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CSAG2 is a cancer-specific activator of SIRT1

Xu Yang and Patrick Ryan Potts
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Corresponding author(s): Patrick Potts (Ryan.Potts@STJUDE.ORG)

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Reviewer #1 (Evidence, reproducibility and clarity (Required)):

The manuscript entitled "CSAG2 is a cancer-specific activator of SIRT1 that suppresses p53" reports the identification of oncogenic protein CSAG2 as a novel regulator of SIRT1 and p53 in multiple cancers. Through bioinformatic dataset mining, the authors identified the CSAG proteins are highly expression in a variety of cancers and upon manipulations, can alter tumor growth in vitro and in vivo. The authors further found that CSAG2 is selectively bound to SIRT1, and through a series of biochemical and molecular approaches, show that CSAG2 is involved in SIRT1-mediated p53 deacetylation in cancer cells. It is of interest to discover CSAGs as oncogenic protein and novel to reveal the CSAG2-SIRT1 interaction; however, the authors seemed quite restrained to the deacetylation of p53, which is likely being one of the many downstream targets of SIRT1; the dependency on p53 should be examined. Additional comments are as follow:

1. It is interesting to see the tissue expression spectrum of CSAGs as shown in Fig.1. However, whether the expression pattern of these genes is known or was newly identified by the authors?

2. The authors should revise available protein-protein interaction data sets from cancer cells to see if CSAG-SIRT1 protein-protein interaction is also observed. It is also important to analyze of this protein is part of a SIRT1 core protein complex. This is important because SIRT1 is an enzyme, so this is a core member of the complex should be investigated.

3. Whether CSAG2 expression in CRC and melanoma correlates with survival or other clinical features? Or otherwise the authors might need to provide a justification why those two tumor types were selected for functional studies as in Fig 2.

4. Based on the authors' model, CSAG2 promotes tumor growth and enables resistance to genotoxic stress through SIRT1-p53 axis. Indeed, the cellular models used are all with a wild-type p53; however, the TCGA dataset showing survival includes tumors with both wt and mutant p53, questioning the SIRT1-mediated p53 deacetylation pathway as the mechanism underlying CSAG2 for tumors in general. It is recommended for the authors to either narrow down to specific tumor types/subsets, or explore other potential SIRT1-dependent mechanism.

5. The way Fig 3 was organized is somehow confusing. It makes sense to discover and validate CSAG2-SIRT1 interaction; however, why the authors considered p53 as the downstream pathway is not clear. And the p53 deacetylation data in this figure do not convincingly support the involvement of

SIRT1.

Reviewer #1 (Significance (Required)):

See above

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In the current manuscript, the authors demonstrate that previously uncharacterized testis protein CSAG2, overexpressed in different types of cancers, show a pro-oncogenic activity in different assays ranging from proliferation and colony assays formation to anchorage-independent growth and tumor formation in mouse xenografts. This pro-oncogenic effect correlates with worse prognosis in different types of cancers. CSAG2 activates the activity of the NAD-dependent deacetylase SIRT1 in cancer cells through binding of its N-terminal region (1-37) to the catalytic domain of SIRT1. This activation results in hypoacetylation of the SIRT1 target K382 in p53, which has been linked to p53 transcriptional inhibition. Furthermore, they link this mechanism to the increased resistance induced by CSAG2 to genotoxic stress in these cells.
The authors have done a good job demonstrating that CSAG2 has a pro-oncogenic activity, which they correlate with SIRT1 activation and an effect on p53ac K382ac levels. However, I have several major issues that I believe could strengthen the claims of the authors. My main overall concerns are that the authors need to demonstrate that the observed effect of CSAG2 on SIRT1 has a functional meaning and that p53 is the real target of this observed effects on oncogenesis. In this sense, I think there are several aspects that would be worth to develop:

1) First, the authors need to show some more evidence to demonstrate that CSAG2 is an in vivo regulator of SIRT1 activity beyond overexpression. This is not obvious since in the vast majority of molecular experiments the authors need to inhibit the non-Sirtuin HDACs to detect the K382ac levels. In this sense, the only direct evidence pointing to SIRT1 in the cell studies is the use in very few experiments of inhibitor EX-527, and a SIRT1 siRNA in 4A. In my opinion other known targets of SIRT1 could be tested to demonstrate unequivocally that the effect of CSAG2 is similar. Moreover, is SIRT1 endogenous activity altered in testis cell lines upon downregulation of CSAG2? To provide evidences in this line would strongly support an in vivo role of CSAG2 on SIRT1.

2) In the same lane, if CSAG2 binds to the catalytic domain of SIRT1, can still bind to the catalytic-dead point mutant H363Y? If this is the case, would overexpression of this mutant revert the effect of CSAG2 overeexpression? This would support a direct effect of CSAG2 on SIRT1. Similarly, If CSAG2 exerts its effect through binding to the N-terminal residues 1-37, could the overexpression

of this peptide, or of a fusion between this sequence and other unrelated protein be enough to activate SIRT1 activity?

