SHORT RESEARCH ARTICLE

Regulation of Cancerous inhibitor of PP2A (CIP2A) by small molecule inhibitor for c-Jun NH_2-Terminal Kinases (JNKs), SP600125, in Human Fibrosarcoma (HT1080) cells [version 1; referees: 1 approved with reservations]

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v1 **First published:** 14 Aug 2013, **2**:174 (doi: 10.12688/f1000research.2-174.v1)
Latest published: 11 Dec 2013, **2**:174 (doi: 10.12688/f1000research.2-174.v2)

Abstract

Background: Protein phosphatase 2A inhibition is one of the pre-requisites for human cell transformation. Previously, we have identified an endogenous inhibitor of PP2A, CIP2A (Cancerous Inhibitor of Protein Phosphatase 2A) in human fibrosarcoma cells (HT1080) using tandem affinity purification. CIP2A over expression has been demonstrated in almost every tumour type studied so far. However, our understanding on the mechanisms regulating CIP2A expression in human cancers, especially in sarcomas, is still emerging.
Methods: Human fibrosarcoma (HT1080) cells were treated with small molecule inhibitors against the three major signalling pathways, namely p38, MEK and JNK pathways to identify the pathway regulating CIP2A expression in the sarcoma cells. This was followed by verification of the results using small interfering RNAs (siRNA) for the kinases.
Results: In line with previous observations, small molecule inhibitor for MEK pathway (PD98059) decreased CIP2A mRNA and protein expression. Interestingly, small molecule inhibitor for the JNK pathway, SP600125 decreased mRNA and protein levels of CIP2A oncoprotein with negligible effect of SB203580 (p38 kinase) inhibitor on CIP2A expression in HT1080 cells. However, siRNAs specific to either JNK1 or JNK2 kinases did not result in decrease in CIP2A expression. Contrarily, two different CIP2A siRNAs, which were used as positive controls, decreased JNK2 expression in HT1080 cells.
Conclusion: Although it is well established that SP600125 inhibits JNK kinases, it has also been shown to inhibit a spectra of other kinases. SP600125 inhibits CIP2A protein expression both in time and concentration dependent manner. However, depletion of both JNK1 and JNK2 kinases using specific siRNAs fails to decrease CIP2A protein expression levels, thereby indicating the need to verify the results obtained by treatment with small molecular inhibitors of kinases by independent approaches like two different target specific siRNAs. Finally, fortuitously we identify JNK2 as a CIP2A downstream target in HT1080 cells.

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Referee Status: ? ?

	Invited Referees 1	2
REVISED version 2 published 11 Dec 2013	? report	? report
version 1 published 14 Aug 2013	? report	

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How to cite this article: Khanna A. **Regulation of Cancerous inhibitor of PP2A (CIP2A) by small molecule inhibitor for c-Jun NH $_2$ -Terminal Kinases (JNKs), SP600125, in Human Fibrosarcoma (HT1080) cells [version 1; referees: 1 approved with reservations]** *F1000Research* 2013, **2**:174 (doi: 10.12688/f1000research.2-174.v1)

Grant information: The work was funded by Academy of Finland through the Tampere Graduate Program in Biomedicine and Biotechnology.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: No competing interests were disclosed.

First published: 14 Aug 2013, **2**:174 (doi: 10.12688/f1000research.2-174.v1)

Introduction

It has been recently established that regardless of phenotypic variability between different cancer types, perturbation of a limited number of genetic elements is sufficient to induce transformation in different human cell types[1]. Experimentally, it was demonstrated that activation of RAS and telomerase (TERT), along with inactivation of the tumour suppressor proteins P53 and Retinoblastoma protein (RB) can immortalize a variety of human cell types, which can subsequently transform to a tumourigenic state in response to inhibition of protein phosphatase 2A (PP2A)[1,2]. Various independent studies have shown that inhibition of PP2A activity is a pre-requisite for human cell transformation[1,3–5]. Therefore, understanding the mechanisms by which PP2A is inhibited in cancer cells is vital for developing new anti-cancer therapies.

