nature portfolio

Peer Review File

Mutational scanning reveals substrate-assisted autoregulation of the WNT destruction complex

Corresponding Author: Dr Rajat Rohatgi

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Padmanarayana et al. entitled "Mutational scanning reveals substrate-assisted autoregulation of the WNT destruction complex" presents an exhaustive study of mutations in CTNNB1, AXIN1, APC, and GSK3B on the assembly of the β-catenin destruction complex. Using a comprehensive base-editor screen, the authors identified both well-characterized mutations and a number of novel missense mutations.
Following identification, the authors attempt to reconcile these mutations with the known interaction motifs of β-catenin, Axin, and APC. This in itself represents a tour de force, as there is extensive and sometimes conflicting literature on the subject. Nevertheless, the authors present a logical and, wherever possible, structurally informed interpretation of these mutations.
I have a concern regarding the interpretation of a certain novel mutation with drastic amino acid substitutions. The authors do not appear to consider that they might alter protein folding and/or degradation rather than representing novel regulatory sites. Several mutations appear to alter the hydrophobic core or perturb the structure of β-catenin, such as L644P, L621P, and L497P. I would expect that proline substitutions affect much more than the local environment and could drastically alter the conformation of β-catenin, which in turn would affect protein stability. While it is not necessary for this study to test this biochemically, it would be useful to discuss this possibility or provide it as an alternative explanation. I would suggest either avoiding the designation of these sites as "regulatory" per se, or clearly distinguishing them from the mutations that were experimentally validated as functional.
Minor comments:
• It would be valuable to show or discuss whether the novel mutations occur in human populations.
• I would expect a stronger correlation between the increased z-score in the "top" population and the decrease in the "bottom" population (Fig. 1g), but I am not a expert in the base editor screens. A brief discussion addressing this apparent discrepancy would be appreciated.
Overall, this is an exhaustive and very valuable study with extensive validation of the genetic screens. Most importantly, it offers an explanation for the long-standing phenomenon of the "just right" amount of APC required for signaling. The authors' model convincingly demonstrates that two independent sites on APC are important for β-catenin interaction and that their progressive loss through truncation has differential effects on β-catenin stability and degradation.
Unfortunately, without a high-resolution structural biology model, full understanding of the destruction complex remains incredibly difficult. However, this study provides a unifying interpretation of existing data and represents a treasure trove of information for further characterisation and understanding of this complex.

(Remarks on code availability)
This is very much outside of my area of expertise.

Reviewer #2

(Remarks to the Author)
Padmanarayana et al use base editing screens to mutagenize 4 genes involved in WNT signaling in HAP1 cells using a WNT reporter assay as a read out. B-catenin is an important therapeutic target in cancer but has been difficult to drug due to complex regulation by multiple protein-protein interactions. Systematic base editing screens maintain physiological gene expression levels and therefore provide important insights into dynamic structure function relationships in cells. The authors employ an impressive range of biochemical and biophysical assays to explore sites governing WNT activity, revealing an

interesting mode of homeostatic regulation of the BDC where AXIN1 and APC are bridged by the substrate, b-catenin. There are some technical concerns regarding the screens that should be addressed, and some of the claims around therapeutic relevance need to be further clarified. Finally, focusing on only two genes (of 4) is limited in scope in comparison with much larger published base editing screens in less tractable cell models (e.g. PMID: 38093011).

Main comments
• Haploid HAP1 cells makes technical sense for efficient genome editing, but the translational relevance of HAP1 (or HEKs) for WNT signaling in cancer is questionable. I would recommend performing validation of variant effects in colorectal cancer models. Combining this with a reporter of WNT signaling without measuring the effect on a cancer cell models dependent on WNT or APCmut, for example, weakens translational relevance. What does 20 % reduction in reporter activity translate to?
• Validation of 95/370 sgRNAs in HAP1 (Ext Fig4) is not reassuring; false positives of 74%? This may relate to why only AXIN1 and b-catenin were followed up and the other genes dropped.
• The explanation to focus on AXIN1 and b-catenin does not rule out technical issues with editing i.e. are these sgRNAs simply not editing? Are there positive control guides in GSK-b, for example? These are important questions to address false negatives.
• Some of the alpha fold prediction structural analyses can be superficial or speculative (e.g. Fig. 2a), and can be confusing to follow (e.g. Ext.fig5). The authors should focus on fewer key sites with validation (biophysics) to back up their claims and remove superfluous structural models.
• I enjoyed Fig. 3 and can see that b-catenin abundance is unaltered by missense variants here (the IP is informative and well-preformed), but for other variants, loss of function phenotypes could be due to loss of protein expression or stability. Abundance should be measured or claims should be removed where this is not known to avoid overinterpreting the function of such sites.
• Replicate correlation of read counts in Ext Data Fig1d is encouraging but not sufficient. L2FC or z-score correlation between replicates (biological effect), and significant depletion of control sgRNAs should be shown. I also note the authors show correlation between technical duplicates, which is not as robust as independent biological replicates. Statistical analysis of the base editing screen is required to measure uncertainty and account for experimental noise - the authors mention the exclusion of outliers. E.g. MAGeCK would be suitable.
• The identification of an AXIN1 peptide (affinity enhanced) for B-catenin is interesting and raises the question of whether a peptide can be expressed or delivered to cells to compete with the PPI (as opposed to expressing an entire AXIN1 transgene in Fig. 7) to act as a WNT agonist. Although interesting, the therapeutic relevance of this finding in cancer is limited (and perhaps overstated?) as it would promote oncogenic signaling. To reduce signaling, one would require therapeutic genome editing to strengthen the interaction.

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• It would be informative to compare patient tumor mutations from clinical databases (e.g. TCGA, COSMIC) with those found here. How many overlap or are missing as hits? This would increase translational relevance.
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• The discussion lacks sufficient references to prior work on the assembly and regulation of the BDC to contextualise how their model compares to current understanding. The authors do not explore the role of these variants in subcellular localisation of BDC subunits, a key regulatory feature.
• For Fig. 7, SW480 proliferation experiments would be a useful addition.
• I recommend the authors deposit their data as a resource on MAVE db to increase accessibility.

(Remarks on code availability)
Detailed instructions and comments for pre-processing sequencing data but stops short of providing code to reproduce figures.

Reviewer #3

(Remarks to the Author)
In the submitted manuscript, Ganesh and his colleagues performed base-editor screens on several components of the β-catenin destruction complex to better understand the molecular interfaces between these components. The beta-catenin destruction complex is key to the canonical Wnt signaling pathway, and its dysregulation has been associated with many types of human cancer. Therefore, a detailed analysis of mutations and their impact is of broad interest, particularly since inhibition of the pathway has yet to be addressed by therapeutic interventions. The topic is timely and insights of this study could have a broad impact on the design of novel therapeutic modalities.

The authors performed comprehensive adenosine and cytosine base editor screens of four components of the β-catenin destruction complex (BDC) and followed up with a detailed mechanistic analysis of the identified mutations and their functional implications. In addition to β-catenin, they covered AXIN1, APC, and GSK3β with their libraries, achieving approximately 80-90% residue coverage. The authors further focused on approximately 150 previously uncharacterized mutations that led to functional changes, including loss- and gain-of-function alleles. The primary screens were performed in haploid HAP1 cells, and follow-up experiments were conducted in the HEK293 and SW480 cell lines, which are typical models for studying Wnt signaling activity.

