3b α-sense
Brn-3b(l) >
Brn-3b(s) >
Actin >
(ii)
Cyclin D1 protein levels (equalized for actin)
Brn-3b(+)
LTR
3b antisense
(iii)
Breast cancer cell line
normalised values
Brn-3b
CyclinD1
pLTR
α-sense Brn3b
LTR1
3b α-sense
Brn-3b >
Actin >
B
(i)
NB tumour: n = 48;
R = 0.55
Brn-3b mRNA
Cyclin D1 mRNA
(ii)
NB tumour: n = 48;
R = 0.0002
cyclin E mRNA
Brn-3b mRNA
C
Breast tumours: n = 42;
R = 0.85
Cyclin D1 mRNA (fg)
Brn-3b mRNA
(fg)

promoter, electrophoretic mobility shift assay (EMSA) was performed using ^{32}P-radiolabeled oligonucleotides probes (corresponding to this sequence). Figure 4a(ii) shows retarded protein/DNA complexes formed when the probe was incubated with Brn-3b (+) protein extracts (indicated by *) but not present when using

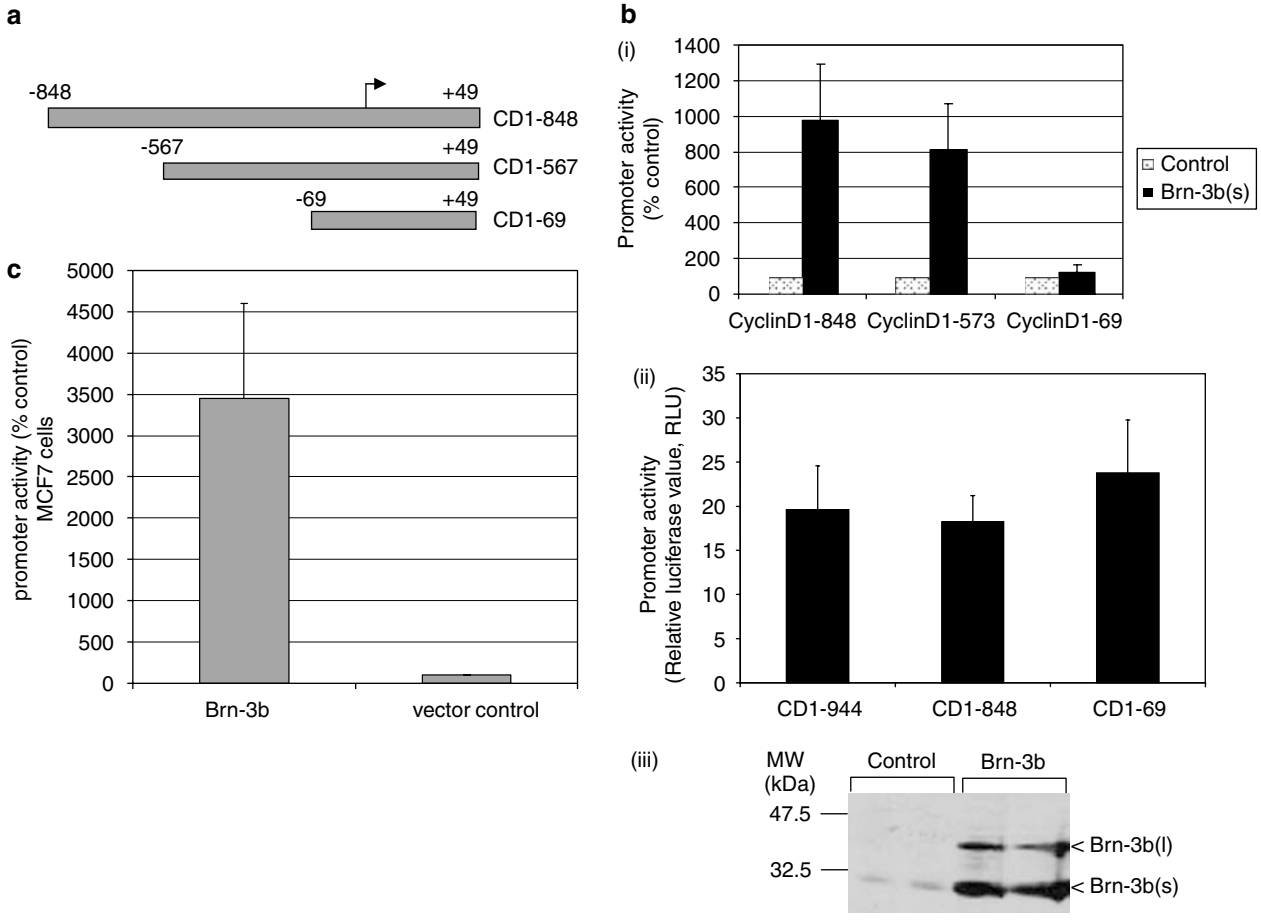
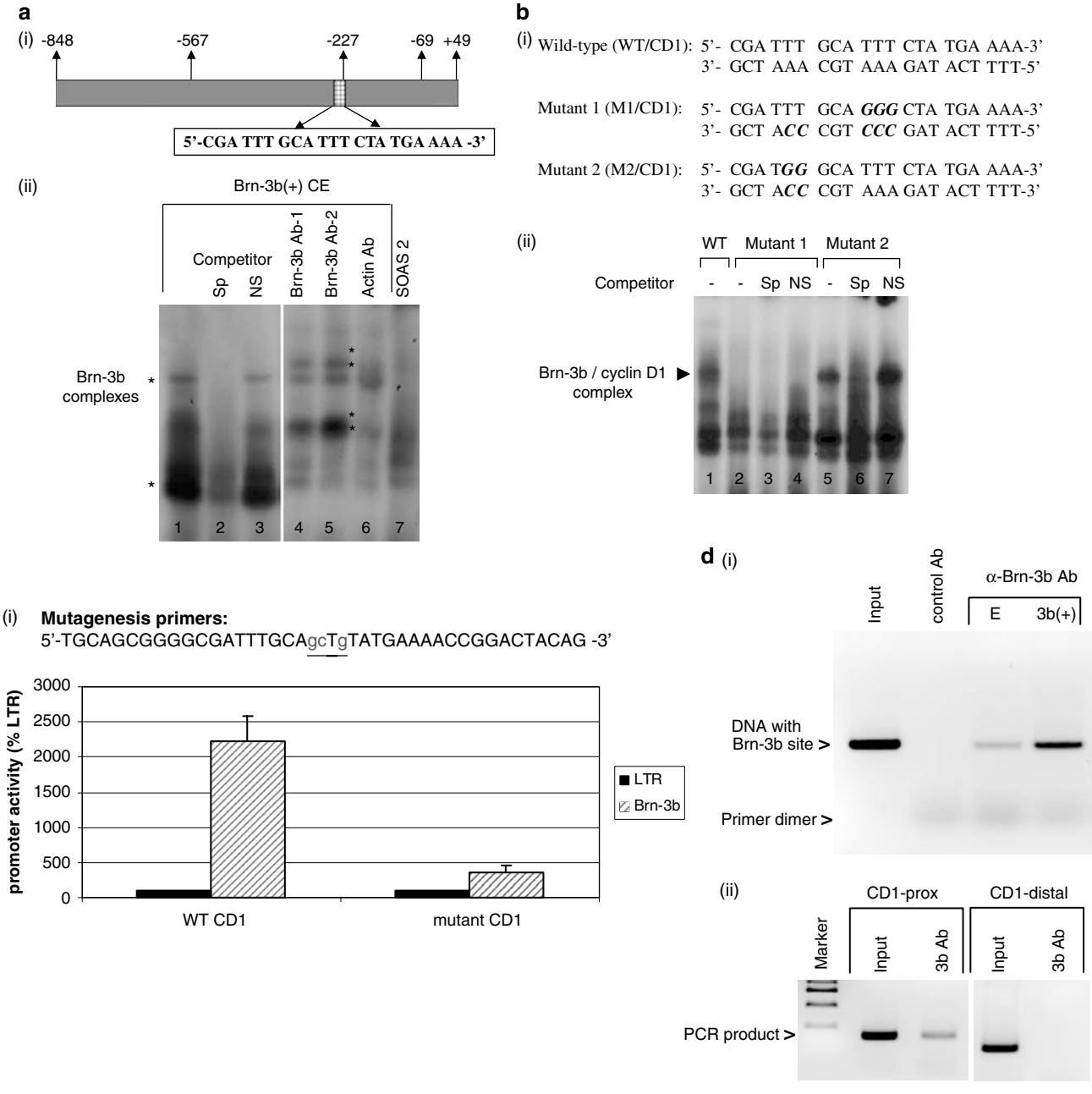


