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ORIGINAL ARTICLE

PR55α-containing protein phosphatase 2A complexes promote cancer cell migration and invasion through regulation of AP-1 transcriptional activity

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The proto-oncogene c-Jun is a component of activator protein-1 (AP-1) transcription factor complexes that regulates processes essential for embryonic development, tissue homeostasis and malignant transformation. Induction of gene expression by c-Jun involves stimulation of its transactivation ability and upregulation of DNA binding capacity. While it is well established that the former requires JNK-mediated phosphorylation of S63/S73, the mechanism(s) through which binding of c-Jun to its endogenous target genes is regulated remains poorly characterized. Here we show that interaction of c-Jun with chromatin is positively regulated by protein phosphatase 2A (PP2A) complexes targeted to c-Jun by the PR55α regulatory subunit. PR55α-PP2A specifically dephosphorylates T239 of c-Jun, promoting its binding to genes regulating tumour cell migration and invasion. PR55α-PP2A also enhanced transcription of these genes, without affecting phosphorylation of c-Jun on S63. These findings suggest a critical role for interplay between JNK and PP2A pathways determining the functional activity of c-Jun/AP-1 in tumour cells.

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INTRODUCTION

The activator protein-1 (AP-1) transcription factor is a central regulator of genetic responses invoked by a multitude of physiological and pathological stimuli, including cellular stresses, growth factors, cytokines and oncogenes. It consists of dimers formed by members of the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, FRA1, FRA2), ATF and MAF families, whose expression and activities are dynamically regulated by contextual cues.[1]

The reconfiguration of signalling networks in cancer cells can dramatically alter AP-1 functionality. Oncogenic RAS-ERK signalling drives overexpression and/or activation of FRA1 and c-Jun, promoting assembly of highly active FRA1/c-Jun dimers.[2,3] These complexes can induce oncogenic transformation of rodent fibroblasts in the absence of mutant Ras proteins, and are thought to have a critical role in mediating the pro-malignant actions of the pathway in a variety of epithelial cancers, including its ability to drive cell proliferation, survival, transformation, migration, invasion and metastasis.[4–9]

The proto-oncogene c-Jun is an essential regulator of embryonic development, tissue homeostasis (for example, intestine, liver, brain, skin), inflammation and malignant transformation.[10–14] A central mechanism through which extracellular signals and oncogenes control c-Jun function is by regulating its phosphorylation. Phosphorylation of S63/S73 within the c-Jun transactivation domain by JNK is critical for activation, modulating interactions with transcriptional co-activators and repressors.[15,16] Another important, but lesser studied region of c-Jun under phosphorylation-dependent control is located adjacent to its C-terminal bZIP domain. Unlike S63/S73, several residues in this region undergo dephosphorylation during c-Jun activation, including T239 and S243.[17] The latter is targeted by ERK or DYRK2, which is thought to prime T239 for subsequent phosphorylation by GSK3.[18–21] These modifications appear to repress c-Jun function through multiple mechanisms, including promoting its degradation and disrupting its ability to bind synthetic DNA templates containing an AP-1 consensus motif *in vitro*.

In the present study, we identified a heterotrimeric protein phosphatase 2A (PP2A) complex associated with FRA1/c-Jun dimers in cancer cells using mass spectrometry. The PR55α regulatory subunit of the complex targets its catalytic core to c-Jun to mediate dephosphorylation of T239, promoting binding of c-Jun to pro-migratory and invasive target genes and enhancing their expression independently of changes in transactivation domain phosphorylation. These findings suggest that functional activation of c-Jun/AP-1 is modulated by the cooperative actions of two phosphoregulatory complexes, JNK and PR55α-PP2A.

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RESULTS

PR55α targets PP2A to c-Jun/FRA1 complexes

Persistent activation of the ERK–MAPK pathway in cancer cells has been shown to promote formation of FRA1-containing AP-1 dimers, which can act as potent regulators of cell migration, invasion and metastasis.[4,5,8,22,23] To better understand how phosphorylation events regulate these complexes, we used proteomics to identify kinases or phosphatases associating with epitope-tagged FRA1 stably expressed in BE colorectal cancer (CRC) cells,[5] or a stabilized variant of the protein (FRA1$^{\Delta 3}$)[24] transiently expressed in HEK293 cells. FRA1 complexes isolated from both cellular settings contained three PP2A subunits, the PR65α scaffold, PR55α regulatory subunit and the catalytic (PP2Ac) subunit (Supplementary Table S1). When coexpressed in HEK293 cells, HA-FRA1$^{\Delta 3}$ associated with all three PP2A subunits, binding most efficiently to PR55α (Figure 1a). We also confirmed that FRA1, PR55α and PP2Ac formed endogenous complexes in BE cells (Figure 1b).

