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Structural Basis of Divergent Cyclin-Dependent Kinase Activation by Spy1/RINGO proteins

Denise A. McGrath, Bre-Anne Fifield, Aimee H. Marceau, Sarvind Tripathi, Lisa A. Porter, and Seth M. Rubin

Corresponding author: Seth M. Rubin, University of California, Santa Cruz

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Editorial Decision:	06 April 2017
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 06 April 2017

Thank you for submitting your manuscript on CDK activation by Spy1/RINGO to The EMBO Journal. We have now received the comments of two expert referees, copied below for your information. I am pleased to inform you that both of them appreciate your findings and their potential importance, and that we shall therefore be happy to consider this work further for publication, pending satisfactory revision of a number of specific issues. Most of these points refer to presentational aspects, but it will also be important to improve the biological assays in present Figure 4C/D/E as requested by referee 2. Regarding presentation, the manuscript may benefit from some streamlining and sub-structuring, and you may also want to consider a combined "Results & Discussion" section to minimize redundancies.

REFEREE REPORTS

Referee #1:

This manuscript uses structural, biochemical, biophysical and cell biology approaches to address the question of how the S/R domain of the Spy proteins activates Cdks. Spy proteins activate Cdks independently of cyclins, and are over-expressed in tumours and are a means by which viruses deregulate the cell cycle. Spy activation of Cks occurs without T-loop (activation segment phosphorylation). Additionally, Cdk-Spy complexes are not subject to inhibition by Cdk inhibitors such as p27Kip and display relaxed substrate specificity.

The study is extremely interesting. The structural data comprehensively address the above questions indicating (i) how the S/R domain promotes a conformational change of Cdk that resembles that caused by CycA and T160 phosphorylation (ii) how the S/R domain resembles a cyclin fold although shares no sequence similarity (iii) how the S/R domain interacts with the PSTAIR helix and mimics T160 phosphorylation and (iv) why the Spy does not bind Cdk inhibitors. The structural

data are strongly supported by biochemical and cell biological experiments. Overall the manuscript was a pleasure to read, and the results will be of interest to the cell cycle and cancer biology communities. This is a well-written manuscript with clear figures. The manuscript certainly warrants publication in EMBO J and rapid publication is recommended.

Minor comments:

1. Table 1 lists three X-ray data sets, two for the Cdk2-Spy1 complex, with resolutions of 3.2 ≈ and 2.7 ≈. However in the text (page 5, my page numbering), the structure is stated as being a 2.4 ≈ resolution.

2. Page 5. A figure showing Glu51 would be helpful.

3. Fig. 3A. Show T160.

4. Page 8. 'Cyclin A' is missing in sentence starting 'Consistent with...'

5. Page 8. 'Fig. S2' not 'Fig. 2'.

Referee #2:

This manuscript reports the x-ray crystallographic structure at a resolution of 2.4 A of the noncanonical CDK activator protein referred to as Speedy/RINGO. The manuscript in general is well written and clearly describes the work, and it represents a major advance in our understanding of how Spy1 is able to activate Cdk2 in the absence of binding by a typical cyclin, and in the absence of T-loop phosphorylation on Cdk2.

In response to the questions posed above on the referee's page, I would suggest a title change so that it is clear that this manuscript presents the structure of the protein. If permitted by EMBO, for example, the title might be: "Divergent Activation of Cyclin-Dependent Kinase by Spy1 in Development and Cancer Revealed by Structural Determination," or some permutation thereof.

In terms of the organization of the manuscript, the Introduction at present seems too lengthy, and some of the information would be better suited for the Discussion (eg. some of the information in the 2nd paragraph which describes non-Cyclin activators more generally, or the information in the last paragraph of the Intro.

The images presented in Fig 1A and 1B are really interesting to view, and EMBO might consider using one or the other as a cover photo. The extent to which the T Loop is "pulled away from the active site and contacts Spy1" is remarkable. The authors might wish to emphasize this by actually stating how many A the tip of the T-loop has moved compared with the "CDK2 alone" structure.

Analysis of the kinetic data in Fig 3 is nicely done and good to see included.

One weakness of the ms. is the data presented in Fig 4D looking at the ability of Spy1 and mutant Spy1(DE) to stimulate HEK293 cell proliferation. These proteins are transiently over expressed and cell numbers are determined over a 24-hr time period. It would be preferable to use a cell assay using a cell line that displays more normal cell cycle parameters, assayed over a longer time course and perhaps including MTT quantitation in addition to just cell counts. Similarly, this reviewer has doubts as to whether the data in Fig 4E convincingly demonstrate the ability of Spy1 to overcome UV-activated checkpoint and would strongly prefer the inclusion of data that do not rely on transient over expression. Another option might be to consider ommitting Fig 4CDE, as the data in Fig4B compellingly demonstrate the importance of D97 and E135 for the ability of Sp1 to activate Cdk2.

The last section of the Results: "Spy1 lacks the canonical Cyclin binding cleft..." although fascinating, its interest level decreases due to the length of this section. This reviewer would suggest paring this section judiciously so that the overall flow throughout the Results section is maintained.

The Discussion would be improved by the inclusion of several subheadings. The Discussion should also include a more thorough treatment of the importance of Spy1 expression, and other non-Cyclin activators, in various human cancers. This information should not be in the Intro.

Note that the legend to Fig 6 is inconsistent with the figure itself and with the description: "Figure 6... Rb771-928 and Rb771-874 both contain 7 consensus Cdk sites but only Rb771-928 contains the

RxLF sequence that docks to the MRAIL site in CycA." But the Fig shows data for RbC and RbC-F877A. Please correct.

