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# Erk/p90RSK/14-3-3 signalling impacts on expression of PEA3 Ets transcription factors via the transcriptional repressor capicúa

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**Running title:** Growth factors regulate capicúa and ETV1 **Key words:** 14-3-3/ capicúa/ ETV1/ cancer/ spinocerebellar ataxia type 1 (SCA1)

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#### Abstract

Compounds that inhibit signalling upstream of Erk are promising anti-cancer therapies, motivating research to define how this pathway promotes cancers. Here, we show that human capicúa represses mRNA expression for PEA3 Ets transcription factors ETV1, ETV4 and ETV5, and this repression is relieved by multisite controls of capicúa by Erk, p90RSK and 14-3-3 proteins. Specifically, 14-3-3 binds to p90RSK-phosphorylated Ser173 of capicúa thereby inhibiting DNA binding to its HMG box, while Erk phosphorylations prevent binding of a Cterminal NLS to importin alpha 4 (KPNA3). ETV1, 4 and 5 mRNA levels in melanoma cells are elevated by siRNA knockdown of capicúa, and decreased by inhibiting Erk and/or expressing a form of capicúa that cannot bind to 14-3-3s. Capicúa knockdown also enhances cell migration. Our findings give further mechanistic insights into why ETV1 is highly expressed in certain cancers, indicate that loss of capicúa can desensitise cells to the effects of Erk pathway inhibitors, and highlight interconnections among growth factor signalling, spinocerebellar ataxias and cancers.

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#### Introduction

Capicúa is a transcriptional repressor discovered in several developmental contexts in *Drosophila* [1]. Signalling via specific receptor tyrosine kinase/Ras/Raf/Erk pathways relieves repression by capicúa leading to the transcription of genes that specify differentiation in wing veins, imaginal eye discs, head and tail [1-6], hence the name capicúa meaning head-and-tail in Catalan. Lack of capicúa enables *Drosophila* cells to grow without Ras function, but does not compensate for growth defects due to mutations in insulin/PKB(Akt) signalling [6].

Mammalian capicúa (also known as CIC) is highly expressed during development of the granule layers of the cerebellum [7], and has been linked circumstantially to two disorders of neural crest cell origin, namely spinocerebellar ataxia 1 (SCA1) and Ewing's family tumours [8-10].

SCA1 is a motor disorder caused by a polyglutamine expansion mutation of ataxin-1, with phosphorylation of Ser776 potentiating the disease [11, 12]. Phosphorylated Ser776 of ataxin-1 binds to 14-3-3s, which are dimeric proteins that regulate many cellular processes by docking onto specific phosphorylated serine and threonine residues on their targets [13, 14]. 14-3-3 binding to phosphorylated Ser776 is proposed to regulate the interactions of ataxin-1 with two protein complexes, one including splicing factors and the other containing capicúa [10, 15, 16]. The polyglutamine mutation of ataxin-1 that underlies SCA1 may disturb the balance between the two complexes [10, 15].

The suggestion of a connection between capicúa and cancer comes from two cases of Ewing-like sarcoma that were found to express transforming CIC-DUX4 fusion proteins, comprising most of capicúa and part of the double homeodomain protein DUX4 [8]. True Ewing-family tumours more commonly arise from fusions of the EWS gene with genes encoding Ets transcription factors [17], which raised the question of whether the CIC-DUX4 fusion promotes the expression of Ets transcription factors. Indeed, the HMG box of the CIC binds to a DNA sequence within the promoters of genes encoding the PEA3 subfamily Ets transcription factors (ETV1, ETV4 and ETV5, also known as ER81, PEA3 and ERM respectively), while the attached DUX4 portion enhances the transcription of ETV1 and ETV5 [8]. One inference of these findings is that normal capicúa might repress expression of ETV1, ETV4 and/or ETV5.

The three PEA3 Ets transcriptional activators have roles in development of many tissues and also in cancer progression. For example, ETV1 targets include genes needed to synthesise the neurotransmitter dopamine, as well as genes involved in cell migration and cancer metastases [18]. In addition to Ewing's sarcomas, chromosomal rearrangements involving gene fusions and amplifications that lead to overexpression of ETV1, ETV4, ETV5 have also been identified in breast and prostate cancers, with ETV1 in particular being linked to aggressive prostate tumours and pinpointed as a driver mutation in melanomas [17, 19-23]. Enhanced growth factor/Erk signalling has also been linked to ETV1, ETV4 and ETV5 mRNA expression in developing tissues, cultured melanoma cells, and in gastrointestinal stromal tumours that are positive for the receptor tyrosine kinase KIT, but no underlying mechanisms have been described [24-27]. Phosphorylations of ETV1 protein by the Erk-activated p90RSK and/or MSK1 have however been found to activate this transcription factor [28].

Overall, these findings raise questions about potential regulatory links between Erk signalling, capicúa and expression of the PEA3 Ets transcription factors in human cells. Specifically, does Erk signalling inhibit human capicúa by analogy with the *Drosophila* protein? Does capicúa repress ETV1, ETV4 and/or ETV5 expression? If so, the sum of these two negatives would be a positive effect of Erk signalling on transcription of ETV1, ETV4 and/or ETV5. We therefore began this study by testing the effects of Erk-activating growth factors, Erk pathway inhibitors and capicúa knockdown on the levels of ETV1, ETV4 and ETV5 mRNAs in HEK293 and melanoma cells.

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### Materials and methods Materials

Synthetic peptides were from Dr Graham Bloomberg, University of Bristol, Molecular mass markers from Bio-Rad, MG132 from Calbiochem, ECL kit from GE Healthcare. PD184352, PLX4720, BI-D1870 from DSTT (Division of Signal Tranduction Therapy, University of Dundee, Scotland, UK), SDS/polyacrylamide precast gels and EGF from Invitrogen, and PMA (phorbol 12-myristate 13-acetate) from Sigma.