Eukaryotic cells express various combinations of at least 25 PP2A regulatory subunits, representing the largest and most diverse component of the phosphatase.[25] They target the catalytic core to specific substrates, hence acting as key determinants of biological outcomes. Consistent with playing such a targeting role for AP-1, knockdown of PR55α reduced the abundance of FRA1/PP2A complexes in BE cells (Figure 1c). Analysis of FRA1 mutants revealed that the ability to dimerize with Jun, rather than DNA binding, was necessary for association with PR55α (Figures 1d and e). Consistent with this finding, PR55α co-purified with endogenous c-Jun in BE cells (Figure 1f), and was required for binding of PP2A to c-Jun (Figure 1f). In contrast to PR55α, we did not detect significant association of c-Jun with several other PP2A regulatory subunits, including PR55δ, PR56ε and PR72 (Supplementary Figure S1). The formation of PR55α/c-Jun complexes, however, did not require FRA1 (Figure 1g). Further analysis of the interactions using *in vitro*-translated proteins revealed direct association of PR55α with c-Jun, but not c-Fos or FRA1 (Figure 1h). These data indicate that PR55α binds to c-Jun, targeting PP2A to AP-1 complexes in tumour cells.

PR55α-PP2A dephosphorylates T239 of c-Jun

To investigate if c-Jun was a substrate for PP2A, we determined the effects of a PP2A inhibitor (okadaic acid) and PR55α depletion on several key c-Jun phosphoregulatory sites (Figure 2a). While okadaic acid enhanced phosphorylation of S63, T239 and S243 (Figure 2b), PR55α knockdown specifically increased phospho-T239 levels in BE colorectal carcinoma (Figures 2c and d),

