THE EMBO JOURNAL

Thalidomide and its metabolite 5-hydroxythalidomide induce teratogenicity via the cereblon neo-substrate PLZF

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DOI: 10.15252/embj.2020105375

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Review Timeline:

Submission Date:	22nd Apr 20
Editorial Decision:	12th Jun 20
Editorial Correspondence:	26th Jun 20
Revision Received:	26th Aug 20
Editorial Decision:	22nd Oct 20
Revision Received:	28th Oct 20
Editorial Decision:	16th Nov 20
Revision Received:	17th Nov 20
Accepted:	30th Nov 20

Editor: Ieva Gailite

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 12th Jun 2020

Thank you for submitting your manuscript for consideration by the EMBO Journal. I apologise for the protracted review process due to delays in review submission. We have now received two referee reports on your manuscript, which are included below for your information. We will forward the comments of the third referee to you as soon as we receive them, but since the two reports are in a good agreement, I am taking a decision based on the comments at hand to avoid further delays.

As you will see from the comments, both reviewers appreciate the presented identification of PLZF as a thalidomide-induced CRBN substrate, but also indicate a number of concerns that have to be addressed before they can support publication of the manuscript. Based on the overall interest expressed in the reports, I would like to invite you to submit a revised version of your manuscript in response to reviewers' comments. Both reviewers have also indicated that the manuscript would benefit from detailed language editing, so I recommend having the manuscript checked by a native speaker or a language editing service.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and I would be happy to discuss the revision in more detail via email or phone/videoconferencing.

Please feel free to contact me if have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Referee #1:

Yamanaka et al. identify PLZF as a zinc-finger (ZF) neo-substrate for the CRBN E3 ubiquitin ligase. The initial discovery was made using protein arrays, followed by extensive cellular and biochemical validation. The authors report that PLZF is drug-induced CRBN substrate where degradation is induced by thalidomide, as well as 5-hydroxythalidomide. The latter compound is a frequently observed metabolite of thalidomide, with currently little information available concerning protein substrates targeted by 5-hydroxythalidomide. The detailed mapping of the PLZF degron required for degradation implicated PLZF zinc-fingers ZF1 and ZF3. The authors then continue to demonstrate that PLZF/ZBTB16 protein loss in a chicken-limb developmental system results in limb deformation, a phenotype reminiscent of thalidomide-induced phocomelia. Yamanaka & colleagues go on to make the case that a previously identified phocomelia substrate, SALL4, does not fully explain thalidomide teratogenicity across different animal models, while PLZF would offer a broader explanation.

The identification of additional candidate proteins that in addition to SALL4 could contribute to thalidomide-induced limb deformation, is an important discovery; as is the implication that thalidomide metabolites could be involved in this process. The experiments in the manuscript appear carefully conducted, and this body of work is undoubtedly going to be of great interest to the field. This reviewer considers this manuscript perfectly suited for publication in EMBO following careful experimental revision; this should also include the text, which is full of spelling and gramatical mistakes and is illegible in places.

Major points:
- The rationale why only PLZ/ZBTB16 ZF1-3 were examined is not clear; It would be paramount to carry out experiments where each ZF is deleted and cellular degradation is assessed. The argument that ZF1-3 are conserved and only those were tested is not convincing, as even ZFs with little conservation can contribute to degradation. It is important that this is experimentally addressed.
- P. 14, lines 306-310; while the cellular experiments are convincing, they are based on over-expression; to use these cellular findings to explain SALL4 vs PLZF phenotypes in different animals is very far-fetched for my liking and should be more carefully discussed.
- Fig 4E. the effect of thalidomide and 5-Hydroxythalidomice on PLZF in THP-1 cells seems very minor; this needs to be done more quantitative i.e. using LICOR Western-blotting? Same with Fig. 7C! These are key figures of the paper and require extra attention, revised experiments and further quantification.
- Is PLZ/ZBTB16 degradation evident in published proteomic data-sets, e.g by Celgene, the Ebert or Fischer labs? To what extent were PLZF ZF1-3 binding tested by Sievers et al., 2018?

Minor points:
- P. 3, line 64; There is significant concern in the field whether zebra-fish is a useful model system for studying phocomelia (see: doi: 10.1073/pnas.1307684110); either cite this reference and point of the controversial nature, or remove arguments relating to zebrafish all together.
- P. 3, line 62/63; what is meant here? .. it was reported that the forelimb of SALL4-CKO were not abnormality (?); also see above. Overall the manuscript needs to be carefully revised for language and spelling; I gave up correcting spelling mistakes after a while…
- P. 4, line 76; the full-length
- P. 4, Fig 4A is mentioned before other main text figures?
- P. 5, line 111; for substrate proteins of protein kinases … E3 ligases
- P. 7, line 150; see Petzold et al., 2016 for detailed measurements of CK1a-CRBN binding in the presence of other IMiDs, CK1A binding in vitro is also observed with thalidomide, lenalidomide and pomalidomide and small differences in binding Kd translate into almost binary degradation/no-degradation choices in cells; the Matyskiela et al., 2016 reference refers to GSPT1; this should be corrected and PMID: 26909574 should be cited instead.
- P.7, line 157; Donovan et al., 2018 used a TF-FRET set-up and not an alpha-screen; all references should be carefully checked
- P.17 line 370; include Sievers et al., 2018 reference here

Referee #3:

Using a cell-free protein screening assay, Yamanaka and colleagues identified PLZF as an additional thalidomide-induced neo-substrate of the CRBN-CRL4 E3 ligase. PLZF was convincingly validated as a pan-IMiD substrate by a large series of protein analyses in different cell lines, IPs, and ubiquitination assays. The zinc finger domain critical for the CRBN-thalidomide-PLZF interaction was successfully mapped by cloning and testing different PLZF forms. The authors could further show that 5-hydroxythalidomide, a major metabolite of thalidomide in human but not in mice, is also able to degrade PLZF and SALL4, a previously identified CRBN neo-substrate involved in thalidomide-induced teratogenicity. As shown in the current work and by previous publications, thalidomide treatment of chicken embryos results in limb malformation. The authors demonstrate that PLZF, but not SALL4, is degraded by thalidomide and 5-hydroxythalidomide in chicken limb

buds and that knockdown of PLZF by shRNAs resembles the thalidomide phenotype. This implies that the teratogenicity observed in chicken embryos is induced by PLZF degradation suggesting that it contributes to thalidomide teratogenicity in human in addition to SALL4.

Identification of PLZF as a thalidomide-induced CRBN neo-substrate likely involved in teratogenicity is a new important finding adding another piece to the puzzle of the molecular mechanism for thalidomide teratogenicity. Furthermore, PLZF in addition to SALL4 may serve as an easily assessable marker for drug-associated teratogenicity what may be useful for future drug development.

Major concerns:

-Although the results are highly suggestive that PLZF is involved in thalidomide-induced teratogenicity in chicken, the finding would be strengthened by a rescue experiment by overexpressing PLZF or even better a degradation resistant PLZF mutant in analogy to the transfection of the CRBN YWAA mutant as performed by Ito et al. 2010.

Minor concerns:
-As the authors mention, PLZF is involved in several biological processes including immune function. Would degradation of PLZF be in line with other properties of IMiDs like effects on T cells and monocytes?

-5-hydroxythalidomide is degrading PLZF but not IKZF1. What is the impact of 5-hydroxythalidomide on other CRBN neo-substrates (e.g. CK1a, ZFP91, GSPT1)?

-Is 5-hydroxythalidomide in general toxic to cells as compared to thalidomide?

-Is thalidomide converted to 5-hydroxythalidomide in chicken?

-The manuscript text has to be thoroughly revised. There are some inconclusive sentences e.g., abstract: "However, it remains unclear." and many grammatical mistakes, e.g. "it was reported that forelimb of SALL4-CKO mice were not abnormality".

I am herewith forwarding you the delayed report on your manuscript (EMBOJ-2020-105375) from reviewer #2. Additionally, the reviewer has provided a Word document with suggestions for grammatical corrections. The general assessment by the reviewer is positive and largely complementary to the reports by the other two reviewers. I would therefore ask you to include these comments in your revision of the manuscript.

Referee #2:

OVERVIEW

The authors constructed a human protein array consisting of 1,118 human recombinant transcription factor (TF) and other zinc finger proteins, and used this array to discover a novel TF, promyelocytic leukemia zinc finger (PLZF), that binds to the cereblon (CRBN) component of the CRL4 E3 ubiquitin ligase complex in the presence of the teratogenic sedative/immunosuppressive drug thalidomide, its derivatives (lenalidomide, pomalidomide) and its 5-hydroxythalidomide metabolite. In a series of comprehensive biochemical studies using cell-free and cellular assays together with related biochemical and developmental studies in chick embryos, the authors show that the resulting PLZF-CRBN-thalidomide complex results in the ubiquitination and proteasomal degradation of PLZF, resulting in developmental disorders including limb abnormalities in chick embryos, as previously shown in various species for other developmental important TFs and other binding proteins such as SALL4. They also show in several knockdown studies that PLZF itself is necessary for normal limb development. Remarkably, PLZF but not SALL4 was reduced in the limb buds of thalidomide-exposed chick embryos, in contrast to previous studies of rabbit embryos in which SALL4 was degraded, revealing species-dependent, CRBN-mediated mechanisms of susceptibility.

These studies not only identify PLZF as a new developmentally important CRBN substrate, but also provide a novel molecular basis for an unsubstantiated chemical mechanism previously proposed for thalidomide teratogenesis. In the latter case, the phthalimido ring of thalidomide is oxidized by cytochromes P450 enzymes (CYPs) to form a 5-hydroxythalidomide metabolite. Years ago, this reaction was postulated to involve the formation of an unstable arene oxide (epoxide) reactive electrophilic intermediate that covalently binds to developmentally important cellular macromolecules, causing developmental disorders. The authors' studies support a teratogenic role for the 5-hydroxythalidomide metabolite, but suggest that the mechanism involves binding of the stable 5-hydroxythalidomide metabolite itself to CRBN, rather than the covalent binding of a reactive epoxide precursor.