Figure 3 Brn-3b activates cyclin D1 (CD1) promoter in NB and breast cancer cell lines. (**a**) Schematic representation of cyclin D1 promoter constructs used in the study. The longest construct (CD1-848) comprises sequences −848 to +49 cloned into pGL2 luciferase vector and the deletion constructs were obtained by removing specific regions from 5′ end of the promoter; that is to positions −567 (CD1-567) and −69 (CD1-69), respectively (kind gifts from Dr Gerard Redeuilh, Saint-Antoine Hospital, Paris). (**b**)(i) Reporter assays showing the effect of co-transfecting Brn-3b expression vectors with cyclin D1 promoter constructs into ND7 neuroblastoma cells. Changes in promoter activity in the presence of Brn-3b were expressed as percentage of the empty vector control (set at 100%) after adjusting for any variability in transfection efficiency (using Renilla Luciferase reporter gene, pRL-TK). Values shown represent the result of three experiments (±s.d.). (ii) Reporter assay showing basal promoter activity of deletion construct CD1-69 compared with full-length CD1-848 promoter. Luciferase activity of each promoter, measured following transfection into neuroblastoma cells were normalized to TK-Renilla. (iii) Western blot analysis to show increased expression of Brn-3b proteins following transfection of Brn-3b expression vector compared with vector control-transfected cells. (**c**) Reporter assays showing transactivation of cyclin D1 promoter (CD1-848) by Brn-3b in MCF7 breast cancer cells compared with empty vector control (set at 100%). Values are adjusted using internal control renilla reporter gene and represent mean (±s.d.) of three experiments.

Figure 2 Brn-3b correlates with cyclin D1 in cell lines and tumour biopsies. (**A**)(i)(a) Representative western blot analysis showing increased cyclin D1 protein in IMR32 cells overexpressing Brn-3b (Brn-3b+) and lower levels in cells with reduced Brn-3b (3b α-sense) compared with LTR1 (vector) control. (b) Brn-3b protein expression in stably transfected cells lines either overexpressing Brn-3b or with antisense to reduce its levels. (ii) Quantification of cyclin D1 protein in IMR32 cells expressing different levels of Brn-3b by scanning densitometry of three independent experiments. (iii) Reduction of Brn-3b in MCF7 cells using Brn-3b antisense results in corresponding decrease in cyclin D1 protein levels compared with vector control cells. (**B**)(i) Correlation of *Brn-3b* mRNA with *cyclin D1* levels in tumour samples. qRT–PCR was used to quantify human *cyclin D1* (TaqMan Gene Expression Assay, hCG2016647 (Applied Biosystems)) and *GAPDH* levels were used to adjust for variability. Regression analysis was carried out using Sigma plot. (i) The significant relationship between *cyclin D1* and *Brn-3b* ($R = 0.55$) in NB biopsies, which was unique since (ii) shows poor correlation between *Brn-3b* and *cyclin E* mRNA in these samples ($R = 0.0002$). (**C**) Significant correlation between *Brn-3b* mRNA with *cyclin D1* in breast cancer biopsies following qRT–PCR ($R = 0.85$). Breast cancer RNAs were obtained from Candis Tissue Bank (Liverpool, UK) and CR-UK Hedley Atkins Breast Pathology Laboratory (Guy's Hospital, London). LTR, long terminal repeat; NB, neuroblastoma.

SAOS2 cell extracts (lacking endogenous Brn-3b). These bands were specifically competed upon addition of unlabeled Brn-3 binding site (specific competitor) but not by nonspecific oligonucleotide (NS). Addition of Brn-3b antibodies (but not anti-actin antibodies) resulted in supershift of the protein/DNA complexes (indicated by double *). These results confirm that Brn-3b proteins bind to this region of *CD1* promoter, specifically and strongly, and the presence of complexes with different mobilities suggests that Brn-3b may bind to DNA in different configuration (for example, monomer or hetero-/homo-dimer).

We next tested which of two A/T sites, found within this region, was required for binding of Brn-3b to CD1 promoter by introducing mutations into each site (mutant 1 and mutant 2) (Figure 4b(i)). ^{32}P-labeled mutant oligonucleotide probes were used in EMSA and compared with WT probe. Figure 4b(ii) shows the expected complexes following incubation of cell extracts with WT probe but no specific bands were seen following incubation of cell extract with labeled mutant 1 probe (TTT > > GGG at positions 240–237) and no changes occur upon addition of specific or nonspecific competitor. In contrast, incubation of cell extract with mutant 2 probe (TTT > > TGG at positions 246–243) resulted in a similar pattern of complexes seen with WT probe, that is, formation of complexes that were competed by specific competitor but not nonspecific



competitor. Thus, TTT at positions 240–237 appears to be critical for binding of Brn-3b to the *CD1* promoter and suggests that sequence 5′-ATTTCTAT-3′ found between −248 and −228 (from transcriptional start site), in *CD1* promoter is likely to be the octamer site bound by Brn-3b.