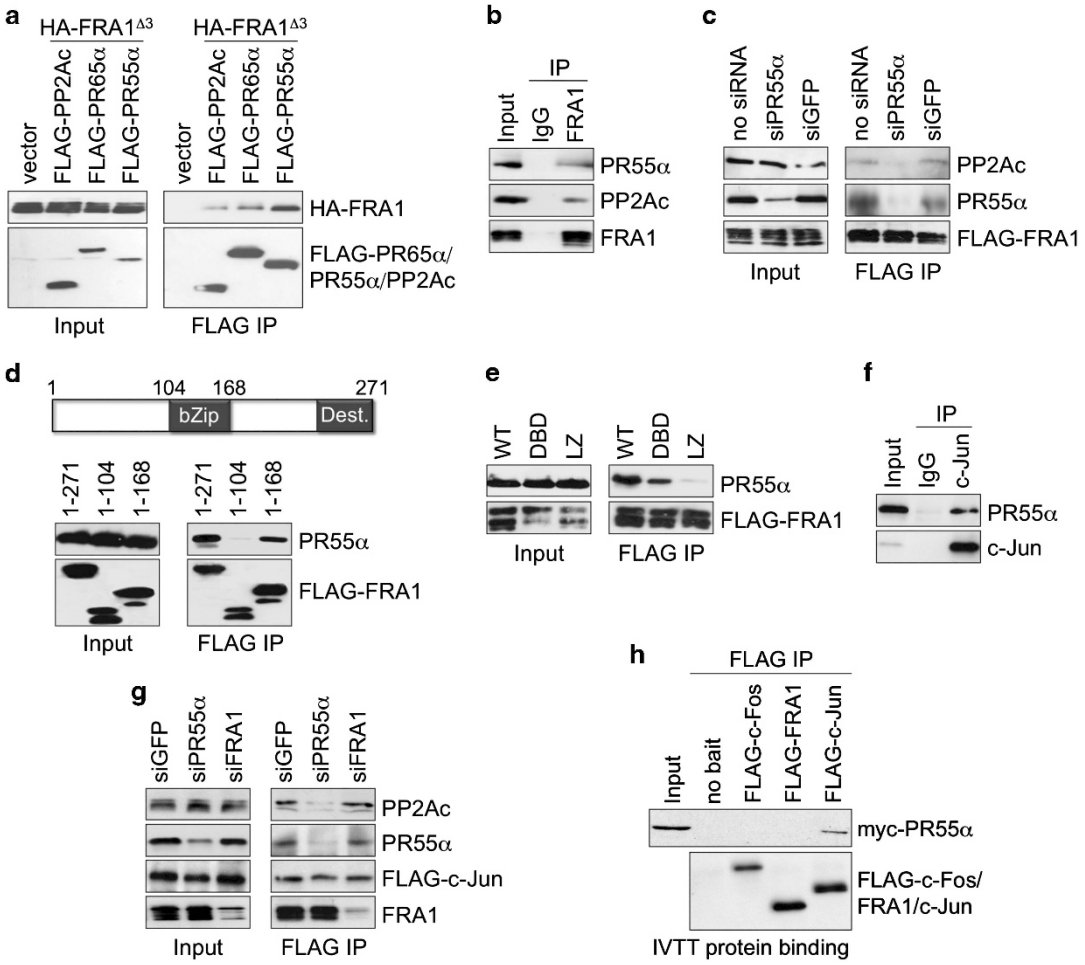

Figure 1. A PR55α–PP2A complex associates with c-Jun/FRA1 AP-1 dimers. (**a**) Co-immunoprecipitation of HA-FRA1$^{\Delta 3}$ with FLAG-PP2A subunits in HEK293 cells. (**b**) Co-immunoprecipitation of endogenous FRA1 with PR55α and PP2Ac in BE colorectal carcinoma cells. (**c**) Effect of PR55s knockdown on FRA1/PP2Ac association in BE cells stably expressing FLAG-FRA1. (**d**) Endogenous PR55α association with full-length and truncated FLAG-FRA1 proteins in HEK293 cells. (**e**) Endogenous PR55α association with wild-type FLAG-FRA1 and mutants defective in DNA binding (DBD) or dimerization (LZ) in HEK293 cells. (**f**) Co-immunoprecipitation of endogenous c-Jun with PR55α in BE cells. (**g**) Effects of PR55α or FRA1 knockdown on c-Jun/PP2Ac association in BE cells stably expressing FLAG-c-Jun. (**h**) Analysis of interactions between *in vitro* transcribed and translated myc-PR55α and FLAG-c-Fos, FLAG-FRA1 and FLAG-c-Jun.

