nature portfolio

Peer Review File

Limb reduction in an *Esco2* cohesinopathy mouse model is mediated by p53-dependent apoptosis and vascular disruption

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

A well designed, well written and noteworthy study looking for how ESCO2 causes limb malformations. Many studies looking at ESCO2 (and other genes that cause limb malformations) focus on the gene target itself but not how the loss of that gene actually causes the limb malformation/s. Hence there has been a missed opportunity for some time to determine if there are commonalities between limb syndromes and teratogen induced limb syndromes. However, several studies and review articles have questioned or proposed commonalities in the past. So it is refreshing to see a study that shows how the ESCO2 variant causes limb malformations and elegantly shows ESCO2 variant causes p53 induced cell death which then impacts on vascular development resulting in limb malformations. The Authors validate this finding by showing vascular and limb rescue following p53 inhibition and go on to show striking similarity in some genetic targets of ESCO2 and Thalidomide – hinting at commonality between the syndromes – which has only been proposed in the past.

The data presented is convincing and significant showing how ESCO2 causes the limb reduction phenotypes – thru p53 induction, cell death and vascular disruption. The Authors show ESCO2 downregulates a range of genes linked to other limb reduction syndromes including those targeted by Thalidomide, demonstrating a common pathway between these limb reduction conditions. Interestingly and something the Authors might want to consider expanding to strengthen their Commonality point, are several studies showing Thalidomide also targets blood vessels, causes cell death, localized gene expression changes and tissue damage. The limb damage results either through loss of chrondrogenic precursors or the resulting vascular changes result in the vessels being in the wrong place such that the bony elements cant form normally. For example see following Review and references therein (Vargesson 2019, doi: 10.1177/1753193418805249). There are also a range of studies suggesting loss of vessels or mispositioning of vessels during development results in limb reduction phenotypes via a variety of injury situations, for example, see the following Review and references therein (Vargesson, Hootnick 2017 doi: 10.1016/j.reprotox.2016.10.005).
Thus, given the similarities between Thalidomide induced limb reduction and limb reductions in genetic syndromes like RBS, and the evidence in this manuscript for RBS for a vascular aetiology in these conditions, this Reviewer supports the Authors proposing this as a commonality for limb reduction between these syndromes (and may likely be the same for other limb reduction syndromes).

Of course, RBS (and Thalidomide) affect multiple tissues and organs, not just limbs. Do the Authors think/hypothesise that the other organ/tissue damages seen in these syndromes are through the same mechanism or through multiple mechanisms?

The finding that chrondrogenesis was not rescued following p53 inhibition suggests p53/ESCO2 not directly involved/required for chrondrogenesis – did you leave the p53 inhibitor treated limbs to E15+ to see if bony pattern was rescued compared to the mutant limbs? It maybe that chrondrogenesis rescue takes time perhaps for precursors to be

repopulated etc? However, given the vascular disruption was rescued were vascular patterns actually normal? If not, I wonder if this is enough to still impact on chrondrogenesis recovery as vessels still in wrong places/missing etc?

Mention p53 upregulation in E9.5 could be explained by DNA damage induced by ROS build up and discuss some genes identified in the scRNA Seq screen that appear to support this. Could you attempt to prevent ROS build up by using a ROS inhibitor to confirm this is the case?

Did you identify Cereblon in the scRNA Seq screens? I think Supplementary Table 5 indicates CRBN is downregulated, I think this should also be stated clearly in the manuscript. CRBN is required to mediate Thalidomide actions via CRL4. Your scRNA Seq analysis shows most of the components of the CRL4/CRBN complex are downregulated in the ESCO2 variant – suggesting a shared molecular aetiology. Is ESCO2 acting via Cereblon complex (as proposed by Citation 15) or can ESCO2 act independently of CRL4 and Cereblon? Could there be tissue specific actions and mechanisms? This might be the case for Thalidomide.

