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ORIGINAL ARTICLE

Identification of *PUMA* as an estrogen target gene that mediates the apoptotic response to tamoxifen in human breast cancer cells and predicts patient outcome and tamoxifen responsiveness in breast cancer

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Recognition of the pivotal role of estrogen in the aetiology of breast cancer has led to the development of antiestrogens (AE), such as tamoxifen (TAM) as effective therapies for the treatment and prevention of this disease. However, despite their widespread clinical efficacy, response to AEs is often short-lived, and acquired or innate therapeutic resistance remains a major obstacle in the successful treatment of breast cancer. Thus, delineating the intracellular pathways that mediate the cellular response to estrogen could potentially lead to new, more effective approaches to the treatment of breast cancer, particularly endocrine-resistant disease. Here, we have identified the BCL-2 homology 3 (BH3)-only, pro-apoptotic regulator, *PUMA* (*p*53 *u*pregulated *m*odulator of *a*poptosis) as an estrogen target gene that is acutely downregulated in response to estrogen in breast cancer cell lines, independently of their p53 status. *PUMA* is transcriptionally upregulated following treatment with TAM, and knock down of PUMA expression in these cells attenuates the apoptotic response to TAM. Furthermore, low *PUMA* expression in breast carcinomas is significantly associated with breast cancer-specific death ($P = 0.0014$ and $P = 0.0115$, for mRNA and protein, respectively), and worse outcome in TAM-treated patients (mRNA, $P = 1.49e\text{-}05$). These findings suggest that the dysregulation of apoptotic signaling pathways such as those executed through PUMA, can significantly impact on both the progression and therapeutic responsiveness of breast cancer. Moreover, they provide a convincing rationale for exploring new therapeutic approaches involving endocrine and non-endocrine therapies that target apoptotic pathways as an effective strategy for tackling endocrine refractory disease.
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Introduction

Breast cancer remains the most common cancer of women and the second most frequent cause of cancer deaths, despite a major decline in breast cancer mortality in past decades. Aberrations in molecular pathways regulating estrogen synthesis and action are primary etiological factors in the pathogenesis of breast cancer (Jordan, 2009). This dependence on the estrogen drive to cell proliferation and increased cell survival, and an understanding of the underlying molecular mechanisms, has led to the routine use of the estrogen receptor (ER) as a biomarker of hormone responsiveness, and the development of antiestrogens (AEs), for example, tamoxifen (TAM), and aromatase inhibitors (AIs) as effective therapies for the treatment and prevention of breast cancer (Swaby *et al*., 2007). Indeed, the routine use of adjuvant endocrine therapy is one of the major contributors to the recent decline in breast cancer mortality that has occurred preferentially in ER-positive disease (Hurvitz and Pietras, 2008). However, despite its widespread clinical efficacy, response is often short-lived, and resistance to endocrine therapy remains a major obstacle to the successful treatment of this disease (Musgrove and Sutherland, 2009). A major challenge in understanding the molecular basis of endocrine resistance is that although estrogen has been implicated as a major factor in the development and progression of breast cancer, the downstream effectors of its mitogenic and pro-survival actions remain to be fully characterized. Thus, the identification and elucidation of intra-

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cellular pathways that mediate the cellular response to estrogen could potentially lead to new, more effective approaches to the treatment of breast cancer, particularly endocrine-resistant disease.

In addition to its well-characterized mitogenic actions, estrogen has direct, inhibitory effects on the apoptotic machinery in breast cancer cells, including transcriptional repression of the proapoptotic molecules BAK, BIK and caspase-9 (Frasor *et al*., 2003), and increased expression of anti-apoptotic BCL-2 through estrogen-response elements (EREs) in the *BCL-2* coding sequence (Perillo *et al*., 2000). There is also evidence of ligand-independent cross-talk between estrogen and growth factor intracellular signaling cascades, particularly those mediated by the erbB family of receptor tyrosine kinases, leading to the activation of survival pathways through activation of phosphatidylinositol 3-kinase (Butt *et al*., 2005). Although the involvement of aberrant apoptotic signaling in the endocrine response is relatively understudied (Butt *et al*., 2007), such a concept is strongly supported by several clinical studies that have revealed associations between expression of the death receptor, Fas and Fas ligand (Reimer *et al*., 2002), and expression of the proapoptotic protein, BAD (Cannings *et al*., 2007) and resistance to TAM (Musgrove and Sutherland, 2009), and others that have reported a decrease in expression of survival genes in breast cancers following neoadjuvant treatment with AIs (Ellis *et al*., 2003). More recently, the intracellular domain (4ICD) of the receptor tyrosine kinase, HER4/ErbB-4 was identified as an effector of TAM-induced apoptosis through its activity as a BCL-2 homology 3 (BH3)-only, proapoptotic protein (Naresh *et al*., 2008). TAM treatment disrupts the interaction between ERα and 4ICD, leading to an accumulation of the latter at the mitochondria where it blocks BCL-2-mediated survival signals. The clinical relevance of these observations was emphasized by demonstrating a significant association between suppressed HER4 expression and TAM-resistance in breast cancer patients (Naresh *et al*., 2008).

In a recent study aimed at an unbiased identification of functional classes of estrogen-responsive genes that might be implicated in AE resistance, we defined an apoptotic/cell survival gene signature distinct from three other functional signatures representing cell proliferation, cell growth and transcriptional regulation (Musgrove *et al*., 2008). This apoptotic signature not only predicted response to TAM in breast cancer patients, but was predictive independent of the proliferative signature, providing strong evidence that aberrations in pathways driving apoptosis/survival define a distinct, clinically-relevant mechanism of the endocrine responsiveness in a subset of patients (Musgrove *et al*., 2008). Indeed, our more recent studies have demonstrated that individual genes mediating cellular survival, such as BAG-1 can predict outcome and TAM responsiveness in breast cancer patients (Millar *et al*., 2008), with *in vitro* evidence that they may also have a role early on in breast cancer development (Anderson *et al*., 2010).

Herein, we have focused on a candidate in the apoptosis signature, PUMA/BBC3 (*p*53-*u*pregulated *m*odulator of *a*poptosis/*B*cl-2 *b*inding *c*omponent-*3*; referred to hereafter as PUMA), whose role as an estrogen target gene has not previously been explored. PUMA is a proapoptotic, BH3-only, BCL-2 family member and an essential mediator of p53-dependent and -independent apoptosis, induced by a diverse range of signals, including genotoxic stress, deregulated oncogene expression and growth factor withdrawal (Han *et al*., 2001; Nakano and Vousden, 2001; Yu *et al*., 2001). Once activated, typically by transcriptional upregulation, PUMA acts at the mitochondria orchestrating an intrinsic apoptotic response through the activation of BAX and/or BAK. Current evidence suggests that PUMA initiates apoptosis by sequestering anti-apoptotic binding partners, such as BCL-2 and BCL-X_L away from BAX and BAK, although some suggestion of direct interaction has also been proposed (reviewed in Yu and Zhang, 2009). Once BAX and/or BAK are activated, PUMA-induced apoptosis follows the classical mitochondrial pathway of membrane depolarisation, release of apoptogenic molecules such as cytochrome c and SMAC, and caspase activation.

Here we report that PUMA is transcriptionally downregulated in response to estradiol in human breast cancer cells. *In vitro* analyses have demonstrated that PUMA is robustly upregulated following treatment with TAM, with evidence that it may influence the apoptotic response to TAM. Furthermore, low PUMA mRNA and protein expression is associated with poor patient outcome in cohorts of breast cancer patients. Thus in summary, this study has identified PUMA as a potentially important molecule in breast cancer progression and therapeutic response.