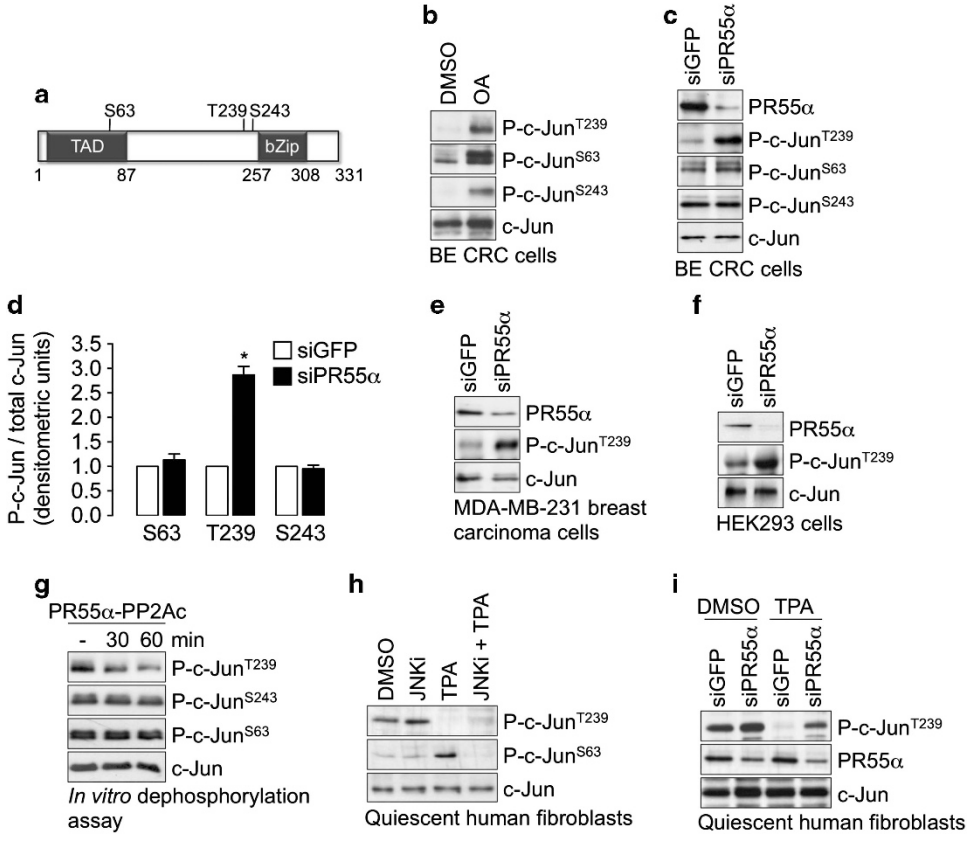

Figure 2. PR55α-PP2A regulates dephosphorylation of c-Jun T239. (**a**) Schematic of major c-Jun functional domains and phosphorylation sites examined in this study. TAD, transactivation domain; BR, basic region; LZ, leucine zipper. (**b**) Effects of okadaic acid (10 nM for 2 h) on c-Jun phosphorylation in exponentially growing BE cells. (**c**) Effects of PR55α knockdown on c-Jun phosphorylation in BE cells. (**d**) Semi-quantitative analysis of data from (**c**). Error bars represent s.e.m ($n = 3$). *$P < 0.05$ (Student's *t*-test). (**e**, **f**) Effect of PR55α knockdown on endogenous c-Jun T239 phosphorylation in MDA-MB-231 breast carcinoma and HEK293 cells. (**g**) Dephosphorylation of c-Jun by PR55α-PP2A *in vitro*. (**h**) Effects of TPA (30 min) and/or a JNK inhibition (30 min) on c-Jun phosphorylation in quiescent human fibroblasts. (**i**) Effect of transient PR55α silencing on TPA-induced changes in c-Jun phosphorylation in quiescent human fibroblasts.

as well as MDA-MB-231 breast carcinoma and HEK293 cells (Figures 2e and f).

To further characterize the apparent selectivity of PR55α-PP2A for phospho-T239, we tested the ability of immunopurified PR55α complexes to dephosphorylate c-Jun *in vitro* (Figure 2g). The complexes reduced phospho-T239 levels within 30 min, but did not significantly alter phosphorylation on S63 or S243, supporting the notion that T239 was a highly specific substrate for PR55α-PP2A.

c-Jun activation is stimulated by a vast array of extracellular signals, such as growth factors, mitogens, cytokines and stresses. To determine if PR55α-PP2A regulates dephosphorylation of T239 in response to a c-Jun activating agent, we treated quiescent human diploid fibroblasts with the mitogen TPA.[17] As expected, TPA induced robust phosphorylation of S63 that coincided with dephosphorylation of T239 (Figure 2h). These modifications were, however, not coupled, as JNK inhibition selectively repressed induction of phospho-S63, whereas PR55α depletion specifically antagonized dephosphorylation of T239 (Figure 2i). Thus phosphorylation of S63 (by JNK) and dephosphorylation of T239 (by PR55α-PP2A) are independently regulated events occurring during c-Jun activation.

Dephosphorylation of T239 promotes c-Jun/AP-1 function by enhancing target gene occupancy

The ability of c-Jun to activate transcription requires its dimerization with another AP-1 protein, binding to DNA and upregulation of transactivation capacity. To determine if PR55α-PP2A regulates the activity of c-Jun/FRA1 dimers, we examined three genes containing binding sites co-occupied by FRA1 and c-Jun in BE cells, *VIM*, *AXL* and *ZEB2* (Figures 3a and b). These genes were previously identified as FRA1 targets in various carcinoma cells, where they act as regulators of cell migration, invasion and epithelial–mesenchymal transitions (EMT).[26–28] Stable depletion of PR55α reduced mRNA levels of all three genes, which correlated with impaired *in vitro* migration and invasion by the cells (Figures 3c–f). An impairment of *in vitro* migration and invasion was also observed upon PR55α knockdown in RKO and SW480 colorectal carcinoma cells (Supplementary Figure S3).

Phosphorylation of T239 has been reported to negatively regulate both c-Jun stability and DNA binding *in vitro*.[17,21] Despite increasing phospho-T239 levels, PR55α depletion did not affect steady-state c-Jun protein levels in several cell lines (Figure 2). However, PR55α knockdown impaired enrichment of c-Jun at *VIM*, *AXL* and *ZEB2* (Figure 3g), without affecting dimerization of c-Jun with FRA1 (Supplementary Figure S2).

