

p62 overexpression in breast tumors and regulation by prostate-derived Ets factor in breast cancer cells

H Garrett R Thompson¹, Joseph W Harris¹, Barbara J Wold², Fritz Lin³ and James P Brody¹

¹Department of Biomedical Engineering, University of California, Irvine 204 Rockwell Engineering Center, Irvine, CA 92697-2715, USA; ²Department of Biology, California Institute of Technology, 1200 E. California Blvd., Pasadena, CA 91125, USA; ³Irvine Medical Center, University of California, 101 The City Drive South Orange, CA 92868, USA

p62 is a multifunctional cytoplasmic protein able to noncovalently bind ubiquitin and several signaling proteins, suggesting a regulatory role connected to the ubiquitin–proteasome pathway. No studies to date have linked p62 protein expression with pathological states. Here we demonstrate the overabundance of p62 protein in malignant breast tissue relative to normal breast tissue. The proteasome inhibitor PSI increased p62 mRNA and protein; however, PSI treatment of breast epithelial cells transfected with the p62 promoter did not affect promoter activity. High levels of prostate-derived Ets factor (PDEF) mRNA have been identified in breast cancer compared to normal breast. Only the PSA and maspin promoters have been identified as targets of this transcription factor. Here we show that PDEF stimulates the p62 promoter through at least two sites, and likely acts as a coactivator. PSI treatment abrogates the PDEF-stimulated increase of p62 promoter activity by 50%. Thus, multiple mechanisms for the induction of p62 exist. We conclude that (1) p62 protein is overexpressed in breast cancer; (2) p62 mRNA and protein increase in response to PSI, with no change of basal promoter activity; (3) PDEF upregulates p62 promoter activity through at least two sites; and (4) PSI downregulates PDEF-induced p62 promoter activation through one of these sites.

Oncogene (2003) 22, 2322–2333. doi:10.1038/sj.onc.1206325

Keywords: breast cancer; P62; PDEF; promoter; proteasome inhibitor; SQSTM1

Introduction

A milestone in the field of cancer research was the discovery that the cell cycle is deregulated in breast cancer tissue and human breast tumor cell lines (Keyomarsi and Pardee, 1993). A key regulatory mechanism of the cell cycle is specific degradation of the cyclins by the ubiquitin–proteasome pathway (Hochstrasser, 1996; DeSalle and Pagano, 2001). This pathway is often

regarded as a simple method that the cell employs for the destruction of incorrectly folded proteins, or as a general means of regulating the degradation of proteins. However, the proteasome has recently been recognized to play a crucial role in many different fundamental biochemical processes, including cell cycle, apoptosis, metabolism, immune response, signal transduction, stress-response and cell differentiation (reviewed in Kornitzer and Ciechanover, 2000). It is now well established that the ubiquitin–proteasome pathway can be the target of cancer-related deregulation and can lead to the transformation of normal cells to cancer cells, drug resistance and tumor progression (Spataro *et al.*, 1998).

In gastric cancer cells (Fan *et al.*, 2001), prostate (An *et al.*, 1998), transformed fibroblasts (An *et al.*, 1998), rat neuronal cells (Pasquini *et al.*, 2000; Kuusisto *et al.*, 2001) and breast cancer cells (this report), cell proliferation is inhibited by proteasome inhibitors (PIs) in a time- and dose-dependent manner. When assessed, it was shown that decreased cell proliferation was the result of apoptosis, suggesting that PIs have potential use as anticancer drugs. In fact, there are several PIs in clinical trials, including PS-341 (Adams, 2001) now in phase III and others that demonstrate promising results as cancer therapies (Owa *et al.*, 2001).

The product of the SQSTM1 gene, p62, is a widely expressed multifunctional protein with diverse functions. With respect to the ubiquitin–proteasome pathway, p62 has been shown to noncovalently bind free ubiquitin (Vadlamudi *et al.*, 1996), serving as an ubiquitin sink (Shin, 1998) and may represent an ubiquitination-mediated regulatory mechanism during cell proliferation and differentiation (Lee *et al.*, 1998). Several diverse roles for p62 have been proposed based on the rat p62 homolog zeta protein kinase C (PKC)-interacting protein (ZIP) and the mouse proteins A170 and signal transduction adapter protein (STAP), which share approximately 90% homology to each other (Geetha and Wooten, 2002). These functions include acting as a scaffold linker protein in the NF- κ B signal transduction pathway (Sanz *et al.*, 1999, 2000), K⁺ channel modifier (Gong *et al.*, 1999), and nuclear factor (Rachubinski *et al.*, 1999; Sudo *et al.*, 2000; Geetha and Wooten, 2002). The ability of p62 to bind noncovalently to ubiquitin and several signaling proteins suggests that

*Correspondence: JP Brody; E-mail: jpbrody@uci.edu
Received 16 July 2002; revised 6 December 2002; accepted 10 December 2002

p62 may play a regulatory role connected to the ubiquitin pathway.

Some researchers have analysed the expression of p62 under basal and stimulated conditions. For instance, reports have found that the p62 mRNA is ubiquitously expressed in a variety of tissue types, including normal tissues, by Northern blot analysis (Joung *et al.*, 1996) and computational analysis of cDNA libraries (Ghadersohi and Sood, 2001). Upregulation of p62 mRNA and protein has been shown in reference to neuronal apoptosis (Kuusisto *et al.*, 2001), and stimulation of peripheral blood mononuclear cells with phorbol 12-myristate 13-acetate (PMA)/ionomycin increased protein levels by stabilizing mRNA as well as stimulating *de novo* transcription (Lee *et al.*, 1998). No studies to date have correlated p62 protein expression with cancer. In this report, we assess the relative abundance of p62 protein between normal and malignant breast tissue.

Many ETS-domain transcription factors have been linked to cancer by increased or decreased expression, as well as their involvement in signal transduction cascades resulting in the regulation of proto-oncogenes or tumor-suppressor proteins (reviewed in Dittmer and Nordheim, 1998). This is in accordance with the understood role of transcription factors, in which deregulation uncouples the tight cellular control between signaling pathways and controlled cell growth. Generally, Ets family members are nuclear targets of signal transduction and function in concert with other nuclear proteins (Wasylyk *et al.*, 1998).

Prostate-derived Ets factor (PDEF) is a recently described member of the Ets transcription factor family (Oettgen *et al.*, 2000). High levels of PDEF transcript have been identified in breast tumor cDNA libraries compared to normal breast and was found to be upregulated in 14 of 20 primary breast tumors examined and in the peripheral blood of one patient suffering from metastatic breast cancer (Ghadersohi and Sood, 2001). To date, only the PSA promoter (Oettgen *et al.*, 2000) regulated by NKX-3.1 protein binding (Chen *et al.*, 2002) and the maspin promoter (Yamada *et al.*, 2000) have been identified as targets of PDEF. Recently, a consensus DNA-binding sequence based on the core GGAT motif has been proposed for PDEF based on 11 potential binding sites in the PSA promoter (Oettgen *et al.*, 2000), although the maspin sequence does not match the consensus.

Results

p62 Protein overexpression in breast cancer

The seemingly diverse functions of p62 suggest that transcription of the p62 gene or translation of the protein is tightly controlled. To investigate the differential expression of this protein in breast cancer, we assessed relative p62 protein concentrations in 13 normal and malignant breast tissues by Western blot (Figure 1). In total, 40 μ g total cell lysate from three invasive ductal carcinoma samples, one secondary metastatic liver tumor and nine normal samples, three

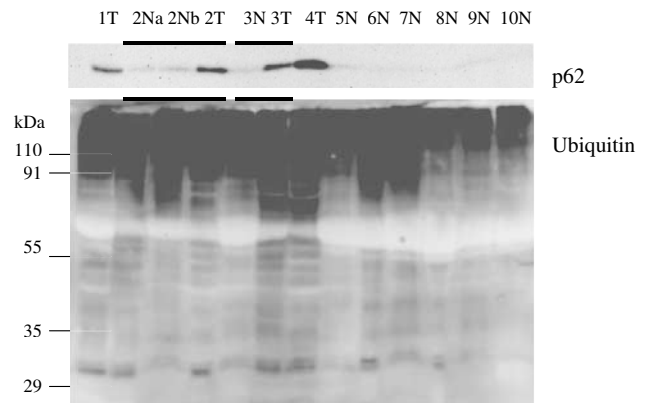


Figure 1 p62 Protein expression in normal and cancerous breast tissue. (a) A measure of 40 μ g total cell lysate from nine normal and four tumor tissue samples, extracted in RIPA buffer, was subjected to Western blot analysis for p62 and ubiquitinated proteins. Results are visualized by chemiluminescence. Molecular weight markers are indicated. Underscores indicate matched samples from the same patient. Samples were obtained from 10 different individuals

matched to tumors, were analysed. Each of the four tumor samples exhibited a dramatically higher expression level of p62 relative to normal breast tissue, up to a 122-fold increase (Table 1). Samples were normalized to total protein content, as determined by the method of Bradford.