1st Revision - authors' response 27 May 2017

Enclosed please find our revised manuscript titled “Structural Basis of Divergent Cyclin-Dependent Kinase Activation by Spy1/RINGO proteins” that we are re-submitting for publication as a Research Article in The EMBO Journal. We are pleased with the enthusiasm of the reviewers and grateful for their insightful comments. We have improved the manuscript according to their suggestions. Details of changes in the revision can be found in our Response to Reviewers document.

Point-By-Point Response:

We thank the reviewers for their effort, and we appreciate the constructive criticisms that were offered. Our responses to their recommendations are detailed below:

Reviewer #1:

1. Table 1 lists three X-ray data sets, two for the Cdk2-Spy1 complex, with resolutions of 3.2 Å and 2.7 Å. However in the text (page 5, my page numbering), the structure is stated as being a 2.4 Å resolution.

We corrected the typo in the text; 2.7 Å is correct.

2. Page 5. A figure showing Glu51 would be helpful.

Glu51 is now shown in Fig. 1A.

3. Fig. 3A. Show T160.

We added T160 to each T-loop in the figure.

4. Page 8. 'Cyclin A' is missing in sentence starting 'Consistent with...'

We corrected the typo and changed “Cdk2” to “Cdk2-CycA.”

5. Page 8. 'Fig. S2' not 'Fig. 2'.

We corrected the typo; Fig EV2 (Fig S2) is correct.

Reviewer #2:

In response to the questions posed above on the referee's page, I would suggest a title change so that it is clear that this manuscript presents the structure of the protein. If permitted by EMBO, for example, the title might be: "Divergent Activation of Cyclin-Dependent Kinase by Spy1 in Development and Cancer Revealed by Structural Determination," or some permutation thereof.

Similar to as suggested by the editor, we changed the title to “Structural Basis of Divergent Cyclin-Dependent Kinase Activation by Spy1/RINGO proteins.”

In terms of the organization of the manuscript, the Introduction at present seems too lengthy, and some of the information would be better suited for the Discussion (eg. some of the information in the 2nd paragraph which describes non-Cyclin activators more generally, or the information in the last paragraph of the Intro.

The introduction has been shortened by moving the suggested information to the discussion.

The images presented in Fig 1A and 1B are really interesting to view, and EMBO might consider using one or the other as a cover photo. The extent to which the T Loop is " pulled away from the active site and contacts Spy1" is remarkable. The authors might wish to emphasize this by actually stating how many A the tip of the T-loop has moved compared with the "CDK2 alone" structure.

Depending precisely on how one defines the "tip" of the loop, we measure a 5-6 Å distance between different Cα positions in the aligned structures. We now estimate this distance change as follows:

"The T-loop contacts Spy1 and is pulled ~5-6 Å away from the active site relative to its position in the structure of Cdk2 alone." We would be thrilled for this figure to be considered for a cover photo.

One weakness of the ms. is the data presented in Fig 4D looking at the ability of Spy1 and mutant Spy1(DE) to stimulate HEK293 cell proliferation. These proteins are transiently over expressed and cell numbers are determined over a 24-hr time period. It would be preferable to use a cell assay using a cell line that displays more normal cell cycle parameters, assayed over a longer time course and perhaps including MTT quantitation in addition to just cell counts. Similarly, this reviewer has doubts as to whether the data in Fig 4E convincingly demonstrate the ability of Spy1 to overcome UV-activated checkpoint and would strongly prefer the inclusion of data that do not rely on transient over expression. Another option might be to consider ommitting Fig 4CDE, as the data in Fig4B compellingly demonstrate the importance of D97 and E135 for the ability of Sp1 to activate Cdk2.

It is a good point that the Spy1(DE) mutant may only delay the effects on cell proliferation and that it is a good measure to use multiple cell lines. We included measurements of NIH3T3 cells, which have wild-type p53, display more "normal" cell cycle parameters, and are frequently used for the study of checkpoint control (including previous proliferation and checkpoint papers with Spy1 – of which we have added into the text). We reproduced the proliferation assay in both HEK293 and NIH3T3 cells over a 5 day time course. We added RT-qPCR data to ensure that expression levels between wildtype and mutant proteins are similar. As suggested, we removed the irradiation data and kept the focus on Cdk2 activity and cell proliferation.

The last section of the Results: "Spy1 lacks the canonical Cyclin binding cleft..." although fascinating, its interest level decreases due to the length of this section. This reviewer would suggest paring this section judiciously so that the overall flow throughout the Results section is maintained.

To improve the readability of this section, we pared down some of the introductory description of MRAIL-binding by Cdk substrates, and we separated out the description of Fig 6 into a new section.

The Discussion would be improved by the inclusion of several subheadings. The Discussion should also include a more thorough treatment of the importance of Spy1 expression, and other non-Cyclin activators, in various human cancers. This information should not be in the Intro.

As suggested, we have added subheadings to the discussion and have expanded our treatment of Spy1 in cancer. Some of this discussion was moved from the introduction as noted above.

Note that the legend to Fig 6 is inconsistent with the figure itself and with the description: "Figure 6... Rb771-928 and Rb771-874 both contain 7 consensus Cdk sites but only Rb771-928 contains the RxLF sequence that docks to the MRAIL site in CycA." But the Fig shows data for RbC and RbC-F877A. Please correct.

The figure caption has been corrected.

2nd Editorial Decision - Acceptance 02 June 2017

Thank you for submitting your revised manuscript for our consideration. We have now had a chance to go through your responses to the referees' comments and to look into your revised files, and I am pleased to inform you that we have now accepted the article for publication in The EMBO Journal.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Seth Rubin
Journal Submitted to:	EMBO Journal
Manuscript Number:	EMBOJ-2017-96905

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Methods Section, pages 13-14.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes
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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Page 14

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	PDB codes on page 13
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA