nature portfolio

Peer Review File

Ongoing genome doubling promotes evolvability and immune dysregulation in ovarian cancer

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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:

Referee #1

(Remarks to the Author)
In their manuscript, McPherson et al. investigated the role of whole genome doubling (WGD) in the evolution of high-grade serous ovarian cancer (HGSOC) using scWGS-based single-cell copy number analysis. Based on the analysis of 29,481 cells from 65 specimens from 40 patients, the authors first investigated the evolutionary history of these tumors and suggest that WGD is a continuous process that drives tumor evolution, which is mainly presented in three modes:
1) unitary evolutionary origin followed by significant diversification
2) independent WGD events on a pre-existing background of copy number diversity, and
3) evolutionarily late clonal expansions of WGD populations.
Then they classified each tumor cells into three categories, according to the WGD state; 0 x WGD, 1x WGD, and 2 x WGD (and based on the fraction of cells with WGD, classified each tumor cells into Pervasive WGD (>50% 1x/2xWGD cells) and Rare WGD (≤50% 1x/2xWGD cells) tumors according to the frequency of WGD-positive cells therein. Then, a number of features that were relevant to evolutionary processes, such as cell-to-cell diversity (NND), frequency of divergent cells, the number of copy number changes along evolution, MNs surrogated by cGAS expression, HL amp, rate of chromosomal instability, gene expression, proinflammatory responses, etc. were compared between the two groups to see the effect of WGD on tumor evolution. Based on the results of these analyses, they concluded that WGD is a driving force of the evolution of HGSOC.
These conclusions are potentially of interest and may provide new insight into the role of WGD in tumor evolution. However, this reviewer raises several concerns that should be addressed before this study is considered for publication. The main problem is a significant overlap between Pervasive WGD and Rare WGD groups and HRD-Del and FBI subtypes, which the same authors have shown to drive tumor evolution and immune responses. In their previous study (Funnell, et al., Nature, 2022), they performed scWGS for TNBC and HGSOC and revealed the large variation within tumors in terms of focal amplifications (HLAMPs), copy number alterations and lengths of the copy number segments. They then classified TNBC and HGSOC into 4 categories based on the underlying mutational processes using bulk sequencing data for SNV and SVs, which were enriched for small tandem duplications and BRCA1 mutations (HRD-Dup), deletions and BRCA2 mutations (HRD-Del), fold-back inversions and CCNE1 amp (FBI), and tandem duplications and CDK12 mutations (TD). In particular, the authors reported that FBI tumors showed increased polyploidy, chromosomal mis-segregation, variations in copy numbers, and high-level amplifications, and immune evasion, compared with HRD-Dup tumors. Meanwhile, Rare WGD was highly enriched for HRD-Dup tumors, while Prevalent WGD tumors were enriched for HR-proficient FBI tumors. Therefore, it is unclear whether the observed difference between Prevalent WGD and Rare WGD tumors is due to WGD events, underlying mutational processes, or both. It is also unclear whether there is any difference between WGD(+) cells in Rare WGD and Prevalent WGD tumors. Comparisons should also be made for WGD cells across different tumors.

Major comments
#1. WGD at single-cell resolution
- As mentioned above, the dichotomous grouping of Prevalent vs. Rare WGD substantially overlaps with the previous one, which is based on the underlying mutational processes the authors claim to dictate evolutionary process of HGSOC. Most FBI/HRD-Del tumors were classified in Prevalent WGD, while Rare HGD was highly enriched for HRD-Dup tumors (Fig. 1G). In addition, patients with Prevalent WGD were older than Rare WGD, which might have influenced the observation that the WGD acquisition time calculated from the SNV number was later in Prevalent WGD tumors than in Rare WGD tumors. Thus, it is important to show that WGD events are independently associated with many of the features that the authors used to support the role of WGD in tumor evolution in multivariable analyses.

#2. Evolutionary histories of WGD clones
- The authors reconstructed phylogenetic trees and estimated evolutionary time based on sparse SNV matrices. However, this inference could be affected by several factors. For example:
SNVs were detected more sensitively in larger cell populations than in smaller populations. Thus, the length of peripheral branches tended to be underestimated.
Due to the small insert size will cause false mutation calls will result from incorrect mapping. The authors used a training data set to exclude mutations in subclones across different CNA clones. However, this could not exclude many other artifacts, such as those found across all samples, leading to an overestimation of trunk length. In addition, these artifacts would show lower VAFs, which might have led to an underestimation of WGD timing.
L.193-4: Given the fact that the length of peripheral branches is likely to be underestimated, it is unclear to what extent the inference of the WGD timing is valid.
- SNVs are shown as a matrix in Fig. 2A but this reviewer wants to see the raw data showing the distribution of SNVs on phylogenetic trees.
- WGD events in subclones are interesting but have already been reported in a lung cancer study (Nature, 616, 525-533).
- Fig. 2C: The authors speculate that the independent WGD events in OV-025 and OV-045 approximately coincided. However, two of the three WGD events in OV-045 were mapped just after the branch bifurcation and therefore could therefore represent a single event and if so, the timing of this event could be substantially different from that of the remaining WGD event. In addition, judging from the substantially different branch lengths to which two WGD events in OV-025 were mapped, the two WGD events could be substantially separated.

#3. Post WGD genomic diversification
-Interpretation of 'divergent cells'
-L228-230: It is unclear in what respect these divergent HGSOC cells resemble the 'hopeful monsters' of ref #21. It is still premature to speculate that divergent cells are unstable because these near-divergent cells explain substantial fractions of the tumor cell population, as much as 20% of the population (Fig. 3D), suggesting that at least some of these divergent cells may be more stable than the authors speculate (L.240). To clarify this point, cell fate tracking, even in in vitro cultures of primary HGSOC cells, would be needed to determine whether divergent cells are being newly generated while those of previous generations are disappearing, or whether they are being stably maintained in the tumor population.
- It is important to show a significant correlation between the fraction of WGD cells and that of divergent cells directly, rather than comparing Prevalent and Rare WGD tumors, because WGD cells are rarely seen in the latter tumors.

- Inference of rates of cell-specific copy number changes
-The authors calculated the copy number alterations accumulated since the immediate ancestor in a phylogenetic tree and showed an increase between 0xWGD and 1xWGD and 1xWGD and 2xWGD. However, is there a possibility that this step-up could be largely explained by the copy number changes associated with WGD events? To clarify this, Figure 3F and Figure 4G should be plotted for branches within 0xWGD, 1xWGD, and 2xWGD, and for those between 0xWGD and 0xWGD, and 1xWGD and 2xWGD.
- In addition, many cells that acquired more chromosomal changes died and were not observed. Thus, the inferred number may depend more on the selective process/pressure than on the actual rate of copy number changes. Finally, the difference in copy number losses in 1x WGD cells between Rare vs. Prevalent WGD is marginal (p=0.016). Given the multiple testing, the difference may not be real.

#4. Evolutionary dynamics of WGD and non-WGD clones
- The descriptions of copy number abnormalities assigned to ancestral branches in the main text and Method are inconsistent and confusing.
- Fig. 4B: Pre-WGD and non-WGD should be shown separately. Again, the difference may simply represent the copy number changes that occurred at the time of the WGD.

#5. WGD-specific cell intrinsic and tumor microenvironmental phenotypes
- In their previous study, the authors demonstrated HRD-Dup tumors retained inflammatory response, whereas in FBI tumors show increased TGFb signaling and immune evasion. Thus, it is unclear whether the difference in immune response between Prevalent WGD and Rare WGD groups was due to WGD or the difference in the underlying mutational process.
- It is curious that Prevalent WGD cells showed lower STING expression than Rare WGD cells, even though the former showed more cGAS+ cells in the previous section. The authors speculate that STING transcriptional repression may be prerequisite for clonal expansion. This is a possible explanation but lacks experimental evidence. It is important to evaluate SING expression in 1xWGD cells between Prevalent and Rare WGD cells. Did the STING expression correlate with the frequency of 1xWGD and 2xWGD cells? This should be evaluated in multivariable analysis. Please show the data showing higher cGAS expression in Prevalent WGD cells in scRNA seq as well.
- In Ext Fig 6L, low STING expression in WGD(+) RPE1 cell that appeared in the late passage is claimed to support the presence of immune evasion. However, STING expression should be compared with WGD-negative cells in the same passage.

#6. Discussion
The following discussions are not fully supported by data.

1) L397: "These findings suggest that the evolutionary history of WGD in HGSOC is characterized by the rapid expansion of WGD clones, likely driven by changes in the fitness landscape that favor their proliferation." A long trunk in many tumors

rather indicate that the evolution of WGD clones seems to be rather a slow process?

2) L403: "Evolutionary analysis of our data indicates that gradual losses, rather than punctuated evolution, shape the post-WGD evolution of many WGD clones, suggesting historical adaptation and tolerance for the high CIN levels associated with WGD." The basis of this statement is unclear, despite the descriptions in L294-325. More explicit explanation is needed why the results in the first and second paragraphs in page 10-11 can support this statement.

Minor issues:
There are many mis-citations. These are some examples:
- Fig. 1D and 1E are mis-cited and so are Ext Figs. 3H and I (-> 2H and 2I; L878).
- Fig2-F: with no explanation for this, the authors' claim here is unknown.
- L944-948: 2 mutant copies in both clones (post-WGD and post-divergence) -> mutant copies in one clone (pre-WGD and post-divergence)?
- ED Fig. 3D is mis-cited (L267).
- ED Figs. 2O: no legend for colors used.
- L318: ED Fig. 4J -> 4I
- L361 Ext Fig. G -> ED FIg. 6G. Lack of explanation of colors in Figs. 6C, D.
- ED Fig. 4B right panel: it does not look like 1 x WGD but near tetraploid.
- L316-318: "In clonal WGDs, the number of chromosome losses and arm gains and losses were significantly correlated with the age of the WGD as estimated using mutations (Methods, Extended Data Fig. 4J)." This reviewer failed to identify the relevant description in Method. Does "age of the WGD" mean the number of mutations after WGD?

Referee #2

(Remarks to the Author)
Whole-genome doubling is prevalent in human cancers, and has been associated with increased rates of metastasis, drug resistance, and poor patient outcomes. Understanding the dynamics of WGD may shed new light on the mechanisms underpinning it, and, moreover, whether it is selected or not during tumour development.

In this work McPherson et al. harness a unique cohort of high grade serous ovarian cancer samples and 29,481 tumour cell genomes to explore WGD. They delineate three modes of WGD: 1) early fixation event; 2) late fixation of parallel WGD; 3) emergence of late WGD clones.

Despite its prevalence and links to poor outcome, a deep understanding of WGD dynamics during cancer evolution is lacking. While I was impressed by the work, I think there is scope for improvement (further details below). Key aspects for improvement include: a) why single-cell sequencing is required (e.g. what cannot be inferred with previous approaches vs. with single-cell); b) how many of the results are a result of WGD vs. increased chromosomal instability; c) further demonstration that the WGD calls are accurate; cd this is a dense manuscript, with a lot of information – some of the terminology chosen adds to the confusion.

Comments:

1. It would be useful to have an overview of how many samples have been previously published, and how many are unique to this cohort and analysis.

2. Some of the terminology is a little confusing, e.g. 'Prevalent WGD patients' – it is the tumour and the cancer cells that harbour the WGD, not the patient – so I would be inclined to make this clearer. I also think a better name than 'Prevalent WGD' would avoid confusion (also see comment 7).

3. The authors state that "The distribution of allele-specific copy number features showed clear separation between WGD states, permitting assignment of per-cell WGD multiplicities of 0 (0×WGD, 46% of cells), 1 (1×WGD, 53%) and 2 (2×WGD, 1%) (Extended Data Fig. 2H-I)". While there does appear to be relatively clear separation, I think it should be acknowledged that this is a heuristic method, and it is perfectly possible for cells to never have 'genome-doubled' but simply a large fraction of chromosome arms has independently been gained. The authors might wish to evaluate the likelihood of this – e.g. through simulations based on their observed gain/loss frequency

4. Related to the above, the premise of the manuscript relies on accurately estimating ploidy from single cell data. Indeed, when analysing bulk data, determining correct ploidy and purity is essential to be able to infer WGD correctly. Different tools may estimate these quite differently. And, they are quite frequently wrong. While I appreciate the purity question should not be an issue with single-cell data, the low coverage may render accurate ploidy determination difficult. Thus, there is a need for further demonstration that the ploidy calls (and thereby WGD inference) are correct throughout the manuscript. This is particularly pertinent for samples with mixed WGD – how many cells support this? And, how robust are the WGD calls to down-sampling?

5. Related to the above, I don't understand Figure 2B. What does the fraction #WGD mean? Isn't it impossible to detect residual cells without WGD in the case of a truncal WGD? (This would imply an absence of a clonal sweep, and no such thing as an MRCA). And, I don't understand what Fraciton Cells means?

6. Does the number of C>T mutations at CpG sites correlate with patient age? How confident can the authors be this truly represent a constant mutation rate?

7. The authors have different numbers of cancer cells sequenced for different patients. The number of cells sequenced may have an impact on the likelihood of detecting parallel WGD or cases with mixed WGD. It would be useful to evaluate how robust the results are to sampling bias?

8. The authors state that for "23 out of 25 Prevalent WGD patients, a single ancestral WGD event was common to the dominant 1×WGD population of cells (Fig. 2B)" Does this mean in the prevalent WGD cases there is almost never a 0x WGD population? I think this should be made clearer – the previous paragraph implies that many previous bulk analyses were wrong, and potentially wrong with regards to overstating WGD as a truncal event. As an aside, given that if there is a truncal WGD all subsequent cells will necessarily inherit the WGD – if the authors are confident in the truncal WGD – they can assess their false negative rate of WGD calls in these tumours.

9. Rather than define the tumours as Prevalent of Rare WGD, I would suggest defining the tumours by the mode of WGD evolution; e.g. ancestral truncal WGD; parallel WGD, subclonal WGD etc. And then tumour may have multiple different types, and be grouped accordingly. Prevalent and Rare obscures key differences between tumours.

10. doubleTime will clearly depend a lot on the accuracy of Articull and the associated mutations. Given that we are not reviewing Articull it would be useful to demonstrate that the tool is reasonably robust.

11. I find the statement “In total, 5% of all tumour cells (n=1,481 cells), were part of non-dominant WGD states across the cohort (median of 2.6% of cells per patient Extended Data Fig. 2L)”, a little confusing. By non-dominant, do you mean <50%? I think this needs to be clearly defined in the main text.

12. I'm not sure how much disagreement there is between the previous bulk analyses and the single cell data here. This is implied throughout the manuscript. But, a proper exploration of the benefits/disadvantages of single-cells vs. bulk is lacking. For instance, I'm not sure I understand why a difference would be expected in the case of clonal WGD? Likewise, the logic of timing in single cell and DLP+ pseudo-bulk from a clonal event should be similar – are the authors suggesting previous analysis was wrong due to lack of single-cell? Thus, while I appreciate the resolution should be better with single-cell, I think the authors should consider: 1. Avoiding over-stating differences (unless they're very confident this is significantly different from previous reports). 2. Directly evaluate what differences can be inferred with single cell compared to bulk.

13. When the authors say 'late' WGD patients, do they mean late on the trunk? Late in evolutionary time? The term ‘late’, here, is subject to some confusion. The fact that extant populations with 0xWGD are observed suggests the WGD cannot be in the MRCA – this should be made clearer.

14. Differences in the karyotype landscape between WGD and non-WGD cells will reflect both the rate of chromosome missegregation events, but also tolerance of losses/gains following a missegregation rate. This needs to be acknowledged in Fig 3E and Ex4F. For instance, the observation that ploidy- adjusted chromosome losses were 4.1 times more abundant in Rare WGD 1×WGD cells compared to Rare WGD 0×WGD cells (p=5.6×10-4, Mann-Whitney U test), 2.3 times more abundant in Prevalent WGD 1×WGD cells compared to Rare WGD 0×WGD cells (p=5.6×10-4, Mann-Whitney U 258 test), and 1.8 times more abundant in Rare WGD 1×WGD cells compared to Prevalent WGD 1×WGD cells (p=0.016, Mann-Whitney U test), is likely not as simple as purely a difference in rate.

15. In Figures 4B and C – would the authors expect whole genome doubled chromosomes/arms to have an increased probability of losing more DNA? It would be interesting to see if losses are more prevalent in/specific to certain genes/genomic regions post-WGD.

16. The final part of the analysis focusses on tumour cell phenotypes and microenvironment remodelling in the context of WGD. Here, I think it would be useful to attempt disentangle the extent to which the results being observed truly reflect WGD vs. increased chromosomal instability (potentially as a cause or consequence of WGD).

17. Overall, the flow of the manuscript is a bit jagged. There are often long sentences, spanning multiple lines of text, with few punctuations, making it difficult to distinguish key points from the abundance of results presented. For example, confusing terminology across lines 284-285, “significantly higher number of low-CCF high level amplifications per cell”, required several rereads to understand. I would be inclined to make such statements clearer.

Minor points:

1. Extended Data Figure 2J – p-values would be useful.

2. Figure 2A – Schematic could be more representative of the actual methodology described in the methods section (the synopsis in the main text is also a bit confusing). It is also unclear what “T” and “S” stand for in the rightmost panel.

3. Figure 2D – I am a bit confused about the lack of orange colouring, denoting WGD, in the phylogenies track of the figure because the track underneath suggests the presence of WGD events

4. Figure 3I – Only one p-value is significant, which along with the other p-values, probably would not hold after multiple-test

correction. Describing it as a “modest correlation” seems to be overselling the association.

5. Extended Data Figure 4D – Might be better to facet by rare vs prevalent WGD to show significance of divergent cells

6. Extended Data Figure 4I
1. Wrongly referenced on line 318 as “Extended Data Figure 4J”
2. The authors state that “In clonal WGDs, the number of chromosome losses and arm gains and losses were significantly correlated with the age of the WGD as estimated using mutations”. However, looking at the figure panels, only the correlations with the losses seem to be significant. Also, stating the R2 values might be helpful for better interpretation of the actual correlation.

7. Sample processing (Methods) – in point 2, the authors state scRNA-seq was performed on samples from 32 patients, but in point 3, “for each specimen with scRNA-seq”, this increases to 37

(Remarks on code availability)
I appreciate the effort for making the code available.

Referee #3

(Remarks to the Author)
Summary
In the manuscript ‘Ongoing genome doubling promotes evolvability and immune dysregulation in ovarian cancer’, the authors present a new bioinformatics tool, doubleTime, and the findings from applying this method to single cell whole genome sequencing (scWGS) data from 65 primary tumor samples from 40 high grade serous ovarian cancer (HGSC) patients that are part of the MSK SPECTRUM cohort. The authors combine this data with immunofluorescence data to examine DNA sensing / micronuclei formation, and previously generated single cell RNA sequencing (scRNAseq) and to a lesser extent bulk WGS data and MSK-IMPACT clinical sequencing data. The key findings of the study include:
(1) Through analysis of single cell genomes the evolutionary history of copy number aberrations (CNA) and whole genome doubling (WGD) can be determined. Multiple WGD states are frequently identified within individuals, and through analysis of the timing of CNA events compared to WGD distinct evolutionary patterns are discernable.
(2) Cases with WGD have higher numbers of ruptured micronuclei, as determined by cGAS immunofluorescence than cases with rare WGD, these cases also had higher numbers of subclonal CNA that are not detected in bulk WGS.
(3) Cases with high prevalence of WGD cells have a higher proportion of cancer cells in the G1 phase of the cell cycle compared to cases with rare WGD, and similar findings to recent work that HGSC cases with WGD have reduced expression of immune signalling pathways.

