The oncogenic transcription factor FOXQ1 is a differential regulator of Wnt target genes

Giulia Pizzolato, Lavanya Moparthi, Simon Söderholm, Claudio Cantù and Stefan Koch
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Original submission

First decision letter

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MS TITLE: The oncogenic transcription factor FOXQ1 is a bimodal regulator of Wnt target genes

AUTHORS: Giulia Pizzolato, Lavanya Moparthi, Simon Soderholm, Claudio Cantu, and Stefan Koch
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Previous work has identified FOXQ1 as a target candidate and positive regulator of WNT signaling pathway. In this study by Pizzolato et al, further insight is provided for FOXQ1 and its functional regulation of b-catenin-dependent WNT signaling. A primary discovery reported is the unexpected and interesting functional and physical relationships for FOXQ1 N- and C-terminal domains on WNT

target genes. Depending on the terminus tested, this occurs in positive and negative polarities and either in complex with WNT transcriptional complexes or not. While interesting, an overall mechanistic model is not provided. The authors also report the first and robust BioID proximity network, which provides a valuable dataset for the community. Together, this manuscript is fairly well-written and provides a foundation for future FOXQ1 study. Several weaknesses are identified, which if addressed would significantly increase the importance and impact of the work.

Comments for the author

Previous work has identified FOXQ1 as a target candidate and positive regulator of WNT signaling pathway. In this study by Pizzolato et al, further insight is provided for FOXQ1 and its functional regulation of b-catenin-dependent WNT signaling. A primary discovery reported is the unexpected and interesting functional and physical relationships for FOXQ1 N- and C-terminal domains on WNT target genes. Depending on the terminus tested, this occurs in positive and negative polarities and either in complex with WNT transcriptional complexes or not. While interesting, an overall mechanistic model is not provided. The authors also report the first and robust BioID proximity network, which provides a valuable dataset for the community. Together, this manuscript is fairly well-written and provides a foundation for future FOXQ1 study. Several weaknesses are identified, which if addressed would significantly increase the importance and impact of the work.

Major critiques:
1. The authors produce physical and functional datasets which contribute to a deeper understanding of FOXQ1 in WNT signaling. However, the significance and impact of the work as a whole is incremental. How does FOXQ1 impact cancer cell biology, either in the context of WNT signaling or independently of WNT signaling? Do the N-term or C-term of FOXQ1 differentially impact biology, for example WNT-driven colonocyte stem cell growth? Or mesenchymal stem cells. How does FOXQ1 increase transcription of b-cat/TCF-dependent target genes?
2. FOXQ1 has many ascribed functions in the literature. From an unbiased global perspective, how relevant or pronounced is its contribution to the WNT pathway? For example, RNAseq GSEA— does this point to WNT signaling? WNT appears to be very weakly present within the BioID network. The BioID network is strong yet the validation and mechanistic insights gained have not been reported.
3. Several aspects of data presentation, data depth and statistical analyses could be improved. First, the degree of CRISPRa and CRISPRi-based FOXQ1 expression does not correlate with TOP/Ren. CRISPRi was not tested on endogenous FOXQ1 transcript. Did CRISPRi not work in HCT116 cells? Second, much rests on qPCR of target gene, however these data are hidden in ratios and might be better presented with individual data points rather than the average (box and whisker).
Do the qPCR effect sizes result in protein-level changes? Third, the statistical test is not articulated. Fourth, several experiments include addition of exogenous WNT ligand while others do not. Protein-protein proximity and interaction studies should look at the impact of WNT ligand on dynamics. Fifth the levels of protein expression for all reporter and qPCR data are not shown.
Minor: additional referencing would better support some the author's claims.
For example, claimed that half of the FOX proteins function in WNT signaling. At times, the description over-states importance. For example and while the literature supports a role for FOXQ1 in cancer and in WNT signaling, I do not believe FOXQ1 is yet worthy of being referred to as a "major oncogene in several types of carcinoma". The discussion opens with a statement that "FOXQ1 is a selective regulator of WNT target gene". I do not believe selectivity has been addressed.
In Figure 1, the importance of Fig1A is questionable to the study. The relevance of the WNT ligand qPCR is minor to the main focus of the study.
The term "bimodal" in this reviewer's mind does not describe differential impacts on signaling between a protein's N- and C-terminus.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, the authors address regulation of Wnt/bcatenin pathway by the forkhead box transcription factor FOXQ1. Though previous work has implicated FOXQ1 in the regulation of