3) How different is CSAG2 activation mechanism compared to the other described SIRT1 protein activator AROS? As mentioned below, AROS was also shown to activate SIRT1 specifically in cancer. This issue should be discussed.

4) Another relevant issue is the functional effect of CSAG2 over p53 function through SIRT1. The authors demonstrate the effect of CSAG2 on p53 K382ac, and show the effect of CSAG2 on different features of the oncogenic phenotype including colony assay, anchorage independent assays and xenografts. However, with the exception of an unquantified WB of some p53 targets they do not demonstrate that CSAG2 exerts this effect through p53. Considering the authors' claims this is a very important issue and should be developed further. I would suggest several options:

i) To test the effect of protein and/or expression levels of these p53 targets showing a quantification and statistical significance;

ii) Maybe it would be worth to demonstrate that the effect of CSAG2 is p53-dependent by downregulating p53 in experiments in figure 5;

iii) Besides the cancer phenotype experiments, maybe other p53 associated functions such apoptosis or senescence could be tested.

5) Some WBs of p53 K283ac/p53 need quantification to be convincing because the signal of p53 is saturated in many of them and the ratio is not easy to assess. For instance s mentioned, fig 3D, but also fig 3H, where the last lane seem to have lower levels of p53 compared with the previous ones.

Reviewer #2 (Significance (Required)):

The identification and characterization of a novel SIRT1 protein activator, particularly relevant in the context of cancer, should be of interest to a wide range of researchers of different fields, not only because it represents a novel way to activate SIRT1 activity, but also because it could be potentially used as a target in cancer prognosis and therapeutics. In some contexts where SIRT1 activity has a protective effect, like in neurodegenerative diseases, diabetes or even aging, upregulation of CNAG2 may also be an interesting approach. So far, only one SIRT1 protein activator, AROS, have been described, but in this case there are some contradictory evidences suggesting that may also act as SIRT inhibitor. As mentioned above, AROS was also shown to activate SIRT1 activity in the context of cancer. If the authors can demonstrate that CSAG2 is a true in vivo SIRT1 activator, and characterize further this activity as mentioned earlier, this would in my opinion increase significantly the relevance of the work.

Response to Reviewers

Point by point response to each reviewer is below. We have added 28 new experimental figure panels (Fig. 3E, 3G, 3I, 3K, 3L, 3M, 3N, 4C, 4D, 4E, 4I, 5E, 5F, 5I, 5J, 5K, 5L, 5M, 5N, 6H, 6I, 6J, 6K, S1A, S2I, S2J, S2K, and S2L) in response to the reviewers' suggestions. Importantly, in response to both reviewers we have examined additional SIRT1 targets and p53 dependency for CSAG2 activities. Additionally, the text has been edited as suggested, including removal of model figure and p53 from title. We believe these substantial revisions provide additional support for our conclusions and broaden the impact and scope of our findings.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

The manuscript entitled "CSAG2 is a cancer-specific activator of SIRT1 that suppresses p53" reports the identification of oncogenic protein CSAG2 as a novel regulator of SIRT1 and p53 in multiple cancers. Through bioinformatic dataset mining, the authors identified the CSAG proteins are highly expression in a variety of cancers and upon manipulations, can alter tumor growth in vitro and in vivo. The authors further found that CSAG2 is selectively bound to SIRT1, and through a series of biochemical and molecular approaches, show that CSAG2 is involved in SIRT1-mediated p53 deacetylation in cancer cells. It is of interest to discover CSAGs as oncogenic protein and novel to reveal the CSAG2-SIRT1 interaction; however, the authors seemed quite restrained to the deacetylation of p53, which is likely being one of the many downstream targets of SIRT1; the dependency on p53 should be examined. Additional comments are as follow:

We would like to thank the reviewer for their insightful comments that have yielded a much stronger story. In response to your suggestions, we have investigated 1) additional SIRT1 targets affected by CSAG2 and 2) dependency on p53 for CSAG2 phenotypes. In this revised manuscript we provide compelling evidence that 1) CSAG2 is a bona fide activator of SIRT1 that promotes deacetylation of multiple targets, including p53 K382, H3K14, and H4K16; and 2) the ability of CSAG2 to mediate anchorage-independent growth and chemoresistance is dependent on p53 regulation. Each of these points are addressed in more detail below. We feel these new data provide a compelling argument for our conclusions.

