Check for updates

SHORT RESEARCH ARTICLE

REVISED Regulation of Cancerous inhibitor of PP2A (CIP2A) by small molecule inhibitor for c-Jun NH₂-Terminal Kinases (JNKs), SP600125, in Human Fibrosarcoma (HT1080) cells [version 2; peer review: 2 approved with reservations]

Anchit Khanna¹⁻³

¹Adult Cancer Program, Lowy Cancer Research Centre and Prince of Wales Hospital, UNSW Medicine, University of New South Wales, Sydney, Australia

²Institute of Biomedical Technology and BioMediTech, University of Tampere and Tampere University Hospital, Tampere, Finland

³Tampere Graduate Program in Biomedicine and Biotechnology (TGPBB), University of Tampere, Tampere, Finland

v2 First published: 14 Aug 2013, 2:174
<https://doi.org/10.12688/f1000research.2-174.v1>
 Latest published: 11 Dec 2013, 2:174
<https://doi.org/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

Open Peer Review**Approval Status** ? ?

	1	2
version 2 (revision) 11 Dec 2013	 view	 view
version 1 14 Aug 2013	 view	

1. **Kristopher Clark**, University of Dundee, Dundee, UK

2. **Tony Hunter**, Salk Institute for Biological Studies, La Jolla, CA, USA

Any reports and responses or comments on the article can be found at the end of the article.

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.

Keywords

Fibrosarcoma, HT1080, SP600125, CIP2A, JNK2, siRNA, PP2A

Corresponding author: Anchit Khanna (a.khanna@unsw.edu.au)

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.

Copyright: © 2013 Khanna A. This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

How to cite this article: Khanna A. **Regulation of Cancerous inhibitor of PP2A (CIP2A) by small molecule inhibitor for c-Jun NH₂-Terminal Kinases (JNKs), SP600125, in Human Fibrosarcoma (HT1080) cells [version 2; peer review: 2 approved with reservations]** F1000Research 2013, 2:174 <https://doi.org/10.12688/f1000research.2-174.v2>

First published: 14 Aug 2013, 2:174 <https://doi.org/10.12688/f1000research.2-174.v1>

REVISED Amendments from Version 1

The manuscript has now been revised based on the comments received from the respectable reviewer. New data showing additional controls, along with the specific explanation to clarify the siRNA based specificity issues, has been now added. Notably, an additional small siRNA screen involving kinases previously demonstrated to be sensitive to SP600125 has been carried out, and resulted in identification of CHK1 as the kinase positively regulating CIP2A expression in HT1080 cells. This is in accordance to recent observations seen in gastric, prostate, breast and cervical cancer cells (Khanna *et al.*, Cancer Research 2013). Additionally, the requested p-values and text have been modified to accommodate and explain the new findings.

See referee reports**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 types¹. 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 prerequisite 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 cells⁶. Several layers of evidence, both from us and others, have shown CIP2A to be required for malignant cell growth and *in vivo* tumour formation⁶. In addition, the prognostic role of CIP2A has been demonstrated in several human tumours⁶. Moreover, since CIP2A overexpression has been observed at a high frequency in most human cancers studied so far^{6,7}, identification of mechanisms regulating its expression in human cancers becomes important to address.

Although several transcription factors like MYC⁸, ETS1⁹, E2F1¹⁰ and ATF2¹¹ 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 sarcomas¹². In addition, since CIP2A was identified using HT1080 (human fibrosarcoma cell line) cell extracts¹³ 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'-CUGUGUUGUGUUUGCACUTT-3', and CIP2A.2, 5'-ACCAUUGAU-AUCCUAGAATT-3'. As a control, a scrambled siRNA with the sequence 5'-UAACAAUGAGACACGGCTT-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% nonfat 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 antibody⁸ 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.

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

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 (12 h timepoint; Shown is mean values + S.D., or representative results from three independent experiments. Student T-test was used to 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 (24 h 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.

(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'-CGAG-CACAGAGCCTCGCCTTTGC-3', and Actin reverse: 5'-CATAG-GAATCCTTCTGACCCATG-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 cells¹⁴. 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 to 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 maybe involved in regulating CIP2A expression in human fibrosarcoma, HT1080 cells were treated with small molecule inhibitors for the p38 (SB203580; 20 μ M), JNK (SP600125; 10 μ M) and ERK (PD98059; 20 μ M) signalling pathways. As previously observed in gastric cancer cells⁹, PD98059 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.

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, 72 h 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 72 h 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.