bcatenin-mediated transcription, few mechanistic details exist. This work used CRISPR tools and truncation mutants, proteomics, and ChIP to explore how FOXQ1 regulates subsets of Wnt-target gene expression. They conclude that FOXQ1 has bcatenin-dependent and independent functions in the regulation of Wnt-target genes.

Comments for the author

This work clarifies previous studies on FOXQ1 and Wnt signaling and should be of interest to researchers in signaling, cancer, and transcriptional regulation. This manuscript is clearly written and of sufficient novelty and interest to warrant publication in JCS. Its major weakness is all work was done in HEK293 cells; critical findings should be demonstrated in additional cell lines or organoid models. It is not clear how broadly applicable to other cell types and tissue the "bimodal" regulation of Wnt target genes by FOXQ1 is. Minor weaknesses are 1) to complement the work in Fig. 2, it would be good to see how FOXQ1 synergizes (or does not synergize) with the LEFdeltaN-VP16 construct (Aoki, M., … Vogt, P.K.
Proc. Natl. Acad. Sci. USA 96, 139–144 (1999)) that is bcatenin independent. 2)
Fig. 5I Input/IP labels are missing, and the data is not convincing.

First revision

Author response to reviewers' comments

Referee response

We thank the referees for their constructive comments. Based on these suggestions, we have revised our manuscript to include new experimental data in support of our conclusions. A point- by-point response to the individual comments follows below. Most importantly, we now included a new RNA-seq dataset performed in HCT116 cells, which further illustrates that FOXQ1 differentially regulates the transcription of Wnt target genes in colorectal cancer cells. Moreover, we have expanded our results on the FOXQ1-associated proteome during active Wnt signalling. We hope that these changes sufficiently address the referees' concerns.

Reviewer 1 Comments for the Author:

Previous work has identified FOXQ1 as a target candidate and positive regulator of WNT signaling pathway. In this study by Pizzolato et al, further insight is provided for FOXQ1 and its functional regulation of b-catenin-dependent WNT signaling. A primary discovery reported is the unexpected and interesting functional and physical relationships for FOXQ1 N- and C- terminal domains on WNT target genes. Depending on the terminus tested, this occurs in positive and negative polarities and either in complex with WNT transcriptional complexes or not. While interesting, an overall mechanistic model is not provided. The authors also report the first and robust BioID proximity network, which provides a valuable dataset for the community. Together, this manuscript is fairly well-written and provides a foundation for future FOXQ1 study. Several weaknesses are identified, which if addressed would significantly increase the importance and impact of the work.

Major critiques:

1. The authors produce physical and functional datasets which contribute to a deeper understanding of FOXQ1 in WNT signaling. However, the significance and impact of the work as a whole is incremental. How does FOXQ1 impact cancer cell biology, either in the context of WNT signaling or independently of WNT signaling? Do the N-term or C-term of FOXQ1 differentially impact biology, for example WNT-driven colonocyte stem cell growth? Or mesenchymal stem cells. How does FOXQ1 increase transcription of b-cat/TCF-dependent target genes?

Reply: To address the comment regarding the functional impact of FOXQ1 on Wnt signalling, we have now performed cell proliferation assays using HCT116 and 293T cell lines (**new Figure 4F**).

FOXQ1 significantly increased the proliferation of CRC cells, as opposed to a decreased proliferation of 293T cells. The loss of FOXQ1 N terminus, in particular, seems to reduce the proliferation of 293T cells. To investigate this further, we are currently working on establishing functional assays in intestinal organoids to study the role of FOX transcription factors in stem cells. However, since these organoids strictly depend on active Wnt signalling for propagation, results on the possible functional role of FOXQ1 have been inconclusive so far. We therefore propose to address this question more comprehensively in a follow-up study.