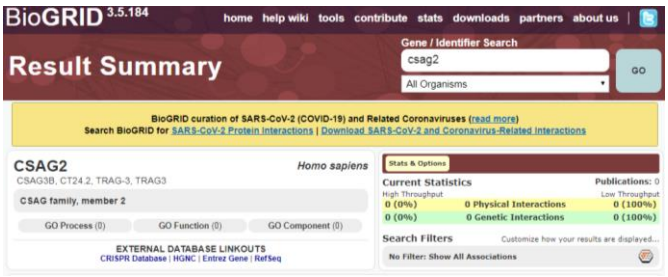
1. It is interesting to see the tissue expression spectrum of CSAGs as shown in Fig.1. However, whether the expression pattern of these genes is known or was newly identified by the authors?

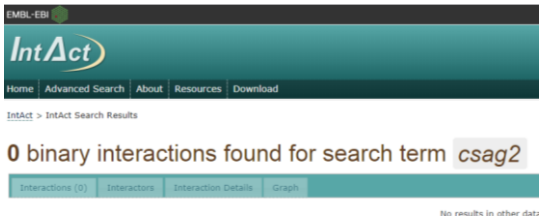
To our knowledge, no previous publications have comprehensively analyzed CSAG expression in normal tissue and cancer. There are anecdotal studies showing one off expression in a particular set of tumors and correlation with poor patient prognosis, but nothing to our knowledge broadly examining its expression.

2. The authors should revise available protein-protein interaction data sets from cancer cells to see if CSAG-SIRT1 protein-protein interaction is also observed.

We have examined several protein-protein interaction data sets, including the BioPlex, BioGrid, IntAct, Human reference interactome research project, NCBI Gene, and Uniprot, and have not seen any CSAG2 interactions reported. This speaks to the novelty of our work. In none of those

studies was CSAG2 used as a bait for pulldown and many used cell lines that do not express endogenous CSAG2. In addition, CSAG2 is a small protein that doesn't have many peptides that are readily detectable by traditional mass spectrometry methods. Therefore, they would have missed this interaction.





It is also important to analyze of this protein is part of a SIRT1 core protein complex. This is important because SIRT1 is an enzyme, so this is a core member of the complex should be investigated.

It is not clear to us what the reviewer is asking in relation to whether CSAG2 is a component of the SIRT1 core protein complex. To our understanding, SIRT1 does not form a core complex. Rather it interacts with a number of regulators, cofactors, and substrates. This includes, but is not limited to, the PPARa/SIRT1 complex in transcriptional regulation, LSD1/SIRT1 co-repressor complex, BMAL/CLOCK/SIRT1 in circadian rhythm, and BCL6/BCOR/SIRT1 complex in transcriptional control of neurogenesis. In addition, SIRT1 has also been reported to bind DBC1 and AROS to alter its activity. However, these none of these interactions are thought to be constitutive and universally important for SIRT1 function that would suggest a core complex. We did not detect any of these other SIRT1 interactors in our TAP-MS experiments (Table S1). This could be for several reasons, including lack of expression of these regulators (some cell type and tissue specific) and/or CSAG2 binding is mutually exclusive with these other regulators.

3. Whether CSAG2 expression in CRC and melanoma correlates with survival or other clinical features? Or otherwise the authors might need to provide a justification why those two tumor types were selected for functional studies as in Fig 2.

We appreciate this suggestion. Unfortunately, our data visualization tool did not have sufficient data for analysis of CSAG2 expression correlation with clinical feature of CRC and melanoma. However, we have included 4 additional tumor types were data was available and CSAG2 correlated with poor prognosis in Fig. S1A. Thus, CSAG2 expression correlates with poor overall survival in 6 different tumor types. These findings suggest our results have implications beyond CRC and melanoma.

Cell lines used in this study have been carefully considered and selected based on previous literature and cell line availability. We chose cell lines based on several criteria given the experimental setup and biological relevance. This includes, CSAG2 negative cells for gain of function studies, endogenous CSAG2 positive cells for loss of function studies. Additionally, p53 mutational status expression level, existence of knockout cells, and prior functional studies were decision drivers. We feel that the inclusion of multiple cell lines spanning different tumor

types adds value to our study showing the reproducibility of our work and impact in multiple tumor types.

4. Based on the authors' model, CSAG2 promotes tumor growth and enables resistance to genotoxic stress through SIRT1-p53 axis. Indeed, the cellular models used are all with a wild-type p53; however, the TCGA dataset showing survival includes tumors with both wt and mutant p53, questioning the SIRT1-mediated p53 deacetylation pathway as the mechanism underlying CSAG2 for tumors in general. It is recommended for the authors to either narrow down to specific tumor types/subsets, or explore other potential SIRT1-dependent mechanism.

Our apologies as we now see how the model would suggest the primary downstream pathway regulated by CSAG2-SIRT1 is p53. This is not our intention and thus we have removed the model (Fig. 7), deleted p53 from the title, and altered the abstract. However, in new data added, we now show CSAG2-mediated anchorage-independent growth (Fig. 3N) and resistance to genotoxic stress (Fig. 5I-N) is dependent on p53 regulation (discussed below). Additionally, we examined additional targets of SIRT1 for regulation by CSAG2 as suggested by the reviewer (discussed below).

