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Peer Review File

Comprehensive RAD51C ovarian cancer variant analysis uncouples homologous recombination and replicative functions

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Identification and characterization of functional impaired variants of unknown significance (VUS) of homologous recombination genes are valuable for clinical diagnosis and treatment of cancers with HR deficiency, such as, breast cancer and ovarian cancer. RAD51C genetic alterations have been identified in ~3% of familial ovarian cancers. In this study, Rein et al. have screened 27 ovarian cancer-derived VUS of RAD51C, a paralog of Rad51 recombinase, and examined the impact of these mutations on the biochemical character and the function of RAD51C.

Using yeast-three-hybrid, the authors identified five RAD51C variants with 50% or more reduced interaction with RAD51D (RAD51C-T120A, -G162A, F164L, -V239G, -A279P). They went on to test the effect of several VUS variants on sister chromatid recombination and found that five of the seven HR deficient variants were modestly affected with a 12-30% HR reduction (T120A, G162A, F164L, V166G, and V239G). Strikingly, the L238R and A279P variants exhibited a very defective HR repair ability and are highly sensitive to cisplatin and olaparib. Interestingly, the two key variants, L238R and V239P, are in the Walker B motif, while the variant A279P resides around the Walker B motif. The authors further showed that the L238R variant of RAD51C has a defect in the formation of a stable BCDX2 complex, the A279P variant has a moderate defect in DNA binding, while the V239G variant exhibited a reduction in ATPase activity.

The work reveals the importance of Walker B domain in RAD51C function and identified two important HR deficient RAD51C variants and several hypomorphic RAD51C variants. They provided evidence that RAD51C Walker B region is functionally critical for HR repair and response to therapy. The classification of RAD51C VUS is important for risk management and effective treatment. However, the direct evidence showing the contribution of the key RAD51C VUS to tumor formation or growth is lacking. In general, the novel information that this study brings to the field and the depth of the work are insufficient to justify the publication of this work by Nat Commn. It is more suitable for a specificized journal.

Reviewer #2

(Remarks to the Author)
RAD51 paralogs, in addition to their canonical HR mediated DSB repair function, have evolved with DNA damage signaling, FA pathway of ICL repair, and replication fork stability and restart functions. In the recent years, RAD51C germline mutations have been identified in FA-like disorder and breast and ovarian cancers. Authors in this manuscript have characterized 27 RAD51C VUS that were identified in ovarian cancer patients to test its interactions with other RAD51 paralogs, HR efficiency, ATP hydrolysis, and Olaparib sensitivity. Except for 2 RAD51C mutants (L238R and A279P) none of the other mutants showed severe HR defect and Olaparib sensitivity. Moreover, the authors fail to provide molecular mechanism of defect associated with these mutants leading to ovarian cancer. For example, why A279P variant proficient in ATP hydrolysis is severely defective for HR. D242A in the Walker B motif defective for ATPase, is also critical for HR as characterized by Hu et al., 2023 using D242N pathogenic mutation. Indeed, this study has characterized large no. of RAD51C mutants including G189R and A279P that have been examined in this study. Why authors have examined only 12 mutants in HR function out of initially selected 27. Out of 12, only 2 mutants exhibit severe HR defects. How are the other mutants related to disease? Since RAD51C participates in replication stress responses, examining these mutants with their

new functions might provide more information. In fact, such studies have been carried out recently (Longo et al., 2023; Kolinjivadi et al., 2023). Indeed, RAD51C pathogenic mutations A126T and G264S that were HR proficient showed increase in replication stress markers, defective fork stability and restart functions (Longo et al., 2023). These observations certainly indicate that RAD51C is multifunctional in genome maintenance. Based on the recent structural data with BCDX2 (Greenhough et al., 2023; Rawal et al., 2023) and CX3 complexes (Longo et al., 2023), in depth analyses with RAD51C variants can be performed. The Fig. 2b and c data should be presented as supplementary. The manuscript is also not written well. Overall, the manuscript doesn't provide any new information about RAD51C HR functions, and the molecular mechanism of defects associated with RAD51C mutants leading to cancer.

Reviewer #3

(Remarks to the Author)
Rein et al present a comprehensive analysis of RAD51C VUS in ovarian cancer. This study provides much needed information on the impact of variants in RAD51C with detailed functional studies using multiple platforms.