With regard to the regulation of Wnt target genes, our data collectively indicate that FOXQ1 acts as a beta-catenin-independent transcription factor that functions by recruiting similar co- factors as TCF/LEF to promote or inhibit gene expression. These effects appear to be gene and cell type-specific, and will require further investigation with a focus on molecular mechanisms on the chromatin level.

2. FOXQ1 has many ascribed functions in the literature. From an unbiased global perspective, how relevant or pronounced is its contribution to the WNT pathway? For example, RNAseq GSEA—does this point to WNT signaling? WNT appears to be very weakly present within the BioID network. The BioID network is strong, yet the validation and mechanistic insights gained have not been reported.

Reply: As suggested, we have now done RNA sequencing of FOXQ1-expressing HCT116 cells in absence or presence of Wnt3a (**new Figures 6 and S6**). In summary, GSEA suggests that regulation of Wnt signalling is a minor function of FOXQ1, at least compared to other possible functions such as regulation of KRAS and MYC signalling. Nonetheless, the data additionally indicate that FOXQ1 and Wnt signalling converge on shared transcriptional targets that drive epithelial-to-mesenchymal transition. Thus, the combinatorial action of FOXQ1 and Wnt/beta-catenin may promote tumour progression in colorectal cancer.

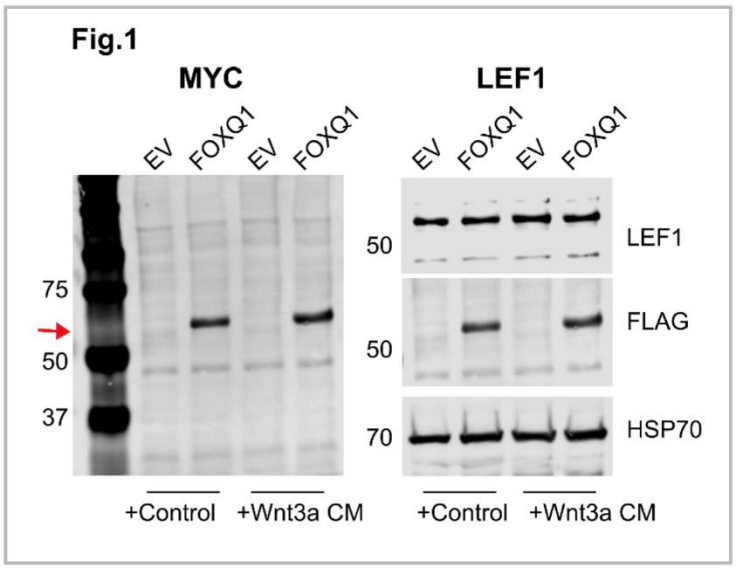
3. Several aspects of data presentation, data depth and statistical analyses could be improved.

First, the degree of CRISPRa and CRISPRi-based FOXQ1 expression does not correlate with TOP/Ren. CRISPRi was not tested on endogenous FOXQ1 transcript. Did CRISPRi not work in HCT116 cells?

Reply: It is true that there is no apparent quantitative correlation in these CRISPRa/i data. We are unsure at this point whether this is simply due to experimental variation, or other factors such as potential off-target effects of one or more of the guide RNAs. It is important to note, however, that qualitatively the results were fully consistent across multiple cell types and experiments.
CRISPRi did indeed not work in HCT116 in our hands. HCT116 have exceedingly low FOXQ1 levels, which has been attributed to epigenetic silencing in these cells (PMID 21346143). We therefore only showed CRISPRi of endogenous FOXQ1 in 293T cells in our initial submission (original Figure 1E). We have now repeated this experiment in Caco-2 CRC cells (**new Figure S1E**). Although results were somewhat variable, presumably due to poor transfection efficiency, the results support that endogenous FOXQ1 regulates Wnt signalling.

Second, much rests on qPCR of target gene, however these data are hidden in ratios and might be better presented with individual data points rather than the average (box and whisker). Do the qPCR effect sizes result in protein-level changes?