While there are no technical or experimental flaws that preclude publication, there are a number of key points that should be addressed:
1. While I appreciate that scWGS is not a straightforward and cost-effective method to use, I have concerns around the inclusion of the 8 patients with fewer than 200 cells when examining clonality and heterogeneity, or a lack of discussion of the limitations in making conclusions regarding these patients.
2. Although only a small component of the study, the in vitro work only utilises a single cell line limiting the confidence in the results from this experiment. It is also unclear why retinal pigment epithelial cells were chosen rather than a more biologically relevant model such as untransformed fallopian tube cell lines or even HGSC lines without WGD.
I also think that there are a number of missed opportunities in the manuscript as currently presented, and I would encourage the authors to consider including some additional analysis or discussion of the following areas.
1. As most of the analyses have combined the data to the patient level there is limited examination of heterogeneity across tumor sites within individual patients, despite recent papers finding genetic heterogeneity in HGSC patients (eg Cunnea et al Cell Reports Medicine, Burdett et al Nature Genetics).
2. There was limited discussion of the genes involved in the CNA or mutations in relation to events prior to WGD – can the authors explain which events may be causing WGD to occur? It would also be interesting to consider how the WGD timing relates to p53 signature and STIC formation and discuss how the findings relate to those made by Cheng et al (J Path 2024).
3. The decreased STING expression in the prevalent WGD cases despite the high prevalence of cGAS staining at the MN is an interesting finding that could have been explored further, for example are there mutations or decreased expression of upstream regulators of STING?

Additional points for clarification
1. Title: I would recommend that the title be reconsidered, as ‘ongoing genome doubling’ suggests that the focus of the manuscript is the 2nd WGD which it is not. Consider removing the word ongoing from the title or rearrange to Genome doubling promotes ongoing evolvability and immune dysregulation in ovarian cancer. Also consider being more specific with regards to the disease as this study only examines high grade serous ovarian cancers and not all ovarian cancers.
2. The abstract and introduction mention quiescence in the prevalent WGD cases but this term is not used in the results or discussion, for simplicity for the reader please be consistent in the terminology used throughout the manuscript.
3. The introduction has limited discussion about HGSC, and in particular does not describe the actual frequency of WGD in HGSC (either at primary disease or during disease recurrence/end stage), nor recent papers that have looked at WGD in HGSC (eg Cheng et al 2024 J Path, Burdett et at 2023 Nature Genetics). Additionally, the second paragraph of the introduction has a lot of detail regarding the results that are not appropriate for the introduction.
4. Methods
a. Some sections of the results are written in present tense and others in past tense, please update so it is all in past tense.

b. With regards to the flow-sorting of low purity samples, how was purity determined and what purity cutoff was used?
c. In the sample processing section is a reference to Supp Tab 2 – I could not find a table with metrics for numbers of sequenced cells
d. More details of the sequencing itself for the scWGS would be helpful – for example what read length, single or paired end.
e. The paragraph about the spontaneous RPE1 subclone with WGD in the methods (lines 765-769) may be more appropriate in the results section of the manuscript.
f. Some of the bioinformatics tools used do not have citations and the version used is not described (eg bwa-mem, Picard).
g. In the alignment section, the authors mention that local indel realignment can be performed if required. Was it required and if so for which samples?
h. With regards to the haplotype-specific copy number section, more detail is needed for this section to be reproducible, for example is only a single matched normal used or one for each patient and is that bulk WGS data?
i. If possible consider having the cell filtering steps in the methods and the supplementary note in the same order.
j. With regards to the filtering of the S-phase cells – as custom thresholds have been utilised for each patient without the thresholds being provided this section is currently not reproducible.
k. Are the results comparing the bulk WGS copy number inferred by FACETs to the scWGS provided? If not please include.
l. In the section on detecting WGD in single cells is a reference to Ext Data Fig 3H,I – is this correct or meant to be Ext Data Fig 2?
m. In the patient level WGD classifications what is meant by cells classified as “1WGD>1”?
n. For subclonal WGD classification – please clarify what fraction of cells was used as the cutoff for comprising ‘the majority population’, and is this different to the 25% mentioned for patients 081 and 125 with 0x WGD?
o. In the SNV calling section please clarify what is mean by “variant loci that are detected by either caller” when only the Mutect2 tool is described as being run.
p. In the section Enumerating events on ancestral branches please confirm if this sentence is correct as written as the pre-WGD and post-WGD text appears to be the same. “Events were classified as non-WGD if they were predicted to occur on the root branch of a Rare WGD patient, pre-WGD if they were predicted to occur prior to the WGD event on the root branch of a Prevalent WGD patient, and post-WGD if they were predicted to occur prior to the WGD event on the root branch of a Prevalent WGD patient.”
q. In the section on WGD classification of the single-cell RNA sequencing, please provide the data showing that the copy number profiles are highly concordant to the scWGS as Extended Data Fig 5A is a UMAP of the scRNAseq data and does not appear to refer to copy number profiles. If this is meant to be Ext Data Fig 6A please consider also providing additional information – either showing some copy number plots or a heatmap for each patient. Also, is the reference to Extended Data Fig 5B in this section correct?
r. In the Immunofluorescence section, please provide more detail on how the ROIs were defined – ie how were regions of tumor foci identified? Will all of the raw images and the segmentation model for identifying the micronuclei be made available?
s. Some of the information in the section on the identification of WGD subclones in the RPE1 line may be more appropriate in the results than the methods.
5. Results
a. Ovarian cancer patient cohort section – consider clarifying whether the mutations and mutational signatures shown in Ext Data Fig 1B are seen in all tumour sites from a patient or if any limited to a single site.
b. A figure or table comprising the number of cells per sample before and after filtering, and the scWGS coverage range per sample would aid in interpreting the results, particularly with regards to the patients that had >200 cells. This table could also include the number of cells per sample of each WGD state which is hard to interpret from Fig 1G.
c. Considering that cell size correlated with WGD in the DLP+, did the authors examine if nuclei size in the IF correlated with the WGD status from the scWGS?
d. Please consider adding a rationale for classifying cases as having ‘prevalent WGD’ when 50% of cells have a WGD. Did the authors consider classifying samples as high, intermediate or rare WGD rather than the 2 categories?
e. The terminology around classification of tumors or patients as having WGD as currently written is confusing (lines 161 – 164). Initially the text states that each tumor was dichotomized, then only referring to the number of patients with prevalent WGD (ie 40 patients not 65 tumors). On lines 163 and 164 – the authors state that 26/40 patients have been classified as prevalent WGD, then state 60% are classified as having prevalent WGD, and on line 176 23 out of 25 prevalent WGD patients are mentioned – which is correct?
f. Were there any cases with multiple tumors profiled where sites would have been classified differently if the classification was done at a per sample rather than per patient level?
g. The use of two terminologies regarding the WGD is not clear – do all prevalent cases have clonal WGD and rare cases have subclonal WGD? Please consider either expanding the definition of and the differences between clonal/subclonal and rare/prevalent or using only one terminology.
h. Was there statistics performed for Ext Data Figure 2M-P? Please include in text or figure.
i. On line 169 please consider stating which cases did not have multiple WGD states, as it is very difficult to determine from Fig 1G.
j. For the section on the evolutionary histories, please clarify if doubleTime can time the second WGD and if so why there is only a single orange triangle shown in Fig 2B even in cases with 2 WGD. Please also clarify how the cases are split into Fig 2B, C and D in the figure legend. If no orange arrows are shown in Fig 2D because there are not sufficient cells with the WGD to time the event, please consider describing this in more detail in the methods or the limitations section of the discussion.
k. Are theVAF plots shown in Fig 2C available for the other patients?
l. On line 180 the authors refer to Ext Data Fig 3A-B after mentioning patients 025 and 045 – however the figure shows patients 045 and 075. Please clarify in the text or figure legends why certain cases are discussed/shown in the Figures or have the figures match the text.

m. Lines 192 – 194 require some clarification: I'm not sure that the Gerstung paper found all ovarian tumors had early WGD, and a recent Nature Communications paper (Burdett et al) described 24 of 36 HGSC cases in the Gerstung paper as having 'late' WGD. Additionally, the authors state that "later WGD clonal expansions … were common, with 7/25 WGD events" – 7 out of 25 is less than 30% so is this really common? Furthermore, consider indicating in the text or a figure which are the 7 cases with late WGD as it is not easy to determine which cases they are from Fig 2B.
n. For the three evolutionary modes – do they relate to panels Fig 2 B, C and D? If not could an annotation be added to the figure as to which mode each patient fits in? Did the authors examine if there were any clinical or molecular characteristics of the patients that are associated with the WGD evolution mode?
o. Are the 8 patients with highly divergent cells (line 224) listed or depicted anywhere? Is it possible to comment on whether the highly divergent cells had one or two WGD, or were there any associations with the number of divergent cells and clinical or molecular features? Can the highly divergent cells be identified in the scRNAseq data?
p. Why has the non-ploidy adjusted copy number change plot been shown in Fig 3F and the adjusted data in Ext Data Fig 4G when most of the text regarding these results focus on the ploidy adjusted data?
q. With regards to the comment on line 287 that the mutational processes that generate the HL amps might be increased in prevalent WGD cases – is it possible to examine the abundance of copy number signatures such as those described by Macintyre and colleagues (Nature 2018) in these cases in the scWGS or bulk WGS to provide further evidence for this statement?
r. With regards to categorising the CNA as pre- or post-WGD (lines 298-300) please consider explaining why no pre-WGD events were sought in the prevalent WGD cases, especially in cases with late but prevalent WGD.
s. It is unclear why the focus of the discussion of the result in Fig 4B (lines 303-305) is on the gains when the losses are also significantly different but with higher counts than the gains.
t. Why are different thresholds used for calling events clonal – line 278 describes >90% for HL amps, line 309 states >95% for post-WGD events.
u. Figure 3 shows that cells with WGD in the rare WGD cases do have CNA etc, so perhaps consider rephrasing the statement in lines 289-292 as it does not appear that it is only the cases with prevalent WGD that have genomic diversification.
v. Line 318 refers to Ext Data Fig 4J – is this a mistake, as the version of Ext Data Fig 4 that I received only goes up to panel I.
w. Do MEDICC2 phylogenetic trees look similar to the doubleTime trees?
x. A substantial amount is made regarding the findings of a single cell with a distinct copy number profile in one patient (lines 319 – 324, Fig 4E). Please consider moderating the language of the conclusions made regarding this cell/case. And also provide further information regarding the case shown in Fig 4F – for example how many cells have these different events?
y. There do not appear to be statistical tests performed for many of the statements and figure panels of the single cell RNAseq – please add.
z. Line 361 refers to Ext Data Fig G – is this 6G? And line 382 refers to Ext Data Fig 5C – should this be 6C?
aa. Please consider adding additional relevant references regarding immune evasion associated with WGD in HGSC on line 371 – the Burdett et al Nature Communications paper seems particularly relevant but there may be others too.
6. Discussion
a. Lines 396-397 – it is unclear if this is referring specifically to the first WGD or any WGD – but there did not appear to be evidence presented regarding the timing of the second WGD. Considering that the second WGD seem to be subclonal doesn't this suggest that there are co-existing clones with WGD that developed at different times?
b. Please consider comparing your results around CNA following WGD with the Baker et al GRITIC paper.
7. Figures
a. Figure 1) Have the copy number and BAF plots in panels D,E been swapped - the 1xWGD plot looks to have more copy 2 regions than in panel D for 0xWGD. The Fraction Subclonal WGD at the bottom of panel G has not been described in the legend – is this the first or second WGD?
b. Figure 2) The legend for panels B-D states that "Each patient is annotated with mutation signature and age at diagnosis" – where is this shown?
c. Figure 4) In panels B and D – how can the count of CNA be less than 1 and what do the error bars represent?
d. Figure 6) Please add a colour legend for panels C and D and a description of the line in the plot, for the x-axis of panel F is the direction of the differences the same as in panel A? In panel E please add the direction of the fold change ie is read higher in prevalent WGD?
e. Extended Data Figure 1) What is meant in panel B legend for mutational signature undetermined as there do not appear to be any grey boxes in the oncoprint?
f. Extended Data Figure 2) Please consider splitting/coloring the bars in panel A by sample not just patient. Please add details regarding the line and shading to the legend for panels F and G, and also add the correlation and p values. What is meant in the legend for panel P about the "Ovarian Metacohort"? Consider changing the colors in panels N and P for non-WGD and WGD so they are distinct from prevalent and rare WGD. Does panel L only show the 2xWGD cells in patients with predominantly 1xWGD cells or does it also include the 1xWGD cells in patients with predominantly 0xWGD cells?
g. Extended Data Figure 3) The legend for panels A-C states "0xWGD subpopulations" are shown but the y-axis of these graphs suggests both non-WGD and WGD clones are shown. Please provide the color legend for the CN and BAF plots as well as the tumor site.
h. Extended Data Figure 5) Please clarify why there are cells of undetermined WGD class in panel A – if they are from patients where no scWGS data is available why are they shown? Panel F does not appear to be referred to in the text, it would also be interesting to know if the different patterns are associated with the proportion of cells with WGD or the number of WGD detected. It is unclear what the data points in panel G represent – are the points each patient?
8. Data availability – the Synapse site does not mention the scWGS or IF data described for the first time in this manuscript
9. The code availability statement only refers to tools, but does not include code for analysing the IF data or generating any of the figures.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
In their revised manuscript, the authors addressed most of the comments. This reviewer has only one remaining issue regarding the response to [C1].

[C1] The authors try to show the independent effect of WGD and mutational signature using linear models. However, this reviewer is not fully convinced the conclusion. Regardless of the methods/models used, there is a limitation in the statistical power for such a small number of cases (27 WGD-high tumors and 14 FBI tumors), for which these models might not be reliably used to separate the effects of WGD and FBI. So it would be safe to attenuate the statements regarding the effects of WGD-high.

Referee #2

(Remarks to the Author)
The authors have done an excellent job of addressing my comments and improving the manuscript. I have included a few additional comments:

- I appreciate the simulations to show that the WGD heuristic is likely accurate. Although I think a more accurate simulation should include lots of losses before any WGD (apologies if I missed this). This is where things can be a bit trickier.

- I agree that in most cases all chromosomes suggest a similar timing of WGD. However, there are a few notable exceptions (e.g. in OV-044 and OV-052). Does this reflect earlier copy number events, or suggest that it isn't really a single whole genome doubling event?

Few additional comments:
WGD-high tumors comprised 66% of the cohort, were in older patients, and were enriched for FBI and HRD-Del mutation signatures, consistent with previous bulk genome sequencing studies10,18 173 (Extended Data Fig. 2N-Q). --> worth defining what 'older' is here.

Was there any association with the treatment regime and likelihood of observing WGD (and type of WGD?)

Can the authors further disentangle whether the decoupling of CIN and immune related programmes is entirely independent of the association with cell-cycle?

(Remarks on code availability)
I have no issues with the code.

Referee #3

(Remarks to the Author)
The authors have provided a very comprehensive response to all of the reviewer comments which has improved the manuscript and I have no further comments.

Reply to reviewer reports on Nature submission of the manuscript:

“Ongoing genome doubling promotes evolvability and immune dysregulation in ovarian cancer”

Summary of response

We greatly appreciate the reviewers' detailed and thoughtful reading of the original submitted manuscript. We acknowledge this work is data intensive and highly technical requiring careful reading. The comments have helped to improve our presentation of new insights on genome doubling in cancer. We have carefully considered each of the points raised and adjusted the manuscript accordingly. We respond point by point to each comment below. Briefly the major changes are as follows:

1. We have improved the exposition of WGD calling in single cells. This includes additional orthogonal covariates, a clearer description of the heuristic approach, and a description of limitations in the Methods and Discussion.
2. We provided a thorough statistical robustness analysis on the impact of cell population size on phylogenetic and doubleTime inference. Where possible we have added additional scWGS data to improve robustness. Neither of these changes led to different conclusions from the original text, however we appreciate the suggestions from the reviewers as readers will undoubtedly look for veracity of our analyses.
3. We added HGSOC context-appropriate FNE-1 cells (epithelial cells from the fallopian tubes) in the *in vitro* validation of the transcriptional impacts of WGD. In this cell line, we induced *TP53* mutation which eventually led to a spontaneous WGD population. This enabled comparison of WGD and non-WGD cells in the FNE-1 substrate to complement the existing data we had presented in RPE-1 cells. As such, the STING1 repression result is now present in two distinct cell lines.
4. We repeated and improved the IF data generation to obviate the need for batch correction. Improvements in experimental design led to uniform batches and increased image resolution, which greatly improved the detection of cGAS+ ruptured micronuclei, estimation of STING1 protein levels, and localization of tumor areas (the new design included panCK and p53) in the images. As a result, we detected and analyzed an order of magnitude more primary nuclei (approx. 21M) and $cGAS^{+}$ micronuclei (approx. 896k).
5. We completely reworked the terminology with clear definitions and a Glossary of Terms to guide the reader. While our conclusions remain the same, we appreciate that the previous version had confusing and at times conflicting terms.
6. Some speculative or only weakly supported findings were removed in order to focus on the salient findings and improve readability.

In all, the paper's conclusions regarding ongoing WGD, its evolutionary consequences, and its phenotypic impacts remain largely unchanged. However, the claims are now supported by additional data, more robust analyses, and streamlining of the text - resulting in a greatly improved contribution. We maintain these findings will be of great interest to the cancer genomics and genomic/chromosomal instability communities and we look forward to its presentation in the literature.

Referees' comments:

[C=comment, R=reply]

Referee #1

In their manuscript, McPherson et al. investigated the role of whole genome doubling (WGD) in the evolution of high-grade serous ovarian cancer (HGSOC) using scWGS-based single-cell copy number analysis. Based on the analysis of 29,481 cells from 65 specimens from 40 patients, the authors first investigated the evolutionary history of these tumors and suggest that WGD is a continuous process that drives tumor evolution, which is mainly presented in three modes:
1) unitary evolutionary origin followed by significant diversification
2) independent WGD events on a pre-existing background of copy number diversity, and
3) evolutionarily late clonal expansions of WGD populations.
Then they classified each tumor cells into three categories, according to the WGD state; 0 x WGD, 1x WGD, and 2 x WGD (and based on the fraction of cells with WGD, classified each tumor cells into Pervasive WGD (>50% 1x/2xWGD cells) and Rare WGD (≤50% 1x/2xWGD cells) tumors according to the frequency of WGD-positive cells therein. Then, a number of features that were relevant to evolutionary processes, such as cell-to-cell diversity (NND), frequency of divergent cells, the number of copy-number changes along evolution, MNs surrogated by cGAS expression, HL amp, rate of chromosomal instability, gene expression, proinflammatory responses, etc. were compared between the two groups to see the effect of WGD on tumor evolution. Based on the results of these analyses, they concluded that WGD is a driving force of the evolution of HGSOC.
These conclusions are potentially of interest and may provide new insight into the role of WGD in tumor evolution. However, this reviewer raises several concerns that should be addressed before this study is considered for publication. The main problem is a significant overlap between Pervasive WGD and Rare WGD groups and HRD-Del and FBI subtypes, which the same authors have shown to drive tumor evolution and immune responses. In their previous study (Funnell, et al., Nature, 2022), they performed scWGS for TNBC and HGSOC and revealed the large variation within tumors in terms of focal amplifications (HLAMPs), copy number alterations and lengths of the copy number segments. They then classified TNBC and HGSOC into 4 categories based on the underlying mutational processes using bulk sequencing data for SNV and SVs, which were enriched for small tandem duplications and BRCA1 mutations (HRD-Dup), deletions and BRCA2 mutations (HRD-Del), fold-back inversions and CCNE1 amp (FBI), and tandem duplications and CDK12 mutations (TD). In particular, the authors reported that FBI tumors showed increased polyploidy, chromosomal mis-segregation, variations in copy numbers, and high-level amplifications, and immune evasion, compared with HRD-Dup tumors. Meanwhile, Rare WGD was highly enriched for HRD-Dup tumors, while Prevalent WGD tumors were enriched for HR-proficient FBI tumors. Therefore, it is unclear

whether the observed difference between Prevalent WGD and Rare WGD tumors is due to WGD events, underlying mutational processes, or both. It is also unclear whether there is any difference between WGD(+) cells in Rare WGD and Prevalent WGD tumors. Comparisons should also be made for WGD cells across different tumors.

We thank the reviewer for the thorough reading of the manuscript and for the helpful suggestions which have surely improved the paper. Each specific comment [C*] is addressed below inline [R*, blue font for our response].