Site-directed mutagenesis of octamer site within −248 to −228 in CD1 promoter prevented transactivation by Brn-3b

To test whether this sequence was essential for Brn-3b-mediated activation of *CD1* promoter, site-directed mutagenesis was used to introduce mutations into three bases within this site in the longest CD1-848 promoter reporter (Figure 4c(i)). The resultant mutated promoter was co-transfected with Brn-3b into ND7 cells and promoter activity compared with WT *CD1* promoter activity in the same experiment. Figure 4c(ii) shows that Brn-3b transactivated WT CD1 promoter (15- to 20-fold), but this effect was significantly reduced on the mutated promoter (three- to five-fold). Thus, we have identified the sequence that is necessary for effective Brn-3b-mediated transactivation of the *CD1* promoter.

Brn-3b is bound to CD1 promoter in intact cells

Chromatin immunoprecipitation (ChIP) assays were undertaken to test whether Brn-3b is bound to the *CD1* promoter *in vivo*, in intact cells (Lee *et al*., 2005). Proteins bound to chromatin in MCF7 cells were immunoprecipitated, using Brn-3b antibody or control actin antibody. Figure 4d shows that PCRs (with primers to amplify *CD1* promoter flanking the octamer site), using ChIP DNA immunoprecipitated with Brn-3b antibody, give rise to a product that corresponded to the size seen with the input sample (positive control), but this was not observed when using the ChIP DNA obtained with anti-actin antibody. The increased intensity of the amplified product when using DNA obtained following ChIP from Brn-3b overexpressing cells (Brn-3b+) (Figure 4d(i)) shows that more *CD1* promoter was immunoprecipitated in presence of higher levels of Brn-3b. Brn-3b is specifically bound to the proximal promoter because amplification of the distal region of the *CD1* promoter (~2.7 kb from binding site), using the same chromatin DNA (immunoprecipitated with Brn-3b Ab), did not give rise to the product which was seen when using the input (positive control) (Figure 4d(ii)). These results confirm that Brn-3b binds directly to proximal CD1 promoter in intact cells, thus facilitating promoter activation.

Our results therefore suggest that the correlation between Brn-3b and CD1 in breast cancers and NBs is likely to arise from the ability of Brn-3b to directly regulate CD1 expression. This relationship also exist in non-transformed cells since Brn-3b and CD1 have overlapping expression during retinal development and loss of Brn-3b result in reduction of CD1 in Brn-3b KO retinas (Mu *et al*., 2001). Interestingly, also, both CD1 and Brn-3b mutants are blind (Sicinski *et al*., 1995; Gan *et al*., 1996). Thus, expression of Brn-3b and CD1 are closely associated in different tissues, suggesting that Brn-3b-mediated transactivation of CD1 expression is important in distinct cell types.

The proliferative effects of CD1 relate to its control of G_1 transition in specific cell types (Arnold *et al*., 1991), and although it functions primarily by forming active complexes with CDK4 (Israels and Israels, 2000), CD1 can also act via mechanisms that are independent of CDK4 (Zwijsen *et al*., 1997; Knudsen *et al*., 1999; Bienvenu *et al*., 2001). Elevated CD1 in tumours can be achieved through different mechanisms including deregulated gene expression, mutations or amplifications (Weinberg, 1995). In NB cell lines and tumours,