The finding that in ESCO2 variants many genes linked to causing other limb reduction syndromes are downregulated will help (in time) identify shared/common mechanisms between other limb syndromes. Particularly the finding that multiple members of the CRL4/CRBN complex are downregulated in ESCO2 variants is important and will provide a platform to determine if CRL4/CRBN is a common factor involved in a range of diseases or if this is the result of something else and is coincidental.

The Methods are detailled and clearly explained and are detailled enough to allow reproducibility.

Minor Points:

Citations need looking at as some appear to be missing eg: end of Discussion there is reference to citations 198 and 199? And duplicated citations eg: Citation 23 and 82?

Reviewer #2 (Remarks to the Author):

It was a pleasure to read the work by Strasser, Jabs and colleagues.
The team set to investigate the mechanism of limb reduction in ESCO2 related Roberts syndrome. They created a conditional knock out mice for a known human mutation that replicated the human phenotype. They identified vascular defects in mutant limbs linked to p53-related signaling. Vascular changes were rescued by pifithrin-alpha. They also observed significant enrichments among genes associated with limb reduction defects (several syndromes with skeletal defects) and suggest a common vascular etiology for these group of conditions, including thalidomide embryopathy.
The results are presented well and the flow of the manuscript is excellent. The introduction and discussion are succinct and methods and results are exhaustive (with good supplementary information).

I would like to congratulate the authors for such an important work and suggest mentioning the ClinVar ID of the variant in the manuscript.

Response to Reviewers' Comments
Nature Communications manuscript: NCOMMS-23-41250-T

We thank the reviewers' for their recommendations toward improving the manuscript. Our responses to the reviewers' comments are in blue and underlined.

Reviewer #1 (Remarks to the Author):

A well designed, well written and noteworthy study looking for how ESCO2 causes limb malformations. Many studies looking at ESCO2 (and other genes that cause limb malformations) focus on the gene target itself but not how the loss of that gene actually causes the limb malformation/s. Hence there has been a missed opportunity for some time to determine if there are commonalities between limb syndromes and teratogen induced limb syndromes. However, several studies and review articles have questioned or proposed commonalities in the past. So it is refreshing to see a study that shows how the ESCO2 variant causes limb malformations and elegantly shows ESCO2 variant causes p53 induced cell death which then impacts on vascular development resulting in limb malformations. The Authors validate this finding by showing vascular and limb rescue following p53 inhibition and go on to show striking similarity in some genetic targets of ESCO2 and Thalidomide – hinting at commonality between the syndromes – which has only been proposed in the past.

The data presented is convincing and significant showing how ESCO2 causes the limb reduction phenotypes – thru p53 induction, cell death and vascular disruption. The Authors show ESCO2 downregulates a range of genes linked to other limb reduction syndromes including those targeted by Thalidomide, demonstrating a common pathway between these limb reduction conditions. **Interestingly and something the Authors might want to consider expanding to strengthen their Commonality point, are several studies showing Thalidomide also targets blood vessels, causes cell death, localized gene expression changes and tissue damage. The limb damage results either through loss of chrondrogenic precursors or the resulting vascular changes result in the vessels being in the wrong place such that the bony elements cant form normally. For example see following Review and references therein (Vargesson 2019, doi: 10.1177/1753193418805249). There are also a range of studies suggesting loss of vessels or mispositioning of vessels during development results in limb reduction phenotypes via a variety of injury situations, for example, see the following Review and references therein (Vargesson, Hootnick 2017 doi: 10.1016/j.reprotox.2016.10.005).**

Thus, given the similarities between Thalidomide induced limb reduction and limb reductions in genetic syndromes like RBS, and the evidence in this manuscript for RBS for a vascular aetiology in these conditions, this Reviewer supports the Authors proposing this as a commonality for limb reduction between these syndromes (and may likely be the same for other limb reduction syndromes).