A high level of care and attention to detail is apparent throughout the study. This is apparent with consideration of confounding factors such as growth rate which was both measured and mitigated through the use of colony formation assays that are moderately robust to its impact.

The results are well presented with clarity and using informative figures such as the annotated structures to explain the conclusion that the authors have drawn.

The statistics used are appropriate for the study, and all the methods are explained in sufficient detail.

Although only one variant effect predictor is applied, REVEL is an appropriate choice and representative of the state of the art.

I would like to see some discussion of alternative mechanisms of RAD51C action other than HR. These results apply equally to RAD51C's role in replication fork protection.

The authors could also comment on the suitability of the different assays they applied for future higher throughput approaches (ie MAVEs). It seems that at least the yeast three hybrid assay would be eminently suited to this approach, and this lower throughput comprehensive study would provide excellent calibration.

I also consider it essential that at least a supplementary table uses full validated HGVS strings to describe the variants to facilitate searching and robust integration into databases.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this revised manuscript, the authors screened 27 ovarian cancer-derived RAD51C VUS to identify those affecting the assembly of functional tetrameric RAD51B-C-D-XRCC2 (BCDX2) complex. They identified a mutation cluster of the RAD51C Walker B region affecting the interactions with other RAD51 paralogs. The authors further analyzed these variants for their capacities in HR repair, replication, DNA binding and ATPase activity, and RAD51 filament formation, and identified separation-of-function alleles that uncouple RAD51C distinct enzymatic activities with HR and replication. In general, the authors identified additional VUS with functional defects, which provides valuable information for pathogenicity classification and inform future strategies to treat individuals with RAD51C loss-of-function alleles. It is now suitable for publishing in Nat Commun.

Reviewer #3

(Remarks to the Author)
This revised manuscript greatly improves on the original paper. All my original comments have been addressed, and the additional work included now makes this a more significant advance in the field.

While the original version showed an important additional domain, the new assessment of HR vs replication function provides novel insight into the partitioning of function and resolves the issue of discordant results between ATP hydrolysis and HR function.
The comparison to the high throughput DMS data shows the complementarity of this more detailed approach, highlighting the limitation of a single growth assay in HAP1 for understanding some of these variants, and providing valuable calibration data for future efforts.

Reviewer #4

(Remarks to the Author)

It is a difficult paper to read and the data presentation is a bit sloppy - but overall, after adding the extra analyses on the impact of the mutations on "DNA replication fork protection" and RAD51 nucleoprotein filament formation, plus the additional classification tables, it seems to me that this elevates the impact of the paper to help variant classification in the clinic.

I agree with the reviewers' comments stating that the work does not bring up a new mechanistic insight on the functions of the various RAD51 paralogs complexes.

One notion that is not clear to me is that Walker A and B motifs are part of the same ATPase catalytic pocket, aren't they? Why would Walker B motif mutations specifically act as separation-of-function? Wouldn't you expect Walker A motif mutations to also result in such separation-of-function?

Note: References 6 and 24 refer to the same publication.

Response to Reviewer Comments:

Reviewer #1:

The work reveals the importance of Walker B domain in RAD51C function and identified two important HR deficient RAD51C variants and several hypomorphic RAD51C variants. They provided evidence that RAD51C Walker B region is functionally critical for HR repair and response to therapy. The classification of RAD51C VUS is important for risk management and effective treatment. However, the direct evidence showing the contribution of the key RAD51C VUS to tumor formation or growth is lacking. In general, the novel information that this study brings to the field and the depth of the work are insufficient to justify the publication of this work by Nat Commn. It is more suitable for a specificized journal.

"…the direct evidence showing the contribution of the key RAD51C VUS to tumor formation or growth is lacking."

While we agree with the reviewer that mouse models would be interesting and useful for understanding direct impact on tumor pathogenicity, there are several important technical and logistical considerations. First, we would like to point out that clinicians and clinical geneticists follow current American College of Medical Genetics/Association for Molecular Pathology (ACMG/AMP) guidelines for variant pathogenicity classification. These guidelines focus on a host of pathogenicity evidence but relies heavily on patient data like co-segregation and population frequency. Aside from the clinical data and pedigrees provided in this study, our analysis of RAD51C variants falls under the functional category of evidence criteria. Mouse and xenograft models of RAD51C variants would also provide functional evidence but would only serve to support our current results and do not provide any further evidence of pathogenicity in the ACMG/AMP classification system. Therefore, these studies would have limited clinical utility on variant classification despite considerable time and expense.