As part of an effort to identify a suitable cell line for studying the regulation of p62, expression levels of p62 were assessed in four normal and four tumorigenic breast epithelial cell lines. Representative Western blots are shown in Figure 2. A measure of 15 μ g total cell lysate, determined by Bradford protein assay was loaded in duplicate lanes. Two of four transformed cell lines expressed greater than twofold higher levels of p62 protein than the four normal breast epithelial cell lines (Table 2). Values are normalized to β -actin protein expression and differ little when normalized to total protein content.

Since p62 has been shown to bind ubiquitin non-covalently, it has been proposed that increased p62 protein levels may be a protective mechanism in response to increased ubiquitinated proteins. Thus, concentrations of ubiquitinated proteins were assessed by Western blot using a monoclonal antiubiquitin antibody for the tissue samples (Figure 1) and cell lines (data not shown). No correlation between ubiquitinated protein concentration and p62 concentration was observed.

PDEF and *p62* mRNA expression levels in normal breast and malignant breast epithelial cells

p62 mRNA levels in three cancer cell lines and two normal cell lines were assessed by real-time PCR in an effort to account for observed differences in p62 protein levels. All five cell lines showed similarly high levels of p62 mRNA regardless of p62 protein concentration (Figure 3a). This finding is in accordance with previous

Table 1 p62 Protein expression in normal and cancerous breast tissue

Sample	Patient #	Description	Relative p62
1T	1	Invasive ductal carcinoma	16-fold
2Na	2	Normal liver tissue, matched	2.0-fold
2Nb	2	Lymph node, metastatic breast cancer, matched	4.8-fold
2T	2	Liver tumor, metastatic breast cancer	19-fold
3N	3	Normal breast tissue, matched	3.1-fold
3T	3	Invasive ductal carcinoma	22-fold
4T	4	Invasive ductal carcinoma	122-fold
5N	5	Normal breast tissue	2.9-fold
6N	6	Normal breast tissue	1.7-fold
7N	7	Normal breast tissue	1.3-fold
8N	8	Normal breast tissue	2.0-fold
9N	9	Normal breast tissue	2.1-fold
10N	10	Normal breast tissue	onefold

Samples are identified as tumor (T) or normal (N). Matched normal samples are denoted with an N following T number and are identifiable by patient number. All nonmatched normal breast tissue samples were derived from breast reduction surgeries. Matched normal tissues are described as grossly unremarkable. Relative p62 protein values are determined by densitometry from data presented in Figure 1 and are normalized to 10N, a normal breast tissue sample

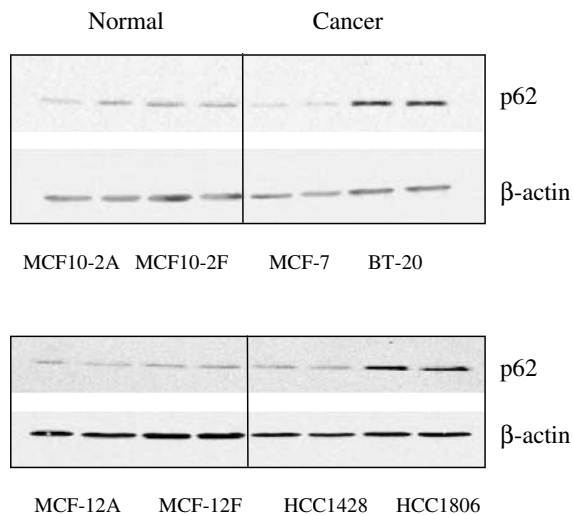


Figure 2 p62 Protein expression in normal and transformed breast epithelial cell lines. (a) A measure of 15 μ g total cell lysate, extracted in RIPA buffer, was subjected to Western blot analysis in duplicate lanes. Results of p62 and β -actin expression are presented for four normal breast epithelial cell lines: MCF10-2A, MCF10-2F, MCF-12A and MCF-12F and four transformed cell lines: MCF-7, BT-20, HCC1428 and HCC1806. Results are visualized by chemiluminescence. Figure is representative of three independent Western blots

studies that found p62 mRNA ubiquitously expressed in numerous tissues, both normal and cancer.

It has previously been shown that PDEF mRNA is significantly increased in breast cancer cells. We assessed PDEF mRNA levels in normal breast and transformed breast epithelial cell lines by real-time PCR (Figure 3a) and Northern blot analysis (Figure 3b). Northern blot analysis is not sensitive enough to detect PDEF mRNA levels from the normal cell lines. By quantitative real-time PCR, we demonstrate greater than a 100-fold increase in PDEF mRNA levels in two of the breast cancer cell lines relative to normal cells. Note however, that this does not necessarily translate into 100-fold

increase in PDEF protein, or upregulation of p62 100-fold. Interestingly, HCC1806 cells express the highest p62 protein levels, while expressing the lowest PDEF mRNA levels, 1000-fold lower than the other tumorigenic cell lines studied. While mRNA levels do not necessarily correlate to protein levels, it should be noted that expression of PDEF can be regulated translationally in prostate epithelial cells, as PDEF was detected in normal prostate glandular epithelial cells, but not in carcinoma cells with hPSE/PDEF transcripts, using anti-hPSE antiserum (Nozawa *et al.*, 2000). Based on this apparent discrepancy, we used HCC1806 cells to transfect PDEF and the p62 promoter to study the regulation of this promoter.

p62 Promoter analysis in the presence and absence PDEF

While p62 mRNA and protein expression data are suggestive of increased mRNA stability or altered translation rates, an increase in p62 transcription could not be ruled out. Based on the identification of a preferred Ets-1 consensus sequence for PDEF and the high-PDEF mRNA levels in transformed cells, we analysed whether PDEF acts as a regulator of p62 gene expression. To test this hypothesis, transfection experiments were performed using p62 promoter-luciferase constructs. Briefly, two potential PDEF binding sites were identified in the -1.8 kb promoter region of the p62 gene. The putative PDEF binding site furthest from the transcription start site (Ets-1a) located -1175 to -1166 most closely resembles the proposed consensus binding sequence for PDEF (Oettgen *et al.*, 2000). The likelihood of the proximal site (Ets-1b) located -516 to -509 being a preferred PDEF binding site could not be determined by visual inspection as it differed from the sequences analysed in that study.

When transfected into HCC1806 cells in culture, the 1.8 kb region upstream of the SQSTM1 gene increased luciferase activity approximately 10-fold over control transfections (Figure 4). Cotransfection with PDEF increased promoter activity another 3.5-fold. Mutating

Table 2 p62 Protein expression in normal and transformed breast epithelial cell lines

Cell line	Description	Relative p62/ β -actin
MCF-7	Pleural effusion from mammary adenocarcinoma	0.9-fold \pm 0.1
BT-20	Tumorigenic mammary epithelial cells	2.3-fold \pm 0.2
HCC1428	Epithelial breast adenocarcinoma and pleural effusion cells from a metastatic site	1.2-fold \pm 0.1
HCC1806	Primary acantholytic squamous cell carcinoma	6.3-fold \pm 0.1
MCF12-F	Nontumorigenic epithelial cells from normal mammary tissue	1.4-fold \pm 0.1
MCF12-A	Nontumorigenic epithelial cells from normal mammary tissue	0.9-fold \pm 0.2
MCF10-2F	Nontumorigenic mammary epithelial cells	1.1-fold \pm 0.1
MCF10-2A	Nontumorigenic mammary epithelial cells	Onefold \pm 0.2

Relative p62/ β -actin expression ratios are presented normalized to MCF10-2A. Values represent the mean expression ratios of three independent experiments. Error bars indicate s.e.m. MCF-7, BT-20, HCC1428 and HCC1806 are transformed tumorigenic breast epithelial cells. MCF12-F, MCF12-A, MCF10-2F and MCF10-2A are cell lines derived from normal breast epithelial cells