Cancerous Inhibitor of Protein Phosphatase 2A (CIP2A) is a recently identified oncogene, which has been demonstrated to inhibit the endogenous tumour suppressive activity of PP2A in cancer cells[6]. Several layers of evidence, both from us and others, have shown CIP2A to be required for malignant cell growth and *in vivo* tumour formation[7–12]. In addition, the prognostic role of CIP2A has been demonstrated in several human tumours[8,11–13]. Moreover, since CIP2A overexpression has been observed at a high frequency in most human cancers studied so far[6–9,13–20], identification of mechanisms regulating its expression in human cancers becomes important to address.

Although several transcription factors like MYC[8], ETS1[17], E2F1[9] and ATF2[19] have been identified as positive regulators of CIP2A in various carcinomas, factors influencing CIP2A expression in non-hematopoietic mesenchymal cells or sarcomas, are yet to be discovered. Notably, CIP2A amplification has been observed in soft-tissue sarcomas[21]. In addition, since CIP2A was identified using HT1080 (human fibrosarcoma cell line) cell extracts[6] this cell line was selected to dissect the mechanisms for high CIP2A expression in sarcomas. Since p38, ERK and JNK signalling pathways are commonly perturbed in cancers, we assessed the role of these pathways in CIP2A expression in HT1080 cells. To this end, respective small molecule kinase inhibitors, namely SB203580 (p38 pathway inhibitor), PD98059 (MEK pathway inhibitor) and SP600125 (JNK pathway inhibitor) were used to inhibit signalling through these pathways in HT1080 cells.

Material and methods

Chemicals

SP600125 was purchased from Calbiochem (Cat No. - 420119, Merck-Millipore CAS 129-56-6, San Diego, CA) and stocked as a 20 mM solution in DMSO. PD98059 was purchased from Calbiochem (Cat No. - 513000, Merck-Millipore, San Diego, CA) and stocked as 40 mM stock in DMSO. SB203580 was purchased from Calbiochem (Cat No. - 559389, Merck-Millipore, San Diego, CA) and stocked as 20 mM.

RNAi

The siRNAs to inhibit CIP2A expression were obtained from Eurofins MWG operon (Ebersberg, Germany). Either of the following double-stranded oligonucleotides was transiently transfected into HT1080 cell line as CIP2A siRNAs: CIP2A.1, 5´-CUGUGGUUGUGUUUGCACUTT-3´, and CIP2A.2, 5´-ACCAUUGAUAUCCUUAGAATT-3´. As a control, a scrambled siRNA with the sequence 5´-UAACAAUGAGAGCACGGCTT-3´ was used instead. HP-validated siRNAs for human JNK1 and JNK2 were purchased from Qiagen Sciences (Germantown, MD). Either of the following oligonucleotides were transiently transfected into HT1080 at 30%–50% confluency in a six-well plate were transfected with the siRNA in antibiotic free medium using RNAiMAX Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

Immunoblotting

Proteins were extracted in hot Laemmli sample buffer and subjected to immunoblot analysis. Thirty micrograms of total protein extracts was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Helsinki, Finland) and transferred to nitrocellulose membranes (Thermo Scientific Pierce Protein Biology Products, Rockford, USA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS; 20 mM Trizma Base and 150 mM NaCl dissolved in distilled water and adjusted with HCl to pH 7.5) containing 0.1%-NP40 (Igepal Ca-630; Sigma-Aldrich)[8]. Nitrocellulose membranes (Thermo Scientific Pierce Protein Biology Products, Rockford, USA) were incubated with antibodies to JNK1 (Cat. No. sc-1648: 1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), JNK2 (Cat. No. sc-827: 1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) in 5% milk in TBS-NP40 (Igepal Ca-630; Sigma-Aldrich) at 4°C overnight, with a 1:5000 dilution of the rabbit polyclonal anti-CIP2A antibody[8] at 4°C overnight, or with a 1:1000 dilution of goat polyclonal anti-β-Actin antibody (Cat. No. sc-47778, Santa Cruz Biotechnology) at room temperature for 1 hour. The proteins were visualized by enhanced chemiluminescence (ECL) with Proteome Grasp ECL Kit (Thermo Scientific Pierce Protein Biology Products, Rockford, USA).

