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EZH2 cooperates with gain-of-function p53 mutants to promote cancer growth and metastasis

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1st Editorial Decision 8th Jun 2018

Thank you again for the submission of your manuscript (EMBOJ-2018-99599) to The EMBO Journal and in addition providing us with a preliminary revision plan. Thank you also for your patience with my response, which got delayed due to detailed discussions in the team regarding your preliminary point-by-point response. As mentioned earlier, your study has been sent to four referees, and we received reports from all of them, which I enclose below.

The referees acknowledge the potential interest and novelty of your work, although they also express major concerns. In particular, referee #2 raises reservations regarding conclusive demonstration of IRES-dependent EZH2 activity (ref#2, pts.1,2), and states that your claims on direct engagement of EZH2 with the translational machinery are not sufficiently well supported by the data in his/her view (ref#2, pt.4). Referee #3 is critical regarding the EZH2 deletion experiments and asks you to employ more rigorous and precise measures to provide unequivocal proof for independence from EZH2 methyltransferase activity (ref3, pt.1, see also, ref#2, pt. 6 and ref#4, pts1,3). Referee #1 raises issues regarding the human/mouse tumorigenesis data and differential susceptibility of WT vs p53 GoF tumors and states that direct versus indirect effects are not sufficiently explored functionally (ref#1, pts 1,2,6).

We realise that you would - judging from the information provided in the point-by-point letter - be potentially able to address the issues raised by the referees in a revised version of the manuscript.

I judge the comments of the referees to be generally reasonable and can - based on your sensible preliminary response - offer to invite you to revise your manuscript experimentally to address the referees' concerns. I agree that in particular the aspect of in IRES- and methyltransferase activity dependence, and p53 mutant in vivo selectivity of EZH2's effects would need to be conclusively addressed in a revised version of the manuscript to move towards publication.

REFEREE REPORTS:

Referee #1:

In the submitted manuscript, Zhao et al perform RNA immunoprecipitation (RIP) in prostate cancer cell lines to identify RNAs interacting with EZH2, the catalytic subunit of polycomb repressive complex 2 (PRC2) and show that EZH2 binds to 5' UTR of p53 mRNA and thereby increases the translation of both WT and mutant p53 mRNA. They identify a region within EZH2, which is distinct from its SET domain, that mediates the interaction with p53 5'UTR. Using RNAi mediated EZH2 knockdown in various systems as well as rescue experiments with different EZH2 fragments they show that the effect of EZH2 on p53 translation is independent of EZH2 catalytic activity as EZH2 lacking the SET domain shows similar effect on p53 translation as WT EZH2 whereas EZH2 lacking the mRNA binding domain cannot rescue the reduced p53 translation upon EZH2 knockdown. Furthermore, the authors show strong correlation between EZH2 and p53 levels in a mouse model of prostate cancer as well as in various human tumors including prostate, brain, colorectal and pancreatic cancer. Lastly, by inhibiting EZH2 through different reagents in one p53 mutant and one p53 WT prostate cancer cell line, they show that although both p53 WT and mutant cell lines are sensitive to depletion of EZH2 levels, p53 mutant cell line is less sensitive to catalytic inhibition of EZH2. Based on this, the authors hypothesize that targeting EZH2 levels rather than its enzymatic activity would be more effective in treatment of advanced cancer especially those harboring p53 mutation.

The manuscript provides compelling evidence to support an interaction of EZH2 and p53 5'UTR, and its effect on p53 levels. The biochemical data provided in the manuscript to support this is convincing and well controlled. However, the data obtained from the experiments on mouse and human tumors is largely correlative and does not provide conclusive evidence supporting the authors' hypothesis that EZH2 directly affects p53 levels in the tumors and/or tumors harboring p53 mutation are more sensitive to EZH2 depletion.

1. One of the key data in the manuscript is provided in Figure 7C and 7D, which show the sensitivity of p53 WT and p53 mutant prostate cancer cell lines to EZH2 inhibition/depletion. In contrast to the authors' interpretation that there is synthetic lethality between EZH2 depletion and p53 mutation, both WT and mutant p53 cell lines show significant sensitivity to EZH2 depletion. It is also surprising that p53 WT cell line show reduced cell viability despite reduced WT p53 and p21 levels upon EZH2 depletion. This suggest that the effect on tumor cells observed upon EZH2 depletion might not be through changes in p53 or p21 levels but be dependent on the expression of other proteins. Therefore, testing the effect of EZH2 depletion on a large panel of characterized cell lines would help to determine if p53 status is involved in regulating differential sensitivity.
2. Similarly, data in Figure 7E and 7F show that both the tumors with either p53 loss or p53 mutation are sensitive to EZH2 depletion. Although tumors with p53 mutation look more sensitive to EZH2 depletion, the two tumors are very different and it would be difficult to infer anything regarding their differential sensitivity.
3. It is not clear if EZH2 binds to p53 5' UTR independent of PRC2 and whether depletion of other PRC2 members would have similar effects on p53 level. The authors should address this.
4. Figure 6 shows the increased tumor cell growth as well as metastatic potential upon ectopic expression of EZH2 without a SET domain. Assuming the continued expression of endogenous WT EZH2, EZH2deltaSET expression would lead to partial loss of PRC2 activity. Incomplete loss of PRC2 activity has been shown to be sufficient to promote tumor growth (Wassef et al., Genes Dev. 2015 Dec 15; 29(24): 2547-2562) and that would explain the increased tumor cell growth observed upon EZH2deltaSET expression. What are the H3K27me3 levels in these cells? Would expression of WT EZH2 have a similar positive effect on tumor cell growth?
5. How does deletion of the mRNA binding domain affect the catalytic activity of EZH2? Does EZH2deltamRBD form a stable PRC2 complex?
6. In Figure 4, the authors show high EZH2 and p53 levels in a Pten-/- mouse prostate tumor model. The authors claim that it is WT p53 that they are detecting in Pten-/- tumors. If it is, then how can the tumors tolerate such high levels of p53 and Bax? Also, data in Figure 4 show that the deletion of Ezh2 in Pten-/- tumor model does not block tumor development, which is consistent with previous reports (Wassef et al., Genes Dev. 2015 Dec 15; 29(24): 2547-2562) but that would be contradicting to the authors own observation in Figure 7D that show sensitivity of p53 WT cancer cell line to EZH2 inhibition/depletion.

The authors argue that Ezh2 deletion leads to downregulation of WT p53 in Pten-/- tumors and that would potentiate tumorigenesis and would explain why Ezh2 deletion does not affect tumor development in Pten-/- model. However, according to Figure 4D, p53 levels do not drop lower than in WT tissues and thus p53 levels might not contribute to tumor development in this setting.
7. The authors should provide a protocol for the purification of proteins associated with EZH2? Moreover, they should address if DNA or RNA is required for the interactions observed between EZH2 and the proteins shown in Figure 3A. The authors should also write the composition of the BC100 buffer that is used in co-immunoprecipitation experiments. Since this is a buffer that normally contains 100 mM KCl, the co-immunoprecipitation experiments appear to have been done under very low stringency, and it would therefore be meaningful if the authors increased the salt concentrations in the washes to investigate how stable the interactions are between EZH2 and the core members of the PRC2 complex versus the ribosomal proteins reported in the manuscript.
8. How do the authors explain the Myc band in the control lane of the western blot shown in Figure 1F?

Referee #2:

The manuscript from Zhao and colleagues reports that the PRC2 subunit EZH2 increases p53 protein level by recruiting translation factors to the IRES within the 5'UTR of the mRNA. The authors go on to show that, through this mechanism, EZH2 synergises with p53 gain-of-function mutants to promote prostate cancer cell invasion and metastasis.

The study is essentially split into two halves. The first half describes a novel molecular mechanism through which EZH2 increases protein abundance. The authors use native RIP-seq to identify mRNAs that co-precipitate with EZH2 in prostate cancer cell lines. Focusing on p53 mRNA, they report that EZH2 binds to the 5' IRES and that this requires the central region of EZH2. They find that this region of EZH2, and not the methyltransferase domain, is required for EZH2-mediated increase in p53 protein levels. They propose that EZH2 binding to the IRES stimulates translation through an interaction with PABP1 and translation initiation factors. In addition, they show that EZH2 increases p53 mRNA levels.

The second half of the paper seeks to demonstrate a role for this mechanism in cancer. The authors show that EZH2 is necessary for the increase in p53 protein levels in prostate tumours in PTEN KO mice. They then report that EZH2 cooperates with gain of function (GOF) mutant p53 to promote cancer cell growth, invasion and metastasis and that this requires the central RNA binding portion of EZH2 and not its methyltransferase activity. They go on to show that EZH2 depletion is synthetically lethal in cells with GOF mutant but not WT p53 and suggest the dependence on EZH2 as a function of p53 mutant status as a potential explanation for EZH2 functioning as either an oncogene or tumour suppressor in different types of cancer.

The definition of a new potential new mechanism through which EZH2 operates and contributes to oncogenesis, especially one that may explain the dichotomous roles of EZH2 in cancer, is potentially of great interest to the chromatin and cancer fields. However, such a mechanism is quite far outside of our current understanding of EZH2 function and so requires high levels of experimental evidence that this study does not yet provide. In particular, the study does not conclusively demonstrate that the EZH2 increases p53 protein levels through binding to the p53 IRES and increasing ribosomal engagement. The use of superior methods, more controls, and further dissection of the mechanism are required to support the authors' conclusions.

Major concerns

1. EZH2 binding to p53 IRES1. UV-crosslinking based approaches such as PAR-CLIP and iCLIP are universally acknowledged as being superior to the more old fashioned native RNA IP approach used here (and there is no evidence that crosslinked-based approached are susceptible to contamination with non-specific RNAs as stated on p.5. See Brockdorff N. 2013. Noncoding RNA and Polycomb recruitment. RNA 19: 429-442). Native RNA IP is limited to physiological salt, no reducing agents and no ionic detergents and thus lacks stringency. Furthermore, protein-RNA

interactions identified may be indirect and also may only occur after the cell is lysed. Thus, the method does not tell us whether a protein interacts with a particular RNA in cells. Fig 1B shows that EZH2 RIP only enriches for p53 exons, i.e. the mature mRNA. However, EZH2 PAR-CLIP (Kaneko et al., 2014) and iCLIP (Beltran et al., 2016) clearly demonstrate that PRC2 binds in introns, indicating interaction with nascent RNA. The RIP experiments performed in this study also lack necessary controls, including RIP in EZH2 KO or KD cells to show the RNA precipitation is dependent on EZH2. The authors should confirm that EZH2 interacts with p53 mRNA in cells using UV-RIP using lysis and wash conditions that removes any non-crosslinked protein-RNA interactions that may be indirect or non-specific. That EZH2 interacts with p53 RNA and the IRES sequence in vitro is not surprising because the protein interacts with most RNAs in vitro, including bacterial RNA species (Davidovich et al., 2013), and so measurements of EZH2-RNA interaction in vitro is not a sufficiently robust demonstration of EZH2 RNA binding specificity.

2. Requirement for IRES1 for EZH2 mediated upregulation. No data are included to support the reqirement of IRES1 for EZH2 to upregulate p53 protein levels. Also, does EZH2 impact the ratio of full-length vs the N-terminally truncated form of p53 (translated from IRES2)?