The manuscript would be improved by providing more context for the teratological relevance of 5-hydroxythalidomide, and by providing a more complete mechanistic context within which to consider CRBN-dependent mechanisms.

MAJOR COMMENTS

1. The relevance of the comprehensive biochemical data rests upon the associated changes in limb bud development, the latter of which would benefit from more explicit and quantitative details.
• The Methods (pp. 31-32) should indicate more clearly for the studies of limb reduction (figs. EV9 & EV10), exactly what HH stage and embryonic day the chick embryos were treated, and what drug solution concentration (both molar and ug/ul) and volume were administered for both thalidomide or 5-hydroxythalidomide, and what HH and embryonic day the embryos were evaluated.
• For both figs. EV9 and EV10, the measurement of limb reduction should be quantified (length, area, etc.), the data for the mean + SD for reduction should be presented for each treatment group in a new bar graph, and the differences should be tested for statistical significance and shown in the figure. If a precise measurement is not possible, then a clear definition of a substantial reduction (e.g. greater than 50% less than the mean of control group?) should be provided, and the mean number of animals exhibiting this reduction should be compared to that for the control group in a new bar graph, and differences analyzed for statistical significance that is shown in the figure. The same is true for the fgf8 studies in panel B of fig. EV9 (see below).
• Given the fundamental importance of limb reduction to the authors' hypothesis, I believe that these two EV figures or some variant (the relevant panels from these figures?) would be better placed in the main body of the manuscript.

2. Indicate at least in the Methods (p. 31), if not in the Discussion, why different volumes of drug solution were applied to chick embryos in the biochemical studies (30 ul) versus the morphological studies of limb reduction (10 ul). In the latter case, additional 10 ul volumes were applied twice more (for a total application of 30 ul), but at 12-hour intervals, which would result in substantially lower peak exposure concentrations to the limb bud. If the higher volume (and hence dose) in the former was necessary to elicit the observed outcomes, then discuss why the outcomes are still relevant to the changes observed in limb reduction with the lower dose.

3. In the Results on p. 12, the authors assert that thalidomide and its 5-hydoxythalidomide metabolite were similarly efficacious in cells in causing the degradation of PLZF and SALL4, based upon representative immunoblot analyses shown in fig. 4D and 4E. To support this assertion, these two panels should each be accompanied by a complementary graph that shows the mean + SD values for the replicates for each treatment in line diagrams or bar graphs, along with the level of significance for differences and the number of cell assays sampled. The Results should be expanded to comment on the added panels and content.

4. Similarly on p. 12, the authors state that "these results suggest that 5-hydroxythalidomide has potential similar to that of thalidomide for PLZF and SALL4 degradation in humans". At least in the Discussion, they should provide a more nuanced context in which to apply their in vitro data. Important issues include: (a) their own data in a cell-free assay (fig. 4B) show that

5-hydroxythalidomide is much less effective that thalidomide in enhancing PLZF binding to CRBN; (b) in humans, thalidomide will be metabolized by CYPs to its 5-hydroxythalidomide metabolite primarily in the mother's liver rather than in the embryo, which has relatively low CYP levels, and most of that 5-hydroxythalidomide will be enzymatically glucuronidated by the mother and eliminated in her urine without reaching the embryo; (c) thalidomide is rapidly and non-enzymatically hydrolyzed to 12 major metabolites, independent of its CYP-catalyzed metabolism to 5-hydroxythalidomide, so what is the percentage of thalidomide that is metabolized to its 5-hydroxythalidomide metabolite?; (d) what molar concentration range of 5-hydroxythalidomide is found in the plasma of an adult taking thalidomide, and what molar concentration would be estimated in the embryo?; and, (e) how do the molar concentrations of thalidomide and 5-hydroxythalidomide in the authors studies, including chick embryos, compare to those achieved in adults taking thalidomide and embryos exposed in utero? This discussion is also important to understanding the context of the authors' novel and important discovery that 5-hydroxythalidomide may contribute to not only the teratogenicity of thalidomide, but also its immunomodulatory role.

5. p. 14 and fig. 5: The data presented suggest but do not confirm the authors' novel and important assertion that "thalidomide-dependent PLZF degradation occurs in many animals, ... while in contrast, SALL4 degradation from thalidomide occurs in a limited number of animals (species)". To confirm this discovery, at least the key panels from fig. 5 showing results from representative samples should each be accompanied by a complementary graph that shows the mean + SD values for the replicates for each treatment using line diagrams or bar graphs, along with the level of significance for differences and the number of cell assays sampled. The same issue applies their assertions relating to the panels in the same figure showing the remarkable effects of 5-hydroxythalidomide in initiating SALL4 and PLZF degradation. The Results should be expanded to comment on the added panels and content.

6. p. 14, lines 311-315: The authors should include a cautionary comment that the evidence they cite for thalidomide causing limb defects in mice "humanized" to express human CYP3A isoforms is based upon a single in vitro (embryo culture) study showing at best only modest evidence of limb defects, and the same authors found no evidence of limb abnormalities in in vivo studies in these humanized CYP3A mice. The absence of limb defects in this humanized model may be due in part to maternal glucuronidation and elimination of the 5-hydroxythalidomide metabolite formed by the human CYP3A isoforms, as noted above.

7. p. 16 and fig. 7: The authors assert in the Results that thalidomide induces the degradation of PLZF but not SALL4 in chick embryos, unlike other species susceptible to thalidomide-induced limb abnormalities (e.g. rabbits) which have been shown by other labs to exhibit SALL4 degradation. To prove this assertion, the immunohistochemical staining should be quantified, and panels 7A, 7B and 7C, each showing results from one representative sample, should each be accompanied by a complementary graph that shows the mean + SD values for the replicates for each treatment using line diagrams or bar graphs, along with the level of significance for differences and the number of cell assays sampled. In panel B, the meaning of "normal", "weak" and "strong" should be specified. To complement the biochemical schemes shown respectively for chick and human limb buds in panels 7D and 7E, it would be useful to add a parallel scheme for rabbits to illustrate the major difference between that species (published by others) and the authors' results in chick embryos. In addition to the ug/ml values, add the molar concentrations of thalidomide and 5-hydroxythalidomide in the figure legend. The Results should be expanded to comment on the added panels and content.

8. In the Discussion, the authors say that 5-hydroxythalidomide showed higher "potential" (efficacy?) than thalidomide in inducing the degradation of both PLZF and SALL4 from humanized mice and chick embryos in HEK294T cells. Given the potential importance of 5-hydroxythalidomide, the authors should add a short paragraph to the Discussion briefly outlining the old hypothesis for thalidomide teratogenicity being due to its "bioactivation" by CYP isozymes to a reactive epoxide intermediate which, if not detoxified by epoxide hydrolase, will covalently bind to developmentally important proteins resulting in developmental disorders. Some relevant references are as follows:
Gordon et al., Proc. Natl. Acad. Sci. USA 78: 2545-25-48, 1981.
Fort et al., Teratogen. Carcinogen. Mutagen. 20: 35-47, 2000.
Chowdhury et al., Chem. Res. Toxicol. 23: 1018-1024, 2010.

9. As noted above, thalidomide rapidly and non-enzymatically hydrolyzes to 12 major products, which are in addition to the 5-hydroxythalidomide metabolite produced by the CYP-catalyzed metabolism of thalidomide. Two of the hydrolysis products, 2-phthalimidoglutaramic acid and 2-phthalimidoglutaric acid, cause limb abnormalities in rabbit embryo culture via a mechanism involving oxidative stress (Lee et al., FASEB J. 25: 2468- 2483, 2011), which is supported by in vivo studies of thalidomide (Parman et al., Nat. Med. 5: 582-585, 1999). This mechanism should be mentioned either in the Introduction on p. 4 with the other mechanisms, or in the Discussion, to provide a more complete context.

10. Fig. EV9.
• Panel B, fgf8 mRNA expression: provide an additional panel with a bar graph showing mean + SD data and statistical analysis of significance between control and thalidomide.
• Panel C, limb abnormalities: provide an additional panel with a bar graph showing mean + SD data and statistical analysis of significance for some quantitative measurement of limb reduction (see comments for fig. 6). Also, in panel C and/or its legend, add the molar concentration of thalidomide to complement the ug/ul concentration-each will be selectively familiar to different readers.

11. Fig. EV10. Add molar concentrations-this is particularly relevant for 5-hydroxythalidomide, which has a higher molecular weight than thalidomide, so the same ug/ul concentration delivers a different molar amount of the parent compound and its metabolite.

12. Fig. 6 and related section of the Results. As noted above for figs. EV9 and EV10, at a minimum the frequency data shown in panel E should be analyzed for statistical significance of the apparent differences between shControl and shPlzf for various limb abnormalities, and the levels of significance provided in a new column of the table. It would be worthwhile if the authors could also quantify the severity of limb reduction, as indicated above for figs. EV9 and EV10, and provide these data in a new bar graph showing the mean + SD for the severity of limb reduction in replicate samples and the statistical significance of apparent differences.

MINOR COMMENTS

1. Throughout the manuscript and figure legends, consistently use upper case font for proteins (e.g. PLZF, SALL4, CRBN), and use lower case italic font for animal genes (e.g.

Plzf, Sall4, Crbn).