We thank the reviewer for their suggestions to further strengthen our conclusions. We have modified the discussion to include the highlighted studies, as follows:

> **Lines 466-484**: "Vascular etiologies have been reported in teratogenically induced limb reduction, such as thalidomide embryopathy, but not in most genetic limb reduction disorders[82,109]. Our data show how loss of ESCO2 causes limb reduction phenotypes, through p53 induction, cell death, and vascular disruption. We also demonstrate that loss of ESCO2 causes the downregulation of several genes linked to other limb reduction syndromes, including genes targeted by thalidomide, demonstrating commonality between these limb reduction conditions. In our *Esco2*$^{fl/fl}$*;Prrx1-Cre* mouse model, we observed significant enrichments for genes previously established to regulate thalidomide's angiogenic response (e.g. *Igfbp-3, Tgfbr3, Hand1, Col5a2*, and *Efnb2*), suggesting RBS and other cohesinopathies potentially share similar downstream targets with thalidomide. Thalidomide also targets blood vessels, causes cell death, localized gene expression changes, and tissue damage. This limb damage results either through loss of chondrogenic precursors or the resulting vascular changes that induce the mispositioning of vessels such that the bony elements cannot form normally[82,109]. There are also other studies suggesting loss or mispositioning of vessels during development results in limb reduction phenotypes via a variety of injury situations, as reviewed in Vargesson & Hootnick (2020)[109]. The combination of these studies and our analyses provide evidence for a shared pathogenic etiology between RBS, thalidomide embryopathy, and potentially other limb reduction conditions."

Of course, RBS (and Thalidomide) affect multiple tissues and organs, not just limbs. **Do the Authors think/hypothesise that the other organ/tissue damages seen in these syndromes are through the same mechanism or through multiple mechanisms?**

Our paper was specifically focused on RBS limb reduction, as it is the most striking feature of this syndrome as mentioned in lines 114-115, "Internal organs were not significantly affected where *Prrx1* was not expressed[51,52] (Fig. 1a)." We have considered studying other organs and tissues, but believe that many additional experiments of tissues in RBS and thalidomide embryopathy are needed considering the varying levels of *ESCO2*

expression across tissues and development stages. For this reason we have not commented on this hypothesis in the discussion because it is beyond the scope of this paper.

The finding that chrondrogenesis was not rescued following p53 inhibition suggests p53/ESCO2 not directly involved/required for chrondrogenesis – **did you leave the p53 inhibitor treated limbs to E15+ to see if bony pattern was rescued compared to the mutant limbs? It maybe that chrondrogenesis rescue takes time perhaps for precursors to be repopulated etc? However, given the vascular disruption was rescued were vascular patterns actually normal? If not, I wonder if this is enough to still impact on chrondrogenesis recovery as vessels still in wrong places/missing etc?**

We chose to use a p53 inhibitor as proof-of-principle that the p53 pathway was an underlying cause of the phenotypes observed, and to specifically test whether it would restore or ameliorate the abnormal vasculature phenotype. In our conditional knockout mouse model, the embryos presented with disorganized vessels and hemorrhage at E12.5, hence why we only performed the rescue experiment to this stage and not thereafter. While we do not know why chondrogenesis was not recovered in the rescue experiments, we now state your suggestion as a possibility on lines 444-447:

> "Although it is possible that more time was needed to allow repopulation of the chondrocytes, the vasculature was still not entirely recovered to normal and therefore it is unlikely that chondrogenesis would be fully recovered as well."

We have also qualified the following statement in the results section on lines 357-358:

> "In sum, our results identified p53 inhibition as a **direct** mediator of vascular hemorrhage, but not chondrogenesis in mutant limbs."

Mention p53 upregulation in E9.5 could be explained by DNA damage induced by ROS build up and discuss some genes identified in the scRNA Seq screen that appear to support this. **Could you attempt to prevent ROS build up by using a ROS inhibitor to confirm this is the case?**

We targeted the p53 pathway given our transcriptomic evidence and drug repositioning data specifically identifying a p53-inhibitor. We did not have significant histological or transcriptional evidence suggesting a primary role for ROS compared to other pathways (i.e. p53), however, this can be a focus of future studies that are beyond the scope of the current manuscript.