Results

PUMA mRNA and protein expression in human breast cancer cell lines

PUMA mRNA and protein expressions were examined in a panel of cancer (both ER-positive and ER-negative), normal and immortalized breast cancer cell lines by quantitative real-time PCR and immunoblot, respectively. Figure 1 demonstrates that *PUMA* transcripts (Figure 1a) and protein (Figure 1b) were detected in both normal and cancer cells to varying degrees. However, although there was a trend towards increased PUMA expression in cancer cell lines compared with normal and immortalized breast epithelial cell lines, this was not significant at either the mRNA or the protein level. In addition, no significant difference was observed in PUMA mRNA expression between cell lines expressing mutant or wild-type p53 protein, but there were significantly higher levels of PUMA protein in wild type versus mutant p53-expressing cells (Figure 1b).

Downregulation of PUMA expression by estradiol in human breast cancer cell lines

Recently, we utilized an *in vitro* model in the steroid hormone-responsive human breast cancer cell line MCF-7, to identify novel, estrogen-regulated targets in breast cancer cells (Musgrove *et al*., 2008). Briefly,

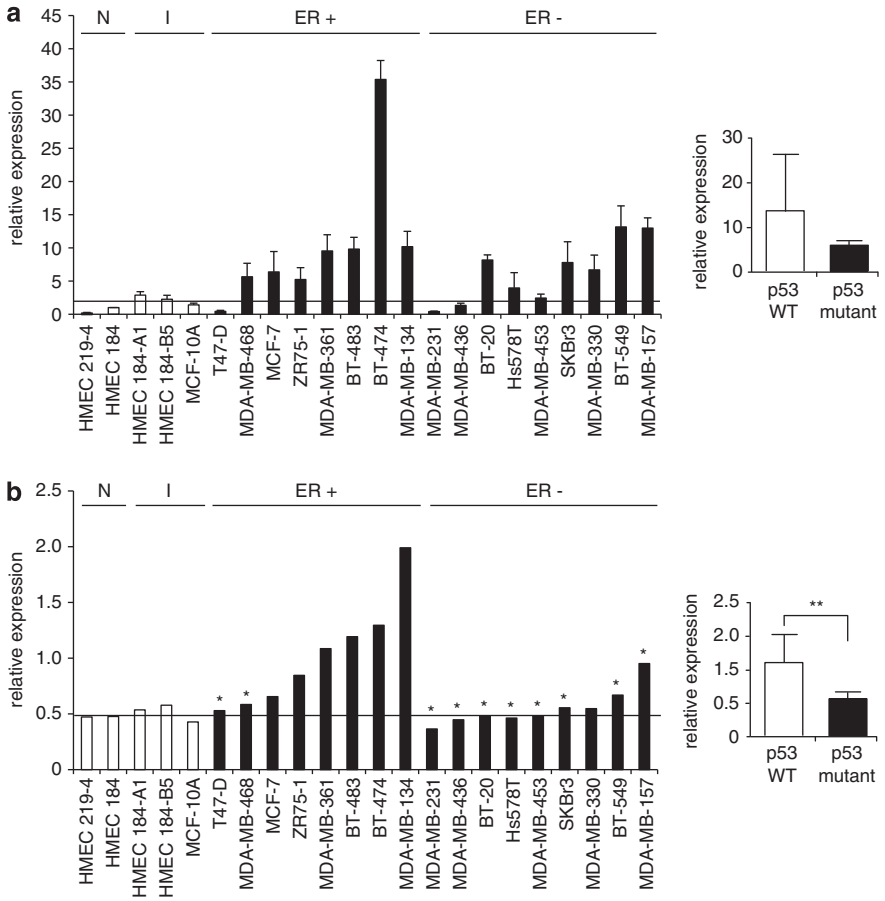


Figure 1 PUMA mRNA and protein expression levels in human breast cancer cell lines compared with normal and immortalized breast cell lines. (**a**) *PUMA* mRNA expression in a panel of ER-positive and ER-negative breast cancer cell lines (black bars) and normal and immortalized breast epithelial cells (white bars), normalized to RPLPO loading control. Bars are means of triplicate samples. Representative data from two independent experiments are shown. Line indicates the mean *PUMA* expression level in normal and immortalized cells. (**b**) Densitometric analysis of PUMA protein expression in breast cancer cell lines (black bars) and normal and immortalized breast epithelial cells (white bars), normalized to β-actin loading control. Cell lines with known mutant p53 status are indicated (*). Representative data from two independent experiments are shown. Graphs represent levels of *PUMA* mRNA or protein in cells expressing wild-type p53 or mutant p53 protein. Error bars indicate 95% confidence intervals, $^{**}P<0.01$ for wild-type p53 versus mutant p53 protein.

MCF-7 cells were arrested in G_1 phase by treatment with the AE ICI 182780, then cell cycle progression was reinitiated by treatment with 17β-estradiol. RNA was collected 6 h after estrogen treatment, and differential gene expression was determined using Affymetrix GeneChip Arrays (HG-U133 Plus V2.0; Millennium Science, Box Hill, VIC, Australia) and Bayesian linear modeling methods in the limma package. Figure 2a shows the intensity of a representative probe set for *PUMA*, which was significantly downregulated following estrogen treatment.

We confirmed the downregulation of PUMA expression by estrogen over a time course of treatment in estrogen-responsive MCF-7 and T-47D cells. ICI-arrested cells were treated with 17β-estradiol (10 nM) or vehicle, and PUMA mRNA and protein expression was determined by quantitative real-time PCR or immunoblot, respectively. Figure 2b shows an acute and sustained downregulation of *PUMA* mRNA within 3–6 h of estradiol treatment compared with controls in both MCF-7 and T-47D cells. Estradiol treatment also resulted in a downregulation of PUMA protein in both cell lines (Figure 2c). Densitometric analyses of immunoblots from three independent experiments demonstrated that this effect was significant following 9 h of treatment in MCF-7 cells and after 12 h in T-47D (Figure 2d). This observed downregulation of PUMA by estrogen is independent of functional p53, as MCF-7 and T-47D cells express wild-type and mutant p53, respectively (Runnebaum *et al*., 1991). Interestingly, estradiol-mediated PUMA downregulation also followed a similar time course to the downregulation of another BH3-only protein, BIK (Figure 2e; Hur *et al*., 2004), suggesting it may form part of a coordinated, anti-apoptotic signaling cascade acutely initiated in response to estrogen.

PUMA is a direct transcriptional target of estrogen

The rapid effects of estrogen on *PUMA* mRNA led us to investigate if *PUMA* is a direct transcriptional target of estrogen. Treatment with cycloheximide did not affect the estradiol-mediated downregulation of *PUMA*