2. In fig. 4, The labels "A" and "C" are missing from their respective panels, unless they were cut off in my version.

3. p. 10, lines 218-220: In this sentence of the Results, the authors' interpretation of the minimal PLZF protein degradation in GCB-DLBCL (SU-DHL-4) cells exposed to immunomodulatory (ImiD) drugs should be explained more clearly. The explanation needs to be distinguished from the sentence that follows, which says that, "These results strongly suggest that PLZF degradation is caused by IMiD drugs in various cells." In related figure EV5A, the abbreviations for DMSO (DM), lenalidomide (Le) and pomalidomide (Po) should be specified in the figure legend.

4. p. 13: For mouse (Mm) and chicken (Gg) proteins in the cell-free assay, the authors indicate that CRBN with thalidomide "did not bind" Mm SALL4 or PLZF, nor Gg SALL4 (fig. EV7). This should be changed to "minimally bound", as the figure shows measurable binding.

5. p. 14, line 300: Similarly for protein degradation (fig. 5D), "almost never" should be changed to, "minimal", and "observed" should be deleted.

6. There are numerous errors in grammar, and occasionally in word selection, throughout the manuscript that should be corrected. An accompanying Microsoft Word file, provided separately, will show some of these errors indicated by track changes in the Abstract, Introduction, Results and Discussion. The Authors should seek similar editorial assistance for the Methods and Legends for both the manuscript and EV figures.

Point-by-Point Responses to the Reviewers' Critiques (EMBOJ-2020-105375)

We deeply appreciate the thorough analysis and constructive suggestions provided by the three reviewers to guide us to further improve our manuscript. As described in more detail below, we have addressed all the reviewers' concerns. With this extensive revision, we hope that the reviewers will concur with us that we have addressed all of the raised concerns in a satisfactory manner and, consequently, substantially strengthened our paper.

Reviewer #1

Yamanaka et al. identify PLZF as a zinc-finger (ZF) neo-substrate for the CRBN E3 ubiquitin ligase. The initial discovery was made using protein arrays, followed by extensive cellular and biochemical validation. The authors report that PLZF is drug-induced CRBN substrate where degradation is induced by thalidomide, as well as 5-hydroxythalidomide. The latter compound is a frequently observed metabolite of thalidomide, with currently little information available concerning protein substrates targeted by 5-hydroxythalidomide. The detailed mapping of the PLZF degron required for degradation implicated PLZF zinc-fingers ZF1 and ZF3. The authors then continue to demonstrate that PLZF/ZBTB16 protein loss in a chicken-limb developmental system results in limb deformation, a phenotype reminiscent of thalidomide-induced phocomelia. Yamanaka & colleagues go on to make the case that a previously identified phocomelia substrate, SALL4, does not fully explain thalidomide teratogenicity across different animal models, while PLZF would offer a broader explanation.

The identification of additional candidate proteins that in addition to SALL4 could contribute to thalidomide-induced limb deformation, is an important discovery; as is the implication that thalidomide metabolites could be involved in this process. The experiments in the manuscript appear carefully conducted, and this body of work is undoubtedly going to be of great interest to the field. This reviewer considers this manuscript perfectly suited for publication in EMBO following careful experimental revision; this should also include the text, which is full of spelling and gramatical mistakes and is illegible in places.

Response: We thank the reviewer for the considerate comments on the importance of identifying PLZF in order to understand thalidomide teratogenicity.

Major points:

- The rationale why only PLZ/ZBTB16 ZF1-3 were examined is not clear; It would be paramount to carry out experiments where each ZF is deleted and cellular degradation is assessed. The argument that ZF1-3 are conserved and only those were tested is not convincing, as even ZFs with little conservation can contribute to degradation. It is important that this is experimentally addressed.

Response: We thank you for pointing out an important concern about the interaction region of CRBN-PLZF with thalidomide. In response to the reviewer's comment, we experimentally addressed this concern by transfection of truncated PLZF in cultured cells. The result has been added to Fig 3C in the revised manuscript. As shown in this revised figure, all truncated ZNFs of PLZF (ZNF1-9, ZNF1-8, ZNF1-7, ZNF1-6, ZNF1-5, ZNF1-4 and ZNF1-3) were degraded by thalidomide treatment. However, AlphaScreen-based biochemical analysis showed that only ZNF1-3 did not interact with CRBN (Fig 3B in the revised manuscript). Therefore, we considered artificial effects caused by truncated protein. All five ZNFs (ZNF1 – 5) were swapped with ZNF7, since it has been reported that ZNF7 does not interact with CRBN with IMiD (Sievers et al., 2018). All swapped full-length PLZFs were evaluated (Fig 3E in the revised manuscript). The findings indicated that ZNF1 and ZNF3 are critical for interaction of PLZF-CRBN. The binding ability of each GA mutant of ZNF1 or ZNF3 was significantly lower than that of PLZF-WT (Fig 3G in the revised manuscript). Double GA mutations involving ZNF1 and ZNF3 abrogated binding (Fig 3G in the revised manuscript). These findings were confirmed by cell-based protein degradation (Fig 3H in the revised manuscript). Based on these results, we concluded that ZNF1 and ZNF3 are required for the interaction between PLZF and CRBN with thalidomide and its derivatives. We added the result of the new experiment to Fig 3B and C and discuss the findings in the revised manuscript on lines 234-244.

- P. 14, lines 306-310; while the cellular experiments are convincing, they are based on over-expression; to use these cellular findings to explain SALL4 vs PLZF phenotypes in different animals is very far-fetched for my liking and should be more carefully discussed.

Response: Thank you for pointing out an important concern about the degradation of the SALL4 and PLZF proteins in vivo. We fully agree with your comment that our

results cannot explain the SALL4 phenotype compared to the PLZF phenotype in different animals because our results are based on cell experiments. We have toned down the relevant text and added the in vivo results from additional experiments that explored the degradation of SALL4 and PLZF proteins in different animals. The resulting revision on lines 321-323 of the revised manuscript addresses the reviewer's comment.

- Fig 4E. the effect of thalidomide and 5-Hydroxythalidomice on PLZF in THP-1 cells seems very minor; this needs to be done more quantitative i.e. using LICOR Western-blotting? Same with Fig. 7C! These are key figures of the paper and require extra attention, revised experiments and further quantification.

Response: Thank you for pointing out the experimental problems in our study. We repeated the experiments and tried to quantify the band intensity since we did not have access to a LICOR device. Thalidomide and 5-hydroxythalidomide induced PLZF degradation in THP-1 cells. In addition, the results in the revised Fig 7G were also quantified using the same method. We added bar graphs based on the band intensity of each protein in Fig 4E and Fig 7G in the revised manuscript.

- Is PLZ/ZBTB16 degradation evident in published proteomic data-sets, e.g by Celgene, the Ebert or Fischer labs? To what extent were PLZF ZF1-3 binding tested by Sievers et al., 2018?

Response: Thank you for the important comment about the identification of PLZF. In cell-based mass spectrometry regimens like SILAC, PLZF was has not been identified as a neosubstrate (An et al., 2017; Donovan et al., 2018; Krönke et al., 2015; 2014; Matyskiela et al., 2018). Sievers et al. (2018) used single ZNFs for screening and did not identify PLZF as neosubstrate. We believe that PLZF was not identified as a neosubstrate for two reasons. The first is the low protein expression levels of PLZF. The second is because multiple ZNFs are required for the interaction of CRBN-PLZF. These points are raised in the Discussion section of the revised manuscript on lines 409-415.

Minor points:

- P. 3, line 64; There is significant concern in the field whether zebra-fish is a useful model system for studying phocomelia (see: doi: 10.1073/pnas.1307684110); either cite this reference and point of the controversial nature, or remove arguments relating to zebrafish all together.

Response: Thank you for your valuable and important suggestions about the in vivo model of thalidomide teratogenicity. We agree that the zebrafish model is a controversial choice for the analysis of thalidomide teratogenicity in limbs. In response to the reviewer's comment, we cited the reference (doi: 10.1073/pnas.1307684110) and mention the zebrafish model for analysis of thalidomide teratogenicity in the revised manuscript (lines 60-63).

- P. 3, line 62/63; what is meant here? .. it was reported that the forelimb of SALL4-CKO were not abnormality (?); also see above. Overall the manuscript needs to be carefully revised for language and spelling; I gave up correcting spelling mistakes after a while...

Response: Thank you for the comment about SALL4 involvement on thalidomide teratogenicity. In the original manuscript, we intended to communicate that SALL4-conditional knockout mice (generated by two lines: TCre and Hoxb6Cre; Sall4 mutant) showed teratogenic phenotypes in the hind limb, while teratogenic forelimb phenotypes were not observed (Akiyama et al., 2015). Since this information could be confusing for readers, we modified the explanation in the Discussion section of the revised manuscript (lines 439-442).

- P. 4, line 76; the full-length

Response: We corrected the mistake in the revised manuscript (line 74).

- P. 4, Fig 4A is mentioned before other main text figures?

Response: We have removed Fig. 4A.

- P. 5, line 111; for substrate proteins of protein kinases ... E3 ligases

Response: We have corrected the mistake in the revised manuscript (lines 111-112).

- P. 7, line 150; see Petzold et al., 2016 for detailed measurements of CK1a-CRBN binding in the presence of other IMiDs, CK1A binding in vitro is also observed with thalidomide, lenalidomide and pomalidomide and small differences in binding Kd translate into almost binary degradation/no-degradation choices in cells; the Matyskiela et al., 2016 reference refers to GSPT1; this should be corrected and PMID: 26909574 should be cited instead.

Response: Thank you for highlighting an important point regarding the neosubstrate specificity of IMiD. We changed our representation of CK1α protein degradation and interaction by referring to the correct references in the revised manuscript (lines 149-153) and added additional references (PMID: 26909574).