3. EZH2 RNA binding region. The authors identify EZH2 residues 336-554 as necessary for RNA binding and also necessary for upregulation of p53 protein levels. However, this is quite a large deletion, removing the MCSS, SANT2 and CXC domains. This region also contacts SUZ12 in the crystal structure (Justin et al,. 2016; Brooun et al., 2016) and is necessary for interaction with CDYL (Yang et al,. 2011). Thus, this deletion could remove other aspects of EZH2 function, or indeed cause loss of all functions. It is also possible that the mutant acts as a dominant negative, and this leads to p53 upregulation.

4. EZH2 interaction with PABP1 and eIF proteins. The interactome of EZH2 has been determined by a number of previous studies and PABP and eIF proteins have not previously been observed (eg. Xu et al., 2015; Kloet et al., 2016; Hauri et al., 2016; Conway et al., 2018). The reporting of the mass-spec data is inadequate - whether peptides for these proteins were enriched in the SFP-EZH2 pull-downs vs the empty vector pull-downs is not shown. There is also no evidence to support the model in Fig 3C that EZH2 directly contacts eIF4G2 but only indirectly contacts the other factors. The domain of EZH2 that interacts with PABP1 and eIF4G2 is not identified and the requirement for these interactions for EZH2 to increase p53 mRNA translation is not investigated.

5. EZH2 increases p53 mRNA levels. After spending time building a model that EZH2 increases p53 protein levels by directly increasing p53 mRNA translation, the authors then go on to show that EZH2 also increases p53 mRNA levels and that this depends on the putative RNA binding domain (p. 11 and Fig EV3F). It is postulated this this is due to EZH2 promoting the formation of a closed mRNA loop but no data is provided to support this model. That EZH2 knockdown might instead be affecting p53 gene transcription (measured using by RT-PCR with intronic primers, or Pol II ChIP or BrU RNA labelling), and this causes the effects on p53 protein levels, is not considered.

6. Requirement for methyltransferase activity. The authors use an EZH2 delta SET mutant and also the EZH2 methyltransferase inhibitor GSK-126 to show that the ability of EZH2 to increase p53 protein levels is independent of its methyltransferase function. However, the methyltransferase inhibitor DZNep reduces p53 protein levels (Fig 7C) and has the same effect as EZH2 ASO on VCaP cell viability (Fig 7D). How do the authors explain this result? Could this because due to effects of DZNep on EZH1? DZNep could be having the same, non-RNA-dependent, repressive effect on PRC2 as the EZH2 mRBD deletion mutant.

Minor concerns.

1. Page 5 states that "Increasing evidence suggests that interactions with long non-coding RNAs (lncRNAs), such as HOTAIR and XIST, are important for the PcD activity of EZH2". However, PRC2 has recently been shown to have no preference for lncRNAs (Kaneko et al., 2014; Beltran et al., 2016), not to bind Xist when measured by RAP or CHART (Chu et al., 2015; McHugh et al., 2015), and not to be required for effects of HOTAIR (Portoso et al., 2017).

2. Fig 1F. The first sample is labeled as IgG RIP from cells lacking Myc-EZH2-WT yet there is a band at the size of EZH2 in the anti-MYC western blot.

3. Fig 2A. The legend does not describe what the sample labeled "Control" is.

4. p.7 states that a "120-nucleotide (nt) region (-120 to -1 nt) immediately adjacent to the translation start in the 5'UTR of p53 mRNA is critical for EZH2 binding (Figure 2B)". However, Fig 2B does not show this region, instead indicating that 81-120, 122-160 and 161-202 are all required.

5. This sentence on p.13 is unclear: "Following Pten deletion, even though Ezh2 protein levels were markedly elevated in Pten-knockout prostate tumors (Figures 4A and 4B), there were a significant portion of acini remained at the high grade prostatic intraepithelial neoplasia (HGPIN)/cancer stage after co-deletion of Ezh2 in the Pten- deleted tumors (Figures 4F and 4G). "

6. p. 13 and Fig 5A. The correlation between p53 and EZH2 protein levels across all cancers is reported to only be 0.2 and the scatter plot in Fig 5A essentially shows no correlation. The correlations in prostate cancer shown in Figs 5B and 5D are more convincing.

7. p. 19, line 2. Should read "These data are consistent...".

8. p. 21 line 9. mRNA not message RNA.

9. p. 29, Figure 1 legend. It is unclear what "generated according to the distinct reads on chromosomal window from RIP-seq" means.

Non-essential suggestions

1. It would have been nice to include WT EZH2 in the experiments show in Figures 6, EV5M and EV5N.

2. p.18 and Fig 7 E and F. To determine the effect on EZH2 on tumours with GOF p53 mutants, cells with WT p53 would be a better control.

3. p. 20. The authors suggest that their findings might explain the dichotomous roles for EZH2 in different types of cancer. Does p53 mutation status correlate with EZH2 overexpression / GOF mutation vs EZH2/SUZ12 deletion?

Referee #3:

This is a huge paper containing 48 separate sub figures in total and as such has proven hard to evaluate in detail. The fundamental hypothesis is that the EZH2 protein has a novel function that does not require its methytransferase activity. This function is to bind to the mRNA of p53 and enhance its translation. This binding is localized to the IRES domain in the 5' UTR of the p53 mRNA and involves the mRBD domain (amino acids 336-554) of the EZH2 protein. In general the studies are well performed and the data is convincing The results are important because they show that EZH2 not only enhances the expression of wild type p53 but also mutant p53, as both, of course, contain the IRES element. This means that high levels of EZH2 can drive the synthesis of large amounts of mutant p53 . As many mutant p53 proteins show a Gain of Oncogenic Function (GOF) this means that the combination of high level activity of EZH2 and mutation in p53 can lead to especially aggressive and invasive tumors . They present some good data to support this in figures 6 and 7 and this EZH2 could emerge as an important modifier gene in the p53 pathway. The main issues are
the degree of specificity of the effect . Does EZH2 bind many mRNA's and how selective is the p53 effect ? Much of the work is driven by overxpression and by correlation so it would be good to see more direct evidence of endogenous interaction for example using proximity ligation approaches.

1) It would be more convincing if they used a point mutant that kills methyltransferase activity to divorce this activity from observed phenotype. While the sub domain work is convincing it does depend on over-expression

2) Some more controls to show p53 mRNA specificity would be useful in Figure 2E (maybe blot for a larger group of non p53 regulated genes/ proteins as at the moment ERK2 levels are the sole control). Using the same experimental system they should also over express EZH2 in these cells and looked for increased p53 levels.They should also do the now standard control of rescuing the si effect with a si resistant variant of EZH2 achieved using third base position variation to retain the correct coding sequence but escape si interaction . This control has proved very valuable in si RNA based studies .

3) They should ectopically express p53 in H1299 (or other p53-null cell line) cells from a plasmid that does not encode 5'UTR (basically pCMV-p53) and show that EZH2 over expression has no effect on p53 levels.

Referee #4:

In this paper, the authors report that EZH2 binds to IRES1 of p53 mRNA, and thereby augments the translation of p53 mRNA as well as the stability of p53 mRNA. This does not require the methyl-transferase activity of EZH2, but rather relies on a distinct RNA-binding domain of EZH2. They further show that in cells and tumors harboring mutant p53, this positive effect of EZH2 on p53 protein expression is important for exerting mutant p53 gain-of-function (GOF). Accordingly, they show that depletion of EZH2 selectively attenuates the growth of tumors driven by mutant p53 GOF, suggesting that EZH2 downregulation may be considered as a selective treatment for such tumors.
Overall, this is a novel and very interesting study, with potential major clinical implications. The work is generally well performed, and many possible pitfalls are addressed by appropriate controls. However, there remain a number of aspects that need to be addressed in order to make the main conclusions more robust, as listed below.

1. Fig. 2E. The authors need to rule out that the reduction in p53 protein upon EZH2 knockdown may be due to decreased p53 protein stability. Furthermore, to show that the effect is dependent on IRES1, the authors should express p53 from expression plasmids that either retain or do not retain IRES1, and show that EZH2 regulates p53 levels in the former but not in the latter case.
2. Fig. 5A,B and Fig. 7B. Is the correlation between p53 and EZH2 RNA levels equally strong in tumors that carry TP53 mutations as compared to those that retain wild type p53? As discussed by the authors in page 17, one might expect that the selective pressures will favor this correlation particularly in mutant p53 tumors.
3. Fig. 6. The authors should express the same mutant p53 from an expression plasmid that either retains or does not retain IRES1, and show (in vitro) that the GOF of mutant p53 is augmented by EZH2 only in the former but not the latter case.

Minor comments:
1. Fig. 2. The numbering of p53 mRNA positions in the various deletion mutants is confusing: whereas Fig. 2C shows numbering based on the translation start site, Fig. 2B uses numbering starting from the 5' end of the 5' UTR. The authors should adopt a single numbering method consistently throughout the paper.

1st Revision - authors' response 30th Sep 2018

EMBOJ-2018-99599
Referee comments:

We would like to thank the Editor and Reviewers for the time to evaluate our work and recognition of the novelty and significance of our study and insightful comments on our manuscript. All the concerns have been considered thoroughly in generating the revised manuscript. Please see our point-by-point response to each comment below.

Referee #1:

In the submitted manuscript, Zhao et al perform RNA immunoprecipitation (RIP) in prostate cancer cell lines to identify RNAs interacting with EZH2, the catalytic subunit of polycomb repressive complex 2 (PRC2) and show that EZH2 binds to 5' UTR of p53 mRNA and thereby increases the translation of both WT and mutant p53 mRNA. They identify a region within EZH2, which is distinct from its SET domain, that mediates the interaction with p53 5'UTR. Using RNAi mediated EZH2 knockdown in various systems as well as rescue experiments with different EZH2 fragments they show that the effect of EZH2 on p53 translation is independent of EZH2 catalytic activity as EZH2 lacking the SET domain shows similar effect on p53 translation as WT EZH2 whereas EZH2 lacking the mRNA binding domain cannot rescue the reduced p53 translation upon EZH2 knockdown. Furthermore, the authors show strong correlation between EZH2 and p53 levels in a mouse model of prostate cancer as well as in various human tumors including prostate, brain, colorectal and pancreatic cancer. Lastly, by inhibiting EZH2 through different reagents in one p53 mutant and one p53 WT prostate cancer cell line, they show that although both p53 WT and mutant cell lines are sensitive to depletion of EZH2 levels, p53 mutant cell line is less sensitive to catalytic inhibition of EZH2. Based on this, the authors hypothesize that targeting EZH2 levels rather than its enzymatic activity would be more effective in treatment of advanced cancer especially those harboring p53 mutation.

The manuscript provides compelling evidence to support an interaction of EZH2 and p53 5'UTR, and its effect on p53 levels. The biochemical data provided in the manuscript to support this is convincing and well controlled. However, the data obtained from the experiments on mouse and human tumors is largely correlative and does not provide conclusive evidence supporting the authors' hypothesis that EZH2 directly affects p53 levels in the tumors and/or tumors harboring p53 mutation are more sensitive to EZH2 depletion.

1. One of the key data in the manuscript is provided in Figure 7C and 7D, which show the sensitivity of p53 WT and p53 mutant prostate cancer cell lines to EZH2 inhibition/depletion. In contrast to the authors' interpretation that there is synthetic lethality between EZH2 depletion and p53 mutation, both WT and mutant p53 cell lines show significant sensitivity to EZH2 depletion. It is also surprising that p53 WT cell line show reduced cell viability despite reduced WT p53 and p21 levels upon EZH2 depletion. This suggest that the effect on tumor cells observed upon EZH2 depletion might not be through changes in p53 or p21 levels but be dependent on the expression of other proteins. Therefore, testing the effect of EZH2 depletion on a large panel of characterized cell lines would help to determine if p53 status is involved in regulating differential sensitivity.