The pan-14-3-3 antibody (K-19), anti-GFP (sc-8334) and anti-CIC (goat; sc-67528) were from Santa Cruz. Antibodies that recognise phosphorylated Thr202/Tyr204 on Erk1/2 (#9101) and anti-Erk1/2 (#9102) were from Cell Signaling Technology. The anti-KPNA3 is A301-626A from Bethyl Laboratories Inc. and recognises an epitope between residues 1 and 50 of human KPNA3 (NP\_002258.2), which is identical in KPNA4. Anti-CIC (rabbit; ab61860) was from Abcam.

Purified recombinant protein kinases generated in the DSTT (Division of Signal Tranduction Therapy, University of Dundee, Scotland, UK) were His-PKCalpha (protein kinase C alpha; residues 1 to 672 of the human protein) expressed in insect cells (constitutively active); His-PKCzeta (protein kinase C zeta; residues 2 to 592 of the human protein) expressed in insect cells (constitutively active); His-PKCzeta (protein kinase C zeta; residues 2 to 592 of the human protein) expressed in insect cells (constitutively active); His-RSK1 (p90 ribosomal S6 kinase 1; residues 1 to 735 of the rat protein) expressed in insect cells and activated by phosphoErk2 and PDK1; His-RSK2 (p90 ribosomal S6 kinase 1; residues 2 to 740 of the rat protein) expressed in insect cells and activated by phosphoErk2 and PDK1.

#### Plasmids

Full-length human CIC (Q96RK0) was amplified from IMAGE consortium EST 100014433 and cloned into a derivative of pcDNA5 FRT/TO (Invitrogen) that contains the coding sequence for GFP upstream of the multiple cloning site, to express N-terminally tagged GFP-CIC. C-terminal truncations of CIC were generated from this plasmid by inserting a stop codon at the appropriate position by mutagenesis. N-terminal truncations were made by re-amplifying the appropriate fragments of CIC and cloning into the same vector. Point mutations of CIC were introduced using KOD Hot Start DNA polymerase (Novagen). GST-tagged CIC fragments were generated by re-amplifying the appropriate fragment from existing plasmids and cloning into the pGEX6P vector (GE Healthcare).

Full-length human KPNA3 (O00505) was amplified from human brain total RNA (Stratagene) and cloned into a derivative of pcDNA5 FRT/TO (Invitrogen) that contains the coding sequence for the HA tag upstream of the multiple cloning site.

All plasmids were sequence-verified and plasmid transfections of human cells were performed using Lipofectamine 2000 (Invitrogen). DNA sequencing was performed by The Sequencing Service, College of Life Sciences, University of Dundee (www.dnaseq.co.uk). Cell stimulations, lysis and isolation of GFP-tagged proteins

Human HEK (human embryonic kidney)-293 cells cultured on 10-cm-diameter dishes in DMEM medium containing 10% (v/v) serum were used untransfected, or 24 to 36 h after transfection with the indicated plasmids. Cells were serum-starved for a further 4 to 8 h (unstimulated), then stimulated with 100 ng/ml EGF for 15 min and 100 ng/ml PMA for 30 min. Where indicated, cells were incubated PD184352 (2  $\mu$ M for 1 h) and BI-D1870 (10  $\mu$ M for 30 min) prior to stimulations with EGF and PMA. After stimulations, the medium was aspirated and cells lysed in 0.3 ml of ice-cold lysis buffer comprising 25 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM benzamidine, 0.2 mM PMSF, 0.1% (v/v) 2-mercaptoethanol, 1  $\mu$ M microcystin-LR, 0.27 M sucrose and one mini CompleteTM proteinase inhibitor cocktail tablet (Roche) per 10 ml of lysis buffer. Lysates were clarified by centrifugation, snap frozen and stored at -80°C. Protein concentrations were determined with Coomassie Protein Assay Reagent (Thermo Scientific).

To isolate GFP-tagged proteins, 5  $\mu$ l of GFP-Trap-agarose (<u>www.chromotek.com</u>) was mixed with 1 to 3 mg lysate on an orbital shaker at 4 °C for 1 h, and washed by centrifugation (12,500 rpm for 30 sec) and resuspension, twice in 1 ml 25 mM Tris-HCl pH 7.5, 150 mM NaCl and twice in 1 ml TBS-T. Proteins were extracted into 20 to 40  $\mu$ l LDS sample buffer (Invitrogen) containing 1% 2-mercaptoethanol.

### Western blots and 14-3-3 Far-Westerns

Membranes were incubated in 50 mM Tris-HCl pH 7.5, 0.15 M NaCl and 0.2% (v/v) Tween 20 containing 5% (w/v) dried skimmed milk powder (Marvel) and immunoblotted at 4 °C for 16 h (or room temp for 1 h for anti-GFP and anti-HA) using the indicated antibodies at 1  $\mu$ g/ml. Detection was performed using horseradish-peroxidase-conjugated secondary antibodies (Promega) and ECL® (enhanced chemiluminescence reagent; Amersham Biosciences) for Western blots of endogenous proteins, except phospho- and total Erk1/2 for which secondary antibodies were labelled with IRDye and detected by Li-Cor infrared imaging. 14-3-3 Far-Westerns assays are similar to Western blots, but use digoxygenin (DIG)-labelled 14-3-3 in place of primary antibody, and are used to test the ability of phosphorylated proteins on the membranes to bind directly to 14-3-3 [13].

### **In-vitro phosphorylations**

Phosphorylation reactions were performed at 30°C in a total volume of 20  $\mu$ l. The protein kinase reaction buffer contained 50 mM Tris-HCl pH 8.0, 1 mM mM EGTA, 10 mM EDTA, 100 mM MgAc, 1 mM ATP with or without 50 mU/ul of protein kinases where 1 unit is nmoles of phosphate incorporated per min at 30°C into standard substrate peptides. Reactions were stopped with LDS sample buffer (Invitrogen).