- P.7, line 157; Donovan et al., 2018 used a TF-FRET set-up and not an alpha-screen; all references should be carefully checked

Response: Thank you for the comment about the biochemical assay system in the previous study. We now clear stated that Donovan et al. (2018) performed TR-FRET analysis in the revised manuscript (lines 156-158).

- P.17 line 370; include Sievers et al., 2018 reference here

Response: We have added the references in the revised manuscript (line 402).

Referee #2:

OVERVIEW

The authors constructed a human protein array consisting of 1,118 human recombinant transcription factor (TF) and other zinc finger proteins, and used this array to discover a novel TF, promyelocytic leukemia zinc finger (PLZF), that binds to the cereblon (CRBN) component of the CRL4 E3 ubiquitin ligase complex in the presence of the teratogenic sedative/immunosuppressive drug thalidomide, its derivatives (lenalidomide, pomalidomide) and its 5-hydroxythalidomide metabolite. In a series of comprehensive biochemical studies using cell-free and cellular assays together with related biochemical and developmental studies in chick embryos, the authors show that the resulting PLZF-CRBN-thalidomide complex results in the ubiquitination and proteasomal degradation of PLZF, resulting in developmental disorders including limb abnormalities in chick embryos, as previously shown in various species for other developmental important TFs and other binding proteins such as SALL4. They also show in several knockdown studies that PLZF itself is necessary for normal limb development. Remarkably, PLZF but not SALL4 was reduced in the limb buds of thalidomide-exposed chick embryos, in contrast to previous studies of rabbit embryos in which SALL4 was degraded, revealing species-dependent, CRBN-mediated mechanisms of susceptibility.

These studies not only identify PLZF as a new developmentally important CRBN substrate, but also provide a novel molecular basis for an unsubstantiated chemical mechanism previously proposed for thalidomide teratogenesis. In the latter case, the phthalimido ring of thalidomide is oxidized by cytochromes P450 enzymes (CYPs) to form a 5-hydroxythalidomide metabolite. Years ago, this reaction was postulated to involve the formation of an unstable arene oxide (epoxide) reactive electrophilic intermediate that covalently binds to developmentally important cellular macromolecules, causing developmental disorders. The authors' studies support a teratogenic role for the 5-hydroxythalidomide metabolite, but suggest that the mechanism involves binding of the stable 5-hydroxythalidomide metabolite itself to CRBN, rather than the covalent binding of a reactive epoxide precursor.

The manuscript would be improved by providing more context for the teratological relevance of 5-hydroxythalidomide, and by providing a more complete mechanistic context within which to consider CRBN-dependent mechanisms.

Response: We thank the reviewer for the considerate comments on the importance of the study findings concerning thalidomide teratogenicity, including metabolites.

MAJOR COMMENTS

1. The relevance of the comprehensive biochemical data rests upon the associated changes in limb bud development, the latter of which would benefit from more explicit and quantitative details.

Response: We thank the reviewer for pointing out important concerns about in vivo experiments using chicken embryos.

· The Methods (pp. 31-32) should indicate more clearly for the studies of limb reduction (figs. EV9 & EV10), exactly what HH stage and embryonic day the chick embryos were treated, and what drug solution concentration (both molar and ug/ul) and volume were administered for both thalidomide or 5-hydroxythalidomide, and what HH and embryonic day the embryos were evaluated.

Response: We mentioned the HH stage and embryonic day chicken embryos used in all experiments in the Methods section and figure legends in the revised manuscript.

· For both figs. EV9 and EV10, the measurement of limb reduction should be quantified (length, area, etc.), the data for the mean + SD for reduction should be presented for each treatment group in a new bar graph, and the differences should be tested for statistical significance and shown in the figure. If a precise measurement is not possible, then a clear definition of a substantial reduction (e.g. greater than 50% less than the mean of control group?) should be provided, and

the mean number of animals exhibiting this reduction should be compared to that for the control group in a new bar graph, and differences analyzed for statistical significance that is shown in the figure. The same is true for the fgf8 studies in panel B of fig. EV9 (see below).

Response: We quantified the expression area of *Fgf8* (Fig 7A in the manuscript) and measured the length of each bone (Fig 7B in the revised manuscript) and the length of each limb bud (Fig 7D and F in the revised manuscript).

· Given the fundamental importance of limb reduction to the authors' hypothesis, I believe that these two EV figures or some variant (the relevant panels from these figures?) would be better placed in the main body of the manuscript.

Response: We replaced Figs. EV9 and EV10 in the original manuscript with Fig 7A-D and F in the revised manuscript.

2. Indicate at least in the Methods (p. 31), if not in the Discussion, why different volumes of drug solution were applied to chick embryos in the biochemical studies (30 ul) versus the morphological studies of limb reduction (10 ul). In the latter case, additional 10 ul volumes were applied twice more (for a total application of 30 ul), but at 12-hour intervals, which would result in substantially lower peak exposure concentrations to the limb bud. If the higher volume (and hence dose) in the former was necessary to elicit the observed outcomes, then discuss why the outcomes are still relevant to the changes observed in limb reduction with the lower dose.

Response: We thank the reviewer for pointing out an important concern about the exposure level of thalidomide in the in vivo experiments using chicken embryos. In the in situ hybridisation (Fig 7A in the revised manuscript), IHC (Fig 7C in the revised manuscript), or immunoblot experiments (Fig 7E and G in the revised manuscript) experiments, 30 µl of thalidomide or 5-hydroxythalidomide was used at HH18 (E3) and the embryos were collected at HH23 (E4) to obtain robust phenotypes following the short-term thalidomide exposure. For bone staining, chicken embryos developed up to E10 were required. However, almost all chicken embryos treated with 30 µl of

thalidomide did not develop to E10. Therefore, to increase the collection rate of chicken embryos at E10, we treated the embryos with thalidomide three times. In response to the reviewer's comment, we explained the difference in thalidomide treatment in the Methods section of the revised manuscript (lines 857-860).

3. In the Results on p. 12, the authors assert that thalidomide and its 5-hydoxythalidomide metabolite were similarly efficacious in cells in causing the degradation of PLZF and SALL4, based upon representative immunoblot analyses shown in fig. 4D and 4E. To support this assertion, these two panels should each be accompanied by a complementary graph that shows the mean + SD values for the replicates for each treatment in line diagrams or bar graphs, along with the level of significance for differences and the number of cell assays sampled. The Results should be expanded to comment on the added panels and content.

Response: We thank the reviewer for the suggestion about the protein degradation level of SALL4 and PLZF by thalidomide or 5-hydroxythalidomide. In response, we quantified the band intensities of SALL4 and PLZF, and added bar graphs in the revised manuscript (revised Fig 4D and 4E). 5-Hydroxythalidomide induced protein degradation of SALL4 more potently than did thalidomide. In contrast, protein degradation of PLZF by 5-hydroxythalidomide was slightly weaker than that caused by thalidomide. These differences in protein degradation levels were consistent with the biochemical analysis (Fig 4B in the revised manuscript). Therefore, we added quantified data in the form of bar graphs (Fig 4D and 4E in the revised manuscript) and have mentioned the differences between thalidomide and 5-hydroxythalidomide in both biochemical interaction and protein degradation in the revised manuscript (lines 264-274).

4. Similarly on p. 12, the authors state that "these results suggest that 5-hydroxythalidomide has potential similar to that of thalidomide for PLZF and SALL4 degradation in humans". At least in the Discussion, they should provide a more nuanced context in which to apply their in vitro data. Important issues include: (a) their own data in a cell-free assay (fig. 4B) show that 5-hydroxythalidomide is much less effective that thalidomide in enhancing PLZF binding to CRBN;

Response: Thank you for an important comment. We clearly note that the interaction of CRBN-PLZF in the presence of 5-hydroxythalidomide was weaker than that of thalidomide in the revised manuscript (lines 269-272).

(b) in humans, thalidomide will be metabolized by CYPs to its 5-hydroxythalidomide metabolite primarily in the mother's liver rather than in the embryo, which has relatively low CYP levels, and most of that 5-hydroxythalidomide will be enzymatically glucuronidated by the mother and eliminated in her urine without reaching the embryo;

Response: This is a very good question. We agree with your comments that CYPs metabolise thalidomide to 5-hydroxythalidomide in the liver of the mother, rather than in the embryo, and that 5-hydroxythalidomide is further enzymatically glucuronidated by the mother and eliminated in her urine without reaching the embryo. The first metabolic step from thalidomide to 5-hydroxythalidomide is slow, while the second step from 5-hydroxythalidomide to glucuronidated products via reactive arene oxide intermediate is relatively fast (Chowdhury et al., Chem. Res. Toxicol. 23:1018-1024 (2010); Yamazaki et al., Chem. Res. Toxicol. 29, 1279-1281 (2016); Yamazaki et al., Chem. Res. Toxicol., 29, 1903-1911 (2016).). Thus, arene oxide derived from 5-hydroxythalidomide is a potentially active species of teratogenic mechanisms induced by thalidomide. Although the first metabolite, 5-hydroxythalidomide, is further metabolised to arene oxide, the lifetime of 5-hydroxythalidomide is still relatively long due to the level of detection in plasma. Plasma concentrations of thalidomide are approximately 10 uM initially, 13 uM at 4 h, 10 uM at 8 h and 0 uM at 24 h. The levels of 5-hydroxythalidomide at the same respective times are 0.25 uM, 0.25 uM, 0.20 uM and 0.1 uM in chimeric TK-NOG mice with humanised liver cells harbouring cytochrome P450 3A5 after oral administration of thalidomide (100 mg/kg) (Guengerich et al, Chem. Res. Toxicol. 2015, 28, 2088−2090). Thus, the concentration of 5-hydroxythalidomide is approximately 1-5% that of thalidomide.