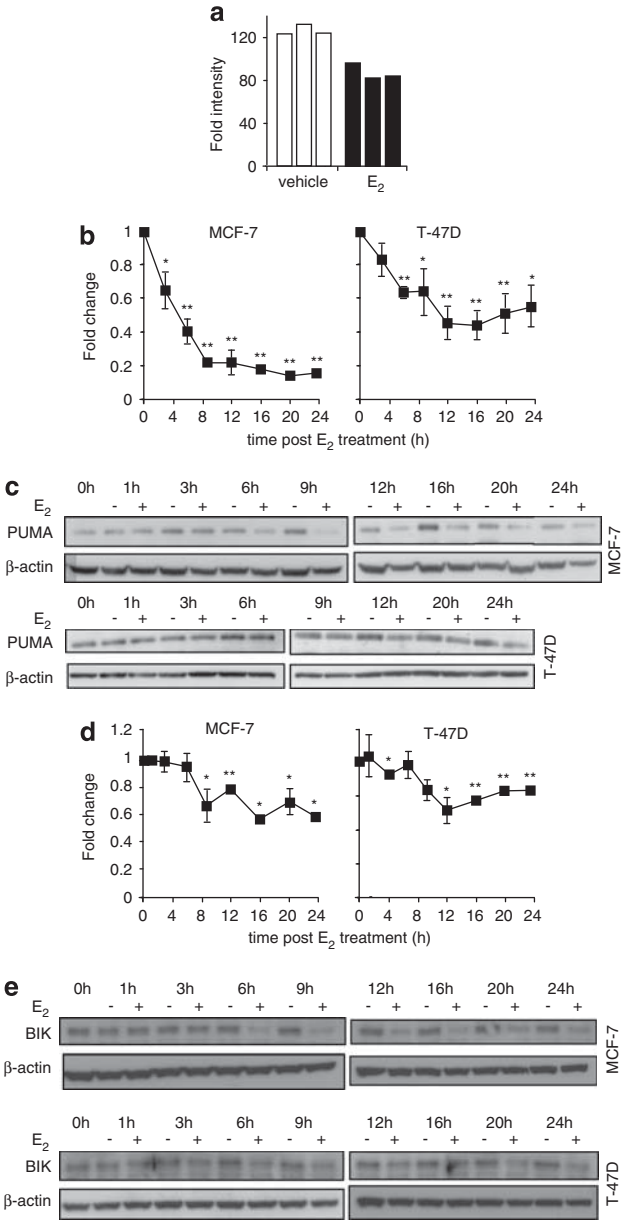


Figure 2 Transcriptional downregulation of *PUMA* by estrogen in human breast cancer cell lines. (**a**) *PUMA* mRNA expression in a representative probe set from HG-U133 Plus V2.0 microarrays, following 48 h pretreatment with ICI 182780, then 6 h stimulation with estradiol (E2) or vehicle. (**b**) Cells were pretreated with ICI 182780 for 48 h, then stimulated with estradiol and RNA isolated at various time points as indicated. Samples were analyzed in triplicate by reverse transcription PCR with *PUMA*-specific primers and expression of *PUMA* is presented normalized to *RPLPO* and relative to vehicle controls at 0 h. Data shown is the mean of triplicate samples from four independent experiments ± s.e. $*P<0.01$, $**P<0.001$ for estradiol treatment versus vehicle treatment. (**c**) Representative immunoblot analysis of endogenous PUMA expression in whole cell lysates at time points post-estradiol treatment up to 24 h. β-actin was used as a loading control. (**d**) Densitometric analyses showing PUMA expression normalized to β-actin and relative to vehicle controls at 0 h, from three independent experiments ± s.e. $*P<0.01$, $**P<0.001$ for estradiol treatment versus vehicle treatment. (**e**) Repression of BIK protein by estrogen in human breast cancer cells. Representative immunoblots of endogenous BIK expression in whole cell lysates at time points post-estradiol treatment up to 24 h. β-actin was used as a loading control.

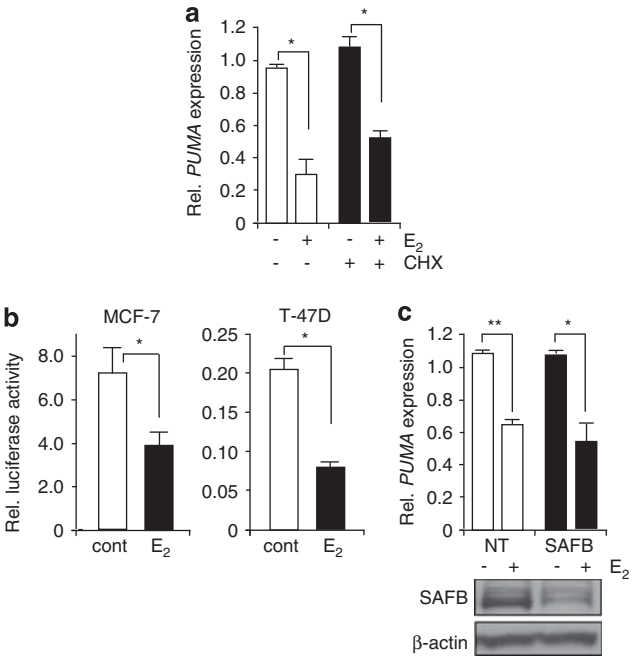


Figure 3 PUMA is a transcriptional target of estrogen. (**a**) MCF-7 cells were arrested with ICI 182780 for 48 h, then treated with cycloheximide (black bars) or control (white bars) before addition of estradiol or vehicle as indicated for 8 h; levels of PUMA mRNA were determined by quantitative real-time PCR. Data shown is the mean of triplicate samples from three independent experiments ± s.e. $*P<0.001$ for estradiol treatment versus vehicle treatment. (**b**) MCF-7 and T-47D cells were transfected with a PUMA-luciferase reporter construct, pretreated with 10 nM ICI 182780 for 24 h and then stimulated with estradiol (100 nM) or vehicle for 24 h. Luciferase activity was normalized to the activity of the pGL3-Basic reporter. Data shown is the mean of triplicate samples from three independent experiments ± s.e. $*P<0.001$ for estradiol treatment versus vehicle treatment. (**c**) MCF-7 cells were transfected with scaffold attachment factor (SAFB)-specific siRNA or non-targeting control (NT) overnight then arrested with ICI 182780 for 48 h before addition of estradiol or vehicle for a further 8 h; levels of *PUMA* mRNA were determined by quantitative real-time PCR. Data shown is the mean of triplicate samples from two independent experiments ± s.e. $*P<0.05$; $**P<0.01$ for estradiol treatment versus vehicle treatment.

mRNA expression (Figure 3a), suggesting that it is independent of ongoing protein synthesis. Analysis of the human *PUMA* genomic sequence 1.1 kb upstream of the transcriptional start site revealed two sequences similar to the ERE consensus (5′-GGTCAnnnTGACC-3′). We further investigated the activation of PUMA by estrogen, using a reporter plasmid containing the *PUMA*-promoter upstream of a luciferase cDNA in the promoter-less luciferase reporter vector, pGL3 (Han *et al*., 2001). Figure 3b demonstrates a significant decrease in luciferase activity, following treatment with estradiol compared with control cells in both MCF-7 and T-47D, suggesting that *PUMA* is a direct transcriptional target of estrogen. However, using chromatin immunoprecipitation (ChIP) assays to determine if estradiol binds directly to the endogenous *PUMA* promoter, we were unable to detect any significant binding compared with positive control ERE sequences (data not shown).

As previous studies have reported that estrogen-mediated regulation of *PUMA* requires the co-repressor SAFB1 (Hammerich-Hille *et al*., 2010), we investigated the effects of SAFB1 suppression on *PUMA* expression following estradiol treatment. However, Figure 3c demonstrates that knockdown of SAFB expression using specific siRNA did not significantly affect *PUMA* repression in the presence of estradiol, suggesting additional, as yet undefined mechanisms may regulate *PUMA* expression in this experimental model.

TAM-induced apoptosis is associated with an upregulation of PUMA

To further explore the functional consequences of estrogen-regulated PUMA expression, we determined the effects of the AE, TAM on PUMA expression, using an established model of TAM-induced apoptosis in breast cancer cells. Cells were treated with a proapoptotic concentration of an active metabolite of TAM, 4-hydroxytamoxifen (4-OHT; 7.5 μM), resulting in a significant induction of apoptosis after 24 h (Figure 4a). The proapoptotic effects of 4-OHT in this model are associated with a downregulation of ERα expression (Figure 4a), suggesting that they are predominantly mediated through ER-dependent signaling pathways. Similar results were obtained in T-47D cells (data not shown). Using this model, we examined the expression of PUMA mRNA and protein (Figures 4b and c, respectively) over a time course of 4-OHT treatment. Figures 4b and d demonstrate that a significant induction of PUMA mRNA and protein preceded the induction of apoptosis in 4-OHT-treated cells. Furthermore, this induction of PUMA appears to be specifically associated with the apoptotic response to 4-OHT, as titration experiments in MCF-7 cells demonstrated that only at pro-apoptotic concentrations of 4-OHT was an induction of PUMA observed (Figure 4e), with similar results obtained in T-47D cells (data not shown). A comparison of the effects of TAM on other members of the BCL-2 family, demonstrates that although BIM is also upregulated, other members of the family are not (Figure 4f), suggesting that there is some specificity in the pro-apoptotic, AE response in these cells.