Cell culture

HT1080 cells originally were obtained from ATCC and were cultured in DMEM (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Bio-Whittaker Europe, Verviers, Belgium).

mRNA analysis

Total mRNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA) and converted to cDNA by using the M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant cDNA synthesis kit (Promega Corporation, Madison, WI). cDNAs were subjected to quantitative real-time polymerase chain reaction (PCR) by using the Light Cycler (Roche Diagnostics, Mannheim, Germany) and SYBR Green PCR Master Mix kit (Roche Diagnostics). Primer sequences (Sigma-Proligo, St Louis, MO) used for PCR of CIP2A were as follows: CIP2A forward, 5´-CTGGTGAGATAATCAGCAATTT-3´ and CIP2A reverse, 5´-CGAAACATTCATCAGACTTTTCA-3´. Transcript levels were normalized to levels of TATA-binding protein (TBP) or β-Actin expression, which were determined by PCR of the same samples using the following primers: TBP forward, 5´-GAATATAATCCCAAGCGGTTTG-3´, and TBP reverse, 5´-ACTTCACATCACAGCTCCCC-3´; Actin forward, 5´-CGAGCACAGAGCCTCGCCTTTGC-3´, and Actin reverse: 5´-CATAGGAATCCTTC TGACCCATG-3´.

Bioinformatics

Cancer cell line encyclopaedia (http://www.broadinstitute.org/software/cprg/?q=node/11) was used to get the expression levels of JNK1 and JNK2 in HT1080 cells[22]. This is a resource which provides analysis and visualization of DNA copy number, mRNA expression, mutation data and more, for 1000 cancer cell lines.

Statistics

Student T-test was used obtain the statistical significance value using Graph Pad software.

Results

SP600125 inhibits CIP2A mRNA and protein expression in a time and concentration dependent manner in HT1080 cells

To determine the oncogenic signalling pathways that may be involved in regulating CIP2A expression in human fibrosarcoma, HT1080 cells were treated with small molecule inhibitors for the p38 (SB203580; 20 μM), JNK (SP600125; 10 μM) and ERK (PD98058; 20 μM) signalling pathways. As previously observed in gastric cancer cells[17], PD98058 reduced CIP2A mRNA and protein expression in HT1080 cells (Figure 1A and B). Importantly, while SB203580 showed negligible effect, SP600125 potently inhibited CIP2A mRNA and protein expression in HT1080 (Figure 1A and B) cells. Furthermore, inhibition of CIP2A protein expression by SP600125 was observed to be both time and concentration dependent (Figure 1C and D).

Neither JNK1 nor JNK2 regulate CIP2A protein expression in HT1080

SP600125-mediated inhibition of CIP2A expression suggested the involvement of the c-Jun N-Terminal Kinases (JNKs) in regulation of CIP2A expression in HT1080 cells. Since the JNK3 isoform is well known to be neural-specific[23], specific validated siRNAs against JNK1 and JNK2 isoforms were transfected into HT1080 cells and CIP2A expression estimated. Although, both JNK1 and JNK2 siRNAs reduced their target proteins (JNK1 and JNK2 respectively), there was no change in CIP2A protein expression with either of them individually (Figure 2A and B) or in combination