We have examined whether the ability of CSAG2 to promote anchorage-independent growth is dependent on p53 regulation. Indeed, we find that expression of CSAG2 in p53-null HCT116 cells fails to induce soft agar growth. This data is now shown in Fig. 3N.

Additionally, we have addressed whether CSAG2 expression or knockdown alters the sensitivity of p53 null HCT116 or p53 mutant H1299 cells to doxorubicin or H_2O_2. We find that in all conditions and unlike in p53 wild-type HCT116 and H460 cells, upregulation or downregulation does not alter sensitivity to these genotoxic stressors. This data is now shown in Fig. 5I-N.

These data provide compelling evidence that the ability of CSAG2 to promote anchorage-independent growth and chemoresistance is mediated through its regulation of p53 (and SIRT1). However, this should not discount potential other pathways and cellular phenotypes that CSAG2 likely promotes through regulation of SIRT1 and its targets. Anchorage-independent growth (avoidance of anoikis) and sensitivity to DNA damaging agents are well established to be tightly controlled by p53. Thus, it is not surprising CSAG2 regulation of these processes proceeds in a p53-dependent manner, although other CSAG2-SIRT1 functions that are p53-independent are very likely. A discussion of this point has been added to the text (p14) and the model (Fig. 7) removed.

Finally, as suggested by the reviewer we have examined whether CSAG2 alters acetylation levels of other known SIRT1 targets, ac-H3K14 and ac-H4K16. We find that in HeLa, U2OS, and HCT116 cells expression of CSAG2 significantly reduced the levels of ac-H3K14 and ac-H4K16 by 50-75% (Fig. 6H-J and S2K). Furthermore, knockdown of endogenous CSAG2 elevated ac-H4K16 levels in A375 cells (Fig. 6K and S2L). These findings, in addition to the regulation of p53 ac-K382 levels, provide strong evidence that CSAG2 affects multiple SIRT1 targets. Additionally, these results are in line with our mechanistic studies suggesting that CSAG2 regulates SIRT1 kcat, independent of altering substrate binding affinity (Km) (Fig. 6G). Therefore, we feel confident in the conclusion that CSAG2 is an activator of SIRT1.

5. The way Fig 3 was organized is somehow confusing. It makes sense to discover and validate CSAG2-SIRT1 interaction; however, why the authors considered p53 as the downstream pathway is not clear. And the p53 deacetylation data in this figure do not convincingly support the involvement of SIRT1.

Our apologies for the confusion. p53 ac-K382 is the most well established SIRT1 target. It has reproducibly shown by many labs as bona fide, cellular target of SIRT1. In addition, given that CSAG2 is a cancer-specific protein, regulation of a key tumor suppressor like p53 is of prime interest and likely meaningful. As discussed above, we have now provided additional evidence that CSAG2-induced anchorage-independent growth and chemoresistance are dependent on p53 regulation. However, other targets of SIRT1 are likely important for CSAG2 actions. We now provide new data as discussed above showing that indeed CSAG2 alters acetylation levels of other SIRT1 targets (ac-H3K14 and ac-H4K16). Thus, CSAG2 enhances SIRT1 activity towards multiple targets, not just p53.

We have quantified the western blots in Figure 3 examining p53 ac-K382 levels and normalized to total p53 to provide a quantitative view on the robustness and reproducibility of CSAG2 regulation of p53 ac-K382 levels. We provide compelling evidence in Fig. 4A, 4B, 4C, 4D, and 4E that CSAG2-induced changes in p53 ac-K832 levels are dependent on SIRT1 as genetic knockdown of SIRT1 or chemical inhibitor of SIRT1 blocks CSAG2 action. Furthermore, we provide new data showing that knockdown of endogenous CSAG2 with two independent shRNAs does not increase p53 ac-K382 levels in cells treated with SIRT1 inhibitor (Fig. 4C). Additionally, overexpression of SIRT1 catalytic dead H363Y mutant that binds CSAG2 (Fig. 4D) blocks CSAG2 regulation of p53 ac-K382 (Fig. 4E). Finally, we have shown that CSAG mutant (Δ37) that fails to bind SIRT1 (Fig. 4H-I) also fails to regulate p53 ac-K382 levels (Fig. 4I). These data in aggregate provide a compelling argument that CSAG2 regulation of p53 ac-K382 levels is dependent on SIRT1.