Secondly, RAD51C is essential and knockout mice are embryonic lethal. The more severe homologous recombination deficient (HRD) variants, like those caused by Walker B disruption, would likely result in embryonic lethality as we have observed in other non-transformed cell lines.

"In general, the novel information that this study brings to the field and the depth of the work are insufficient to justify the publication of this work by Nat Comms."

We respectfully disagree with the reviewer that our study is insufficient to justify publication in *Nature Communications*. Our study is the only analysis to date that examines every known RAD51C ovarian cancer variant for its homologous recombination function. The unique combination of cell-based functional assays, biochemical studies and structural analysis enabled us to identify the Walker B region as a functionally critical region of RAD51C. This finding was left unidentified by several recent publications examining RAD51C variants (Hu et al., 2023; Prakash et al., 2022) and underscores the significance of the BCDX2 complex structure published in two independent *Nature* papers (Greenhough et al., 2023 and Rawal et al., 2023 which we co-authored). Our study is thus extremely timely, with an increased interest in the RAD51 paralogs, RAD51C and their connection to breast and ovarian cancer. Since the initial publication we have added experimental data demonstrating that RAD51C replicative and HR functions can be uncoupled and show that RAD51C ATPase activity is critical replication. Our study will have significant ramifications for the future of RAD51C variant classification, diagnosis and patient care and is currently being used to inform the creation of the RAD51C variant classification guidelines through ClinGen's Hereditary Breast, Ovarian and Pancreatic Cancer Variant Curation Expert Panel (VCEP). This group, of which I belong to, comprises of world- leaders in the field of variant analysis and works to both officially classify all genetic variants associated with HBOC and pancreatic cancer and develop guidelines for classification in each associated gene. The addition of our findings will now enable swift and accurate classification of variants in and around the Walker B region. Ovarian cancer prevention and treatment strategies are currently lacking as exemplified through average stage of diagnosis and low five-year survival rates. Thus, reclassification of these variants will have a significant impact on patients and their families through improved risk assessment and expanded availability of targeted treatment strategies.

Reviewer #2:

"Authors in this manuscript have characterized 27 RAD51C VUS that were identified in ovarian cancer patients to test its interactions with other RAD51 paralogs, HR efficiency, ATP hydrolysis, and Olaparib sensitivity. Except for 2 RAD51C mutants (L238R and A279P) none of the other mutants showed severe HR defect and Olaparib sensitivity."… "Why authors have examined only 12 mutants in HR function out of initially selected 27. Out of 12, only 2 mutants exhibit severe HR defects."

In our initial screen, we examined a total of 27 RAD51C ovarian cancer variants using our yeast-three-hybrid system. This assay examines the interaction between RAD51C and RAD51D and was found to be highly indicative of HR proficiency in our previous analysis of 50+ RAD51C

variants (Prakash et al., 2022; Pearson correlation r = 0.8949). Therefore, we utilized this system in an effort to quickly parse out likely HRD variants. To further increase the accuracy of our screen, we have now analzyed all the remaining ovarian cancer variants for HR deficiency and have now identified additional HR deficient mutants.

Additionally, the reviewer makes several comments on the lack of severe HRD variants uncovered in our analysis. This low HRD variant yield is not unexpected given a high rate of passenger mutations that are unrelated to the disease and plague the field. For example, similar findings were reported in an analogous *Nature Communications* publication examining PALB2 VUS (Wiltshire et al., 2020). Of the 84 total variants screened, the authors only found four of these variants to be HRD. Although the identification of dysfunctional variants is important for patients harboring these specific variants, the identification of the critical region is vital for the reclassification of any current or future variants found in and around the Walker B. Thus, the focus should not be on the number of HRD variants, but rather the resulting implications of dysfunctional variant clustering.

"Moreover, the authors fail to provide molecular mechanism of defect associated with these mutants leading to ovarian cancer. For example, why A279P variant proficient in ATP hydrolysis is severely defective for HR."