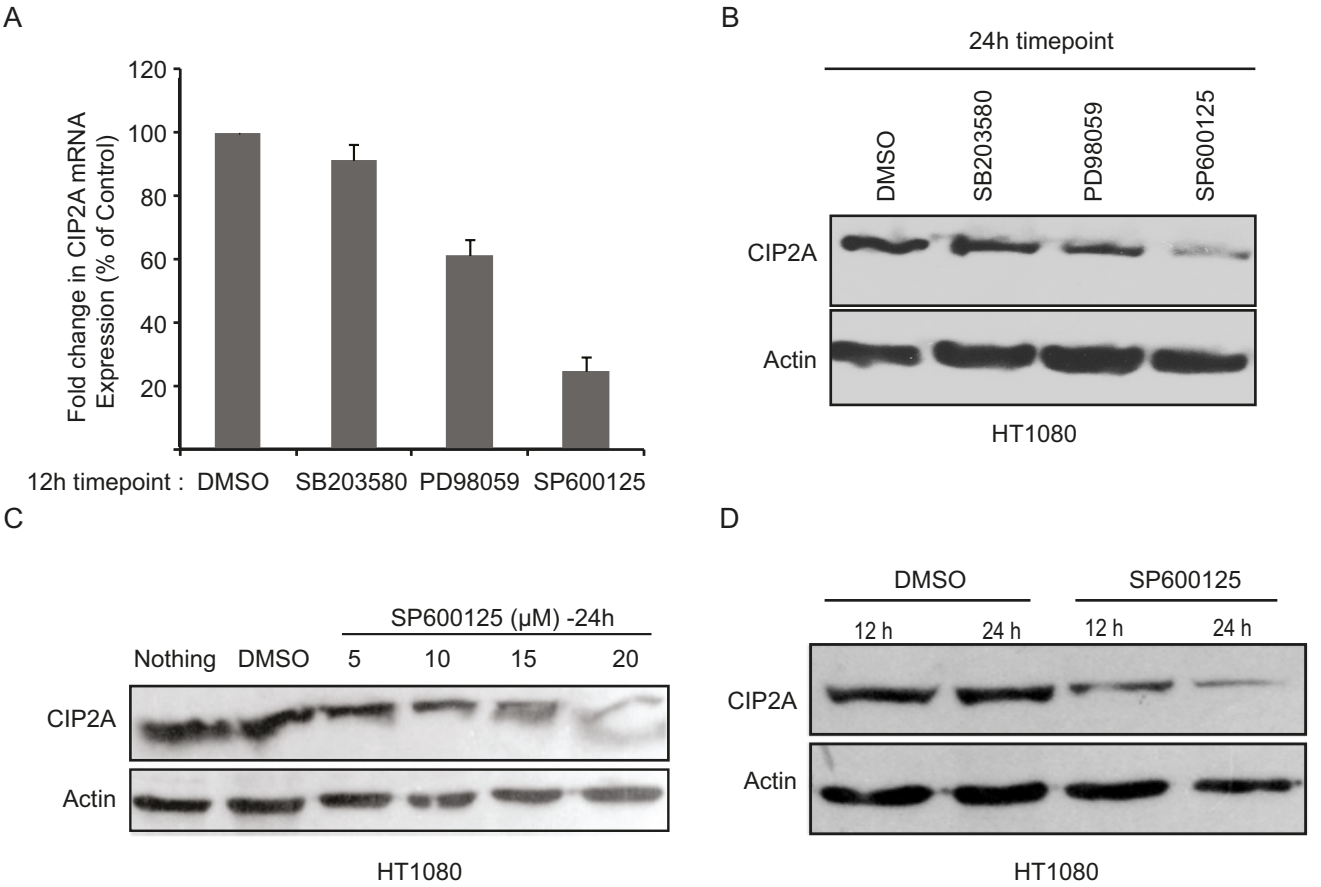

Figure 1. SP600125 positively regulates CIP2A expression in HT1080 cells. A. qRT-PCR showing the effect of small molecule inhibitors against the p38 (SB203580), MEK-ERK (PD98059) and JNK (SP600125) pathways on CIP2A mRNA expression (12h timepoint; Shown is mean values ± S.D., of representative results from three independent experiments. Student T-test was used obtain the statistical significance value) **B**. Western blot showing the effect of small molecule inhibitors against the p38 (SB203580), MEK-ERK (PD98059) and JNK (SP600125) pathways on CIP2A protein expression (24h timepoint). Shown is the representative picture of two independent experiments. **C** and **D**. Effect of small molecule inhibitors against JNK (SP600125) pathway on CIP2A protein expression in concentration (**C**) and time (**D**) dependent manner. Shown is the representative picture of two independent experiments.