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-specific¹⁵, 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,B and Supplementary Figure 1A) or in combination (Figure 2C). Since the JNK2 isoform

is expressed more than 10-fold higher than the JNK1 isoform in the HT1080¹⁴ (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). SP600125 has been demonstrated to inhibit various kinases like CDK2, DYRK1 and AMPK even more effectively than JNK kinase itself¹⁶ (Supplementary Table 1). Interestingly, recently CHK1 kinase (also inhibited by SP600125; Supplementary Table 1) was shown to inhibit CIP2A levels both *in vitro* and *in vivo*^{6,17,18}. Therefore, we transfected HT1080 cells with 2 different siRNAs against CHK1 (Kinase almost as sensitive to SP600125 as JNK) and with siRNAs

against CDK2 and DYRK1 (two other kinases that are more sensitive to SP600125 than JNK). As shown in [Supplementary Figure 1B](#), two different siRNAs against CHK1 decreased CIP2A expression in HT1080 cells.

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^{19–21}. 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 NH₂-terminal kinase (JNKs) activity^{22,23}, 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 kinases¹⁶. The study revealed that SP600125, although a JNK inhibitor, could inhibit the activity of several other kinases like CDK2, DYRK1 and CHK1¹⁶. Notably, we here identify CHK1 as the potential SP600125 sensitive kinase that positively regulates CIP2A expression in HT1080 cells ([Supplementary Figure 1B](#)).

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, HeLa²⁴. Interestingly, JNK2 has been shown to regulate CIP2A

expression via ATF2 transcription factor in mouse embryo fibroblasts (MEFs)¹¹ 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 systems⁸.

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 material

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)

A

B

Supplementary Figure 1. A. Western blot showing JNK1, CIP2A and Actin protein levels in HT1080 cells transfected with either control (Scr.) or combination of siRNAs against JNK1 and JNK2. The numbers below the western blot indicate the quantification of CIP2A and JNK1 protein levels after normalization to Actin levels. **B.** Western blot showing CIP2A and Actin levels in HT1080 cells transfected with siRNAs indicated for specific kinases previously known to be sensitive to SP600125 chemical inhibitor. The numbers below the western blot indicate the quantification of CIP2A protein levels after normalization to Actin levels.

