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AP-2γ promotes proliferation in breast tumour cells by direct repression of the *CDKN1A* gene

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Overexpression of the activator protein (AP)-2γ transcription factor in breast tumours has been identified as an independent predictor of poor outcome and failure of hormone therapy. To understand further the function of AP-2γ in breast carcinoma, we have used an RNA interference and gene expression profiling strategy with the MCF-7 cell line as a model. Gene expression changes between control and silenced cells implicate AP-2γ in the control of cell cycle progression and developmental signalling. A function for AP-2γ in cell cycle control was verified using flow cytometry: AP-2γ silencing led to a partial G1/S arrest and induction of the cyclin-dependent kinase inhibitor, p21cip/*CDKN1A*. Reporter and chromatin immunoprecipitation assays demonstrated a direct, functional interaction by AP-2γ at the *CDKN1A* proximal promoter. AP-2γ silencing coincided with acquisition of an active chromatin conformation at the *CDKN1A* locus and increased gene expression. These data provide a mechanism whereby AP-2γ overexpression can promote breast epithelial proliferation and, coupled with previously published data, suggest how loss of oestrogen regulation of AP-2γ may contribute to the failure of hormone therapy in patients.

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Introduction

Activator protein-2 (AP-2) factors are a family of developmentally regulated DNA-binding transcription factors conserved from *Drosophila* to man. In mammals, five family members have been identified: AP-2α-ε, each encoded by a separate gene (reviewed in Eckert *et al*, 2005). Although studies in model organisms have shown that these factors have important functions during embryogenesis, particularly in the formation of critical organs and body structures, they are minimally expressed in most adult tissues. However, overexpression, of the AP-2α and AP-2γ family members, in particular, has been associated with certain tumour types, including breast cancer (reviewed in Pellikainen and Kosma, 2007).

Despite their high degree of homology, these two AP-2 family members appear to have distinct functions in breast cancer. AP-2α expression was seen to decline in level from normal breast through to DCIS and primary invasive cancers and show a positive correlation with expression of the universal cell cycle inhibitor *CDKN1A*, encoding p21cip (Gee *et al*, 1999). Reduced nuclear AP-2α staining has been associated with more aggressive breast cancers (Pellikainen *et al*, 2002) and *TFAP2A* mRNA expression was correlated inversely with increased tumour grade (Sotiriou *et al*, 2006). Furthermore, AP-2α expression in breast cancer is associated with favourable prognostic markers, namely ERα expression, ErbB2 negativity and reduced proliferation. Thus, AP-2α may be acting in a tumour suppressive function. Consistent with this, loss of AP-2α expression in invasive breast cancer has been significantly correlated with hypermethylation of a CpG island over exon 1 of the *TFAP2A* gene (Douglas *et al*, 2004).

Although its exact function has been the subject of some controversy (see Pellikainen and Kosma, 2007 for a discussion), an increasing body of evidence suggests that AP-2γ expression has an opposite function to that of AP-2α in breast carcinoma. AP-2γ overexpressing transgenic mice did not develop tumours; however, epithelial hyperplasia and impaired differentiation were observed, implicating AP-2γ in the promotion of proliferation (Jager *et al*, 2003). In human disease, a link between high levels of *TFAP2C* mRNA and reduced disease-free survival was observed (Zhao *et al*, 2003) and *TFAP2C* mRNA expression also has been associated with advancing clinical grade in gene expression profiling studies (Sotiriou *et al*, 2006). Recently, studies using a specific AP-2γ antibody to examine expression in human breast tumour tissues have shown a strong correlation between elevated levels of AP-2γ and reduced patient survival, both independently and in the context of indicators of good prognosis: namely ERα expression and ErbB2 negativity (Gee *et al*, 2009). In addition, an association between high AP-2γ levels and reduced response to hormone therapy was also noted and an independent study, looking specifically for a relationship with resistance to tamoxifen therapy, showed similar findings, particularly in post-menopausal patients (Guler *et al*, 2007).

Thus, in summary, AP-2α expression generally is correlated with reduced proliferation and good prognosis in breast cancer patients, whereas AP-2γ expression has been associated with continued proliferation, disease progression and resistance to endocrine therapy. A number of cancer-related target genes have been linked with each of these transcription factors; however, the overall mechanism whereby they

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contribute to tumourigenesis remains unclear. To examine which cellular pathways are activated by AP-2γ in breast cancer, we describe here the generation of an AP-2γ-dependent expression profile, which, together with functional data, demonstrates that this transcription factor is able to sustain proliferation of breast epithelial cells through direct repression of the *CDKN1A* gene that encodes p21cip.

Results

Gene expression changes in MCF-7 cells on AP-2γ silencing

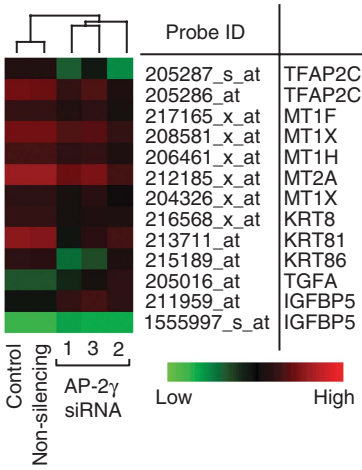
AP-2γ overexpression, particularly in ER positive breast tumours, is a poor prognostic marker (Guler *et al*, 2007; Gee *et al*, 2009). To gain insight into which cellular pathways are active in such tumours, we used the MCF-7 breast tumour line as a model for ER positive, ErbB2 negative tumours with AP-2γ overexpression and negligible levels of the other AP-2 family members (see Supplementary Figure S1A; Orso *et al*, 2004). Three distinct self-interfering RNA sequences across the *TFAP2C* coding sequence were identified (siRNA1-3; see Materials and methods) and short-term transfection procedures optimised to achieve effective silencing of AP-2γ. Levels of AP-2 cofactors were not diminished during AP-2γ silencing, indicating a large degree of specificity; of particular note, levels of ER were constant in the silenced cells over all time points used in this study (see Supplementary Figure S1B and C).

We compared the expression profiles of cells separately transfected with the three AP-2γ targeting sequences with those from control cells treated with transfection reagent alone or a non-silencing control (NSC) siRNA on Affymetrix arrays. Statistical analysis (see Materials and methods) identified 302 probesets whose intensity changed significantly following AP-2γ silencing (FDR corrected; $P<0.01$). This represented 254 distinct genes, of which 190 genes were down-regulated and 64 genes were up-regulated (see Supplementary Table S1). As shown in Figure 1, both AP-2γ probesets present on the array featured in this list: *TFAP2C* 205286_at; fold change −2.09 ($P=0.00439$); *TFAP2C* 205287_s_at; fold change −2.39 ($P=0.00646$). The apparent fold change between control and experimental samples compared with an average value of 5.3-fold change observed by real-time PCR (Supplementary Figure S2); this is in line with the underestimation of fold-change expression commonly observed using microarrays (Yuen *et al*, 2002). We also examined the list of genes for those already known to be associated with AP-2 biology. A number of genes, including members of the metallothionein and keratin superfamilies, were shown to be significantly altered (Figure 1) in expression level in silenced MCF-7 cells. To validate the dataset further, 11 of the 254 significantly regulated genes were analysed by real-time PCR using RNA from cells transfected with a fourth AP-2γ siRNA. All of the genes were up- or down-regulated to an extent consistent with the array data (see Supplementary Figure S2), strongly suggesting that these data represent a reliable assessment of changes in transcript abundance on AP-2γ depletion.