To directly establish the role of T239 phosphorylation in regulating c-Jun target gene binding, T239 was replaced with an alanine residue. When stably expressed in BE cells, the mutant protein demonstrated higher enrichment on *VIM*, *AXL* and *ZEB2*, and enhanced cell migration (Figures 3h–j). Together, these findings indicate that PR55α-PP2A positively regulates c-Jun/AP-1-dependent processes by dephosphorylating T239 to promote c-Jun target gene occupancy and expression.

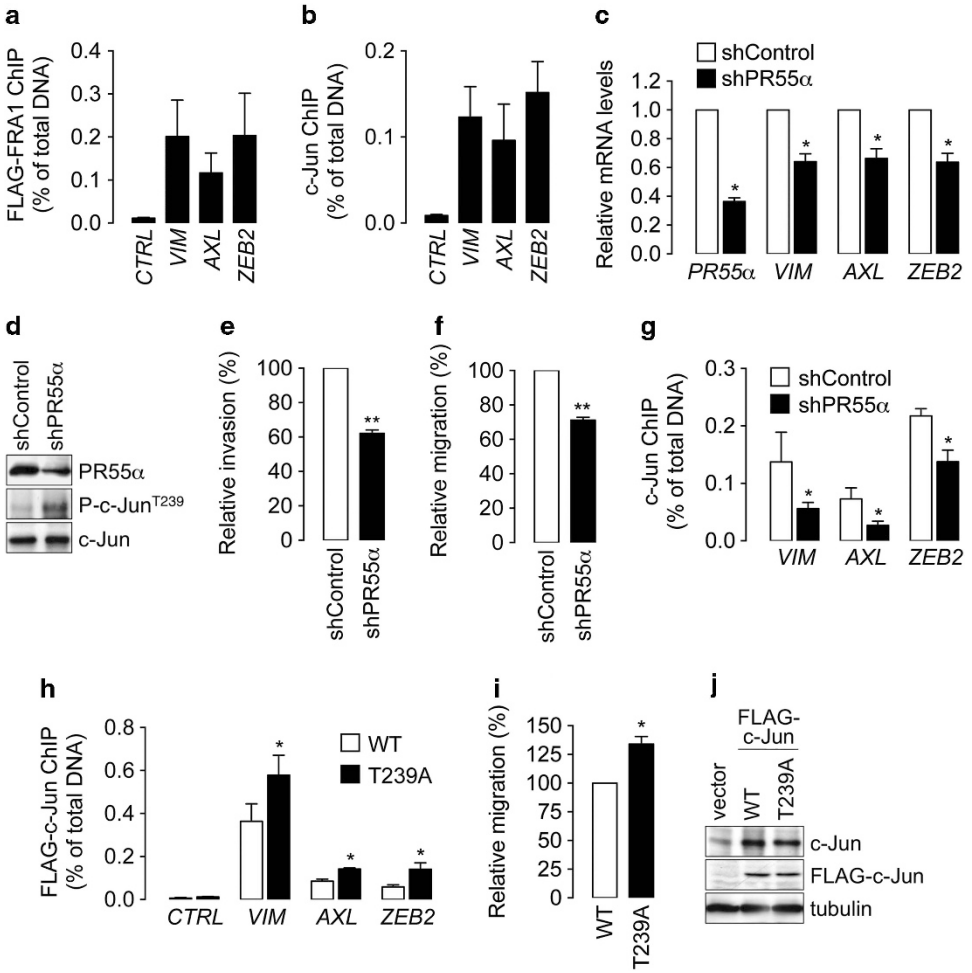

Figure 3. Dephosphorylation of T239 by PR55α-PP2A positively regulates c-Jun target gene binding and expression in CRC cells. (**a**) ChIP-qunatitative PCR analysis of FLAG-FRA1 binding to genomic regions of *VIM, AXL* and *ZEB2* in BE cells. (**b**) ChIP-quantitative PCR analysis of endogenous c-Jun binding to genomic regions of *VIM, AXL* and *ZEB2* in BE cells. (**c**) *VIM, AXL, ZEB2* expression in BE cells stably transduced with control or PR55α-targeting small hairpin RNA. lData was normalized using glyceraldehyde 3-phosphase dehydrogenase as endogenous control and represent expression relative to shControl cells. (**d**) Immunoblot analysis of cells from (**c**). (**e**, **f**) *In vitro* migration and invasion assays on cells from (**c**). (**g**) ChIP-quantitative PCR analysis of endogenous c-Jun binding to genomic regions of *VIM, AXL* and *ZEB2* in cells from (**c**). (**h**) ChIP-quantitative PCR analysis of c-Jun binding to genomic regions of *VIM, AXL* and *ZEB2* in BE cells stably expressing FLAG-c-Jun WT or FLAG-c-Jun T239A. The intergenic spacer region of ribosomal DNA was used as negative control (*CTRL*) for ChIP experiments. (**i**) *In vitro* migration assays on cells from (**h**). (**j**) Immunoblot analysis of cells from (**h**). Error bars represent s.e.m ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test).