### Identification of proteins and phosphorylated residues by mass spectrometric analyses

Protein identification by tryptic mass fingerprinting analysis of selected protein gel bands was performed by LC-MS-MS using a linear ion trap-orbitrap hybrid mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific) equipped with a nanoelectrospray ion source (Thermo) and coupled to a Dionex Ultimate 3000 nano-HPLC system. Peptides were typically injected into a PepMap 100 reverse phase  $C_{18}$  3 µm column (Dionex) with a flow of 300 nl/min and eluted with a 40 min linear gradient of 95% solvent A (2% acetonitrile, 0.1% formic acid in H<sub>2</sub>O) to 50% solvent B (90% acetonitrile, 0.08% formic acid in H<sub>2</sub>O). The instrument was operated with the 'lock mass' option to improve the mass accuracy of precursor ions and data were acquired in the data-dependent mode, automatically switching between MS and MS-MS acquisition. Full scan spectra (m/z 300-1800) were acquired in the orbitrap with resolution R = 60,000 at m/z 400 (after accumulation to a target value of 500,000). The 5 most intense ions, above a specified minimum signal threshold of 50,000, based upon a low resolution (R = 15,000) preview of the survey scan, were fragmented by collision induced dissociation and recorded in the linear ion trap (target value of 10,000).

Phosphopeptides were identified by LC-MS and MS-MS mass spectrometry on an **Biosystems** Applied 4000 **Q**Trap coupled to Dionex/LC Packings a Famos/Switchos/Ultimate HPLC. The MS was set to use a precursor ion scan of m/z -79 in negative ion mode followed by an ion trap high resolution scan (enhanced resolution scan) and a high sensitivity MS-MS scan (enhanced product ion scan) in positive mode. Peptides were typically injected onto a PepMap 100 reverse phase  $C_{18}$  3 µm column with a flow of 300 nl/min and eluted with a 40 min linear gradient of 95% solvent A (2% acetonitrile, 0.1% formic acid in H<sub>2</sub>O) to 50% solvent B (90% acetonitrile, 0.08% formic acid in H<sub>2</sub>O). A Harvard syringe pump was used to deliver isopropanol at a flow of 100 nl/min with mixing occurring at a T-junction after the LC and prior to the MS [29].

Melanoma cell culture and cell migration

The human melanoma cell lines were kindly gifted by Richard Marais (The Institute of Cancer Research, London, UK). SKMEL13 melanoma cells were maintained in RPMI containing 10% FCS, and SBCL2 and PWMK cells in DMEM containing 10% FCS in a humidified incubator with 5%  $CO_2$  at 37°C. The ability of cells to migrate was tested in a growth factor-reduced MATRIGEL<sup>TM</sup> invasion chamber (BD Biosciences).

### Immunofluorescence and microscopy

Cells grown on cover slips were fixed in -20°C methanol, permeabilised with 0.2% Triton X-100, rinsed with PBS, stained with 4,6-diamidino-2-phenylindole (DAPI) and mounted. The slides were taken on a Zeiss LSM700 microscope using an alphaPlan-Apochromat 100x NA 1.46 objective and digital images analysed in Adobe Photoshop.

### **Quantitative RT-PCR analysis**

Melanoma cells (250,000 per well) were seeded in 6-well culture plates and total RNA isolated using the RNeasy minikit (Qiagen) and treated with RNase-Free DNase (Qiagen). RNA (1  $\mu$ g) was converted to cDNA using Quanta BIOSCIENCES qScript<sup>TM</sup> cDNA SuperMix (95048-500) using following primer sequences: CIC (Forward) 5'-GGTACTGGCAAGAAGGTGAAGG-3'; CIC (Reverse) 5'-ACTCAGGCAACTCAGCAAAGC-3'. 5'-ETV1 (Forward) CCCTCCATCGCAGTCCATACC-3' :ETV1 (Reverse) 5'-CTTGGCATCGTCGGCAAAGG-3'. ETV4 (Forward) 5'-5'-CAGGCGGAGGTTGAAGAAAGG-3'; ETV4 (Reverse) AAGGGCAGAAGAAAGGCAAAGG -3'. **ETV5** (Forward) 5'-5'-ETV5 GGGAAATCTCGATCAGAGGACTG-3'; (Reverse) GGAGCATGAAGCACCAAGTT-3'. B2M (Forward) 5'- TGCCGTGTGAACCATGTG -3'; B2M (Reverse) 5'- ACCTCCATGATGCTGCTTACA-3'. GAPDH (Forward) 5'-5'-CAATGACCCCTTCATTGACC-3'; GAPDH (Reverse) GACAAGCTTCCCGTTCTCAG-3'. B-Actin (Forward) 5'-TCTACAATGAGCTGCGTGTG-3'; ß-Actin (Reverse) 5'- TAGATGGGCACAGTGTGGGT-3'. PCR cycling steps for Bio-Rad iCycler iQ<sup>®</sup> of PCR products were 30 s at 95°C for initial denaturation; 30 s at 60°C for annealing; and 30 s at 72°C for extension. All assays were performed with the comparative threshold cycle  $(C_t)$  method, the average of three analyses for each gene was calculated and

# data normalized to an internal standard gene, B2M ( $\beta$ microglobulin). siRNAs

ON-TARGET plus SMARTpool siRNA for human *capicúa* consisting of four individual siRNAs (GCUUAGUGUAUUCGGACAA, CGGCGCAAGAGACCCGAAA, GAGAAGCCGCAAUGAGCGA, CGAGUGAUGAGGAGCGCAU) was from Dharmacon. siRNA oligos were transfected into cells at 40 to 60% confluency using Lipofectamine 2000 (Invitrogen).

### Electrophoretic mobility shift assays (EMSA)

Biotin-labeled and unlabeled forms of the following oligonucleotide and complementary sequence synthesised (Invitrogen): were **Biotin-**Responsive element (CRE) in the ETV4 and ETV5 promoters [8] in bold type). (The CRE in the ETV1 promoter has an A in place of G where underlined [8]). The LightShift® Chemiluminescent EMSA Kit (Pierce, Rockford, IL) was used for the EMSA assays. The binding reactions (30 min at room temp in 20  $\mu$ l) were carried out in the presence of 2.5 ng poly(dl-dC) in 1× binding buffer using 100 pmol biotin-labelled duplex probe, and where indicated 20-fold molar excess of unlabelled probe. Reactions were stopped by adding 5x loading buffer. DNA/protein complexes were separated on 5% Novex DNA Retardation gels (Invitrogen). The complexes were blotted onto positively charged Biodyne nylon membrane (Thermo Scientific), membrane reacted with Spectrolinker XL-1500 UV cross-linker

(Spectronics Corporation, Westbury, NY) and probes visualised according to the LightShift® Chemiluminescent EMSA Kit (Pierce).