Figure 4 Identification of the putative Brn-3b binding site in the cyclin D1 promoter. (**a**)(i) Schematic representation of cyclin D1 promoter, showing location of putative Brn-3 binding site (−248 and −227). EMSAs were performed as described (Lee *et al*., 2005), and protein–DNA complexes were resolved by polyacrylamide gel electrophoresis. (ii) Protein–DNA complexes formed when cell extracts prepared from Brn-3b overexpressing (Brn-3b (+)) NBs were incubated with labeled probe (lane 1; indicated by *) but not with SAOS2 cell extract, lacking Brn-3b (lane 7). The Brn-3b–DNA complexes were specifically competed by addition of 'cold' competitor (unlabeled oligonucleotide) (lane 2) but not upon addition of 100-fold molar excess of nonspecific competitor (lane 3). Incubation with Brn-3b antibody resulted in supershift of the Brn-3b-containing complexes bound to DNA (lanes 4 + 5 – double *), but not in the presence of actin antibody (control) (lane 6). (**b**)(i) Sequence showing the WT oligonucleotide from cyclin D1 promoter and mutations introduced into 'TA'-rich sites (indicated in bold italics). (ii) Complexes formed when Brn-3b (+) cell extracts were incubated with WT oligonucleotide (lane 1) were not seen when labeled mutant 1 oligonucleotides were incubated with same cellular extract (lanes 2–4). Lane 5 shows labeled mutant 2 oligonucleotides incubated with Brn-3b (+) cell extract, which give rise to similar complexes seen in lane 1. These were competed by addition of specific competitor (lane 6) but not nonspecific competitor (lane 7). (**c**) Mutation of the octamer site by site-directed mutagenesis. (i) Sequence of primers used to introduce mutations into 'TA' site at positions 240–237 within CD1-848 promoter construct (base changes indicated by grey lower-case letters) which resulted in formation of *Pvu*II restriction enzyme site in the promoter (using QuikChange II site-directed mutagenesis kit). (ii) Results of reporter assays using either WT CD1 promoter or mutated CD1 promoter following co-transfection, into ND7 cells, with or without Brn-3b into neuroblastoma cells. The activity of each promoter in the presence of empty vector control was set at 100% and effects of Brn-3b are expressed as percentage of this control. Values were adjusted for renilla control and represent the result of three experiments (±s.d.). (**d**) ChIP assay showing association of Brn-3b to cyclin D1 promoter *in vivo*. ChIP was carried out as described (Lee *et al*., 2005) in MCF7 cells using Brn-3b antibody or control actin antibody. Primers were designed to amplify the proximal promoter or distal region ~2.7 kb upstream of Brn-3 site in standard PCR and resolved on 2% agarose gel. (i) PCR products resulting from amplification for proximal cyclin D1 promoter containing the putative Brn-3 site. Lane 1 shows the fragment amplified from input sample (cell extract before IP), which was not observed when IP was undertaken with control anti-actin antibody (lane 2). Lanes 3 and 4 show bands amplified when using chip DNA obtained following IP with Brn-3b antibody from cells expressing endogenous Brn-3b (**e**) or from stable cell lines overexpressing Brn-3b protein. (ii) PCR products resulting from amplification of ChIP DNA using primers designed to amplify either the proximal cyclin D1 promoter or at a distal site 2.7 kb upstream of putative Brn-3 site. ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; IP, immunoprecipitation; WT, wild type.

increased CD1 is not associated with mutations or amplifications (Molenaar *et al*., 2003) and are likely to result from deregulation of pathways that control CD1 expression in these cells. Given that elevated Brn-3b can significantly enhance growth and proliferation in breast cancer and NB cells and reducing endogenous expression slows their growth, it is likely that the Brn-3b transcription factor mediates its effects, in part, by transactivating effectors of the cell cycle such as *CD1* and *CDK4* (Samady *et al*., 2004), thus allowing cell cycle progression and promoting cellular proliferation.

Materials and methods

Plasmids
Reporter constructs containing cyclin D1 promoter (CD1-848) or deletion constructs (CD1-567 and CD1-69) cloned into pGL2 luciferase vector were kind gifts from Dr Gerard Redeuilh (Saint-Antoine Hospital, Paris, France). Brn-3b expression vectors contain cDNA encoding either Brn-3b(s) or genomic DNA capable of encoding both Brn-3b(l) and Brn-3b(s) (Lee *et al*., 2005). pRL-TK (Promega, Southampton, UK) containing Renilla Luciferase reporter gene under control of herpes simplex virus-thymidine kinase promoter was used to control for transfection efficiency.

Antibodies
α-Brn-3b antibody (goat polyclonal), α-cyclin D1 mAb (DCS-6) and α-actin (I-19) (goat polyclonal) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary antibodies were obtained from Dako (Cambridgeshire, UK); used at 1:2000–1:3000).

Tumour samples
RNAs from neuroblastomas were obtained from the Italian Neuroblastoma Society (Genoa, Italy). Neuroblastoma biopsies were provided by Pathology Department, Great Ormond Street Hospital (London, UK) or UKCCSG, UK. RNA from breast cancer biopsies were provided by Candis Tissue Bank (Liverpool, UK), while breast cancer biopsies were provided by CR-UK Hedley Atkins Breast Pathology Laboratory (Guy's Hospital, London, UK).