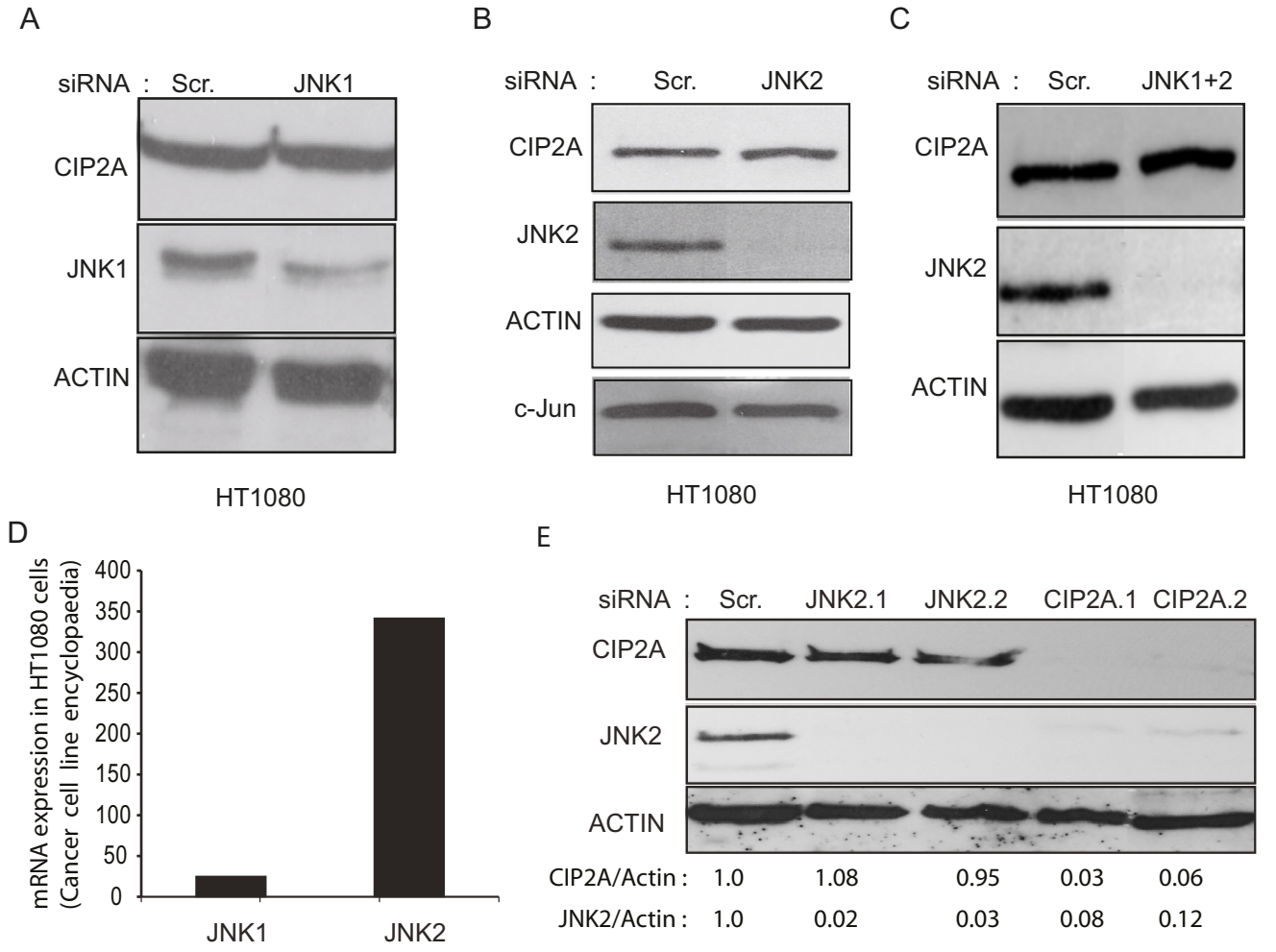

Figure 2. JNK1 and JNK2 do not regulate CIP2A expression in HT1080 cells. A, **B**, and **C**. Western blots showing the effect of JNK1 (**A**), JNK2 (**B**) and combination of both JNK1 and JNK2 (**C**) siRNAs on CIP2A protein expression, 72h post-transfection. Shown is a representative result from two independent experiments. **D**. mRNA expression of JNK1 and JNK2 in HT1080 cell line from the cancer cell encyclopaedia study. **E**. Western blot showing the effect of two different siRNAs specific for JNK2 and CIP2A proteins and their protein expression levels 72h post-transfection. The numbers below the blot are the quantified values for CIP2A and JNK2 protein levels normalized to Actin protein levels, relative to the levels in Scrambled (control) transfected cells. Shown is a representative result of two independent experiments.