Major comments

[C1]: WGD at single-cell resolution

[C1]: As mentioned above, the dichotomous grouping of Prevalent vs. Rare WGD substantially overlaps with the previous one, which is based on the underlying mutational processes the authors claim to dictate evolutionary process of HGSOC. Most FBI/HRD-Del tumors were classified in Prevalent WGD, while Rare HGD was highly enriched for HRD-Dup tumors (Fig. 1G). In addition, patients with Prevalent WGD were older than Rare WGD, which might have influenced the observation that the WGD acquisition time calculated from the SNV number was later in Prevalent WGD tumors than in Rare WGD tumors. Thus, it is important to show that WGD events are independently associated with many of the features that the authors used to support the role of WGD in tumor evolution in multivariable analyses.

[R1]: We agree with the reviewer that analysis of the signature-based classification and its relationship to WGD is an important component that should be better emphasized in the manuscript. To address this comprehensively, we added two sets of analysis:

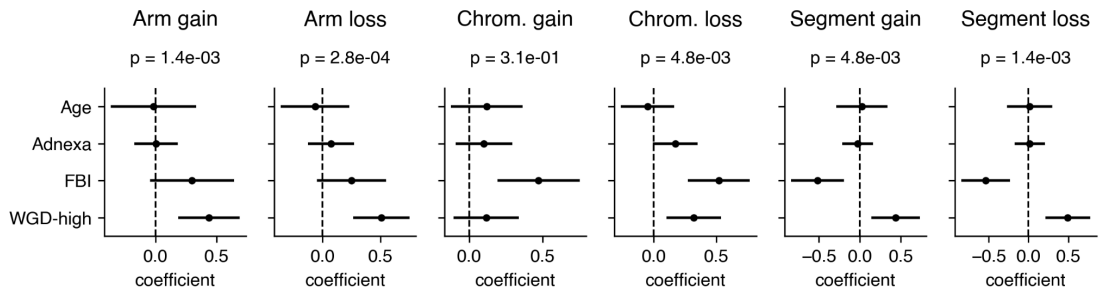
1. **Linear models using GEE:** We fit linear models to test the impact of WGD *while controlling for mutation signature, anatomic site, and age of the patient*. Models were fit with Generalized Estimating Equations (GEE).
2. **HRD-Dup subset analysis:** We subset the patients that were HRD-Dup - a mutation signature subtype for which there were sufficient numbers of WGD-low and WGD-high tumors and repeated the analyses - especially for the phenotypic correlations

Below we present the results of each of these analyses.

1. Linear models using GEE

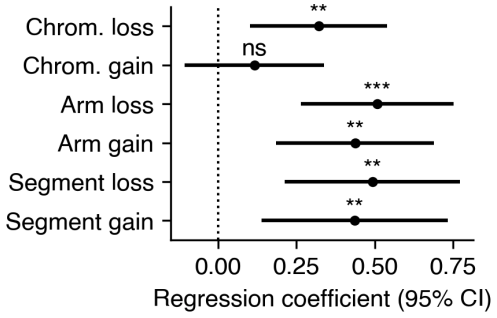
For cell-specific copy-number change, we constructed a linear model with WGD, age, anatomic site, and mutation signature as covariates. For cell cycle, pathway activity, and cell type composition, we investigated the impact of WGD in HRD-Dup patients controlling for patient age and anatomic site.

Focusing first on cell-specific copy-number changes, rates of each type of copy-number change with covariates: mutation signature, age, anatomic site, and WGD, encoding WGD as a binary indicator of WGD-high vs. WGD-low, mutation signature as FBI vs other, and anatomic site as Adnexa vs other were fit (**Rebuttal Figure 1**).



Rebuttal Figure 1: GEE model for rates of cell-specific copy-number change. Shown are the 95% CI coefficient estimates for a GEE model that includes mutation signature (FBI vs other), age, anatomic site (Adnexa vs other), and WGD (high vs low). FDR corrected p-values of the WGD coefficient are shown above each plot.

WGD showed significant positive coefficients for every change with the exception of chromosome gains (Benjamini/Hochberg multiple test corrected). We now show a summary of the WGD coefficients for this model in Extended Data Figure 4H, shown below.



Extended Data Figure 4I: Coefficients for the WGD term of a GEE model of chromosome, arm and segment loss and gain rates (counts per cell, normalized for genome size). The GEE model includes patient age, WGD status (high vs low), mutation signature (FBI vs non-FBI) and site (Adnexa vs non-Adnexa). Significance (FDR corrected) is annotated as 'ns': 5.0×10-2 < p <= 1.0, '*': 1.0×10-2 < p <= 5.0×10-2, '**': 1.0×10-3 < p <= 1.0×10-2, '***': 1.0×10-4 < p <= 1.0×10-3, '****': p <= 1.0×10-4.

2. HRD-Dup subset analysis

Next, to assess mutational signature independent transcriptional features of WGD tumors, we reanalyzed the HRD-Dup subtype, as there were enough samples of both WGD and non-WGD cases to model (31 samples from 8 patients for WGD-high and 16 samples from 5 patients for WGD-low). The results from these analyses are largely consistent with our initial findings, which confirm that WGD is associated with cancer-cell-intrinsic signaling changes, particularly immune-related pathways, cell-cycle phase shifts, and immune cell abundance differences. This reinforces that WGD specific differences are at least partly independent of mutation signature subtype and suggests that differences observed between signature subtypes arise from a combination of WGD-driven effects and the unequal distribution of WGD across signature subtypes.

Focusing on cell cycle changes, we adjusted our cohort-wide model associating WGD with cell cycle phase to account for the interaction between WGD and age, WGD and mutational

signature subtype, and anatomic tumor site. We similarly modeled this within the HRD-Dup subset. Consistent with our original findings, we observe that WGD tumors are associated with a decrease in S phase cells and increase in G1 phase cells, reflective of altered progression through the cell cycle (**Figure 5A** in the revised manuscript, reproduced below).

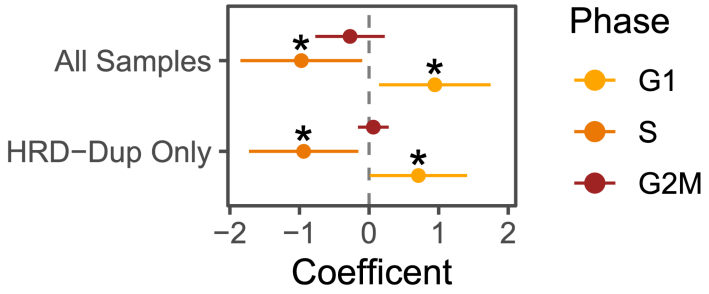
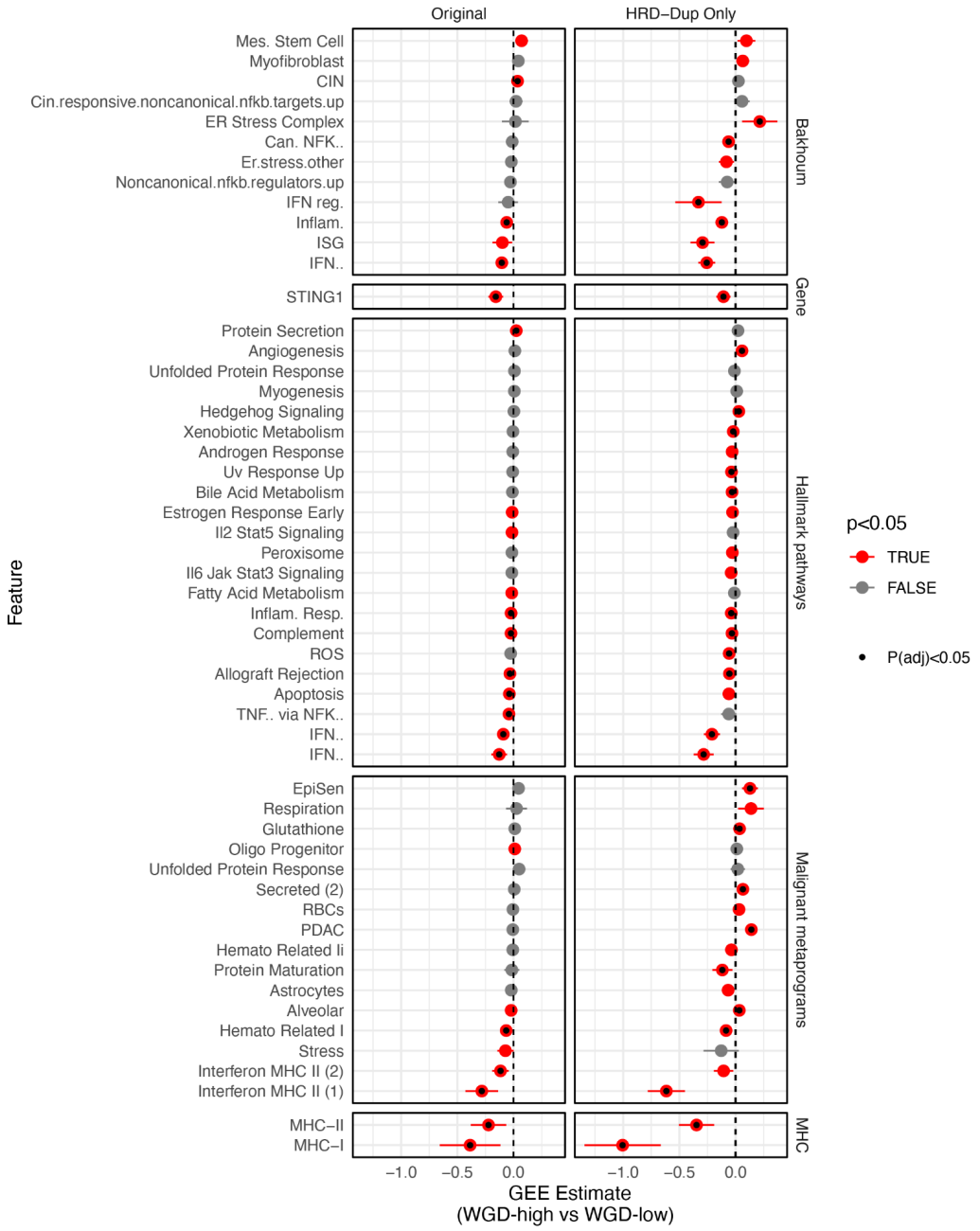


Figure 5A: Coefficients (x-axis) of a Generalized Estimation Equation (GEE) fit to the difference in cancer cell cycle fractions between WGD-low and WGD-high samples, corrected for patient effects, age and tumor site and mutational signature subtype. * indicates $p<0.05$.

We similarly reanalyzed expression of cancer-cell-intrinsic signaling using the HRD-Dup only model. These models demonstrated that the predominant signal driving differential immune-related signaling was the WGD status of these tumors, with significant decreases in MHC-I and II, IFNa and IFNy, STING1, inflammation, and others, consistent with our prior results (**Rebuttal Figure 3**). We additionally see new pathways that pass statistical thresholds of significance for enrichment in WGD tumors (within HRD-Dup), including epithelial senescence, angiogenic signalling, and ER stress complex signalling.



Rebuttal Figure 3: Coefficients from a GEE modeling cancer-cell-intrinsic signatures and WGD status. The original model accounted for tumor site. The HRD-Dup Only model contains interaction terms with WGD status and age, and accounts for anatomic site. Features included in the plot are those where at least one model has an unadjusted p-value less than 0.05.

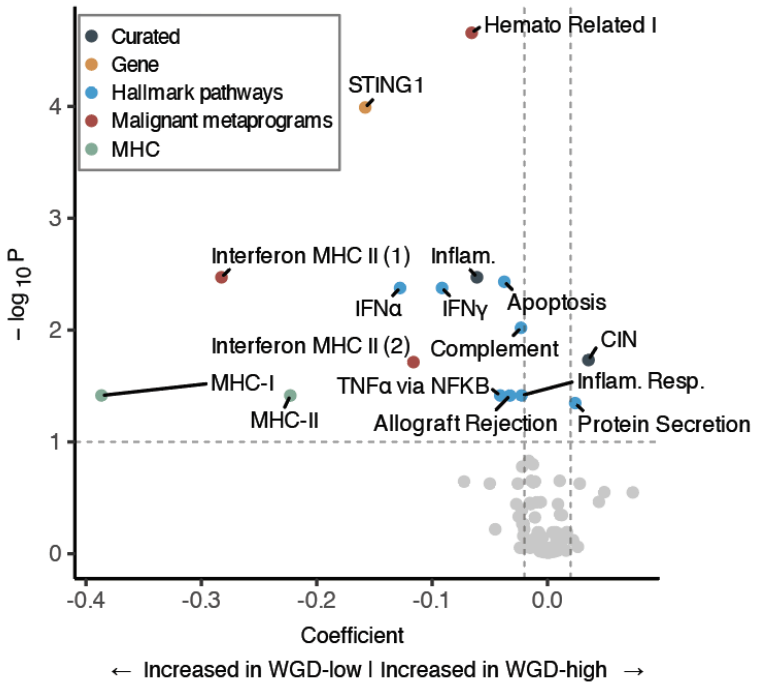
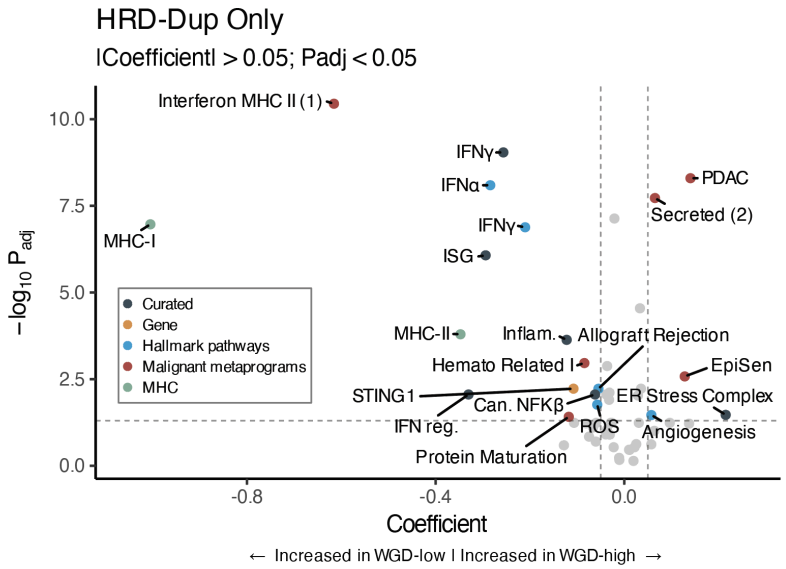


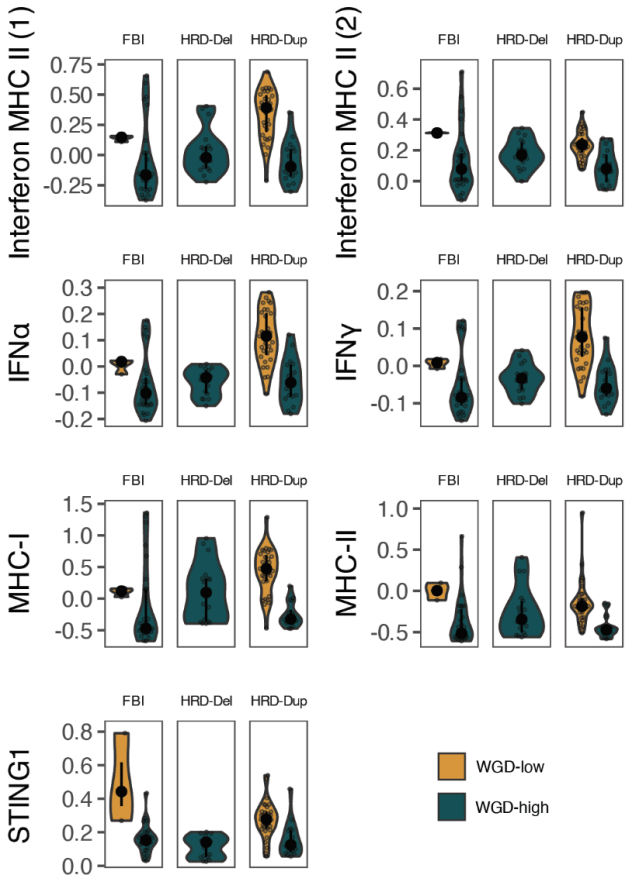
Figure 6A: Scatter plot depicting regression coefficients (x-axis) and significance (y-axis) for selected genes and pathways in WGD-high vs WGD-low tumor cells.



Extended Data Figure 6A: Scatter plot depicting regression coefficients (x-axis) and significance (y-axis) for selected genes and pathways in WGD-high vs WGD-low tumor cells in the HRD-Dup mutation signature subset.

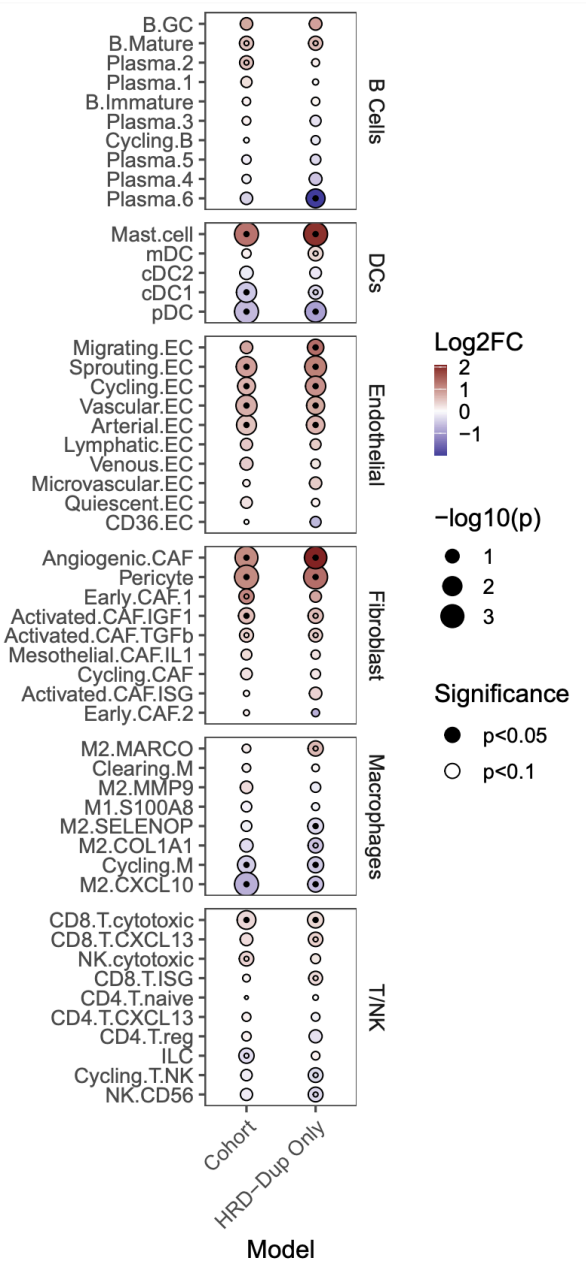
The low number of patients classified as both WGD-low and FBI (2 out of 14 FBI patients) prohibits a robust analysis of the impact WGD has in FBI tumors. Nevertheless, we visualized the difference between WGD-high and WGD-low tumors within FBI for a number of tumor-cell-intrinsic immune-related pathways highlighted in our manuscript (**Extended Data**

Figure 6B), finding that the trend in these patients matched those in HRD-Dup (**Figure 6A**, **Extended Data Figure 6A**). These include decreases in STING1 expression, interferon, and MHC I and II genes.



Extended Data Figure 6B: Violin plots of per sample mean expression for select cancer-cell-intrinsic signaling pathways faceted by mutation signature subset and WGD status.

Finally, regarding microenvironmental composition, we recomputed differential abundance of immune cells between WGD and non-WGD samples (adjusting for anatomic tumor site), for the whole cohort (left) and for HRD-Dup samples only (right). Consistent with our original results, we see a significant enrichment of CXCL10+CD274+ macrophages (M2.CXCL10), IFN-producing plasmacytoid (pDCs), and activated dendritic cells (cDC1) in the microenvironments of WGD-low tumors, whereas WGD-high tumors are characterized by endothelial cells, pericytes and angiogenic CAFs (**Extended Data Figure 6C**).