#### Results

## ETV1, ETV4 and ETV5 expression is blocked by inhibitors of the Erk cascade and enhanced by EGF stimulation and siRNA knockdown of capicúa in melanoma cells

In a 'wild-type' melanoma cell line PMWK, the level of ETV4 and ETV5 but not ETV1 mRNAs was increased in response to EGF stimulation (Figure 1A), which activates prolonged Erk signaling in these cells (Figure 1B), whereas IGF1 did not affect either Erk phosphorylation or expression of the PEA3 Ets transcription factors (Fig. 1A and data not shown). SKMEL13 melanoma cells carry the BRAF(V600E) mutation rendering the Erk signalling pathway constitutively active. In SKMEL13 cells, the mRNA levels for ETV1, ETV4 and ETV5 were markedly decreased by the Erk pathway inhibitor PD184352 [30] and BRAF(V600E) inhibitor PLX4720 [31], and partially decreased by the p90RSK inhibitor BI-D1870 [32] (Figure 1C). p90RSK is activated downstream of Erk in the melanoma and HEK293 cells used in this study (not shown). Similarly, ETV1, ETV4 and ETV5 expression was inhibited by PD184352 and to a lesser extent by BI-D1870 in Sbcl2 cells, which are mutant for N-Ras (supplementary Figure S1).

Downregulating capicúa expression in SKMEL13 melanoma cells (Figure 2A) increased mRNA levels for ETV1, ETV4 and ETV5 (Figure 2B). Furthermore, the knockdown of capicúa lessened the ability of PD184352 to inhibit the expression of ETV1 and ETV5 mRNAs (Figure 2C). Together, these data indicate that capicúa suppresses and Erk activity supports the expression of ETV1, ETV4 and ETV5 mRNAs. Furthermore, when Sbcl2 melanoma cells were allowed to migrate in a Matrigel invasion chamber after knockdown of capicúa, the knockdown cells displayed a 40 to 50% increase in migration compared with the control cells (Figure 2D and supplementary Figure S2).

## EGF and phorbol ester inhibit binding of KPNA3 (importin alpha 4) and promote binding of 14-3-3 to capicúa

We next explored whether capicúa is a target of regulation by Erk signalling. HEK293 cells expressing GFP-capicúa were stimulated with EGF and phorbol 12-myristate 13-acetate (PMA) to activate Erk, and the GFP-capicúa protein was immunoprecipitated from lysates. A ~60 kDa protein was co-immunoprecipitated with GFP-capicua from unstimulated, but not EGF- and PMA-stimulated cells, and was identified as importin alpha 4/karyopherin alpha 3 (KPNA3) by mass spectrometric analysis of tryptic peptides and Western blotting (Figure 3A and 3B). While inhibiting p90RSK with BI-D1870 had no effect on the capicúa/KPNA3 interaction, inhibiting upstream Erk signalling with PD184352 (Figure 3A and 3B) and UO126 (not shown) restored the binding of capicúa to KPNA3 in EGF-stimulated cells (Figure 3A and 3B) and phorbol ester-stimulated cells (not shown).

GFP-capicúa also interacted with 14-3-3s, visualised as two Coomassie-stained bands of which the upper contained 14-3-3epsilon and the lower comprised 14-3-3theta, gamma, alpha/beta, eta and delta/zeta (Figure 3A). 14-3-3sigma is absent from these cells, and was not detected. In contrast to KPNA3, the capicúa/14-3-3 interaction was promoted by EGF and PMA, and prevented when p90RSK was inhibited directly with BI-D1870 and when upstream Erk signalling was blocked with PD184352 (Figure 3A and 3B) and UO126 (not shown). The binding of capicúa to 14-3-3 was also promoted by IGF1, though to a lesser extent than with EGF, and the response to IGF1 was blocked by PI 3-kinase inhibitors (not shown).

Tryptic digests of the extracted GFP-capicúa from EGF-stimulated cells were analysed by precursor ion scanning and MS/MS, revealing the presence of several phosphopeptides (supplementary Figure S3A). Together with previous phosphorylation site

data [13] (www.phosphosite.org), several phosphorylated residues within Rxx(pS) motifs that are potential p90RSK and 14-3-3-binding sites were identified near the HMG box, such as phosphorylated Ser173 (supplementary Figure S3B). In contrast, the C-terminal region of capicúa is phosphorylated mainly on (pS/T)P motifs, which are potential Erk sites, but not the type of motif generally associated with binding to 14-3-3s (supplementary Figure S3A).

### Erk phosphorylations of capicúa blocks binding of a C-terminal NLS to KPNA3

The importin-alpha proteins bind to nuclear localization sequences (NLS) in cargo proteins that are then carried into the nucleus. A cluster of basic residues (1411 KRKMRR1416) in the C-terminal region of capicúa conformed to a potential monopartite NLS (<u>http://cubic.bioc.columbia.edu/db/NLSdb/</u>). Consistent with KRKMRR binding to KPNA3, GFP-capicúa(1-450) did not bind to KPNA3 (Figure 3B), but GFP-capicúa(451-end) did bind to KPNA3 in lysates of unstimulated cells (not shown). Also, KPNA3 binding was lost when K<u>RK</u>MRR was changed to K<u>AA</u>MRR in full-length GFP-capicúa (Figure 4A), and when the C-terminal region containing this sequence was deleted in GFP-capicúa(1-1320) and GFP-capicúa(1-1400) (Figure 4B). The interaction between capicúa and KPNA3 in cell lysates was also blocked by competition with a synthetic peptide containing the canonical NLS sequence from Simian Virus 40 large T-antigen, but not a mutant version [33] (Figure 4C).