Response: We agree with the reviewer that the effect of EZH2 depletion was not only mediated through the changes in p53 or p21 levels but also dependent on the expression of other proteins such as PRC2 function. As we interpreted the model shown in Figure 7A, EZH2 regulates cancer progression through its Polycomb dependent (PcD) and independent function (PcI) as well as its effect on p53 function.

As shown in the original Figure 7C, large amount of EZH2 and p53 remained in C4-2 cells after treated with DZNep or ASO, we decided to repeat the experiments in C4-2 cells as shown in original Figures 7C and 7D by using a higher concentration of GSK-126, DZNep and ASO. As shown in revised Figures 7C and 7D, GSK-126 only inhibited enzymatic activity-dependent PcD and PcI functions of EZH2, while DZNep and ASO inhibited PcD, PcI and p53 functions by causing the depletion of EZH2 and p53 proteins. Thus, it is not surprising that in p53 WT C4-2 cells, the loss of cell viability caused by DZNep or ASO treatment was smaller compared to GSK-126 treatment (Figure 7D). The difference was likely due to the undesired loss of expression of WT p53 tumor suppressor and its downstream growth-inhibitory protein p21 in DZNep- or ASO-treated cells, but not caused by H3K27me3 level, for which no overt difference was detected among the three different treatments (Figure 7C). In p53 mutated VCaP cells, however, the loss of cell viability caused by DZNep or ASO treatment was much greater compared to GSK-126 treatment (Figure 7D), and this was due to the loss of expression of GOF mutated p53 (R248W) in cells treated with DZNep or ASO, but not GSK126 because there was no overt difference in H3K27me3 level among the three different treatments (Figure 7C).

We agree with the reviewer that findings from two cell lines were not sufficient to support the point. By following the reviewer's suggestion, we repeated the experiments using a panel of cell lines of different cancer types, including breast cancer cell lines MCF7 (p53 WT); MDA-MB-231 (R280K); MDA-MB-435 (G266E); prostate cancer cell lines LNCaP (p53 WT); PC3 (p53 loss); 22RV1 (Q331R). Similar to the results shown in Figure 7D, we demonstrated that treatment with DZNep or ASO resulted in much greater inhibitory effect on viability of p53 GOF mutated 22RV1, MDA-MB-231 and MDA-MB-435 cell lines compared to p53 WT LNCaP and MCF7 cells (Figures EV7C and EV7D). Moreover, treatment with GSK-126, DZNep or ASO resulted in similar inhibitory effect on viability of PC3 cells, which is a p53-null cell line, and ectopic expression of p53 GOF mutant R248W in PC3 cells resulted in a similar result seen in cell lines expressing endogenous GOF mutant such as 22RV1, MDA-MB-231 and MDA-MB-435 (Figures EV7C and EV7D). Thus, our new data indicate that the findings in p53 WT (C4-2) and p53 GOF mutated (VCaP) cell lines are fully supported by findings in a large panel of characterized cell lines. Thus, we provide evidence for the selective dependence of EZH2's effect on mutated p53 in a large panel of characterized cell lines.

2. Similarly, data in Figure 7E and 7F show that both the tumors with either p53 loss or p53 mutation are sensitive to EZH2 depletion. Although tumors with p53 mutation look more sensitive to EZH2 depletion, the two tumors are very different and it would be difficult to infer anything regarding their differential sensitivity.

Response: We are very sorry for not being able to provide detailed information about the p53 loss and mutated tumors from genetically engineered mice (GEM) in the original submission. Indeed, we reported very recently (Blee et al., Clin Cancer Res. 2018 Sep 15;24(18):4551-4565), $Pten^{pc-/-}$;$p53^{pc-/R172H}$ and $Pten^{pc-/-}$;$p53^{pc-/-}$ mice were littermates generated by crossing *PB-Cre*4;*Pten*$^{loxP/loxP}$ mice with $p53^{loxP/LSL-R172H}$ mice (via multiple rounds of breeding). Thus, generally speaking p53 loss and p53 mutant mice we used had the similar genetic background. This information has been added to the Materials and Methods section.

To further address this concern, we performed additional experiments using the same cell line but with or without stable expression of p53 mutant. To this end, p53-null, PTEN-inactivated human prostate cancer cell line PC3 was infected with empty lentiviral vector or lentiviral vector for full-length p53 mutant R248W, a prostate cancer-associated GOF mutation of p53 (Song H, et al. Nat Cell Biol, 2007 May;9(5):573-80). We then compared the sensitivity of p53-null and p53 R248-expressing PC3 cells to EZH2 inhibitor GSK-126 or its depletors DZNep and ASO.

Our new data showed that treatment with GSK-126, DZNep and ASO resulted in similar inhibitory effect on cell viability and all treatments had no effect on expression of $p21^{CIP1}$ in p53-null PC3 cells (Figures EV7C and EV7D). In support of our observations in other cell lines examined (Figures 7C, 7D, EV7C and EV7D), treatment with DZNep and ASO resulted in much greater inhibitory effect on viability of p53 R248W-expressing PC3 cells compared to p53-null PC3 cells (Figures EV7C and EV7D). Accordingly, we demonstrated that ectopic expression of p53 R248W increased PC3 xenograft tumor growth in mice, but p53 R248W tumors were more sensitive to EZH2 ASO treatment compared to p53-null PC3 tumors (Figures EV7I and EV7J). Therefore, these new data from in vitro and in vivo studies were consistent with the data from mouse prostate tumors shown in Figures 7E and 7F.

3. It is not clear if EZH2 binds to p53 5' UTR independent of PRC2 and whether depletion of other PRC2 members would have similar effects on p53 level. The authors should address this.

Response: To address this question, we firstly did the PAR-CLIP using EZH2, EED and SUZ12 antibodies separately in C4-2 cells. We found that only EZH2 but not EED and SUZ12 bound to p53 mRNA (Figure EV3D). The data suggest that EZH2 binds to p53 mRNA, but not other components of the PRC2 complex.

We further knocked down (KD) endogenous SUZ12 or EED by gene-specific shRNAs in prostate cancer cell lines C4-2 (p53 WT) and VCaP (p53 mutant – R248W) to determine if EZH2 binds to p53 5' UTR independent of PRC2. Western blot analysis showed that both SUZ2 and EED were effectively knocked down (Figure EV3E). Different from the effect of EZH2 KD, which markedly decreased p53 level, KD of endogenous SUZ12 and EED only slightly decreased p53 protein level

(Figure EV3E), and the effect of SUZ12 and EED KD was likely caused by decreased EZH2 protein levels (Figure EV3E), which is consistent with the previous reports that the level of EZH2 is dependent the intact PRC2 complex (Pasini D et al., EMBO J. 2004 13;23(20): 4061-71; Xu J et al., Mol Cell 2015 57(2): 304-316). Thus, our new data not only show that depletion of other PRC2 members such as SUZ12 and EED do not have similar effects as EZH2 depletion on p53 level, but also suggest that EZH2 regulates p53 level independent of PRC2.

4. Figure 6 shows the increased tumor cell growth as well as metastatic potential upon ectopic expression of EZH2 without a SET domain. Assuming the continued expression of endogenous WT EZH2, EZH2deltaSET expression would lead to partial loss of PRC2 activity. Incomplete loss of PRC2 activity has been shown to be sufficient to promote tumor growth (Wassef et al., Genes Dev. 2015 Dec 15; 29(24): 2547-2562) and that would explain the increased tumor cell growth observed upon EZH2deltaSET expression. What are the H3K27me3 levels in these cells? Would expression of WT EZH2 have a similar positive effect on tumor cell growth?

Response: To determine if EZH2deltaSET expression would lead to partial loss of PRC2 activity, as suggested by the reviewer, we examined the H3K27me3 levels in EZH2deltaSET-expressing cells cultured in vitro. We also overexpressed EZH2 WT in cells to examine the H3K27me3 level by WB and cell viability by MTS assay in comparison to EV and EZH2deltaSET groups.

We demonstrated that only ectopic expression of EZH2-WT induced upregulation in H3K27 methylation (Figure EV5R). However, compared to EV group, no change in H3K27me3 level was observed in EZH2ΔSET- and EZH2ΔmRBD-expressing cells (Figure EV5R). These data suggest that expression of EZH2ΔSET or EZH2ΔmRBD did not induce the partial loss of PRC2 activity in EZH2ΔSET- or EZH2ΔmRBD-expressing cells. No change in PRC2 function (H3K27me3) was consistent with the observation of no change in the protein level of endogenous EZH2 in these cells (Figure EV5P and EV6G). Because there was no change in PRC2 function, the alterations in cell viability can be attributed to the changes in p53 levels. We also found that EZH2 full length and EZH2ΔSET but not EZH2ΔmRBD can increase the cell viability in VCaP cells (Figure EV5R). We further found that EZH2ΔmRBD did bind to EED, but not SUZ12 (Figure EV2G). Thus, our data is consistent with the previous report (Diego Pasini, et al. EMBO J, 2004) that EZH2 cannot promote H3K27 methylation without binding with SUZ12.

5. How does deletion of the mRNA binding domain affect the catalytic activity of EZH2? Does EZH2deltamRBD form a stable PRC2 complex?

Response: Based upon the previous reports (Kaneko S et al., Genes & Dev 2010 Dec 1;24(23):2615-20; Brooun A et al., Nature Communications, 2016 Apr 28;7:11384), we predict that the EZH2-delta-mRBD mutant should not have catalytic activity since it is likely that it cannot forms a functional PRC2 complex by binding to SUZ12. We transfected Myc-tagged WT EZH2 and EZH2-delta-mRBD mutant into C4-2 cells to determine whether EZH2-delta-mRBD mutant can bind to SUZ12 and EED by co-IP and to determine whether expression of EZH2-delta-mRBD mutant affects H3K27me3 levels by WB. We found that EZH2ΔmRBD did bind to EED, but not SUZ12 (Figure EV2G). Thus, EZH2ΔmRBD cannot form a functional PRC2 complex and had no effect on the activity of the PRC2 complex in cells. Indeed, we did not detect any overt change in H3K27me3 level in EZH2ΔmRBD-expressing cells (Figure EV2G). Therefore, consistent with the previous report (Pasini D, et al. EMBO J, 2004 Oct 13;23(20):4061-71), EZH2 can no longer promote H3K27 methylation when lose its binding with SUZ12.

6. In Figure 4, the authors show high EZH2 and p53 levels in a Pten-/- mouse prostate tumor model. The authors claim that it is WT p53 that they are detecting in Pten-/- tumors. If it is, then how can the tumors tolerate such high levels of p53 and Bax? Also, data in Figure 4 show that the deletion of Ezh2 in Pten-/- tumor model does not block tumor development, which is consistent with previous reports (Wassef et al., Genes Dev. 2015 Dec 15; 29(24): 2547-2562) but that would be contradicting to the authors own observation in Figure 7D that show sensitivity of p53 WT cancer cell line to EZH2 inhibition/depletion.
The authors argue that Ezh2 deletion leads to downregulation of WT p53 in Pten-/- tumors and that would potentiate tumorigenesis and would explain why Ezh2 deletion does not affect tumor development in Pten-/- model. However, according to Figure 4D, p53 levels do not drop lower than in WT tissues and thus p53 levels might not contribute to tumor development in this setting.