Extended Data Figure 6C: Dotplot of log2 fold-changes and significance for TME cell type differential abundance testing between WGD-high and WGD-low in the whole cohort and in the HRD-Dup subset.

Taken together, this set of analyses support the findings regarding tumor-cell-intrinsic and microenvironment shifts associated with WGD. However, we do acknowledge that additional cases of FBI and HRD-Del tumors without WGD would allow for a more complete understanding of the phenotypic consequences of mutational signatures and WGD in those subtypes. We also note that the previous work (Funnell et al. 2022) referred to by the reviewer was performed mostly on PDX samples and a mixture of triple negative breast cancer and high grade serous ovarian cancers. This study is on HGSOC primary patient material and therefore reflects the state of this disease encountered in the clinic. Regardless, we thank the reviewer for pointing

this out this important concern and we feel that addressing this question has significantly improved the paper and interpretations of the data.

[C2]: Evolutionary histories of WGD clones

[C2a]: The authors reconstructed phylogenetic trees and estimated evolutionary time based on sparse SNV matrices. However, this inference could be affected by several factors. For example: SNVs were detected more sensitively in larger cell populations than in smaller populations. Thus, the length of peripheral branches tended to be underestimated.
L.193-4: Given the fact that the length of peripheral branches is likely to be underestimated, it is unclear to what extent the inference of the WGD timing is valid.

[R2a]: We agree that some of our analysis is subject to (under)sampling bias. This is explicitly acknowledged in the Discussion (Line 410-411):

> *"Within the limitations of cohort size, sampling bias and genomic coverage, the single-cell analysis we present here enables several key under-studied measures in patient samples: ..."*

And is addressed analytically as follows:

To account for the reduced ability to detect SNVs in smaller cell populations, we conducted a downsampling experiment in which we called SNVs from successively lower-coverage clones, estimated the relationship between a clone's haploid coverage and the sensitivity of SNV calling for clonal SNVs using logistic regression, and used this curve to correct branch lengths. This procedure was included in the results presented in the original manuscript, but we have added the curve to **Fig. 2A** and text to the **Supplementary Note** (reproduced below) to clarify.

> *To evaluate the impact of clone size on the number of clone-specific SNVs recovered, we conducted a simulation experiment in which we randomly subsampled cells, reran our mutation calling pipeline for each random subsample, and then evaluated how many SNVs that were clonal in the original sample were recovered in the subsample. Specifically, for 4 patents with the most cells (OV-004, OV-009, OV-022, and OV-026), we performed the following:*
>
> 1. *Randomly sample [20, 40, 80, 120, 160, 300, 500, 750, 1000] cells 20 times each.*
> 2. *For each sample:*
>   a. *Combine reads from sampled cells into a new BAM file.*
>   b. *Call SNVs using Mutect.*
>   c. *Filter SNVs using ArtiCull.*
>   d. *Measure the proportion of clonal SNVs originally identified in the patient that passed ArtiCull filtering in the new sample.*
>
> *We then computed the haploid coverage for each of these samples, and estimated the relationship between haploid coverage and sensitivity by fitting a logistic curve (**Fig. 2A**). We used this curve to adjust the branch lengths of the doubleTime trees shown in **Fig. 2***

to account for the haploid coverage of each leaf (i.e., by dividing the assigned number of mutations by the expected sensitivity to detect mutations at the corresponding coverage).

We also added the following text to Results to make sure readers are aware that we are performing this correction (Line 185-187):

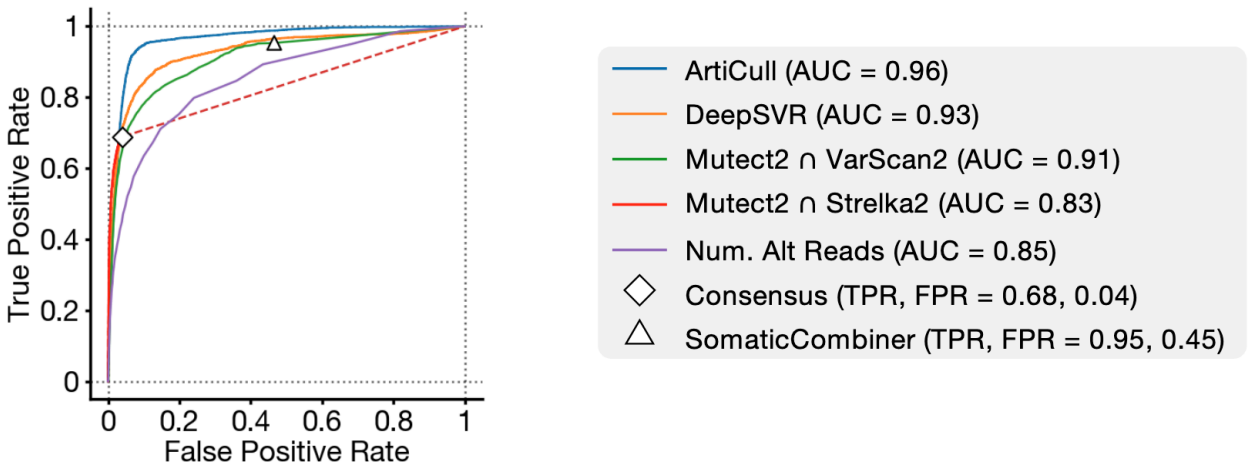
*To control for the effect of small clones on sensitivity to detect mutations, branch lengths were corrected for the total haploid coverage of the corresponding clone (**Supplementary Note**).*

Finally, we are able to relate the WGD timing estimates to orthogonal measurements, providing further evidence of the accuracy of the timing estimates. Specifically, WGD age is associated with the fraction of divergent cells (**Extended Data Fig. 4G**) and the number of accumulated chromosome and arm losses (**Extended Data Fig. 4K**).

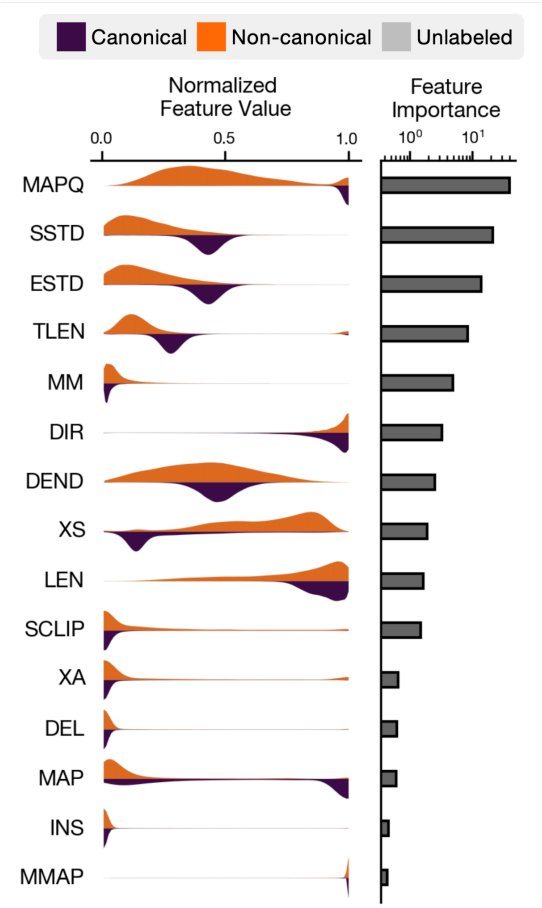
[C2b]: Due to the small insert size will cause false mutation calls will result from incorrect mapping. The authors used a training data set to exclude mutations in subclones across different CNA clones. However, this could not exclude many other artifacts, such as those found across all samples, leading to an overestimation of trunk length. In addition, these artifacts would show lower VAFs, which might have led to an underestimation of WGD timing.

[R2b]: Our group developed a computational method to directly address issues with SNV calling in DLP+ data called ArtiCull (preprint available here: https://www.biorxiv.org/content/10.1101/2025.02.24.639589). The main advance is to leverage the evolutionary structure of the cell populations to identify and remove artifactual mutations. ArtiCull trains a classifier using high-confidence canonical (i.e., conforming to an evolutionary model) and non-canonical SNVs, and then applies this classifier to all SNVs in each dataset. In the ArtiCull preprint, the authors show high precision on a held-out patient (ArtiCull Figure 2F, reproduced as **Rebuttal Fig. 4**) and that non-canonical SNVs are indeed enriched for reads with low mapping quality ("MAPQ" in ArtiCull Figure 2A, reproduced here as **Rebuttal Fig. 5**). For additional details on the method and results, please refer to the preprint. ArtiCull was applied to the combined data from all samples for each patient.

When we inspected the mutations assigned to the trunk, we did not find an enrichment for mutations at low VAFs. For full exposition of this data, we generated patient-specific plots showing the distribution of VAFs for all SNVs assigned to branches of clone trees, faceted by both the branch to which the SNVs were assigned (row) and the clone in which their VAFs are measured (column) (**Supplementary File 2**). In each facet, SNVs are split by the allele-specific copy number of the overlapping region, and vertical lines denote the expected mean of the VAF distribution for each copy-number state. Importantly, low-VAF SNVs assigned to be present across all samples - an indicator of artifacts - are not present in these plots.



Rebuttal Figure 4. Performance of ArtiCull and other mutation callers evaluated on a held-out ovarian cancer sample (OV-022), measured by area under the ROC curve. Experimental details are available in the ArtiCull preprint.



Rebuttal Figure 5. Variant calls from six ovarian and breast cancer samples were used to train an ArtiCull model. Feature distribution and feature importance for labeled canonical (purple) and non-canonical (orange) variants from the training dataset. Feature importance for the trained model is shown alongside the

distribution. Features, from top to bottom in decreasing order of importance, are MAPQ (mean mapping quality), SSTD (standard deviation of start positions), ESTD (standard deviation of end positions), TLEN (mean template length), MM (mean number of mismatches), DIR (bias in directionality), DEND (mean distance of variant to read end), XS (mean alignment score for alternate alignments), LEN (mean read length), SCLIP (proportion of reads with soft-clipped bases), XA (proportion of reads with alternate alignments), DEL (proportion of reads containing a deletion), MAP (mean mappability score in a +/- 300 bp window), and MMAP (proportion of reads with properly mapped mates). Details on these feature definitions are available in Table 1 in the ArtiCull preprint.

[C2c]:- SNVs are shown as a matrix in Fig. 2A but this reviewer wants to see the raw data showing the distribution of SNVs on phylogenetic trees.

[R2c]: Additional visualization of the raw SNV data assigned to phylogenetic trees has been included as **Supplementary File 2**. See above response [R2b] for details.

[C2d]: WGD events in subclones are interesting but have already been reported in a lung cancer study (Nature, 616, 525-533) [https://www.nature.com/articles/s41586-023-05783-5].

[R2d]: We have cited this paper, and do not make claims about being the first to report WGD events in subclones. Our claims are focused on WGD evolution and consequent diversification and are only possible with single-cell approaches.

With the single cell approach we claim the following advances where it is now possible to:

1. Measure multiple WGD states in the same tumor
2. Detect WGD subclones in predominantly diploid samples
3. Detect residual non-WGD cells in predominantly WGD samples which rules out Truncal WGD
4. Identify clones in the same tumor sample that underwent distinct ancestral WGD events
5. Identify cells with rare copy-number profiles (<1-2% of cells)
6. Assign SNVs to specific clones to reason about WGD timing
7. Use cell-specific copy-number changes to estimate rates of aneuploidy-driving processes such as whole-arm and whole-chromosome gains and losses
8. Rather than asking if a tumor is WGD, we can ask how much of the tumor is WGD, and interrogate those specific cells and their subsequent copy number evolution.
9. Map WGD clones in in vitro cell lines to compare expression programs of co-existing 0xWGD and 1xWGD cell populations

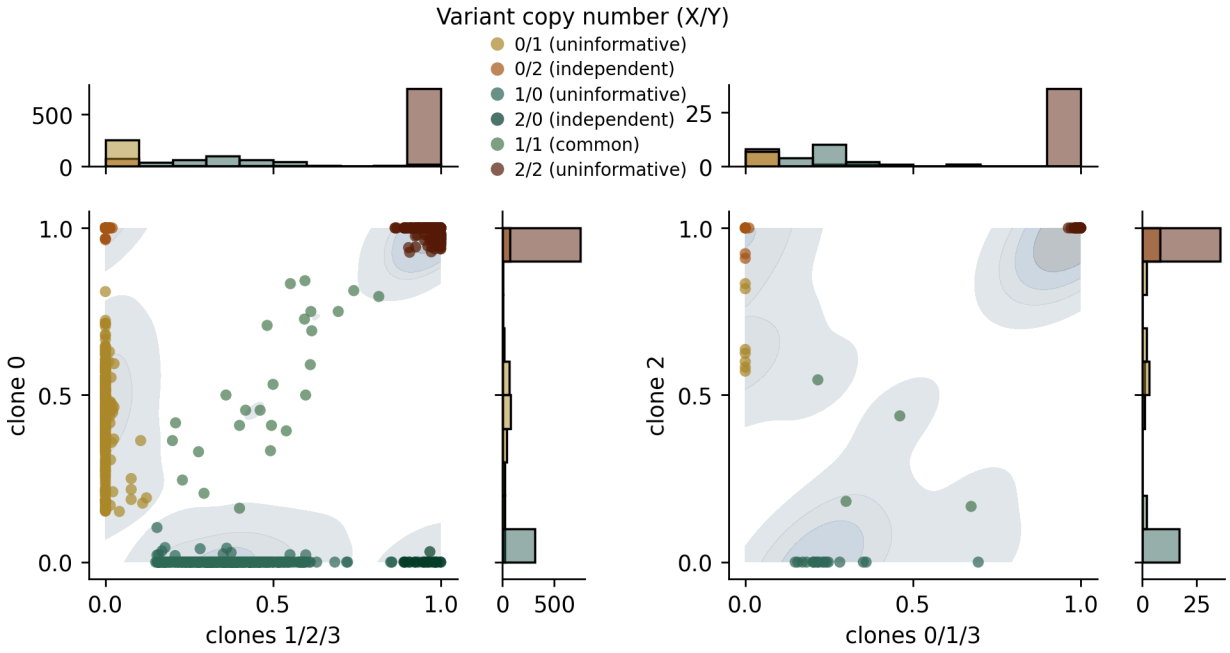
[C2e]: Fig. 2C: The authors speculate that the independent WGD events in OV-025 and OV-045 approximately coincided. However, two of the three WGD events in OV-045 were mapped just after the branch bifurcation and therefore could therefore represent a single event and if so, the timing of this event could be substantially different from that of the remaining WGD event. In addition, judging from the substantially different branch lengths to which two WGD events in OV-025 were mapped, the two WGD events could be substantially separated.

[R2e]: We have adjusted the section on Parallel WGD in the Results section to avoid overly-speculative results. We assert in the paper that the principal support for multiple parallel

WGD events is not branch length (i.e., CpG SNVs assigned to the tree) but rather mutually exclusive 2-variant-copy SNVs in clonal cnLOH regions, including both CpG and nonCpG SNVs. As we describe in Methods section “Discerning parallel from shared WGD,” we assume that SNVs at VAF=1 (i.e., 2 variant copies, genotype 2|0) in these regions were acquired before genome doubling. Thus, if such SNVs are exclusive to one set of clones, this represents evidence that the clones diverged before genome doubling. On the other hand, shared SNVs at VAF=0.5 (i.e., 1 variant copy, genotype 1/1) suggest that genome doubling preceded divergence, with these SNVs having been acquired in the interim. We now make this clearer in **Fig 2A** and **Fig 2C** with improved presentation of the model and visualization and model interpretation of both OV-045 and OV-025 in **Fig 2C**.

In addition, we added analysis showing the support for parallel WGD in patients OV-045 and OV-025 to **Supplementary Note** section 5.2 for the interested reader, reproduced below.

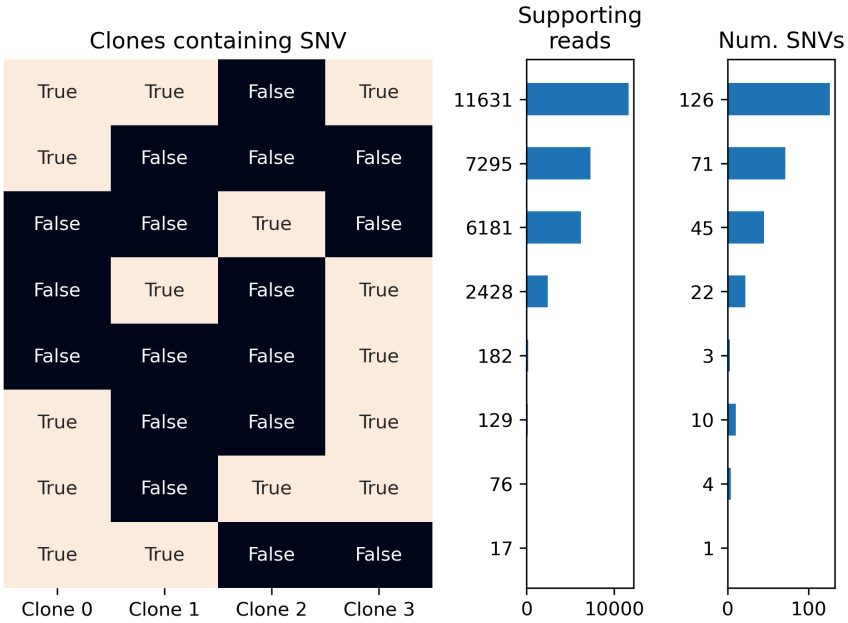
> *In patient OV-045, Clone 0 and Clone 2 were each distinguished from the other clones by a large number of mutually exclusive SNVs in the upper left and bottom right corners (91 and 101 SNVs, respectively), and the small number of SNVs shared at intermediate VAF in the center (54 and 25 SNVs, respectively) were likely affected by subclonal or small undetected copy-number events (**Supplemental Fig. 23**). Support for or against independent WGD depends also on the number of SNV-covering reads for the respective SNV in the relevant clones, which is not shown.*



Supplemental Figure 23. *Pairwise VAFs between parallel WGD events for patient OV-045. Each plot shows a clone inferred to have undergone a unique WGD event on the y-axis, clone 0 (left y-axis) and clone 2 (right y-axis), compared to the remaining 3 clones (x-axis). Each point is an SNV colored by its most likely variant copy number in each partition: mutually exclusive SNVs with 2 variant copies (2/0 or 0/2) support independence, while shared SNVs with 1 variant copy in each partition (1/1) oppose independence. Marginal histograms show total numbers of SNVs. Only those SNVs with at least 10 covering reads in both*

partitions are shown. SNVs that are assigned variant copy numbers 0/0 (absent from both clones) or 2/1 or 1/2 (inconsistent with the simple cnLOH WGD model) are omitted.

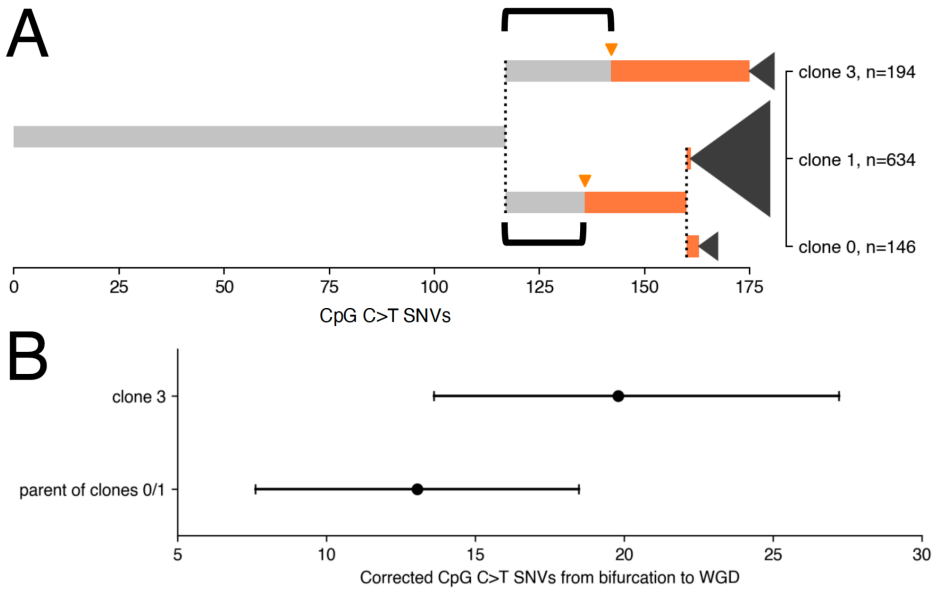
*However, the above analysis overestimated the number of shared SNVs, as it relied on genotyping SNVs in groups of clone(s) rather than individual clones. When we instead examined the 2-variant-copy SNVs at the clone level (**Supplemental Fig. 24**), we found 171 SNVs (supported by 17,812 SNV-covering reads) that distinguished clone 1 from clones 0/2/3 (rows 0 and 2), and 93 SNVs (supported by 9,723 SNV-covering reads) that distinguished clone 0 from clones 1/3 (rows 1 and 3). The lower 4 rows of the table are inconclusive as they include few SNVs and few supporting reads.*



***Supplemental Figure 24.** For patient OV-045, the SNVs present at either 0 or 2 copies for all SBMClone clones are grouped by the subset of clones that harbors them. The left subplot indicates the subset of clones containing each set of SNVs, the left barplot shows the total number of SNVs in the set, and the right barplot shows the supporting reads, i.e., the total number of reads overlapping the SNV positions for all cells. This figure omits the set of 521 SNVs present at 2 copies in all clones with a total of 64339 supporting reads.*

Although we removed the text referring to the simultaneous timing of parallel WGD from the **Results** section, we nevertheless still comment on it in the **Discussion** (lines 435-437). As such, to show that the two WGD events in OV-025 likely have occurred at a similar time, we performed a bootstrap experiment. The time from tumorigenesis to each WGD event is roughly proportional to the number of C>T CpG SNVs that accumulated before the WGD event. (For simplicity, the remainder of this paragraph refers to C>T CpG SNVs simply as “SNVs.”) The two WGD events in OV-025 are assigned to different branches immediately following a bifurcation in the tree, so they share the SNVs on the trunk but do not share the SNVs assigned to the pre-WGD sections of their respective branches (**Rebuttal Fig. 5A**). To compare this latter quantity between the WGD events, we performed a bootstrap estimate by resampling the number of pre-WGD SNVs from the total SNVs assigned to each post-bifurcation branch, with

probability equal to the proportion of SNVs assigned to the pre-WGD section of the branch. After normalizing the sampled counts on each branch for sensitivity (using the total coverage of the descendant clones as described in lines 185-187 and **Supplementary Note** section 8.1), we found that the resulting 95% confidence intervals for WGD timing were highly overlapping for the two branches, and thus we have insufficient statistical support for the two WGD events occurring at different times (**Rebuttal Fig. 5B**).



Rebuttal Figure 5. A. CpG C>T SNV counts on doubleTime tree for patient OV-025. Brackets indicate the SNV quantities being compared in panel B. **B.** Bootstrap intervals for the number of CpG C>T SNVs (y-axis) between the bifurcation in the tree and each of the WGD events (x-axis).

[C3]: Post WGD genomic diversification

-Interpretation of 'divergent cells'

[C3a]: L228-230: It is unclear in what respect these divergent HGSOC cells resemble the 'hopeful monsters' of ref #21. It is still premature to speculate that divergent cells are unstable because these near-divergent cells explain substantial fractions of the tumor cell population, as much as 20% of the population (Fig. 3D), suggesting that at least some of these divergent cells may be more stable than the authors speculate (L.240).

[R3a]: We think that the comparison between divergent cells and the “hopeful monsters” of Bollen et al. (ref #21) is apt. In ref#21, the authors state:

> “Extreme cases were represented by a subset of PDTO-19b cells with highly aberrant karyotypes that could be classified as hopeful monsters, defined as a genome-wide set of changes in ploidy that are likely to substantially alter fitness.”

We believe that the substantial arm- and chromosome-level differences between divergent cells and their nearest neighbors, coupled with increased rates of nullisomy, represent a

"genome-wide set of changes in ploidy that are likely to substantially alter fitness." We have modified the text to more clearly describe in what respect these cells resemble the "hopeful monsters", highlighting their unique profiles and increased nullisomy.

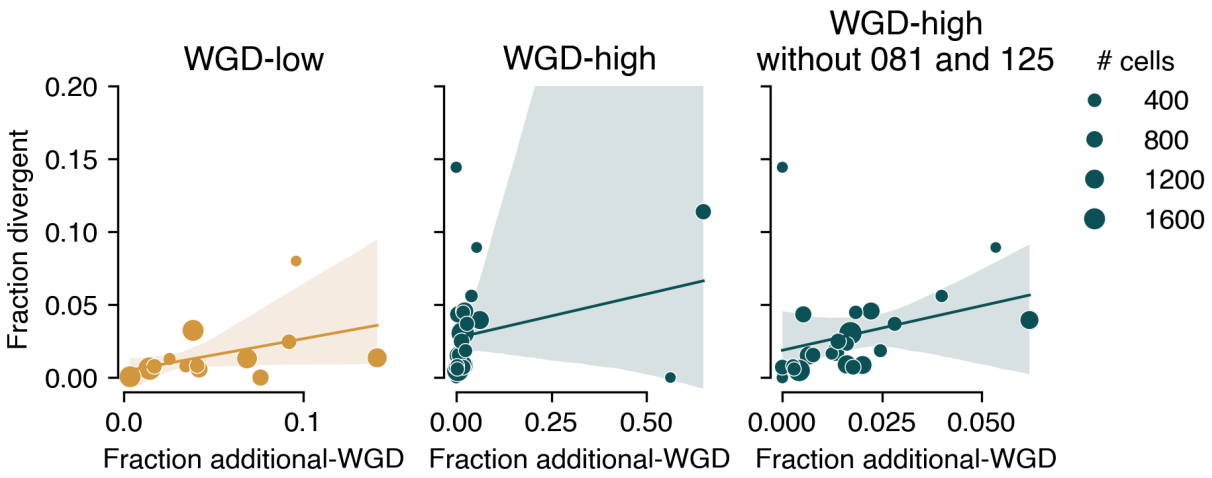
In addition, we believe our use of the term 'unstable' in the initial submission was confusing and imprecise. We no longer describe these cells as unstable, since, as the reviewer correctly suggests, these cells could be persisting in the population even if they are not undergoing successful cell divisions.

[C3b]: To clarify this point, cell fate tracking, even in in vitro cultures of primary HGSOC cells, would be needed to determine whether divergent cells are being newly generated while those of previous generations are disappearing, or whether they are being stably maintained in the tumor population.

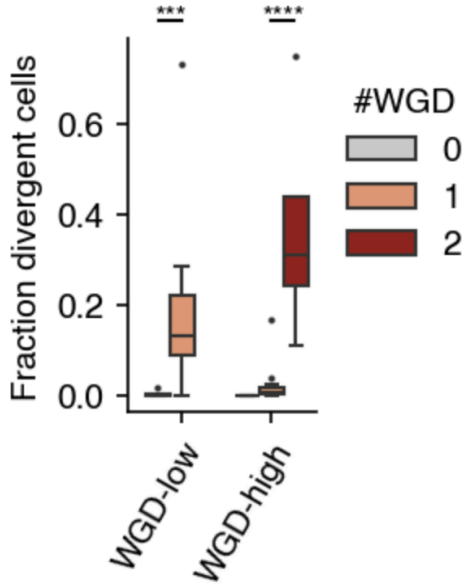
[R3b]: We agree this would be interesting, but it is outside of the scope of this manuscript. Instead we have attempted to understand the fate of divergent cells through analysis of the evolutionary histories of each patient. Specifically, we analyzed examples of WGD subclones as presented in **Fig. 4**, and calculated the number of clone-specific post-WGD chromosome and arm events (**Fig. 4C-D**). We also similarly calculated the average number of events for divergent cells (now annotated on **Fig. 4C)**. In general, the WGD subclones we identified were not characterized by the large number of post-WGD events that characterize divergent cells, suggesting that clonal outgrowth is more likely to occur for WGD subclones with more moderate amounts of post-WGD copy number change.

[C3c]: It is important to show a significant correlation between the fraction of WGD cells and that of divergent cells directly, rather than comparing Prevalent and Rare WGD tumors, because WGD cells are rarely seen in the latter tumors.

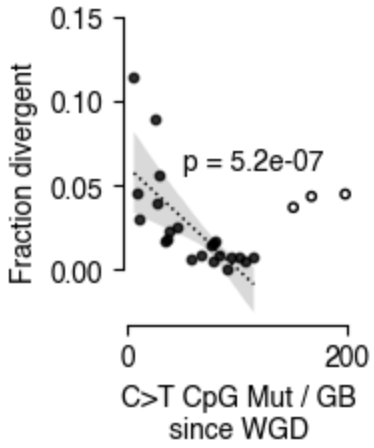
[R3c]: If we understand the reviewer correctly, they are suggesting that we show more directly that divergent cells are the product of high levels of post-WGD CIN. If this hypothesis is true then there should be a correlation between divergent cell fraction and additional-WGD fraction (1xWGD in WGD-low and 2xWGD in WGD-high, previously "subclonal WGD fraction") since divergent cells should also be additional-WGD cells. We do indeed see a weak correlation between fraction divergent and fraction additional-WGD (**Rebuttal Figure 6**). However, we believe a better way of showing the relationship is to compare the divergent cell fraction between majority-WGD and additional-WGD populations as now shown in **Extended Data Fig. 4F** (reproduced below). As shown in the figure, an appreciable fraction of divergent cells are also additional-WGD cells. We now also show the negative correlation between divergent cell fraction and age of clonal WGD for WGD-high tumors in **Extended Data Fig. 4G** (reproduced below), providing further evidence of the association between WGD events and the observed divergent cells.



Rebuttal Figure 6: Fraction of cells that are additional-WGD (x-axis) versus divergent (y-axis) for all WGD-low tumors (left), all WGD-high tumors (center), and all WGD-high tumors excluding patients 081 and 125 (right).



Extended Data Figure 4F. Boxplots comparing fraction of divergent cells between #WGD subpopulations for WGD-low and WGD-high tumors. Mann-Whitney U test significance is annotated as 'ns': 5.0×10-2 < p <= 1.0, '*': 1.0×10-2 < p <= 5.0×10-2, '**': 1.0×10-3 < p <= 1.0×10-2, '***': 1.0×10-4 < p <= 1.0×10-3, '****': p <= 1.0×10-4.



Extended Data Figure 4G. Fraction divergent cells (y-axis) by age of the WGD as measured by C>T CpG mutations gained since WGD (x-axis). Shown is the p-value of a spearman correlation after removing the three patients with the oldest WGDs. Spearman correlation retaining these outliers is ρ=-0.47 p=0.019.

- Inference of rates of cell-specific copy-number changes

[C3d]: The authors calculated the copy number alterations accumulated since the immediate ancestor in a phylogenetic tree and showed an increase between 0xWGD and 1xWGD and 1xWGD and 2xWGD. However, is there a possibility that this step-up could be largely explained by the copy-number changes associated with WGD events? To clarify this, Figure 3F and Figure 4G should be plotted for branches within 0xWGD, 1xWGD, and 2xWGD, and for those between 0xWGD and 0xWGD, and 1xWGD and 2xWGD.

[R3d]: We thank the reviewer for this suggestion. We assume that the reviewer has made a minor typo and meant to say "To clarify this, Figure 3F and Figure 4G should be plotted for branches within 0xWGD, 1xWGD, and 2xWGD, and for those between 0xWGD and ***1xWGD***, and 1xWGD and 2xWGD." The reviewer raises an interesting question regarding the distinction between WGD-associated changes and further changes affecting cells with WGD in their evolutionary history. We do not believe our cohort is powered to answer these questions in small WGD subpopulations, and have refocused our description of the results.

We now highlight the differences between established 0xWGD and 1xWGD clones (WGD-low 0xWGD and WGD-high 1xWGD). When comparing these populations we see an increase in cell-specific copy-number event rates in 1xWGD clones (**Fig. 3F**, reproduced below), even after normalizing for increased ploidy. WGD-associated events are common to the cells in 1xWGD clones and thus would not be included in the cell-specific rates. Thus the increase in ploidy normalized cell-specific rates for 1xWGD clones suggests an increase in instability that is not simply the result of chaotic mitosis immediately post-WGD and not the result of having a larger genome. Instead these rate increases represent a sustained increase in instability for WGD populations. Note that we now only show the rates normalized for increased ploidy and have removed the unnormalized rates figure.

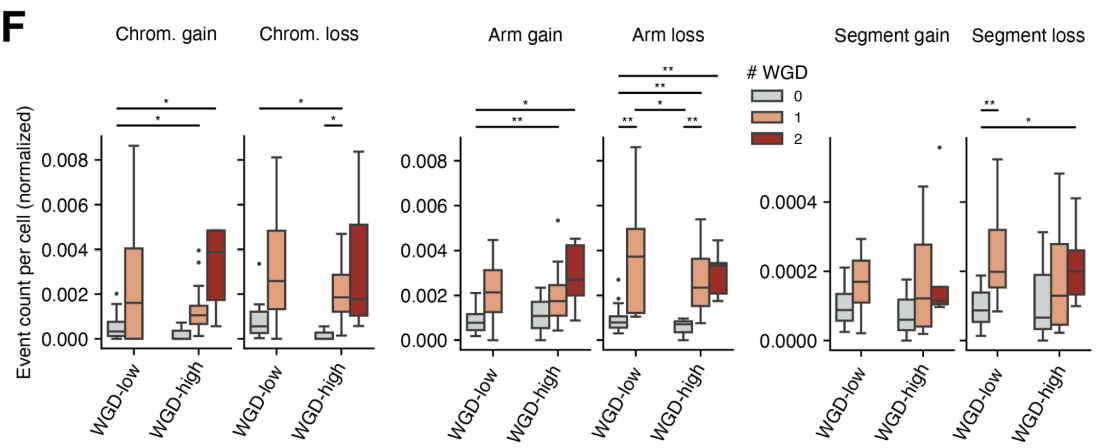


Figure 3F. Event counts per cell for loss and gain of chromosomes, arms, and large segments, split by #WGD state and WGD-high vs WGD-low tumor status. Counts shown are normalized to account for ploidy differences: chromosome rates are calculated as counts per cell per ancestral chromosome number, arm rates are calculated as counts per cell per ancestral arm number, and segment rates are calculated as counts per cell per GB of ancestral genome. Mann-Whitney U test significance (FDR corrected) is annotated as '*': $1.0\times10\text{-}2 < p \leq 5.0\times10\text{-}2$, '**': $1.0\times10\text{-}3 < p \leq 1.0\times10\text{-}2$, '***': $1.0\times10\text{-}4 < p \leq 1.0\times10\text{-}3$, '****': $p \leq 1.0\times10\text{-}4$. Only significant comparisons are shown.

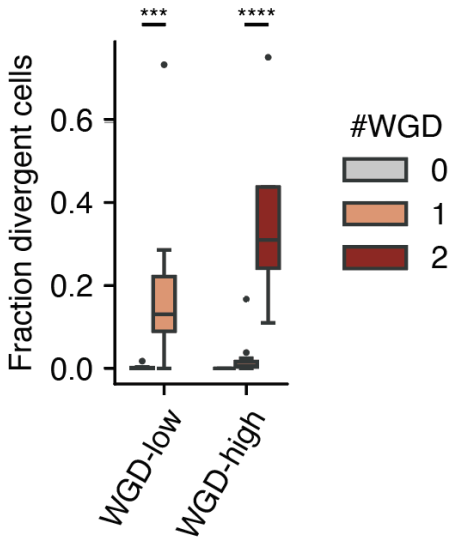
[C3e]: In addition, many cells that acquired more chromosomal changes died and were not observed. Thus, the inferred number may depend more on the selective process/pressure than on the actual rate of copy-number changes.

[R3e]: The reviewers raise an important point. We agree that cells that do not propagate as a result of high levels of chromosomal instability are unobservable in our study. As such, we have added a discussion point as follows (Line 415-419):

> *"We note that since we are sequencing cells in patient tumors, we are still measuring a product of CNA and selection. That we observe cells with considerable homozygous deletions (or nullisomy) suggests we can capture some part of the non-viable population. Future studies in controlled systems that quantify and model cell death could further enhance our understanding of the relationship between WGD, cellular fitness, and clonal expansion."*

[C3f]: Finally, the difference in copy number losses in 1x WGD cells between Rare vs. Prevalent WGD is marginal (p=0.016). Given the multiple testing, the difference may not be real.

[R3f:] As described above, we have reworked **Figure 3F** to highlight only those comparisons that are significant following FDR multiple testing correction. We removed comparison of 1xWGD populations between WGD-low and WGD-high (formerly Rare and Prevalent WGD, respectively) for two reasons. First we agree the statistical significance may have been marginal and underpowered. Second, while our original aim was to explain the increased diversity in additional-WGD populations, we now understand this increased diversity to be the result of divergent cells, which are predominantly additional-WGD (see **Extended Data Fig. 4F**).



Extended Data Fig. 4F: Boxplots comparing fraction of divergent cells between #WGD subpopulations for WGD-low and WGD-high tumors.

[C4]: Evolutionary dynamics of WGD and non-WGD clones

[C4a]: The descriptions of copy number abnormalities assigned to ancestral branches in the main text and Method are inconsistent and confusing.

We have updated the relevant sections of the Results (*Evolutionary dynamics of WGD and non-WGD clones*, Line 288-317) and Methods (*Reconstruction of ancestral copy number*, L1158-1172, and *Enumerating events on ancestral branches*, Line 1209-1215) to improve clarity. In addition, we improved **Fig. 4** with clear diagrams and terms, which we also included in the Glossary of Terms (**Supplementary Note** section 1) to aid the reader.

[C4b]: Fig. 4B: Pre-WGD and non-WGD should be shown separately. Again, the difference may simply represent the copy-number changes that occurred at the time of the WGD.

[R4b]: We adjusted **Fig. 4B** (reproduced below) to show pre-WGD and non-WGD separately as suggested. This indicates chromosome loss and arm loss rates as statistically higher in post-WGD relative to both pre-WGD and non-WGD. In addition, we modified the sentences describing these results (Line 290-296) as follows:

> *We first focused on events predicted to be on the ancestral branches of each patient. These events were divided into those inferred to occur (i) after WGD in the ancestral branches of WGD-high tumors (post-WGD) (ii) before WGD in ancestral branches of WGD-high tumors (pre-WGD) or (iii) on the ancestral branches of WGD-low tumors (non-WGD). We found that gains of chromosomes and arms were rare in general, though chromosome gains were significantly more numerous post-WGD compared to pre-WGD, similar to previous results (Baker et al. 2024) (**Fig. 4B**)*

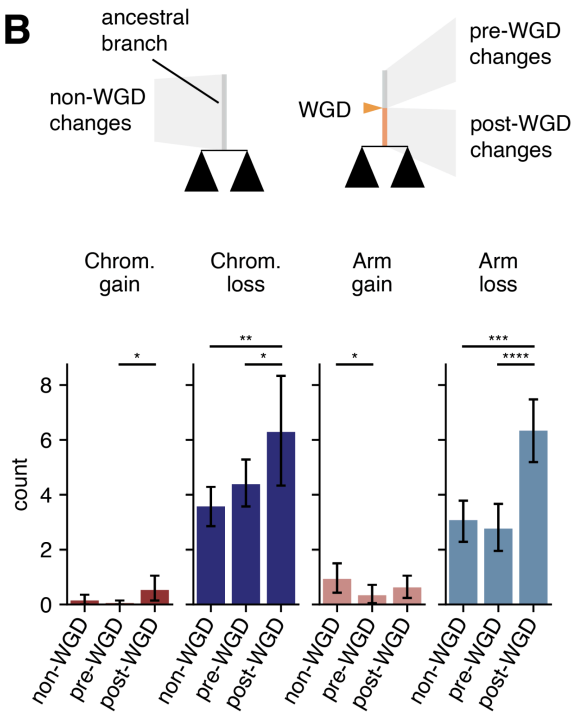


Figure 4B: Counts of ancestral arm and chromosome events detected across the cohort for non-WGD ancestral branches of WGD-low tumors and pre- and post-WGD branches for WGD-high tumors. Each bar and 95% confidence interval shows the distribution of counts on the given type of branch. Mann-Whitney U test significance (FDR corrected) is annotated as '*': 1.0×10-2 < p ≤ 5.0×10-2, '**': 1.0×10-3 < p ≤ 1.0×10-2, '***': 1.0×10-4 < p ≤ 1.0×10-3, '****': p ≤ 1.0×10-4. Only significant comparisons are shown.