References

1. Zhao JJ, Roberts TM, Hahn WC: **Functional genetics and experimental models of human cancer.** *Trends Mol Med.* 2004; **10**(7): 344–50.
[PubMed Abstract](#) | [Publisher Full Text](#)
2. Mumby M: **PP2A: Unveiling a Reluctant Tumor Suppressor.** *Cell.* 2007; **130**(1): 21–4.
[PubMed Abstract](#) | [Publisher Full Text](#)
3. Westermarck J, Hahn WC: **Multiple pathways regulated by the tumor suppressor PP2A in transformation.** *Trends Mol Med.* 2008; **14**(4): 152–60.
[PubMed Abstract](#) | [Publisher Full Text](#)
4. Hahn WC, Dessain SK, Brooks MW, *et al.*: **Enumeration of the simian virus 40 early region elements necessary for human cell transformation.** *Mol Cell Biol.* 2002; **22**(7): 2111–23.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
5. Hahn WC, Weinberg RA: **Rules for making human tumor cells.** *N Engl J Med.* 2002; **347**(20): 1593–603.
[PubMed Abstract](#) | [Publisher Full Text](#)
6. Khanna A, Pimanda JE, Westermarck J: **Cancerous Inhibitor of Protein Phosphatase 2A, an Emerging Human Oncoprotein and a Potential Cancer Therapy Target.** *Cancer Res.* 2013; **73**(22): 6548–53.
[PubMed Abstract](#) | [Publisher Full Text](#)
7. Khanna A, Okkeri J, Westermarck J: Gene Section.2010; 976.
[Reference Source](#)
8. Khanna A, Bockelman C, Hemmes A, *et al.*: **MYC-dependent regulation and prognostic role of CIP2A in gastric cancer.** *J Natl Cancer Inst.* 2009; **101**(11): 793–805.
[PubMed Abstract](#) | [Publisher Full Text](#)
9. Khanna A, Okkeri J, Bilgen T, *et al.*: **ETS1 mediates MEK1/2-dependent overexpression of cancerous inhibitor of protein phosphatase 2A (CIP2A) in human cancer cells.** *PLoS One.* 2011; **6**(3): e17979.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
10. Laine A, Sihto H, Come C, *et al.*: **Senescence sensitivity of breast cancer cells is defined by positive feedback loop between CIP2A and E2F1.** *Cancer Discov.* 2013; **3**(2): 182–97.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
11. Mathiasen DP, Egebjerg C, Andersen SH, *et al.*: **Identification of a c-Jun N-terminal kinase-2-dependent signal amplification cascade that regulates c-Myc levels in ras transformation.** *Oncogene.* 2012; **31**(3): 390–401.
[PubMed Abstract](#) | [Publisher Full Text](#)
12. Barretina J, Taylor BS, Banerji S, *et al.*: **Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy.** *Nat Genet.* 2010; **42**(8): 715–21.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
13. Junttila MR, Puustinen P, Niemela M, *et al.*: **CIP2A inhibits PP2A in human malignancies.** *Cell.* 2007; **130**(1): 51–62.
[PubMed Abstract](#) | [Publisher Full Text](#)
14. Barretina J, Caponigro G, Stransky N, *et al.*: **The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity.** *Nature.* 2012; **483**(7391): 603–7.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
15. Namgung U, Xia Z: **Arsenite-induced apoptosis in cortical neurons is mediated by c-Jun N-terminal protein kinase 3 and p38 mitogen-activated protein kinase.** *J Neurosci.* 2000; **20**(17): 6442–51.
[PubMed Abstract](#)
16. Bain J, McLauchlan H, Elliott M, *et al.*: **The specificities of protein kinase inhibitors: an update.** *Biochem J.* 2003; **371**(Pt 1): 199–204.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
17. Khanna A, Bockelman C, Laine A, *et al.*: **859 Constitutive DNA-damage signaling promotes cancer cell proliferation through Chk1-CIP2A pathway.** *Ejc Supplements.* 2010; **8**(5): 217.
[Publisher Full Text](#)
18. Khanna A, Kauko O, Bockelman C, *et al.*: **Chk1 targeting reactivates PP2A tumor suppressor activity in cancer cells.** *Cancer Res.* 2013; **73**(22): 6757–69.
[PubMed Abstract](#) | [Publisher Full Text](#)
19. Guillemot L, Citi S: **Cingulin regulates claudin-2 expression and cell proliferation through the small GTPase RhoA.** *Mol Biol Cell.* 2006; **17**(8): 3569–77.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
20. Chen Y, Gorelik GJ, Strickland FM, *et al.*: **Decreased ERK and JNK signaling contribute to gene overexpression in “senescent” CD4+CD28- T cells through epigenetic mechanisms.** *J Leukoc Biol.* 2010; **87**(1): 137–45.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
21. Cao Y, Xia DS, Qi SR, *et al.*: **Epiregulin can promote proliferation of stem cells from the dental apical papilla via MEK/Erk and JNK signalling pathways.** *Cell Prolif.* 2013; **46**(4): 447–56.
[PubMed Abstract](#) | [Publisher Full Text](#)
22. Martinez K, Kennedy A, McIntosh MK: **JNK inhibition by SP600125 attenuates trans-10, cis-12 conjugated linoleic acid-mediated regulation of inflammatory and lipogenic gene expression.** *Lipids.* 2011; **46**(10): 885–92.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
23. Tang G, Yue Z, Talloczy Z, *et al.*: **Autophagy induced by Alexander disease-mutant GFAP accumulation is regulated by p38/MAPK and mTOR signaling pathways.** *Hum Mol Genet.* 2008; **17**(11): 1540–55.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
24. Niemela M, Kauko O, Sihto H, *et al.*: **CIP2A signature reveals the MYC dependency of CIP2A-regulated phenotypes and its clinical association with breast cancer subtypes.** *Oncogene.* 2012; **31**(39): 4266–78.
[PubMed Abstract](#) | [Publisher Full Text](#)

Open Peer Review

Current Peer Review Status: ? ?

Version 2

Reviewer Report 26 August 2014

<https://doi.org/10.5256/f1000research.3258.r4566>

© 2014 Hunter T. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Tony Hunter

Salk Institute Cancer Center, Salk Institute for Biological Studies, La Jolla, CA, USA

Here the author showed that the expression of CIP2A (Cancerous Inhibitor of PP2A) in HT080 human fibrosarcoma cells is inhibited by treatment with the PD98059 MEK inhibitor, as previously reported, and also more strongly by the SP600125 JNK MAPK inhibitor, but not by the SB203580 p38 MAPK inhibitor. Although SP600125 does inhibit JNK, it is known to be a rather nonselective protein kinase inhibitor, meaning that its effects on CIP2A expression could be due to inhibition of a protein kinase(s) other than JNK. Indeed, the author found that combined siRNA depletion of JNK1/2 in HT1080 cells did not reduce CIP2A expression, suggesting that SP600125 targets another kinase involved in CIP2A expression. Other kinases known to be inhibited by SP600125 include CHK1, CDK2 and DYRK1. siRNA knockdown of CHK1, but not of CDK2 or DYRK1, led to a reduction in CIP2A protein, suggesting that inhibition of CHK1 might be responsible for the ability of SP600125 to inhibit CIP2A expression. Finally, the author found that two different CIP2A siRNAs, which efficiently depleted CIP2A, led, unexpectedly, to a reduction in JNK2 levels.