While many structure-function studies have examined individual residues or parts of proteins, this study is the first to perform a comprehensive analysis of four BDC components and analyze the impact of numerous mutations in context. The study was conducted with rigorous quality control measures, underlining the high quality of the data. The design of the screens and the analysis are clearly explained, and the authors conducted an impressive set of follow-up experiments to draw molecular conclusions for multiple identified mutations. Among the many interesting findings and comprehensive presentations of resources that will be valuable for the community, one finding stands out in particular: the identification of affinity-enhancing mutations and protein-protein interfaces between Axin1 and β-catenin that only reduce Wnt signaling in the presence of activating mutations. These mutations are of particular interest for designing drugs that target the pathway in tumors without affecting its normal function. These findings could have a broad impact on how to target oncogenic Wnt signaling in colorectal cancers.

Overall, the study is very well executed and I fully support its publication in Nature Genetics. The experiments are well performed and the findings are important for a better understanding of oncogenic Wnt signaling pathways.

I have a few comments that authors should address prior to publication:

1. It would be helpful for readers to have a clearer understanding of the false negative and false positive rates of the screens. Furthermore, it would be helpful if the authors presented and explained these rates in more detail.

2. While it is understandable that the authors focused their further analysis on β-catenin and Axin1, they should add a more detailed explanation of the modest effects of APC and GSK3β mutations. Any additional analyses the authors have regarding the growth defects would also be helpful for understanding the results.

3. Was a systematic analysis performed on gain-of-function mutations in the absence of Wnt3a to determine whether they enhance or activate signaling?

4. Fig. 1g could be improved with regard to labels and color scheme. In its present form, Fig. 1g is slightly confusing since the colors for the genes and mutations/interfacs are very similar. Why are there no hits that increase signaling in the adenine base editor screen?

5. In Fig. 7e, Axin1-AE (affinity enhancing) appears to be more highly expressed in the Western blot. A titration of inducible or titrated Axin1 and its mutant forms would provide more convincing support for the impact.

(Remarks on code availability)
I have looked at the Github repository but have not executed the code.

Decision Letter:

6th Jan 2026

Dear Dr Rohatgi,

Your Article, "Mutational scanning reveals substrate-assisted autoregulation of the WNT destruction complex" has now been seen by 3 referees. You will see from their comments below that while they find your work of interest, some important points are raised. We are interested in the possibility of publishing your study in Nature Genetics, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

*1) Include a “Response to referees” document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

*2) If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions, available
here.
Refer also to any guidelines provided in this letter.

*3) Include a revised version of any required Reporting Summary: https://www.nature.com/documents/nr-reporting-summary.pdf

It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review.
A revised checklist is essential for re-review of the paper.

Please be aware of our guidelines on digital image standards.

EXTENDED DATA FIGURES

When re-submitting your manuscript, please ensure that any supplementary figures and tables that are crucial to the manuscript's conclusions are converted into Extended Data figures and tables to increase visibility of these data. Extended Data figures and tables are online-only (present in the online PDF and full-text HTML versions of the paper), peer-reviewed display items that provide essential background to the article but are not included in the main article due to space constraints. A maximum of ten Extended Data display items (figures and tables) is permitted.

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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We hope to receive your revised manuscript within four to eight weeks. If you cannot send it within this time, please let us know.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

Nature Genetics is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Safia Danovi, PhD
Senior Editor, Nature Genetics
ORCiD: 0009-0007-7822-5479

Referee expertise:

Referee #1: WNT biology

Referee #2: base editing screens

Referee #3: functional genomics, WNT

Reviewers' Comments:

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Overall, the study is very well executed and I fully support its publication in Nature Genetics. The experiments are well performed and the findings are important for a better understanding of oncogenic Wnt signaling pathways.

I have a few comments that authors should address prior to publication:

1. It would be helpful for readers to have a clearer understanding of the false negative and false positive rates of the screens. Furthermore, it would be helpful if the authors presented and explained these rates in more detail.

2. While it is understandable that the authors focused their further analysis on β-catenin and Axin1, they should add a more detailed explanation of the modest effects of APC and GSK3β mutations. Any additional analyses the authors have regarding the growth defects would also be helpful for understanding the results.

3. Was a systematic analysis performed on gain-of-function mutations in the absence of Wnt3a to determine whether they enhance or activate signaling?

4. Fig. 1g could be improved with regard to labels and color scheme. In its present form, Fig. 1g is slightly confusing since the colors for the genes and mutations/interfacs are very similar. Why are there no hits that increase signaling in the adenine base editor screen?

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Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All of my comments have been thoroughly addressed. I congratulate the authors on this impressive study.

(Remarks on code availability)

Reviewer #2

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The reduced emphasis on translational impact, the screen statistical summaries and verification of protein variant expression by Western blotting, are all important additions that have improved the manuscript.
The data in CRC cell lines in Figure 7 have strengthened the relevance of the findings to cancer and demonstrated an effect on cancer cell growth, rather than only reporter expression in HAP1 or HEK cells. However, not showing SW480 proliferation assay data because they did not behave as expected is not satisfactory. Most of the mechanistic work is done with SW480 (e.g. in Fig. 7) - how can the authors reconcile this omission?
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I accept that base editing screens can have high false-negative rates due to sgRNAs not editing, but false-positive rates of 20-30% casts some doubt on the general utility of the screening dataset.

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In the revised manuscript, the authors have fully addressed the comments I provided and added experiments that further support the study's conclusions.

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Decision Letter:

Our ref: NG-A70717R

20th Apr 2026

Dear Dr Rohatgi,

Thank you for submitting your revised manuscript "Mutational scanning reveals substrate-assisted autoregulation of the WNT destruction complex" (NG-A70717R). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Genetics, pending minor revisions to satisfy the referees' final requests (to be addressed textually) and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements soon. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Genetics Please do not hesitate to contact me if you have any questions.

Sincerely,

Safia Danovi, PhD
Senior Editor, Nature Genetics
ORCiD: 0009-0007-7822-5479

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In the revised manuscript, the authors have fully addressed the comments I provided and added experiments that further support the study's conclusions.

Response to Reviewer Comments

Dear editors and reviewers,

Thank you for the thorough evaluation of our manuscript, for your positive comments on the work, and for giving us the opportunity to submit a revision. We appreciate the time commitment required to evaluate both the original manuscript and this revision. Your suggestions have improved the scientific content, logic, and clarity of the manuscript.

Outlined below are our responses to comments from reviewers, including description of new experimental data, new computational analyses, and alterations to the manuscript. If we could not resolve a specific question or address a specific request, we provide a detailed explanation with references from the literature. The manuscript has been significantly shortened by ~1500 words to conform to journal limits.

Reviewer comments are in blue and our responses are in black text. All figure numbers refer to the revised manuscript, unless indicated otherwise. Figure panels with data new to this revised manuscript are called out with bold font.

The major new data and analyses added to this revision (and their associated implications) are as follows:

1. **Figure 7f:** As suggested by Reviewers 2 and 3, we have tested the effects of affinity-tuned AXIN1 alleles on the viability and proliferation of human CRC cell lines. The AXIN1^{AE} allele (which increases AXIN1-β-catenin affinity by 25-fold) is much more effective than an equivalent dose of wild-type AXIN1 at blocking the growth of three different WNT-addicted human CRC cell lines driven by truncating mutations in APC. Supporting data (**Fig.7d**) using a Dox-titratable system (suggested by Reviewer 3) shows that AXIN1^{AE} also suppresses WNT target gene expression in a dose-dependent fashion.