Response: As we indicated in the manuscript, it has been shown previously that p53 protein is upregulated in Pten homozygous deletion prostate tumors in mice (Chen Z et al. Nature 2005 Aug 4;436(7051):725-30). The same study also indicates that increased expression of p53 causes senescence in Pten-null prostate tumors, which therefore prevents further progression of Pten-null tumors (Chen Z et al. Nature 2005 Aug 4;436(7051):725-30).

We believe the data shown in Figure 4 are not entirely contradictory to those shown in Figure 7D. We demonstrated that there were a significant portion of acini remained at the high grade prostatic intraepithelial neoplasia (HGPIN)/cancer stage after co-deletion of Ezh2 in the Pten-deleted tumors (Figures 4F and 4G), indicating that homozygous deletion of Ezh2 failed to completely block Pten deletion-induced tumorigenesis in the prostate with the background of wild-type p53. Consistently, we demonstrated that effective depletion of EZH2 in p53-WT C4-2 cells with DNZep decreased cell growth, but cells still grew faster than those treated with EZH2 enzymatic inhibitor GSK-126 (Original Figure 7D) and this result could be explained by compromised expression of WT (tumor suppressive) p53 and p21 (Original Figure 7C). Similar trend was observed in cells treated with EZH2 ASO (5 μM); however, the difference in growth of cells treated with EZH2 ASO and GSK126 is not statistically significant (Original Figure 7D), and this is likely caused by insufficient depletion of EZH2 by ASO at this concentration (Original Figure 7C). To substantiate this hypothesis, we repeated this experiment by using a higher concentration of ASO to completely deplete EZH2 in C4-2 cells. Western blot analysis showed that H3K27me3 expression was completely abolished by the higher concentration of drugs and ASOs (Figure 7C, right panel). MTS assay showed that cells treated with DZNep and ASO grew relatively faster than those treated with GSK-126 while these cells all grew much slower than control C4-2 cells (Figure 7D, right panel). The data suggest that loss of p53 WT due to EZH2 depletion may partially counteract against the growth inhibitory effect of DZNep and EZH2 ASO.

7. The authors should provide a protocol for the purification of proteins associated with EZH2? Moreover, they should address if DNA or RNA is required for the interactions observed between EZH2 and the proteins shown in Figure 3A. The authors should also write the composition of the BC100 buffer that is used in co-immunoprecipitation experiments. Since this is a buffer that normally contains 100 mM KCl, the co-immunoprecipitation experiments appear to have been done under very low stringency, and it would therefore be meaningful if the authors increased the salt concentrations in the washes to investigate how stable the interactions are between EZH2 and the core members of the PRC2 complex versus the ribosomal proteins reported in the manuscript.

Response: We agree with the reviewer that we should provide a detailed protocol for the purification of proteins associated with EZH2. We employed the protocol described previously (Feng L et al., Genes Dev 2009 Mar 15;23(6):719-28) and used buffer with the concentration of KCl > 100 mM.

Briefly, 293T cells were transfected with SFB backbone vector or SFB-tagged EZH2. 36 h after transfection, cells were lysed by lysis buffer (40 mM Tris-HCl, pH7.5, 300 mM KCl, 2mM EDTA, 0.1% Triton X-100) with 5 mM β-glycerophosphate, 10 mM NaF, and 1μg/mL pepstatin-A at 4°C for 3 h. The supernatant were neutralized with same volume low salt buffer (40 mM Tris-HCl, pH7.5, 25 mM KCl, 2mM EDTA, 0.1% Triton X-100) and incubated with streptavidin sepharose beads (GE) at 4°C overnight. The beads were washed with wash buffer (40 mM Tris-HCl, pH7.5, 150 mM KCl, 2mM EDTA, 0.1% Triton X-100, 5 mM β-glycerophosphate, 10 mM NaF, and 1μg/mL pepstatin-A) for six times and then eluted by 2 mM biotin (Sigma) for 1 h at 4°C twice. The elution products were incubated with S-protein agarose beads (Novagen) overnight at 4°C. The beads were washed by six times with NETN buffer (20 mM Tris-HCl, pH7.5, 100 mM NaCl, 1mM EDTA, 0.5% Nonidet P-40) with 50 mM β-glycerophosphate, 10 mM NaF and 1μg/mL pepstatin-A. The products bound to S-protein agarose beads were subjected to SDS-PAGE and visualized by silver staining. The detailed protein purification protocol has been included in the Materials and Methods section in the revised manuscript.

We also agree with the reviewer that we should address if DNA or RNA is required for the interactions observed between EZH2 and the proteins shown in Figure 3A. We treated cell lysis with DNase or RNase before co-IP assay. We then examined the binding between EZH2 and eIF4G2 using co-IP assay. We found that EZH2 still bound to eIF4G2 and PABP1 after DNase treatment

(Figure EV3B). However, EZH2 failed to bind to eIF4G2 and PABP1 after RNase treatment (Figure 3C). The effectiveness of DNase treatment was evident in the loss of p53 promoter amplification by PCR using genomic DNA as template (Figure EV3B). RNase treatment was evident in the loss of GAPDH mRNA amplification in RT-PCR assay (Figure 3C). These data suggest that EZH2 binding to eIF4G2 and PABP1 is RNA-mediated. Based upon these new findings, we have accordingly removed our working model shown in original Figure 3C.

8. How do the authors explain the Myc band in the control lane of the western blot shown in Figure 1F?

Response: We are very sorry for the incorrect labeling. It was a mislabeling in the original Figure 1F. Myc-EZH2-WT was also transfected in cells of the IgG RIP group and such an error has been corrected in new Figure 1F.

Referee #2:

The manuscript from Zhao and colleagues reports that the PRC2 subunit EZH2 increases p53 protein level by recruiting translation factors to the IRES within the 5'UTR of the mRNA. The authors go on to show that, through this mechanism, EZH2 synergises with p53 gain-of-function mutants to promote prostate cancer cell invasion and metastasis.

The study is essentially split into two halves. The first half describes a novel molecular mechanism through which EZH2 increases protein abundance. The authors use native RIP-seq to identify mRNAs that co-precipitate with EZH2 in prostate cancer cell lines. Focusing on p53 mRNA, they report that EZH2 binds to the 5' IRES and that this requires the central region of EZH2. They find that this region of EZH2, and not the methyltransferase domain, is required for EZH2-mediated increase in p53 protein levels. They propose that EZH2 binding to the IRES stimulates translation through an interaction with PABP1 and translation initiation factors. In addition, they show that EZH2 increases p53 mRNA levels.

The second half of the paper seeks to demonstrate a role for this mechanism in cancer. The authors show that EZH2 is necessary for the increase in p53 protein levels in prostate tumours in PTEN KO mice. They then report that EZH2 cooperates with gain of function (GOF) mutant p53 to promote cancer cell growth, invasion and metastasis and that this requires the central RNA binding portion of EZH2 and not its methyltransferase activity. They go on to show that EZH2 depletion is synthetically lethal in cells with GOF mutant but not WT p53 and suggest the dependence on EZH2 as a function of p53 mutant status as a potential explanation for EZH2 functioning as either an oncogene or tumour suppressor in different types of cancer.

The definition of a new potential new mechanism through which EZH2 operates and contributes to oncogenesis, especially one that may explain the dichotomous roles of EZH2 in cancer, is potentially of great interest to the chromatin and cancer fields. However, such a mechanism is quite far outside of our current understanding of EZH2 function and so requires high levels of experimental evidence that this study does not yet provide. In particular, the study does not conclusively demonstrate that the EZH2 increases p53 protein levels through binding to the p53 IRES and increasing ribosomal engagement. The use of superior methods, more controls, and further dissection of the mechanism are required to support the authors' conclusions.

Major concerns
1. EZH2 binding to p53 IRES1. UV-crosslinking based approaches such as PAR-CLIP and iCLIP are universally acknowledged as being superior to the more old fashioned native RNA IP approach used here (and there is no evidence that crosslinked-based approached are susceptible to contamination with non-specific RNAs as stated on p.5. See Brockdorff N. 2013. Noncoding RNA and Polycomb recruitment. RNA 19: 429-442). Native RNA IP is limited to physiological salt, no reducing agents and no ionic detergents and thus lacks stringency. Furthermore, protein-RNA interactions identified may be indirect and also may only occur after the cell is lysed. Thus, the method does not tell us whether a protein interacts with a particular RNA in cells. Fig 1B shows that EZH2 RIP only enriches for p53 exons, i.e. the mature mRNA. However, EZH2 PAR-CLIP

(Kaneko et al., 2014) and iCLIP (Beltran et al., 2016) clearly demonstrate that PRC2 binds in introns, indicating interaction with nascent RNA. The RIP experiments performed in this study also lack necessary controls, including RIP in EZH2 KO or KD cells to show the RNA precipitation is dependent on EZH2. The authors should confirm that EZH2 interacts with p53 mRNA in cells using UV-RIP using lysis and wash conditions that removes any non-crosslinked protein-RNA interactions that may be indirect or non-specific. That EZH2 interacts with p53 RNA and the IRES sequence in vitro is not surprising because the protein interacts with most RNAs in vitro, including bacterial RNA species (Davidovich et al., 2013), and so measurements of EZH2-RNA interaction in vitro is not a sufficiently robust demonstration of EZH2 RNA binding specificity.

Response: As suggested by the reviewer, we determined EZH2 binding with p53 mRNA in the cells using PAR-CLIP. We pulled down EZH2 associated RNA using PAR-CLIP in C4-2 and VCaP cells with or without EZH2 knockdown. Experimental details were provided in Materials and Methods. Using this approach, we demonstrated that EZH2, but not the IgG bound to p53 mRNA in both C4-2 (Figure 1C) and VCaP (Figure EV5O). Importantly, knocking down EZH2 impaired EZH2 binding with p53 mRNA (Figures 1C and EV5O), suggesting that the binding signal we detected was EZH2-specific.

2. Requirement for IRES1 for EZH2 mediated upregulation. No data are included to support the requirement of IRES1 for EZH2 to upregulate p53 protein levels. Also, does EZH2 impact the ratio of full-length vs the N-terminally truncated form of p53 (translated from IRES2)?

Response: As suggested by the reviewer, we co-transfected EZH2 and pcDNA-p53-FL (5'UTR+CDS+3'UTR) or pcNDA-p53-ΔIRES1 plasmids into p53-null PC3 cells and determine the effect of EZH2 overexpression on p53 protein level by WB. Our new data showed that EZH2 overexpression upregulated protein levels of p53-FL, but not p53ΔIRES1 in PC3 cells (Figure EV2C). These data suggest that EZH2 regulates p53 protein level through IRES1.

To further address this important issue, we generated two new p53 plasmids. One is p53 full length (5'-UTR+CDS+3'-UTR as shown in Figure 1G) in pcDNA backbone vector with a flag tag, and the other is p53 full length with deletion of IRES1 and the first (+1) ATG, which encodes a truncated p53 (p53/47 or ΔNp53) translated from IRES2). IRES2 is located in the first 120 nucleotides of the encoding sequence of the p53 mRNA (Malbert-Colas et al., Mol Cell 2014). We transfected these two plasmids individually or together into PC3 cells with or without EZH2 overexpression and determined the flag-tagged p53 protein expression by WB. We demonstrated that EZH2 overexpression only increased protein levels of full length p53, but not the IRES1 deletion mutant (Figure EV2B). Thus, our new data show that EZH2 does impact the ratio of full-length vs the N-terminally truncated form of p53 translated from IRES2.

3. EZH2 RNA binding region. The authors identify EZH2 residues 336-554 as necessary for RNA binding and also necessary for upregulation of p53 protein levels. However, this is quite a large deletion, removing the MCSS, SANT2 and CXC domains. This region also contacts SUZ12 in the crystal structure (Justin et al,. 2016; Brooun et al., 2016) and is necessary for interaction with CDYL (Yang et al,. 2011). Thus, this deletion could remove other aspects of EZH2 function, or indeed cause loss of all functions. It is also possible that the mutant acts as a dominant negative, and this leads to p53 upregulation.

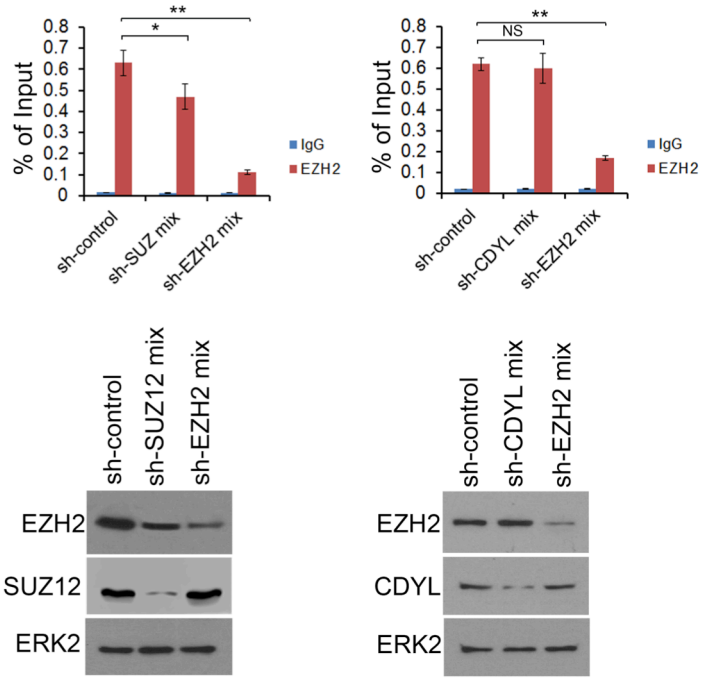
Response: We agree with the reviewer that this deletion removes several domains of EZH2, particularly the region required for EZH2 interaction with SUZ12 and CDYL. To determine if this deletion affects these aspects of EZH2 functions, we transfected expression vectors for Myc-EZH2 WT and Myc-EZH2deltamRBD into C4-2 cells and examined their ability to bind to SUZ12. We found that EZH2ΔmRBD did not bind to SUZ12 and CDYL and had no effect on H3K27me3 level in cells (Figure EV2G). Thus, EZH2ΔmRBD cannot form a functional PRC2 complex and had no effect on the activity of the PRC2 complex in cells. Furthermore, EZH2ΔmRBD was not able to compete with WT EZH2 for SUZ12 binding, suggesting that it may not be able to act as a dominant negative factor to decrease the PRC2 function, which is consistent with our finding that no overt change in H3K27me3 level was detected in EZH2ΔmRBD-expressing cells (Figure EV2G).

To address review's question if SUZ12 or CDYL was involved in regulating p53, we knocked down endogenous SUZ12 and CDYL by shRNA in prostate cancer cell line C4-2 (p53 WT) to determine

if EZH2 binds to p53 5' UTR independent of PRC2 complex or CDYL using PAR-CLIP assay. We demonstrated that knockdown of CDYL had no effect on the protein level of EZH2 and EZH2 binding with p53 mRNA (Review Figure 1, see below).

Different from the effect of EZH2 KD, which markedly decreased p53 level, KD of endogenous SUZ12 only slightly decreased p53 protein level (Figure EV3E and Review Figure 1, see below), and the effect of SUZ12 KD was likely caused by decreased EZH2 protein levels (Figure EV3E and Review Figure 1, see below), which is consistent with the previous report that the level of EZH2 is affected by depletion of SUZ12 (Pasini D et al., EMBO J. 2004 13;23(20): 4061-71; Xu J et al., Mol Cell 2015 57(2): 304-316). Accordingly, we observed that knockdown of SUZ12 only slightly decreased EZH2 binding of p53 mRNA as revealed by PAR-CLIP assay (Review Figure 1, see below).

Together, these data suggest that EZH2 binding of p53 mRNA is not affected by SUZ12 and CDYL.



Review Figure 1. At 40 h after transfection with indicated shRNAs, C4-2 cells treated with 100 μM 4-thiouridine (4SU) for 8 h were subjected to PAR-CLIP assay, followed by RT-qPCR measurement of p53 mRNA immunoprecipitated by IgG or anti-EZH2 antibody (Top). Effectiveness of knockdown was evaluated by western blot (Botton). ERK2 was used as a loading control. Data shown as means ± SD (n=3). * P<0.05, ** P<0.01, NS, no significance.

4. EZH2 interaction with PABP1 and eIF proteins. The interactome of EZH2 has been determined by a number of previous studies and PABP and eIF proteins have not previously been observed (eg. Xu et al., 2015; Kloet et al., 2016; Hauri et al., 2016; Conway et al., 2018). The reporting of the mass-spec data is inadequate - whether peptides for these proteins were enriched in the SFP-EZH2 pull-downs vs the empty vector pull-downs is not shown. There is also no evidence to support the model in Fig 3C that EZH2 directly contacts eIF4G2 but only indirectly contacts the other factors. The domain of EZH2 that interacts with PABP1 and eIF4G2 is not identified and the requirement for these interactions for EZH2 to increase p53 mRNA translation is not investigated.

Response: As we reported in our manuscript, we performed tandem affinity purification and mass spectrometry (TAP-MS) in 293T cells transfected with an empty vector containing S, Flag, and Biotin-binding-protein-(streptavidin)-binding-peptide (SFB) tags or SFB-tagged EZH2. As suggested by the reviewer, we have provided the list of proteins pulled down by SFB-EZH2 and SFB-vector empty vector (Table EV2).

As to why PABP and eIF proteins have not previously been observed by a number of previous studies, we believe this could be caused by the differences in many factors such as tagged proteins used, lysate from different cell lines and/or different purification strategies as we discussed below.

We noticed that in the studies done by Xu et al., and Hauri et al., the authors ectopically expressed three core components of PRC2 (EZH2, EED and SUZ12) simultaneously in cells prior to protein purification (Xu J et al., Mol Cell, 2015 Jan 22;57(2):304-316; Hauri S et al., Cell Rep, 2016 Oct 4;17(2):583-595). In our system, we only expressed SFB-tagged EZH2 alone, but not the other core components of PRC2. According to previously reports (Hauri S et al., Cell Rep, 2016 Oct 4;17(2):583-595), they used two baits for purification, that is, they used EZH2 plus another protein to do pull down assay. The proteins they found were the partners of at least those two baits. It is likely that the proteins identified by Hauri et al. are those that require the presence of the intact PRC2 complex. In contrast, in our system we performed tandem purifications using two tags, but targeting the same protein. The proteins we identified are those that were pulled down by EZH2 alone. Thus, it makes sense that the proteins pulled down in our experiments are different from those reported in these two papers and suggests that the proteins we identified should be broader than those only bind to the intact PRC2 complex, which was the case that we identified several proteins beyond the components of the PRC2 complex. It is also a novel aspect of our study by defining EZH2 solo binding proteins, but not new PRC2 complex members, thereby further reinforcing the concept that we identify a non-canonical PRC2 function of EZH2.

We identified eIF4G2 and PABP1 as the binding proteins of EZH2 using the whole cell lysate whereas Xu et al., Kloet et al. and Conway used the nuclear extracts for protein purification (Xu J et al., Mol Cell, 2015 Jan 22;57(2):304-316; Kloet S et al., Nat Struct Mol Biol, 2016 Jul;23(7):682-690; Conway E et al., Mol Cell, 2018 May 3;70(3):408-421). Additionally, Conway et al. performed protein purification using Flag-tagged PHF1/PCL1, a component of PRC2 and therefore PRC2 interacting proteins and those specifically bind to PHF1 should be identified in their study, but it is not surprising that the proteins they identified are different from ours.

To determine whether EZH2 interacts with PABP1 and eIF4G2 directly or indirectly through other factors such as RNA, we treated lysate of C4-2 cells with RNaseA prior to co-IP assays. The effectiveness of RNaseA treatment was evident by the depletion of GAPDH mRNA in cell lysate (Figure 3C). Importantly, RNaseA treatment completely abolished the interaction of EZH2 with both PABP1 and eIF4G2 (Figure 3C). Additionally, we cloned PABP1 and eIF4G2 into pGEX-4T1 (GST) vector, and Myc-EZH2 into pcDNA3.1 vector, these proteins were purified from bacteria and TNT Quick coupled transcription/translation kit through T7 promoter, respectively. The purified proteins were used for in vitro GST pull down assay to determine if addition of p53 full-length mRNA would facilitate their interaction. We demonstrated that GST-PABP1 or GST-eIF4G2 bound to EZH2 in vitro only in the presence of full-length p53 mRNA (Figure EV3C). The data suggest that EZH2 binds to eIF4G2 and PABP1 in a manner dependent on RNA.

Based upon these new data, we have also removed the model shown in original Figure 3C.

5. EZH2 increases p53 mRNA levels. After spending time building a model that EZH2 increases p53 protein levels by directly increasing p53 mRNA translation, the authors then go on to show that EZH2 also increases p53 mRNA levels and that this depends on the putative RNA binding domain (p. 11 and Fig EV3F). It is postulated this this is due to EZH2 promoting the formation of a closed mRNA loop but no data is provided to support this model. That EZH2 knockdown might instead be affecting p53 gene transcription (measured using by RT-PCR with intronic primers, or Pol II ChIP or BrU RNA labelling), and this causes the effects on p53 protein levels, is not considered.

Response: We agree with the reviewer that we have no data to support that EZH2 increases p53 mRNA levels by promoting the formation of a closed mRNA loop. Since we only have data showing 1) EZH2 binds to the IRES1 in the 5'UTR of p53 mRNA; 2) EZH2 binds to PABP1, a

protein increasing mRNA stability; 3) EZH2 increases p53 mRNA levels. Therefore, the hypothetic model shown in original Figure 3C has been removed.

As suggested by the reviewer, we knocked down endogenous EZH2 in C4-2 cells and then used intronic primers to perform RT-PCR to determine the effect of EZH2 KD on p53 pre-mRNA level. Using these cells, we also performed total Pol II (N20) ChIP in p53 promoter to determine whether EZH2 knockdown affect p53 transcript by affecting Pol II loading at the p53 promoter. Our new data showed that knockdown of EZH2 had little or no effect on p53 pre-RNA level and the total Pol II occupancy in the p53 promoter (Figure EV3G). These new data further suggest that EZH2 regulation of p53 RNA level is not mediated through the transcription level.

6. Requirement for methyltransferase activity. The authors use an EZH2 delta SET mutant and also the EZH2 methyltransferase inhibitor GSK-126 to show that the ability of EZH2 to increase p53 protein levels is independent of its methyltransferase function. However, the methyltransferase inhibitor DZNep reduces p53 protein levels (Fig 7C) and has the same effect as EZH2 ASO on VCaP cell viability (Fig 7D). How do the authors explain this result? Could this because due to effects of DZNep on EZH1? DZNep could be having the same, non-RNA-dependent, repressive effect on PRC2 as the EZH2 mRBD deletion mutant.

Response: As reported previously (Tan et al., Genes Dev. 2007 May 1;21(9):1050-63), DZNep affects both methyltransferase (MTase) activity and protein stability/level of EZH2. Therefore, unlike GSK126, DZNep can also inhibit MTase-independent functions of EZH2 by depleting EZH2 protein, an effect similar to similar to EZH2 ASO. Thus, as shown in Figure 7C, similar to EZH2 ASO, DZNep, but not GSK126 can reduce p53 protein level.

To determine if the effect of DZNep on p53 expression is mediated by its effect on EZH1, we examined the effect of DZNep on p53 protein level in EZH1 knockout (KO) VCaP cells by WB. Our new showed that DZNep treatment almost completely depleted p53 protein even in EZH1 KO cells (Figure EV7E, left panel), suggesting that EZH2 regulate p53 through a mechanism independent of EZH1 function.

Additionally, we knocked down the endogenous EZH2 and rescued the shRNA-resistant EZH2ΔmRBD and then compared the effect of DZNep on H3K27me3 level (PRC2 function) in VCaP cells. We demonstrated that DZNep downregulated both EZH2 protein and H3K27me3 level, but restored expression of EZH2ΔmRBD failed to upregulate H3K27me3 level (Figure EV7E, right panel). These data suggest that functioning as a depletor of EZH2, DZNep can inhibit both Polycomb-dependent and p53-regulatory functions of EZH2. In contrast, the EZH2 mRBD deletion mutant cannot regulate both functions.

Minor concerns.

1. Page 5 states that "Increasing evidence suggests that interactions with long non-coding RNAs (lncRNAs), such as HOTAIR and XIST, are important for the PcD activity of EZH2". However, PRC2 has recently been shown to have no preference for lncRNAs (Kaneko et al., 2014; Beltran et al., 2016), not to bind Xist when measured by RAP or CHART (Chu et al., 2015; McHugh et al., 2015), and not to be required for effects of HOTAIR (Portoso et al., 2017).

Response: We agree with the reviewer. We not only changed wording, but also removed those references we cited originally.

2. Fig 1F. The first sample is labeled as IgG RIP from cells lacking Myc-EZH2-WT yet there is a band at the size of EZH2 in the anti-MYC western blot.

Response: We are very sorry for our incorrect labeling. It was a mislabeling in the original Figure 1F. Cells for IgG RIP experiment were also transfected with Myc-EZH2-WT and such an error has been corrected in new Figure 1F.

3. Fig 2A. The legend does not describe what the sample labeled "Control" is.

Response: We made the correction according to the reviewer's comments. The p53 fragments were subcloned in the backbone vector pcDNA3.1(+). Therefore, we changed "Control" to "pcDNA3.1(+).

4. p.7 states that a "120-nucleotide (nt) region (-120 to -1 nt) immediately adjacent to the translation start in the 5'UTR of p53 mRNA is critical for EZH2 binding (Figure 2B)". However, Fig 2B does not show this region, instead indicating that 81-120, 122-160 and 161-202 are all required.

Response: We made the correction according to the reviewer's comments. In Figure 2B, we adopted a single numbering method for the numbering of p53 mRNA positions in different mutants. Accordingly, we changed "1-40" to *"-202 ~ -182*"; "41-80" to *"-181 ~ -123*"; "81-120" to *"-122 ~ -83*"; changed "122-160" to *"-82 ~ -43"*; changed "161-202" to *"-42 ~ -1*".

5. This sentence on p.13 is unclear: "Following Pten deletion, even though Ezh2 protein levels were markedly elevated in Pten-knockout prostate tumors (Figures 4A and 4B), there were a significant portion of acini remained at the high grade prostatic intraepithelial neoplasia (HGPIN)/cancer stage after co-deletion of Ezh2 in the Pten- deleted tumors (Figures 4F and 4G). "

Response: We re-worded this part as follows: "In Pten deleted tumors, Ezh2 protein levels were substantially elevated (Figures 4A and 4B), suggesting that increased expression of EZH2 may play a causal role in mediating PTEN deletion-induced prostate tumorigenesis. To our surprise, there were a significant portion of acini remained at the high grade prostatic intraepithelial neoplasia (HGPIN)/cancer stage after co-deletion of Ezh2 in the Pten- deleted tumors (Figures 4F and 4G)…."

6. p. 13 and Fig 5A. The correlation between p53 and EZH2 protein levels across all cancers is reported to only be 0.2 and the scatter plot in Fig 5A essentially shows no correlation. The correlations in prostate cancer shown in Figs 5B and 5D are more convincing.

Response: We agree with the reviewer. The onco-mining data originally shown in Figure 5A was not quite convincing and therefore was removed. As indicated by the reviewer, the correlations in prostate cancer shown in Figs 5B and 5D are more convincing and therefore included in the revised Figure 5.

7. p. 19, line 2. Should read "These data are consistent...".

Response: We re-worded this part as instructed on page 22.

8. p. 21 line 9. mRNA not message RNA.

Response: We re-worded this part as instructed on page 24.

9. p. 29, Figure 1 legend. It is unclear what "generated according to the distinct reads on chromosomal window from RIP-seq" means.

Response: We made the correction by changing the legend to "generated based on the distinct RIP-seq reads on specific gene exons".

Non-essential suggestions
1. It would have been nice to include WT EZH2 in the experiments show in Figures 6, EV5M and EV5N.

Response: We performed cell colony formation and western blot assay by including WT-EZH2 in these new experiments. Our new data showed that EZH2 full length and EZH2ΔSET but not EZH2ΔmRBD increased VCaP cell growth as evident by colony formation assays (Figures EV5S).

2. p.18 and Fig 7 E and F. To determine the effect on EZH2 on tumours with GOF p53 mutants, cells with WT p53 would be a better control.

Response: We agree with the reviewer that we did not use WT p53 in our allograft studies as shown in Figure 7E and 7F. Therefore, we repeated the experiments using a panel of cell lines of

different cancer types, two of which express endogenous WT p53: breast cancer cell line MCF7 (p53 WT) and prostate cancer cell line LNCaP (p53 WT). Similar to the results shown in Figure 7D, we demonstrated that treatment with DZNep or ASO resulted in much greater inhibitory effect on viability of p53 GOF mutated VCaP, 22RV1, MDA-MB-231 and MDA-MB-435 cell lines compared to p53 WT cell lines LNCaP and MCF7 (Figures EV7C and EV7D). Thus, our new data indicate that the findings in C4-2 (p53 WT) and VCaP (p53 GOF mutated) cell lines (Figure EV5Q) are fully supported by our new findings from more cancer cell lines that express WT p53.

3. p. 20. The authors suggest that their findings might explain the dichotomous roles for EZH2 in different types of cancer. Does p53 mutation status correlate with EZH2 overexpression / GOF mutation vs EZH2/SUZ12 deletion?

Response: As questioned in the "Minor concern #6" of this reviewer ("The correlation between p53 and EZH2 protein levels across all cancers is reported to only be 0.2 and the scatter plot in original Figure 5A essentially shows no correlation."), more convincing experimental data (originally shown in Figures 5B and 5D) are included, but the onco-mining data originally shown in Figure 5A was removed. However, we agree with the reviewer that it is very important to determine whether p53 mutation status correlates with EZH2 overexpression, especially the correlation between GOF mutation of p53 and EZH2/SUZ12 deletion and therefore it warrants further investigation in the future. However, it is worth noting that our data from patient samples shown in Figure 7B does support the notion that the potential dichotomous roles for EZH2 are likely depend on the status of p53 mutations.

Referee #3:

This is a huge paper containing 48 separate sub figures in total and as such has proven hard to evaluate in detail. The fundamental hypothesis is that the EZH2 protein has a novel function that does not require its methytransferase activity. This function is to bind to the mRNA of p53 and enhance its translation. This binding is localized to the IRES domain in the 5' UTR of the p53 mRNA and involves the mRBD domain (amino acids 336-554) of the EZH2 protein. In general the studies are well performed and the data is convincing The results are important because they show that EZH2 not only enhances the expression of wild type p53 but also mutant p53, as both, of course, contain the IRES element. This means that high levels of EZH2 can drive the synthesis of large amounts of mutant p53 . As many mutant p53 proteins show a Gain of Oncogenic Function (GOF) this means that the combination of high level activity of EZH2 and mutation in p53 can lead to especially aggressive and invasive tumors . They present some good data to support this in figures 6 and 7 and this EZH2 could emerge as an important modifier gene in the p53 pathway. The main issues are
the degree of specificity of the effect . Does EZH2 bind many mRNA's and how selective is the p53 effect ? Much of the work is driven by overexpression and by correlation so it would be good to see more direct evidence of endogenous interaction for example using proximity ligation approaches.

1) It would be more convincing if they used a point mutant that kills methyltransferase activity to divorce this activity from observed phenotype. While the sub domain work is convincing it does depend on over-expression

Response: We agree with the reviewer that it would be more convincing to use a point loss-of-function mutant to divorce the activity of EZH2 from the phenotype we observed. Even though early studies suggest that the tumor associated mutations in Y641 (e.g. Y641F) causes loss of H3K27me3 activity in vitro (Morin RD et al., Nature Genetics 2010 Feb;42(2):181-5), increasing evidence indicates that several cancer associated EZH2 mutations, including Y641F and A677G, are gain of function mutations instead (Yap et al., Blood, 2011 Feb 24;117(8):2451-9; McCabe MT et al., PNAS, 2012 Feb 21;109(8):2989-94). Therefore, we performed further experiments using Y641F, a point mutant, but gain of function, rather than the point loss-of-function mutant. We infected Mia-Pa-Ca2 cells (expressing endogenous p53 GOF mutant R248W) with lentivirus for EV, EZH2-Y641F mutant or EZH2ΔmRBD in combination with or without p53 shRNA. We performed cell viability and cell invasion assay to compare the effects among EV, EZH2ΔmRBD, and EZH2-Y641F. Our new data showed that EZH2 Y641F mutant slightly upregulated H3K27me3 level in Mia-PaCa2 cells, which is consistent with previous report (Yap et al., Blood, 2011 Feb

24;117(8):2451-9). However, EZH2 Y641F mutant, but not EZH2 ΔmRBD mutant increased p53 protein level, cell viability and cell invasion, and this effect was largely abolished by knockdown of p53 R248W even in the presence of slightly elevated H3K27me3 level (Figure EV6I). These data further support the notion that the EZH2 regulates the level of GOF mutant p53 protein in a manner independent of its enzymatic activity.

2) Some more controls to show p53 mRNA specificity would be useful in Figure 2E (maybe blot for a larger group of non p53 regulated genes/ proteins as at the moment ERK2 levels are the sole control). Using the same experimental system they should also over express EZH2 in these cells and looked for increased p53 levels. They should also do the now standard control of rescuing the si effect with a si resistant variant of EZH2 achieved using third base position variation to retain the correct coding sequence but escape si interaction . This control has proved very valuable in si RNA based studies.

Response: We thank the reviewer for the excellent suggestions. In Figure 2E, we added the β-TUBULIN as the internal control which is not known to be regulated by EZH2. As to the siRNA-resistant (SR) overexpression experiments with rescuing of the siRNA effects, we have done these experiments and the data are shown in Figures 2D and 2G.