(c) thalidomide is rapidly and non-enzymatically hydrolyzed to 12 major metabolites, independent of its CYP-catalyzed metabolism to

5-hydroxythalidomide, so what is the percentage of thalidomide that is metabolized to its 5-hydroxythalidomide metabolite?;

Response: Part of the answer to this question has been mentioned above in point (b). We agree that thalidomide is rapidly and non-enzymatically hydrolysed to 12 major metabolites. However, the percentage of 5-hydroxythalidomide metabolites has not been clearly investigated. Some studies suggest that the significant route of thalidomide breakdown in humans and animals is through spontaneous hydrolysis, not CYP-catalyzed metabolism. However, the percentage of 5-hydroxythalidomide is not very important, since the concentration of 5-hydroxythalidomide is highly dependent on the dose.

(d) what molar concentration range of 5-hydroxythalidomide is found in the plasma of an adult taking thalidomide, and what molar concentration would be estimated in the embryo?; and,

Response: The concentrations of 5-hydroxythalidomide in plasma using chimeric TK-NOG mice with humanised liver cells harbouring cytochrome P450 3A5 are mentioned above in point 8. In addition, previously reported data have varied. In myeloma patients, only hydrolysis products were detected in plasma and urine samples. No hydroxylated metabolites were detected at any time point (Chung, Clin. Cancer Res., 2004, 5949). However, Figg and co-workers (Ando et al., Cancer Biol Ther., 2002, 1:669-673) reported that the concentration range of 5-hydroxythalidomide is dependent on the dose and CYP polymorphism, and that 5-OH thalidomide was detected at 46.6 nM (CYP2C19, dose of 600 mg/day). These findings indicate that the molar concentration in the embryo is challenging to estimate.

(e) how do the molar concentrations of thalidomide and 5-hydroxythalidomide in the authors studies, including chick embryos, compare to those achieved in adults taking thalidomide and embryos exposed in utero? This discussion is also important to understanding the context of the authors' novel and important discovery that 5-hydroxythalidomide may contribute to not only the teratogenicity of thalidomide, but also its immunomodulatory role.

Response: We agree with you that the concentration of 5-hydroxythalidomide is vital to the teratogenicity of thalidomide and its immunomodulatory role. However, the concentration is not a salient point in the discussion of our discovery. The importance of our study is that, compared to thalidomide, 5-hydroxythalidomide more strongly induces SALL4 degradation, which is highly related to teratogenicity. The binding of thalidomide to CRBN, which recruits SALL4, was recently reported as a novel hypothesis of the teratogenicity of thalidomide. There is a considerable difference between the two hypotheses. The SALL4/thalidomide/CRBN complex is current more accepted that the arene oxide intermediate from 5-hydroxythalidomide. Our finding concerning SALL4/5-hydroxythalidomide/CRBN could be the key to connect the two independent hypotheses.

5. p. 14 and fig. 5: The data presented suggest but do not confirm the authors' novel and important assertion that "thalidomide-dependent PLZF degradation occurs in many animals, ... while in contrast, SALL4 degradation from thalidomide occurs in a limited number of animals (species)". To confirm this discovery, at least the key panels from fig. 5 showing results from representative samples should each be accompanied by a complementary graph that shows the mean + SD values for the replicates for each treatment using line diagrams or bar graphs, along with the level of significance for differences and the number of cell assays sampled. The same issue applies their assertions relating to the panels in the same figure showing the remarkable effects of 5-hydroxythalidomide in initiating SALL4 and PLZF degradation. The Results should be expanded to comment on the added panels and content.

Response: We thank the reviewer for the suggestion about protein degradation levels of mouse or chicken SALL4 and PLZF by thalidomide or 5-hydroxythalidomide. In response, we quantified the band intensities of SALL4 and PLZF, and added bar graphs in the revised manuscript (Fig 5A-H in the revised manuscript). MmPLZF and MmSALL4 or GgSALL4 did not induce protein degradation by MmCRBN-WT or GgCRBN-WT (Fig 5A, 5B and 5D in the revised manuscript). In contrast, GgPLZF induced protein degradation by thalidomide treatment (Fig 5C in the revised manuscript). Furthermore, humanised-MmCRBN or -GgCRBN more strongly induced protein degradation of PLZF and SALL4 (Fig 5A-D in the revised manuscript). Therefore, we concluded that the differences in the amino acid sequences in both CRBN

and neosubstrate (SALL4 and PLZF) are critical for the species specificity of thalidomide. Given that ZNF1 and ZNF3 in PLZF were highly conserved among many species and that GgPLZF was degraded in chicken embryos, but not GgSALL4, we suggested “thalidomide-dependent PLZF degradation occurs in many animals”. However, further in vivo experiments will be required to validate our suggestion. Concerning protein degradation by 5-hydroxythalidomide, our results suggest the possibility that 5-hydroxythalidomide more strongly induces protein degradation in mouse or chicken SALL4 and PLZF. Again, in vivo analyses will be required to validate the results. We are planning to do these experiments. Based on these results, we added the bar graphs (Fig 5A-H in the revised manuscript) and discussed protein degradation of mouse or chicken protein by thalidomide and 5-hydroxythalidomide (lines 317-323, 332-335).

6. p. 14, lines 311-315: The authors should include a cautionary comment that the evidence they cite for thalidomide causing limb defects in mice "humanized" to express human CYP3A isoforms is based upon a single in vitro (embryo culture) study showing at best only modest evidence of limb defects, and the same authors found no evidence of limb abnormalities in in vivo studies in these humanized CYP3A mice. The absence of limb defects in this humanized model may be due in part to maternal glucuronidation and elimination of the 5-hydroxythalidomide metabolite formed by the human CYP3A isoforms, as noted above.

Response: Thank you for your valuable suggestion. Kazuki et al. (Sci. Rep. 6, 21419, 2016) provided critical evidence that metabolic activation is essential for teratogenicity, which strongly supports the arene oxide hypothesis. Although we did not discuss the “SALL4/5-hydroxythalidomide/CRBB" vs. "arene oxide" in this manuscript, our results support the importance of bio-activation of thalidomide. In response to the reviewer’s comment, we modified the sentences accordingly (lines 325 and 462-483).

7. p. 16 and fig. 7: The authors assert in the Results that thalidomide induces the degradation of PLZF but not SALL4 in chick embryos, unlike other species susceptible to thalidomide-induced limb abnormalities (e.g. rabbits) which have been shown by other labs to exhibit SALL4 degradation. To prove this assertion, the immunohistochemical staining should be quantified, and panels 7A, 7B and 7C,

each showing results from one representative sample, should each be accompanied by a complementary graph that shows the mean + SD values for the replicates for each treatment using line diagrams or bar graphs, along with the level of significance for differences and the number of cell assays sampled. In panel B, the meaning of "normal", "weak" and "strong" should be specified. To complement the biochemical schemes shown respectively for chick and human limb buds in panels 7D and 7E, it would be useful to add a parallel scheme for rabbits to illustrate the major difference between that species (published by others) and the authors' results in chick embryos. In addition to the ug/ml values, add the molar concentrations of thalidomide and 5-hydroxythalidomide in the figure legend. The Results should be expanded to comment on the added panels and content.

Response: We thank the reviewer for mentioning the unresolved and important concerns about the in vivo experiments using chicken embryos. In response, we quantified the immunohistochemical staining (Fig 7C in the revised manuscript) and immunoblot analyses (Fig 7E and 7G in the revised manuscript). The results showed that GgPLZF was degraded by thalidomide treatment, but not by GgSALL4. With regard to phenotypes in Fig 7D and F in the revised manuscript, these phenotypes were based on evaluation by four developmental biology experts. To address the reviewer's concern, we measured limb bud lengths of chicken embryos that were used for immunoblotting, and added the graphed limb bud length of each phenotype in Fig 7D in the revised manuscript. Based on these results and the reviewer's comments, we modified the schematic diagram of protein degradation in the chick and human limb bud (Fig 9A and B in the revised manuscript). In the case of rabbit limb bud, OcCRBN has the same amino acid sequence as HsCRBN (Glu373 and Val384 in rabbit CRBN in Fig. EV4C). Furthermore, the production of 5-hydroxythalidomide in rabbits was previously reported. The findings support the validity of the rabbit model for humans. Therefore, we described human, non-human primate and rabbit in the schematic diagram (Fig 9B in the revised manuscript and lines 454-447 and 500-502).

8. In the Discussion, the authors say that 5-hydroxythalidomide showed higher "potential" (efficacy?) than thalidomide in inducing the degradation of both PLZF and SALL4 from humanized mice and chick embryos in HEK294T cells. Given the potential importance of 5-hydroxythalidomide, the authors should add a short paragraph to the Discussion briefly outlining the old hypothesis for thalidomide

teratogenicity being due to its "bioactivation" by CYP isozymes to a reactive epoxide intermediate which, if not detoxified by epoxide hydrolase, will covalently bind to developmentally important proteins resulting in developmental disorders. Some relevant references are as follows:
Gordon et al., Proc. Natl. Acad. Sci. USA 78: 2545-25-48, 1981.
Fort et al., Teratogen. Carcinogen. Mutagen. 20: 35-47, 2000.
Chowdhury et al., Chem. Res. Toxicol. 23: 1018-1024, 2010.

Response: Thank you for your suggestion. We have previously answered the question in point Response No.4-(b) and No.6. The sentences were modified accordingly with the inclusion of cited references.