To explore which key cellular processes may be influenced by AP-2γ, the significantly regulated genes were interrogated using analyses based on gene ontology (GO) terms (shown graphically in Supplementary Figure S3). From this analysis it was apparent that a large number of the down-regulated genes were classified as being involved in the cell cycle: 35% of down-regulated genes compared with just 3% of up-regulated genes. To provide an objective, statistically controlled measure of enrichment of GO terms from this analysis, GO terms from the dataset were compared with those across the whole HG-U133Plus2 array which again showed that the down-regulated gene set is highly enriched in gene categories relating to all phases of the cell cycle. In addition, the up-regulated gene set was found to be over-represented in cellular processes related to development and morphogenesis (see Supplementary Table S2).

Silencing of AP-2γ in MCF-7 cells causes a partial G1/S arrest

If loss of AP-2γ in MCF-7 cells has such a profound effect on cell cycle-associated gene expression, then silenced cells should show evidence of an altered cell cycle distribution.



Probe ID	Gene description		Fold change	*P*-value
205287_s_at	TFAP2C	transcription factor AP-2 gamma	−2.09	0.00439
205286_at	TFAP2C	transcription factor AP-2 gamma	−2.39	0.00646
217165_x_at	MT1F	metallothionein 1F (functional)	−1.51	0.00934
208581_x_at	MT1X	metallothionein 1X	−1.72	0.00916
206461_x_at	MT1H	metallothionein 1H	−1.68	0.00793
212185_x_at	MT2A	metallothionein 2A	−1.96	0.00511
204326_x_at	MT1X	metallothionein 1X	−1.49	0.0485
216568_x_at	KRT8	Keratin 8	−1.66	0.000383
213711_at	KRT81	keratin 81	−4.02	0.000275
215189_at	KRT86	keratin 86	−2.12	0.00344
205016_at	TGFA	transforming growth factor, alpha	1.78	0.0074
211959_at	IGFBP5	insulin-like growth factor binding protein 5	2.18	0.00817
1555997_s_at	IGFBP5	insulin-like growth factor binding protein 5	1.46	0.0141

Figure 1 Significant gene expression changes related to AP-2 biology in silenced MCF-7 cells. Changes observed in probe sets between the *reference* (NSC siRNA and cells treated with transfection reagent only) and *test* (AP-2γ targeting siRNAs 1, 2 and 3) groups at the indicated False Discovery Rate corrected *P*-values. A full list of significantly regulated probe sets together with normalised expression values, fold-change values (between *test* and *reference* groups) and FDR corrected *P*-values is given in Supplementary Table S1.

MCF-7 control and AP-2γ silenced cells were examined by FACS analysis of fixed, propidium iodide (PI)-stained cells. As illustrated in Figure 2A, cell populations 72 h after transfection with AP-2γ targeting siRNAs showed a significant ($P<0.01$) increase in the proportion of cells in G1 plus a concomitant reduction in the proportion of S-phase cells compared with controls. Additional analyses examining the relative proportion of sub-G1 cells in the transfected cells showed that, compared with controls, AP-2γ silencing was not associated with an increase in genomic DNA fragmentation (data not shown). This, coupled with the absence of morphological changes, suggested that silencing was unlikely to lead directly to programmed cell death (see below). S-phase activity in control and silenced cells was examined by labelling transfected cultures with BrdU immediately before harvest. Silenced cells were clearly still cycling and able to take up BrdU, but there was a significant reduction in the fraction of cells actively undergoing S phase compared with the control populations (see Figure 2B). The percentage of cells undergoing DNA synthesis in this experiment remained comparable to the proportion of cells with S-phase DNA content shown in Figure 2A, indicating that the cycling abnormality in silenced cells occurred before S-phase entry.

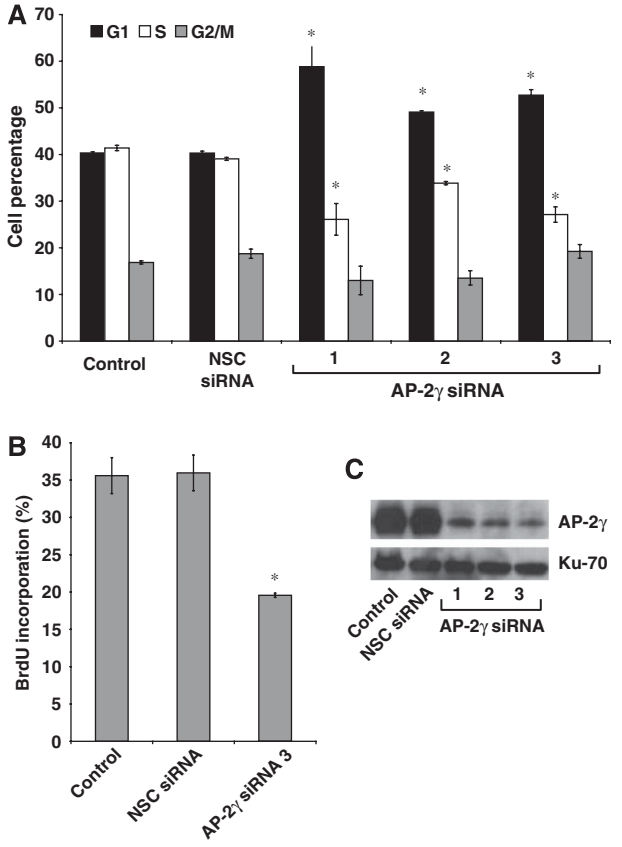


Figure 2 MCF-7 cells transiently transfected with AP-2γ targeting siRNAs show altered cell cycle distribution. (**A**) FACS analysis for DNA content performed 72 h after transfection. Cells were transfected as indicated, split 1 in 2 at 48 h, and harvested for cell cycle analysis 24 h later. (**B**) FACS analysis for BrdU incorporation performed 72 h after transfection. Cells were treated as in (A) but were labelled with BrdU for 20 min just before harvest. (**C**) Western blot analysis demonstrating AP-2γ knock down efficiency 72 h after transfection. Whole cell extracts (WCE; 5 μg/lane) were separated by SDS–PAGE, blotted to membrane and probed with primary antibodies against AP-2γ before being reprobed for the Ku-70 subunit of DNA-dependent protein kinase as a loading control. For A and B, averages from triplicate experiments are shown (± standard error). $^*P<0.01$ compared with controls. For A, representative cell cycle analysis diagrams are shown in Supplementary Figure S4.