Cell culture, transient transfections, luciferase assay
Neuroblastoma ND7 cell line (Budhram-Mahadeo *et al*., 1994a) and MCF7 breast cancer cell lines were grown in the appropriate growth medium (Lillycrop *et al*., 1992; Budhram-Mahadeo *et al*., 1994a; Samady *et al*., 2004) until subconfluent, then plated for experiments. Transient transfections were carried out with the specified reporter/expression vectors in the ND7 cell line using Fugene (Roche, Welwyn Garden City, UK) as described (Hudson *et al*., 2005). The renilla reporter was included in all transfections to provide an internal control for variation in transfection efficiencies. Cells were harvested after 48 h in passive lysis buffer and luciferase reporter analysis undertaken using the dual luciferase assay system (Promega) and TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA) as described (Lee *et al*., 2005).

RNA extraction and cDNA synthesis
Cells were harvested in TRIZOL reagent (Invitrogen, Paisley, UK) and processed according to the manufacturer's protocol. For RNA isolation from tumour biopsies, tissues were homogenized in liquid nitrogen, resuspended in Tirzol and then processed according to the manufacturer's protocol. DNAse1 (RNAse free) treatment was used to remove contaminating genomic DNA (Amersham Pharmacia Biotech, Buckinghamshire, UK) followed by phenol/chloroform extraction and ethanol precipitation of RNA. Total RNA (0.5–1 μg) was used for cDNA synthesis in 20–50 μl reaction using SUPERSCRIPT II, RNase H-Reverse Transcriptase (Invitrogen). This cDNA (1–2 μl) was used in subsequent qRT–PCR for each target gene to be studied or the housekeeping gene, GAPDH.

Quantitative real-time RT–PCR
TaqMan Gene Expression Assay from Applied Biosystems (Warrington, UK) were used for amplification and quantification of human cyclin D1 (hCG2016647) and for human GAPDH (99999905). Total Brn-3b transcripts were measured using qRT–PCR, in which custom probe and primers were designed using ABI assay-by-design file builder programme (http://www.appliedbiosystems.com). QuantiTect Probe PCR Kit used for the qRT–PCR was obtained from Qiagen (West Sussex, UK). Reactions were carried out using the DNA Engine Opticon (MJ Research, GRI Braintree, Essex, UK) and conditions were as stipulated for use with TaqMan Gene Expression Assay. The comparative C_t method ($2^{-\Delta\Delta|C_t}$ method) was used to determine changes in relative levels of different genes (Vandesompele *et al*., 2002). The C_t values of samples of interest were compared with the calibrator, for example, control untreated cell line (ND7) or untreated SHSY5Y human neuroblastoma cells, or MCF7 breast cancer cells were used for tumour samples and normalized to endogenous GAPDH housekeeping gene. For standard curve method, a known amount of each target was prepared as a series of serial dilution and once tested to show that a linear standard curve was produced; aliquots of the each standard were frozen so that the same sets of standards was used for all subsequent experiment to ensure reproducibility in the studies.

Electrophoretic mobility shift assay
EMSA was performed as described previously (Lee *et al*., 2005). Double-stranded oligonucleotides (containing the putative Brn-3 site in the cyclin D1 promoter) comprised of sequence 5′-CGATTTGCATTTCTATGAAAA-3′ (or mutant oligonucleotides, see Figure 4b) were end-labeled with T4 kinase. Labeled probe (1 ng) was incubated with cellular extract prepared either from IMR-32 neuroblastoma cells overexpressing Brn-3b protein or from SAOS2 cells (that do not express Brn-3b) under conditions described (Lee *et al*., 2005). The protein–DNA complex was resolved from the free probe by electrophoresis and detected by autoradiography.

Chromatin immunoprecipitation assay
This technique was carried out as described by Lee *et al*. (2005) using Brn-3b overexpressing MCF7 cells and antibody to either Brn-3b (goat pAb); or actin pAb. Proximal region of cyclin D1 promoter (containing Brn-3 site) was amplified using primers CD1F1: 5′-GACCCACTCGAGGCGGAC-3′ and CD1R: 5′-CGCCAAACGCCGGGAGCA-3′, whereas distal promoter region was amplified using primers CD1f-1.5Kb: 5′-CAATGACCCTCAAAAGCCCAG-3′ and CD1r-1.5Kb: 5′-AACCGGGCTTGCAATTTTGCG-3′. Standard conditions were used for PCR (2.5 mM $MgCl_2$ with cycling parameters of one cycle at 94°C for 15 min; 40 cycles of 95°C for 30 s; 58°C for 30 s and 72°C for 30 s followed by 72°C for 5 min. PCR products were resolved on 2.5% agarose/TBE gel.