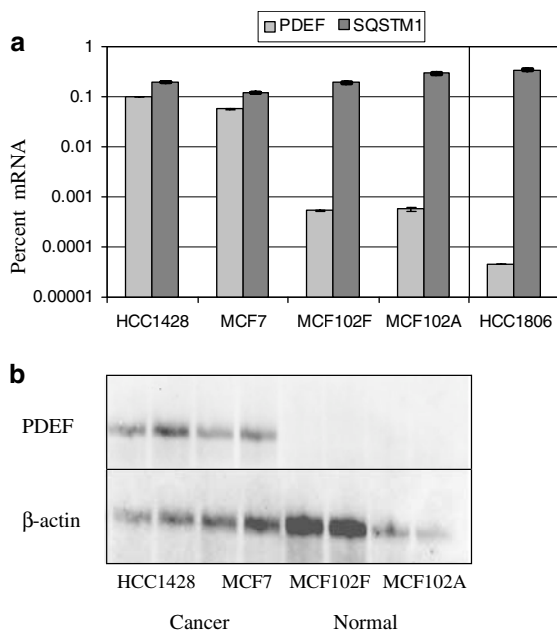


Figure 3 PDEF and SQSTM1 mRNA concentrations in breast cancer cell lines. **(a)** HCC1428, MCF-7 and HCC1806 are malignant; MCF102F and MCF102A are nontransformed. Quantitation of SQSTM1 and PDEF mRNA by real-time PCR. Data are normalized to mRNA concentration and is represented as a percentage of total mRNA on a log scale. Samples were assayed in triplicate in three separate experiments, with error bars indicating the s.e.m. typically less than 5%. **(b)** Northern blot of PDEF and β -actin mRNA. HCC1428 and MCF-7 are malignant; MCF102F and MCF102A are nontransformed. Samples assayed in duplicate

the core sequence of the distal Ets-1a site from GGAT to GGAA had no effect on promoter activity in the absence or presence of the PDEF construct. In contrast, however, a single-point mutation of the Ets-1b site from GGAT to GGAA decreased the basal promoter activity by approximately 70%. As expected, the double mutant construct yielded results identical to the Ets-1b mutant data. Interestingly, for the Ets-1b and double mutant transfections, cotransfection with PDEF increased activity 3.5-fold, recovering the basal wild-type promoter level. Removal of the eight nucleotide Ets-1b site altogether restored basal promoter activity; however, cotransfection with PDEF resulted in only a 2.3-fold

increase, thereby abrogating the PDEF effect by approximately half (Figure 5).

Regulation of p62 protein levels by PSI in a time-dependent manner

Previous studies have assessed the role of PIs on cellular death in a number of different cell types (An *et al.*, 1998; Pasquini *et al.*, 2000; Fan *et al.*, 2001; Kuusisto *et al.*, 2001). An appropriate PI and dose was determined through the use of cell proliferation assays. Dramatic cell death (50–80%) was observed in a dose-dependent manner after 24 h over the concentrations of the three PIs assayed: 1–16 μ M PSI, 5–20 μ M MG-132, 5–20 μ M lactacystin (Figure 6).

p62 Protein levels as well as ubiquitinated proteins were assessed from cultured normal (MCF10-2A, MCF10-2F) and cancer cell lines (MCF-7, HCC1428 and HCC1806) treated with 10 μ M PSI for 0, 8, 16 or 32 h. A 32 h DMSO vehicle treatment was included as a control. A measure of 15 μ g total cell lysate, extracted in RIPA buffer, was subjected to Western blot analysis for p62 and total ubiquitinated substrates in duplicate lanes and visualized by chemiluminescence. For each cell line, PSI increased p62 protein expression in a time-dependent manner, with a rapid accumulation of ubiquitinated substrates. Representative data for three of these cell lines are presented in Figure 7. Similar results were found for the cell lines not shown. To show equal loading of cell lysate, β -actin is included as a control.

PDEF and p62 mRNA concentrations in breast cancer cells in the presence and absence of PSI

To study whether the increase of p62 protein in PSI-treated cells was because of increased mRNA, mRNA expression levels of p62 from HCC1806 cells treated with 10 μ M PSI was measured by real-time PCR over 32 h (Figure 8). DMSO-treated vehicle values are shown for each time point. Addition of PSI to cultured HCC1806 cells increased p62 mRNA in a time-dependent manner, with the greatest stimulation observed at 32 h. Thus, the effects of PSI on PDEF-stimulated p62 promoter activity were assessed.

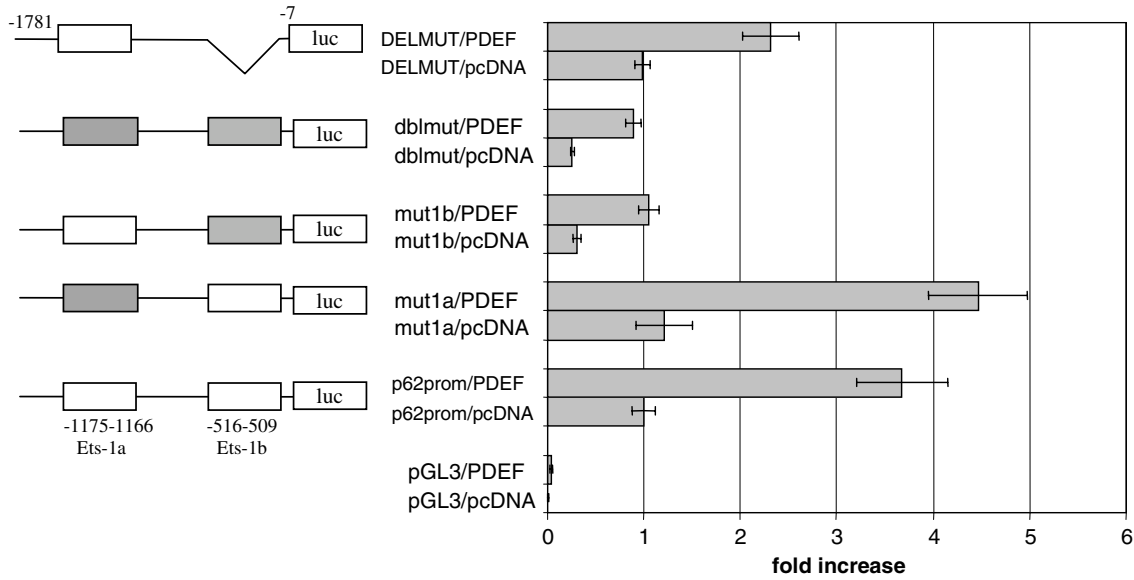


Figure 4 SQSTM1 promoter analysis. SQSTM1 promoter (from -1781 to -7) cloned in the pGL3 basic luciferase reporter construct was transfected into HCC1806 cells in the absence and presence of the PDEF/pcDNA3.1 construct. Point mutations (gray boxes) were generated in the core binding sequence for each of the Ets-1 sites (white boxes). In each case, the mutation altered the core sequence GGAT to GGAA. Positions of Ets-1 sites are indicated relative to the transcription start site. 'luc' denotes luciferase gene. Results are presented as the luciferase/ β -gal mean for three independent transfections, each plated in triplicate wells, each well assayed in triplicate. Error bars are s.e.m.