These results suggest that MCF-7 cells are unable to cycle efficiently in the absence of physiological levels of AP-2γ and this was also reflected by experiments attempting to establish sublines with stable expression of AP-2γ targeting siRNAs. Sequences representing siRNA1-3 were used to derive shRNA expression constructs; control constructs bearing a single base mismatch to each siRNA sequence were also generated. Each construct was separately transfected into MCF-7 cells and stable puromycin-resistant sublines were selected. Compared with the empty vector and the mismatch controls, there were significantly fewer colonies on the plates transfected with AP-2γ shRNA constructs. Moreover, when the few surviving colonies were expanded and examined for AP-2γ expression, all were found to express wild-type levels of the protein (see Supplementary Figure S5), indicating that the shRNA expression cassette either had been lost or otherwise rendered non-functional in these colonies.

Attempts to establish stable AP-2γ shRNA lines also failed using ZR75-1 and MCF10A breast lines. Only in T47D cells were two sublines identified (from over 40 examined) that initially showed reduced AP-2γ expression; however, this was not sustained during passage. Thus, the loss of AP-2γ expression in breast epithelial cells is incompatible with sustained proliferation in either a p53 wild-type (MCF-7, ZR75-1, MCF10A) or mutant (T47D) background. This was also true for both ER negative (MCF10A) and ER positive (MCF-7, T47D, ZR75-1) cell lines; levels of ER remained constant during silencing in all lines examined.

The proliferation deficiency was confirmed by establishing MCF-7 lines with doxycycline-inducible shRNA expression (see Materials and methods) and comparing the growth of such lines with that of controls using cell counting assays. Figure 3B and C illustrates growth curves over 6 days for two representative AP-2γ-shRNA clones in the presence and absence of inducer. Significantly reduced cell numbers were recorded for silenced, compared with untreated cultures and also compared with a line expressing the NSC shRNA (Figure 3A). These inducible lines were also used to examine whether AP-2γ silencing results in increased cell death by assaying induced and uninduced cells for Annexin V binding, a recognised hallmark of early apoptotic cells. Neither inducible line demonstrated a significant change in the percentage of apoptotic cells when treated with doxycycline for up to 96 h compared with controls (Supplementary Figure S6). Thus, taking all of these results together, we conclude that AP-2γ silenced cells have a reduced proliferation rate because of their failure to efficiently negotiate the G1/S checkpoint.

AP-2γ silencing leads to induction of p21cip expression

A more detailed analysis of the subset of cell cycle-related genes down-regulated following AP-2γ silencing showed that the majority display cell cycle periodicity (Whitfield *et al*, 2002) with almost all showing their lowest expression at G1 or S phase, again consistent with a perturbation at the G1/S transition. The majority of these changes probably occurred as a consequence of the cell cycle arrest and the genes are unlikely therefore to be direct targets of AP-2γ. Consistent

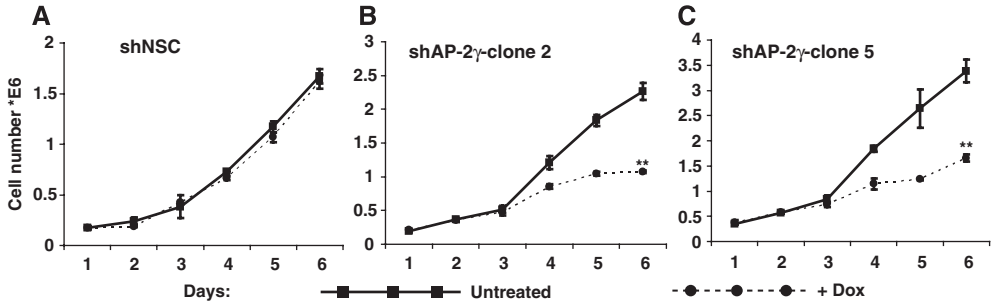


Figure 3 Impaired proliferation on induction of shAP-2γ in inducible cell lines. Individual MCF-7 lines stably transfected with doxycycline-inducible vectors expressing NSC (**A**) or AP-2γ shRNA (**B**, **C**) were plated on day 1 at 100 000 cells per well in six-well plates in normal growth media in the presence or absence of 1 μg/ml dox, as indicated. Triplicate wells for each condition and each line were harvested for cell counting (CASY) daily for 6 days. Error bars represent standard errors between the triplicates. (B, C) *P*-values for cell counts at day 6 in induced compared with untreated cells were $^{**}P<0.01$ (Students *t*-test). Successful AP-2γ silencing was verified using quantitative RT–PCR (see Supplementary Figure S6A).

with this hypothesis, many of these genes are known to be transcriptionally regulated by E2F family members at the G1/S transition (Ishida *et al*, 2001; Polager *et al*, 2002; Ren *et al*, 2002) and therefore AP-2γ may have a function in the regulation of genes that act upstream of this point.

Non-contact-inhibited cells from control and AP-2γ silenced populations were harvested 72 h post-transfection and examined for expression of cell cycle regulatory proteins by western blotting. Levels of the G1-cyclins (D1, D3 and E) and their associated kinases (cdk4/6 and cdk2) did not alter significantly. However, their activity, as evidenced using antibodies against phosphorylated forms of pRb, was diminished in silenced cells, whereas levels of the cyclin-dependent kinase inhibitor proteins, p21cip and p27kip, both increased (see Supplementary Figure S7). Regulation of p27kip occurs largely at the post-translational level (Nakayama *et al*, 2001), but p21cip is transcriptionally regulated, notably in response to p53 stabilisation (el-Deiry *et al*, 1993; reviewed in Gartel and Radhakrishnan, 2005). Further analysis of our control and silenced cells demonstrated that loss of AP-2γ led to increased levels of p21cip at the level of both mRNA and protein (Figure 4A and B) but the expression of endogenous wild-type p53 in MCF-7 cells was not altered after AP-2γ silencing (Figure 4C) so could not be responsible for the observed increase. A number of additional factors have been shown to activate transcription from the *CDKN1A* promoter, including AP-2α (Zeng *et al*, 1997); however, levels of this factor also remained very low and constant on AP-2γ silencing (Figure 4C). Additional analyses using the inducible shAP-2γ lines also revealed an induction of *CDKN1A* expression in doxycycline-treated cells with kinetics in accord with the slowed proliferation rate observed for the silenced cells (see Supplementary Figure S8 and Figure 3).