[C5]: WGD-specific cell intrinsic and tumor microenvironmental phenotypes

[C5a]: In their previous study, the authors demonstrated HRD-Dup tumors retained inflammatory response, whereas in FBI tumors show increased TGFb signaling and immune evasion. Thus, it is unclear whether the difference in immune response between Prevalent WGD and Rare WGD groups was due to WGD or the difference in the underlying mutational process.

[R5a]: We thank the reviewer for pointing this out. Please refer to the response above regarding the co-variation with WGD and mutational signature. This is detailed in the section entitled '**HRD-Dup subset analysis'.** We agree this was an important omission in the previous version of the study. The new results reflect that within the HRD-Dup subset of patients, the inflammatory signalling is likely reduced in the WGD-high population (see **Extended Data Fig. 6**). We modified the text to include this important analysis in the relevant sections (Line 340-344, Line 388-392):

> *"Cells in WGD-high tumors showed a significant decrease in Type I (IFN-α/IFN-β) and Type II (IFN-γ) interferon, inflammatory pathways, and TNFa/NF-κB signaling, both cohort wide (**Fig. 6A**) and within the HRD-Dup subset (**Extended Data Fig. 6A**). FBI tumors showed similar trends (**Extended Data Fig. 6B**), suggesting that the impact of WGD on immuno-phenotypic signaling may be independent of mutation signature."*

> *"Consistent with the increased expression of IFN-stimulated genes (ISGs) observed in the cancer cells of WGD-low tumors, we observed enrichment of CXCL10+CD274+ macrophages (M2.CXCL10), IFN-producing plasmacytoid (pDCs), and activated dendritic cells (cDC1) in the microenvironments of WGD-low tumors in both cohort-wide (**Fig. 6H-I**) and HRD-Dup-specific analysis (**Extended Data Fig. 6C**)."*

We speculate this has implications for determining how mutational processes impact anti-tumor immunity and suggests that WGD may induce immunosuppressive phenotypes independently of mutational process. This is further elaborated in the Discussion (Line 463-465):

> *"Intriguingly, the genomic and phenotypic consequences of WGD were evident even within HRD subtypes, indicating the potential for composite biomarkers involving mutational process and WGD to stratify patients."*

[C5b]: It is curious that Prevalent WGD cells showed lower STING expression than Rare WGD cells, even though the former showed more cGAS+ cells in the previous section. The authors speculate that STING transcriptional repression may be a prerequisite for clonal expansion. This is a possible explanation but lacks experimental evidence.

[R5b]: We appreciate further inquiry into this important point. We respond below with i) evidence from the literature from some of our group's recent papers in experimental systems; ii) improved analysis of IF data including cGAS, STING and p53 and iii) additional experiments in FNE-1 cells comparing 0xWGD and 1xWGD STING expression.

Evidence from literature

We appreciate the reviewer's suggestion regarding the correlation between STING expression and the frequency of 1xWGD and 2xWGD cells. We would like to refer the reviewer to our recent findings in Li et al. (Nature 2023), where we observed a dynamic, context-dependent "yin and yang" relationship between cGAS and STING protein levels. Specifically, our study revealed that increased cGAS expression in cells undergoing chromosomal instability, such as WGD-high (formerly Prevalent WGD) cells, often coincides with a compensatory downregulation of STING protein levels, potentially as a mechanism to prevent excessive immune activation.

More broadly, it is important to note that our hypothesis stipulating that reductions in STING-dependent pro-inflammatory signaling may facilitate clonal expansion is based on extensive work our group has conducted demonstrating how chromosomally unstable cancer cells evade STING-dependent immune surveillance.

For instance, we have shown that chromosomal instability (CIN) induces cGAS activation via the accumulation of cytosolic DNA, yet many cancer cells, particularly those with prevalent whole-genome doubling (WGD), develop mechanisms to repress STING signaling to avoid deleterious immune responses (Bakhoum et al. 2018; Li et al. 2021, 2023; J. Zhang et al. 2021; Ritchie and Li 2024; Konno et al. 2018). In this context, we propose that repression of STING in

WGD-high cells is a critical adaptive mechanism, allowing them to bypass the immune surveillance typically induced by cGAS activation.

This evasion of immune surveillance has been further explored in studies from our group and others, demonstrating how chromosomally unstable cells modulate STING expression to prevent immune detection, thus enabling clonal expansion and tumor progression. Importantly this body of published work supports the hypotheses we present from patient samples.

Accordingly we have added the following in the Discussion to point the reader to the corroborating published results (Line 449-451):

> *While WGD was associated with increased cGAS+ ruptured micronuclei, as expected given the higher levels of CIN, WGD-high tumors showed decreased cell-intrinsic and cell-extrinsic interferon signaling, consistent with previous findings on chronic-CIN induced immunosuppression (Li et al., Nature 2023).*

Improved IF analysis

As noted in the manuscript, our IF data further support these findings, showing higher cGAS protein activity in WGD-high cells. New IF data generated for the revised manuscript show a decrease in STING protein measured for WGD-high cells, providing orthogonal validation of the STING expression data (**Fig 6E,F**). The balance between cGAS and STING is crucial in the context of chromosomal instability, and further underscores the adaptive responses these cells use to evade immune surveillance. In this contribution we show this association as a function of WGD.

New FNE-1 in vitro data

In addition to our previous published work, in this revision we also added further *in vitro* association in fallopian tube epithelium FNE-1 cells, where following TP53 knock out we observed a spontaneously arising WGD populations leading to a mixture of 0xWGD and 1xWGD cells. We performed DLP+ and scRNA on the mixed population and found that the 1xWGD population indeed showed a reduction in *STING1* in WGD cells. This is presented in **Extended Data Fig. 7** and elaborated in responses below.

[C5c]: It is important to evaluate STING expression in 1xWGD cells between Prevalent and Rare WGD cells.

[R5c]: Unfortunately, we are unaware of any reliable method to infer ploidy from scRNA without specific distinguishing CNAs, so we cannot identify 1xWGD cells in WGD-low tumors to perform this comparison. However, we are able to compare STING expression between 0xWGD and 1xWGD in the *in vitro* systems we studied, and were able to confirm lower STING expression in 1xWGD as shown in **Extended Data Fig. 7G** reproduced below in response to your later comment.

[C5d]: Did the STING expression correlate with the frequency of 1xWGD and 2xWGD cells? This should be evaluated in multivariable analysis. Please show the data showing higher cGAS expression in Prevalent WGD cells in scRNA seq as well.

[R5d]: We do not observe a correlation between STING expression and the frequency of 1xWGD and 2xWGD subpopulations in WGD-low and WGD-high tumors. We think it likely that we would need to sequence an order of magnitude more cells by DLP+ in order to see such a relationship given the low prevalence of additional-WGD subpopulations.

Regarding cGAS expression, it is important to note that cGAS activity is primarily regulated at the post-translational level rather than through changes in transcription (Yu and Liu 2021). cGAS is constitutively expressed and becomes functionally active upon binding to double-stranded DNA—such as in ruptured micronuclei—leading to phase separation and cytoplasmic puncta formation. This feature of cGAS is why we can utilize cGAS spatial localization to examine MN rupture rates. We did confirm that cGAS was expressed in all patient samples in which we have transcriptomic data, and we do not find variation in expression coincident with WGD status. We do, however, find evidence of WGD-related variation in cGAS activity in the form of increased rate of MN rupture in WGD-high (**Fig. 3I,J**, reproduced below). In order to examine cGAS/STING pathway activity on a transcriptional level we focused on assessing the downstream transcriptional interferon signalling response.

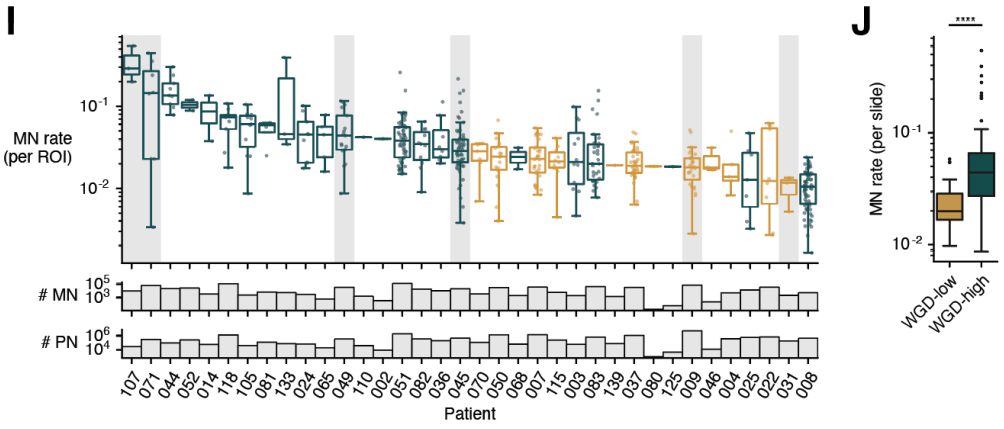


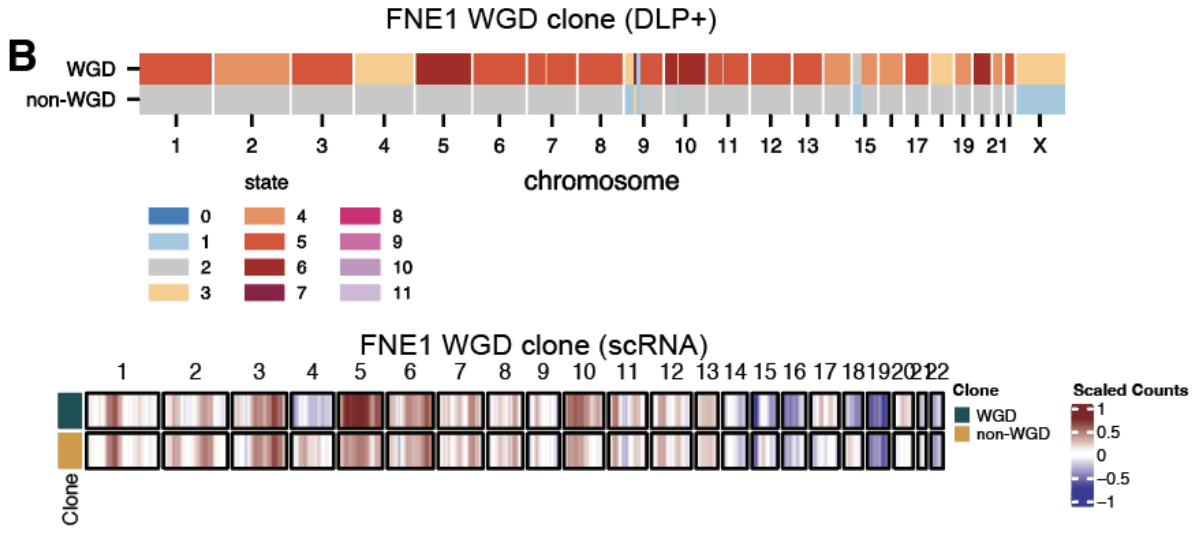
Figure 3. I. Box plot showing MN rates per slide across all samples from each patient, colored by WGD-high vs WGD-low tumor status. Each point is a sample. Bar plots below the box plots show the total number of cGAS+ MN across all tumor ROIs (upper) and the total number of PN across all tumor ROIs (lower). **J.** MN rate per slide, grouped by WGD-high vs WGD-low tumor status. Significance of WGD-high vs. low calculated using a GEE model with patients as groups is annotated as '****': $p \leq 1.0 \times 10\text{-}4$.

[C5e]: In Ext Fig 6L, low STING expression in WGD(+) RPE1 cell that appeared in the late passage is claimed to support the presence of immune evasion.

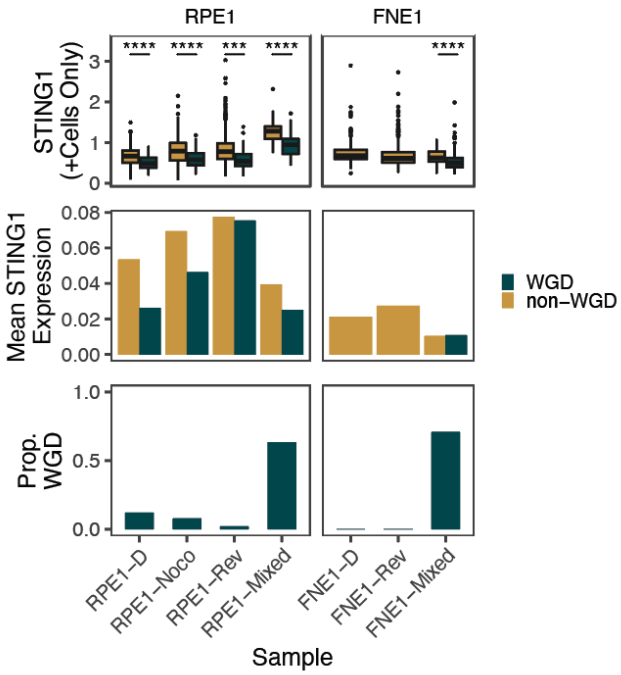
[R5e]: We would like to clarify that while we examined cell-intrinsic STING1 expression in the *in vitro* data with the RPE1 cell line, this data is not used to support claims about the presence of immune evasion. In these experiments, RPE1 cells are not co-cultured or grown in the presence

of an immune selective pressure (see response to comment [C5b] above regarding immune-related claims). Rather, we find that STING expression is down-regulated in WGD RPE1 cells compared to the co-existing non-WGD cells (of the same passage) in a cGAS- and immune-microenvironment-independent manner.

Furthermore, we have replicated the *in vitro* experiments using the immortalized fallopian tube epithelial cell line FNE1. In brief, we generated *TP53-/-* cells and subjected cells to reversine to induce chromosomal missegregation, sequencing both DMSO control and reversine-treated cells using 10x scRNA and DLP+. In addition, we propagated the TP53-/- FNE1 cells and monitored for the existence of WGD clonal outgrowth by DNA FISH, sequencing the resulting mixed population using 10x scRNA and DLP+. As in the RPE1 data, we found a 1xWGD clone in DLP+ with characteristic copy-number aberrations which we were able to detect in scRNA-seq (**Extended Data Fig. 7B,** reproduced below). As seen in the RPE-1 context, expression of STING1 was decreased in the 1xWGD population (**Extended Data Fig. 7G** (right)**,** reproduced below). Taken together, these results suggest an association between WGD and cell-intrinsic suppression of STING1 in two distinct cell line systems.



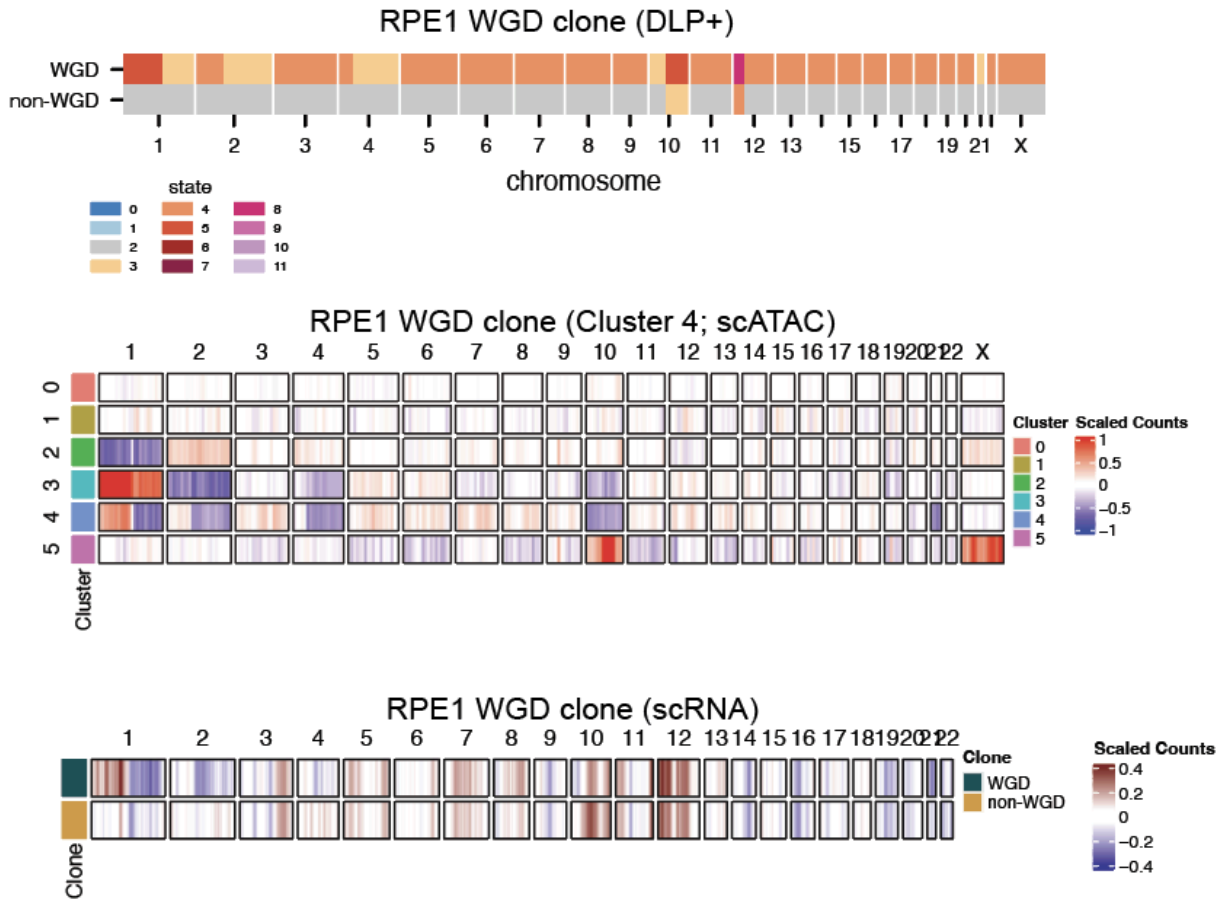
Extended Data Fig. 7B: Clone copy number inferred from DLP (top) and scRNA-seq (bottom) for FNE1 cells. Two clones were identified in both modalities: one WGD and one non-WGD.



Extended Data Fig. 7G: STING1 expression in STING1 positive cells (top), mean STING1 expression (middle), and proportion of WGD cells (bottom) for non-WGD and WGD RPE1 (left) and FNE1 (right) cells by treatment condition.

[C5f]: However, STING expression should be compared with WGD-negative cells in the same passage.

[R5f]: We did indeed compare STING1 expression between WGD and non-WGD cells in the same passage. For these experiments, we had serially passaged p53-deficient RPE1 cells and monitored for emergence of WGD cells every 5 passages. We deliberately chose a passage for analysis in which approximately 50% of the cells were WGD and 50% of the cells were diploid. Using DLP+ we were able to characterize the copy-number profile of a WGD subclone that included a large clone-specific copy-number alteration on chromosome 1 **(Extended Data Fig. 7A**). That same copy number alteration on chromosome 1 was detectable by inferCNV analysis in the scRNA and scATAC data.



Extended Data Figure 7A: Clone copy number inferred from DLP (top), scATAC-seq (middle), and scRNA-seq (bottom) for RPE1 cells across treatment conditions. Two clones were identified in all modalities: one WGD and one non-WGD.