(Figure 2C). Since the JNK2 isoform is expressed more than 10-fold higher than the JNK1 isoform in the HT1080[22] (Figure 2D) cell line we transfected two validated and specific siRNAs against JNK2. In addition, we also transfected the HT1080 cells with two different CIP2A siRNA as positive controls. In line with our previous observation (Figure 2B and C), the two different siRNAs for JNK2 knocked out JNK2 expression (Figure 2E), while CIP2A expression remained unaltered. Surprisingly, two different CIP2A siRNA, which worked well as positive controls, efficiently decreased JNK2 expression (Figure 2E).

Discussion

Even though small molecule inhibitors are an emerging therapeutic option against cancers, the specificity issues limit their potential to be used in clinics. They have been extensively used to study various cell signalling pathways. In particular SP600125 has been used to study the effect of c-Jun N-Terminal Kinases (JNKs) in various processes[24–26]. Our results suggest that even though we were able to see decrease in CIP2A expression in HT1080 cells on treatment with SP600125 (Figure 1), at doses used previously to inhibit c-Jun NH_2-terminal kinase (JNKs) activity[27,28], we were not able to validate the findings using two different siRNAs specific to the JNK2 kinase (Figure 2E). Interestingly, a decade ago a previous study emphatically demonstrated the effect of SP600125 on different kinases[29]. The study revealed that SP600125, although a JNK inhibitor, could inhibit the activity of several other kinases[29]. In fact, SP600125 was demonstrated to inhibit kinases like CK1, DYRK1 and AMPK even more effectively than JNK kinase itself[29] (Supplementary Table 1).

Surprisingly, two different CIP2A siRNAs used as positive controls, decreased JNK2 expression levels in HT1080. This has also been observed in a separate study in an epithelial origin cell line, HeLa[20].

Interestingly, JNK2 has been shown to regulate CIP2A expression via ATF2 transcription factor in mouse embryo fibroblasts (MEFs)[19] in the pre-transformed stage. Since we observe the vice versa in fully transformed HT1080 cells, it can suggest that there may be a molecular switch between JNK2 and CIP2A which may have a possible role in the RAS-transformation of mesenchymal cells. Nevertheless, the functional consequence of CIP2A-mediated JNK2 expression in mesenchymal cells would require further exploration.

Altogether, our study highlights the need for the validation of results obtained by small molecule treatments with independent approaches like two or more target specific siRNAs, shRNAs or use of inducible systems like RNAi or Tamoxifen/Tetracycline-induced overexpression systems[8].

Competing interests
No competing interests to declare.

Grant information
The work was funded by Academy of Finland through the Tampere Graduate Program in Biomedicine and Biotechnology.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements
Professor Jukka Westermarck is acknowledged for his guidance.

Supplementary table

Supplementary Table 1.

	List of SP600125-sensitive kinases (with their kinase activity as a percentage of control incubations at 10 μM of SP600125 in parenthesis)
1.	CK1 (10 ± 1)
2.	DYRK1 (16 ± 6)
3.	CDK2 (20 ± 1)
4.	SGK (22 ± 7)
5.	AMPK (26 ± 1)
6.	PHK (34 ± 1)
7.	JNK (38 ± 5)
8.	CHEK1 (39 ± 0)
9.	PRAK (39 ± 1)
10.	PKCα (79 ± 8)

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Current Referee Status: ?

Version 1

Referee Report 20 August 2013

doi:10.5256/f1000research.2042.r1558

? **Kristopher Clark**
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Inhibition of the protein phosphatase PP2A contributes to the acquisition of a cancerous phenotype in cells. One mechanism by which the inhibition of PP2A can be achieved is through the up-regulation of endogenous inhibitors of PP2A such as CIP2A. However, the signaling pathways regulating CIP2A expression in cancer cells remain poorly defined. In this article, Anchit Khanna has studied the role of the different mitogen-activated protein (MAP) kinase cascades in regulating the expression of CIP2A in the HT1080 human fibrosarcoma cell line. Treatment of HT1080 cells with the pharmacological inhibitor SP600125 led to a drop in the mRNA and protein expression of CIP2A in HT1080 cells. SP600125 has often been used to implicate the protein kinases of the JNK family in the cellular process under study, but as pointed out by the author, this compound has many off-target effects. Anchit Khanna therefore attempted to validate the results using siRNA mediated knock-down of JNK1 and JNK2 expression in HT1080 cells. Unfortunately, CIP2A expression was normal in the cells in which JNK1 and/or JNK2 were depleted using siRNA oligos. The author concluded that the effects of SP600125 on CIP2A expression were not due to JNK1 or JNK2 inhibition, and stated the importance of validating experimental results using independent methods.