The results in Figures 3A and 3B had indicated that phosphorylation of capicúa and/or KPNA3 by Erk prevents the interaction between these two proteins. To determine which is the relevant Erk target, GFP-capicúa and HA-KPNA3 were expressed separately in HEK293 cells that were stimulated or not with EGF +/-PD184352. When the various cell lysates were mixed and GFP-capicúa extracted, only GFP-capicúa from unstimulated and PD184352-treated cells was bound to HA-KPNA3 (Figure 4D). In contrast, HA-KPNA3 could bind to GFP-capicúa irrespective of whether the HA-KPNA3-expressing cells had been stimulated with EGF (Figure 4D). This means that Erk phosphorylation of capicúa blocks its interaction with KPNA3.

When the eleven (phosphoSer/Thr)Pro sites identified on capicúa were mutated, only Ala mutations of Ser1382 and Ser1409, which are closest to the NLS, prevented the EGF-induced loss of binding of capicúa to KPNA3 (Figure 4E and not shown). These results suggest that Erk phosphorylations of Ser1382 and Ser1409 mask the NLS and prevent its binding to KPNA3.

We hypothesised that disruption of the NLS, and EGF-stimulated dissociation of capicúa from KPNA3, would prevent capicúa from entering the nucleus. On the contrary however, full-length GFP-capicúa was nuclear under all conditions tested even when the NLS was mutated (not shown). However, the C-terminal part of GFP-capicúa (residue 451 to end) required the intact NLS for its nuclear localisation (Figure 4F and supplementary Figure S4). These data indicate that while the NLS/importin interaction has the potential to direct localisation, capicúa also has other mechanisms for getting into the nucleus. Indeed, the N-terminal half of the protein was also nuclear even though it does not bind to KPNA3, indicating that this part of the protein also specifies nuclear targeting (supplementary Figure S4).

## 14-3-3 binds to the evolutionarily-conserved p90RSK-phosphorylated Ser173, adjacent to the HMG box of capicúa

Both full-length protein and truncated GFP-capicúa (residues 1 to 450) bound to 14-3-3s in response to EGF and PMA (Figures 3A, 3B, 5A and data not shown). However, GFP-capicúa(Ser173Ala) showed only a trace of EGF- and PMA-stimulated binding to 14-3-3s (Figure 5A), implicating phosphoSer173 (KRRTQ( $pS_{173}$ )LS) in 14-3-3 binding. Consistent with the cellular analyses, GST-capicúa (1 to 450) that was phosphorylated *in vitro* on Ser173 and other residues by p90RSK could bind to 14-3-3s (Figure 5B), whereas Ser173Ala-substituted protein could not (not shown).

Ser173 is conserved in metazoan forms of capicúa (supplementary Figure S5). When expressed in human cells, *Drosophila* capicúa interacted with 14-3-3 in response to EGF and PMA, and this interaction required Ser461, corresponding to Ser173 in the human protein (Figure 5C). Thus, 14-3-3 binding to p90RSK-phosphorylated *Drosophila* capicúa may help explain how its repressor activity is relieved by Erk signalling in flies [1].

14-3-3s are dimeric and often bind to two phosphorylated sites on their targets [14], and we therefore searched for a second 14-3-3-binding site on human capicúa. Single Ser to Ala substitution of other phosphorylated sites identified between residues 1 and 450 (supplementary Figure S3A) had no obvious effect on 14-3-3 binding. A truncated GFP-capicúa(1-370) with Ser173Ala did not show even trace binding to 14-3-3s however (Figure 5D), and we tentatively conclude that phosphoSer431 (SQRAA(pS<sub>431</sub>)ED) might provide a second low-affinity binding site for a 14-3-3 dimer.

This proposed configuration of a 14-3-3 dimer straddling phosphorylated Ser173 and possibly phosphoSer431 on either side of the DNA-binding HMG box (residues 200 to 268) suggests that the 14-3-3 might directly affect the binding of capicúa to DNA. Consistent with this hypothesis, we found that capicúa binds to double-stranded DNA containing the minimal CIC response element (CRE; 5'-TGAATGAA-3') that was previously identified to bind to capicúa [8], and addition of exogenous 14-3-3 to p90RSK-phosphorylated capicúa decreases the capicúa interaction with DNA (Figure 5E). 14-3-3 had no effect on DNA binding of unphosphorylated capicúa (Figure 5E).

## Expression of ETV1, ETV4 and ETV5 in melanoma cells is inhibited by a form of capicúa that cannot bind to 14-3-3

Endogenous capicúa also binds to 14-3-3 in lysates of melanoma cells containing constitutively active Erk, and this interaction is prevented by PD184352 (Figure 6A), whereas in wild-type melanoma cells the capicúa/14-3-3 interaction depends on EGF (Figure 6B). This finding suggested that the p90RSK- and 14-3-3-mediated regulation of capicúa might contribute to the Erk-dependent expression of ETV1, ETV4 and ETV5 (Figure 1A), and we tested this hypothesis. Overexpressing capicúa(90-450), which includes the DNA-binding HMG box, had no obvious effect on mRNA levels for these Ets transcription factors (Figure 6C). However, their expression, particularly for ETV5, was decreased by expressing capicúa(90-450) with Ser173 substituted by Ala, and more-so by a form of capicúa lacking all five of the phosphorylated sites identified in the N-terminal half of the protein (Figure 6C and 6D). These data are consistent with the hypotheses that capicúa contributes to the repression of transcription ETV1, ETV4 and ETV5, and p90RSK-mediated binding of 14-3-3 to capicúa inhibits its repression of these Ets transcription factors.