Did you identify Cereblon in the scRNA Seq screens? I think Supplementary Table 5 indicates CRBN is downregulated, I think this should also be stated clearly in the manuscript.

We did identify Cereblon in the scRNA screen and have changed the text accordingly to reflect its downregulated expression, as follows:

> **Lines 396-401**: "We observed that *Cul4b, Rbx1, Ddb1, and Crbn1* were significantly downregulated in many clusters: *Cul4a* and *Cul4b* in 8/16 and 14/16 clusters, respectively; *Ddb1* in 15/16 clusters, including all DM clusters; *Rbx1* in 11/16 clusters; and *Crbn1* in 13/16 clusters (Supplementary Table 2). In DM8 specifically, *Cul4b, Rbx1, Ddb1, and Crbn1* were significantly downregulated. DM1 was the only cluster in which all genes were downregulated (Supplementary Table 2)."

CRBN is required to mediate Thalidomide actions via CRL4. Your scRNA Seq analysis shows most of the components of the CRL4/CRBN complex are downregulated in the ESCO2 variant – suggesting a shared molecular aetiology. **Is ESCO2 acting via Cereblon complex (as proposed by Citation 15) or can ESCO2 act independently of CRL4 and Cereblon? Could there be tissue specific actions and mechanisms?** This might be the case for Thalidomide.

The finding that in ESCO2 variants many genes linked to causing other limb reduction syndromes are downregulated will help (in time) identify shared/common mechanisms between other limb syndromes. Particularly the finding that multiple members of the CRL4/CRBN complex are downregulated in ESCO2 variants is important and will provide a platform to determine if CRL4/CRBN is a common factor involved in a range of diseases or if this is the result of something else and is coincidental.

We thank the reviewer for raising this important question. We certainly know that ESCO2 can act independently of CRL4 and cereblon, for example, by acetylating the Smc3 subunit of the cohesin complex (its canonical role), as indicated in the manuscript's introduction. Whether it acts *via* the $CRL4^{CRBN}$ complex, similar to thalidomide, is unknown to date. Citation 15 (Sanchez et al., 2022) does not suggest that it acts *via* the complex, rather that *ddb1* transcription (a component of the $CRL4^{CRBN}$ complex) is downregulated upon *esco2*-morpholino knockdown. Minamino et al., (2018) (PMID: 30100344) and Sun et al., (2019) (PMID: 30779731) show that the CUL4-DDB1-DCAF1 complex does target ESCO2 for degradation in normal, healthy tissues to temporally regulate ESCO2 and cohesion formation during mitosis. However, directionality matters

here: in these studies, the CUL4 complex targets ESCO2, while thalidomide targets the CRL4 complex, and specifically binds CRBN. Many of ESCO2's molecular functions and pathways have yet to be elucidated, so there could certainly be tissue specific actions and mechanisms dependent on that, but this is outside the scope of this study.

To address this comment, we have added in the following on lines 459-461:

> "It remains unknown whether *ESCO2* acts via the CRL4 complex in the affected limb bud tissues as does thalidomide, but the CRL4 complex does target and promote ESCO2 degradation during mitosis to prevent excess cohesion formation[25]."

The Methods are detailled and clearly explained and are detailled enough to allow reproducibility.

Minor Points:

Citations need looking at as some appear to be missing eg: end of Discussion there is reference to citations 198 and 199? And duplicated citations eg: Citation 23 and 82?

We have reviewed the references and made corrections accordingly. We also added both Vargesson citations that Reviewer #1 mentioned earlier in the new text (citations: 82 and 109).

Reviewer #2 (Remarks to the Author):

It was a pleasure to read the work by Strasser, Jabs and colleagues.
The team set to investigate the mechanism of limb reduction in ESCO2 related Roberts syndrome. They created a conditional knock out mice for a known human mutation that replicated the human phenotype. They identified vascular defects in mutant limbs linked to p53-related signaling. Vascular changes were rescued by pifithrin-alpha. They also observed significant enrichments among genes associated with limb reduction defects (several syndromes with skeletal defects) and suggest a common vascular etiology for these group of conditions, including thalidomide embryopathy.