The data as presented does not confirm or refute a role for the JNKs in regulating CIP2A expression in HT1080 cells, as alternative explanations for the data remain possible. For instance, the knock-down of JNK1 or JNK2 may not be sufficiently effective, leaving residual JNK1/2 catalytic activity in cells which drives the expression of CIP2A. Indeed, the data presented in figure 2A show that JNK1 expression was suppressed by only 50% and the author failed to show the level of JNK1 expression in the experiment depicted in figure 2C. In addition to blotting for the respective kinase, it would have also been informative in both the inhibitor and RNAi studies to measure the phosphorylation of a substrate such as c-Jun to verify that these treatments indeed blocked JNK function in HT1080 cells.

It is also good practice to use structurally-unrelated protein kinase inhibitors to reduce the chance of off-target effects contributing to the phenotype under study. Since SP600125 entered the market, much improved JNK inhibitors have been developed such as JNK-in-8 (Zhang *et al*. 2012 Chem Biol). It would be worthwhile to test the effect of such compounds on CIP2A expression, which could help establish whether a role for JNKs exist in this pathway.

The author is correct in stating that one must validate results using different approaches. I was therefore very surprised to read that the author concluded that CIP2A is involved in regulating the expression of JNK2 in HT1080 using a single approach. RNAi technology, like other methodologies, is fraught with off-target problems. It is essential to validate the results of these experiments by re-expressing the protein

using an RNAi-resistant cDNA of CIP2A. This control experiment would strongly support the author's conclusion that CIP2A controls JNK2 expression in HT1080 cells. I acknowledge that these experiments are challenging and the author may be able to provide supporting evidence using alternative approaches to the one used in this article.

Although the experiments are well performed, the study is incomplete and does not allow one to make a strong case for or against involvement of the JNKs in regulating CIP2A expression. Based on this assessment, I have scored this paper 'Approved with Reservations'.

Additional Minor Points:

1. The author omitted to indicate the statistical significance of the data in Figure 1A.
2. In a single author paper, one should not use the terms 'we' and 'us' rather 'I' and 'me.'

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 11 Dec 2013

Anchit Khanna, University of New South Wales, Australia

- *"The data as presented does not confirm or refute a role for the JNKs in regulating CIP2A expression in HT1080 cells, as alternative explanations for the data remain possible. For instance, the knock-down of JNK1 or JNK2 may not be sufficiently effective, leaving residual JNK1/2 catalytic activity in cells which drives the expression of CIP2A."*

  I thank Dr. Clark for his prompt and valuable comments on the manuscript. However, I would like to highlight Figures 2B, C and E wherein almost 100% knockdown of JNK2 kinase was achieved. In fact, two different siRNAs for JNK2 isoform, which is the isoform that is majorly expressed in HT1080 (Fig. 2D) cells, completely abolished JNK2 expression leaving little room for the existence of residual JNK2 activity in these cells for maintaining CIP2A levels. Additionally, 10 fold higher expression of JNK2 isoform in comparison to the JNK1 isoform in Ht1080 cells (Fig 2D), suggests it to be more relevant functionally.

- "*...the data presented in figure 2A show that JNK1 expression was suppressed by only 50% and the author failed to show the level of JNK1 expression in the experiment depicted in figure 2C.*"

  I thank Dr. Clark for this relevant observation. I have now attached the JNK1 levels from the experiment shown in Fig 2C as supplementary figure 1A with quantifications. As shown in the figure, despite complete knockdown of JNK1 in these cells, CIP2A levels remain almost unaltered, thereby re-emphasizing the fact that JNK1 does not regulate CIP2A expression in HT1080 cells. Complete knockdown of JNK1 also rules out the possibility of residual JNK1 activity driving CIP2A expression and the use of c-Jun as marker of JNK function in this experiment. With reference to SP600125, the concentrations used in Figure 1 have been demonstrated previously in several studies to inhibit JNK activity (p-Jun levels). Few examples of the studies that have used similar concentrations of SP600125 to inhibit JNK

activity are Yue *et al.,* 2011 (used 20uM of SP600125), Duan *et al.,* 2011 (5uM of SP600125 in HT1080 cells), and Kong *et al*., 2013 (10uM of SP600125).