3) They should ectopically express p53 in H1299 (or other p53-null cell line) cells from a plasmid that does not encode 5'UTR (basically pCMV-p53) and show that EZH2 over expression has no effect on p53 levels.

Response: As suggested by the reviewer, we co-transfected plasmids for EZH2 and pCMV-driven pcDNA-p53-FL (5'UTR+CDS+3'UTR) or the mutant without 5'UTR (pcNDA-p53-ΔIRES1) into p53-null PC3 cells and determined the effect of EZH2 overexpression on p53 protein level by WB. Our new data showed that EZH2 overexpression upregulated protein level of p53-FL, but not p53ΔIRES1 in PC3 cells (Figure EV2C). These data suggest that EZH2 regulates p53 protein level through IRES1.

Referee #4:

In this paper, the authors report that EZH2 binds to IRES1 of p53 mRNA, and thereby augments the translation of p53 mRNA as well as the stability of p53 mRNA. This does not require the methyl-transferase activity of EZH2, but rather relies on a distinct RNA-binding domain of EZH2. They further show that in cells and tumors harboring mutant p53, this positive effect of EZH2 on p53 protein expression is important for exerting mutant p53 gain-of-function (GOF). Accordingly, they show that depletion of EZH2 selectively attenuates the growth of tumors driven by mutant p53 GOF, suggesting that EZH2 downregulation may be considered as a selective treatment for such tumors.
Overall, this is a novel and very interesting study, with potential major clinical implications. The work is generally well performed, and many possible pitfalls are addressed by appropriate controls. However, there remain a number of aspects that need to be addressed in order to make the main conclusions more robust, as listed below.

1. Fig. 2E. The authors need to rule out that the reduction in p53 protein upon EZH2 knockdown may be due to decreased p53 protein stability. Furthermore, to show that the effect is dependent on IRES1, the authors should express p53 from expression plasmids that either retain or do not retain IRES1, and show that EZH2 regulates p53 levels in the former but not in the latter case.

Response: We thank the reviewer for the excellent suggestions. Firstly, we determined the protein half-life of p53 in C4-2 cells. We found that EZH2 knockdown did not result in overt change in p53 protein half-life in C4-2 cells (Figure EV3H). We also examined the effect of the proteasome inhibitor MG132 and lysosome inhibitor chloroquine (CQ) on EZH2 regulation of p53 protein level in C4-2 cells. Our new data showed that both inhibitors failed to block EZH2 depletion-induced downregulation of p53 protein in C4-2 cells (Figure EV3I).

Secondly, to determine if the effect is dependent on IRES1, we constructed two p53 plasmids. One is p53 full length (FL) (5'-UTR+CDS+3'-UTR) as shown in Figure 1G in pcDNA backbone vector

with a flag tag, and the other is flag-tagged p53 full length with IRES1 deleted (p53ΔIRES1). We co-transfected EZH2 and pcDNA-p53-FL (5'UTR+CDS+3'UTR) or pcNDA-p53-ΔIRES1 plasmids into p53-null PC3 cells and determined the effect of EZH2 overexpression on p53 protein level by WB. Our new data showed that EZH2 overexpression can upregulate protein level of p53-FL, but not p53 ΔIRES1 in PC3 cells (Figure EV2C). This new data further suggests that EZH2 ruglates p53 protein level through IRES1.

2. Fig. 5A,B and Fig. 7B. Is the correlation between p53 and EZH2 RNA levels equally strong in tumors that carry TP53 mutations as compared to those that retain wild type p53? As discussed by the authors in page 17, one might expect that the selective pressures will favor this correlation particularly in mutant p53 tumors.

Response: This is an excellent point. We re-analyzed the TCGA data by focusing on the correlation of EZH2 with wild-type or mutated p53 in tumor samples. The data showed that the correlation between p53 and EZH2 RNA levels was stronger in tumors that carry TP53 mutations as compared to those that retain wild type p53 (Figure EV5I), supporting the notion that the selection pressures may favor the correlation in p53 mutant tumors.

3. Fig. 6. The authors should express the same mutant p53 from an expression plasmid that either retains or does not retain IRES1, and show (in vitro) that the GOF of mutant p53 is augmented by EZH2 only in the former but not the latter case.

Response: As suggested by the reviewer, we introduced GOF mutants R273H and R248W into flag-tagged p53FL and p53deltaIRES1 plasmids. We co-transfected EZH2 and pcDNA-p53-mut-FL (5'UTR+CDS+3'UTR) or pcNDA-p53-mut-ΔIRES1 plasmids into p53-null PC3 cells and determined the effect of EZH2 overexpression on mut p53 protein level by WB. Our new data showed that EZH2 overexpression can upregulate protein level of mut p53-FL, but not p53 ΔIRES1 mutant in PC3 cells (Figure EV5N). These new data suggest that EZH2 ruglates mut p53 protein level through IRES1.

Minor comments:
1. Fig. 2. The numbering of p53 mRNA positions in the various deletion mutants is confusing: whereas Fig. 2C shows numbering based on the translation start site, Fig. 2B uses numbering starting from the 5' end of the 5' UTR. The authors should adopt a single numbering method consistently throughout the paper.

Response: We adopted a single numbering method for the numbering of p53 mRNA positions in different mutants. Accordingly, we changed "1-40" to *"-202 ~ -182*"; "41-80" to *"-181 ~ -123";* "81-120" to *"-122 ~ -83"*; changed "122-160" to *"-82 ~ -43"*; changed "161-202" to the *"-42 ~ -1*".

2nd Editorial Decision 13th Dec 2018

Thank you for submitting your revised manuscript for consideration by The EMBO Journal, as well as for providing additional source data. My apologies for getting back to you with this unusual delay, which is due to protracted referee input and the extensive nature of this study. Your revised manuscript was sent back to all four original reviewers for re-evaluation, and we have received comments from three of them, which I enclose below. Please note that while referee #3 was not able to look back into the work at this time, we have editorially assessed your response to his-her concerns.

As you will see the referees find that their concerns have been largely addressed and they are now in favour of publication, pending minor revision. Thus, we ask you to revise your manuscript according to the minor remaining issues as indicated below.

Please note that once the above matters are adjusted at re-submission, we want to swiftly move on with acceptance and publication of your work.

REFEREE REPORTS:

Referee #2:

My comments are numbered according to my original review.

1. Although improved, the issue of whether EZH2 directly binds p53 mRNA in cells has not been fully addressed. I previously suggested that the authors confirm that EZH2 interacts with p53 mRNA in cells using UV-RIP, using lysis and wash conditions that remove any non-crosslinked protein-RNA interactions that may be indirect or non-specific.

In the revised manuscript the authors have performed IPs from UV-crosslinked cells and also in cells in which EZH2 levels have been reduced by RNAi. These show EZH2 binding to p53 mRNA. However, critically, they have not demonstrated that the lysis and wash conditions remove non-crosslinked protein-RNA interactions and therefore that the interaction is direct. To do this, the experiment must be performed with and without UV crosslinking to show that the RNA is only pulled down by EZH2 from UV-crosslinked cells.

The description of the experimental method also requires correction. This is a UV-RIP experiment, not PAR-CLIP. Although cells were labeled with 4sU, as in the PAR-CLIP procedure, the other steps of the PAR-CLIP protocol were not followed. In PAR-CLIP, cells are crosslinked at 365nm UV, which specifically crosslinks protein to 4sU (Hafner et al., 2010). After immunoprecipitation and RNAseT1 treatment, the RNP of interest is separated from other proteins and RNAs by SDS-PAGE and the crosslinked RNA visualized by autoradiography. RNPs are then transferred to membranes, the RNP of interest isolated, RNA purified, and reverse transcribed. The cDNA is then amplified and sequenced and the sites of 4sU incorporation identified by C to T transitions. In contrast, the authors crosslinked cells at 254nm UV, which crosslinks unmodified RNA bases to proteins, did not perform the SDS-PAGE purification step or sequencing. The description of the method should therefore be changed to UV-RIP.

2. The authors have now demonstrated that the increase in p53 levels in response to EZH2 overexpression depends on IRES1.

3. I previously expressed concern that the large region of EZH2 the authors label as the RNA binding domain (based on their own data) overlaps with the region of the protein known to bind SUZ12. Their revised data show that this is indeed the case and that deleting this region disrupts PRC2. The question then is, is the loss of RNA binding and p53 upregulation independent of SUZ12 and other PRC2 subunits and I'm not convinced this has been demonstrated. The authors provide new data showing that SUZ12 knockdown reduces p53 RNA co-precipitation in EZH2 UV-RIP nad reduces p53 protein level. They ascribe this to the reduced levels of EZH2 in the cells, consistent with previously published observations. This reduction in EZH2 levels is because EZH2 is unstable when not bound to SUZ12. However, in the authors' model, there are two pools of EZH2, one bound to SUZ12 and one not bound to SUZ12. They ascribe the p53 RNA binding and p53 upregulation activities to the non-SUZ12-bound pool of EZH2. If this is the case, why does EZH2 RNA binding and p53 protein levels decrease in the absence of SUZ12? The functions of the non-SUZ12 bound pool of EZH2 shouldn't be affected by the loss of SUZ12. If anything, the pool of non-SUZ12 bound EZH2 should increase in the absence of SUZ12 and one should see greater p53 RNA binding and greater p53 protein upregulation.

Given these issues, rather than concluding that EZH2 binds p53 RNA and increases its translation is independent of PRC2, the safer conclusion would be that intact PRC2, independent of its methyltransferase activity, is required for RNA binding and for p53 upregulation via IRES1. I don't think removing the conclusion that this aspect of EZH2 function is independent of PRC2 would significantly detract from the novelty of the paper.

In this regard, to represent the core function of the 336-554 domain accurately in the paper, the authors should refrain from referring to it as a pure RNA binding domain, instead labeling the recognized EZH2 domains (eg MCSS, SANT and CXC) in Fig 1D and changing "deltamRBP" to "delta336-554" in the other figures.

4. The authors have shown that PABP1 and eIF4G2 are not identified in their control SFP pull-down. Ideally the levels of proteins in the EZH2 and control pull-downs should be compared by their intensities - could these be provided for the control experiment? The new Fig 3C showing that the interaction of EZH2 with these factors is dependent on RNA is convincing.

5. This issue has been addressed.

All minor concerns and non-essential suggestions have been addressed.

Referee #4:

Actually, in my major comment 3, I requested to show a differential effect of EZH2 on gain-of-function (GOF) of mutant p53, not on levels of mutant p53 protein. Assessment of mutant p53 GOF should be performed by functional assays, such as those employed in Fig. 6A. Hence, the authors have not satisfied this request. Nevertheless, the revised version is significantly stronger than the original one. I therefore agree to forego the above request and support the publication of the revised paper as is.

2nd Revision - authors' response 19th Dec 2018

All editorial changes requested were made.

Referee #1:

The authors have provided further evidence that cells expressing p53 mutant protein are more sensitive to EZH2 depletion than EZH2 enzymatic inhibition. They have analyzed the effect of EZH2 depletion/inhibition on multiple tumor cell lines expressing WT or mutant p53 as well as on cells expressing exogenous WT/mutant p53.

1. In a new Figure 7, panels C and 7D, the authors have used higher amounts of drugs and ASO in C4-2 cells in order to see the differential effect on cell viability upon EZH2 depletion/inhibition. The authors should state the concentration of drugs/ASO used in these experiments and why the increased concentrations only were used in C4-2 (p53 WT) cells and not in VCaP (p53 mutant) cells? To compare the effects of the drugs/ASO on different cell lines one has to treat the cell lines with the same concentrations.