AP-2γ represses CDKN1A expression

To examine the effect of AP-2γ on p21cip expression, luciferase reporter assays were used to assess *CDKN1A* promoter activity in AP-2 non-expressing cells. Genomic sequences from −2325 to +8 relative to the start of transcription of the *CDKN1A* gene have been shown to be important for its regulation (el-Deiry *et al*, 1993). As shown in Figure 5A, basal activity from a reporter construct containing these sequences was reduced in a dose-dependent manner following co-transfection with an AP-2γ expression construct. This contrasted with the increase in activity observed after co-transfection with an AP-2α expression plasmid (see Figure 5B) and suggests that AP-2γ may directly repress *CDKN1A* promoter activity.

A series of 5′ deletion mutant reporter constructs were evaluated in control and AP-2γ silenced MCF-7 cells to narrow down which region of the *CDKN1A* promoter was mediating AP-2γ repression. An increase in luciferase activity following AP-2 silencing was observed for all of the reporter constructs, suggesting that the minimal region assayed (from −111 to +8 relative to the *CDKN1A* start of transcription) is sufficient to mediate repression by AP-2 (see Supplementary Figure S9). A single AP-2-binding site has been mapped within this region. As illustrated in Figure 6, mutation of this site in the context of the −2325/+8 construct resulted in increased reporter activity in MCF-7 cells, consistent with the prevention of AP-2γ binding leading to a relief of repression at the *CDKN1A* promoter.

To show unequivocally that AP-2γ has a direct function in p21cip transcriptional regulation, chromatin immunoprecipitation (ChIP) combined with quantitative PCR were used to investigate AP-2γ occupancy at the endogenous *CDKN1A* promoter. Four sets of qPCR primers were designed to amplify specifically known regulatory regions across the *CDKN1A* promoter, chosen at a spacing so as not to amplify overlapping chromatin fragments, as illustrated in Figure 7A. Primers to a centromeric region consisting of satellite repeat (SAT2) sequences (Jiang *et al*, 2004) on Chromosome 1 were used as a negative control to determine antibody specificity. Figure 7B shows the relative enrichment of *CDKN1A* promoter-specific sequences measured in AP-2γ, p300 and acetylated histone 4 (acH4) ChIP. None of the antibodies used in these ChIP experiments were enriched for amplification from the SAT2 heterochromatin region, implying that the activity observed at the *CDKN1A* locus was specific for each protein target. These data revealed that AP-2γ occupancy within the *CDKN1A* endogenous promoter region was confined to the region amplified by the proximal promoter primer set, in agreement with the site of AP-2γ activity deduced from the promoter deletion reporter assays described above (Supplementary Figure S9; Figure 6).

Further studies, to explore whether AP-2γ silencing was accompanied by changes in AP-2γ occupancy at the −21/+44 region, used ChIP assays on MCF-7 cells transfected

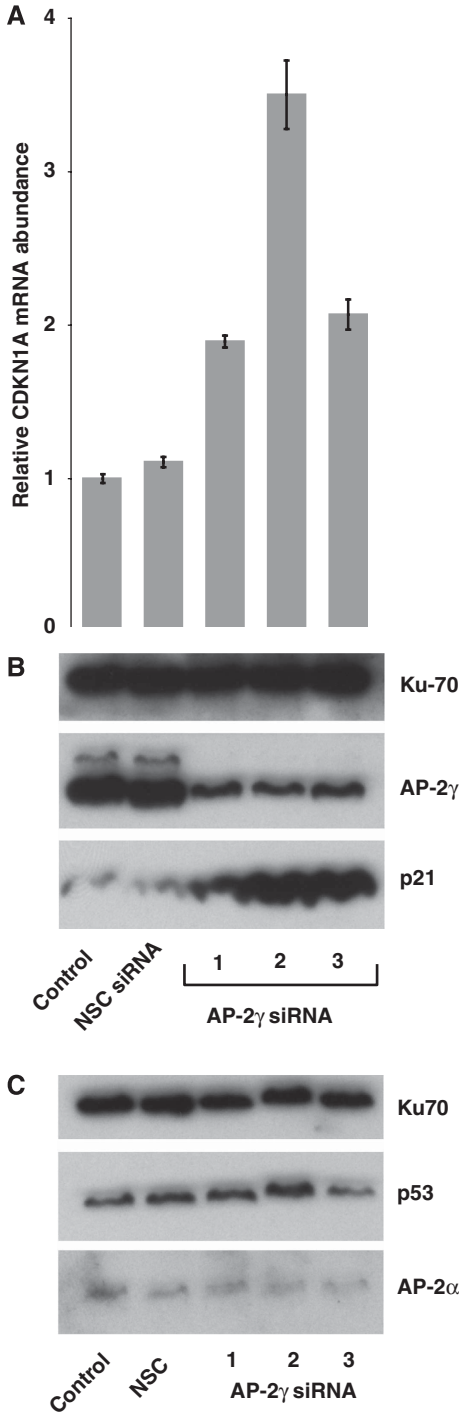


Figure 4 Induction of p21cip following transient transfection with AP-2γ targeting siRNAs. MCF-7 cells were transfected with control or AP-2γ silencing siRNAs, passaged at 48 h and harvested at 72 h. Parallel samples were processed for RNA or WCE. (**A**) Quantitative PCR analysis of *CDKN1A* mRNA levels. Data were normalised to *GAPDH* mRNA levels and the transfection reagent only (control) values, and were set at 1 to generate relative mRNA levels. The graph represents data averaged from three PCR replicates, ± standard error. (**B**) WCE (5 μg/lane) were separated by SDS–PAGE, blotted and probed with primary antibodies against Ku-70, p21cip and AP-2γ. (**C**) A separate western blot was used to examine expression of p53 and AP-2α in the same samples.

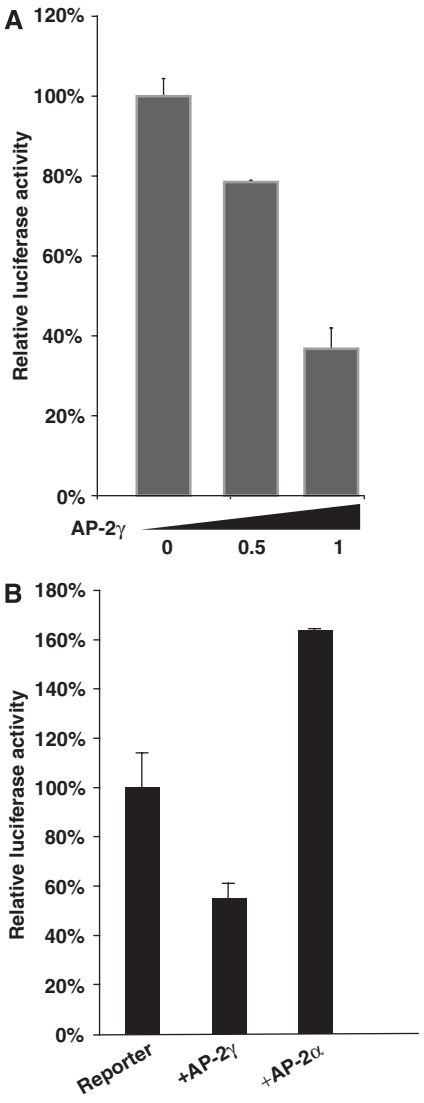


Figure 5 AP-2γ repressed basal activity from the *CDKN1A* promoter in HepG2 cells. The AP-2 non-expressing cell line, HepG2, was transfected transiently with 1.0 μg of the *CDKN1A* luciferase reporter construct (containing genomic sequences from −2325 to +8 relative to the transcription start site) and additional plasmids as follows: (**A**) the indicated amounts (μg) of an AP-2γ expression construct; (**B**) 1.0 μg of control, AP-2γ or an AP-2α expression constructs as indicated. A renilla luciferase construct was co-transfected in each experiment and variations in plasmid concentration were compensated for using pcDNA3 plasmid. Cells were assayed for luciferase activity 48 h after transfection. Results (mean ± s.d. of three independent experiments) are presented as relative luciferase activity, corrected for renilla luciferase activity with activity in cells transfected with the *CDKN1A* reporter alone set at 100%.