- *"It is also good practice to use structurally-unrelated protein kinase inhibitors to reduce the chance of off-target effects contributing to the phenotype under study*"

  I thank Dr. Clark for sharing his expertise in the field and totally agree that structurally–unrelated kinase inhibitors have decreased off target effects and their use would definitely further confirm the negative findings. However, the main aim of this study is to highlight the un-specificity of SP600125 compound which has been used extensively as specific JNK inhibitor. In addition, using two different siRNAs (to overcome off-target effects due to one siRNA) that knock down JNK2 expression almost completely (Fig 2E) without changing the levels of CIP2A significantly strongly suggest that JNK2 does not regulate CIP2A expression in HT1080 cells.

- "*The author is correct in stating that one must validate results using different approaches. I was therefore very surprised to read that the author concluded that CIP2A is involved in regulating the expression of JNK2 in HT1080 using a single approach. RNAi technology, like other methodologies, is fraught with off-target problems. It is essential to validate the results of these experiments by re-expressing the protein using an RNAi-resistant cDNA of CIP2A. This control experiment would strongly support the author's conclusion that CIP2A controls JNK2 expression in HT1080 cells. I acknowledge that these experiments are challenging and the author may be able to provide supporting evidence using alternative approaches to the one used in this article."*

  I thank Dr. Clark for highlighting the issue of siRNA specificity and totally agree that there are off-target effects that have to be ruled out with this approach. It is for this reason most journals and the general scientific community requests the use of two independent siRNAs for verifying the effects due to depletion of the target gene. Accordingly, as shown in Fig 2E, there have been two independent siRNAs against both JNK2 and CIP2A used which not only almost completely deplete their target proteins but also rule out the element of un-specificity which usually arises by the use of a single siRNA against the target protein. Additionally, an independent study (Niemelä *et al.,* 2012) using microarray approach identified JNK2 as downstream target of CIP2A in HeLa cells, thereby lending further confidence to these findings. Moreover, as mentioned in the manuscript this finding surfaced just by chance as CIP2A siRNAs were being used as positive controls in the experiment. Additionally, I agree and have now further highlighted in the manuscript as well that CIP2A–mediated regulation of JNK2 would need further exploration. Finally, I would like to emphasize that the main aim of this study is to highlight that SP600125, concentrations at which it has been demonstrated to inhibit JNK activity, may also inhibit other kinases as highlighted by an elegant study previously (kinases that it inhibits have been listed in supplementary table 1). Interestingly, we recently identified CHK1 kinase (which is almost as sensitive as JNK to SP600125) as a positive regulator of CIP2A expression (Khanna *et al*., 2013). Therefore, to explore the possible role of CHK1 and other SP600125 sensitive kinases in regulating CIP2A expression, we did a pilot screen and transfected HT1080 cells with 2 different siRNAs against CHK1 (Kinase almost as sensitive to SP600125 as JNK according to previously published study) and with siRNAs against CDK2 and DYRK1 (two other kinases that are more sensitive to SP600125 than JNK2). As shown in supplementary figure 1B, two different siRNAs against CHK1 decreased CIP2A expression

as observed previously, while CDK2 and DYRK1 didn't change CIP2A expression. This suggests that most likely the SP600125 kinase regulating CIP2A expression in HT1080 is CHK1, as observed in other cancers cells like prostate, breast, gastric and cervical cancers (Khanna *et al*., 2013).

- *"Although the experiments are well performed, the study is incomplete and does not allow one to make a strong case for or against involvement of the JNKs in regulating CIP2A expression."*

  I thank Dr. Clark for his positive and encouraging comments and hope that the my response, new data and changes in the text mitigate his previous concerns and are enough to conclude that there are un-specificity issues that may exist with SP600125, the small molecule inhibitor of JNK2 kinase as highlighted by the study.

Additional Minor Points:

1. The revised figure with the p-values using student t-test is now attached and is in accordance to the figure legend.

2. I thank Dr. Clark for bringing this to my notice and have revised this in the modified manuscript.

Competing Interests: No competing interests were disclosed.