Further investigations have suggested that this AE-mediated stimulation of *PUMA* is reversible; when the AE, ICI 182780 is removed after 48 h treatment, *PUMA* mRNA levels significantly decrease (Figure 4g). However, interestingly, the addition of estradiol following AE removal results in a decrease in *PUMA* expression to significantly lower levels than merely removing ICI alone. Furthermore, the effects of estradiol in this context are irreversible (Figure 4g). These data suggest that estrogen is directly repressing *PUMA* expression, rather than merely counteracting the stimulatory effects of AEs.

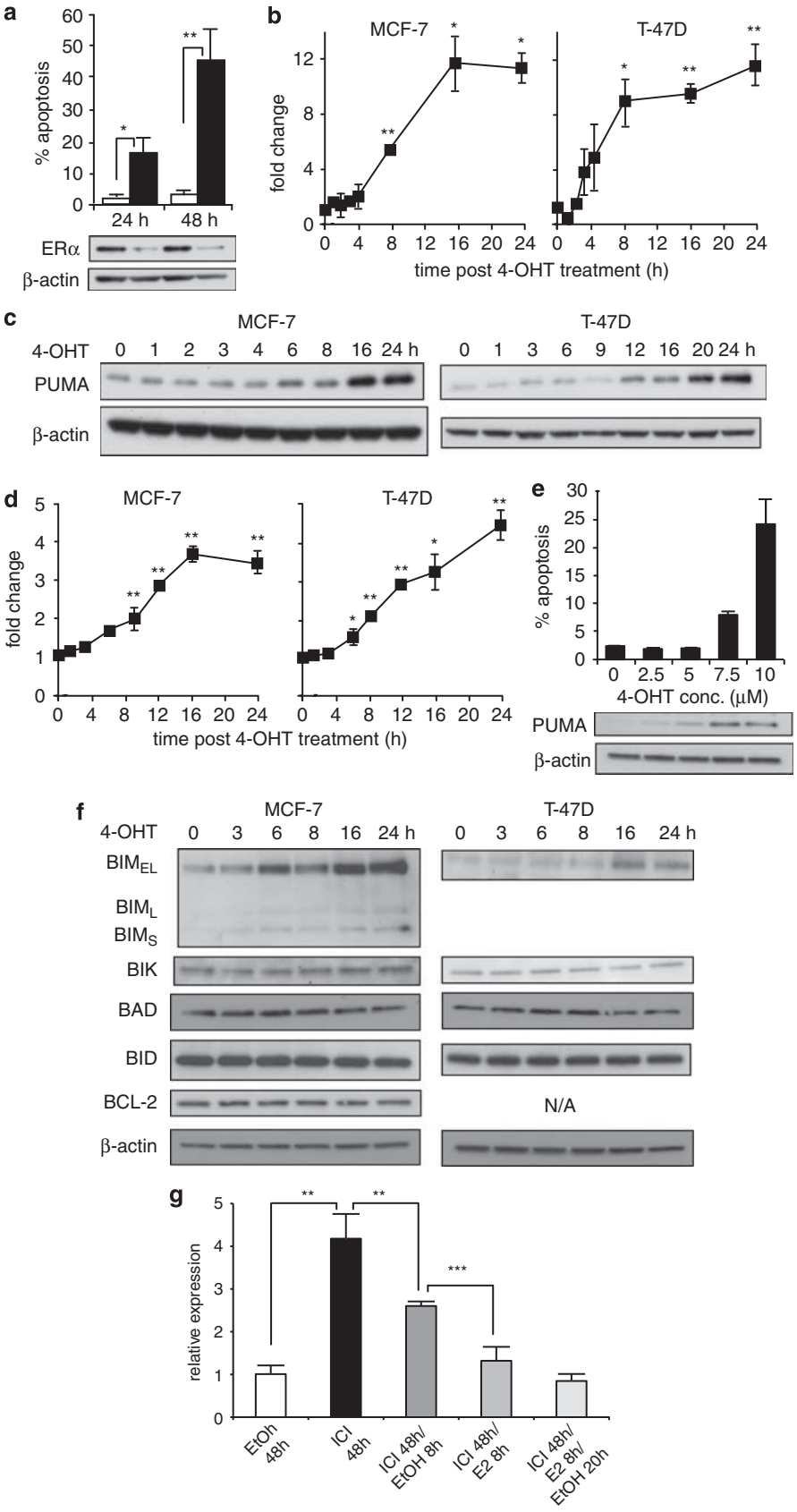
PUMA modulates the apoptotic response to 4-OHT in breast cancer cells

To further delineate a mechanistic role for TAM-induced PUMA expression, we determined the effects of PUMA knockdown on 4-OHT-induced apoptosis. MCF-7 cells were treated with either non-targeting (NT) siRNA or PUMA-specific siRNA, in the presence or absence of 4-OHT, and analyzed for PUMA expression by immunoblot. Figure 5a demonstrates that treatment with PUMA-specific siRNA significantly reduced expression of PUMA compared with the NT and mock-transfected controls. This downregulation was maintained in the presence of 4-OHT. PUMA knockdown significantly attenuated the apoptotic response to 4-OHT compared with cells transfected with NT control (Figure 5b), comparable to levels observed in cells overexpressing antiapoptotic BCL-2 (Figure 5c). Levels of apoptosis following mock or siRNA transfection were typically slightly higher than the ~3–4% usually observed basally, and increased to approximately 30% following treatment with 4-OHT in mock and NT controls and ~15–18% in the presence of PUMA siRNA (Supplementary Figure 1).

PUMA mRNA expression and patient outcome

The relationship between *PUMA* mRNA expression and breast cancer outcome was explored using pub-