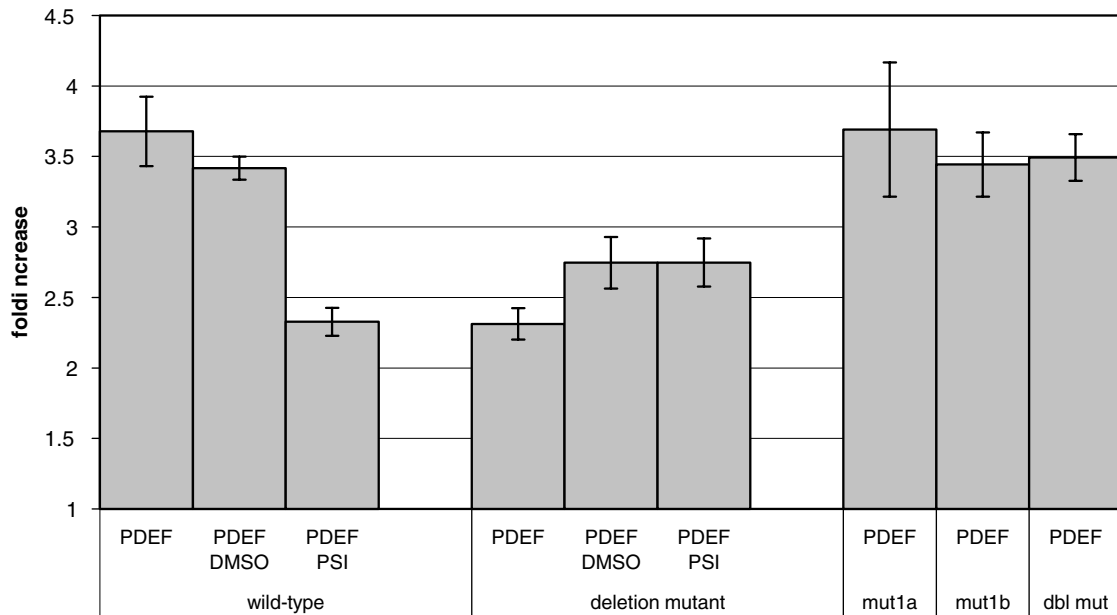


Figure 5 PDEF stimulation of p62 promoter activity. Graphical representation of PDEF effect on p62 promoter activity compared to pcDNA3.1+ empty vector transfected cells. Data are presented as fold increase because of PDEF. Addition of PSI attenuates the PDEF effect similar to deletion of site Ets-1b. DMSO-treated transfected cells are presented as a vehicle control for PSI treatment

p62 Promoter analysis in the presence and absence of PSI

The question as to whether the observed PSI-stimulated increase of p62 mRNA was because of increased transcription was studied using the p62 promoter construct. Addition of PSI increased p62 mRNA levels (Figure 8), but did not increase basal promoter

activity after 8 h compared to DMSO vehicle control-treated cells (Figure 9), suggesting that increases in mRNA levels are likely because of increased mRNA stability rather than increased transcription. PSI attenuated the PDEF-induced increase of promoter activity compared to wild-type or vehicle-treated cells (Figure 5), an effect that is reproduced by deleting the Ets-1b site.

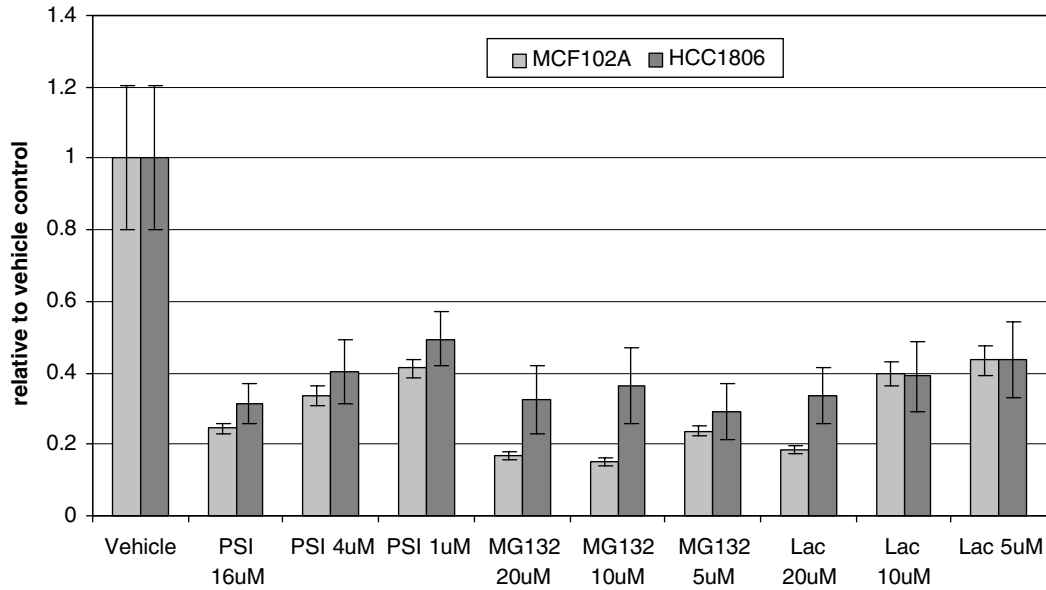


Figure 6 Cell proliferation of MCF102A and HCC1806 cells in the presence of PIs. Cells were cultured in the presence of three concentrations of PSI, MG132 and lactacystin or vehicle control (1 : 1000 dilution DMSO) for 24 h. Results are presented as relative cell number to vehicle control. Error bars represent the s.e.m. for eight replicates

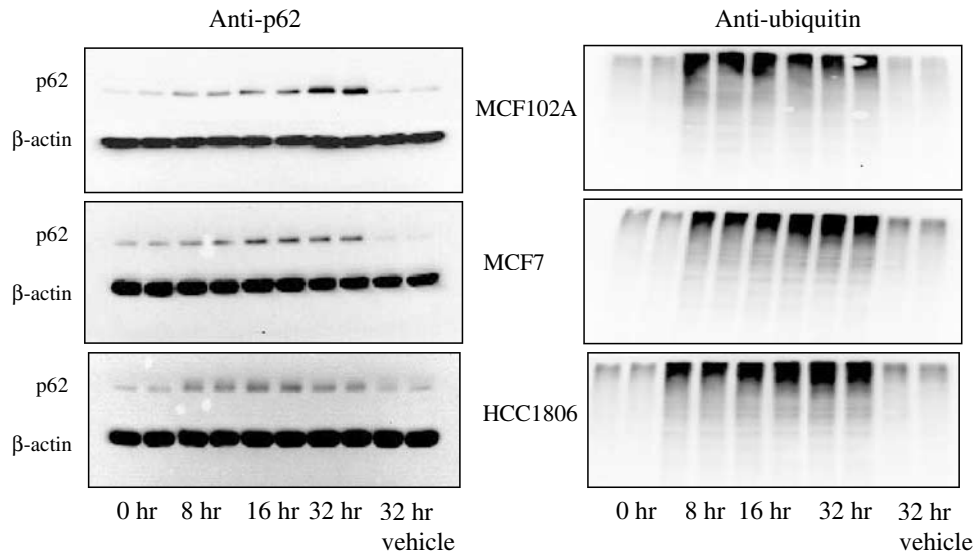


Figure 7 Anti-p62 and anti-ubiquitin Western blots of PSI-treated cells. Cultured cells were incubated with 10 μ M PSI, or DMSO vehicle, for the times indicated. A total of 15 μ g total cell lysate, extracted in RIPA buffer, was subjected to Western blot analysis in duplicate lanes. Results were visualized by chemiluminescence. P62 is the top band in left panels; β -actin is the bottom band in left panels. Ubiquitinated substrates for each cell line are shown in the right panels. Data are presented for one nontransformed cell line, MCF10-2A and two transformed cell lines, MCF7 and HCC1806. In all three PSI leads to increased p62

Discussion

We report here for the first time that p62 protein is overexpressed in cancerous tissue. In this study we demonstrate basal promoter activity of the 1.8 kb upstream region of the SQSTM1 gene and the ability to stimulate promoter activity with PDEF, a proposed candidate breast tumor marker. We show that while treatment of cultured breast epithelial cells with PSI

increases p62 mRNA and protein, there is no effect on basal promoter activity, suggesting a stabilization of mRNA. Furthermore, PSI treatment of cells transfected with the PDEF construct attenuated the PDEF effect to approximately half. Deletion of the Ets-1b site altogether has the identical effect as treatment with PSI, and PSI treatment of cells transfected with this construct had no additional effect. These results show (1) PDEF upregulates the p62 promoter, (2) PDEF certainly