2. **Supplementary Table 2:** As suggested by Reviewers 1 and 2, we now cross-reference missense mutations nominated by our screen in CTNNB1 and AXIN1 to human disease variants found in the ClinVar database. This analysis is also mentioned in the text and discussed in the **Supplementary Notes**.

3. **Extended Data Fig.1:** As suggested by Reviewers 2 and 3 we provide additional metrics of screen quality, including an estimation of false positive and false negative rates, z-score correlations between replicates, and z-score distributions for different guide classes. This analysis is discussed in detail in the Supplementary Notes.

4. **Extended Data Fig. 6b**: As suggested by Reviewers 1 and 2, we measured the steady-state abundances for 55 mutants of β-catenin identified by the screen as changing the strength of WNT signaling. The mutations chosen for in-depth validation did not change β-catenin protein levels, making global misfolding unlikely. Protein abundances for all AXIN1 mutants discussed in the text were already provided.

Reviewer #1

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We are grateful for these positive comments on our mutational screen and follow-up mechanistic analysis. As noted in this comment, we have made every attempt to integrate (and reconcile when necessary) our results with the extensive structural and mechanistic literature on the BDC.

Comment 1.1. I have a concern regarding the interpretation of a certain novel mutation with drastic amino acid substitutions. The authors do not appear to consider that they might alter protein folding and/or degradation rather than representing novel regulatory sites. Several mutations appear to alter the hydrophobic core or perturb the structure of β-catenin, such as L644P, L621P, and L497P. I would expect that proline substitutions affect much more than the local environment and could drastically alter the conformation of β-catenin, which in turn would affect protein stability. While it is not necessary for this study to test this biochemically, it would be useful to discuss this possibility or provide it as an alternative explanation. I would suggest either avoiding the designation of these sites as "regulatory" per se, or clearly distinguishing them from the mutations that were experimentally validated as functional.

Response 1.1. We agree with this important point and apologize for failing to clearly describe our efforts to determine if specific mutations altered β-catenin protein abundances (which we take as a proxy for protein stability). We have now included immunoblots (**new Extended Data Fig.6B**) used to measure steady-state β-catenin levels for ~55 mutations that were tested in Phase I of our validation pipeline in eHAP1 cells. These abundances were measured in the same mutant eHAP1 cell lines for which WNT signaling assays are shown in Fig.2b and Extended Data Fig.6A. As part of the validation decision-tree for β-catenin (Extended Data Figure 4a), we excluded any mutations that lowered protein abundances >25%. These β-catenin blots confirm that the novel mutations we chose to follow-up in mechanistic studies did not dramatically change in protein abundances. Note that mutations that target the β-catenin degron (e.g. D32G) lead to expected increases in β-catenin abundance.

Additionally, for several mutations mentioned in the above comment we do provide additional data on both β-catenin abundance and integrity:

Figures 3C and 4C: Immunoblots show that protein abundances (see input) of β-catenin K435G/M436V, L497P/L498P, E571K, and I579T/L580P, C619Y/E620K, L621P, L643S/L644P, and D624N/E626K/E629K mutants are similar to WT β-catenin. Additionally, IP experiments show that most mutants retain the ability to bind E-Cadherin, suggesting integrity of the ARM domain. The one exception is L621P (Fig.4C), which shows reduced (but not abolished) binding to E-cadherin but accumulated normally in response to WNT signal activation (Extended Data Fig.7d). We now acknowledge this in the manuscript: "*The only exception was β-catenin L621P, which showed a modest decrease in abundance (but still accumulated in response to signal activation)*."

In the case of AXIN1 mutations, protein abundances for all mutations investigated in detail are shown in Fig.7a, Extended Data Fig.8b, Extended Data Fig.9a, and Extended Data.9c.

Finally, we describe several gain-of-function mutations in β-catenin (C619Y/E620K and L643S/L644P) and AXIN1 (Y822H/Y823H, V475I/L479W) that are very unlikely be due to global conformational disruption, misfolding or instability.

Overall, we completely agree with the more careful use of the term "regulatory" to describe mutations that change signaling activity and have been more judicious in the revised manuscript. We reserve the use of this term to describe sites or residues where mutations have bi-directional effects on activity. We acknowledge this limitation in the revised manuscript and state that mutations can influence function through indirect mechanisms (e.g. local structural perturbation):
"We cannot exclude the possibility that some mutations have broader effects on local protein conformation beyond disrupting specific binding interfaces. However, for all mutations analyzed in-depth below, the preservation of β-catenin abundance and E-cadherin interactions argues against global misfolding."

Comment 1.2. It would be valuable to show or discuss whether the novel mutations occur in human populations.

Response 1.2: We thank all reviewers for suggesting this analysis. In our original manuscript (Fig.1e, f) we did compare our screen results to oncogenic mutations identified in TCGA/cBioPortal. We show that our screen accurately identified truncating mutations in APC (before the first SAMP repeat) as well as missense mutations in the β-catenin degron (Fig.1e,f).

In the revised manuscript (see **new Extended Data Table 2 and new Supplementary Notes)**, we have now cross-referenced missense *AXIN1* and *CTNNB1* (β-catenin) mutations identified in our screens against the ClinVar database of human variants. A separate column in **Extended Data Table 2** also notes the oncogenic potential of variants. This resource will allow a reader to determine whether a residue change in the ClinVar database was also targeted by a base-edit, whether the amino acid change is predicted to be the same or different, and whether the associated sgRNA was validated in eHAP1 and 293T cells. For β-catenin, 44/221 "uncertain" variants and 11/20 "pathogenic" variants in ClinVar were at residues also targeted by the base-editing screen (a difference with a p-value of ~0.025 by Fisher's exact test). Benign β-catenin variants were too scarce (7) to assess statistically but were absent from screen hits.

Interestingly, several β-catenin variants found in intellectual disability syndromes are at the same residues that were targeted by loss-of-function screen hits: β-catenin R469H is found in "Severe intellectual disability-progressive spastic diplegia syndrome|CTNNB1-related disorder" and is listed as a variant of "uncertain significance" in ClinVar. Our data in the manuscript (see Fig.2d, f) shows that this variant impairs responses to WNT ligands and reduces the affinity of β-catenin for TCF4 by ~30-fold, showing that this variant is likely to be pathogenic (and thus may be causal for the disease). This point is now briefly noted in the manuscript text.

Comment 1.3. I would expect a stronger correlation between the increased z-score in the "top" population and the decrease in the "bottom" population (Fig. 1g), but I am not a expert in the base editor screens. A brief discussion addressing this apparent discrepancy would be appreciated.