Given the continuing and indiscriminate use of SP600125 as a JNK MAP kinase inhibitor, even though it is well known to be a very nonspecific kinase inhibitor, it is reassuring to see a paper where the author has carried out a careful analysis and reports that the inhibitory effects of SP600125 treatment they observed on CIP2A expression in HT1080 cells were not due to inhibition of JNK1/2, and must therefore have been due to the action of SP600125 on another protein kinase, such as CHK1. However, there are a number of concerns about the paper as it stands.

1. PD98059 MEK inhibition only reduced CIP2A expression ~50%, as did CHK1 knockdown. To confirm the results obtained using CHK1 siRNA knockdown (this really needs validation by expression of an siRNA-resistant form of CHK1), the author should test the effects of a small molecule CHK1 inhibitor (there are several selective CHK1 inhibitors, e.g. PF 477736). This would then allow him to test the effects of combined MEK and CHK1 inhibition, i.e., are they additive, which would indicate that they activate CIP2A expression by independent pathways.

2. Does the inhibitory effect of SP600125 occur at the CIP2A RNA level? The author needs to

determine this. They have previously reported that CIP2A is regulated downstream of ERK by ETS1-driven transcription, but the effects of SP600125 on CIP2A protein levels do not have to occur at the RNA level (although this seems likely). What is known about the response elements in the CIP2A promoter region that respond to CHK1 activation? One might expect CIP2A to be induced by DNA damage, which causes CHK1 activation. Is this the case?

3. How are JNK2 levels regulated downstream of CIP2A – does this occur at the RNA level, and what pathway is involved in JNK2 expression downstream of CIP2A?
4. Do these results hold true for other fibrosarcoma cell lines?
5. Since the data in Figure S1B demonstrate that CHK1 is likely to be the key target for SP600125 inhibition of CIP2A expression, these data should be included in the main body of the paper.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and 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.

Reviewer Report 07 February 2014

<https://doi.org/10.5256/f1000research.3258.r2719>

© 2014 Clark K. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Kristopher Clark

MRC Protein Phosphorylation Unit, University of Dundee, Dundee, UK

In the revised version, Dr Khanna has re-emphasized the important drawbacks of using non-specific kinase inhibitors such as SP600125 to support a role for the c-Jun N-terminal Kinases in cells. The new data help demonstrate that the author could deplete the levels of JNK1/2 to very low levels without any effect on CIP2A expression. In contrast, treatment of cells with siRNA oligos against CHK1 (another SP600125 sensitive kinase) did reduce the expression of CIP2A in HT1080. While these data help support the author's claims, there remains an important issue from my previous report that needs rectification. It is absolutely essential when performing experiments looking at the inhibition of a kinase to verify that the phosphorylation of a physiological substrate is blocked. In the case of JNK1/2, c-Jun is an authenticated substrate with fantastic reagents commercially available for its study. I encourage the author to use the literature as a guide to identify the concentrations of a drug required for the experiments but the author should then validate these findings in the system under study. There is often batch-to-batch variation and the quality of inhibitors can vary greatly between suppliers. It is also important for any RNAi experiment because the depletion of the protein is always incomplete and some kinases can fully

phosphorylate their substrate even when expressed at very low levels. This data would demonstrate that the RNAi knockdown had the desired functional effect of blocking substrate (c-jun) phosphorylation without affecting CIP2A expression and clearly show that JNK1/2 are not involved in this process. It is a simple control experiment and I do not fully understand the author's reluctance to provide such data.

It has come to my attention that the data presented in Supplementary Table 1 was originally published by Bain *et al.* (Biochem J 2003). In the original version of the manuscript, I had assumed that the authors had performed their own assays to confirm what had been published in the above mentioned article. It is absolutely essential that the author clarifies the source of the data and includes it into the legend of the table.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and 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.

Version 1

Reviewer Report 20 August 2013

<https://doi.org/10.5256/f1000research.2042.r1558>

© 2013 Clark K. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Kristopher Clark

MRC Protein Phosphorylation Unit, University of Dundee, Dundee, UK

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.'

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and 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.

Author Response 11 Dec 2013

Anchit Khanna, University of New South Wales, Sydney, 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 (Niemiälä *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.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research