To further test this model, we examined the extent to which PR55α-PP2A-mediated dephosphorylation of T239 regulates expression of the c-Jun target *ZEB2*, encoding a transcriptional repressor of the epithelial differentiation marker, *CDH1*. Transient silencing of either c-Jun or PR55α expression reduced *ZEB2* levels in BE CRC and MDA-MB-231 breast carcinoma cells, accompanied by induction of *CDH1* (Figures 4a and b and Supplementary Figure S4). By contrast, transient knockdown of JunB, which we found to bind poorly to PR55α, had no effect on *ZEB2* expression in BE cells (Supplementary Figure S5). The impact of PR55α depletion on *ZEB2* and *CDH1* levels was significantly attenuated in cells stably expressing c-Jun T239A (Figures 4c and d), suggesting that modulation of T239 phosphorylation is the primary mechanism through which PR55α-PP2A modulates c-Jun/AP-1 transcriptional responses.

DISCUSSION

In contrast to its well-studied regulation by a multitude of kinases, the identity and extent to which phosphatases control c-Jun activity has remained unclear. Previous studies examining PP2A regulation of c-Jun yielded conflicting findings, demonstrating both enhancement[29] and inhibition[30] of transcriptional activity in reporter gene assays. These discrepancies may have arisen in part because they focused on modulating PP2Ac activity, which can indirectly affect c-Jun activity by modulating upstream signalling events, or because reporter assays do not always reflect the endogenous chromatin at target gene promoters. By contrast, the present study examined the role of PP2A by manipulating its targeting to endogenous c-Jun/AP-1 complexes and by examining effects on endogenous transcriptional targets.

T239 was originally identified as one of several residues dephosphorylated during c-Jun activation.[17] While its phosphorylation by GSK3 requires priming at S243, dephosphorylation of T239 is not affected by acute GSK3 inhibition,[18] implying that phosphatases have the dominant role in mediating this event during activation. Although phosphorylation of S243 and T239 is coupled, their regulation by phosphatases differs. For example, TPA-induced activation of c-Jun correlates with dephosphorylation