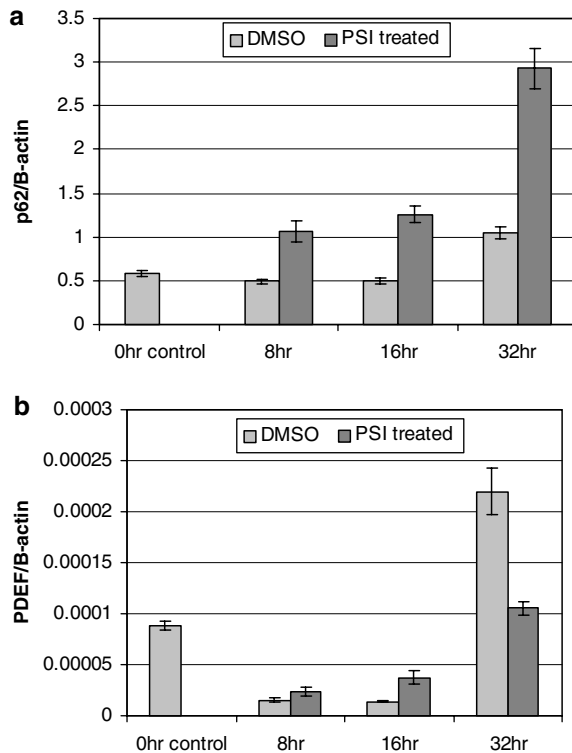


Figure 8 p62 and PDEF mRNA concentrations in PSI-treated HCC1806 breast cancer cells. Cultured cells were incubated with 10 μ M PSI, or DMSO vehicle, for the times indicated. Message RNA was purified from total RNA, reverse-transcribed to cDNA, quantitated by real-time PCR and normalized to β -actin mRNA concentration. Values are the means of three independent experiments, each assayed in triplicate. Error bars represent s.e.m. **(a)** Relative p62 mRNA expression with PSI treatment over 32h. DMSO vehicle effects were assessed for each time point and are presented for reference. **(b)** Relative PDEF mRNA expression with PSI treatment over 32h. DMSO vehicle effects were assessed for each time point and are presented for reference

affects approximately half, but not all, of its regulation at the Ets-1b site, (3) PSI specifically blocks PDEF action at the Ets-1b site, but not elsewhere.

PDEF mRNA is 100-fold higher in two human breast cancer cell lines compared to untransformed cultured epithelial cells. However, PDEF mRNA was not highly expressed in HCC1806 cells, making this a suitable cell line for PDEF cotransfection studies. Furthermore, in our efforts to identify a cell line suitable for studying the regulation of p62, we found that MCF-7 and HCC1806, two commercially available tumorigenic breast epithelial cell lines, do not overexpress p62 protein like their tissue counterparts. Thus, HCC1806 cells were chosen for PDEF cotransfection studies because they express relatively high levels of p62 protein and very low levels of PDEF mRNA.

p62 Promoter activity has been previously described by large-scale deletion analysis (Vadlamudi and Shin, 1998). Primary nucleotide sequence analysis of the p62 promoter identified two putative Ets-1 transcription factor binding sites. Based on the PDEF consensus sequence proposed by Oettgen *et al.* (2000), we identified

these Ets-1 sites as possible PDEF binding sites. The distal site resides in a 500 bp region responsible for approximately two-thirds of the basal promoter activity, with the proximal site in a 500 bp region showing no further reduction of promoter activity when deleted (Vadlamudi and Shin, 1998). When PDEF is cotransfected with the wild-type p62 promoter, 3.5-fold stimulation of basal promoter activity is observed. It was expected that mutation of the distal site would affect basal promoter activity based on its location within the key 500 bp element and also the high degree of homology with the proposed PDEF consensus sequence. The negative result from mutating this sequence demonstrates that the GGAT core at that site is not a crucial element for basal transcription and also that PDEF does not mediate any activity through that site. However, mutation of the core GGAT of the proximal site (-516 to -509) to GGAA dramatically decreased basal promoter activity but did not abolish the PDEF stimulation. Interestingly, deletion of the entire site restored basal promoter activity, but abrogated the PDEF effect by approximately 50%.

Since deletion of this site does not affect basal promoter activity, this site is likely unbound during unstimulated conditions. Furthermore, mutating the core sequence must introduce an inhibitory element that is bound during basal conditions. Addition of PDEF appears to recover repressed promoter activity back to basal levels, which approximates a 3.5-fold increase. Thus, the mechanism of PDEF stimulation may be because of reversing the inhibition, perhaps by binding a repressor protein, or maintaining its stimulation by another means.

Deletion of the entire proximal site attenuates the PDEF effect suggesting that PDEF indeed exerts some mechanism of action at this site, but not through direct DNA binding. This is supported by the finding that PDEF stimulates the mutated promoter. Furthermore, *in vitro* translated PDEF protein was unable to bind wild-type or mutant Ets-1a or Ets-1b digoxigenin-labeled double-stranded 40-mer oligonucleotides as determined by electrophoretic mobility shift assay (data not shown). Also, it is unlikely that PDEF represses an inhibitor already present at the site, as deletion of the site does not affect basal promoter activity. Therefore, we conclude that PDEF acts as a coactivator at this site and that PDEF acts by at least one other site in the promoter.

The notion of Ets family members acting as coactivators is not novel and may represent an important means of regulation (Wasylyk *et al.*, 1998). One mechanism is by *cis*-acting autoinhibitory modules that are thought to act by blocking promiscuous DNA binding in the absence of appropriate regulatory cues. For example, DNA binding by PEA3, the mouse homolog of the ETS-domain protein E1A-F, is inhibited by two short amino-acid motifs located on either side of the ETS DNA-binding domain (Bojovic and Hassell, 2001). Binding of USF-1 to PEA3 relieves the inhibitory action, and promotes DNA binding (Greenall *et al.*, 2001). In addition to basic helix-loop-helix proteins (e.g. USF-1), hZIP proteins, GATA proteins, nuclear