9. As noted above, thalidomide rapidly and non-enzymatically hydrolyzes to 12 major products, which are in addition to the 5-hydroxythalidomide metabolite produced by the CYP-catalyzed metabolism of thalidomide. Two of the hydrolysis products, 2-phthalimidoglutaramic acid and 2-phthalimidoglutaric acid, cause limb abnormalities in rabbit embryo culture via a mechanism involving oxidative stress (Lee et al., FASEB J. 25: 2468- 2483, 2011), which is supported by in vivo studies of thalidomide (Parman et al., Nat. Med. 5: 582-585, 1999). This mechanism should be mentioned either in the Introduction on p. 4 with the other mechanisms, or in the Discussion, to provide a more complete context.

Response: Thank you for your suggestion. We understand your comments concerning arene oxide, including the oxidative stress hypothesis via bio-activation. One of our authors (N. Shibata) has collaborated with Wells (Toxicol. Sci. 2011,122, 157) and Chowdhury et al. (Chem. Res. Toxicol. 2015, 28, 2088−2090; Chem. Res. Toxicol. 2016, 29, 1279−1281; Chem. Res. Toxicol. 2017, 30, 1622−1628; J. Toxicol. Sci. 2017, 42,343; Chem. Res. Toxicol. 2012, 25, 274−276; Chem. Res. Toxicol. 2013, 26, 486−489; Chem. Res. Toxicol. 2010, 23, 1018–1024; Chem. Res. Toxicol. 2014, 27, 147−156; Chem. Res. Toxicol. 2014, 27, 304−308) for more than a decade. In the present manuscript, our intention is to show the importance of a metabolite, 5-hydroxythalidomide, with a stable 5-hydroxythalidomide metabolite to CRBN, in SALL4 degradation. We deliberately have avoided discussion on the important hypothesis of “covalent binding of a reactive arene oxide/glucuronidation/oxidative stress” because we still believe the potential of the hypothesis. To avoid a detailed

discussion of the mechanism of teratogenicity at the molecular level, we modified the discussion section with brief comments on arene oxide intermediates.

10. Fig. EV9.

· Panel B, fgf8 mRNA expression: provide an additional panel with a bar graph showing mean + SD data and statistical analysis of significance between control and thalidomide.

Response: We thank the reviewer for the comment. As mentioned in response No.1, we measured *Fgf8* mRNA expression area and added the bar graph (Fig. 7A in the revised manuscript).

· Panel C, limb abnormalities: provide an additional panel with a bar graph showing mean + SD data and statistical analysis of significance for some quantitative measurement of limb reduction (see comments for fig. 6). Also, in panel C and/or its legend, add the molar concentration of thalidomide to complement the ug/ul concentration-each will be selectively familiar to different readers.

Response: We thank the reviewer for the comment. As mentioned in response No. 1, we measured bone length and added the bar graph (Fig 7B in the revised manuscript). In addition, we explained the thalidomide concentration using both µg/µl and molar in the figure legend.

11. Fig. EV10. Add molar concentrations-this is particularly relevant for 5-hydroxythalidomide, which has a higher molecular weight than thalidomide, so the same ug/ul concentration delivers a different molar amount of the parent compound and its metabolite.

Response: We thank the reviewer for the comment. In response, we described the thalidomide and 5-hydroxythalidomide concentrations using both µg/µl and molar in the figure legend.

12. Fig. 6 and related section of the Results. As noted above for figs. EV9 and EV10, at a minimum the frequency data shown in panel E should be analyzed for statistical significance of the apparent differences between shControl and shPlzf for various limb abnormalities, and the levels of significance provided in a new column of the table. It would be worthwhile if the authors could also quantify the severity of limb reduction, as indicated above for figs. EV9 and EV10, and provide these data in a new bar graph showing the mean + SD for the severity of limb reduction in replicate samples and the statistical significance of apparent differences.

Response: We thank the reviewer for an important comment about the knockdown experiments. In response, we measured bone lengths of each chicken embryo and analysed the statistical significance (Fig 6E in the revised manuscript). We added a new graph to Fig 6E, and quantified the data in Figs. EV9 and EV10 (Fig 7A, B, D, and F in the revised manuscript).

MINOR COMMENTS

1. Throughout the manuscript and figure legends, consistently use upper case font for proteins (e.g. PLZF, SALL4, CRBN), and use lower case italic font for animal genes (e.g. Plzf, Sall4, Crbn).

Response: We modified the fonts for proteins as genes throughout the revised manuscript.

2. In fig. 4, The labels "A" and "C" are missing from their respective panels, unless they were cut off in my version.

Response: We addressed these concerns.

3. p. 10, lines 218-220: In this sentence of the Results, the authors' interpretation of the minimal PLZF protein degradation in GCB-DLBCL (SU-DHL-4) cells exposed

to immunomodulatory (ImiD) drugs should be explained more clearly. The explanation needs to be distinguished from the sentence that follows, which says that, "These results strongly suggest that PLZF degradation is caused by IMiD drugs in various cells." In related figure EV5A, the abbreviations for DMSO (DM), lenalidomide (Le) and pomalidomide (Po) should be specified in the figure legend.

Response: We now have clarified the explanation of PLZF degradation in various cell lines and CRBN expression level for PLZF degradation in the revised manuscript (lines 221-225). In addition, we have identified the abbreviations in all legends in the revised manuscript.

4. p. 13: For mouse (Mm) and chicken (Gg) proteins in the cell-free assay, the authors indicate that CRBN with thalidomide "did not bind" Mm SALL4 or PLZF, nor Gg SALL4 (fig. EV7). This should be changed to "minimally bound", as the figure shows measurable binding.

Response: In the biochemical analysis in Fig EV7 (Fig EV5 in the revised manuscript), the vertical axis in each bar graph indicates the relative AS signal, which is a thalidomide-dependent interaction between CRBN and PLZF or SALL4. Thalidomide-dependent interactions were not evident between MmCRBN-WT and MmPLZF, MmCRBN-WT and MmSALL4 or GgCRBN-WT and GgSALL4 (Fig EV5A in the revised manuscript). Therefore, the luminescent signal in these protein pairs did not increase in the presence of thalidomide compared with dimethylsulfoxide. To address the reviewer's comment, we explained the interactions in the revised manuscript more clearly (lines 291-297).

5. p. 14, line 300: Similarly for protein degradation (fig. 5D), "almost never" should be changed to, "minimal", and "observed" should be deleted.

Response: We modified the explanation in Fig 5D in the revised manuscript (line 315).

6. There are numerous errors in grammar, and occasionally in word selection, throughout the manuscript that should be corrected. An accompanying Microsoft

Word file, provided separately, will show some of these errors indicated by track changes in the Abstract, Introduction, Results and Discussion. The Authors should seek similar editorial assistance for the Methods and Legends for both the manuscript and EV figures.

Response: We thank the reviewer for the important suggestion about the quality of the manuscript. The comment was valuable in improving the manuscript. We thoroughly revised the manuscript with the assistance of a native English-speaking science editor contracted to Editage (URL: https://www.editage.com).

Reviewer #3

Using a cell-free protein screening assay, Yamanaka and colleagues identified PLZF as an additional thalidomide-induced neo-substrate of the CRBN-CRL4 E3 ligase. PLZF was convincingly validated as a pan-IMiD substrate by a large series of protein analyses in different cell lines, IPs, and ubiquitination assays. The zinc finger domain critical for the CRBN-thalidomide-PLZF interaction was successfully mapped by cloning and testing different PLZF forms. The authors could further show that 5-hydroxythalidomide, a major metabolite of thalidomide in human but not in mice, is also able to degrade PLZF and SALL4, a previously identified CRBN neo-substrate involved in thalidomide-induced teratogenicity. As shown in the current work and by previous publications, thalidomide treatment of chicken embryos results in limb malformation. The authors demonstrate that PLZF, but not SALL4, is degraded by thalidomide and 5-hydroxythalidomide in chicken limb buds and that knockdown of PLZF by shRNAs resembles the thalidomide phenotype. This implies that the teratogenicity observed in chicken embryos is induced by PLZF degradation suggesting that it contributes to thalidomide teratogenicity in human in addition to SALL4.

Identification of PLZF as a thalidomide-induced CRBN neo-substrate likely involved in teratogenicity is a new important finding adding another piece to the puzzle of the molecular mechanism for thalidomide teratogenicity. Furthermore, PLZF in addition to SALL4 may serve as an easily assessable marker for drug-associated teratogenicity what may be useful for future drug development.

Response: We thank the reviewer for their comments about the importance of our study in clarifying thalidomide teratogenicity.

Major concerns:

-Although the results are highly suggestive that PLZF is involved in thalidomide-induced teratogenicity in chicken, the finding would be strengthened by a rescue experiment by overexpressing PLZF or even better a degradation resistant PLZF mutant in analogy to the transfection of the CRBN YWAA mutant as performed by Ito et al. 2010.

Response: We thank the reviewer for the valuable suggestion to strengthen our argument that PLZF is a neosubstrate involved in thalidomide teratogenicity. We fully agree with the reviewer's comment that rescue experiments in chicken embryos will strengthen the data for the involvement of PLZF and thalidomide teratogenicity. We tried to perform rescue experiments by observing phenotypes in chicken embryos transfected with GgPLZF before we submitted the original manuscript. However, it was experimentally difficult to distinguish rescued embryos and only a weak phenotype caused by thalidomide. Therefore, in the revised manuscript, we investigated whether GgPLZF overexpression rescued mRNA expression of *Fgf10* and *Fgf8*, since the downregulation of these genes produces thalidomide teratogenicity. GgPLZF overexpression rescued both thalidomide-induced teratogenic phenotypes and reduced *Fgf10* and *Fgf8* expression by thalidomide treatment (Fig 8 in the revised manuscript). These results strengthen our argument that PLZF is a neosubstrate involved in thalidomide teratogenicity. We have added the new experimental results in Fig 8 in the revised manuscript.

Minor concerns:

-As the authors mention, PLZF is involved in several biological processes including immune function. Would degradation of PLZF be in line with other properties of IMiDs like effects on T cells and monocytes?