#### Discussion

The Erk cascade regulates cellular proliferation, differentiation and survival, and is commonly activated in human cancers where it is associated with tumour aggression and metastases. Here, we provide mechanistic and functional evidence of a regulatory pathway linking Erk signalling via inhibition of the transcriptional repressor capicúa to expression of the PEA3 Ets transcription factors that promote the transcription of pro-metastatic genes. Our findings could therefore explain why mRNA levels of ETV1 in particular are often elevated in breast, prostate, melanoma, gastrointestinal and other tumours, even when ETV1 expression is not driven by fusion to another gene [17, 19, 20, 22-24, 27]. In particular, our data are consistent with the identification of ETV1 and ETV5 in screens for mRNAs whose expression was decreased by BRAF(V600E)/MEK inhibitors in melanoma cells [25, 26]. It is also tempting to speculate that the mechanism that we describe here could contribute to the KIT receptor tyrosine kinase/Erk-driven expression of ETV1 in gastrointestinal stromal tumours (GIST) [27]. Moreover, it was reported that high ETV1 expression could support

growth of GIST cell lines, whether they contain KIT that is sensitive or resistant to imatinib (Gleevec) [27]. Placing capicúa and ETV1/4/5 in a transcriptional axis downstream of Erk therefore raises the pressing question of whether loss or inactivation of capicúa, and hence expression of ETV1/4/5, could desensitise tumours to the therapeutic effects of Erk pathway inhibitors (such as Gleevec) as anti-cancer drugs. Indeed, here we found that capicúa knockdown caused significant desensitization of melanoma cells to the inhibitory effect of PD184352 on ETV1 and ETV5 mRNA expression (Figure 2C). Capicúa knockdown also enhanced cell invasion in a 2-D assay (Figure 2D and supplementary Figure S2), and how ETV1 and/or other downstream targets mediate the effects of capicúa on proliferation and migration is a critical question for future research.

With these data inferring that deregulated capicúa could conceivably contribute to tumour progression, we focused on the underlying mechanistic details, and our findings are summarised in Figure 6E. Capicúa was discovered to be regulated by Erk itself, and also via Erk-activated p90RSK working together with 14-3-3s. The effect of Erk-mediated phosphorylations releasing the importin-alpha protein KPNA3 from the NLS in the Cterminal region of capciúa is clear, though how this regulation impacts on subcellular localization of capicúa is not. Our data indicate that the protein has at least one further nuclear localization mechanism mediated by the N-terminal part of the protein, within or close to the DNA-binding HMG box. We also found that 14-3-3 binds to p90RSKphosphorylated Ser173, and possibly a second phosphorylated residue to the C-terminal side of the DNA-binding HMG box, and initial results indicate that p90RSK phosphorylation and 14-3-3 binding inhibits the binding of capicúa to the minimal CIC response element (CRE; 5'-TGAATGAA-3') [8]. However, the effects of 14-3-3 on capicúa might be more complicated than these initial data suggest. In future, a more thorough study of the interplay between capicúa, 14-3-3 and the promoter regions of each of the PEA3 transcription factors will be required. The evolutionary conservation of p90RSK-mediated binding of 14-3-3s to phosphorylated Ser173 in human and Ser461 in Drosophila capicúas respectively, suggests that this mechanism may contribute to the relief of capicúa action triggered by receptor tyrosine kinase signalling during Drosophila development. However, no obvious matches for the NLS or other phosphorylations in the human protein could be identified in the Drosophila protein.

Our findings suggest that p90RSK and 14-3-3 should at least be considered as potential drug targets for tumours that depend on Erk signaling, provided that the drugs could be sufficiently well targeted to the cancers. As well as capicúa, further proteins bind to 14-3-3 via p90RSK-phosphorylated sites (Silvia Synowsky, Olof Olsson and Carol MacKintosh, unpublished). We also note that 14-3-3eta has been reported to contribute to prostate cancer, based on a study in which 14-3-3eta enhanced androgen- and EGF-induced androgen receptor transcriptional activity [34]. Prototypic antagonists that block binding of phosphorylated proteins to 14-3-3s promote apoptosis in cell-based experiments [35], which is consistent with indications that 14-3-3s may mediate many downstream effects of PI 3-kinase/PKB and Erk/p90RSK signalling.

Our findings also highlight interconnections between spinocerebellar ataxia (SCA) and growth factor signalling networks. PolyGln mutations in ataxin-1 cause SCA1, and ataxin-1 protein interacts with capicúa [10, 15]. In this connection, it was interesting to notice that *Drosophila* capicúa contains polyGln stretches that are absent in the human protein, though whether this is coincidental or has any connection with polyGln disease is obscure. It is also intriguing that capicúa represses the ETV1 specifier of dopaminergic neurons [18], given that defects in these neurons underlie other movement disorders, namely Parkinson's disease and DOPA-responsive dystonia. Interestingly, SCA types 14 and 27 were recently discovered to arise from defects in PKCgamma and the FGF14 growth factor respectively,

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which impact on Erk signalling [36-38]. Thus, there are signs of intertwining of the molecular networks underpinning spinocerebeller ataxias and certain tumours. It would be interesting to dissect how the gene networks impacted by mutations in ataxin-1 overlap with the gene targets of capicúa and ETV1, 4 and 5. Perhaps, faulty growth factor regulation of the ataxin-1/capicúa/ETV1 axis is a common denominator that interconnects spinocerebellar ataxias, dopaminergic disorders and cancers derived from neural crest progenitor cells?

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#### Figure legends

Figure 1 Effect of EGF, PD184352 and capicúa knock-down on expression of ETV1, ETV4 and ETV5 mRNAs in BRAF(V600E) SKMEL13 and/or wild type PMWK melanoma cells

(A) qRT-PCR of ETV1, ETV4 and ETV5 mRNA from PMWK cells stimulated with EGF (100 ng/ml) for the number of hours indicated (black bars), IGF1 (50 ng/ml) for 24 h (black bars), and with no stimulus (grey bars). Gene expression is normalised relative to the internal control gene  $\beta_2$  microglobulin, assigning a value of 1.0 for the zero time point with no stimulus. Data represent means  $\pm$  s.d. (4 different sets of analyses for each data point).

(B) Western blot of whole cell lysate of PMWK cells stimulated with EGF for the times indicated, and immunoblotted with indicated antibodies to monitor the phosphorylation status of Erk.

(C) The effect of the Erk pathway inhibitor PD184352 (2  $\mu$ M, [30]), BRAF(V600E) inhibitor PLX4720 (10  $\mu$ M, [31]) and p90RSK inhibitor BI-D1870 (10  $\mu$ M, [32]) on expression of ETV1, ETV4 and ETV5 in SKMEL13 cells at the times indicated in hours (black bars). Data for cells with no inhibitor are shown in grey bars. Gene expression is normalised to the internal control gene  $\beta$ 2 microglobulin, assigning a value of 1.0 for the zero time point with no inhibitor. Data represent the mean  $\pm$  s.d. (4 different sets of analyses for each data point).