Response 1.3: We agree with this astute observation: for sgRNAs that elevate signaling, enrichment z-scores in the top gate are greater than corresponding depletion z-scores in the bottom gate (Fig. 1g). We emphasize that our computational pipeline used enrichment metrics only since enrichment-based selection is considered to be more robust and to yield stronger statistical signals compared to depletion-based selection in pooled genetic screens[1]. Depletion scores were used only for the visualization in Fig.1g but not for screen analysis. Some possible reasons for the z-score asymmetry noted above is as follows:

1. Gate asymmetry: As shown in Fig.1d, our bottom gate was set at 15% but the top gate was set to 10%. The less stringent bottom gate would be expected to lead to weaker depletion signals. We chose a less stringent gate to identify a broad spectrum of loss-of-function mutants, including those with mild-to-moderate effects on WNT signaling.
2. Screens were done in the presence of high WNT ligand, which leads to high baseline reporter fluorescence in the overall population. sgRNAs that elevate activity even more beyond this high baseline will be enriched in the top gate but may be poorly depleted in the bottom gate due to a limited dynamic range. In contrast, sgRNAs that reduce WNT responsiveness (enriched in the bottom gate, e.g. sgRNAs that truncate β-catenin) will lead to a very large decrease in reporter fluorescence (compared to the overall population) and hence show stronger (and more symmetric) statistical signals for both enrichment and depletion.

Comment 1.4. Overall, this is an exhaustive and very valuable study with extensive validation of the genetic screens. Most importantly, it offers an explanation for the long-standing phenomenon of the "just right" amount of APC required for signaling. The authors' model convincingly demonstrates that two independent sites on APC are important for β-catenin interaction and that their progressive loss through truncation has differential effects on β-catenin stability and degradation.

Unfortunately, without a high-resolution structural biology model, full understanding of the destruction complex remains incredibly difficult. However, this study provides a unifying interpretation of existing data and represents a treasure trove of information for further characterisation and understanding of this complex.

Response 1.4: We greatly appreciate this positive comment on our work, particularly on our effort to provide a working model for the decades-long enigmatic observation that colorectal cancer is driven by "just-right" levels of WNT signaling.

Reviewer #2

Padmanarayana et al use base editing screens to mutagenize 4 genes involved in WNT signaling in HAP1 cells using a WNT reporter assay as a read out. B-catenin is an important therapeutic target in cancer but has been difficult to drug due to complex regulation by multiple protein-protein interactions. Systematic base editing screens maintain physiological gene expression levels and therefore provide important insights into dynamic structure function relationships in cells. The authors employ an impressive range of biochemical and biophysical assays to explore sites governing WNT activity, revealing an interesting mode of homeostatic regulation of the BDC where AXIN1 and APC are bridged by the substrate, b-catenin.

We appreciate these positive comments and the reviewer's note that we followed-up our screens with detailed mechanistic studies using cellular assays and *in vitro* reconstitution of key PPIs using purified proteins.

Comment 2.1. There are some technical concerns regarding the screens that should be addressed, and some of the claims around therapeutic relevance need to be further clarified. Finally, focusing on only two genes (of 4) is limited in scope in comparison with much larger published base editing screens in less tractable cell models (e.g. PMID: 38093011).

Response 2.1: The base-editing screening literature includes both broad gene-set screens and focused single-gene screens. Indeed, many studies in this field have focused on single genes or very small gene sets with extensive mechanistic follow-up (see Table 1 in [2] for a summary). Lue and Liau note in this review that "Base editor scanning has been used to explore individual cancer targets like BRCA1, BRCA2, and DNMT3A, as well as larger collections of disease-relevant proteins." Hence, there is value in our approach-- the in depth validation and mechanistic analysis of the BDC, a complex critical to both WNT signaling and colorectal cancer. Our focused analysis is distinguished by the following:

1. Extensive secondary validation of mutations. Our effort to individually validate ~370 sgRNAs (Extended Data Figure 4a), rarely seen in other base editing screening campaigns, provides a high confidence view of mutations across β-catenin (summarized in Extended Data Figure 6a).
2. Extensive biochemical validation of screen-identified mutations using purified proteins in combination with quantitative binding measurements (BLI). This effort is notable because it allowed us to correlate quantitative affinity measurements with cellular WNT signaling phenotypes.
3. To characterize the biochemical impact of mutations identified in our screens in cells, we performed immunoprecipitations for both endogenous beta-catenin and AXIN1. Unlike the vast majority of studies in the WNT field that rely on the overexpression of tagged proteins—which can mask subtle phenotypes through stoichiometric artifacts—our approach preserves the physiological concentrations of these components.
4. Generation of precise affinity-tuned alleles of AXIN1 at endogenous loci, enabling us to determine how changes in affinity at a single PPI interface in a multi-protein complex influences complex assembly, signaling output and (in this revised manuscript) cancer cell proliferation.
3. Discovery of substrate-assisted autoregulation as the biochemical mechanism behind the long-noted concept of "just-right" WNT signaling in colorectal cancer-- as noted by Reviewer #1, Comment 1.4.

In terms of therapeutic impact, we note that our manuscript is timely since there is a recent resurgence of interest in drugs targeting β-catenin (described in a very recent highlight in Nature Reviews Drug Discovery[3]), driven partly by positive early-phase results from a stapled peptide inhibitor of the β-catenin-TCF complex. Indeed, our work (see Figures 3C and 4C) identified several new β-catenin residues that are critical for this therapeutically relevant interaction. Consequently, our high-resolution mutational data and follow-up biochemical studies, interpreted through the lens of structures (as noted by other Reviewers), will be useful for expanding drug discovery efforts targeting beta-catenin.

Comment 2.2. Haploid HAP1 cells makes technical sense for efficient genome editing, but the translational relevance of HAP1 (or HEKs) for WNT signaling in cancer is questionable. I would recommend performing

validation of variant effects in colorectal cancer models. Combining this with a reporter of WNT signaling without measuring the effect on a cancer cell models dependent on WNT or APCmut, for example, weakens translational relevance. What does 20 % reduction in reporter activity translate to?

Response 2.2: While our work has translational relevance (as noted by Reviewer #3), it is not primarily a translational study. We are focused on genetic analysis using base-editing technology of a signaling complex that plays a key role in the WNT pathway, a pathway whose relevance extends beyond cancer to development, stem cell function, and tissue homeostasis. As shown in Fig.1e,f, screens identified many of the known driver mutations in APC and β-catenin found in CRC (TCGA/cBioPortal).

In the revised manuscript, we use human CRC cell lines to test some of the mutations beyond HAP1 and HEK293T cells:

1. **Extended Data Fig. 7g and 7h** shows that novel screen-identified β-catenin mutations reduce constitutively elevated WNT reporter activity in two CRC cell lines: RKO (engineered to carry a truncating mutation at APC residue 1320) and DLD-1 (APC 1417*).
2. In **Figures 7c-7e**, we test the impact of the affinity-tuned AXIN1 variants (AXIN1^{WT}, AXIN1^{AR} and AXIN1^{AE}) on BDC assembly, abundance of BDC components, and endogenous *AXIN2* target gene transcription (not reporter fluorescence) in a human CRC cell line carrying an APC truncation (SW480). We show that the affinity enhancing *AXIN1^{AE}* allele can rescue the phosphorylation of endogenous β-catenin in these cells (Fig.7c), providing direct biochemical evidence that the BDC has been partially repaired in this human cancer cell line.
3. In new data (**Figure 7f**), we show that expression of AXIN1^{AE} is much more effective than AXIN1^{WT} or AXIN1^{AR} at suppressing both colony formation and proliferation of three human CRC cell lines carrying various *APC^{mcr}* alleles (DLD-1, LoVo, HT29). These cell lines were chosen because they have been shown to be highly addicted to WNT signaling in the DepMap database. As a control, we use RKO cells, which carry full-length, wild-type APC. In RKO cells, all three affinity tuned alleles show comparable effects. These data support our model (Fig.8) that increasing AXIN1-β-catenin affinity increases BDC activity (and decreases WNT signaling) more effectively in cells carrying protein-truncating *APC^{mcr}* alleles. These data fulfill a key prediction of our auto-regulatory model and the proposed critical role played by the AXIN1-β-catenin PPI in the oncogenic BDC (Fig.8).

Comment 2.3. Validation of 95/370 sgRNAs in HAP1 (Ext Fig4) is not reassuring; false positives of 74%? This may relate to why only AXIN1 and b-catenin were followed up and the other genes dropped.

Response 2.3: We appreciate the opportunity to clarify our validation pipeline and the meaning of these numbers. These numbers do not represent the false positive rate in our screens, but rather our decision to select only the sgRNAs with the largest effects for validation in a second cell line (HEK293T). Without this step to filter out sgRNAs with smaller effects, the work required for individual sgRNAs in a second cell line would be paralyzing. There are several ways in which we can assess the false positive rate in our screens:

1. For the overall screen, Extended Data Fig.4a now shows false positive rates based on individual guide validation in both HAP1 cells and 293T cells. Out of the 370 sgRNAs selected for validation, 72% showed the expected effect on WNT reporter activity in HAP1 cells. Out of these, only the top 76 were chosen for validation in 293T cells to limit our efforts to evaluate the most highly performing sgRNAs (>50% change in WNT reporter fluorescence). In 293T cells, 84% of these showed the expected effect on WNT signaling strength. (Please note that the original Extended Data Fig.4a contained an error that has been corrected-- 76 sgRNAs (not 95) were validated in HEK293T cells). Thus, the false positive rate based on extensive, individual validation of nearly 400 sgRNAs in two different cell lines is ~20-30%.
2. When looking at all sgRNAs targeting only β-catenin alone (See Extended Data Fig.6a), the protein for which we did the most in-depth analysis, the false positive rate is ~15% based on validation studies in HAP1 and HEK293T cells.

3. Our libraries also include ~280 non-targeting control sgRNAs that can be used to assess false positive rates. As shown in **new Extended Data Fig.1d,** false positive rates for these sgRNAs are extremely low for ABE and CBE screens in both the top and bottom gates: 1-3%
4. Finally, we can use knowledge of *APC* genetics to estimate a false positive rate for sgRNAs that target *APC*. As shown in Fig.1e, our screen identified (as expected) numerous sgRNAs predicted to produce nonsense mutations in APC proximal to the first AXIN1-binding SAMP domain (showing that the *APC* locus was edited in our screen). Similar truncating mutations are a hallmark of human cancer. However, our CBE libraries also included ~90 sgRNAs predicted to make truncating mutations after the first SAMP domain, and these sgRNAs should not elevate WNT signaling (truncations distal to the first SAMP repeat are not seen in human cancers). Indeed, none of these sgRNAs (0%) were enriched in the top gate (see **new Extended Data Fig.1g**), again suggesting a very low false positive rate.

In summary, considering non-targeting sgRNAs, our false positive rate is <5%. Considering our extensive experimental validation efforts covering nearly 400 sgRNAs in two different cell lines (which we note is rare in other published base editing screens), we estimate our false positive rate to be ~20%. A discussion of false positive and false negative rates is now provided in the **new Supplementary Notes** section.

Comment 2.4. The explanation to focus on AXIN1 and b-catenin does not rule out technical issues with editing i.e. are these (APC and GSK3B) sgRNAs simply not editing? Are there positive control sgRNAs in GSK-b, for example? These are important questions to address false negatives.
Response 2.4: We thank the reviewer for pushing us to evaluate false positive rates (in the last comment) and false negative rates in this comment. First, we would like to address the question of why our screen did not nominate missense mutations in *APC* and *GSK3B* (a question also asked by Reviewer #3). We believe the reasons are different for the two genes and these reasons reflect the underlying biology, not a technical failure:

1. GSK3B: A recent study from Lebensohn and colleagues (now confirmed by us) has shown that GSK3B is functionally redundant with GSK3A in HAP1 cells (see Figure S3B and S3C in [4]). Thus we should not find mutations in GSK3B that impact WNT signaling and indeed we did not find any (confirming a low false positive rate). We did test four sgRNAs from our library and confirmed that they are capable of introducing base-edits at the *GSK3B* locus in HAP1 cells at frequencies that are similar to those observed at other gene loci.

2. APC: As one of the most commonly mutated tumor suppressor genes in human cancer, *APC* has been sequenced extensively in human cancers (TCGA/cBioPortal). It is notable that only truncating mutations (but not missense mutations) in *APC* have been identified as potential driver mutations. Thus, it is not surprising that we did not identify missense mutations in APC that increase (or decrease) WNT signaling. As has been noted previously, this resilience to missense mutations in APC is likely because it contains multiple, redundant motifs to engage its key interaction partners (β-catenin and AXIN1, Fig.1b). However, we did identify plenty of sgRNAs predicted to make nonsense or splice site mutations in APC that were top hits in our screen (see Fig.1e, Extended Data Fig.3d), demonstrating that our library successfully edited the *APC* locus.

Additionally, we note that our library was a pooled library including sgRNAs targeting all four genes. At two steps (plasmid library and mutant cell library prior to sorting) deep sequencing was used to ensure that sgRNAs targeting all 4 genes were represented proportionally; there was no evidence that the sgRNAs targeting *APC* and *GSK3B* were selectively lost. Given the pooled nature of the library and the screen, it is unlikely that only sgRNAs targeting two of the four genes failed to introduce base edits.

Finally we turn to providing estimates of false negative rates. We did not include control sgRNAs targeting essential genes (as has been done in some other screens) so cannot use depletion of these sgRNAs to estimate false negative rates. However, an equally good strategy (and one that is arguably more relevant to the specific gene loci being targeted) is to analyze sgRNAs predicted to make nonsense mutations (only in the

CBE library) or splice-site mutations (both ABE and CBE libraries): under perfect conditions, most truncating mutations in β-catenin should diminish WNT signaling, while truncating mutations in APC should elevate WNT signaling. As shown in **new Extended Data Fig. 1e,f** and described in the new **Supplementary Notes**, ~20% of nonsense sgRNAs in *APC* were enriched in the top gate with a *z* score>2 and ~42% of nonsense sgRNAs targeting β-catenin were enriched in the bottom gate with *z* score>2. Based on these metrics, we estimate that the overall false negative rate in our screens is ~60-80%. Such high false negative rates are a weakness of base editing screens and have been reported previously[2,5,6]. These rates are expected to be even higher for relaxed specificity Cas9 variants (like the NG-PAM Cas9 used in our screens).

Comment 2.5. Some of the alpha fold prediction structural analyses can be superficial or speculative (e.g. Fig. 2a), and can be confusing to follow (e.g. Ext.fig5). The authors should focus on fewer key sites with validation (biophysics) to back up their claims and remove superfluous structural models.