Figure 4 Tamoxifen-induced apoptosis is associated with an upregulation of PUMA. (**a**) Induction of apoptosis by 4-OHT. MCF-7 cells were incubated in the presence (black bars) or absence (white bars) of 4-OHT (7.5 μM) for 24 h, then attached and floating populations were analyzed for M30-FITC positivity by flow cytometry. Values shown are means of triplicate wells from three independent experiments ± s.e. $*P<0.001$, $**P<0.0001$ for 4-OHT-treated cells versus untreated controls. Samples were also analyzed for ERα expression by immunoblotting and a representative blot is shown. β-actin was used as a loading control. (**b**) Cells were treated with 4-OHT and RNA isolated at various time points as indicated. Samples were analyzed in triplicate by reverse transcription PCR with *PUMA*-specific primers and expression of *PUMA* is presented normalized to *RPLPO* and relative to vehicle controls at 0 h. Data shown is the mean of triplicate samples from four independent experiments ± s.e. $*P<0.01$, $**P<0.001$ for 4-OHT treatment versus vehicle treatment. (**c**) Representative immunoblot analysis of endogenous PUMA expression in whole cell lysates at time points post-4-OHT treatment up to 24 h. β-actin was used as a loading control. (**d**) Densitometric analyses showing PUMA expression normalized to β-actin and relative to vehicle controls at 0 h, from three independent experiments ± s.e. $*P<0.01$, $**P<0.001$ for 4-OHT treatment versus vehicle treatment. (**e**) MCF-7 cells were incubated with various concentrations of 4-OHT as indicated for 24 h, then attached and floating populations were analyzed for M30-FITC positivity by flow cytometry. Values shown are means of triplicate wells from two independent experiments ± s.e. Samples were also analyzed for PUMA expression by immunoblotting and a representative blot is shown. β-actin was used as a loading control. (**f**) Response of BCL-2 family members to 4-OHT treatment. Representative immunoblots of endogenous BCL-2 family members in whole cell lysates at time points post-4-OHT treatment (7.5 μM, MCF-7; 10 μM, T-47D) up to 24 h. β-actin was used as a loading control. T-47D cells do not express detectable levels of BCL-2 protein or the BIM_L and BIM_S isoforms. (**g**) Effects of ICI/E2 removal on PUMA mRNA expression. Proliferating MCF-7 cells were arrested with the AE, ICI 182780 (10 nM) or EtOH for 48 h, then stimulated with estradiol (E2; 100 nM) or EtOH for 8 h and PUMA mRNA expression determined by RT-PCR, or E2 removed for a further 20 h before analysis. Data shown is the mean of triplicate samples ± s.e. $**P<0.01$, $***P<0.001$.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% apoptosis
24 h
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ERα
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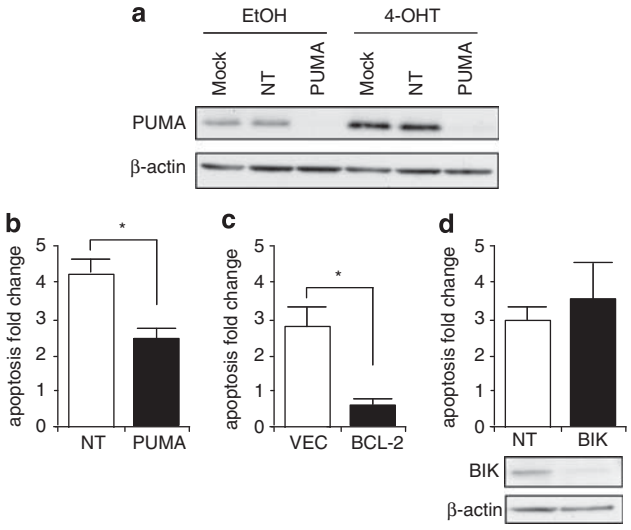

Figure 5 PUMA modulates the apoptotic response to TAM in breast cancer cells. (**a**) MCF-7 cells were transfected with PUMA-specific siRNA, NT control or mock transfected overnight, then treated with 4-OHT or EtOH control for a further 24 h. Levels of PUMA protein expression were determined by immunoblot. Expression of β-actin was used as a loading control. (**b**) Cells were treated with siRNAs as described above in the presence or absence of 4-OHT for 24 h, then induction of apoptosis was determined by M30-FITC labelling and analyzed by flow cytometry. (**c**) MCF-7 cells stably overexpressing *BCL-2* cDNA or vector controls were treated with 4-OHT for 24 h, then apoptosis determined. (**d**) MCF-7 cells treated with BIK-specific siRNA or NT, then treated with 4-OHT for a further 24 h, as described above. The level of apoptosis induced by 4-OHT is shown as fold change relative to vehicle controls, from at least two independent experiments ± s.e. $*P<0.05$ for VEC or NT siRNA versus BCL-2 expressing or PUMA siRNA, respectively.

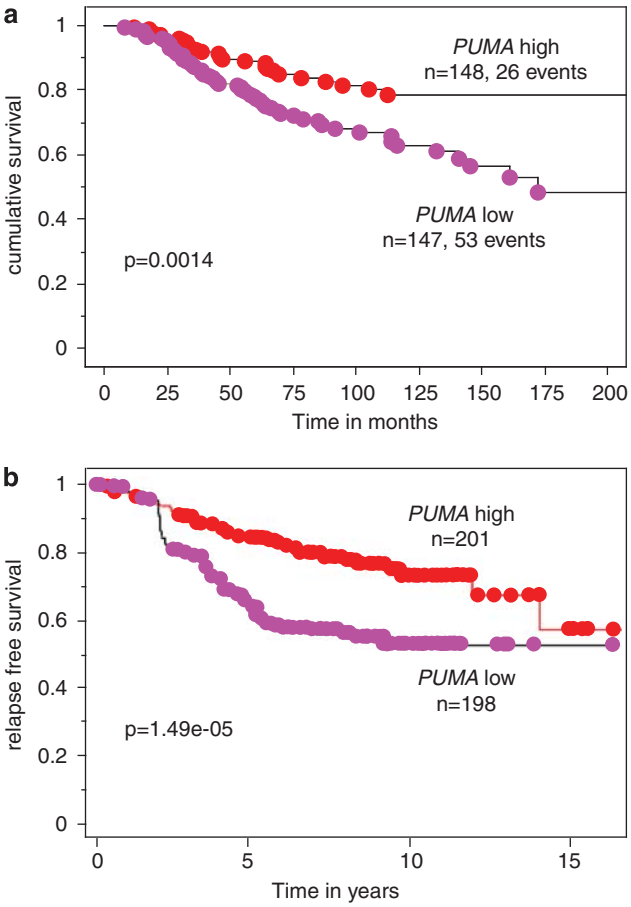

Figure 6 Breast cancer-specific death as a function of *PUMA* mRNA expression. Kaplan–Meier analyses (log rank test) are shown for (**a**) all patients in the NKI cohort (van de Vijver *et al*., 2002) and (**b**) ER+, endocrine-treated patients using KM plotter (http:www.kmplot.com/breast/), from publicly-available microarray data (Győrffy *et al*., 2009). The data were dichotomized at the median value into high and low expressing groups.

lically available gene expression data from van de Vijver *et al*. (2002), chosen because of its similar clinicopathological composition to our clinical cohort (Millar *et al*., 2008). Examination of the distribution frequency of *PUMA* mRNA showed a normal distribution that was dichotomized using the median value (0), into high or low expressing groups. Using this cut point, 147 out of 295 (49.8%) patients were classified as *PUMA* high. High *PUMA* expression correlated with ER+, low tumor grade (1 and 2, all $P<0.0001$) and small tumor size <20 mm ($P=0.023$). Kaplan–Meier analysis for breast cancer-specific death showed that high expression was associated with a favorable prognosis and low expression with a poor prognosis ($P=0.0014$; Figure 6a). Cox proportional hazards models were subsequently constructed to include traditional clinicopathological variables, with step-wise removal of redundant variables until resolution. Using this approach, high *PUMA* expression remained an independent predictor of outcome for breast cancer-specific death (HR 0.534, 95% CI 0.331–0.861, $P=0.01$; Table 1) in a resolved model that also included grade 3 and HER2 amplification.

We further explored the prognostic significance of *PUMA* mRNA expression in ER+, endocrine-treated patients using KM plotter, an online tool that incorporates publicly-available microarray data from 1809 clinically annotated breast cancers (Győrffy *et al*., 2009). Figure 6b shows that high *PUMA* expression remained a highly significant indicator of favorable prognosis in ER+, endocrine-treated patients ($P=1.49\times10^{-5}$).