Response: We thank the reviewer for the important comment about the involvement of PLZF degradation on the immunomodulatory effects of IMiD. It has been reported that PLZF functions as an important transcription factor in many immune responses, including development and function of immune cells. Therefore, we also believe that PLZF is a neosubstrate involved in teratogenicity and immunomodulatory effects, as the reviewer pointed out. However because IKZF1 and IKZF3 are also important transcription factors in immune responses, further analyses are required to understand the effect of PLZF degradation on the immunomodulatory effect. These analyses are beyond the scope of this study and will be thoroughly analysed in future research.

-5-hydroxythalidomide is degrading PLZF but not IKZF1. What is the impact of 5-hydroxythalidomide on other CRBN neo-substrates (e.g. CK1a, ZFP91, GSPT1)?

Response: We thank the reviewer for the suggestion concerning the neosubstrate specificity of 5-hydroxythalidomide. In response, we investigated whether 5-hydroxythalidomide induced protein degradation of CK1α and ZFP91. Immunoblot analysis showed that CK1α and ZFP91 were degraded by lenalidomide and pomalidomide, respectively, although CK1α and ZFP91 were not degraded by 5-hydroxythalidomide (Fig 4F in the revised manuscript). We added the new experimental results to Fig 4F in the revised manuscript and mention the neosubstrate specificity of 5-hydroxythalidomide (lines 264-265).

-Is 5-hydroxythalidomide in general toxic to cells as compared to thalidomide?

Response: We thank the reviewer for the comment concerning the cytotoxicity of 5-hydroxythalidomide. In the THP-1, HuH7 and HEK293T cells, 5-hydroxythalidomide was not cytotoxic. Lenalidomide and pomalidomide are reportedly active against multiple myeloma by protein degradation of IKZF1 and IKZF3. Therefore, it is important to investigate whether 5-hydroxythalidomide also is active against multiple myeloma. This research is beyond the scope of this study and will be investigated in future research.

-Is thalidomide converted to 5-hydroxythalidomide in chicken?

Response: We thank the reviewer for an important comment about the generation of 5-hydoxythalidomide in chicken. The generation of 5-hydroxythalidomide in chickens has not been reported. However, in humans, mice and rabbits, the generation of 5-hydoxythalidomide was reported (Lu, et al., J. Pharmacol. Exp. Ther. 2004, 310, 571−577). Since 5-hydroxythalidomide was generated by P450 in these species, it is anticipated that 5-hydroxythalidomide is also generated in chickens. This remains to be demonstrated.

-The manuscript text has to be thoroughly revised. There are some inconclusive sentences e.g., abstract: "However, it remains unclear." and many grammatical mistakes, e.g. "it was reported that forelimb of SALL4-CKO mice were not abnormality".

Response: We thank you for the comments about critical issues in the original manuscript, including grammatical mistakes. The manuscript was revised with the assistance of a native English-speaking science editor contracted to Editage (URL: https://www.editage.com).

1st Revision - Editorial Decision 22nd Oct 2020

Dear Dr. Sawasaki,

Thank you for submitting a revised version of your manuscript. I sincerely apologise for the delay in the review process. Your study has now been seen by all original referees, who find that most of their main concerns have been addressed and are now in favour of publication of the manuscript after further textual revisions. There are also a couple of editorial issues that have to be addressed before I can extend formal acceptance of the manuscript:

2. In Author Checklist, please clarify point 3b on randomisation. Did you intend to say that all thalidomide treated embryos showed teratogenicity, and thus randomisation was not necessary? The current text could also be perceived as stating that thalidomide-treated embryos that did not show any phenotype were treated as controls.
3. Figures 8A and Appendix Fig. S2 are not referred to in the text.
4. Please correct the callouts to Table EV1 in the manuscript text (currently Table S1 is mentioned on page 25).
5. Please add a brief legend to Table EV1 in a separate tab.
6. Please update Appendix Figure nomenclature to Appendix Figure S1 etc. in the manuscript text and in the figure legends.
7. Please assemble the two Appendix Figures and their legends into a single Appendix file prefaced by a short table of contents.
8. Many Western blot panels and the corresponding source data appear rather pixelated. Please clarify how were the Western blot images acquired and submit better resolution images.
9. Source data panels for most Western blot panels do not fully match the provided source data. Please send us full images of the original blots, as currently most of source data images are cropped. Please note that the figures should fully match the source data.
10. During a standard image analysis we detected potential aberrations in the figure 6E. We would like to clarify these issues and we are kindly requesting the unmodified source data for these panels.
11. Papers published in The EMBO Journal are accompanied online by a 'Synopsis' to enhance discoverability of the manuscript. It consists of A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height, jpeg or png format). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Our publisher is currently performing their pre-publication check of data information provided in figure legends, and I will send you their comments once the check has been completed to allow you to start preparation of the revision.

Please let me know if you have any further questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

Referee #1:

My concerns from the previous submission have been addressed. The ZF mapping as well as the chicken teratogenicity experiments have substantially added to the paper.

Given a recent manuscript by the same corresponding author,

https://www.nature.com/articles/s41467-020-18488-4

it would be desirable to cite this work and to clearly state that 5'-hyrdoxythalidomide is able to recruit SALL4 (and indicate this as already known).

Referee #2:

Second round of reviews:

The authors have satisfactorily addressed the majority of my concerns, particularly including the revision of several figures to include bar graphs with quantitative comparisons (means + SD) and statistical analysis, which was a major concern. Moreover, they have added an important new study showing that overexpression of PLZF partially protects embryos from thalidomide-initiated abnormalities in Fgf8/10 expression and limb bud development. In light of the authors' revisions, this manuscript highly merits publication in the EMBO Journal.

However, there remain several important concerns that the authors did not satisfactorily address, including some errors in their revisions.

Please note that:

• The line numbers in the text below are based upon my copy of the Word version of the manuscript, and may be somewhat inaccurate as the numbers changed due to my editing of the text in the manuscript.
• Most of my recommendations for revision have been made directly in the accompanying Word version of the manuscript, shown by track changes.

LINE COMMENT
#

27-29 Revise and combine two sentences into one as follows: "Human cytochromes P450 convert thalidomide to two monohydroxylated metabolites that are considered to contribute to thalidomide effects, although the mechanism is unclear." (Note that the "s" is on "cytochromes" and not "P450".)

82-85 The sentence in blue font concerning a cited study of embryos from mice expressing human CYP3A enzymes needs revision, as do several related statements throughout the manuscript. A claim of "teratogenicity" cannot be made based upon embryo culture data because the defect may be resolved prior to the end of gestation. Also in the cited paper, CYP3A protein was in the placenta rather than the embryo, and an electrophilic reactive intermediate formed in that organ may not be sufficiently stable to reach the embryo. More importantly, the claimed limb abnormalities were at best only modest, no other embryopathies were observed (unlike in other species like rabbits), and no limb abnormalities occurred with in vivo studies in the same mouse strain by the same authors, so the basic premise is controversial. I suggest revising this sentence to a more cautious statement something like the following: "Mouse embryos that express placental human CYP3A isoforms exhibited modest thalidomide-induced limb defects in culture compared to wild-type controls, suggesting that placental CYP3A isoforms may convert thalidomide to an embryopathic reactive intermediate via the formation of 5-hydroxythalidomide, although no limb defects were observed in vivo (Kazuki et al., 2016)." This wording in part reflects a paper cited by the authors that indicates it is not clear whether a reactive electrophilic epoxide intermediate is produced during the formation of 5-hydroxythalidomide, or following its subsequent oxidation, but its presence in either case is supported by the presence of a glutathione conjugate, since glutathione is known to react with and detoxify many different types of electrophilic reactive intermediates.

326-328 I have revised this sentence in the manuscript for some of the important reasons noted above for 82-85.

852-853 Should the second "thalidomide" read "5-hydroxythalidomide", and the existing "5-hydroxythalidomide" be deleted, as I have shown in my revision in the accompanying manuscript?

922 Fully define "EGFP" and explain its relevance. Also fully define this abbreviation in the legend for fig. 8 (line 1305).

1258 vs. 1265 In the legend for fig. 7, the vehicle for thalidomide is listed as DMSO in 7(A), and as HBC in 7(B-G). Is the use of these two different vehicles correct, and if so why were different vehicles used in each case? It would also help to spell out the full name for the HBC abbreviation with its first use in 7B.

1302-1317 In the legend for fig. 8, define the arrowheads as they are in the Results-yellow for Fgf10 and pink for Fgf8.

1319-1321 Revise figure title and subtitles in the legend for fig. 9 to read, "... thalidomide teratogenicity based upon limb bud development in embryo culture". (see rationale above for lines 82-85)

Referee #3:

In their revised manuscript Yamanaka and colleagues addressed all my critical points by introducing additional experimental data and answered all questions in detail in their response letter. Most importantly, the authors now performed the critical GgPLZF overexpression rescue experiment that strongly supports the hypothesis of the work. I have no further comments to the content.

The language has markedly improved in the revised manuscript. However, there are still some grammatical mistakes and illogical sentences. Please again carefully check the manuscript.

Examples:
We have identified substrate proteins for protein kinase (Tadokoro et al., 2010) and E3 ligase (Takahashi et al., 2016) protein arrays.

In conclusion, our findings support we the hypothesis that the degradations of PLZF and SALL4 by thalidomide and 5-hydroxythalidomide produce CRBN-dependent severe teratogenic phenotypes in species sensitive to thalidomide (Fig 9B).

Point-by-Point Responses to the Reviewers' Critiques (EMBOJ-2020-105375R)

We deeply appreciate the constructive suggestions and kind comment to publish this study. As described in more detail below, we have addressed all the reviewers' concerns.