with control or AP-2γ targeting siRNA. The data presented in Figure 8A record the relative occupancy at this region for AP-2γ itself plus a number of other relevant factors and epigenetic markers of transcriptional activity. Notably, a significant reduction in AP-2γ and p300 occupancy was accompanied by increased histone 4 acetylation, which is consistent with the hypothesis that loss of AP-2γ from the *CDKN1A* locus leads to relief from repression and hence gene activation, as confirmed by the increased levels of p21cip mRNA and protein observed in the same experiment (Figure 8B). Numerous studies have shown that repression of *CDKN1A* transcription requires histone deacetylases, notably HDAC1 and 2 (reviewed in Ocker and Schneider-Stock,

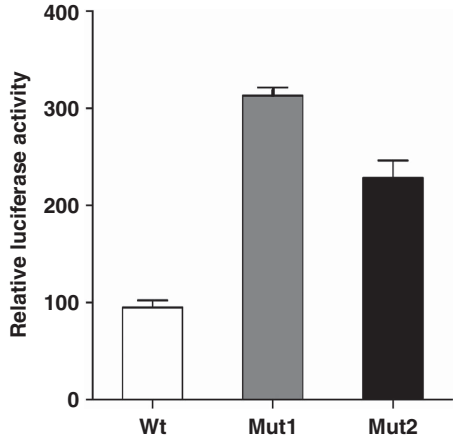


Figure 6 Mutation of the AP-2-binding site in the *CDKN1A* promoter results in relief from repression. MCF-7 cells were transfected with either the wild-type −2325/+8 *CDKN1A* luciferase reporter plasmid, or versions (Mut1 and Mut2) with 2 bp substitutions within the AP-2-binding site that abrogate AP-2γ DNA binding. Cells were harvested after 48 h and assays were carried out and controlled as in Figure 5. Luciferase activity with the wt construct was set at 100% and activity of the mutants is expressed relative to the wt.

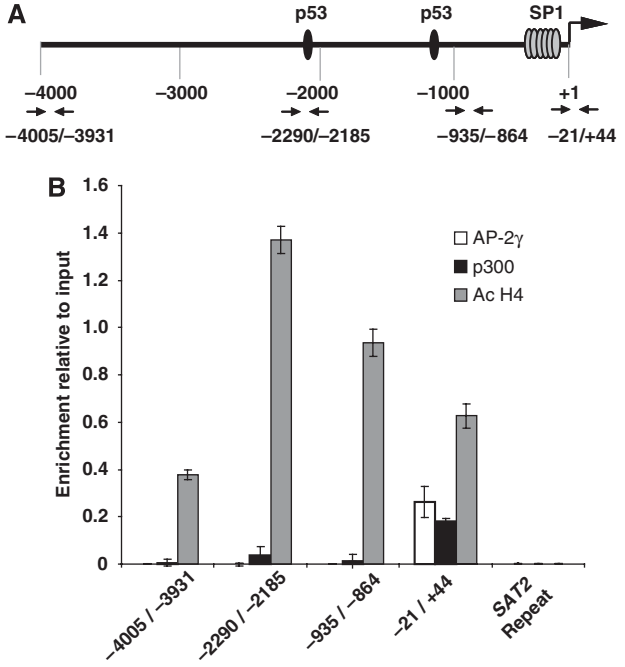


Figure 7 ChIP assay showing endogenous AP-2γ and p300 occupancy and AcH4 levels across the *CDKN1A* promoter region. (**A**) Summary of the location of key regulatory elements across the *CDKN1A* promoter (adapted from Gartel and Radhakrishnan, 2005) in relation to the primers used for the quantitative PCR. (**B**) ChIP assay for AP-2γ, p300 and acetylated histone 4 (AcH4) in wild-type MCF-7 cells. Cells were passaged (1 in 2) 24 h before use in ChIP assays to ensure cells were not contact inhibited. Results were normalised to a no antibody (background) control and are expressed relative to a 1/1000 dilution of the total input chromatin. Chromatin samples were analysed by qPCR using −4005/−3931, −2290/−2185, −935/−864 and −21/+44 *CDKN1A* promoter-specific primers and SAT2 heterochromatin-specific primers (see Supplementary data). Data were averaged from three PCR replicates, ± standard error.

2007). Interestingly, loss of AP-2γ from the *CDKN1A* locus in silenced cells was accompanied by reduced occupancy of HDAC2. In contrast, the level of Sp1 occupancy at the proximal promoter remained largely unchanged between control and AP-2 silenced cells (Figure 8A).

In an attempt to place these findings in a therapeutic context, we also have examined AP-2γ interaction at the *CDKN1A* locus during anti-oestrogen treatment of MCF-7 cells. *TFAP2C* is an oestrogen-regulated gene whose expression levels decline in ER positive breast tumour lines treated with anti-oestrogens (tamoxifen, faslodex; Orso *et al*, 2004) thus mimicking the effect of siRNA studied here. We therefore examined, using ChIP, whether anti-oestrogen treatment can also lead to loss of AP-2γ from the *CDKN1A* promoter. Compared with vehicle-treated controls, MCF-7 cells incubated with anti-oestrogen for 24 h displayed reduced AP-2γ protein expression and a sharp decline in the relative levels of AP-2γ occupancy at the *CDKN1A* proximal promoter (Figure 9A). As expected, levels of p21cip mRNA and protein increased and the treated cells demonstrated additional hallmarks of growth arrest including reduced levels of ppRb (Figure 9B). To extend this study, we have similarly treated other ER positive breast cell lines, that express significant levels of both AP-2γ and AP-2α, with anti-oestrogens and examined AP-2 occupancy at the endogenous *CDKN1A* proximal promoter using ChIP. As shown in Figure 10A, we found that relative AP-2γ binding at the promoter again declined in treated cells as expected, whereas in contrast, AP-2α binding increased and these changes in occupancy were accompanied by a significant elevation in p21cip mRNA levels (Figure 10B).