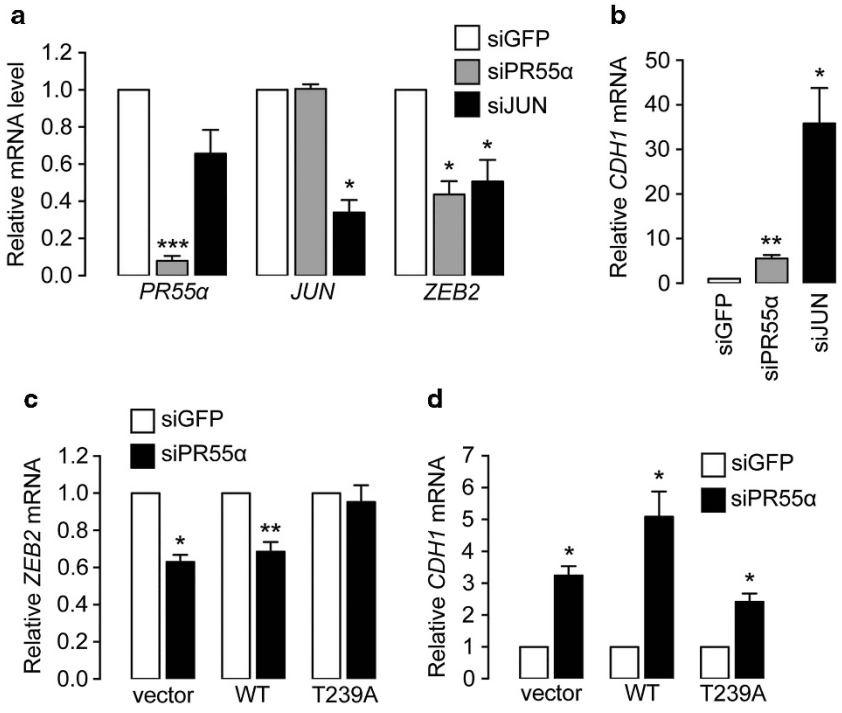

Figure 4. c-Jun T239A antagonizes the effects of PR55α depletion on *ZEB2* and *CDH1*. (**a**, **b**) Effects of transient c-Jun or PR55α knockdown on *ZEB2* and *CDH1* expression in BE cells ($n=3$). (**c**, **d**) Effects of transient c-Jun or PR55α knockdown on *ZEB2* and *CDH1* expression in BE cells stably transduced with empty vector, FLAG-c-Jun WT or FLAG-c-Jun T239A retroviral contructs ($n=4$). Data from the qRT–PCR analysis was normalized using glyceraldehyde 3-phosphase dehydrogenase as endogenous control and represent expression relative to cells transfected with siRNAs targeting GFP. Error bars represent s.e.m. $^*P<0.05$, $^{**}P<0.01$ (Student's *t*-test).

of T239 but not S243.[18] While our results indicate that the former requires PR55α-PP2A, the latter is mediated by PP2B.[31] This region adjacent to the bZIP domain of c-Jun thus appears to be subject to tight control by the relative balance of phosphatase activities, which may contribute to its reported ability to modulate various facets of c-Jun function. For example, phosphorylation of S243 but not T239 controls interaction with Sp1, whereas both sites have been implicated in negative regulation of c-Jun stability during cell cycle progression and in tumour cells.[21,32] Interestingly, we did not observe any significant effect of PR55α depletion or T239 mutation on steady-state levels c-Jun in carcinoma cells. This may indicate that the two sites cooperatively modulate c-Jun stability, or that their destabilizing influence is muted upon S63 phosphorylation, which has been reported to prevent c-Jun ubiquitylation.[33] However, we found that T239 phosphorylation did not affect expression of either wild-type c-Jun or a S63A mutant, but was associated with a reduction in the *in vitro* DNA binding capacity of both proteins (Supplementary Figure S6).

Both c-Jun and FRA1 are frequently overexpressed and/or persistently activated in epithelial cancers, and are important regulators of pro-malignant processes such as invasion, migration and EMT. By enhancing transcription of c-Jun/FRA1 targets involved in these processes, including *VIM, ZEB2* and *AXL*, PR55α-PP2A may thus have a role in tumour progression. Although widely thought to act as a tumour suppressor, growing evidence suggests that PP2A can also have oncogenic and pro-metastatic actions, depending on which regulatory subunit(s) are involved.[34–39] In CRC, frequent hypermethylation of the PR55β was reported to promote tumourigenesis and rapamycin resistance.[40] Using published microarray data, we found that while expression of PR55β and many other PP2A regulatory subunits were reduced in CRC tumours, PR55α transcript levels were modestly elevated, with highest levels occurring in tumours exhibiting microsatellite instability (Supplementary Figure S7). In addition, we also found that PR55α transcript levels were elevated in prostate, breast and ovarian carcinomas (Supplementary Figure S8).

The possibility that PR55α-PP2A regulates substrates contributing to malignant growth of CRCs is further supported by recent work showing that it can enhance Wnt pathway activity through dephosphorylation of residues promoting degradation of the transcriptional effector β-catenin.[36] Interestingly, PR55α-PP2A targeted sites in both c-Jun and β-catenin are GSK3β targets, raising the possibility that this phosphatase complex may be important for opposing the actions of this kinase. PR55α-PP2A has also been identified as a positive regulator of TGFβ signalling,[41] which together with the Wnt and Ras pathways, has key roles in regulating tumorigenesis and EMT in carcinoma cells.[42–44] It is thus tempting to speculate that PR55α-PP2A may contribute to integration of transcriptional outputs specified by these pathways in cancers.

MATERIALS AND METHODS

Cell culture and reagents

Cell lines were maintained as described previously.[24] Stable BE colorectal lines were cultured with 0.8 μg/ml puromycin (Sigma, St Louis, MO, USA). BJ human fibroblasts were serum deprived for 40 h prior to treatment with TPA (100 ng/ml), JNKi/SP-600125 (30 nM) or okadaic acid (10 nM).