Reviewer #1 (Significance (Required)):

See above

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In the current manuscript, the authors demonstrate that previously uncharacterized testis protein CSAG2, overexpressed in different types of cancers, show a pro-oncogenic activity in different assays ranging from proliferation and colony assays formation to anchorage-independent growth and tumor formation in mouse xenografts. This pro-oncogenic effect correlates with worse prognosis in different types of cancers. CSAG2 activates the activity of the NAD-dependent deacetylase SIRT1 in cancer cells through binding of its N-terminal region (1-37) to the catalytic domain of SIRT1. This activation results in hypoacetylation of the SIRT1 target K382 in p53, which has been linked to p53 transcriptional inhibition. Furthermore, they link this mechanism to the increased resistance induced by CSAG2 to genotoxic stress in these cells.

The authors have done a good job demonstrating that CSAG2 has a pro-oncogenic activity, which they correlate with SIRT1 activation and an effect on p53ac K382ac levels. However, I have several major issues that I believe could strengthen the claims of the authors. My main overall concerns are that the authors need to demonstrate that the observed effect of CSAG2 on SIRT1 has a functional meaning and that p53 is the real target of this observed effects on oncogenesis. In this sense, I think there are several aspects that would be worth to develop:

1) First, the authors need to show some more evidence to demonstrate that CSAG2 is an in vivo regulator of SIRT1 activity beyond overexpression. This is not obvious since in the vast majority of molecular experiments the authors need to inhibit the non-Sirtuin HDACs to detect the K382ac levels. In this sense, the only direct evidence pointing to SIRT1 in the cell studies is the use in very few experiments of inhibitor EX-527, and a SIRT1 siRNA in 4A.

We have added additional data showing SIRT1 dependency for CSAG2 regulation of p53 ac-K382 in Fig. 4C. We show that unlike control treated cells, knockdown of CSAG2 in EX-527 treated cells has no effect on p53 ac-K382 levels. This combined with the data presented in Fig. 4A and 4B showing that overexpression of CSAG2 fails to modulate p53 ac-K382 levels in SIRT1 knockdown cells or EX-527 treated cells provides strong evidence for SIRT1-dependent regulation of p53 ac-K382 by CSAG2. Furthermore, overexpression of SIRT1 catalytic dead H363Y mutant blocks CSAG2 regulation of p53 ac-K382 (Fig. 4D-E). Finally, our finding that N-terminal deletion mutation of CSAG2 that fails to interact with SIRT1 does not affect p53 ac-K382 levels (Fig. H-I) further supports our conclusions.

In my opinion other known targets of SIRT1 could be tested to demonstrate unequivocally that the effect of CSAG2 is similar.

We thank the reviewer for this important suggestion. We have addressed this question by examining whether CSAG2 regulates the acetylation levels of two additional SIRT1 targets, ac-H3K14 and ac-H4K16. We find that in HeLa, U2OS, and HCT116 cells expression of CSAG2 significantly reduced the levels of ac-H3K14 and ac-H4K16 by 50-75% (Fig. 6H-J and S2K). Furthermore, knockdown of endogenous CSAG2 elevated ac-H4K16 levels in A375 cells (Fig. 6K and S2L). These findings, in addition to the regulation of p53 ac-K382 levels, provide strong evidence that CSAG2 affects multiple SIRT1 targets. Additionally, these results are in line with our mechanistic studies suggesting that CSAG2 regulates SIRT1 kcat, independent of altering substrate binding affinity (Km) (Fig. 6G). Therefore, we feel confident in the conclusion that

CSAG2 is an activator of SIRT1. To make sure readers are not distracted from this point and focused entirely on p53 regulation by CSAG2, we have removed the model figure (Fig. 7), deleted p53 from the title, and altered the abstract accordingly.

Moreover, is SIRT1 endogenous activity altered in testis cell lines upon downregulation of CSAG2? To provide evidences in this line would strongly support an in vivo role of CSAG2 on SIRT1.

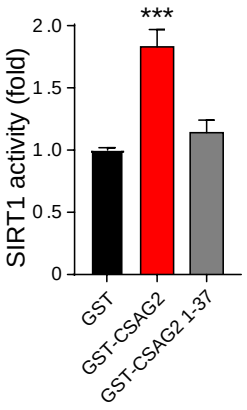
We are not familiar with any human testis cell lines that can be grown and manipulated in culture. Most studies on germ cells are performed using rodent cell culture models. There are no robust methods for growth of human germ cells. Unfortunately (and interestingly), CSAG2 has evolved independent of the rodent lineage and thus the rodent germ cell culture models are not feasible for this study.

2) In the same lane, if CSAG2 binds to the catalytic domain of SIRT1, can still bind to the catalytic-dead point mutant H363Y? If this is the case, would overexpression of this mutant revert the effect of CSAG2 overeexpression? This would support a direct effect of CSAG2 on SIRT1.

We thank the reviewer for this insightful idea. We found that CSAG2 binds SIRT1 H363Y similarly to SIRT1 wild-type (Fig. 4D). Furthermore, expression of SIRT1 H363Y blocked CSAG2-mediated regulation of p53 ac-K382 (Fig. 4E). These data are consistent with genetic ablation of SIRT1 and SIRT1 inhibitor blocking CSAG2 regulation of p53 ac-K382 levels and further confirm our conclusions that CSAG2 regulates p53 ac-K382 levels through altering SIRT1 activity.