Statistical analysis
Data points from independent experiments were compared using Student's *t*-test analysis and the level of significance of the test is given as *P*-value in the text. Regression analysis was undertaken using SigmaPlot 2001 programme to establish correlation of different factors.

Abbreviations

CD1, cyclin D1; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; LTR, long terminal repeat; NB, neuroblastoma; POU, Pit-Oct-Unc; RA, retinoic acid.

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References

Arnold A, Motokura T, Bloom T, Kronenberg H, Ruderman J, Juppner H *et al*. (1991). The putative oncogene PRAD1 encodes a novel cyclin. *Cold Spring Harb Symp Quant Biol* **56**: 93–97.

Barnes DM, Gillett CE. (1998). Cyclin D1 in breast cancer. *Breast Cancer Res Treat* **52**: 1–15.

Bienvenu F, Gascan H, Coqueret O. (2001). Cyclin D1 represses STAT3 activation through a Cdk4-independent mechanism. *J Biol Chem* **276**: 16840–16847.

Brodeur GM. (2003). Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* **3**: 203–216.

Budhram-Mahadeo V, Lillycrop KA, Latchman DS. (1995). The levels of the antagonistic POU family transcription factors Brn-3a and Brn-3b in neuronal cells are regulated in opposite directions by serum growth factors. *Neurosci Lett* **185**: 48–51.

Budhram-Mahadeo V, Morris PJ, Ward T, Weber B, Sassone-Corsi P, Latchman DS. (2001). The closely related POU family transcription factors Brn-3a and Brn-3b are expressed in distinct cell types in the testis. *Int J Biochem Cell Biol* **33**: 1027–1039.

Budhram-Mahadeo V, Ndisang D, Ward T, Weber BL, Latchman DS. (1999). The Brn-3b POU family transcription factor represses expression of the BRCA-1 anti-oncogene in breast cancer cells. *Oncogene* **18**: 6684–6691.

Budhram-Mahadeo V, Theil T, Morris PJ, Lillycrop KA, Moroy T, Latchman DS. (1994). The DNA target site for the Brn-3 POU family transcription factors can confer responsiveness to cyclic AMP and removal of serum in neuronal cells. *Nucleuc Acid Res* **22**: 3092–3098.

Budhram-Mahadeo VS, Latchman DS. (2006). Targeting Brn-3b in breast cancer therapy. *Expert Opin Ther Targets* **10**: 15–25.

Dennis JH, Budhram-Mahadeo V, Latchman DS. (2001). The Brn-3b POU family transcription factor regulates the cellular growth, proliferation, and anchorage dependence of MCF7 human breast cancer cells. *Oncogene* **20**: 4961–4971.

Erkman L, McEvilly RJ, Luo L, Ryan AK, Hooshmand F, O'Connell SM *et al*. (1996). Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. *Nature* **381**: 603–606.

Fantl V, Stamp G, Andrews A, Rosewell I, Dickson C. (1995). Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. *Genes Dev* **9**: 2364–2372.

Gan L, Xiang M, Zhou L, Wagner DS, Klein WH, Nathans J. (1996). POU domain factor Brn-3b is required for the development of a large set of retinal ganglion cells. *Proc Natl Acad Sci USA* **93**: 3920–3925.

Guo Y, Yang K, Harwalkar J, Nye JM, Mason DR, Garrett MD *et al*. (2005). Phosphorylation of cyclin D1 at Thr 286 during S phase leads to its proteasomal degradation and allows efficient DNA synthesis. *Oncogene* **24**: 2599–2612.

Irshad S, Pedley RB, Anderson J, Latchman DS, Budhram-Mahadeo V. (2004). The Brn-3b transcription factor regulates the growth, behavior, and invasiveness of human neuroblastoma cells *in vitro* and *in vivo*. *J Biol Chem* **279**: 21617–21627.

Israels ED, Israels LG. (2000). The cell cycle. *Oncologist* **5**: 510–513.

Knudsen KE, Cavenee WK, Arden KC. (1999). D-type cyclins complex with the androgen receptor and inhibit its transcriptional transactivation ability. *Cancer Res* **59**: 2297–2301.