The results are presented well and the flow of the manuscript is excellent. The introduction and discussion are succinct and methods and results are exhaustive (with good supplementary information).

I would like to congratulate the authors for such an important work and suggest **mentioning the ClinVar ID** of the variant in the manuscript.

We thank the reviewer for their constructive comments. We have added in the ClinVar ID for the pathogenic variation cited. See below for the edited text:

> **Lines 98-102**: "We introduced a deletion in exon 4 resulting in a premature stop codon, replicating the effect of a documented human pathogenic variant in *ESCO2* (c.879_880delAG (p.D292fsX47); **ClinVar ID: 21250)** that is predicted to produce a truncated *Esco2* transcript with nonsense-mediate decay (Supplementary Fig. 1a-c)[10]."

In addition to the above changes, we have edited the authorship order and affiliations. Drs. Ethylin Wang Jabs and Meng Wu have moved to Mayo Clinic. We edited the manuscript to incorporate their new affiliations and contributions. We have also added Meng Wu as a corresponding author.

We have added the bolded text for clarification in the abstract, on line 40: "Lastly, significant enrichments were identified among genes associated with RBS, thalidomide embryopathy, and other genetic limb reduction disorders, suggesting a common vascular etiology **among these conditions."**

We have changed the text in figure legend 6e to align with the box and whisker plot (formerly a bar graph), on lines 1134-1137: **e.** Box and whisker plot showing the corrected total cell fluorescence (CTCF) of TUNEL+ signal between *Esco2*$^{fl/fl}$ and *Esco2*$^{fl/fl}$;*Prrx1-Cre*$^{Tg/0}$ limbs treated with PBS or pifithrin-α. In box plots, center values are medians, and error bars indicate variability outside the upper and lower quartiles (n=3).

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I thank the Authors for the hard work in addressing the Reviewers comments and the detailled information in the Rebuttal letter.

All of my queries/suggestions have been addressed (apart from two minor points - below). I congratulate the Authors on an elegant and insightful study - showing how ESCO2 gene loss actually causes limb malformations thru cell death induction, p53 activation and vascular disruption. And how gene changes and morphological changes in the mutant limbs indicate shared pathways with other limb malformation syndromes and teratogens.

I only have two minor points:
1. In the discussion line 474 some genes are mentioned that are suggested to regulate thalidomides angiogenic response. Can a reference be added for this statement, as i am unaware of these listed genes being shown to be involved.

2. Reference 109 i think the publication date needs to be corrected?

Reviewer #2 (Remarks to the Author):

The manuscript is revised satisfactorily

Response to Reviewers' Comments #2

We thank Reviewer #1 for their thorough reading of the manuscript, and their additional recommendations toward improving the discussion section. Reviewer #2 had no further suggestions. Our responses to the Reviewers' comments are in blue and underlined.

Reviewer #1 (Remarks to the Author):

I thank the Authors for the hard work in addressing the Reviewers comments and the detailled information in the Rebuttal letter.

All of my queries/suggestions have been addressed (apart from two minor points - below). I congratulate the Authors on an elegant and insightful study - showing how ESCO2 gene loss actually causes limb malformations thru cell death induction, p53 activation and vascular disruption. And how gene changes and morphological changes in the mutant limbs indicate shared pathways with other limb malformation syndromes and teratogens.

I only have two minor points:
1. In the discussion line 474 some genes are mentioned that are suggested to regulate thalidomides angiogenic response. Can a reference be added for this statement, as i am unaware of these listed genes being shown to be involved.

We added the reference for the genes listed (reference #109), and removed *COL5A2* from the list.

2. Reference 109 i think the publication date needs to be corrected?

To accommodate the reference above, the original reference #109 is now reference #110. We corrected the year to 2017.

Reviewer #2 (Remarks to the Author):

The manuscript is revised satisfactorily

No comment because the reviewer didn't request any changes.