Reply: We have changed the qPCR data presentation and show in all figures the individual data points. In addition, we are providing all the Ct values as supplemental material for transparency (**new Table S4)**. We have now also tested the protein levels of two high abundance Wnt targets, MYC and LEF1, upon FOXQ1 overexpression. The MYC antibody did not produce any conclusive results, and we did not observe any apparent changes in LEF1 levels after 24 hours of transfection (see referee Figure 1). Despite this, we hope that the combined reporter assay, qPCR data, and new RNA sequencing results sufficiently support regulation of Wnt signalling by FOXQ1.



Third, the statistical test is not articulated.

Reply: Thank you for pointing it out. The details on the statistical test (type of test, number of replicates, p-values) have been reported in the revised figure legends.

Fourth, several experiments include addition of exogenous WNT ligand while others do not. Protein-protein proximity and interaction studies should look at the impact of WNT ligand on dynamics.

Reply: We appreciate this suggestion and have now performed an additional TurboID experiment for FOXQ1 (**new Figure 5C-G**). For better comparability with the Tcf7l1-BioID data included in these analyses, cells were also treated with 5 µM CHIR99021 to activate Wnt signalling. The results indicate highly dynamic rearrangement of the FOXQ1 proximity interactome upon Wnt pathway activation.

Please find below the account details to access the proteomics data in the ProteomeXchange Consortium:

- FOXQ1 + CHIR samples

Username: reviewer_pxd035624@ebi.ac.uk

Password: uEYchUJ3

- FOXQ1 samples

Username: reviewer_pxd030464@ebi.ac.uk

Password: CWWwcjFW

Fifth, the levels of protein expression for all reporter and qPCR data are not shown.

Reply: We do not routinely perform immunoblot validation of reporter and qPCR experiments once our workflow is solidly established, so protein levels for these specific experiments are not available. However, these assays are done under the same conditions as, for example, the immunoblot experiments in figures 3 and 5, which are often done in parallel and always show high levels of over-expression. This is also reflected in consistently high FOXQ1 induction in our RNA sequencing data (see new Figure 6A). Nonetheless, if required, we can repeat some key experiments with these controls included.
Minor: additional referencing would better support some the author's claims. For example,

claimed that half of the FOX proteins function in WNT signaling. At times, the description over-states importance.

Reply: Thank you for this and the following comments. We have now changed the text accordingly, by including additional references and revising some statements, as suggested. We have changed this statement to "numerous FOX family members have been shown to regulate Wnt signalling".

For example and while the literature supports a role for FOXQ1 in cancer and in WNT signaling, I do not believe FOXQ1 is yet worthy of being referred to as a "major oncogene in several types of carcinoma".

Reply: We agree with the comment and have changed the text to "a putative oncogene in several types of carcinomas".

The discussion opens with a statement that "FOXQ1 is a selective regulator of WNT target gene". I do not believe selectivity has been addressed.

Reply: Thank you for this comment. We realise that the term may be misinterpreted and changed the text to "FOXQ1 is a differential regulator of Wnt target genes".

In Figure 1, the importance of Fig1A is questionable to the study.

Reply: We have removed this panel, which we included for illustration purposes only.
The relevance of the WNT ligand qPCR is minor to the main focus of the study.

Reply: It is true that WNT ligand regulation is not the main finding of this study, and we have considered moving these data to the supplement. However, considering that Wnt ligand induction has been proposed as a mode of action for multiple FOX proteins including FOXQ1, we believe that results are nonetheless relevant for the overall conclusions of our study.

The term "bimodal" in this reviewer's mind does not describe differential impacts on signaling between a protein's N- and C-terminus.

Reply: Thank you for this observation. We used the term "bimodal" to refer to the apparent TCF-dependent and independent functions of FOXQ1 in the Wnt pathway, but realise that this statement was ambiguous. To avoid any misunderstandings, we have exchanged the term "bimodal" with "differential" throughout the revised manuscript.