Response 2.5: The structural models shown in Figure 2a and Extended Data Fig.5 are not Alpha Fold models but rather derived from previously published real crystal structures (with PDB ID numbers and references noted in the figure legends). The use of AlphaFold in Fig.2a is to model the disordered N- and C-terminal domains (which helps present them in a compact way but are not a focus of our work). As noted by Reviewers 1 and 3, our work helps to conceptualize and reconcile a large body of genetic, structural and biochemical data on the BDC. As such, we feel it is valuable for the field (and an important acknowledgement of prior structural work) to show the residues identified as hits in our screens on experimentally derived structures. While we can only validate a small fraction of these screen nominated residues, presenting them on structures (rather than as simple lists) will stimulate further experimental work on the many PPI interfaces found in the BDC. Note that Fig.6a uses an AlphaFold model because it provides a more complete picture of the PPI interface and allows us to visualize a greater number of mutants; otherwise the model is superimposable on the crystal structure of this interface.

Comment 2.6. I enjoyed Fig. 3 and can see that b-catenin abundance is unaltered by missense variants here (the IP is informative and well-preformed), but for other variants, loss of function phenotypes could be due to loss of protein expression or stability. Abundance should be measured or claims should be removed where this is not known to avoid overinterpreting the function of such sites.

Response 2.6: Please see our detailed Response 1.1 to Comment 1.1, which addresses this question with additional data and discussion.

Comment 2.6. Replicate correlation of read counts in Ext Data Fig1d is encouraging but not sufficient. L2FC or z-score correlation between replicates (biological effect), and significant depletion of control sgRNAs should be shown. I also note the authors show correlation between technical duplicates, which is not as robust as independent biological replicates. Statistical analysis of the base editing screen is required to measure uncertainty and account for experimental noise - the authors mention the exclusion of outliers. E.g. MAGeCK would be suitable.

Response 2.6: Thank you for this very constructive comment. It has prompted us to substantially expand our description of the statistical evaluation methods used in our study and also led us to perform additional analyses to address screen quality and estimate uncertainty and false positive and negative rates (see **new Extended Data Fig.1c-g** and **Supplementary Notes** in the revised manuscript). Specific responses to this comment are as follows:

1. Replicates: Our methods note the procedure used to perform each screen twice: “To generate mutant library cells, eHAP1-7TS cells were transduced in two biological replicates at a low MOI of approximately 0.5” and “For each biological replicate screen, mutant library cells were thawed in 15-cm dishes, split and seeded for the FACS sorting stage.” We have now added the missing detail that the screens were performed on separate

days. Consequently these represent biological replicates. As requested, we have now provided z-score correlation plots in addition to read count correlation plots in **new Extended Data Fig.1c**.

2. Control guides: As noted in Response 2.4 above, we did not include control sgRNAs targeting essential genes in our libraries. However, we use other equally valid metrics to address false negative and false positive rates, now presented in **new Extended Data Fig.1d-g** and **Supplementary Notes**. These data (**Extended Data Fig.1d**) show the distribution of *z*-scores for non-targeting controls, confirming that these are centered around zero. Additionally, **Extended Data Fig.1e,f** show z-score distributions for other classes of sgRNAs in our libraries.

3. Statistical analysis: We have described statistical analysis of screen data in the methods section, which is based on generating a control distribution using the ~280 non-targeting guides: "The log-fold change (LFC) between sorted and unsorted populations was then calculated for each sgRNAs . To provide a standardized score (z-score) of enrichment or depletion, we used the LFC values from control sgRNAs (non-targeting) to compute a mean and standard deviation, which were then used to calculate z-scores for each sgRNAs in the respective sorted populations as described previously." Similar z-score centric approaches to pooled CRISPR screen analysis have been used in prior publications[5–7]. We apologize for not providing the raw and FDR corrected *p*-values for this comparison to quantify uncertainty in the original manuscript; these values have now been added to a **revised Supplementary Table** 1. Finally, as requested above, we have re-analyzed all our screening data using MAGeCK and provide this parallel analysis for interested readers in new tabs within **Supplementary Table 1.**

4. Outliers: With respect to outliers, we explicitly state the criteria for outlier exclusion in the methods: "We filtered out any sgRNAs with $\log_2$-normalized reads per million in the unsorted sample falling outside 1.5 times the interquartile range (IQR) from the first and third quartiles, effectively removing outliers and sgRNAs with extremely low or high representation." Most analysis methods (including MAGeCK and the foundational base editing screen papers we used as a guide[5,7]) use some criteria for outlier exclusion, so our decision to exclude outliers is not unusual. We used a standard method for outlier detection: the 1.5X IQR method is the classic fence established by John Tukey and its application to $\log_2$-transformed read counts is reasonable given the log-normal distribution of sequencing data. The 1.5×IQR fences correspond roughly to ±2.7 standard deviations from the mean, capturing about 99.3% of the data. Tukey chose 1.5 empirically as a good balance between sensitivity and specificity for detecting anomalous values.

Comment 2.6. The identification of an AXIN1 peptide (affinity enhanced) for B-catenin is interesting and raises the question of whether a peptide can be expressed or delivered to cells to compete with the PPI (as opposed to expressing an entire AXIN1 transgene in Fig. 7) to act as a WNT agonist. Although interesting, the therapeutic relevance of this finding in cancer is limited (and perhaps overstated?) as it would promote oncogenic signaling. To reduce signaling, one would require therapeutic genome editing to strengthen the interaction.

Response 2.6: We have revised the discussion to de-emphasize the direct therapeutic implications of the affinity tuned AXIN1 alleles. An isolated AXIN1^{AE} peptide would likely inhibit WNT signaling by blocking the interaction between β-catenin and TCF/LEF transcription factors. Our intention was not to suggest therapeutic genome editing to introduce the *AXIN1^{AE}* allele into tumor cells. However, our model in **Figure 8d** suggests that a molecular glue that increases the affinity between β-catenin and either AXIN1 or APC could increase the activity of the oncogenic BDC shown. As Reviewer 3 notes, our study does have timely therapeutic implications: "The topic is timely and insights of this study could have a broad impact on the design of novel therapeutic modalities." Indeed, **new data in Fig.7f** shows that AXIN1^{AE}, which has 25-fold higher affinity for β-catenin compared to wild-type AXIN1, is also more effective in suppressing the growth of CRC cell lines. Hence, it is not unreasonable to speculate that a molecular glue that accomplishes the same represents a therapeutic strategy.

Comment 2.7. ThIt would be informative to compare patient tumor mutations from clinical databases (e.g. TCGA, COSMIC) with those found here. How many overlap or are missing as hits? This would increase translational relevance.

Response 2.7: Please see Response 1.2 to Comment 1.2. We now present (**new Supplementary Table 2** and **new Supplementary Notes**) a comparison of our screen hits to human ClinVar data. Additionally, Fig.1e,f shows the overlap between our screen hits and two of the major driver mutations seen in human CRC: APC truncations and β-catenin degron mutations. We find that ~20% of predicted truncating mutations in APC are enriched in the Top 10% gate in our screens and ~60% of predicted missense mutations in the β-catenin degron are enriched in the Bottom 15% gate in our screens (based on a *z*>2 threshold). As for AXIN1, no missense mutations are annotated as drivers in TCGA/cBioPortal so this comparison is not relevant.

As noted above[2], base editing screens have high false negative rates so we do not expect to find comprehensive coverage of oncogenic mutations in our screens. Adenine and cytosine base editors can only make a subset of mutations and often individual sgRNAs can make multiple mutations, limiting comparisons to human variant databases. Use of prime-editing, HDR or deep mutational scanning (DMS) is required to comprehensively interrogate and functionally test human variants[8], a worthy goal but one that constitutes an entirely different project.

Comment 2.8. Fig2b – the validation experiments do not show any replicates or error bars. Same with Figure 5a.