Reviewer #1

My concerns from the previous submission have been addressed. The ZF mapping as well as the chicken teratogenicity experiments have substantially added to the paper.

Given a recent manuscript by the same corresponding author,

https://www.nature.com/articles/s41467-020-18488-4

it would be desirable to cite this work and to clearly state that 5'-hyrdoxythalidomide is able to recruit SALL4 (and indicate this as already known).

Response: We thank the reviewer for the kind comments on our revised manuscript. As according to the reviewer's comment, we cited our recent article involving structural analysis between SALL4 and 5-hydroxythalidomide in the revised manuscript (lines 477-483).

Referee #2:

Second round of reviews:

The authors have satisfactorily addressed the majority of my concerns, particularly including the revision of several figures to include bar graphs with quantitative comparisons (means + SD) and statistical analysis, which was a major concern. Moreover, they have added an important new study showing that overexpression of PLZF partially protects embryos from thalidomide-initiated abnormalities in Fgf8/10 expression and limb bud development. In light of the authors' revisions, this manuscript highly merits publication in the EMBO Journal.

However, there remain several important concerns that the authors did not satisfactorily address, including some errors in their revisions.

Response: We thank the reviewer for the kind comments on our revised manuscript. As according to the reviewer's comment, we modified the revised manuscript as described in detail below.

Please note that:
· The line numbers in the text below are based upon my copy of the Word version of the manuscript, and may be somewhat inaccurate as the numbers changed due to my editing of the text in the manuscript.
· Most of my recommendations for revision have been made directly in the accompanying Word version of the manuscript, shown by track changes.

LINE COMMENT
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27-29 Revise and combine two sentences into one as follows: "Human cytochromes P450 convert thalidomide to two monohydroxylated metabolites that are considered to contribute to thalidomide effects, although the mechanism is unclear." (Note that the "s" is on "cytochromes" and not "P450".)

Response: Thank you for the kind suggestion about generation of thalidomide metabolite in abstract. As according to the reviewer's comment, we combined the two sentences in the revised manuscript (lines 26-28).

82-85 The sentence in blue font concerning a cited study of embryos from mice expressing human CYP3A enzymes needs revision, as do several related statements throughout the manuscript. A claim of "teratogenicity" cannot be made based upon embryo culture data because the defect may be resolved prior to the end of gestation. Also in the cited paper, CYP3A protein was in the placenta rather than the embryo, and an electrophilic reactive intermediate formed in that organ may not be sufficiently stable to reach the embryo. More importantly, the claimed limb abnormalities were at best only modest, no other embryopathies were observed (unlike in other species like rabbits), and no limb abnormalities occurred with in vivo studies in the same mouse strain by the same authors, so the basic premise is controversial. I suggest revising this sentence to a more cautious statement something like the following: "Mouse embryos that express placental human CYP3A isoforms exhibited modest thalidomide-induced limb defects in culture compared to wild-type controls, suggesting that placental CYP3A isoforms may convert thalidomide to an embryopathic reactive intermediate via the formation of 5-hydroxythalidomide, although no limb defects were observed in vivo (Kazuki et al., 2016)." This wording in part reflects a paper cited by the authors that indicates it is not clear whether a reactive electrophilic epoxide intermediate is produced during the formation of 5-hydroxythalidomide, or following its subsequent oxidation, but its presence in either case is supported by the presence of a glutathione conjugate, since glutathione is known to react with and detoxify many different types of electrophilic reactive intermediates.

Response: We thank the reviewer for the kind comments and important suggestion about phenotypes in humanized CYP3A mice. As according to the reviewer's comment, we modified the sentence in the revised manuscript (lines 81-87).

326-328 I have revised this sentence in the manuscript for some of the important reasons noted above for 82-85.

Response: We thank the reviewer for the kind suggestion. As according to the reviewer's comment, we modified the sentence in the revised manuscript (lines 325-326).

852-853 Should the second "thalidomide" read "5-hydroxythalidomide", and the existing "5-hydroxythalidomide" be deleted, as I have shown in my revision in the accompanying manuscript?

Response: Thank you for the kind comment. We corrected the mistake in the revised manuscript (line 855).

922 Fully define "EGFP" and explain its relevance. Also fully define this abbreviation in the legend for fig. 8 (line 1305).

Response: We thank the reviewer for pointing out lack of explain about rescue experiments. As according to the reviewer's comment, we explained a reason of use of EGFP expression vector and fully defined the abbreviation of EGFP in the figure legend in the revised manuscript (lines 924 - 925).

1258 vs. 1265 In the legend for fig. 7, the vehicle for thalidomide is listed as DMSO in 7(A), and as HBC in 7(B-G). Is the use of these two different vehicles correct, and if so why were different vehicles used in each case? It would also help to spell out the full name for the HBC abbreviation with its first use in 7B.

Response: We thank the reviewer for pointing out the mistake about vehicles in Fig 7. In all vivo experiments including Fig 7, the vehicle for thalidomide is HBC. We corrected the mistake in the figure legend and spell out the full name for the HBC in the revised manuscript (line 1264 -1265).

1302-1317 In the legend for fig. 8, define the arrowheads as they are in the Results-yellow for Fgf10 and pink for Fgf8.

Response: We thank the reviewer for the kind suggestion. As according to the reviewer's comment, we defined the yellow and pink arrowhead in the legend for Fig 8 in the revised manuscript (lines 1314-1317).

1319-1321 Revise figure title and subtitles in the legend for fig. 9 to read, "... thalidomide teratogenicity based upon limb bud development in embryo culture". (see rationale above for lines 82-85)

Response: We thank the reviewer for the kind suggestion. As according to the reviewer's comment, we modified figure title and subtitles in the legend for Fig 9 in the revised manuscript (lines 1328 - 1333).

Reviewer #3

In their revised manuscript Yamanaka and colleagues addressed all my critical points by introducing additional experimental data and answered all questions in detail in their response letter. Most importantly, the authors now performed the critical GgPLZF overexpression rescue experiment that strongly supports the hypothesis of the work. I have no further comments to the content.

The language has markedly improved in the revised manuscript. However, there are still some grammatical mistakes and illogical sentences. Please again carefully check the manuscript.

Examples:
We have identified substrate proteins for protein kinase (Tadokoro et al., 2010) and E3 ligase (Takahashi et al., 2016) protein arrays.

In conclusion, our findings support we the hypothesis that the degradations of PLZF and SALL4 by thalidomide and 5-hydroxythalidomide produce CRBN-dependent severe teratogenic phenotypes in species sensitive to thalidomide (Fig 9B).

Response: We thank the reviewer for kind comments and pointing out grammatical mistakes. As according to the reviewer's comment, we carefully checked and corrected the mistakes in the revised manuscript (lines 112 and 504).

2nd Revision - Editorial Decision 16th Nov 2020

Thank you for addressing most of the final issues in the revised manuscript and for providing an explanation to the source data issues. I would like to ask you to use the link below to update the figure panels and the source data for Fig. EV2 and Appendix Fig. S1 in the final manuscript.

Please let me know if you have any questions regarding these points, and I look forward to receiving the final version of the manuscript!

3rd Authors' Response to Reviewers 20th Nov 2020

The authors performed the requested changes.

3rd Revision - Editorial Decision 30th Nov 2020

Editor accepted the revised manuscript.

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: Tatsuya Sawasaki
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2020-105375R

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?	No statistical analysis was performed to determine sample size. Most experiments were repeated more than three times. Statistics methods or results were stated in figure legends or methods in this article.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical analysis was performed to determine sample size. Sample sizes were determined based on previous studies in the field to enable statistical analyses and ensure reproducibility.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from analysis.
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.	No
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used in this study. In chicken experiments, HBC-treated chicken embryos showing normal phenotype were used as a control invdividuals, and thalidomide-treated chicken embryos showing teratogenicity were used as thalidomide-treated chicken individuals.
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.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was not necessary as we were observing large differences in staining or morphology.
5. For every figure, are statistical tests justified as appropriate?	Yes. See Materials and Methods section in this article.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. See Materials and Methods section and figure legends in this article.
Is there an estimate of variation within each group of data?	Yes. See figure legends in this article.

USEFUL LINKS FOR COMPLETING THIS FORM

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

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-tumo

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/

Is the variance similar between the groups that are being statistically compared?	Yes. See Materials and Methods section in this article.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies were stated to be able to detect each endogenous protein in supplier's datasheets and these antibodies were used according to supplier's protocol. Anti-PLZF (GeneTex) and anti-SALL4 (abcam) were predicted to be able to detect chicken Plzf and Sall4, and expression of Plzf and Sall4 in chicken limb bud was validated by in situ hybridization in this article. Immunoblot analysis using chicken limb bud showed that the antibodies be able to detect chicken Plzf and Sall4. AGIA antibody has been validated in published paper (Yano, et al., PLoS ONE 11: e0156716). cited in Methods. The catalogue numbers and manufacture of all antibodies used in this article were stated in Materials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T, THP-1, and HuH7 cells were purchased from Riken BioResource Research Center (Riken BRC). DF-1 cells were purchased from ATCC. MT-4 and Raji cells were purchased from Japanese Cancer Research Resources Bank (JCRB) Cell Bank. TK, HT, BJAB, SU-DHL-4 cells were kindly provided from Prof. Fuminori Tokunaga and Dr. Daisuke Oikawa.

* 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.	See Materials and Methods section in this article.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All chicken experiments were properly conducted in accordance with the guidelines of Tohoku University and Nagoya University.
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.	We confirmed the ARRIVE guidelines.

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	See Materials and Methods section in this article.
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).	Raw data for Expanded View and Appendix was provided in the Supplementary Information.
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.	NA

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.	No