Latchman DS. (1998a). Regulation of neuronal differentiation and apoptosis by Brn-3 POU family transcription factors: potential use in gene therapy. *Gene Ther Mol Biol* **2**: 95–101.

Latchman DS. (1998b). The Brn-3a transcription factor. *Int J Biochem Cell Biol* **30**: 1153–1157.

Lee SA, Ndisang D, Patel C, Dennis JH, Faulkes DJ, D'Arrigo C *et al*. (2005). Expression of the Brn-3b transcription factor correlates with expression of HSP-27 in breast cancer biopsies and is required for maximal activation of the HSP-27 promoter. *Cancer Res* **65**: 3072–3080.

Lillycrop KA, Budhram-Mahadeo V, Lakin ND, Terrenghi G, Wood JN, Polak JM *et al*. (1992). A novel POU family transcription factor is closely related to Brn-3 but has a distinct expression pattern in neuronal cells. *Nucleic Acids Res* **20**: 5093–5096.

Molenaar JJ, van Sluis P, Boon K, Versteeg R, Caron HN. (2003). Rearrangements and increased expression of cyclin D1 (CCND1) in neuroblastoma. *Genes Chromosomes Cancer* **36**: 242–249.

Motokura T, Bloom T, Kim HG, Juppner H, Ruderman JV, Kronenberg HM *et al*. (1991). A novel cyclin encoded by a bcl1-linked candidate oncogene. *Nature* **350**: 512–515.

Mu X, Zhao S, Pershad R, Hsieh TF, Scarpa A, Wang SW *et al*. (2001). Gene expression in the developing mouse retina by EST sequencing and microarray analysis. *Nucleic Acids Res* **29**: 4983–4993.

Redeuilh G, Attia A, Mester J, Sabbah M. (2002). Transcriptional activation by the oestrogen receptor alpha is modulated through inhibition of cyclin-dependent kinases. *Oncogene* **21**: 5773–5782.

Sabbah M, Courilleau D, Mester J, Redeuilh G. (1999). Estrogen induction of the cyclin D1 promoter: involvement of a cAMP response-like element. *Proc Natl Acad Sci USA* **96**: 11217–11222.

Samady L, Dennis J, Budhram-Mahadeo V, Latchman DS. (2004). Activation of CDK4 gene expression in human breast cancer cells by the Brn-3b POU family transcription factor. *Cancer Biol Ther* **3**: 317–323.

Sicinski P, Donaher JL, Parker SB, Li T, Fazeli A, Gardner H *et al*. (1995). Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* **82**: 621–630.

Smith MD, Latchman DS. (1996). The functionally antagonistic POU family transcription factors Brn-3a and Brn-3b show opposite changes in expression during the growth arrest and differentiation of human neuroblastoma cells. *Int J Cancer* **67**: 653–660.

Turner EE, Jenne KJ, Rosenfeld MG. (1994). Brn-3.2: a Brn-3-related transcription factor with distinctive central nervous system expression and regulation by retinoic acid. *Neuron* **12**: 205–218.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A *et al*. (2002). Accurate normalization of real-time quantitative RT–PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**(7): RESEARCH0034; 1–12.

Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A, Schmidt EV. (1994). Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* **369**: 669–671.

Weinberg RA. (1995). The retinoblastoma protein and cell cycle control. *Cell* **81**: 323–330.

Xiang M. (1998). Requirement for Brn-3b in early differentiation of postmitotic retinal ganglion cell precursors. *Dev Biol* **197**: 155–169.

Xiang M, Zhou L, Nathans J. (1996). Similarities and differences among inner retinal neurons revealed by the expression of reporter transgenes controlled by Brn-3a, Brn-3b, and Brn-3c promotor sequences. *Vis Neurosci* **13**: 955–962.

Xiang M, Zhou L, Peng YW, Eddy RL, Shows TB, Nathans J. (1993). Brn-3b: a POU domain gene expressed in a subset of retinal ganglion cells. *Neuron* **11**: 689–701.

Zwijsen RM, Klompmaker R, Wientjens EB, Kristel PM, van der BB, Michalides RJ. (1996). Cyclin D1 triggers autonomous growth of breast cancer cells by governing cell cycle exit. *Mol Cell Biol* **16**: 2554–2560.

Zwijsen RM, Wientjens E, Klompmaker R, van der SJ, Bernards R, Michalides RJ. (1997). CDK-independent activation of estrogen receptor by cyclin D1. *Cell* **88**: 405–415.