Similarly, If CSAG2 exerts its effect through binding to the N-terminal residues 1-37, could the overexpression of this peptide, or of a fusion between this sequence and other unrelated protein be enough to activate SIRT1 activity?

We have tested whether Myc-CSAG2 1-37 fragment is necessary and/or sufficient for regulation of p53 ac-K382 in cells. We found that Myc-CSAG2 1-37 is necessary, but not sufficient. This data is shown in Fig. 4I. Additionally, we also tested whether CSAG2 1-37 fragment is sufficient to activate SIRT1 *in vitro*. Consistent with our cell based data, CSAG2 1-37 fragment was not sufficient to enhance SIRT1 activity (data below). These data suggest that CSAG2 1-37 is necessary, but not sufficient for stimulating SIRT1 activity in vitro and in cells.



3) How different is CSAG2 activation mechanism compared to the other described SIRT1 protein activator AROS? As mentioned below, AROS was also shown to activate SIRT1 specifically in cancer. This issue should be discussed.

Our apologies for not discussing AROS in more depth in our original submission, as its function as a SIRT1 regulator is controversial. AROS was originally reported as a binding partner of SIRT1 that could enhances its activity in vitro and in cells towards p53 ac-K382 (Kim et al., 2007, PMID: 17964266). Deletion analysis showed that amino acids 114–217 of SIRT1 are sufficient for the interaction between SIRT1 and AROS. However, three subsequent studies failed to reproduce the results, drawing questions into whether AROS is truly a SIRT1 activator. They found that AROS did have weak affinity to SIRT1. However, they failed to reproduce activation of SIRT1 in multiple assays. AROS did not stimulate, but in some cases inhibited, SIRT1 *in vitro* towards multiple substrates in multiple reaction setups. Furthermore, AROS did not regulate p53 ac-K382 levels in cells. PMID: 23548308, PMID: 24258275, PMID: 24681097. A synopsis of these observations are now included in the manuscript.

4) Another relevant issue is the functional effect of CSAG2 over p53 function through SIRT1. The authors demonstrate the effect of CSAG2 on p53 K382ac, and show the effect of CSAG2 on different features of the oncogenic phenotype including colony assay, anchorage independent assays and xenografts. However, with the exception of an unquantified WB of some p53 targets they do not demonstrate that CSAG2 exerts this effect through p53. Considering the authors' claims this is a very important issue and should be developed further. I would suggest several options:

We thank the reviewer for these suggestions that have strengthen interpretation of our results.

i) To test the effect of protein and/or expression levels of these p53 targets showing a quantification and statistical significance;

p53 targets have now been quantified in Fig. 3E. Importantly, our results show that upregulation of PUMA, Bax and p21 upon doxorubicin treatment is suppressed by CSAG2 back to baseline levels. These results are consistent with the conclusions that CSAG2 enhances SIRT1-mediated deacetylation of p53 K382 to downregulate its transcriptional activity.

ii) Maybe it would be worth to demonstrate that the effect of CSAG2 is p53-dependent by downregulating p53 in experiments in figure 5;

We thank the reviewer for this helpful suggestion. We have addressed this through examining whether CSAG2 expression or knockdown alters the sensitivity of p53 null HCT116 or p53 mutant H1299 cells to doxorubicin or H_2O_2. We find that in all conditions and unlike in p53 wild-type HCT116 and H460 cells, upregulation or downregulation does not alter sensitivity to these genotoxic stressors. This data is now shown in Fig. 5I-N.

In addition, we have examined whether the ability of CSAG2 to promote anchorage-independent growth is dependent on p53 regulation. Indeed, we find that expression of CSAG2 in p53-null HCT116 cells fails to induce soft agar growth. This data is now shown in Fig. 3N.

These data in combination provide compelling evidence that the ability of CSAG2 to promote anchorage-independent growth and chemoresistance is mediated through its regulation of p53 (and SIRT1). However, this should not discount potential other pathways and cellular phenotypes that CSAG2 likely promotes through regulation of SIRT1 and its targets. Anchorage-independent growth (avoidance of anoikis) and sensitivity to DNA damaging agents are classically tightly controlled by p53. Thus, it is not surprising CSAG2 regulation of these processes proceeds in a p53-dependent manner, although other CSAG2-SIRT1 functions that are p53-independent are very likely. A discussion of this point has been added to the text (p14).

iii) Besides the cancer phenotype experiments, maybe other p53 associated functions such apoptosis or senescence could be tested.