## Figure 2 Effect of capicúa knock-down on expression of ETV1, ETV4 and ETV5 mRNAs in BRAF(V600E) SKMEL13 melanoma cells

(A) The expression of capicúa (CIC) mRNA in SKMEL13 cells treated with CIC siRNAs (siCIC, black bars) compared with mock-transfected controls (grey bars). Data represent mean  $\pm$  s.d. (4 different sets of analyses for each data point).

(B) The expression of ETV1, ETV4 and ETV5 mRNA in the same SKMEL13 cells treated with CIC siRNAs that are shown in (A), with data for siRNA-treated cells in black and mock-transfected controls in grey. Data represent mean  $\pm$  s.d. (4 different sets of analysis for each data point).

(C) The effect of CIC knockdown followed by exposure to 2  $\mu$ M PD184352 on ETV1, ETV4 and ETV5 mRNA expression in SKMEL13 cells. Black bars represent data for cells treated with siCIC and PD184352 for the times indicated, whereas grey bars are for mock transfected cells treated with PD184352. Data represent means ± s.d. (4 different sets of analyses for each data point).

(D) Cells at 30 to 40% confluency in 6-well plates were transfected with CIC siRNAs (siCIC), non-targeting siRNA that does not target any known genes as a control (siNT) and mock transfection. After 24 h, cells were serum-deprived for 2 h, detached with cell dissociation buffer (GIBCO), counted, and added in migration buffer (750  $\mu$ l DMEM containing 1% BSA) to the upper chambers of a growth factor-reduced MATRIGEL<sup>TM</sup> invasion device (BD Biosciences) with chemoattractant (DMEM containing 10% FCS) in the bottom wells. The cells that did not migrate were removed from the upper face of the filters using cotton swabs, and cells that had migrated to the lower face of the filters were fixed with Reastain Quick-Diff kit (Reagena). Images (X10) were captured (examples shown in supplementary figure S2), migrated cells counted in five random fields, and the histogram shows the number of cells that migrated to the lower face of the filters, presented as mean values  $\pm$  s.d. for the two experimental conditions (that is experiments starting with 2.5 x 10<sup>5</sup> and 1 x 10<sup>5</sup> cells, respectively).

### Figure 3 Identification of regulated phosphorylated sites on GFP-capicúa and its regulated interactions with KPNA3 and 14-3-3

(A) GFP-capicúa (CIC) and GFP (control) proteins were isolated from lysates of transfected cells using GFP-Trap® (an immobilised single chain antibody) coupled to agarose. Cells were stimulated with EGF (100 ng/ml for 30 min) and PMA (100 ng/ml for 30 min) with or without preincubation with the p90RSK inhibitor BI-D1870 (10  $\mu$ M for 30 min). Captured proteins were subjected to SDS-PAGE, gels stained with colloidal Coomassie Blue, and proteins and phosphorylated sites identified by mass spectrometry as described in Materials and Methods and Supplementary figure S3. Arrows point to the proteins identified as GFP-capicúa (GFP-CIC), 14-3-3s and KPNA3.

(B) Effects of inhibitors of the Erk pathway and p90RSK on the EGF-regulated binding of KPNA3 and 14-3-3 to GFP-capicúa. HEK293 cells were transfected to express GFP-CIC (full length, WT) and GFP-CIC (1-450), and stimulated or not with EGF +/-PD184352 (2  $\mu$ M inhibitor for 60 min) and +/-BI-D1870 (10  $\mu$ M inhibitor for 30 min). EGF was used at 100 ng/ml for 30 min. Lysates (2.5 mg) were subject to GFP-Trap® immunoprecipitation, followed by immunoblotting with the indicated antibodies. In addition, the ability of the GFP-CIC (1-450) to bind directly to 14-3-3 was tested with a 14-3-3 Far-Western assay.

# Figure 4 Mechanism of regulated binding of an NLS in GFP-capicúa to KPNA3 (importin alpha 4)

(A) HEK293 cells were transfected to express GFP-capicúa (GFP-CIC) and GFP-capicúa with a double R1412A and K1413A substitution in the putative NLS (GFP-CIC (NLS\*)), and stimulated or not with EGF +/-PD184352. Lysates were subjected to GFP-Trap® immunoprecipitation, and immunoprecipitates and whole cell lysates were immunoblotted with the indicated antibodies.

(B) HEK293 cells were transfected to express the indicated GFP-capicúa proteins and stimulated or not with EGF. Cell lysates and GFP-Trap® immunoprecipitates were analysed with the indicated antibodies.

(C) HEK293 cells were transfected to express GFP-capicúa and cell lysates were incubated with indicated concentrations of synthetic wild type (CASPKKKRKV-OH) or mutant (CASPKTKRKV-OH) SV40 large T-antigen NLS peptides for 30 min at 4°C followed by GFP-Trap immunoprecipitation and analysis by immunoblotting.

(D) GFP-capicúa and HA-KPNA3 were expressed separately in HEK293 cells that were stimulated or not with EGF +/-PD184352. Different combinations of the lysates were mixed as indicated, and GFP-capicúa was immunoprecipitated from the mixtures and immunoblotted with the indicated antibodies.

(E) HEK293 cells were transfected to express wild-type and mutated forms of GFP-capicúa as indicated, and stimulated or not with EGF +/-PD184352 followed by GFP-Trap® immunoprecipitation. Immunoprecitated proteins were analysed by Western blotting using anti-GFP and anti-KPNA3 antibody.

(F) DAPI staining and GFP immunofluorescence of HEK293 cells transfected with indicated GFP-capicúa constructs. Scale bar (bottom right), 10 µm.

# Figure 5 Identification of the p90RSK-phosphorylated and 14-3-3-binding residues on human and *Drosophila* capicúa proteins

(A) HEK293 cells were transfected with the indicated GFP-capicúa constructs and stimulated or not with EGF +/-BI-D1870. Lysates were subject to GFP-Trap® immunoprecipitation, and immunoprecipitates and lysates were immunoblotted with the indicated antibodies. The ability of GFP-capicúa proteins to bind to 14-3-3 in vitro was assessed by a 14-3-3 Far Western assay.