Discussion

We have suggested earlier that high levels of the developmentally regulated transcription factor, AP-2γ are correlated with poor survival in breast cancer. This correlation occurred both independently and in the context of the generally considered indicators of good prognosis: namely ERα expression and ErbB2 negativity (Gee *et al*, 2009). The purpose of the current study was to investigate which pathways are activated in AP-2γ expressing breast cancer cells and how they might contribute to poor patient prognosis.

The largest functional category of transcripts significantly regulated following AP-2γ silencing in MCF-7 cells belonged to genes related to the cell cycle (Supplementary Figure S3), which correlated with the partial G1/S arrest exhibited by silenced cells (Figures 2–4).

These observations are also in accord with those made on mice homozygously deleted for *tfcap2c*: AP-2γ is an essential gene in the extra-embryonic tissues and its loss was shown to result in a marked reduction in cell proliferation in these tissues, ultimately leading to early embryonic death because of inadequate development of the placenta (Werling and Schorle, 2002).

As the majority of genes identified from the array study are known to display cell cycle periodicity, it seems highly likely that expression changes occurred as a consequence of the cell cycle arrest and therefore these are probably not direct AP-2γ target genes. Consistent with this hypothesis, many of these genes are known to be transcriptionally regulated by E2F family members at the G1/S transition. Intriguingly, however, a computational study (Jin *et al*, 2006) found an AP-2

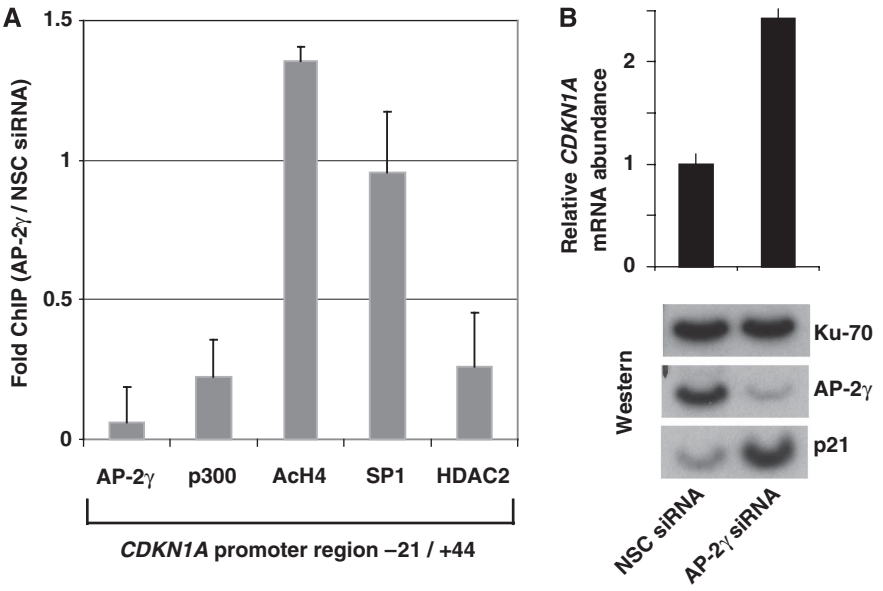


Figure 8 ChIP assay showing fold change in transcription regulator occupancy at the *CDKN1A* proximal promoter after AP-2γ silencing. MCF-7 cells were transfected with NSC control siRNA or AP-2γ siRNA_3, split 1 in 2 at 48 h, then harvested for use in ChIP, qPCR or western blot 24 h later. (**A**) ChIP assay for AP-2γ, p300, SP1, HDAC2 and AcH4 was performed as in Figure 7 using the −21/+44 PCR primers; results are presented as a fold ratio of signal from AP-2γ silenced cells compared with control cells. (**B**) p21 mRNA and protein levels 72 h following AP-2γ silencing in MCF-7 cells. *CDKN1A* levels were analysed by qPCR and normalised to *GAPDH* mRNA levels. The transfection reagent alone (control) value was set at 1 to generate relative mRNA levels. The graph represents data averaged from three PCR replicates, ± standard error. WCE (5 μg/lane) were separated by SDS–PAGE and blotted to a membrane. Blots were probed with primary antibodies against Ku-70, p21cip and AP-2γ.

consensus transcription factor binding site (TFBS) adjacent to 55% of functional E2F1 sites, compared with E2F1 sites in non-E2F target promoters. It seems debatable, however, that this apparent association of E2F1 and AP-2 TFBS has a functional consequence; both E2F and AP-2 factors bind GC-rich sequences, thus there is an increased likelihood of chance co-localisation in GC-rich promoter regions. Unfortunately, ChIP-chip experiments examining the functional consequence of this association *in vivo* (Jin *et al*, 2006) used a pan-AP-2 antibody with a high level of non-specific binding to heterchromatic (SAT2) sequences (KF, unpublished results). Our own TFBS analysis failed to detect any enrichment for the presence of the AP-2 consensus sequence in promoter regions of genes within our AP-2γ dataset compared with a similar region upstream of all the genes on the array (see Supplementary data) and this agrees with earlier studies (Bajic *et al*, 2004) that noted how common the AP-2 consensus TFBS is within gene upstream sequences. Thus, although the potential association of AP-2 family members with regulation of E2F1 target genes is interesting, in the light of the AP-2γ-mediated regulation of *CDKN1A*, this suggestion remains unconfirmed.

Here, we show that cell cycle perturbation at the G1/S transition in AP-2γ silenced cells is due largely to an increase in p21cip levels; not only do we observe increased protein and mRNA levels (Figure 4) but also a decrease in Cyclin/CDK activity, a functional consequence of p21cip induction (Supplementary Figure S7). Moreover, the gene expression changes observed on AP-2γ silencing in MCF-7 cells showed remarkable overlap with earlier studies examining changes following induction of p21cip in unrelated cell lines. In human fibrosarcoma cells, ∼45% of the genes down-regulated on p21 induction (Chang *et al*, 2000) were also down-regulated following AP-2γ silencing in MCF-7 cells. In addition, in human ovarian carcinoma cells, ∼47% of genes differentially regulated on expression of exogenous p21 (Wu *et al*, 2002) were similarly regulated following AP-2γ silencing. In each case, the majority of overlapping genes are cell cycle associated and E2F targets, consistent with up-regulation of p21cip being the major factor leading to cell cycle arrest following AP-2γ silencing.