PUMA expression by immunohistochemistry and patient outcome

PUMA protein expression was assessed by immunohistochemistry using tissue microarrays (TMAs) constructed from tumors from a cohort of 292 patients diagnosed with invasive ductal breast carcinoma (Millar *et al*., 2008). From our original cohort of patients, 268 invasive ductal carcinomas were available for analysis because of loss of some tissue cores during processing of the TMAs. Representative immunohistochemistry staining patterns and intensities of PUMA are illustrated in Figures 7a–d. Cytoplasmic staining was present in 237/268 (88.4%) of cancers and was of variable intensity, which ranged from negative to strong (0–3+), with a

Table 1 Cox multivariate analysis for breast cancer specific death (NKI cohort)

Variable	*HR*	*95% CI*	P-*value*
Size >20 mm	1.722	1.061–2.795	**0.0278**
Grade 3	2.219	1.338–3.680	**0.002**
HER2 amplified	2.782	1.529–5.061	**0.0008**
ER+	0.647	0.381–1.099	0.107
Lymph node +	0.887	0.561–1.402	0.607
PUMA high	0.613	0.369–1.021	0.107
Resolved model			
Grade 3	2.869	1.796–4.582	**<0.0001**
HER2 amplified	2.942	1.687–5.131	**0.0001**
PUMA high	0.534	0.331–0.861	**0.01**

Abbreviations: ER, estrogen receptor; CI, confidence interval; HER, human epidermal growth factor; HR, hazards ratio; NKI, Nederlands Kanker Instituut cohort; PR, progesterone receptor; PUMA, p53-upregulated modulator of apoptosis.
Bold indicates significant *P*-value.

range of 'H score' from 0–270 (Figure 7e). We used the median H score (50) as the cut point to dichotomise the data into high- and low-expressing groups. Using this cut point, 132 of 268 patients (49.3%) were PUMA high. In keeping with the mRNA data, high PUMA expression was positively correlated with ER+, progesterone receptor (PR)+ (both <0.0001), low tumor grade ($P=0.0004$), small tumor size <20 mm ($P=0.0001$), luminal A phenotype ($P<0.0001$), and negatively with p53 status ($P=0.0152$) and the basal subtype ($P<0.0001$). There was no correlation with lymph node status, luminal B or HER2 intrinsic subtypes of breast cancer. Kaplan–Meier analysis showed high PUMA expression to be associated with a good prognosis for all recurrences ($P=0.0126$), distant metastases ($P<0.0001$) and breast cancer-specific death (Figure 7f; $P=0.0115$). However, high PUMA expression was not significant in multivariate analysis for any outcome measure (Table 2).

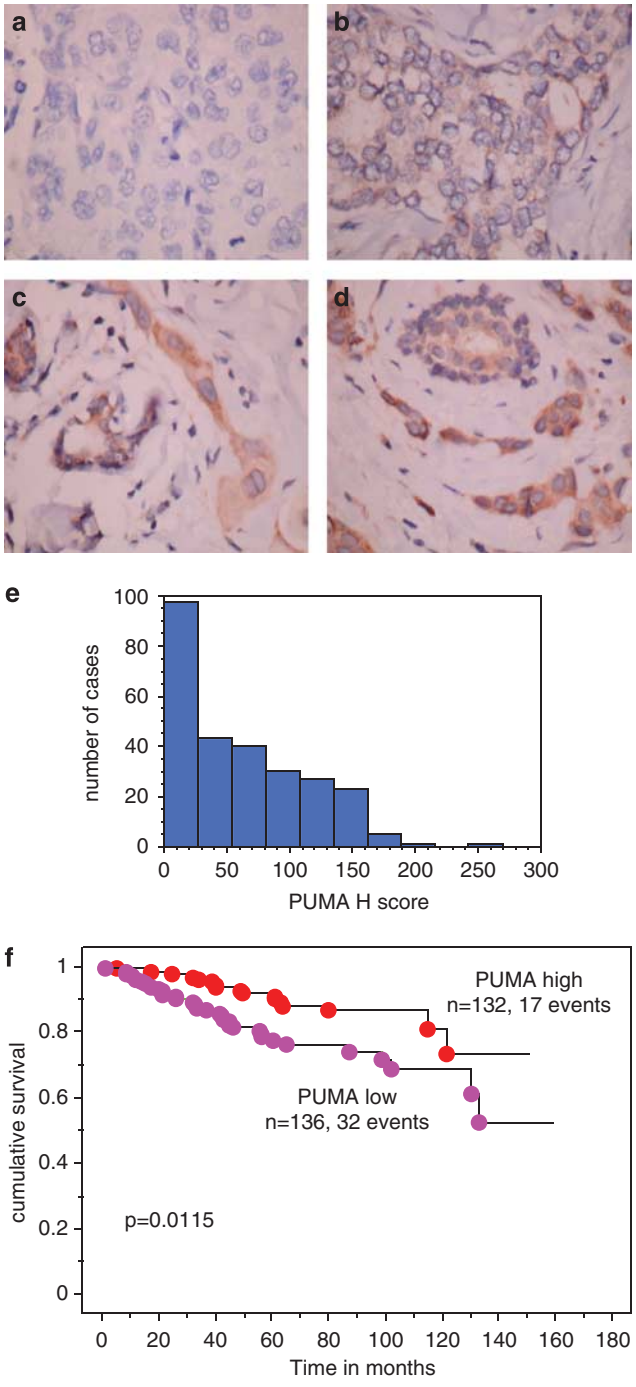

Figure 7 (**a–d**) Representative images of PUMA immunohistochemistry in 268 invasive ductal carcincomas. Cytoplasmic staining was present at variable intensity that ranged from 0 (**a**), 1+ (**b**), 2+ (**c**), 3+ (**d**). The intensity of staining and percentage of positive cells was multiplied to form a modified 'H' (histo) score, the distribution of which is presented in (**e**). (**f**) Kaplan–Meier analysis (log rank test) of breast cancer-specific death in 268 invasive ductal carcinomas. The cohort was dichotomized at the median H score value (50) into high and low expressing groups.

Discussion

Estrogen is a major aetiological factor in the development and progression of breast cancer, and its mitogenic effects on breast cancer cells have been well characterized (Butt *et al*., 2008). However, although the dysregulation of apoptotic/survival pathways is a hallmark of breast cancer like other malignancies (Hanahan and Weinberg, 2000), the influence of disrupted apoptotic signaling on breast tumor growth and response to endocrine therapy is less well understood (Butt *et al*., 2007). Here, we have examined the role of an estrogen target—the BH3-only, pro-apoptotic gene, *PUMA* in this context.

Despite its established role as a p53-upregulated gene and essential mediator of p53-dependent apoptosis (Vousden, 2005), we have demonstrated that *PUMA* mRNA is expressed across a range of breast cancer cell lines, independent of their p53 status, however PUMA protein expression was significantly increased in cells with wild-type p53 compared to those with mutant p53. Interestingly, the acute downregulation of *PUMA* by estradiol was observed in p53 wild-type (MCF-7) and mutant (T-47D) cell lines, suggesting that PUMA's

Table 2 Cox multivariate analysis for breast cancer-specific death

Variable	*HR*	*95% CI*	P-*value*
Size >20 mm	1.078	0.592–1.964	0.805
Grade 3	1.597	0.767–3.325	0.2113
HER2 amp.	2.709	1.461–5.025	**0.0016**
ER+	0.712	0.352–1.442	0.3457
PR+	0.320	0.144–0.710	**0.005**
Lymph node +	3.203	1.703–6.025	**0.0003**
PUMA high	0.719	0.385–1.340	0.2985

Abbreviations: ER, estrogen receptor; CI, confidence interval; HER, human epidermal growth factor; HR, hazards ratio; PR, progesterone receptor; PUMA, p53-upregulated modulator of apoptosis.
Bold indicates significant *P*-value.

regulation and hence, function is independent of p53 in breast cancer. This appears consistent with previous reports that have described p53-independent induction of *PUMA* mRNA by the glucocorticoid, dexamethasone and serum withdrawal (Han *et al*., 2001), and an almost total attenuation of p53-independent, cytokine deprivation-induced apoptosis in *PUMA*-null primary myeloid cells (Jeffers *et al*., 2003).