We have now examined the replication functions and also RAD51 filament formation by EM of the Walker B variants including A279P. We find that this variant is HR deficient while maintaining its function in replication fork progression. Furthermore, A279P results in fewer RAD51 filaments by EM (**Figure 7**). In contrast, V239G, while HR proficient, has a severe defect in replication fork dynamics upon camptothecin treatment (**Figure 6**). Importantly this variant also exhibits an ATPase defect and reduced RAD51 filaments by EM. Together these results suggest that HR and replication functions of RAD51C can be uncoupled and that both HR and replicative roles of RAD51C likely independently contribute to RAD51 filament formation.

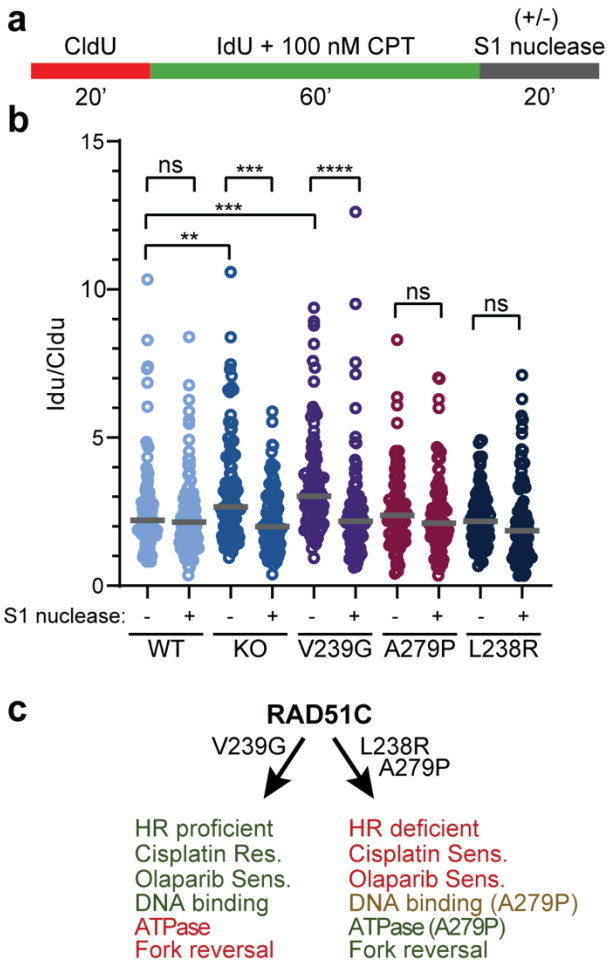


Figure 6. *(a) Schematic of labeling of replication fork progression. The cells were first pulsed with CldU for 20 min followed by IdU with camptothecin (100 nM CPT) for 60 min subsequently medium with or without the S1 nuclease was added for 20 min. Replication fork speed in untreated cells was assessed in Supplementary Fig. 4. (b) RAD51C knockout cells and RAD51C-V239G expressing cells exhibit increased DNA tract lengths and increased ssDNA gaps. RAD51C CRISPR/Cas9 U2OS cells were complemented with the WT RAD51C or the indicated variant and DNA replication fork progression was determined. Each data point represents Idu/Cldu ratio for one fiber measurement. Approximately 200 fibers were analyzed from two experiments and bars represent median of each data set. Statistical significance was determined by Kruskal-Wallis with multiple Dunn's comparison and is indicated (ns is not significant, ** < p 0.01 *** p < 0.001, **** p < 0.0001). (c) RAD51C variants uncouple the enzymatic, HR, and replicative functions. Summary of phenotypes of RAD51C variants V239G and L238R and A279P from Figs. 1-5. Green is indicative of wild-type RAD51C function, yellow is indicative of a partial function, and red is indicative of a reduced function. Note that the DNA binding and ATPase activities were analyzed for V239G and A279P only since L238R formed insoluble aggregates.*