The pcDNA3-FLAG-tagged PP2A subunits, PR55α,[45] PR65α[46] and PP2Ac,[47] pCDNA3-myc-PR55α[36] and FRA1 constructs[24] have been described previously. FLAG-c-Jun was cloned into the pBABE retroviral vector. The PR55a-targeting shRNAmir (5′-GCCAGTCCACGAAGAATAT-3′), cloned into the LMP retroviral vector was from Thermo Scientific (Waltham, MA, USA). The same vector containing a non-silencing small hairpin RNA (shControl) was used as negative control.

The following siRNA oligonucleotides (Dharmacon, Melbourne, VIC, Australia) were transfected into cells using Dharmafect 1 at a final concentration of 25 nM: siFRA1 (5′-CACCAUGAGUGGCAGUCAG-3′), siGFP

Duplex I (P-002048-01), PPP2R2A ON-TARGETplus SMARTpool (L-004824-00-0005), JUN siGENOME SMARTpool (M-003268-03-0005).

The following antibodies were used: Anti-FRA1 (sc-605, Santa Cruz, Dallas, TX, USA), anti-c-Jun (Santa Cruz, sc-44), anti-tubulin (Santa Cruz, sc-8035), anti-PR55α (4953, Cell Signaling, Danvers, MA, USA), PP2Ac (Cell Signaling, 5471), anti-HA (Sigma, V-4505), anti-p-c-Jun^{S63} (Cell Signaling, 9261), anti-p-c-$^{Jun\ T239}$ (Santa Cruz, sc-101720) and anti-p-c-Jun^{S243} (Santa Cruz, sc-1694).

Migration and invasion assay

We seeded 75 000 cells in triplicate into 24-well cell culture inserts (8 μm pore, BD Biosciences, San Jose, CA, USA) for the migration assay or into BD BioCoat invasion chambers (BD Biosciences) for the invasion assay. Fetal Bovine serum 10% in the bottom chamber served as chemoattractant. After 24 h, cells on the upper filter surface were removed with a cotton swab, while those on the lower surface were fixed with 100% methanol and stained with Diff-Quick staining kit (Lab Aids, Sydney, NSW, Australia). Cells that had migrated or invaded were imaged at six random fields per filter using a light microscope (Olympus BX51, Tokyo, Japan).

Protein analysis

Immunoblotting, proteomic analysis of FRA1 complexes and immunoprecipitation analysis were performed as described previously.[24] For *in vitro* dephosphorylation assays, FLAG-PR55α or FLAG-c-Jun proteins were transiently expressed in HEK293 cells. FLAG-c-Jun-expressing cells were treated with okadaic acid (10 nM for 2 h) prior to lysis and incubation with anti-FLAG M2 affinity resin (Sigma), which was then washed with RIPA buffer. Proteins were eluted using FLAG peptide (0.1 mg/ml), diluted dephosphorylation buffer (50 mM Tris–HCl, pH 7.5, 1 mM $CaCl_2$, 100 mM NaCl, 1 mg/ml BSA, 0.025% NP-40 and 1 mM DTT), then incubated at 37 °C. Binding of *in vitro*-translated proteins assessed as described previously.[24]

ChIP and qRT–PCR

Chromatin immunoprecipitation (ChIP) was performed using standard procedures, starting with 4×10^7 BE cells for each c-Jun ChIP assay (10 μg antibody) or 2×10^7 cells for each FLAG ChIP assay (50 μl FLAG-agarose beads). Relative quantification of enriched regions was performed using quantitative real-time PCR (qRT–PCR) and represented as a percentage compared with the negative control.

For real-time quantitative PCR (RT-quantitative PCR), RNA was purified from cells using the Isolate RNA Mini kit (Bioline, Alexandria, NSW, Australia) and reverse transcribed using ThermoScript RT–PCR system for first-strand cDNA synthesis (Invitrogen, Carlsbad, CA, USA). The cDNA was PCR amplified in triplicate using the Fast SYBR Green dye and 100 nM primers on the StepOnePlus Real-Time PCR system (Applied Biosystems, Mulgrave, VIC, Australia). Relative expression was determined using the $^{\Delta\Delta}C_t$ method, with glyceraldehyde 3-phosphase dehydrogenase serving as an internal control.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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