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Peer Review

Review of: "CHD3 Regulates BMP Signalling Response During Cranial Neural Crest Cell Specification"

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1. Independent researcher

In this manuscript, Claassen W et al. analyzed the role of CHD3 during the process of Cranial Neural Crest (CNC) specification and differentiation. By using human induced Pluripotent Stem Cells (iPSCs) in which CHD3 was depleted (homozygous KO), they showed that CHD3 is neither required for maintenance (POU5F/OCT4, SOX2, NANOG expression) nor for multi-lineage differentiation (OTX2 for ectoderm, TBXT/BRACHYURY for mesoderm, SOX17 for endoderm). This suggests that cell competence to respond to pro-differentiating cues is not globally dependent on CHD3 activity. Under specific CNC pro-differentiating conditions, the authors showed that CHD3-KO specifically affects CNC specification and EMT transition, and leads to aberrant mesoderm (*EOMES*, *TBXT*, *MIX1L*) specification. By using ATAC-Seq and ChIP-Seq analyses, they found that CHD3-KO affects chromatin accessibility at enhancers, mainly at BMP-responsive genes (DLX, MSX) and gene targets of DLX/MSX -transcription factors. They concluded that CHD3 is required for early CNC specification by regulating cell response to BMP (and WNT) signaling. I consider the findings really interesting, and the experiment well done. I would suggest some points to increase the already high quality of the manuscript.

1. Figure 7C: In my opinion, the model does not fit with the important results the authors obtained, thus reducing the impact of their study.

I do not understand why the authors used the “balance model.” It looks like CHD-KO alters the cell response to WNT and BMP signaling (rendering them more sensitive to WNT activity and less to BMP). Thus, I would expect that, by reducing WNT (CHIRON 1 µM) and increasing BMP (up to 150µg), the CNC differentiation should be (fully or partially) rescued. However, no changes are observed in CNC marker

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expression in response to increasing BMP, as demonstrated in **Fig. S3**, and no mesoderm/CNC induction by CHIRON 1µM, as shown in Fig. 7A).

In my opinion, the "balance model" suggests a kind of "permissiveness" for mesoderm differentiation in the absence of CHD3 (and impaired BMP signaling). However, the results obtained show a clear, valuable, specific, really interesting, and intriguing role for CHD3 in CNC specification, which is weakly appreciable in the "balance model."

Furthermore, CHD3 seems to be instructive for CNC because in Fig. 7A (using CHIRON 2µM), *EOMES* is no longer up-regulated (no significant/ns compared to WT), and no changes in CNC marker expression are observed. Thus, independently of WNT signaling (and the concentration of CHIRON), CNC is never induced in CHD3-KO, so for me, CHD3 does not balance WNT/BMP signaling activity for CNC induction. It can be interpreted as CHD3 is instructive, and not permissive.

The instructive activity of CHD3 is also suggested in Fig. 5A, where mesodermal markers (EOMES, TBXT, TBX3/6) are not precociously activated in CHD3-KO conditions (and prior to CHIRON treatment). So, this excludes that CHD3 represses mesodermal markers (which are dependent on WNT/Chiron, of course), emphasizing that CHD3 is indispensable for BMP signal independently of the (relative) contributions of other signaling pathways (i.e., WNT), as argued by the authors (in the discussion).

In my opinion, the authors should highlight their important findings about CHD3 as a crucial, specific (and instructive?) player in the process of CNC specification (through BMP) by conceiving another and more striking model, rather than the "balance model."

In my opinion, it would be important to answer whether the observed effects in CHD3-KO are linked to OCT4 (as both a pluripotency and mesoderm regulator) and NANOG (a pluripotency regulator). Also, this will help to understand why 3µM CHIRON induces mesoderm and reducing CHIRON plus increasing BMP (Fig. S3) still does not rescue the CNC specification, as recognized by the authors in the discussion.

1. In the absence of CHD3 activity, CNC are never specified independently of the WNT/BMP signaling manipulation (high/low CHIRON and increasing amount of BMP protein). However, using CNC differentiation conditions, mesoderm induction is observed (Fig.3), and POU5F1/OCT4 is still expressed (at d18, Fig. 2D). In Funa NS et al., Cell Stem Cell 2015 (PMID: 25921273), the "pluripotency factor" POU5F1/OCT4 also regulates mesoderm/primitive streak specification together with b-catenin (WNT effector). In contrast, SOX2 is a well-known pluripotency and neural factor. The authors show chromatin accessibility in OCT4 motifs (in Fig. 4D and 5G). How do they explain this?

What are the relative mRNA expression levels of OCT4, SOX2 (and NANOG?) (qPCR) in CHD3-KO conditions with 3µM (mesoderm induction), 2µM, and 1µM of CHIRON (in the absence of mesoderm and CNC induction, Fig. 7)?

What are the relative mRNA expression levels of OCT4, SOX2 (and NANOG?) (qPCR) in CHD3-KO conditions with 1µM of CHIRON *plus* exogenous BMP (in the absence of mesoderm and CNC induction, Fig. S3)?

What are the expression levels of POU5F1/OCT4, SOX2, NANOG (and some other pluripotency factors) in RNA-Seq data (Fig. 5A), at d14 when CNC markers are weakly expressed (and down in CHD3-KO) and mesodermal markers are not yet activated?

What is the status of chromatin accessibility at d14 for POU5F1/OCT4, SOX2, NANOG (and some other pluripotency factors) in WT and CHD3-KO conditions?

In my opinion, it would be important to answer whether the observed effects in CHD3-KO are linked to OCT4 (as both a pluripotency and mesoderm regulator) and NANOG (pluripotency regulator). Also, this will help to understand why 3µM CHIRON induces mesoderm and reducing CHIRON plus increasing BMP (Fig. S3) still does not rescue the CNC specification, as recognized by the authors in the discussion.

This should have an important impact on the interpretation of their results and rule out (or not) whether CHD3 is primarily involved (at d14) in the exit from pluripotency and instructive for CNC induction/specification (with few efforts, all OMICS data and material for qPCR already available).

Declarations

Potential competing interests: No potential competing interests to declare.