We thank the reviewer for this suggestion. We have examined apoptosis upon DNA damage in the setting of CSAG2 depletion by examining cleaved PARP levels. We found that in both HCT116 (Fig. 5E-F) and A375 (Fig. S2I-J) cells that knockdown of CSAG2 increased cleaved PARP levels in response to genotoxic stress (doxorubicin). These results are consistent with our cytotoxicity findings (Fig. 5A-D).

5) Some WBs of p53 K283ac/p53 need quantification to be convincing because the signal of p53 is saturated in many of them and the ratio is not easy to assess. For instance s mentioned, fig 3D, but also fig 3H, where the last lane seem to have lower levels of p53 compared with the previous ones.

We have quantitated all experiments shown in Figure 3 and shown as the mean +/- SD from 3 independent experiments. p53 ac-K382 levels were normalized to total p53 as suggested. We find that p53 ac-K382 levels are upregulated 10-15 fold and this is dramatically blocked by CSAG2 expression. These results can be found in Fig. 3E, 3G, 3I, 3K, 3L and 3M.

Reviewer #2 (Significance (Required)):

The identification and characterization of a novel SIRT1 protein activator, particularly relevant in the context of cancer, should be of interest to a wide range of researchers of different fields, not only because it represents a novel way to activate SIRT1 activity, but also because it could be potentially used as a target in cancer prognosis and therapeutics. In some contexts where SIRT1 activity has a protective effect, like in neurodegenerative diseases, diabetes or even aging, upregulation of CNAG2 may also be an interesting approach. So far, only one SIRT1 protein activator, AROS, have been described, but in this case there are some contradictory evidences

suggesting that may also act as SIRT inhibitor. As mentioned above, AROS was also shown to activate SIRT1 activity in the context of cancer. If the authors can demonstrate that CSAG2 is a true in vivo SIRT1 activator, and characterize further this activity as mentioned earlier, this would in my opinion increase significantly the relevance of the work.

We thank the reviewer for their kind remarks and pointing out the potential for our study to impact a number of fields. We agree that this work has the potential to directly impact research on a number of diseases, not just cancer. As noted, there have been few discoveries into regulators of SIRT1, especially activators. AROS being one, but its validity has been questioned. We believe that the additional data provided support our conclusions that CSAG2 is a true *in vivo* SIRT1 activator. Our *in vitro* and cell based data, in combination with examination of multiple SIRT1 substrates, provides compelling evidence for CSAG2 as a true SIRT1 activator.

1st Editorial Decision 16th Jun 2020

Dear Ryan,

Thank you for the submission of your revised manuscript to EMBO reports. We have now received reports from the two referees that were asked to re-evaluate your study, which can be found at the end of this email.

As you will see, both referees support the publication of your study, but have some remaining concerns and suggestions to improve the manuscript, we ask you to address in a final revised version of the manuscript. Please also provide a point-by-point response that addresses the remaining referee concerns. I would also support point 3 of referee #1, to put back a model image.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details please refer to our guide to authors:
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See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in

the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

5) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. As the Western blot images are all significantly cropped, I would ask you to submit their source data (scans of entire gels or blots) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

6) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

7) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological or technical replicates - please clearly indicate the nature of the replicate) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

8) Please add up to 5 key words to the title page.

9) Please provide the abstract written in present tense.

10) Please note our new reference format and adjust the final manuscript text accordingly: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

11) Please add a conflict of interest statement and a paragraph detailing the author contributions below the acknowledgements.

12) Table S1 is a dataset. Please upload this as Dataset and name this Dataset EV1. Then please use this nomenclature for the call-outs in the manuscript. Please provide a legend for the dataset on the first TAB of the excel files. Finally, please remove the legend for the table from the main manuscript text.

13) Please add scale bars to all microscopic images. Do not write on the bars in the image. Please define the size in the respective figure legend.

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Achim

Achim Breiling
Editor
EMBO Reports

Referee #1:

In the new version of the manuscript, the authors have answered my major concerns satisfactorily. I believe that the new data have strengthened considerably the connection between CSAG2 and SIRT1 and particularly the link CSAG2-p53. However, I do have a few minor issues:

1) In general, the new data is clear. However, in the case of the new figure 4C, the result is not that easy to assess given than p53 levels look saturated and there is some clear loading variability. This is relevant as many of the results in this work are not black or white. The authors should perform a quantification of this experiment and include the graph and statistical analysis.

2) The labeling in the X-axis of figures 6J and 6K is confusing (white or black ovals). I would suggest change it to avoid misunderstanding.

3) The authors have removed the previous model, and I think this is a mistake. I would suggest adding a new model summarizing the work clearly that may deliver the message that p53 plays a role but it may not be the only target involved.

Referee #2:

The authors have addressed most of my concerns of the previous review.

Two minor points:

1- In Fig. 2, although the authors used 2 different shRNAs, it will technically strength the figure to either use a CRISPR and/or a rescue experiments in the depleted cells.

2- In the interactions reported between CSAG2 and SIRT1, are other known interactor proteins such as AROS also in the same immunoprecipitates?