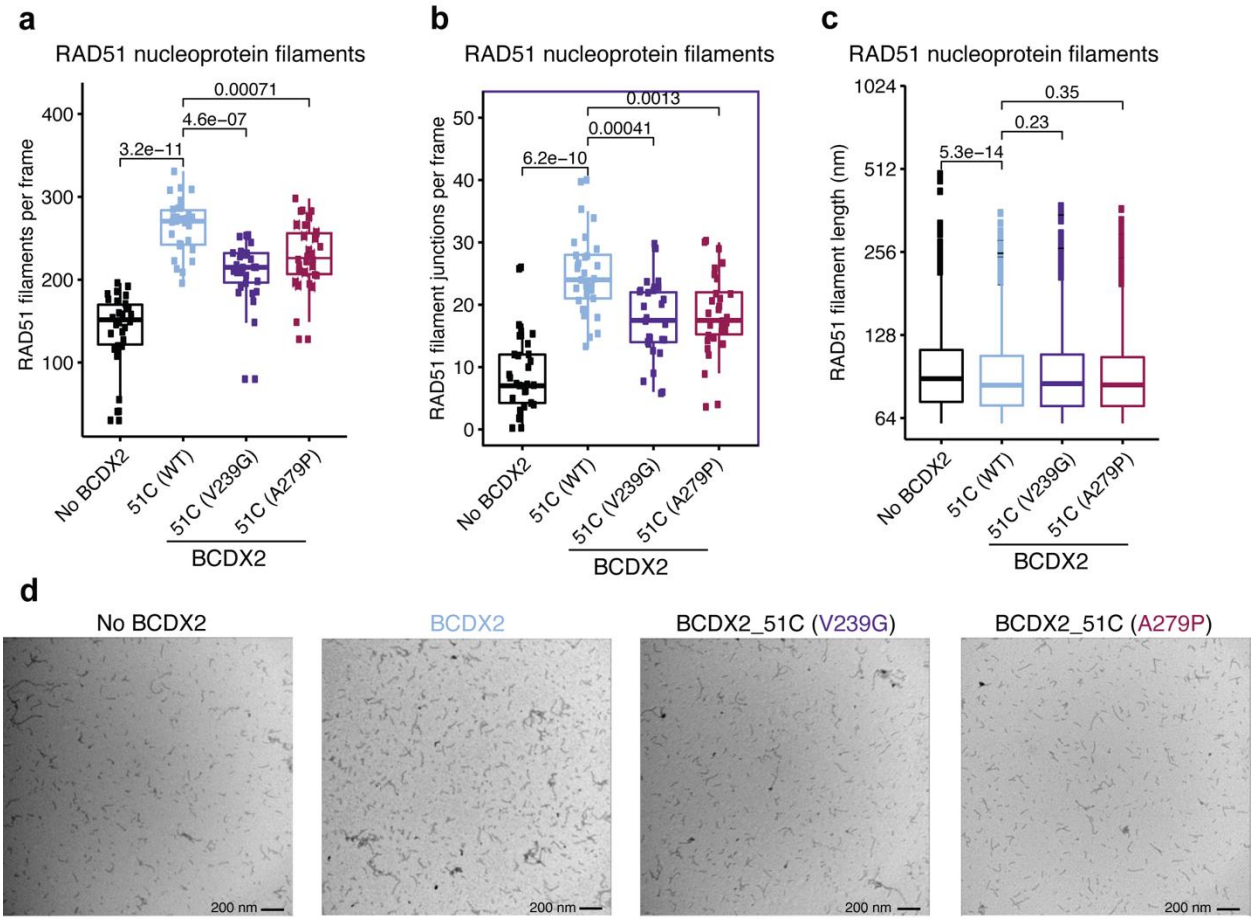


***Figure** 7. (a-c) Box plots depict (a) number of RAD51 nucleoprotein filaments per frame, (b) number of junctions formed by filaments per frame and (c) length of filaments in nanometers measured by negative stain electron microscopy in absence or presence of BCDX2 and CDX2. The p values for the significance of differences in median values were calculated by the Mann-Whitney-Wilcoxon test. (d) Representative micrographs depicting RAD51 nucleoprotein filaments seen by negative stain electron microscopy in absence or presence of BCDX2, and BCDX2 with RAD51C mutants V239G and A279P. Data represent at least 30 electron micrographs for each sample were captured in two independent experiments.*

"Since RAD51C participates in replication stress responses, examining these mutants with their new functions might provide more information. In fact, such studies have been carried out recently (Longo et al., 2023; Kolinjivadi et al., 2023). Indeed, RAD51C pathogenic mutations A126T and G264S that were HR proficient showed increase in replication stress markers, defective fork stability and restart functions (Longo et al., 2023). These observations certainly indicate that RAD51C is multifunctional in genome maintenance. Based on the recent structural data with BCDX2 (Greenhough et al., 2023; Rawal et al., 2023) and CX3 complexes (Longo et al., 2023), in depth analyses with RAD51C variants can be performed."