(B) Purified recombinant constitutively-active forms of PKCalpha, PKCzeta, p90RSK1, and p90RSK2 were used for *in vitro* phosphorylation of GST-capicúa (CIC) (1-450). The reactions were performed with or without kinase in the presence of Mg-ATP. A 14-3-3 Far-Western assay was used to assess the ability of phosphorylated protein to bind to 14-3-3. The second panel is a composite because the GFP-tagged proteins are different sizes.

(C) Lysates from HEK293 cells transfected to express the indicated *Drosophila* capicúa proteins, and stimulated or not with EGF +/-BI-D1870 and PMA +/-BI-D1870 were subjected to GFP pull down assay followed by immunoblotting with indicated antibodies. Direct binding to 14-3-3 was detected in a 14-3-3 Far Western assay.

(D) HEK293 cells were transfected to express the indicated GFP-capicúa proteins and stimulated or not with EGF +/-BI-D1870 and subjected to GFP-Trap® immunoprecipitation. Immunoprecipitates were immunoblotted with the indicated antibodies. 14-3-3 interaction was detected in 14-3-3 Far Western assay.

(E) EMSA assays were performed using biotin-labelled DNA probe containing CRE (capicúa responsive element). Where indicated, unlabelled DNA was also included. DNA was incubated with GST-capicúa (1-450) (1  $\mu$ M) that was phosphorylated in vitro with p90RSK (right hand panel) or not (left panel), and used in the presence and absence of 5  $\mu$ M 14-3-3 dimer. The upper arrow shows the GST-capicúa:DNA complex. Lower bands indicate that the added 14-3-3 has some sequence-non-specific affinity for DNA.

# Figure 6 Effect of over-expressing capicúa that cannot bind to 14-3-3 on regulation of ETV1, ETV4 and ETV5 mRNA expression in BRAF(V600E) SKMEL13 melanoma cells

(A) Western blot of endogenous capicúa isolated by its binding to 14-3-3-Sepharose in lysates of  $^{V600E}$ BRAF SKMEL13 cells, and also the indicated cells with constitutively-active RAS proteins. Cells were treated with 2  $\mu$ M PD184352 for 60 min where indicated, and the phosphorylation status of Erk was monitored in the lysates.

(B) Effect of EGF (100 ng/ml for 60 min) on binding of endogenous capicúa to 14-3-3-Sepharose in lysates of PMWK melanoma cells.

(C) Effect of expressing the indicated GFP-capicúa constructs in SKMEL13 melanoma cells. ETV1, ETV4 and ETV5 mRNA levels were quantified by qRT-PCR 24 and 48 hours post transfection. All constructs encode added NLS sequences to ensure nuclear targeting of all expressed proteins. Data were normalised to the internal control gene ß2 microglobulin, and data from transfected cells are shown by black bars and untransfected cells by grey bars.

(D) For the experiment shown in (C), Western blots of parallel sets of cell lysates were performed to assess the expression levels of the proteins expressed from the constructs 48 h

post-transfection. The top panel is a composite because the GFP-NLS(90-450) and GFP-NLS are different sizes.

(E) Schematic diagram depicting the regulation of human capicúa. Activated p90RSK phosphorylates capicúa residues either side of HMG box creating docking sites for a 14-3-3 dimer, and the binding of 14-3-3 affects the binding of the HMG box to the promoters of PEA3 family transcription factors. In addition, Erk phosphorylation of Ser1409 and other sites prevents binding of importin alpha 4/KPNA3 to the NLS of ERK1/2 capicúa. However, experimental disruption of the capicúa/KPNA3 interaction only prevents nuclear import of the C-terminal part of capicúa, and the N-terminal part of capicúa can also enter the nucleus by another mechanism. ERK 1/2, extracellular signal-regulated kinase 1/2; p90RSK, p90 ribosomal S6 kinase; HMG, High Mobility Group box; NLS, nuclear localisation signal.

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Figure 1 Dissanayake et al



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Figure 3 Dissanayake et al + EGF Α BI-D1870 + PMA В GFP-CIC (WT) GFP-CIC (1-450) Unstimulated GFP Vector BI-D1870 PD184352 + EGF PD184352 + EGF BI-D1870 + EGF PMA Ū. EGF BI-D1870 + Unstimulat • GFP-CIC 250 150 Unstin EGF Ъ 100 14-3-3 75 60.00 Far Western **KPNA3** 50 uwo IB: anti-KPNA3 GFP Pullde 37 GFP 14-3-3-IB: anti-14-3-3 25 20 15 10 IB: anti-GFP NCC .

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Figure 6 Dissanayake et al V600EBRAF RAS melanoma Α SKMEL13 в WM1361 MM485 Wild type Sbcl2 PMWK + EGF + + PD184352 -- + --. IB: anti-CIC 14-3-3 Sepharose IB: anti-CIC Endogenous CIC protein 14-3-3 Sepharose pulldown Endogenous CIC protein pulldown IB: anti-pERK -Cell lysate IB: anti-pERK -IB: anti-ERK -Cell lysate IB: anti-ERK D С ETV1 exbression 1.5 1.5 GFP-NLS (90-450) GFP-NL S173A 5 mut Relative 0.5 24 h post transfection IB: anti-GFP 48 h post transfection IB: anti-pERK 0.0 CTRL WΤ S173A 5 mut Vector IB: anti-total ERK GFP-NLS (90-450) GFP-NLS exbression 1.0 ETV4 **ERK1/2** Е Û p90RSK Relative 0.5 S1409 (Inportin A 24 h post transfection 48 h post transfection 0.0 S173 CTRL S173A 5 mut Vector WΤ GFP-NLS (90-450) GFP-NLS HMG NLS 1.5 ETV5 Relative expression 1410 14171608 1 200 278 1.0 Modulates DNA binding 0.5 24 h post transfection Phosphorylation prevents nuclear trafficking only of 48 h post transfection 0.0 C-terminal fragment CTRL WT S17A 5 mut Vector GFP-NLS (90-450) GFP-NLS

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