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Rev_Com_number: RC-2020-00223
New_manu_number: EMBOR-2020-50912V1
Corr_author: Potts
Title: CSAG2 is a cancer-specific activator of SIRT1

Response to Reviewers' Comments

Referee #1:

In the new version of the manuscript, the authors have answered my major concerns satisfactorily. I believe that the new data have strengthened considerably the connection between CSAG2 and SIRT1 and particularly the link CSAG2-p53. However, I do have a few minor issues:

1) In general, the new data is clear. However, in the case of the new figure 4C, the result is not that easy to assess given than p53 levels look saturated and there is some clear loading variability. This is relevant as many of the results in this work are not black or white. The authors should perform a quantification of this experiment and include the graph and statistical analysis.

Quantitation is now included in Fig 4D.

2) The labeling in the X-axis of figures 6J and 6K is confusing (white or black ovals). I would suggest change it to avoid misunderstanding.

Corrected

3) The authors have removed the previous model, and I think this is a mistake. I would suggest adding a new model summarizing the work clearly that may deliver the message that p53 plays a role but it may not be the only target involved.

New Figure 7 is now included

Referee #2:

The authors have addressed most of my concerns of the previous review.

Two minor points:

1- In Fig. 2, although the authors used 2 different shRNAs, it will technically strength the figure to either use a CRISPR and/or a rescue experiments in the depleted cells.

Although, we agree with the reviewer that CSAG2 knockout cells would be helpful this is extremely difficult. CSAG2 and CSAG3 are identical genes and thus both need to be knocked out. Additionally, simple indels are not feasible as the coding sequence is

contained within a single exon. Thus, deletion of each exon is necessary. However, this is quite challenging as three MAGE-A genes are sandwiched between CSAG2 and CSAG3 (Fig 1A) . Thus, the chances of creating 4 breaks (5'/3' CSAG2 and 5'/3' CSAG3) without deleting the intervening MAGE-A gene is nearly impossible. After several months trying this approach we decided shRNAs were the only viable solution.

2- In the interactions reported between CSAG2 and SIRT1, are other known interactor proteins such as AROS also in the same immunoprecipitates?

Other known SIRT1 interactors, like AROS, were not identified in the purification and mass spectrometry analysis of CSAG2. These results are shown in Dataset EV1.

1st Revision - Editorial Decision 10th Jul 2020

Dr. Patrick Potts
St. Jude Children's Research Hospital
Department of Cell and Molecular Biology
262 Danny Thomas Place
Memphis, TN 38105-3678
United States

Dear Dr. Potts,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Please make sure that the deposited AP-MS data will be public upon publication of the paper.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Rev_Com_number: N/a
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Corr_author: Potts
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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: Patrick Ryan Potts
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2020-50912V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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 iustified
- 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	Sample sizes were determined using accepted methods in the field, including power analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Power analysis was performed
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data was excluded from the study
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.	Samples were randomly distributed into control and experimental groups.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were randomly distributed into control and experimental groups.
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.	Whenever possible, blinding was performed.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The animal studies are double blind.
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, the statistical analyses were performed with unpaired, two-tailed student's t test. The data meet the assumption of normal distribution.
Is there an estimate of variation within each group of data?	Yes, the varation was interpreted by mean ± SD.

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Is the variance similar between the groups that are being statistically compared?	Yes.

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).	Antibodies were validated independently, in published literature, or by the commercial source. Whenever possible, antibodies were validated on knockdown/knockout samples. Antibodies used in this study: anti-GAPDH (Cell Signaling Technology, 2118S), anti-Myc (Roche, 11666606001), anti-Acetyl-p53 Lys382 (Cell Signaling Technology, 2525S), anti-p53 (Santa Cruz Biotechnology, sc-126), anti-p21 (Cell Signaling Technology, 2947S), anti-PUMA (Abcam, ab33906), Bax (Cell Signaling Technology, 2772S), anti-H3 (Abcam, ab1791), anti-H4 (Abcam, ab10158), anti-acetylated-H3K14 (Abcam, ab52946), anti-acetylated-H4K16 (Millipore Sigma, 07329), anti-PARP (Cell Signaling Technology, 9542), donkey anti-Rabbit IgG (GE, NA934V), and sheep anti-Mouse IgG (GE, NA931V).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Source: ATCC or generous gifts from investigators. Authentication by STR analysis. Mycoplasma contamination was routinely tested and confirmed negative before experimentation.

* for all hyperlinks, please see the table at the top right of the document

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.	6-8 week old, male, NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice were obtained from Jackson Lab and housed under standard conditions approved by our animal safety committee.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All studies were approved by the St. Jude Children's Research Hospital institutional review committee on animal safety.
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.	All animal studies followed NIH recommendation guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	N/A
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).	N/A
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G- Dual use research of concern

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