> **Response:** We thank the Reviewer for pointing out this excellent issue. As we indicated in the revised manuscript, we treated VCaP cells (expressing p53 GOF mutant R248W) with different EZH2 inhibitory agents, including GSK126, DZNep and ASOs. We demonstrated that treatment of these agents at 5 μM invariably decreased H3K27me3 levels, increased expression of PcD gene *DAP2IP*, and downregulated expression of PcI genes *CEP76*, *RAD51C* and *TEME48* in VCaP cells (Fig 7C and Appendix Fig S1L). As we stated in the revised manuscript, we found a higher concentration (10 μM) of these inhibitors was needed to achieve the effect on H3K27me3 inhibition and EZH2 target gene expression in p53 WT C4-2 cells that was similar to that in VCaP cells (Fig 7C and Appendix Fig S1L). Per the suggestion of the Reviewer, we have indicated the concentrations (5 μM versus 10 μM) of drugs/ASOs used for these different cell lines in legends of Figures 7C and 7D.
>
> We agree with the Reviewer that one has to treat the cell lines with the same concentrations to compare the effects of the drugs/ASO on different cell lines. Indeed, we expanded our studies by using the same concentration (10 μM) of these drugs in a large panel of eight cancer cell lines expressing either WT or GOF p53, which include C4-2 (p53 WT), LNCaP (p53 WT), MCF7 (p53 WT), MDA-MB-435 (p53 mutant), MDA-MB-231 (p53 mutant), 22RV1 (p53 mutant), PC3 (p53 null) and PC3-p53-248 cells. The data from these experiments are shown in Fig 7C and Appendix Fig S2C.

2. It seems that there is duplication of some western blot pictures e.g. Fig EV7 (C) p53 blot in 22RV1 cell line looks identical (but differently exposed) to EZH2 blot in PC3 +p53 R248W as well as to EZH2 blot in PC3 cell line. Also, in additional figure 7, which is supposed to contain uncropped western blot pictures, EZH2 blot in 22RV1 cell line does not match to EZH2 blot in the actual figure (Figure EV7(C)) and it appears to be identical (but differently exposed) to p53 blot in 22RV1 cell line, EZH2 blot in PC3 +p53 R248W as well as to EZH2 blot in PC3 cell line.

> **Response:** Western blot pictures for p53 in 22RV1 cell line and EZH2 in PC3 +p53 R248W as well as in PC3 cell line are not duplicated from each other and not identical. These western blot photos were originally acquired from the Odyssey Fc Imaging System (LI-COR Biosciences). Given that the original Odyssey images were saved at the resolution of 300 pixels/inch. We went back to the Odyssey images system and re-saved all the images at 600 pixels/inch, and images in these three panels have been replaced with high resolution images in Appendix Fig S2C and the source data for these panels.

3. In the discussion, the authors write:
"A few such inhibitors including GSK126 and EPZ-6438 have been developed and are currently in phase I clinical trials for treatment of B-cell lymphomas and advanced solid tumors (Knutson et al, 2014; McCabe et al, 2012b; Vaswani et al, 2016). To our knowledge, however, no favorable reports have been documented yet."

The authors should change that statement (see e.g. PMID: 29980507, 29650362)

> **Response:** We agree with the Reviewer. We have changed the statement by indicating that these inhibitors are currently being tested in phase I clinical trials…. "To our knowledge, however, no favorable report has been documented yet." on page 23. The references the Reviewer mentioned have been cited in the revised manuscript.

4. In the discussion, the authors write:
"EZH2 is a known RNA-binding protein. By binding to noncoding RNAs (ncRNAs) such as XIST, RepA and HOTAIR, EZH2 has been shown to work together with other PcG proteins to promote X chromosome inactivation, developmental patterning, and maintenance of stem cell pluripotency".

This statement is correct, but the authors should know that these findings are very controversial and should refer to recent papers that EZH2 binds non-specifically to RNA (see e.g. 29185984; 29058709)

> **Response:** We agree with the Reviewer. We have revised our discussion by indicating that "However, the specificity of EZH2 binding of so many RNAs remains a topic for study". Additionally, the two references the Reviewer mentioned have been cited in the revised manuscript.

Referee #2:

My comments are numbered according to my original review.

1. Although improved, the issue of whether EZH2 directly binds p53 mRNA in cells has not been fully addressed. I previously suggested that the authors confirm that EZH2 interacts with p53 mRNA in cells using UV-RIP, using lysis and wash conditions that remove any non-crosslinked protein-RNA interactions that may be indirect or non-specific.

In the revised manuscript the authors have performed IPs from UV-crosslinked cells and also in cells in which EZH2 levels have been reduced by RNAi. These show EZH2 binding to p53 mRNA. However, critically, they have not demonstrated that the lysis and wash conditions remove non-crosslinked protein-RNA interactions and therefore that the interaction is direct. To do this, the experiment must be performed with and without UV crosslinking to show that the RNA is only pulled down by EZH2 from UV-crosslinked cells.

The description of the experimental method also requires correction. This is a UV-RIP experiment, not PAR-CLIP. Although cells were labeled with 4sU, as in the PAR-CLIP procedure, the other steps of the PAR-CLIP protocol were not followed. In PAR-CLIP, cells are crosslinked at 365nm UV, which specifically crosslinks protein to 4sU (Hafner et al., 2010). After immunoprecipitation and RNAseT1 treatment, the RNP of interest is separated from other proteins and RNAs by SDS-PAGE and the crosslinked RNA visualized by autoradiography. RNPs are then transferred to membranes, the RNP of interest isolated, RNA purified, and reverse transcribed. The cDNA is then amplified and sequenced and the sites of 4sU incorporation identified by C to T transitions. In contrast, the authors crosslinked cells at 254nm UV, which crosslinks unmodified RNA bases to proteins, did not perform the SDS-PAGE purification step or sequencing. The description of the method should therefore be changed to UV-RIP.

> **Response:** We thank the Reviewer for carefully examining our new RIP experiments. We agree with the Reviewer that our experiments should be called as UV-RIP rather than PAR-CLIP. Accordingly, we have changed the description of the method to UV-RIP in the main text, Materials and Methods and Figure Legends sections.

2. The authors have now demonstrated that the increase in p53 levels in response to EZH2 overexpression depends on IRES1.

3. I previously expressed concern that the large region of EZH2 the authors label as the RNA binding domain (based on their own data) overlaps with the region of the protein known to bind SUZ12. Their revised data show that this is indeed the case and that deleting this region disrupts PRC2. The question then is, is the loss of RNA binding and p53 upregulation independent of SUZ12 and other PRC2 subunits and I'm not convinced this has been demonstrated. The authors provide new data showing that SUZ12 knockdown reduces p53 RNA co-precipitation in EZH2 UV-RIP nad reduces p53 protein level. They ascribe this to the reduced levels of EZH2 in the cells, consistent with previously published observations. This reduction in EZH2 levels is because EZH2 is unstable when not bound to SUZ12. However, in the authors' model, there are two pools of EZH2, one bound to SUZ12 and one not bound to SUZ12. They ascribe the p53 RNA binding and p53 upregulation activities to the non-SUZ12-bound pool of EZH2. If this is the case, why does EZH2 RNA binding and p53 protein levels decrease in the absence of SUZ12? The functions of the non-SUZ12 bound pool of EZH2 shouldn't be affected by the loss of SUZ12. If anything, the pool of non-SUZ12 bound EZH2 should increase in the absence of SUZ12 and one should see greater p53 RNA binding and greater p53 protein upregulation.

Given these issues, rather than concluding that EZH2 binds p53 RNA and increases its translation is independent of PRC2, the safer conclusion would be that intact PRC2, independent of its methyltransferase activity, is required for RNA binding and for p53 upregulation via IRES1. I don't think removing the conclusion that this aspect of EZH2 function is independent of PRC2 would significantly detract from the novelty of the paper.

> **Response:** We agree with the Reviewer and have drawn the conclusion as suggested by the Reviewer in the revised manuscript on page 12 "..intact PRC2, but independent of its methyltransferase activity, is required for RNA binding and for p53 upregulation via IRES1."

In this regard, to represent the core function of the 336-554 domain accurately in the paper, the authors should refrain from referring to it as a pure RNA binding domain, instead labeling the recognized EZH2 domains (eg MCSS, SANT and CXC) in Fig 1D and changing "deltamRBP" to "delta336-554" in the other figures.

> **Response:** We agree with the Reviewer. We have labeled the recognized EZH2 domains such as MCSS, SANT, and CXC in Fig. 1D and Figure Legends. Also, per the suggestion of the Reviewer, we have changed "ΔmRBD" to "Δ336-554" in all the relevant Figures (Figures 1,2,3,6, EV Figures 2,3,5, and Appendix Figure S1 and S2) and in the main text of the revised manuscript.

4. The authors have shown that PABP1 and eIF4G2 are not identified in their control SFP pull-down. Ideally the levels of proteins in the EZH2 and control pull-downs should be compared by

their intensities - could these be provided for the control experiment? The new Fig 3C showing that the interaction of EZH2 with these factors is dependent on RNA is convincing.

Response: We have already added the intensities in the control group in Table EV2.

5. This issue has been addressed.

All minor concerns and non-essential suggestions have been addressed.

Referee #4:

Actually, in my major comment 3, I requested to show a differential effect of EZH2 on gain-of-function (GOF) of mutant p53, not on levels of mutant p53 protein. Assessment of mutant p53 GOF should be performed by functional assays, such as those employed in Fig. 6A. Hence, the authors have not satisfied this request. Nevertheless, the revised version is significantly stronger than the original one. I therefore agree to forego the above request and support the publication of the revised paper as is.

Response: We are very sorry for our misunderstanding of the point raised by the Reviewer originally. We appreciate very much that the Reviewer finds the revised version is significantly stronger than the original one and agrees to forego the above request and support the publication of the revised paper as is.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Haojie Huang
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-99599R

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 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.

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?	All experiments showed in the manuscript have been repeated for at least two times. And the two-sided student T-test was used to determine if the differences were statistically significant. See Materials and Methods for specifics on each type of experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Minimum of 6 age- and sex- matched animals per group were used.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Animals were excluded from analysis only if they became ill or their weight dropped below 90% of their original weight at the start of the experiment. However, no animals were excluded from the current 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.	Mice were randomly grouped before drug treatment.
For animal studies, include a statement about randomization even if no randomization was used.	See the statement above.
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.	Yes, tumor growth was monitored blindly using living imaging.
4.b. For animal studies, include a statement about blinding even if no blinding was done	See the statement above.
5. For every figure, are statistical tests justified as appropriate?	Yes, see Figure Legends and Materials and Methods
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Two-sided student T-test
Is there an estimate of variation within each group of data?	We show standard deviation or standard error of the mean as described in the figure legend.
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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 used are described in Material and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We described the source of cell lines. All cell lines used in this study were tested for mycoplasma contamination.

* 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-week-old NOD-SCID IL-2-receptor gamma null (NSG) mice were generated in house and used for animal experiments. All mice were housed in standard conditions with a 12 h light/dark cycle and access to food and water ad libitum.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The animal study was approved by the IACUC at Mayo Clinic.
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.	Yes

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 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	RIP-seq data: Gene Expression Omnibus (GEO) number GSE63230.
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).	The data has been deposited into the GEO database.
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. 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.	GSE63230

G- Dual use research of concern

22. 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