Evidence of a connection between RAD51C replication function and pathogenicity has been limited and controversial, particularly in comparison to that of HR function. Therefore, our study initially focused on the HR function of RAD51C. However, due to the recent publication of the

two papers referenced by the reviewer (Kolinjivadi et al., 2023 and Longo et al., 2023 which was published after the submission of our manuscript) we agree that analyzing the replication-based functions of these variants is informative. The addition of this replicative analysis in combination with HR data now complements our understanding of the functional impact of these Walker B region RAD51C variants.

"The manuscript is also not written well."

The paper has now been rewritten with the new data.

"Overall, the manuscript doesn't provide any new information about RAD51C HR functions, and the molecular mechanism of defects associated with RAD51C mutants leading to cancer."

As previously stated in our response to Reviewer #1, we believe that our findings are both novel and invaluable to the future of RAD51C variant classification and now present interesting new findings regarding replication and RAD51 filament formation. We present an analysis of every known RAD51C ovarian cancer variant to date and using a combination of high-throughput assays, HR reporter assays, structural analysis and biochemical studies we have identified a cluster of non-functional variants in and around the Walker B motif. This discovery makes our group the first to determine the essential nature of the Walker B in RAD51C homologous recombination and replication function. Not only will this finding inform classification of RAD51C VUS through the ClinGen VCEP but, it will also provide important context for future studies into RAD51C function.

Reviewer #3:

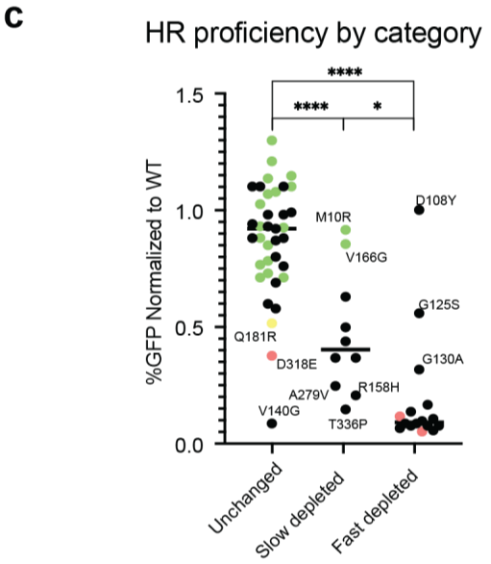
I would like to see some discussion of alternative mechanisms of RAD51C action other than HR. These results apply equally to RAD51C's role in replication fork protection.

We agree with the reviewer and have now included replication analysis and discussed RAD51C HR independent functions throughout.

The authors could also comment on the suitability of the different assays they applied for future higher throughput approaches (ie MAVEs). It seems that at least the yeast three hybrid assay would be eminently suited to this approach, and this lower throughput

comprehensive study would provide excellent calibration.

With the recently published MAVE study in Cell, we have now included a figure showing how our results compare to those studies examining cell viability in RAD51C knockout HAP1 cells (**Supplementary Figure 3c**). We find that while our results are largely congruent, there are some differences.



Supplementary Figure 3(c). *Comparison of growth to HR proficiency from Olvera-León, et al., 2024. Unchanged indicates a variant that has WT growth whereas a slow depleted variant has intermediate growth and a fast depleted variant is inviable. Variants analyzed in this study for HR proficiency are color coded in green (proficient), red (deficient), and yellow (intermediate). The average HR proficiency within the variants categorized as unchanged is significantly higher than the slow-depleted and fast-depleted variants. Statistical significance determined by t-test is indicated (** p-value < 0.01, *** p-value < 0.001, *** p value < 0.0001). Outliers are indicated.*

I also consider it essential that at least a supplementary table uses full validated HGVS strings to describe the variants to facilitate searching and robust integration into databases.

We agree with the reviewer and have now included HGVS strings.