The evidence presented here, from reporter assays and ChIP experiments in normally cycling and in AP-2γ silenced cells, shows that AP-2γ associates directly with the proximal promoter of the *CDKN1A* gene and thereby represses its transcription. Thus, AP-2γ acts in an opposing manner to the related AP-2α factor, which has been shown earlier to activate *CDKN1A* expression (Zeng *et al*, 1997; McPherson *et al*, 2002; Wajapeyee and Somasundaram, 2003). Significantly, AP-2α remained at barely detectable levels following AP-2γ knock-down in MCF-7 cells (Figure 4C). Similarly, p53 levels remained low in cells treated with each of the AP-2γ targeting siRNAs, and we found no evidence for either an induction of p53 or an interaction by AP-2 factors with the classical p53 binding regions of the *CDKN1A* locus (Figure 7) as has been suggested elsewhere (McPherson *et al*, 2002). Moreover, we did not detect any alteration in levels of ER (Supplementary Figure S1) after AP-2γ silencing as reported earlier (Woodfield *et al*, 2007). Instead, we find that AP-2γ is associated with the proximal *CDKN1A* promoter in cycling MCF-7 cells and that on silencing, AP-2γ occupancy falls significantly. A concomitant loss of p300 association and HDAC1/2 binding plus an increase in histone acetylation in the same region suggests that removal of AP-2γ leads to chromatin reorganisation that is required for increased transcription from the *CDKN1A* gene.

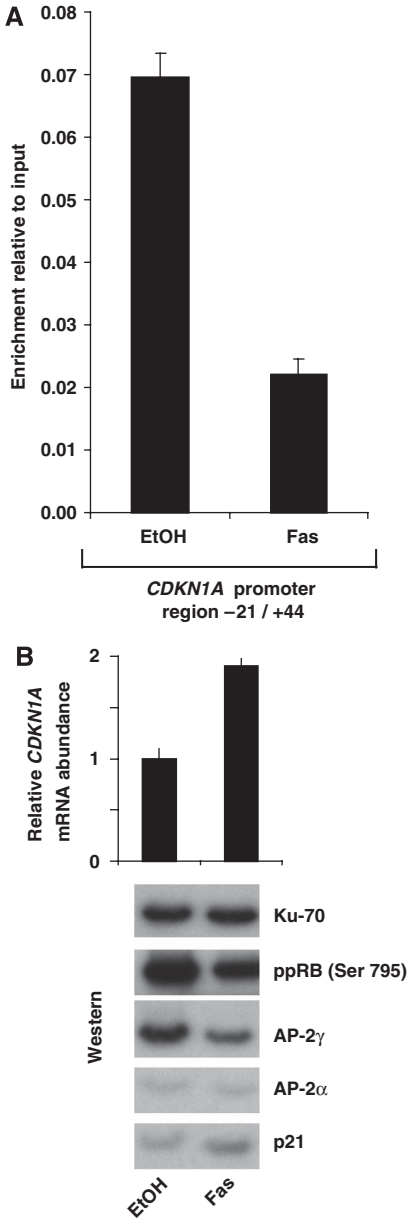


Figure 9 Anti-oestrogen treatment reduces endogenous AP-2γ occupancy at the *CDKN1A* proximal promoter. MCF-7 cells were split one in two into medium containing either the anti-oestrogen faslodex (Fas; 100 nM) or vehicle control (EtOH) and harvested for use in ChIP, qPCR or western blot 24 h later. (**A**) ChIP assay for AP-2γ was performed as in Figure 7. (**B**) p21cip mRNA and protein levels following anti-oestrogen treatment in MCF-7 cells. *CDKN1A* levels were analysed by qPCR as shown earlier (see Figure 8). WCE (5 μg/lane) were separated by SDS–PAGE, blotted to a membrane and probed with primary antibodies against Ku-70, p21cip, AP-2γ, AP-2α and phospho Rb (ser 795) as indicated.

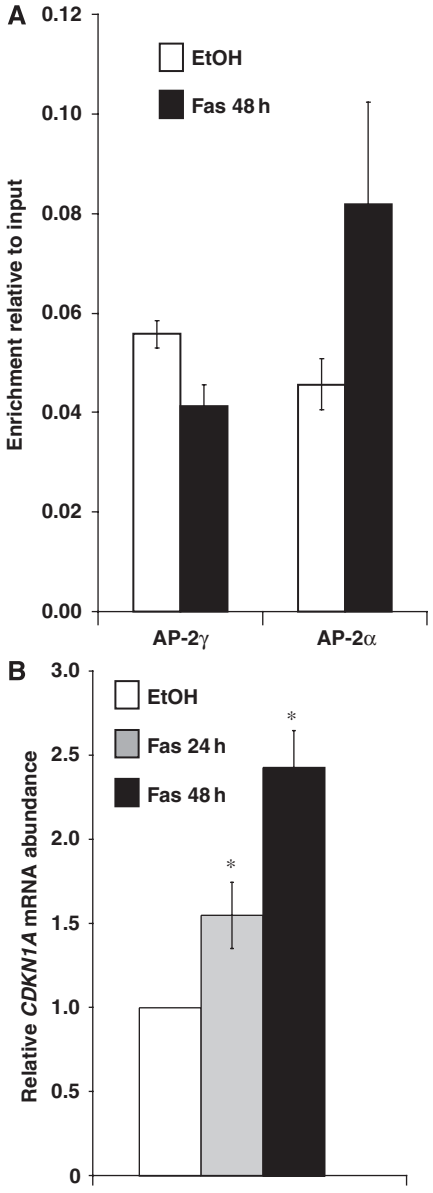


Figure 10 Decreased AP-2γ binding at the *CDKN1A* locus with anti-oestrogen treatment is accompanied by increased AP-2α occupancy in breast lines that express both AP-2 family members. ER positive H3396 cells were split into medium containing either the anti-oestrogen faslodex (Fas) or vehicle control (EtOH) and harvested for use in ChIP or qPCR at 24 or 48 h, as indicated. (**A**) ChIP assay for AP-2 factors was performed as in Figure 7 using primers to the −21/+44 region of *CDKN1A*. Values are the average of three independent ChIP experiments, error bars represent standard errors between the repeats. As for the other ChIP antibodies used in this study, the AP-2α antibody was checked to give a low background at the SAT2 control locus. (**B**) *CDKN1A* mRNA levels following anti-oestrogen treatment in H3396 cells were analysed by qPCR ($*P<0.05$). Similar results were also observed using the ZR75-1 breast cell line (data not shown).

The loss of p300 at a time when histone acetylation is increasing is perhaps unexpected. However, p300 is a known co-factor of AP-2γ, recruited through CITED adapter proteins (Braganca *et al*, 2002, 2003) and, although largely associated with gene activation, it has also been found in repression complexes at regulatory loci of genes that oscillate rapidly between active and inactive states (Simone *et al*, 2004) including *CDKN1A* (Thomas and Chiang, 2005). There are numerous examples in the literature of AP-2 factors acting to repress gene expression, but the precise molecular mechanism has yet to be elucidated. Alterations in the activity or identity of AP-2 cofactors are likely to play a role and it is noteworthy that SUMO-modified p300 has been shown to recruit HDACs (Girdwood *et al*, 2003). AP-2γ itself has also been shown to be a SUMO substrate and modification has been suggested to alter its function (Eloranta and Hurst, 2002). This, or an as yet to be identified modification of AP-2γ, could favour association with co-repressor molecules.