Response 2.8: Given the large numbers of validated sgRNAs (~400) and the consequent need to reduce workload, we did not perform biological replicates for the bar graphs shown in Figs.2b and 5a, only technical replicates. These figure panels show a screening experiment, which was followed in all cases by in-depth validation and mechanistic characterization of selected guides. We now provide error bars, which represent the standard deviation of multiple subsamples of this data.

Comment 2.9. Fig6b, the WNT reporter assay for V475I is not convincingly different. How many times has this been repeated?

Response 2.9: This experiment was repeated three independent times with consistent results. These three biological replicates are shown as individual points on the bar graph, along with analysis showing statistical significance. The effect is small (but reproducible) in two different cell lines (HAP1 and HEK 293T), consistent with our finding that V475I increases AXIN1-β-catenin affinity by only ~2-fold (Fig.6d). This small effect size is the reason we used a double mutant (V475I/L479W) with a greater affinity increase as the *AXIN1*^{AE} allele in the in-cell functional studies shown in Figure 7.

Comment 2.10. Fig. 1e, it doesn't make much sense to compare the number of sgRNAs in the enriched fraction to patient numbers. Perhaps z-scores would be a better measure of phenotypic effect for each sgRNAs ?

Response 2.10: Both metrics provide useful information about the propensity for a specific mutation to produce the desired phenotype that was the basis for selection. Hence, we have represented screen results in both ways in Extended Data Figures 2 and 3. In fact, a plot using z-scores (instead of sgRNA numbers) for APC (as requested in this comment) is shown in Extended Data Figure 3d. Inspection of Extended Data Figures 2 and 3 confirms that these two metrics roughly correlate-- positions targeted by sgRNAs with high z-scores are also often targeted by multiple different sgRNAs (see sgRNAs targeting the β-catenin degron in Extended Data Fig.2c,d).

Comment 2.11. The discussion lacks sufficient references to prior work on the assembly and regulation of the BDC to contextualise how their model compares to current understanding. The authors do not explore the role of these variants in subcellular localisation of BDC subunits, a key regulatory feature.

Response 2.11: The discussion has been truncated to fit the word limitations for the manuscript. However, we have made sure that all prior relevant papers are cited and have now placed our model in the context of prior models from the literature. We have cited (and referenced PDB IDs) for all prior structural studies of the BDC relevant to the PPI interfaces we discuss. In addition, we have included references to other papers that discuss various models for the mechanism of the BDC[9–12].

Comprehensive analysis of the subcellular localization of all studied variants is outside the scope of this manuscript; however, we do report β-catenin subcellular localization in cells carrying the affinity-tuned *AXIN1* alleles in Extended Data Fig.9d.

Comment 2.12. For Fig. 7, SW480 proliferation experiments would be a useful addition.

Response 2.12: Proliferation assays require use of cell lines that are addicted to WNT signaling. As such, we now provide continuous proliferation and colony formation data (**new Fig.7f**) for three CRC cell lines (DLD-1, LoVo, HT29) that carry APC truncations and one control like (RKO) that carries full-length APC. The DepMap database shows that DLD-1, LoVo, and HT29 cells are WNT addicted and thus highly dependent on β-catenin for their growth. (SW480 cells do not show a strong dependence on WNT signaling for growth in our hands despite carrying an APC truncation).

We would like to thank this reviewer (and Reviewer#3) for suggesting proliferation assays as these have significantly improved the paper.

Comment 2.13. I recommend the authors deposit their data as a resource on MAVE db to increase accessibility.

Response 2.13: Thank you for this suggestion and for bringing MAVE db to our attention. We have run into some technical difficulties in our initial attempts to upload our data. However, we are working to resolve the issues and will do our very best to complete the submission prior to publication. Our manuscript (including Supplementary Table 1 containing all the screen results) has been available on *bioRxiv* for several months for interested investigators.

Comment 2.14. Detailed instructions and comments for pre-processing sequencing data but stops short of providing code to reproduce figures.

Response 2.14: We used the software programs GraphPad Prism and Adobe Illustrator, not code, to make most figures. For panels generated using code, the code has now been deposited in GitHub.

Reviewer #3

Comment 3.1In the submitted manuscript, Ganesh and his colleagues performed base-editor screens on several components of the β-catenin destruction complex to better understand the molecular interfaces between these components. The beta-catenin destruction complex is key to the canonical Wnt signaling pathway, and its dysregulation has been associated with many types of human cancer. Therefore, a detailed analysis of mutations and their impact is of broad interest, particularly since inhibition of the pathway has yet to be addressed by therapeutic interventions. The topic is timely and insights of this study could have a broad impact on the design of novel therapeutic modalities.

The authors performed comprehensive adenosine and cytosine base editor screens of four components of the β-catenin destruction complex (BDC) and followed up with a detailed mechanistic analysis of the identified mutations and their functional implications. In addition to β-catenin, they covered AXIN1, APC, and GSK3β with their libraries, achieving approximately 80-90% residue coverage. The authors further focused on approximately 150 previously uncharacterized mutations that led to functional changes, including loss- and gain-of-function alleles. The primary screens were performed in haploid HAP1 cells, and follow-up experiments

were conducted in the HEK293 and SW480 cell lines, which are typical models for studying Wnt signaling activity.

While many structure-function studies have examined individual residues or parts of proteins, this study is the first to perform a comprehensive analysis of four BDC components and analyze the impact of numerous mutations in context. The study was conducted with rigorous quality control measures, underlining the high quality of the data. The design of the screens and the analysis are clearly explained, and the authors conducted an impressive set of follow-up experiments to draw molecular conclusions for multiple identified mutations. Among the many interesting findings and comprehensive presentations of resources that will be valuable for the community, one finding stands out in particular: the identification of affinity-enhancing mutations and protein-protein interfaces between Axin1 and β-catenin that only reduce Wnt signaling in the presence of activating mutations. These mutations are of particular interest for designing drugs that target the pathway in tumors without affecting its normal function. These findings could have a broad impact on how to target oncogenic Wnt signaling in colorectal cancers.

Overall, the study is very well executed and I fully support its publication in Nature Genetics. The experiments are well performed and the findings are important for a better understanding of oncogenic Wnt signaling pathways.

Thank you for this generous comment. We hope that our manuscript will be of interest to the field, especially given the recently expanding interest in β-catenin inhibitors for cancers driven by WNT pathway mutations.

Comment 3.1. It would be helpful for readers to have a clearer understanding of the false negative and false positive rates of the screens. Furthermore, it would be helpful if the authors presented and explained these rates in more detail.

Response 3.1: Please see Responses 2.3 and 2.4 above, where we describe our approach to calculating false positive and negative rates for our screens. In the revision, this analysis is presented in **new Extended Data Fig.1d-g** and estimation of false positive and negative rates discussed in **new Supplementary Notes**.

Comment 3.2. While it is understandable that the authors focused their further analysis on β-catenin and Axin1, they should add a more detailed explanation of the modest effects of APC and GSK3β mutations. Any additional analyses the authors have regarding the growth defects would also be helpful for understanding the results.

Response 3.2: Please see Response 2.4 above, which provides a more detailed discussion of the reasons our screen did not nominate missense mutations in *APC* and *GSK3β*. We emphasize that we did identify plenty of sgRNAs predicted to make nonsense or splice site mutations in *APC* that were top hits in our screen (see Fig.1e, Extended Data Fig.3c,d), demonstrating that our library successfully edited the *APC* locus.