We clustered the inferCNV data and labelled clusters as WGD or non-WGD based on the presence of the chromosome 1 change, and performed differential analysis between WGD and non-WGD cells. Differential expression between the WGD and non-WGD cells found decreased STING in WGD cells, which is presented in **Extended Data Fig. 7G** (reproduced above). We have updated the figure legend for this panel, which was not sufficiently clear and may have led to the misunderstanding.

We also repeated this experiment using the immortalized fallopian tube epithelial cell line FNE1 (see response to above comment [C5e]).

[C6]: Discussion

The following discussions are not fully supported by data.

[C6a]: L397: "These findings suggest that the evolutionary history of WGD in HGSOC is characterized by the rapid expansion of WGD clones, likely driven by changes in the fitness

landscape that favor their proliferation." A long trunk in many tumors rather indicate that the evolution of WGD clones seems to be rather a slow process?

[R6a]: Yes, a long trunk can indicate that tumorigenesis is a slow process. This process likely involves one or more clonal expansions that purify the tumor cell population for cells that share the mutations identified as truncal in our data. What we were referring to in the discussion is the speed with which WGD clones outcompete non-WGD clones and come to be the dominant WGD state in the tumor. The evidence suggesting a rapid expansion of WGD clones is that we observe very few partial expansions: in our cohort of 41 patients, we observe only 2 patients (OV-081 and OV-125) in which at least 15% of tumor cells are WGD and at least 9% of tumor cells are diploid. Even for patient OV-081, one sample consists of 93.1% WGD cells and the other has <1% WGD cells. We therefore speculate the lack of partially completed clonal expansions is consistent with rapid rather than gradual expansions. This is indeed an interpretation and is left to the discussion and not the main results. However, because we do not have direct measurements of this time e.g. via longitudinal samples, we removed the word "rapid" from this sentence in the discussion. We now state the following in the Discussion (Line 435-442):

> *"Intriguingly, in tumors where we observed parallel WGD events, these events occurred at approximately the same time in the evolutionary history of the tumor. This suggests that a WGD-permissive state may have resulted from a cell-extrinsic shift in these tumors, enabling simultaneous expansion of distinct WGD subclones. WGD clones in the remaining tumors were either small or undetectable despite the presence of small fractions of WGD cells generated by ongoing WGD, indicative of a lack of WGD favourable context in those tumors. These observations raise the question of which cell-intrinsic and microenvironmental factors modulate selection of WGD in HGSOC."*

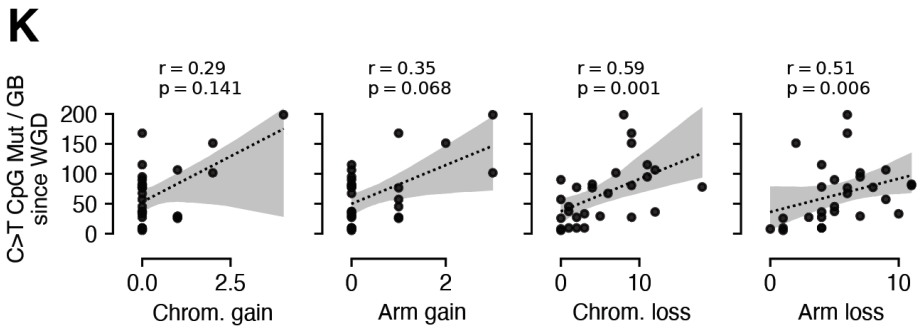
[C6b]: L403: "Evolutionary analysis of our data indicates that gradual losses, rather than punctuated evolution, shape the post-WGD evolution of many WGD clones, suggesting historical adaptation and tolerance for the high CIN levels associated with WGD." The basis of this statement is unclear, despite the descriptions in L294-325. More explicit explanation is needed why the results in the first and second paragraphs in page 10-11 can support this statement.

[R6b]: We have improved the section titled "Evolutionary dynamics of WGD and non-WGD clones". To summarize, two results lead us to conclude that, as we now say in lines 314-317:

> *"These results suggest a fitness model whereby WGD cells are more likely to expand if they are able to avoid the large-scale punctuated changes evident in divergent cells. Under this model, the numerous ancestral post-WGD losses found in truncal WGD clones are more likely to have resulted from gradual genomic attrition following WGD, rather than from catastrophic mitoses post-WGD."*

First, we see a positive correlation between WGD age and the accumulation of post-WGD losses (**Extended Data Fig. 4K**). Second, the WGD subclones we identified have a more moderate amount of post-WGD CNA compared to established clones with truncal WGD or the

examples of individual WGD cells with highly divergent genomes (**Fig. 4C**). The second part of the statement regarding historical adaptation is indeed conjecture, hence its placement in the discussion. Since our results and others suggest that WGD is associated with CIN, and this CIN is ongoing, we do not think it unreasonable to assume that truncal WGD were no different, and that in order for those truncal WGD to have produced a viable tumor, those WGD tumor cells would have had to have adapted to the ongoing CIN that arose post-WGD.



Extended Data Figure 4K: Number of post-WGD chromosome and arm gains and losses (x-axis) compared to the mutation time in C>T CpG counts (y-axis) measured since the WGD event. Spearman correlation coefficients and p-values are shown.

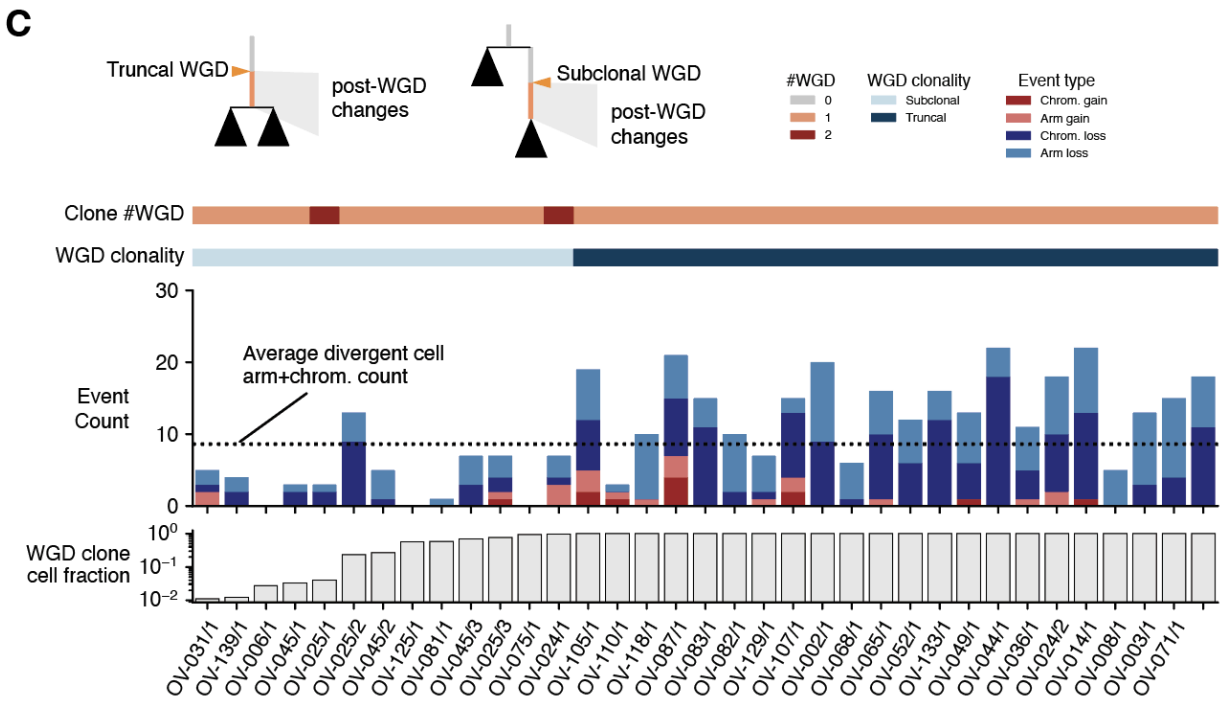


Figure 4C: Bar plots show counts of arm and chromosome events occurring post-WGD for all high-confidence clonal and subclonal WGD events detected across the cohort, split by clonality of the WGD (cell fraction threshold 0.99). Each bar and 95% confidence interval shows the distribution of counts on the root branch of the given type of WGD. Each bar indicates a clone which is labeled below, and annotated above with the number of WGD events ancestral to the clone as well as its clonality. The lower bar plots show the fraction of cells from each patient that the clone represents.

[C7] Minor issues:

There are many mis-citations. These are some examples:
[C7a]: Fig. 1D and 1E are mis-cited and so are Ext Figs. 3H and I (-> 2H and 2I; L878).

[R7a]: All callouts have been reviewed and updated with the new set of figures. Apologies for the errors in the original submission.

[C7b]: Fig2-F: with no explanation for this, the authors' claim here is unknown.

[R7b]: **Fig. 2F** was previously referenced in L206, and has now been removed.

[C7c]: L944-948: 2 mutant copies in both clones (post-WGD and post-divergence) -> mutant copies in one clone (pre-WGD and post-divergence)?

[R7c]: We have corrected this typo.

[C7d]: ED Fig. 3D is mis-cited (L267).

[R7d]: We believe that the callout to **Extended Data Fig. 3D** was correct in the original version (now line 218 referring to **Extended Data Fig. 3C**).

[C7e]: ED Figs. 2O: no legend for colors used.

[R7e]: We have added a legend for WGD status to **Extended Data Fig. 2O**.

[C7f]: L318: ED Fig. 4J -> 4I

[R7f]: The callout now refers to **Extended Data Fig. 4K** (not 4J) and has been corrected in the text.

[C7g]: L361 Ext Fig. G -> ED FIg. 6G. Lack of explanation of colors in Figs. 6C, D.

[R7g]: The figure callout (now **Extended Data Fig. 7G**) has been corrected in the text, and a description of the colors for **Fig. 6C,D** has been added to the legend.

[C7h]: ED Fig. 4B right panel: it does not look like 1 x WGD but near tetraploid.

[R7h]: We assume the reviewer is referring to the right panel of **Extended Data Fig. 4A**. The cell shown in this panel has copy number 4 in many regions, which is likely reached via a doubling from copy number 2 (also many regions with copy number 2, likely doubled from copy number 1). Indeed, it has relatively few regions with odd copy numbers. Note that a cell that has a ploidy near 2 (i.e., diploid) will have a ploidy near 4 (i.e., tetraploid) upon a single

whole-genome doubling. To further clarify this for readers, we have added a schematic of the approximate ploidy corresponding to each genome doubling to the right side of **Fig. 1B.**

[C7i]: L316-318: "In clonal WGDs, the number of chromosome losses and arm gains and losses were significantly correlated with the age of the WGD as estimated using mutations (Methods, Extended Data Fig. 4J)." This reviewer failed to identify the relevant description in Method. Does "age of the WGD" mean the number of mutations after WGD?

[R7i]: Yes, this is what "age of the WGD" refers to. We have updated this text (Line 311-314) to read:

> *"For Truncal WGD clones, the number of chromosome and arm losses were significantly correlated with the age of the WGD as measured by the number of C>T CpG mutations occurring from WGD to time of sample collection (**Methods**, **Extended Data Fig. 4K**)."*

We also added "Age of WGD" to the Glossary of Terms (**Supplementary Note**) to clarify this.

Referee #2

Whole-genome doubling is prevalent in human cancers, and has been associated with increased rates of metastasis, drug resistance, and poor patient outcomes. Understanding the dynamics of WGD may shed new light on the mechanisms underpinning it, and, moreover, whether it is selected or not during tumour development.

In this work McPherson et al. harness a unique cohort of high grade serous ovarian cancer samples and 29,481 tumour cell genomes to explore WGD. They delineate three modes of WGD: 1) early fixation event; 2) late fixation of parallel WGD; 3) emergence of late WGD clones.

Despite its prevalence and links to poor outcome, a deep understanding of WGD dynamics during cancer evolution is lacking. While I was impressed by the work, I think there is scope for improvement (further details below). Key aspects for improvement include: a) why single-cell sequencing is required (e.g. what cannot be inferred with previous approaches vs. with single-cell); b) how many of the results are a result of WGD vs. increased chromosomal instability; c) further demonstration that the WGD calls are accurate; cd this is a dense manuscript, with a lot of information – some of the terminology chosen adds to the confusion.

We thank the reviewer for a careful reading and for their interest in the findings presented. We also appreciate the high level major points - which we respond to at a high level here with details to follow in the point-by-point below.

> a) Why single cell: We note that many of our findings are derived from cell-level features of the genomes - including divergent cells, missegregation rate estimates, analysis of

residual diploid populations, and parallel WGD. None of these results are possible in bulk sequencing. This is elaborated in the discussion.
b) WGD vs CIN: this is improved in **Figure 4** in discussing evolutionary impacts of WGD and is further emphasized in cGAS-STING analysis which shows that WGD decouples the expected association between CIN and STING expression.
c) WGD calls- we added additional analyses supporting the calls and further exposed details of the methods, including limitations; d) we have greatly streamlined terminology including a new Glossary of Terms (**Supplementary Note** section 1) that has improved the readability.

Comments:

[C1]: It would be useful to have an overview of how many samples have been previously published, and how many are unique to this cohort and analysis.

[R1]: The scWGS and IF datasets were newly generated for this manuscript, in addition to all in-vitro scWGS, scRNA, and multiome data (see **Supplementary Table 2** for sample metadata). Multi-site scRNA-seq data, tumor-normal bulk WGS data, tumor-normal MSK-IMPACT, and H&E data were previously reported in Vazquez-Garcia, Uhlitz et al. *Nature* (2022) (see Supplementary Table 2 from that paper for details).

[C2]: Some of the terminology is a little confusing, e.g. 'Prevalent WGD patients' – it is the tumour and the cancer cells that harbour the WGD, not the patient – so I would be inclined to make this clearer. I also think a better name than 'Prevalent WGD' would avoid confusion (also see comment 7).

[R2]: We appreciate the reviewer's comment and have changed the terminology to classify tumors as WGD-high or WGD-low based on the prevalence of WGD cells, rather than "Prevalent WGD" vs "Rare WGD." This is part of a comprehensive update to the terminology used throughout the manuscript that is described in a new Glossary of Terms (**Supplementary Note** section 1).

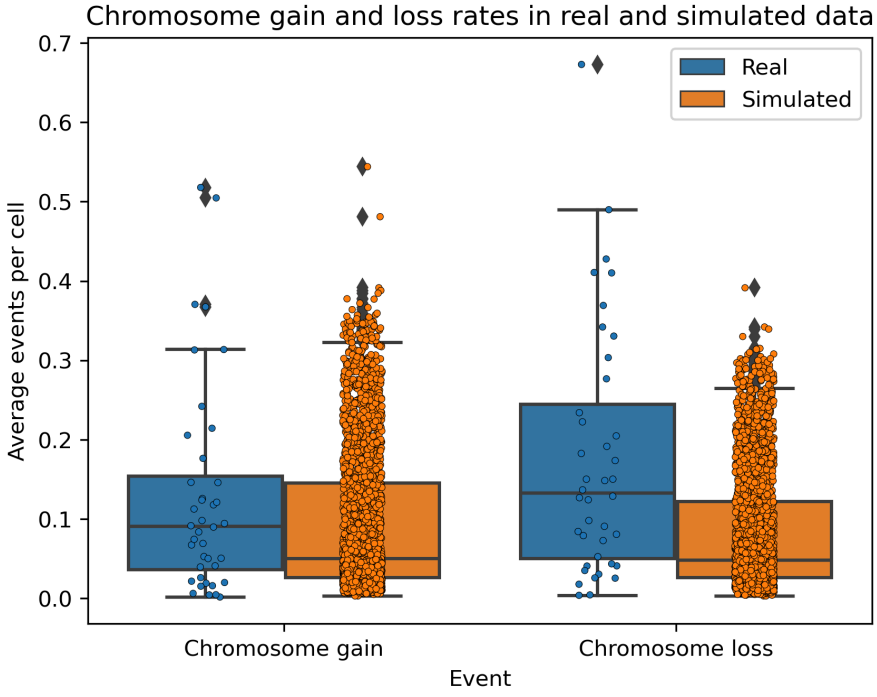
[C3]: The authors state that "The distribution of allele-specific copy number features showed clear separation between WGD states, permitting assignment of per-cell WGD multiplicities of 0 (0×WGD, 46% of cells), 1 (1×WGD, 53%) and 2 (2×WGD, 1%) (Extended Data Fig. 2H-I)". While there does appear to be relatively clear separation, I think it should be acknowledged that this is a heuristic method, and it is perfectly possible for cells to never have 'genome-doubled' but simply a large fraction of chromosome arms has independently been gained. The authors might wish to evaluate the likelihood of this – e.g. through simulations based on their observed gain/loss frequency

[R3]: We added the bolded text in the following passage to the manuscript (Line 148-150, emphasis added):

> *"We inferred the number of WGD events in the evolutionary history of each cell **using a heuristic approach** based on allele-specific copy-number profiles (**Fig. 1B**)."*

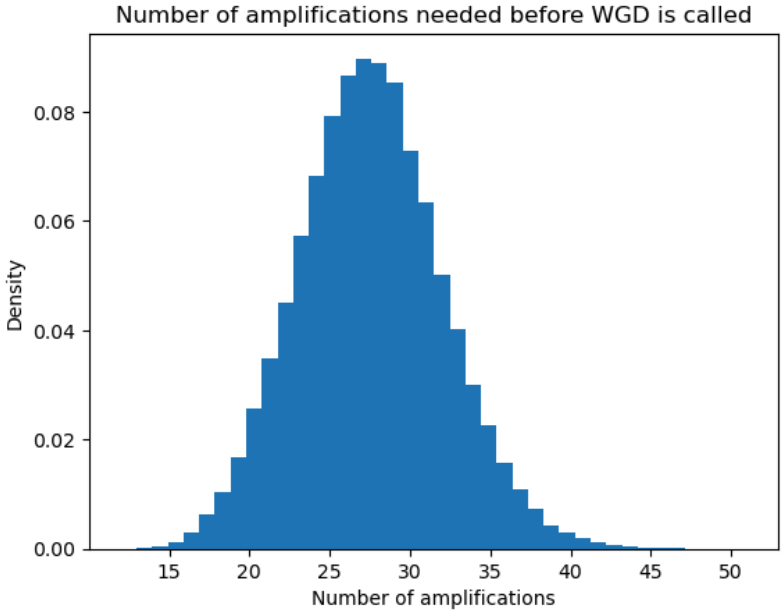
While the method we use to identify WGD is indeed a heuristic, we believe that it is very unlikely that cells identified as WGD are in fact the result of several independent gains. To support this, we analyzed a) WGD calling in existing simulated data, b) performance of the WGD heuristic in a basic model of chromosome gains, and c) `doubleTime` SNV-based evidence that all chromosomes were affected by one simultaneous event. We added these new analyses to the Supplementary Note and reproduced them below.