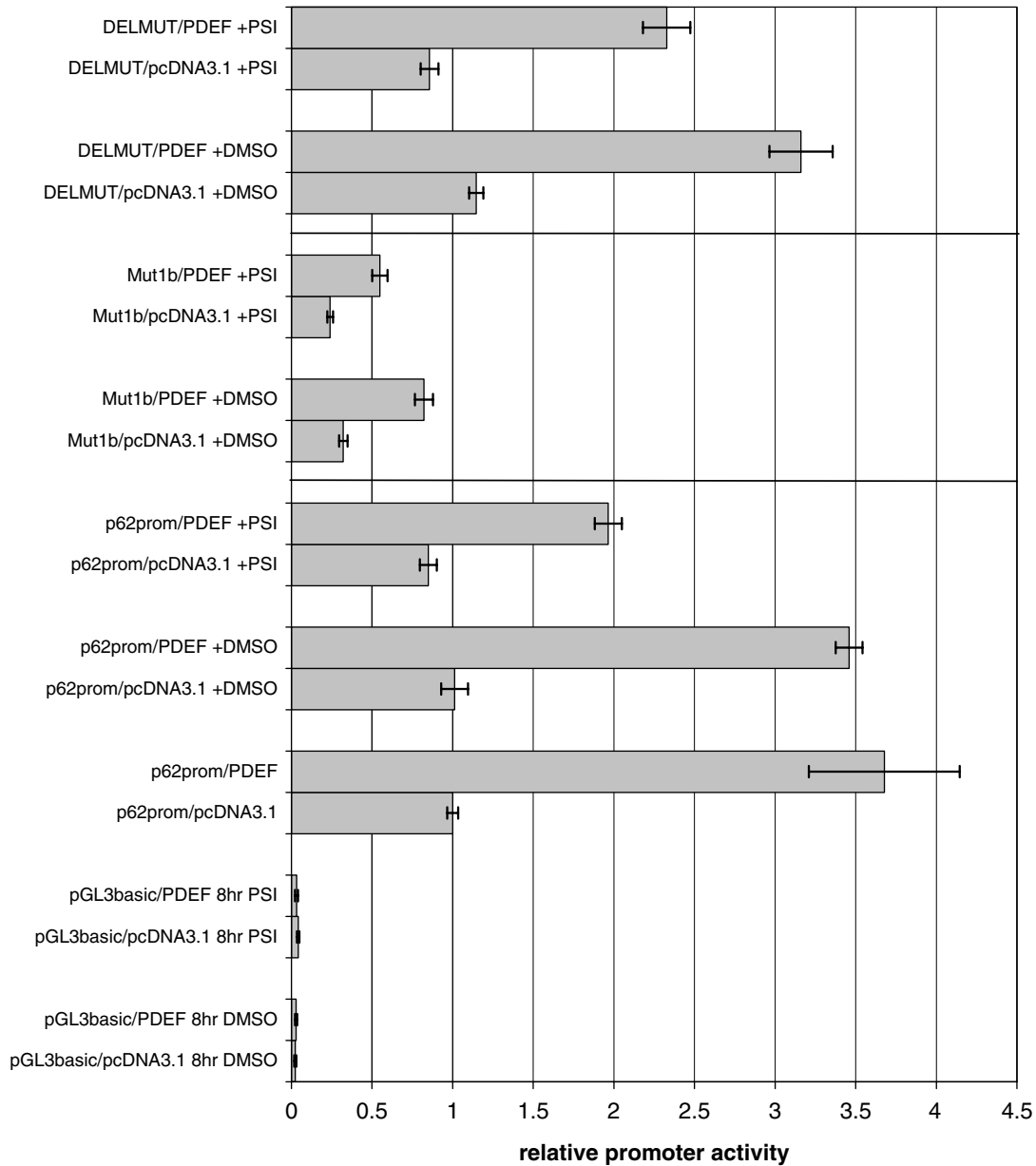


Figure 9 p62 Promoter analysis in the presence of PSI. HCC1806 cells were transfected with the SQSTM1 promoter/luciferase construct in the presence or absence of PDEF/pcDNA3.1+ plasmid in the presence of PSI or DMSO vehicle for 8 h. Control experiments were performed with empty plasmids. Values represent luciferase/ β -gal for three independent transfections, each transfection plated in triplicate wells, with each well assayed in triplicate for both luciferase and β -galactosidase. Error bars represent s.e.m.

hormone receptors and others have been shown to serve as binding partners for ETS-domain transcription factors. These interactions involve various domains and can enhance or repress the activity of the target Ets factor (see Sharrocks, 2001 for review).

Two general scenarios regarding the function of PDEF at this site can be postulated. First, PDEF may be regulating the protein expression of another regulator of p62 promoter activity (e.g. Ets-1). Currently, we cannot discount the possibility of PDEF acting as an upstream effector. Studies are currently underway to investigate this possibility. Second, PDEF is itself acting as a coactivator of the p62 promoter. If this scenario

proves true, our data provide the following mechanisms of action: (1) with respect to the proximal Ets-1b site, PDEF is acting *in trans*, (2) PDEF must be able to bind both the activator, which binds the wild-type site, and the repressor, which binds the mutated site, (3) PDEF acts by at least one other site besides Ets-1b on the p62 promoter, (4) the repressor binds mutant Ets-1b in the absence of PDEF, while the activator requires PDEF to bind the wild-type site, and (5) approximately half of the PDEF stimulation of the p62 promoter is attributable to the Ets-1b site.

In addition to Ets-1 sites, several other Ets family transcription factor binding sites are identified in the

promoter of p62, including six PEA3/E1A-F and two PU.1/Spi-1 sites (Vadlamudi and Shin, 1998). Each of these factors has been demonstrated to be overexpressed in cancer models and directly implicated in tumorigenesis. Ets-1 has been shown to be overexpressed in invasive cancer (Watson *et al.*, 1985). PEA3/E1A-F is overexpressed in metastatic human breast cancer cells and mouse mammary tumors (Trimble *et al.*, 1993; Benz *et al.*, 1997). When ectopically overexpressed in non-metastatic breast cancer cells, they become metastatic with the activation of transcription of matrix metalloproteases (MMPs) or adhesion molecules (de Launoit *et al.*, 2000). In fact, PEA3/E1A-F has been shown to activate the promoters of various MMPs (Higashino *et al.*, 1995) and increase cell motility and invasion in non-small-cell lung cancers (Hiroumi *et al.*, 2001), linking this particular transcription factor to tumor cell invasion and metastasis. PEA3/E1A-F also maintains basal promoter activity of the cancer-related progression elevated gene-3 (PEG-3) (Su *et al.*, 2000), but down-regulates Her-2-neu expression in cancer cells thereby suppressing tumorigenesis (Xing *et al.*, 2000). PU.1/Spi-1 has been shown to be aberrantly overexpressed in erythroleukemia cell lines (Afrikanova *et al.*, 2002) and is implicated in the development of myelomonocytes, B-cells, granulocytes and monocytes/macrophages (Nagamura-Inoue *et al.*, 2001; Yamada *et al.*, 2001).

It has previously been demonstrated that one means of increasing p62 protein expression is through the treatment of cells with PIs (Kuusisto *et al.*, 2001). Like cultured rat neurons, the five breast epithelial cell lines studied here showed an increase in p62 protein levels over time with PSI treatment. However, the mechanism for increasing p62 protein by PSI is PDEF-independent. Two lines of evidence support this conclusion. First, p62 mRNA increases in a time-dependent manner when HCC1806 cells are treated with PSI (Figure 8); however, after 8 h there is no change in the p62 promoter activity, either in the presence of PSI or vehicle control (Figure 9). Thus, the accumulation of p62 mRNA and the subsequent increase in p62 protein is likely because of increased mRNA stability and not increased transcription. In support of this scenario, others have found temporal stabilization of p62 mRNA in response to a variety of extracellular signals (Lee *et al.*, 1998). Secondly, the PDEF-stimulated increase of p62 promoter activity is attenuated by the addition of PSI. Therefore, PDEF and PSI are separate and distinct mechanisms for regulating p62 expression.

Addition of PDEF to the wild-type promoter in the presence of PSI is similar to the effect seen by addition of PDEF to the deletion construct. PDEF may not be able to coactivate the Ets-1 site in either case because either the site is absent in the case of the deletion construct, or the activator is absent in the case of PSI treatment. For instance, it can be hypothesized that the activating protein that binds the Ets-1b site is down-regulated by PSI, yielding essentially the same results as deleting the site. Since this site is a putative Ets-1 site, studies in our lab are underway to determine the role of Ets-1 and PDEF on the p62 promoter.

The ability of p62 to bind ubiquitin and several signaling proteins suggests that p62 may play a regulatory role in the ubiquitin-proteasome pathway. Furthermore, the finding that p62 acts as an immediate-early response gene in that mRNA and protein levels rise rapidly in response to some extracellular stimuli suggests an important role for p62 in cell proliferation and differentiation (Lee *et al.*, 1998). Proteasome inhibition in cultured neuronal cells (Kuusisto *et al.*, 2001) and breast epithelial cells (Figure 7) rapidly increases ubiquitinated substrates and p62 protein levels in a time-dependent manner. Unlike the immediate-early response, PSI treatment requires 32 h to dramatically increase p62 mRNA and protein. Thus, the stabilization of mRNA by PSI acts to increase p62 more slowly in direct contrast to proliferation and differentiation-inhibiting agents. However, the inhibitory effect of the PDEF protein on p62 promoter activity may be illustrative of the usefulness of PSIs in the treatment of cancers.

Materials and methods

Cell culture

The normal breast epithelial cells MCF102A, MCF102F, MCF12A and MCF12F were cultured in 1:1 DMEM/Ham's F12 medium supplemented with 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin and 500 ng/ml hydrocortisone. These four cell lines were cultured with 5% horse serum, and in the case of MCF102F and MCF12F the serum was chelexed prior to use. The transformed cell lines MCF-7, HCC1806, HCC1428 and BT-20 were cultured with 10% FBS supplemented EMEM (MCF-7 and BT-20) or RPMI 1640 (HCC1806 and HCC1428). Cells were routinely cultured in monolayer in 10 cm² culture dishes in the recommended media until <80% confluent and passaged at 1:4 dilution two to three times per week. All cell lines were purchased from ATCC (Manassas, VA, USA).

Three concentrations of proteasome inhibitor 1 (PSI), MG132 or lactacystin were added to cultures for the cell proliferation assay (Calbiochem, San Diego, CA, USA). For studies of PSI stimulated mRNA and protein expression, a final concentration of 10 μ M PSI or 0.075% DMSO (v/v) was added to cultured cells for times indicated.

Measurement of cell proliferation

Cells were grown in 96-well black plates at a density of 1000 cells/well in replicates of eight. After 24 h, PSIs were added to cells in fresh medium and incubated for 24 h, after which the medium was aspirated and cells were rinsed with 1 \times PBS to remove phenol red. Plates were blotted dry and stored at -70°C for 72 h prior to assay. Relative cell number was determined by the CyQuant cell proliferation assay (Molecular Probes, Eugene, OR, USA) as per the manufacturer's instructions. Cell number was normalized to the DMSO vehicle control.