Reviewer 2 Comments for the Author:

This work clarifies previous studies on FOXQ1 and Wnt signaling and should be of interest to researchers in signaling, cancer, and transcriptional regulation. This manuscript is clearly written and of sufficient novelty and interest to warrant publication in JCS.
Its major weakness is all work was done in HEK293 cells; critical findings should be demonstrated in additional cell lines or organoid models. It is not clear how broadly applicable to other cell types and tissue the "bimodal" regulation of Wnt target genes by FOXQ1 is.

Reply: We appreciate this comment and have performed more experiments to expand our main observations in other CRC cell lines (SW48, DLD-1), in addition to our earlier data from 293T and HCT116 (**new Figure 1B**). FOXQ1 significantly increased TOPflash activity in all tested cell lines. Target gene expression was inconsistent in these experiments, presumably due to high basal FOXQ1 expression and Wnt activity. However, we re-analysed a published dataset of FOXQ1-depleted DLD-1 cells (GSE74223, PMID 33330033). Gene set enrichment analysis using Wnt target genes identified in our RNA sequencing data was consistent with our earlier conclusions, i.e., we observed downregulation of Wnt-induced genes upon loss of FOXQ1 (**new Figure S6G**).

Finally, as mentioned in the response to referee 1, we are currently in the process of establishing functional assays in intestinal organoids. However, for technical reasons we have been unable to obtain unambiguous results in time for this revision.

Minor weaknesses are

1) to complement the work in Fig. 2, it would be good to see how FOXQ1 synergizes (or does not synergize) with the LEFdeltaN-VP16 construct (Aoki, M., … Vogt, P.K. Proc. Natl. Acad. Sci. USA 96, 139–144 (1999)) that is bcatenin independent.

Reply: We have now done this experiment (**new Figure 2G, H**). FOXQ1 indeed synergised with constitutively active LEF1 in TOPflash, and in AXIN2 induction in wild-type as well as beta-catenin/TCF/LEF knock-out cells.

2) Fig. 5I Input/IP labels are missing, and the data is not convincing.

Reply: We have now corrected the labels. Despite our best efforts, the CREBBP antibody we used did not produce any better results. To complement these data, we therefore performed an epistasis assay with CREBBP loss-of-function (**new Figures 5J and S5E**). The results suggest that CREBBP may at least in part mediate the function of FOXQ1 in the Wnt pathway. We hope that collectively these data make a sufficiently strong case for CREBBP as a functional interactor of FOXQ1.

Second decision letter

MS ID#: JOCES/2022/260082

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AUTHORS: Giulia Pizzolato, Lavanya Moparthi, Simon Soderholm, Claudio Cantu, and Stefan Koch
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

In this revised manuscript, Pizzolato et al have clarified and extended their characterization of the FOXQ1 transcription factor as a differential regulator of Wnt target genes. Prior review raised several critiques; these have now largely been addressed. The authors have presented an honest synthesis of their data. New RNAseq data are provided which partly supports Wnt involvement although Wnt alteration is minor when compared to the global FOXQ1 gene expression program. New functional data, which were requested, are also included to support a role for FOXQ1 in cell proliferation, although how this ties to Wnt or other FOXQ1 target genes is not explored. In summary, this manuscript has been improved through the addition of experimental detail and statistical analyses. The RNAseq dataset complements well the TurboID data as community resources for FOXQ1. The suggested bifunctional nature of FOXQ1 and b-catenin-independence remains interesting and with much left to learn. It remains to be tested whether FOXQ1 impacts cell biology through the Wnt pathway or Wnt target genes or as suggested, through concerted regulation of EMT.

Comments for the author

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New functional data, which were requested, are also included to support a role for FOXQ1 in cell proliferation, although how this ties to Wnt or other FOXQ1 target genes is not explored. In summary, this manuscript has been improved through the addition of experimental detail and statistical analyses. The RNAseq dataset complements well the TurboID data as community resources for FOXQ1. The suggested bifunctional nature of FOXQ1 and b-catenin-independence remains interesting and with much left to learn. It remains to be tested whether FOXQ1 impacts cell biology through the Wnt pathway or Wnt target genes or as suggested, through concerted regulation of EMT.