Please see Responses 2.2 and 2.12 above for effects on growth and proliferation. In new data (**Figure 7f**), we show that expression of $AXIN1^{AE}$ is much more effective than $AXIN1^{WT}$ or $AXIN1^{AR}$ at suppressing both colony formation and proliferation of three human CRC cell lines carrying various APC^{mcr} alleles (DLD-1, LoVo, HT29). These cell lines were chosen because they have been shown to be highly WNT addicted in the DepMap database. As a control, we use RKO cells, which carry full-length, wild-type APC. In RKO cells, all three affinity tuned alleles show comparable effects. These data support our model (Fig.8) that increasing AXIN1-β-catenin affinity increases BDC activity (and decreases WNT signaling) more effectively in cells carrying protein-truncating APC^{mcr} alleles. These data fulfill a key prediction of our auto-regulatory model and the proposed critical role played by the AXIN1-β-catenin PPI in the oncogenic BDC (Fig.8).

We are grateful to this reviewer for suggesting growth assays as these have considerably strengthened the paper.

Comment 3.3. Was a systematic analysis performed on gain-of-function mutations in the absence of Wnt3a to determine whether they enhance or activate signaling?

Response 3.3: We show in Fig.4b that gain-of-function mutations (C619Y/E620K and L643S/P;L644P) do slightly elevate baseline WNT signaling in the absence of WNT-3a. The difference between these mutations and a control is statistically significant for C619Y/E620K but not for L643S/P;L644P (Fig.4b). However, this baseline activation is much, much lower than that observed in cells carrying the classical phosphodegron mutations in β-catenin. Other gain-of-function mutations (β-catenin E462K in Fig.2g and AXIN1 V475A in Fig.6b) did not show baseline elevation in the absence of ligand stimulation. It would be interesting in the future to see if such activating mutations can be identified by selecting cells with high WNT reporter activity in the absence of WNT3A.

Comment 3.4. Fig. 1g could be improved with regard to labels and color scheme. In its present form, Fig. 1g is slightly confusing since the colors for the genes and mutations/interfacs are very similar. Why are there no hits that increase signaling in the adenine base editor screen?

Response 3.4: We apologize for the visualization, which is challenging given the number of variables we are trying to display. We have now replaced the colors used to designate interfaces in the original manuscript with letter labels and hope this is easier to read.

We do find point mutations in the β-catenin degron (e.g. S45P) that elevate WNT signaling activity (see Fig.1g). However, adenine base editors cannot make nonsense mutations, hence this screen does not identify truncating mutations in APC (which represent a major fraction of the mutations that elevate signaling in CBE screens).

Comment 3.5. In Fig. 7e, Axin1-AE (affinity enhancing) appears to be more highly expressed in the Western blot. A titration of inducible or titrated Axin1 and its mutant forms would provide more convincing support for the impact.

Response 3.5: We have now provided a titration experiment of all three AXIN1 variants in **new Figure 7d**, along with an immunoblot to show AXIN1 abundances. At multiple concentrations of DOX, $AXIN1^{AE}$ suppresses target gene transcription more effectively than $AXIN1^{WT}$. Immunoblotting shows that the abundances of the variant proteins are comparable. Additionally, we repeated the immunoblot shown in Figure 7e of the original manuscript since it was a little overexposed. This new immunoblot (**Figure 7e**) shows that $AXIN1^{WT}$ and $AXIN1^{AE}$ protein abundances are comparable.

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Response to Reviewer Comments (NG-A70717R2)

Thank you for the thorough evaluation of our manuscript and for giving us the opportunity to submit a revision. Please find our responses to remaining comments from Reviewer #2. Reviewer comments are in blue and our responses are in black text.

Comment 1: However, not showing SW480 proliferation assay data because they did not behave as expected is not satisfactory. Most of the mechanistic work is done with SW480 (e.g. in Fig. 7) - how can the authors reconcile this omission?

We were transparent in our prior comments that SW480 is not suitable for WNT-dependent proliferation assays because inhibiting WNT signaling has only a minor effect on SW480 growth. We instead performed two independent proliferation assays (endpoint and real-time) across three WNT-addicted CRC lines (HT29, LoVo, DLD-1) plus an RKO control. This approach mirrors a 2023 *Nature Genetics* paper (PMID: 37749246), which similarly used HT55 and HT29 for proliferation assays without measuring SW480. We also note that mechanistic WNT signaling experiments in Fig.7 span three independent cell lines (HEK293T, HAP1, and SW480), not SW480 alone. We have added the following sentence to address this methodological limitation:

"Note that we did not test SW480 cells since they did not show strong WNT-dependent cell proliferation (and are not represented in DepMap)."

Comment 2: I previously suggested the authors introduce variants into cancer cell lines (e.g. CRC models), rather than simply overexpressing an AXIN variant. The authors sell their approach in the introduction by mentioning the advantage of base editing is studying the BDC at physiological expression levels, so it seems contradictory to revert to overexpression studies (and only for 1-2 variants) in cancer cell lines.

All ~400 variants identified in the screen were introduced at the endogenous locus in HAP1 and HEK293T cells — this principle was not abandoned. Additionally, this comment ignores or fails to acknowledge data in **Extended Data Fig.7e-7h** that tests the most extensively characterized beta-catenin variants in two different cancer cell lines (RKO and DLD-1). We call this out in the following text:

"Several of these novel mutations in β-catenin also reduced the constitutively high level of WNT reporter activity in cancer cell lines carrying the CRC-driving APC^{mcr} oncoprotein (which cannot bind directly to AXIN1) (Extended Data Fig.7e-h)."

The final figures explore a distinct question: whether *affinity-enhancing* AXIN1 variants can suppress oncogenic WNT signaling in cancer lines. Engineering such complex variants at the endogenous gene loci of cancer cells is technically intractable, as most CRC lines carry more than two *AXIN1* alleles. Stable inducible expression is the established method for this type of functional test, used extensively in the field — including in the same 2023 *Nature Genetics* paper cited above. We have now added a sentence acknowledging this methodological limitation:

"*We used stable expression (instead of genome editing at the endogenous locus) to introduce $AXIN1^{AE}$ and $AXIN1^{AR}$ alleles in all cell lines except for eHAP1 because engineering these alleles requires multiple genome edits that are challenging to install at ≥2 AXIN1 alleles present in most cell lines*."

Comment 3: I accept that base editing screens can have high false-negative rates due to sgRNAs not editing, but false-positive rates of 20-30% casts some doubt on the general utility of the screening dataset.

Using the standard method for base editing screens (non-targeting sgRNA controls), our false-positive rate is <4% (see **Extended Data Fig.1d**), consistent with all published screens; in *APC* and *GSK3β* it is 0% (see **Extended Data Fig.1g** and text). The 20–30% figure reflects our unusually comprehensive validation of ~400 variants across the full library — a deliberate effort that most screens do not undertake, and which provides an honest estimate of realistic false-positive rates in the field. We have transparently described these different estimates of the false positive rates in the Supplementary Note (see Note 2) and have now removed a statement that provided a unitary false positive rate: "*In summary, based on the genes that were a target of our screens a conservative estimate of the false positive rate is ~10-20%*."

In summary, none of these points affect the major conclusions. We remain confident in the dataset and believe the mechanistic and translational findings are timely and significant.