SDS-PAGE and Western blots

Tissue samples obtained through the Cooperative Human Tissue Network and our affiliated medical center were homogenized in RIPA buffer (50 mM Tris pH 8.0, 150 mM

NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM sodium ortho-vanadate) supplemented with protease inhibitor cocktail (Sigma, St Louis, MO, USA) using a Polytron hand-held homogenizer. PBS-washed cells were lysed on ice in RIPA buffer with protease inhibitors. Protein concentration was determined by addition of Bradford dye modified for use with detergents (BioRad, Hercules, CA, USA) (Bradford, 1976). Laemmli sample buffer, 5 \times , was added to 15 or 40 μ g protein diluted in gel running buffer prior to boiling for 5 min. Duplicate samples were subjected to SDS-PAGE and transferred to PVDF membrane. Western blot analyses were carried out in the usual way using appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies. Monoclonal mouse anti-p62 (Transduction Laboratories, Lexington, KY, USA) and monoclonal rabbit antiubiquitin (Dako, Glostrup, Denmark) were each diluted 1:500, mouse monoclonal anti- β -actin (Abcam, Cambridge, UK) was diluted 1:5000. HRP-conjugated donkey anti-rabbit IgG and HRP-conjugated sheep anti-mouse IgG secondary antibodies were purchased from Abcam, and diluted 1:2500 and 1:1500, respectively.

Recovery of mRNA from normal and cancer breast cells

Total RNA was harvested from cells solubilized in TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) as per the manufacturer's instructions. RNA extraction, precipitation and solubilization were performed in the usual way, as described by the manufacturer. A measure of 250 μ g total RNA was used for recovery of polyA mRNA from each cell line cultured using a spin-column format (Qiagen, Valencia, CA, USA). Message RNA was quantitated by fluorescence using the RiboGreen RNA Quantitation Reagent (Molecular Probes) in 96-well plate format, assayed in triplicate, using the Fluoroskan Accent FL combination luminometer/fluorometer (LabSystems, Finland). Prostate mRNA was purchased from Clontech (Palo Alto, CA, USA).

Northern blot analysis

A biotinylated probe against PDEF mRNA was generated by first amplifying a short 477 bp region from prostate cDNA with the addition of an SP6 site (ACG ATT TAG GTG ACA CTA TAG AA) downstream of the sequence on the sense strand. The PCR product was used for subsequent *in vitro* transcription reactions with biotinylated dUTP to generate ssRNA antisense probes using the Detector RNA *in vitro* Transcription Biotinylation Kit (KPL, Gaithersburg, MD, USA) as per the manufacturer's instructions. Biotinylated β -actin ssRNA was provided with the kit.

Duplicate 500 ng mRNA samples were subjected to electrophoresis on a 1% low melting point agarose denaturing formaldehyde gel, followed by transfer to BioDyne B positively charged nylon (Pall-Gelman, Ann Arbor, MI, USA). The blot was UV crosslinked and probed with 50 ng/ml biotinylated PDEF. Blot was detected by chemiluminescence using the RNA Detector Northern Blotting Kit (KPL) as per the manufacturer's instructions. The blot was stripped in a high-stringency solution (0.2 \times SSPE, 1% SDS, 60% formamide), followed by probing with β -actin probe and detection.

Reverse transcriptase (RT)-PCR

The ThermoScript RT-PCR System was purchased from GibcoBRL/Invitrogen (Carlsbad, CA, USA). A total of 50 ng of mRNA purified from each cell line was also used as

template for separate reactions. All cDNA samples were stored at -70°C until use.

Real-time quantitative PCR

To generate templates for the standard curves, PDEF and SQSTM1 genes were amplified from prostate cDNA using sequences available at www.ncbi.nlm.nih.gov. For SQSTM1, the forward primer 5'-GTG AAT TCG CTC GCC GCT CGC TAT-3' binds at base pair (bp) 39 and the reverse primer 5'-CGT CTC GAG TGC CTG CTG ACA ACA CCT A-3' binds at bp 1818 of the cDNA. For PDEF, the forward primer 5'-CCC AAG CTT ACC AGG CAG CTA ACA GAC ACA-3' binds at bp 371 and the reverse primer 5'-CGC GGA TCC ATG CCA GGT GTG GTG CAG ATT-3' binds at bp 1812 of the cDNA. For the β -actin control, the forward primer 5'-GCC AGC TCA CCA TGG ATG AT-3' binds at bp 74 of the full-length cDNA whereas the reverse primer 5'-CCT CGG CCA CAT TGT GAA CT-3' binds at bp position 1427 of the gene. PCR products were gel purified and quantitated in triplicate by A_{260} . Serial dilutions of 4 ng/ μ l to 40 ag/ μ l were used to generate a working final concentration range of 20 pg to 20 fg. To reduce pipette error, 5 μ l volumes of each standard were used per reaction. Standards were assayed in duplicate concomitant with p62 and PDEF cDNA of unknown concentrations measured in triplicate.

Real-time PCR primers were designed to amplify small target regions within a full-length cDNA with each primer binding a different exon. This was performed to eliminate any amplification of genomic DNA contamination or immature transcripts. Target regions were kept between 150 and 400 bp in size to maintain reaction efficiency. For SQSTM1, the forward primer 5'-ATC GGA GGA TCC GAG TGT-3' binds at bp 702 and the reverse primer 5'-TGG CTG TGA GCT GCT CTT-3' binds at bp 876 of the cDNA. For PDEF, the forward primer 5'-CCA TCC ACC TGT GGC AGT TC-3' binds at bp 793 and the reverse primer 5'-GAG CGG CTC AGC TTG TCG TA-3' binds at bp 972 of the cDNA. The β -actin forward primer, 5'-ACT GGC ATC GTG ATG GAC TC-3', binds bp position 529 of the full-length cDNA. The reverse primer sequence, 5'-TCA GGC AGC TCG TAG CTC TT-3', binds at position 815. The 10000 \times SYBR Green I (Molecular Probes) was freshly diluted to 2500 \times with 1 \times TBE and then diluted to a working 25 \times stock. The master mix contained 0.2 μ M of each oligonucleotide primer, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 1 \times Promega Mg²⁺-free buffer, 1 \times SYBR Green I, and 2.5 U of Promega Taq in storage buffer B. A measure of 20 μ l master mix was dispersed into each tube to give a final volume of 25 μ l. All reactions were performed on ice to help prevent mis-priming and degradation.

Thermocycling was performed using the Cepheid Smart Cycler (Sunnyvale, CA, USA). DNA product formation was detected during each extension step of the program using the factory-calibrated optics channel for SYBR Green. Upon completion of the amplification program, a melt curve was performed on the samples as well as agarose gel electrophoresis to confirm product formation and purity.

Generation of PDEF construct

PDEF was amplified by PCR from prostate cDNA using the forward primer 5'-CCC AAG CTT ACC AGG CAG CTA ACA GAC ACA-3' containing a *HindIII* restriction site and the reverse primer 5'-CGC GGA TCC ATG CCA GGT GTG GTG CAG AAT-3' containing a *BamHI* restriction site. The PCR product was digested and cloned into pcDNA3.1(+)

(Invitrogen). The construct was transformed into INV α F⁺ precompetent cells (Invitrogen) to generate sufficient plasmid for transfection.

Generation of promoter constructs

The SQSTM1 promoter was cloned from genomic DNA purified from HCC1806 cells based on publicly available sequence data. Nested PCR was necessary to clone the promoter. The forward outside primer sequence was 5'-CCT ATT ACG ACA GCG GTC ATG G-3' and the reverse outside primer was 5'-AGC TGG CGG AAA ATG GG-3'. An aliquot of amplified product was used for subsequent PCR using the nested forward primer 5'-GGA AGA TCT CTG ACT CAC TGC CGC CAG AC-3' containing a *Bgl*II restriction site and the nested reverse primer 5'-CTC AAG CTT TGT AGC GAA CGC GGA GGC-3' containing a *Hind*III restriction site. The PCR product was digested and cloned into pGL3-Basic (Promega) and the insert verified by sequencing. The construct was transformed as above.