For example, nucleophosmin has been suggested to act as an AP-2 co-repressor (Liu *et al*, 2007). More prosaically, AP-2-mediated repression may arise through binding site competition with a much more active DNA binding transcription factor (Ikeda *et al*, 2006). Potentially, in cell lines that express both factors, such a competition may exist between AP-2α, which activates *CDKN1A*, and AP-2γ, which represses it.

That AP-2γ represses, whereas the related AP-2α factor activates, p21cip expression is consistent with a growing literature suggesting that these family members have opposing functions in neoplasia, as reviewed in the Introduction. Tumour expression of AP-2γ has also been associated with a reduced response to anti-oestrogen therapy. However, *TFAP2C* is a ligand-activated ERα target gene, thus, during acute cellular responses to anti-oestrogens AP-2γ levels fall, p21cip is induced (Figure 9) and cell proliferation is arrested, consistent with a response to therapy. In cells that express both AP-2γ and AP-2α, the latter may increase its binding to the *CDKN1A* promoter as we also illustrate here (Figure 10), and thus contribute to p21cip mRNA accumulation. However, anti-oestrogen-resistant lines, derived from ER positive breast cancer cells through long-term exposure to anti-oestrogens, have been shown to restore their AP-2γ expression to pre-treatment levels (Gee *et al*, 2009). This re-expression of AP-2γ in the resistant cells therefore represents a challenge to hormone therapy. The data presented here provide a mechanistic explanation for this challenge, as cells with high levels of AP-2γ will repress *CDKN1A* and will thus be compromised in their ability to arrest their growth in the presence of anti-oestrogens. Extrapolation of these findings to patient data therefore also suggests a mechanism whereby high tumour expression of AP-2γ contributes to the failure of anti-hormone therapy and poor patient survival. Although an independent event (Gee *et al*, 2009), the frequently reported loss of AP-2α activity during tumour progression (Gee *et al*, 1999; Pellikainen *et al*, 2002; Douglas *et al*, 2004), may also contribute to loss of cell cycle control and explain, at least in part, how these two factors can have opposing functions in breast cancer.

Materials and methods

Cell lines and antibodies

MCF-7 and HepG2 cells were cultured in DMEM, 10% FCS; plus insulin (10 μg/ml) for MCF-7 cells. Transfection with siRNA (Oligofectamine; Invitrogen) or plasmid DNA (FuGENE 6; Roche) was carried out according to the manufacturer's directions. H3396 cells (Clarke *et al*, 2004) were cultured in RPMI plus 10% FCS. Faslodex (100 nM; Sigma) was added to normal media where indicated. MCF-T-Rex cells (Applied Biological Materials Inc.) expressing the Tet Repressor were grown in DMEM, 5% Tetracycline-free FCS (Clontech) and G418 (200 μg/ml). After transfection with pSuperior plasmids (see below), single colonies were selected and expanded in puromycin-containing media. Expression of shRNA was induced using 1 μg/ml doxycycline (Dox; Sigma). Western blotting and ChIP antibodies, AP-2γ (6E4/4; Gee *et al*, 2009); AP-2α (3B5); p21cip (DCS60; Cell Signalling); p27kip (2552; Cell Signalling); Ku-70 (C19); ERα (60C; Millipore); p300 (N-15); SP1 (PEP2); AP-2γ (H77); AP-2α (39304, Active Motif); Acetylated-H4 (Upstate); HDAC2 (H54) were from Santa Cruz unless indicated otherwise.

siRNA

AP-2γ-specific siRNAs were selected based on published patterns (Reynolds *et al*, 2004; Ui-Tei *et al*, 2004). BLAST searches verified AP-2γ specificity. AP-2γ_1: AAUGAGAUGGCAGCUAGGAAG; AP-2γ_2: GCGGCCCAGCAACUGUGUAAA; AP-2γ_3: CCACACUGGAGUCGCCGAAUA. A fourth siRNA, J-005238-07 (Dharmacon) referred to herein as AP-2γ_4 was used in some experiments. NSC(AAUUCUCCGAACGUGUCACGU; Qiagen). Short hairpin RNA expressing constructs using the pRetroSuper vector (Brummelkamp *et al*, 2002) were also generated for each silencing sequence plus NSC and *AP-2γ*_4 sequences were also cloned into pSuperior (van de Wetering *et al*, 2003) to permit doxycycline-inducible expression of shRNA.

Gene expression profiling and data analysis

MCF-7 cells at 30–40% confluence were transfected with 25 nM siRNA, harvested after 96 h and extracted for RNA using TRIZOL reagent (Invitrogen) and further purified over an RNeasy Mini column (Qiagen). RNA concentration was estimated by spectrophotometer and MOPS denaturing agarose gel electrophoresis was used to assess RNA quality. Total RNA (8 mg) was prepared for hybridization to Affymetrix GeneChip Human Genome U133 Plus 2.0 oligonucleotide arrays following the manufacturer's recommendations. A complete description of all procedures, statistical analysis and annotation are available in the Supplementary data. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*, 2002) and are accessible through GEO Series accession number GSE15481: (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE15481).

Flow cytometry

Adherent and floating cells were harvested and fixed in 70% ethanol, pelleted and resuspended in 50 μl RNAse A (100 mg/ml; Sigma) and 300 μl PI (50 μg/ml; Sigma). For S-phase labelling, cells were incubated with BrdU (10 μg/ml) for 20 min before harvest and labelled cells were identified using a FITC-conjugated anti-BrdU antibody (Becton Dickinson) and standard procedures. DNA was counterstained with PI as above. Flow cytometry was performed using a FACS Calibur (Becton Dickinson). Fluorochromes were excited by a 488-nm laser and fluorescence was collected between 515 and 545 nm for FITC and above 670 nm for PI. Forward and right angle scatter was used to define the cellular populations and pulse processing of the PI signal was used to eliminate cell doublets. All flow cytometry data were analysed using FlowJo software (Treestar).

Reporter assays

The luciferase reporter construct bearing *CDKN1A* flanking sequences from −2325/+8 (el-Deiry *et al*, 1993), the *CDKN1A* deletion series (Datto *et al*, 1995) and AP-2 expression plasmids (Bosher *et al*, 1996) have been described earlier. Triplicate transfection assays were controlled by co-transfection with phRG-TK Renilla and assayed using Dual Luciferase (Promega) on a Luminoskan Ascent (Thermo Labsystems) luminometer.

ChIP assays

ChIP analysis was performed essentially as described (Metivier *et al*, 2003) with minor modifications (see Supplementary data). Quantification of immunoprecipitated DNA was carried out in triplicate using a 7500 Real-Time PCR System (Applied Biosystems), which contained SYBR Green I Dye and AmpliTaq Gold DNA polymerase. Primer sequences are listed in the Supplementary data. Values were calculated as fold enrichment compared with the IgG control versus a control locus (SAT2).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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