The rapid transcriptional downregulation of *PUMA* in response to estrogen further delineates the mechanisms by which this anti-apoptotic growth factor mediates its survival effects. Estrogen's transcriptional modulation of other apoptotic mediators such as *BAK*, *BIK*, caspase-9 (Frasor *et al*., 2003; Hur *et al*., 2004) and *BCL-2* (Perillo *et al*., 2000) have been reported, and our current data suggest that *PUMA* and other BH3-only proteins, such as BIK may form part of a broad, anti-apoptotic signaling cascade acutely and irreversibly initiated in response to estrogen in breast cancer cells. Indeed, other potent survival factors such as IGF-I and EGF also repress *PUMA* mRNA and protein expression, with evidence that this is mediated through phosphatidylinositol-3-kinase signaling (Han *et al*., 2001). We were unable to demonstrate direct binding of ERα to putative EREs in the PUMA proximal promoter, using ChIP analysis. However, estrogen-mediated repression does not commonly occur through direct ERE binding, but rather indirectly through the sequestration of shared-transcriptional corepressors necessary for maintaining basal gene expression (Carroll *et al*., 2006). Indeed, the maintenance of the transcriptional effect in the presence of the protein synthesis inhibitor, cycloheximide would support the concept that *PUMA* is a primary, yet indirect, target of ERα action in breast cancer cells. Interestingly, recent work by Hammerich-Hille *et al*. not only demonstrated ERα binding to the proximal *PUMA* promoter at a site distinct from the putative EREs, but also showed the involvement of the scaffold attachment factor, SAFB1 as an obligate, ERα corepressor of *PUMA* in response to estrogen in breast cancer cells. However, in our experimental model system, knockdown of SAFB by specific siRNA did not significantly abrogate estradiol-mediated *PUMA* repression, suggesting that additional, as yet undefined mechanisms may regulate *PUMA* expression under these conditions.

PUMA's pivotal role in the induction of stress-induced apoptosis and as a common target of anti-apoptotic growth factors implicates its dysregulation as an important step in the tumorigenic process. Given that functional p53 is lost in the majority of human cancers, PUMA function is indeed compromised indirectly in these malignancies, with evidence that this impinges on the apoptotic response to irradiation and chemotherapeutic drugs (Yu and Zhang, 2005). More directly, loss of PUMA expression has been reported in melanomas (Karst *et al*., 2005), a proportion of Burkitt's lymphomas (Garrison *et al*., 2008), and interestingly, in the tumor stroma associated with breast carcinoma (Finak *et al*., 2008). However, the lack of spontaneous tumor formation in *puma*-knockout mice (Jeffers *et al*., 2003), and the paucity of studies showing reduced PUMA expression in cancer, suggests that it is not commonly directly inactivated during the tumorigenic process. Indeed, our studies reported herein showed no significant decrease in PUMA expression in breast cancer cell lines compared with normal and immortalized breast epithelial cells, and a normal distribution of *PUMA* mRNA and protein in breast cancer tissue.

Despite this lack of evidence for a direct repression of PUMA in cancers, we did observe highly significant correlations between reduced *PUMA* expression at both the mRNA and protein level in primary breast carcinomas, and breast cancer-specific death—suggesting that when *PUMA* repression does occur, it can impact on disease outcome. Furthermore, following the Cox multivariate regression analysis, high *PUMA* mRNA remained an independent predictor of outcome for disease-specific death in a resolved model including grade 3 and *HER2* amplification. Thus, low *PUMA* expression may serve as a molecular marker of poor prognosis in breast cancer, lending further support to the concept that dysregulation of apoptotic pathways can significantly influence the progression of this disease (Butt *et al*., 2008).

Reduced *PUMA* expression may also have important implications for therapeutic response in breast cancers. PUMA is rapidly induced by chemotherapeutic agents that elicit p53-dependent, DNA damage (Han *et al*., 2001), with evidence that it is necessary to educe an apoptotic response to these drugs (Yu *et al*., 2003). However, to our knowledge, this is the first report of the p53-independent, transcriptional upregulation of PUMA by the AE, TAM. PUMA induction occurred before the detectable onset of apoptosis in this cell system and was specifically associated with the cytotoxic, as opposed to the cytostatic, response to TAM (the latter being observed at lower concentrations). This, together with evidence from PUMA-specific siRNA studies, suggests that PUMA expression may, at least in part, mediate the apoptotic response to 4-OHT in breast cancer cells. Importantly, the prognostic significance of these *in vitro* observations is strongly supported by our clinical data showing a highly significant association between low *PUMA* mRNA expression and worse outcome in ER+, endocrine-treated patients.

Interestingly, manipulation of PUMA levels alone did not completely ameliorate TAM-induced apoptosis *in vitro*, possibly reflecting the complex interplay between numerous apoptotic regulators in coordinating the cytotoxic, endocrine response. Indeed, analysis of additional BCL-2 family proteins following TAM treatment demonstrated that although BIM is also upregulated, other members of the family are not. Of interest is the lack of response of BIK to TAM treatment, which differs from its induction following treatment with the pure AE, fulvestrant (Hur *et al*., 2004). These data suggest that there may be a differential apoptotic response pattern to AEs—a theory supported by the observation that PUMA levels remain unchanged following fulvestrant treatment (Hur *et al*., 2004).

In conclusion, we have identified the pro-apoptotic regulator, PUMA as an important target of estrogen in breast cancer cells, and a predictor of outcome and TAM responsiveness in breast cancer patients. In addition, we have demonstrated PUMA's upregulation by the AE, TAM in breast cancer cells and provided evidence that it can mediate TAM's pro-apoptotic effects in this cell system. Given PUMA's broad promiscuity in binding to all anti-apoptotic members of the Bcl-2 family (Chen *et al*., 2005) and its potent efficacy as an apoptotic mediator, its transcriptional regulation by estrogen and AEs is likely to be of considerable relevance in both breast cancer progression and therapeutic response. Thus, our data support the significant potential of identifying approaches to enhance PUMA's activity as a therapeutic strategy to target, in particular, hormone refractory disease.

Materials and methods

Cell lines and reagents
The human breast cancer cell lines MCF-7 and T-47D were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and routinely maintained in RPMI-1640 supplemented with 5% fetal calf serum (FCS), 10 μg/ml insulin and 2.92 mg/ml glutamine under standard conditions. MCF-7 cells stably overexpressing human BCL-2 have been previously described (Butt *et al*., 2006). 4-OHT was purchased from Sigma-Aldrich (St Louis, MO, USA).

Immunoblot analysis
Proteins from whole cell lysates were resolved under reducing conditions on 12% SDS–polyacrylamide gels using standard methods. Resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes and probed with antibodies against ERα (Lab Vision, Fremont, CA, USA), PUMA (Cell Signaling Technology, Danvers, MA, USA), SAFB (Sigma-Aldrich), BIK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BCL-2 (DakoCytomation, Glostrup, Denmark), BIM (Calbiochem, Darmstadt, Germany), BAD and BID (both BD Biosciences Pharmingen, San Diego, CA, USA) overnight at 4 °C or for 2 h at room temperature. Immunoreactive protein bands were detected by the relevant anti-IgG antibodies conjugated with horseradish peroxidase, followed by enhanced chemiluminescence (Pierce, Rockford, IL, USA). Blots were checked for equal loading by reprobing with anti-actin antibody (Sigma-Aldrich).

Measurement of apoptosis by flow cytometry
For M30 analysis, floating and attached cell populations were combined, fixed and permeabilized in ice cold 70% EtOH, then resuspended in phosphate-buffered saline (PBS/0). 5% bovine serum albumin (BSA) with fluorescein isothiocyanate-conjugated M30 CytoDEATH monoclonal antibody (1:100; Alexis Biochemicals, Lausen, Switzerland) before the M30-positive (apoptotic) population was determined by flow cytometry.