The cloned wild-type promoter was used as the template for site-directed mutagenesis by PCR. Primers were designed with a single-point mismatch in the Ets-1 sites. Primers for mutant Ets-1a, at position -1175 to -1166, were 5'-CTC CAC CGC CGG AAG CAG GGA GAG G-3' and its complement. Primers for mutant Ets-1b, at position -516 to -509, were 5'-CAT CAC CGA GGA AGC CAT GCG CTG TAA-3' and its complement. The deletion mutant was generated from the wild-type construct using the forward primer 5'-GGA ATT CAT GCG CTG TAA GAG GG-3' and the reverse primer 5'-GGA ATT CGA TGG GCA GCG CAT A-3' to generate a linear product with *Eco*RI restriction sites on each end. The PCR product was digested with *Dpn*I, gel purified and subsequently digested with *Eco*RI, gel purified, and self-ligated. All constructs were transformed into XL1-

Blue precompetent cells, positive clones identified and grown overnight. Recovered plasmids were quantitated for transfection.

Transfections

HCC1806 cells were plated in six-well culture plates at a density of 350 000 cells/well. After 24 h, cells were transfected with appropriate DNA constructs using the Effectene Transfection Reagent (Qiagen) in triplicate transfections. Control transfections included empty vectors for both the pGL3-basic and pcDNA3.1(+). In total, 2.0 μ g empty vector, wild-type p62 promoter, mutant Ets-1a, mutant Ets-1b, double Ets mutant and deletion mutant were cotransfected with 0.4 μ g PDEF construct or the empty pcDNA3.1(+) vector. In all cases, 0.4 μ g pSV-40 β -gal construct was cotransfected. After 48 h, media were aspirated, cells were rinsed with 1 \times PBS and lysed using CCLR reagent (Luciferase Assay System, Promega). Plates are fast-frozen at -80°C for 5 min and subsequently scraped. Insoluble material was removed by centrifugation and the lysates assayed for luciferase activity in 96-well opaque plates in triplicate. Values are normalized to β -gal activity using the FluoReporter *LacZ*/Galactosidase Quantitation kit (Molecular Probes), according to the manufacturer's instructions, measured in triplicate.

Acknowledgements

Some tissue samples were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. This work was supported by the National Institutes of Health Human Genome Research Institute Grant number 1K22HG00047-01.

References

- Adams J. (2001). *Semin. Oncol.*, **28**, 613–619.
- Afrikanova I, Yeh E, Bartos D, Watowich SS and Longmore GD. (2002). *Oncogene*, **21**, 1272–1284.
- An B, Goldfarb RH, Siman R and Dou QP. (1998). *Cell Death Differ.*, **5**, 1062–1075.
- Benz CC, O'Hagan RC, Richter B, Scott GK, Chang CH, Xiong X, Chew K, Ljung BM, Edgerton S, Thor A and Hassell JA. (1997). *Oncogene*, **15**, 1513–1525.
- Bojovic BB and Hassell JA. (2001). *J. Biol. Chem.*, **276**, 4509–4521.
- Bradford MM. (1976). *Anal. Biochem.*, **72**, 248–254.
- Chen H, Nandi AK, Li X and Biebrich CJ. (2002). *Cancer Res.*, **62**, 338–340.
- de Launoit Y, Chotteau-Lelievre A, Beaudoin C, Coutte L, Netzer S, Brenner C, Huvent I and Baert JL. (2000). *Adv. Exp. Med. Biol.*, **480**, 107–116.
- DeSalle LM and Pagano M. (2001). *FEBS Lett.*, **490**, 179–189.
- Dittmer J and Nordheim A. (1998). *Biochim. Biophys. Acta.*, **1377**, F1–F11.
- Fan XM, Wong BC, Wang WP, Zhou XM, Cho CH, Yuen ST, Leung SY, Lin MC, Kung HF and Lam SK. (2001). *Int. J. Cancer*, **93**, 481–488.
- Geetha T and Wooten MW. (2002). *FEBS Lett.*, **512**, 19–24.
- Ghadersohi A and Sood AK. (2001). *Clin. Cancer Res.*, **7**, 2731–2738.
- Gong J, Xu J, Bezanilla M, van Huizen R, Derin R and Li M. (1999). *Science*, **285**, 1565–1569.
- Greenall A, Willingham N, Cheung E, Boam DS and Sharrocks AD. (2001). *J. Biol. Chem.*, **276**, 16207–16215.
- Higashino F, Yoshida K, Noumi T, Seiki M and Fujinaga K. (1995). *Oncogene*, **10**, 1461–1463.
- Hiroumi H, Dosaka-Akita H, Yoshida K, Shindoh M, Ohbuchi T, Fujinaga K and Nishimura M. (2001). *Int. J. Cancer*, **93**, 786–791.
- Hochstrasser M. (1996). *Annu. Rev. Genet.*, **30**, 405–439.
- Joung I, Strominger JL and Shin J. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 5991–5995.
- Keyomarsi K and Pardee AB. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 1112–1116.
- Kornitzer D and Ciechanover A. (2000). *J. Cell Physiol.*, **182**, 1–11.
- Kuusisto E, Suuronen T and Salminen A. (2001). *Biochem. Biophys. Res. Commun.*, **280**, 223–228.
- Lee YH, Ko J, Joung I, Kim JH and Shin J. (1998). *FEBS Lett.*, **438**, 297–300.
- Nagamura-Inoue T, Tamura T and Ozato K. (2001). *Int. Rev. Immunol.*, **20**, 83–105.
- Nozawa M, Yomogida K, Kanno N, Nonomura N, Miki T, Okuyama A, Nishimune Y and Nozaki M. (2000). *Cancer Res.*, **60**, 1348–1352.
- Oettgen P, Finger E, Sun Z, Akbarali Y, Thamrongsak U, Boltax J, Grall F, Dube A, Weiss A, Brown L, Quinn G, Kas K, Endress G, Kunsch C and Libermann TA. (2000). *J. Biol. Chem.*, **275**, 1216–1225.

- Owa T, Yoshino H, Yoshimatsu K and Nagasu T. (2001). *Curr. Med. Chem.*, **8**, 1487–1503.
- Pasquini LA, Besio Moreno M, Adamo AM, Pasquini JM and Soto EF. (2000). *J. Neurosci. Res.*, **59**, 601–611.
- Rachubinski RA, Marcus SL and Capone JP. (1999). *J. Biol. Chem.*, **274**, 18278–18284.
- Sanz L, Diaz-Meco MT, Nakano H and Moscat J. (2000). *EMBO J.*, **19**, 1576–1586.
- Sanz L, Sanchez P, Lallena MJ, Diaz-Meco MT and Moscat J. (1999). *EMBO J.*, **18**, 3044–3053.
- Sharrocks AD. (2001). *Nat. Rev. Mol. Cell Biol.*, **2**, 827–837.
- Shin J. (1998). *Arch. Pharm. Res.*, **21**, 629–633.
- Spataro V, Norbury C and Harris AL. (1998). *Br. J. Cancer*, **77**, 448–455.
- Su Z, Shi Y and Fisher PB. (2000). *Oncogene*, **19**, 3411–3421.
- Sudo T, Maruyama M and Osada H. (2000). *Biochem. Biophys. Res. Commun.*, **269**, 521–525.
- Trimble MS, Xin JH, Guy CT, Muller WJ and Hassell JA. (1993). *Oncogene*, **8**, 3037–3042.
- Vadlamudi RK, Joung I, Strominger JL and Shin J. (1996). *J. Biol. Chem.*, **271**, 20235–20237.
- Vadlamudi RK and Shin J. (1998). *FEBS Lett.*, **435**, 138–142.
- Wasylyk B, Hagman J and Gutierrez-Hartmann A. (1998). *Trends Biochem. Sci.*, **23**, 213–216.
- Watson DK, McWilliams-Smith MJ, Nunn MF, Duesberg PH, O'Brien SJ and Papas TS. (1985). *Proc. Natl. Acad. Sci. USA*, **82**, 7294–7298.
- Xing X, Wang SC, Xia W, Zou Y, Shao R, Kwong KY, Yu Z, Zhang S, Miller S, Huang L and Hung MC. (2000). *Nat. Med.*, **6**, 189–195.
- Yamada N, Tamai Y, Miyamoto H and Nozaki M. (2000). *Gene*, **241**, 267–274.
- Yamada T, Abe M, Higashi T, Yamamoto H, Kihara-Negishi F, Sakurai T, Shirai T and Oikawa T. (2001). *Blood*, **97**, 2300–2307.