Real-time quantitative PCR
Total RNA was isolated using the RNAeasy kit (Qiagen Victoria, Australia) from cells pretreated with 10 nM ICI 182780 (7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl) nonyl] estra-1,3,5,(10)-triene-3,17β-diol, a kind gift of Dr Alan Wakeling, Astra-Zeneca Pharmaceuticals, Alderly Park, Cheshire, UK) for 48 h and then stimulated with 17β-estradiol (100 nM), and was reverse-transcribed using the Reverse Transcription System (Promega, Sydney, Australia), according to the manufacturer's instructions. Real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using inventoried (pre-made) Taq-Man probes for *PUMA* (Applied Biosytems). Data analyses were performed using the DCt method with *RPLPO* (Applied Biosystems) as an internal loading control. Fold changes in gene expression were calculated relative to untreated controls.

Luciferase reporter assays
MCF-7 and T-47D cells were transfected using Lipofectamine 2000 (Invitrogen Life Technology, San Diego, CA, USA) with a luciferase reporter construct containing either the *PUMA* promoter (a kind gift from Thomas Chittenden, Immunogen, Cambridge, MA, USA), or an ERE sequence as a positive control, in the promoter-less pGL3-Basic vector. 24 h post-transfection, cells were pretreated with 10 nM ICI 182790 for 24 h and then stimulated with 17β-estradiol (100 nM) for 24 h. Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega) and normalized to the activity of the pGL3-Basic reporter.

RNA interference
Small interfering RNAs (siRNA) specific for *PUMA*, *SAFB* or *BIK* (ON-TARGETplus SMARTpool, human BBC3, SAFB and BIK, respectively) and non-targeting controls (ON-TARGETplus siCONTROL) were purchased from Dharmacon. Cells were transfected with LipofectAMINE 2000 in the presence of the siRNAs according to the manufacturer's protocol.

PUMA *gene expression profiling*
Publically available gene expression data sourced from a published study by van de Vijver *et al*. (2002) of breast cancer outcome were analysed to determine a potential relationship between *PUMA* mRNA levels and prognosis. The data set from the Nederlands Kanker Instituut and designated the NKI cohort, comprised 295 patients, 76% of which were ER+, with a median follow-up of 93.6 months (range 0.6–220 months) and was of similar clinicopathological composition to our clinical cohort. Data were generated using Rosetta NKI-spotted oligonucleotide arrays and were downloaded from http://microarray-pubs.stanford.edu/wound_NKI/explore.html as log 2-transformed values in a text table format, as previously described (Millar *et al*., 2008). Raw data were directly transferred to the final output file without further

processing. Data from one probeset were available and expression data were analysed for frequency distribution of mRNA and its association with patient outcome.

Further analyses in ER+, endocrine-treated patients were carried out using KM plotter (http://www.kmplot.com/breast/), an on line tool that incorporates public microarray data from 1809 breast cancer patients and enables filtering based on hormone receptor status and adjuvant endocrine treatment (Györffy *et al*., 2009).

Patient characteristics and clinical cancer cohort

PUMA protein expression was assessed by immunohistochemistry using TMAs constructed from tumors from a cohort of 292 patients diagnosed with invasive ductal breast carcinoma. This cohort has been previously described in more depth elsewhere (Millar *et al*., 2008). Briefly, the cohort consists of cases of invasive ductal carcinoma of no special type, median age 54 (range 24–87) with a median follow-up of 64 months (range 0–152.1). Of these, 68.6% were ER+, 57.1% PR+, 18.7% HER-2 amplified (by FISH), 43.3% lymph node positive. Endocrine therapy (TAM) was given to 49.3% of patients and chemotherapy (AC or CMF) to 38%. Before the approval for this study was obtained from the Human Research Ethics Committee of St Vincent's Hospital, Sydney (HREC SVH H94/080, SVH H00/36).

Immunohistochemistry

4-μm sections were cut from each TMA, mounted on SuperFrost Plus glass slides and baked for 2 h at 70 °C, then dewaxed by passage through xylene (two 5 min washes), cleared and rehydrated in graded alcohol (100, 95 and 70%) ending in a distilled water wash. Antigen retrieval was performed using Dako solution (pH 9.0, s2367, Dako, Carpentaria, CA, USA) in a pressure cooker (Dako Pascal Decloaker) for 30 s, followed by cooling gently for 15 min in a running water bath. Following a thorough wash in distilled water, endogenous peroxidase activity was eliminated with 3% hydrogen peroxide for 5 min. Slides were incubated with PUMA rabbit polyclonal antibody (#4976, Cell Signaling Technology), 1:300 dilution for 60 min at room temperature (RT), then staining was completed in a Dako autostainer. Slides were then rinsed in water and counterstained with haematoxylin, dehydrated through graded ethanol, cleared in xylene and mounted. Negative tissue controls included kidney and prostate, as well as isotype-matched non-specific immunoglobulin substituted for the primary antibody.

All assessments of PUMA immunohistochemical staining were performed by an experienced breast pathologist (EKAM) blinded to the clinical and molecular data and patient outcome. Cytoplasmic staining for PUMA was described in terms of the intensity (0: negative, 1+: weak, 2+: moderate and 3+: strong) and percentage of cells staining positive. From these indices, a simplified '*H* score' (that is, intensity × percentage of positive staining) was calculated for each core and a mean and median score for each parameter calculated for each tumor (range of two to six cores per patient).

ER, progesterone receptor (PR), cytokeratin 5/6 and epidermal growth factor receptor (EGFR) were also stained using the following antibodies: ER (1:100; clone 6F11; Dako), PR (1:200; clone PgR 636; Dako), CK5/6 (1:80; clone MAB1602; Chemicon International, Temecula, CA, USA), EGFR (1:100; clone H11; Dako) and p53 (1:400; clone DO-7, Novocastra, UK). HER-2 FISH was assessed in the Australian National Reference Laboratory (Department of Pathology, St Vincent's Hospital, Sydney, Australia) using the Vysis PathVysion HER-2 DNA dual-colour probe kit (Abbott Laboratories, Abbott Park, IL, USA). A HER2:chromosome 17 ratio >2.2 was classified as HER2 amplification. ER and PR were assessed as positive if they had an H score of >10. CK5/6, and EGFR were assessed as positive if there was any positive cytoplasmic or membranous staining present at any intensity. P53 was considered positive if $>10\%$ staining was present at any intensity.

Statistical analyses

Statistical analyses were performed using Statview 5.0 Software (Abacus Systems, Berkeley, CA, USA). For *in vitro* studies, differences between groups were evaluated by Fisher's protected least significant difference test after analysis of variance (ANOVA) or factorial analysis where appropriate. $P<0.05$ was accepted as statistically significant. *PUMA* mRNA and PUMA protein expression, and its association with clinicopathological variables and intrinsic molecular phenotype of breast cancer were tested by applying the χ^2-test of association in contingency tables. Kaplan–Meier and Cox proportional hazards model were used for univariate analysis and the latter for multivariate analyses. Those factors that were prognostic in univariate analysis were then assessed in a multivariable model to identify factors that were independently prognostic and those that were the result of confounding variables.

Abbreviations

AE, anti-estrogen; TAM, tamoxifen; FCS, fetal calf serum; 4-OHT, 4-hydroxytamoxifen; ER, estrogen receptor; ChIP, chromatin immunoprecipitation; TMA, tissue microarray; PR, progesterone receptor; EGFR, epidermal growth factor receptor; ERE, estrogen response element; HER2, human epidermal growth factor receptor 2.

Conflict of interest

The authors declare no conflict of interest.

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