> *In the existing simulated data, we assessed the performance of the WGD heuristic in identifying cells that had been affected by an ancestral WGD. We found that in this application, the WGD heuristic achieved an accuracy of 100%. While these are relatively simple simulations with only chromosome-level events, the chromosome-level event rates that we estimated from the simulated data are representative of the range of chromosome-level event rates estimated from real data: real data chromosome gains 0.0016-0.52 per cell vs. simulated data chromosome gains 0.003-0.54 per cell, and real data chromosome losses 0.003-0.67 per cell vs. simulated data chromosome losses 0.003-0.39 per cell (**Supplemental Fig. 15**).*



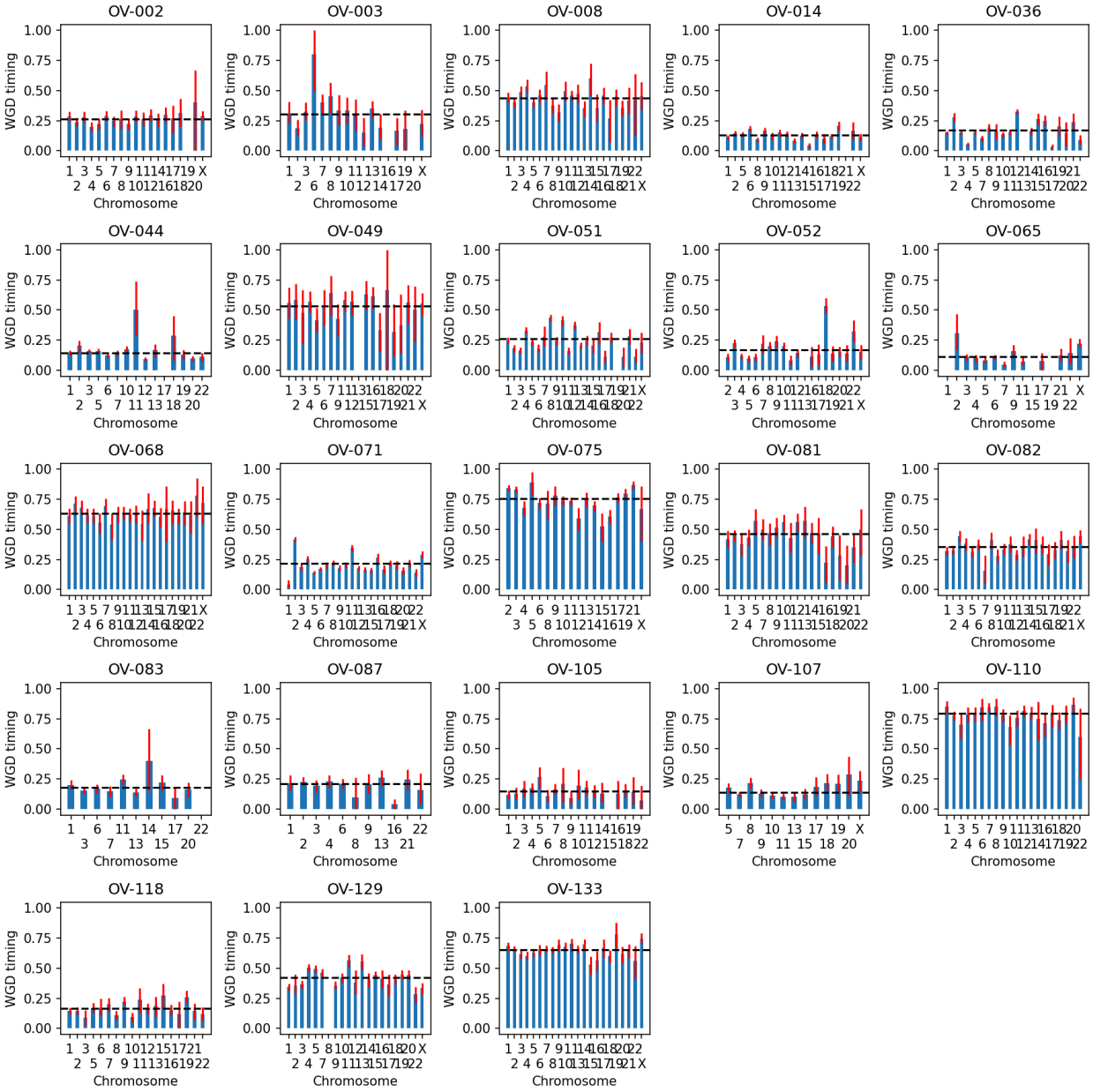
Supplemental Figure 15: Distribution of chromosome-level gain (left) and loss (right) rates for simulated data (blue) vs. real data (orange). Simulated data values are ground truth whereas real data values are inferred.

*To further assess the plausibility of independent chromosome gains, we implemented a very simple model of chromosome amplification in which all chromosomes were treated equally: each chromosome was given equal length in the WGD heuristic, and gain events were uniformly distributed over all chromosomes on both haplotypes. We simulated 100,000 cells in this manner and counted the number of amplifications required before the WGD heuristic would call a cell as WGD (i.e., false positive as no WGD was implemented in the simulation). We found that at least 12 events and an average of 27.5 amplification events were required before a cell reached the threshold for the WGD heuristic (**Supplemental Fig. 16**). Note that this model did not include counteracting deletions, so this is an underestimate of the number of independent events required for a false positive WGD call. Under the principle of maximum parsimony, a cell that is called WGD is much more likely to have undergone a WGD than to have undergone over 20 independent chromosome gains with no counteracting deletions.*



Supplemental Figure 16: Distribution of the number of distinct chromosome-level amplifications required before the WGD classification heuristic incorrectly calls WGD, under a simple model of uniformly random amplifications.

*For the 23 patients analyzed with doubleTime, we inspected the timing inferred by doubleTime on a per-chromosome basis to evaluate the SNV support for WGD, which would be reflected in similar timing estimates for all chromosomes. If instead chromosomes had been gained separately at different times, the doubleTime WGD timing estimates should differ. For each chromosome, we estimated the relative WGD timing on the branch with WGD using the same formula that doubleTime uses internally, but considering only the SNVs located on that chromosome. We found that these estimates were highly concordant, suggesting that the copy-number gains on these chromosomes are likely the result of a single simultaneous event – i.e., WGD (**Supplemental Fig. 17**).*

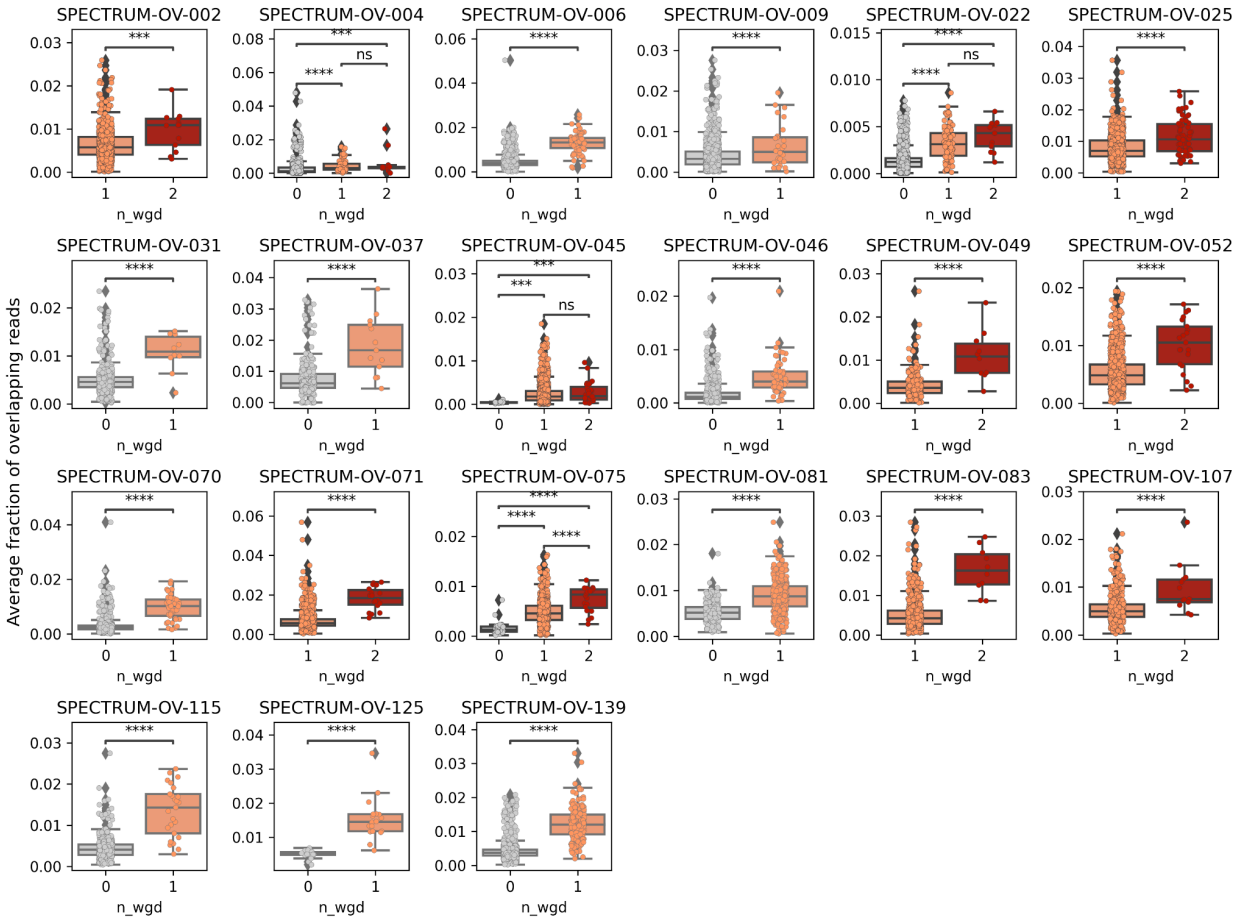


Supplemental Figure 17: doubleTime WGD timing estimates split by chromosome. Only those chromosomes with clonal SNVs that can be used for doubleTime are shown (see Methods for details). Red lines show bootstrapped 95% confidence intervals. Horizontal dashed black lines indicate the overall timing inferred by doubleTime for each patient from SNVs across the genome.

[C4]: Related to the above, the premise of the manuscript relies on accurately estimating ploidy from single cell data. Indeed, when analysing bulk data, determining correct ploidy and purity is essential to be able to infer WGD correctly. Different tools may estimate these quite differently. And, they are quite frequently wrong. While I appreciate the purity question should not be an issue with single-cell data, the low coverage may render accurate ploidy determination difficult. Thus, there is a need for further demonstration that the ploidy calls (and thereby WGD inference) are correct throughout the manuscript. This is particularly pertinent for samples with mixed WGD – how many cells support this? And, how robust are the WGD calls to down-sampling?

[R4]: We agree with the reviewer that accurate ploidy calls are paramount to the findings we present in the manuscript. To ensure correct calling, we applied rigorous filtering to remove normal cells, S-phase cells, and doublets. For doublets in particular, we applied several orthogonal approaches including combining copy number and SNV information to identify and remove tumor-normal doublets, and manually reviewing cell images from the DLP+ nozzle to identify apparent doublets. To further validate ploidy calls, we inspected the support from odd copy-number states in the cells' copy-number profiles (as these typically represent evidence that the cell should be assigned a higher ploidy): we removed any cell called as WGD that did not have a segment of at least 10 MB assigned to copy number 1, 3, or 5. We describe these filtering steps further and provide example copy-number profiles and nozzle images in **Supplementary Note section 1**.

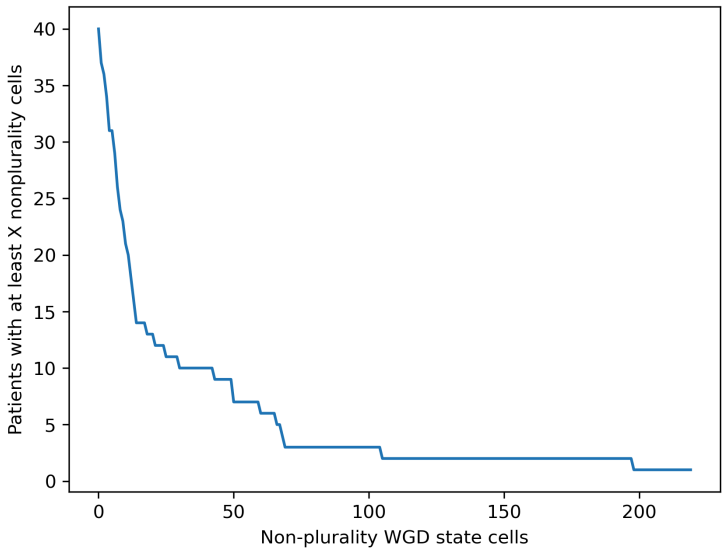
We found that ploidy calls from scWGS were correlated with ploidy calls from IMPACT panel sequencing (**Extended Data Fig. 2F**) and bulk WGS (**Extended Data Fig. 2G**), cell diameter measurements from DLP nozzle images (**Extended Data Fig. 2J**) and mtDNA copy number (**Extended Data Fig. 2K**). To further corroborate our ploidy calls, we analyzed the fraction of overlapping reads as an orthogonal measure of cell ploidy. Because the DLP+ protocol does not include a whole-genome amplification step, the fraction of overlapping reads is directly related to the number of copies of the genome in each cell. We found that cells in which we identified more WGDs had a higher average fraction of overlapping reads than their counterparts from the same patient (**Rebuttal Fig. 7**), and we added a cohort-level summary of this metric as **Extended Data Fig. 2L**. Furthermore, we added orthogonal information from the IF data, and correlated size of primary nucleus in tumor regions with WGD status. This is now included in **Fig 3H**. Thus, two bulk DNA sequencing modalities, biophysical cellular correlation with the nozzle images, mtDNA copy number, overlapping reads, and IF imaging all support the calls at different scales and granularity.



Rebuttal Figure 7: Average fraction of overlapping reads (y-axis) for each cell, grouped by patient (subplot) and WGD state (x-axis). Only patients with at least 10 cells each in at least 2 WGD states are included. Asterisks indicate significant p-values in independent t-test.

Regarding the amount of support for mixed WGD states, we added a Supplementary Note section (reproduced below) which we refer to in the revised Results section.

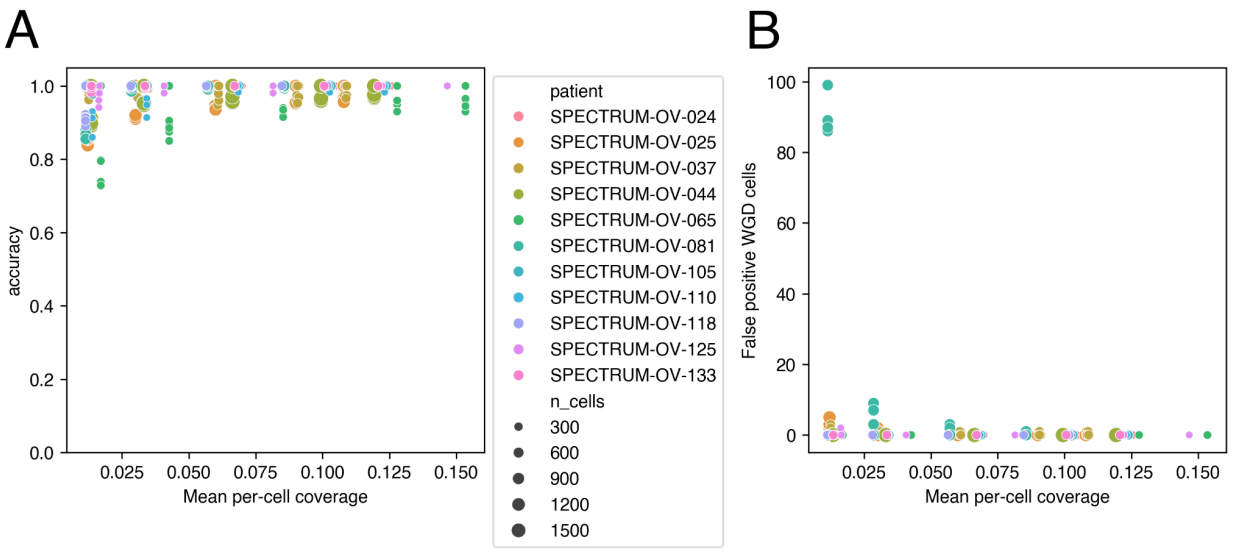
> *While 40/41 patients had at least 1 cell in the non-majority WGD state, most patients had multiple cells supporting their "mixed WGD" status: 37/41 patients had at least 2 cells that are not in the most-abundant WGD state, 30/41 patients had at least 5 cells, 21/41 patients had at least 10 cells, and 12/41 patients had at least 25 cells (**Supplemental Fig. 19**).*



Supplemental Figure 19: Number of cells supporting mixed WGD states. The x-axis shows the number of non-plurality cells, and the y-axis shows the number of patients with at least x cells in the non-plurality WGD state.

To evaluate the WGD calls, we performed a downsampling experiment which we describe in **Supplementary Note** section 2.4, reproduced below.

> *To evaluate the robustness of WGD calls to downsampling, we performed an in silico experiment in which we downsampled read counts from all filter-passing cells for the 10 patients with the highest per-cell coverage, then ran our copy number calling pipeline including WGD calling. We then evaluated the WGD calls from downsampled data, using the original WGD calls as ground truth. We found that the accuracy of WGD calling remained above 80% and the precision remained above 92% at coverage as low as 0.025X (**Supplemental Fig. 18A**). The number of false positive WGD calls was fairly high at the lowest coverage we tested (0.01X-0.03X), but decreased dramatically as coverage increased: no simulated dataset with mean coverage above 0.11X had a single false positive WGD call, and no simulated dataset with mean coverage above 0.06X had more than 1 false positive WGD call (**Supplemental Fig. 18B**). For reference, in the real scWGS data, 41/41 patients have mean coverage above 0.025X, 39/41 patients have mean coverage above 0.05X, and 35/41 patients have mean coverage above 0.06X. Note that this experiment omits the post-hoc filtering that we applied to the real dataset (e.g., inspecting DLP images, requiring a sufficiently large odd-copy-number segment, etc.).*
>
> *We also examined how "mixed WGD" status changed as a function of coverage in this downsampling experiment. 9/10 patients were estimated to have mixed WGD in the original dataset, and for these patients all simulated datasets also yielded mixed WGD. The exception was patient OV-065 whose samples originally consisted entirely of 1xWGD cells, whereas we incorrectly identified 2xWGD cells in 25/36 (70%) of simulated datasets from this patient.*



Supplemental Figure 18: Results from calling WGD on downsampled read counts as measured by accuracy (left y-axis) and the number of false positive WGD cells (right y-axis) compared to the mean per-cell coverage for the simulated dataset (x-axis). The 10 patients with the highest per-cell coverage were included. The size of each point indicates the total number of cells included in the simulated dataset.

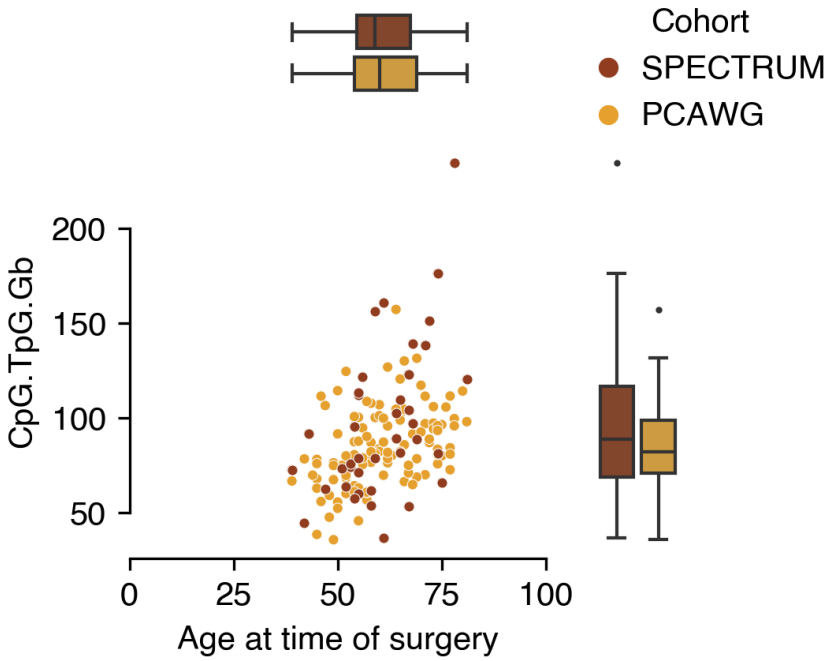
[C5]: Related to the above, I don't understand Figure 2B. What does the fraction #WGD mean? Isn't it impossible to detect residual cells without WGD in the case of a truncal WGD? (This would imply an absence of a clonal sweep, and no such thing as an MRCA). And, I don't understand what Fraction Cells means?

[R5]: 'Fraction #WGD' referred to the fraction of cells in each clone that are inferred to be 0xWGD, 1xWGD or 2xWGD (see **Figure 1A**). 'Fraction cells' referred to the fraction of cells in a clone originating from each site as shown in the colored barplot. We have adjusted the legend and y-axis labels of **Figure 2B** for improved clarity. The y-axis labels now read *"Frac. Cells in Clone by #WGD"* and *"Frac. Cells in Clone by Site"*.

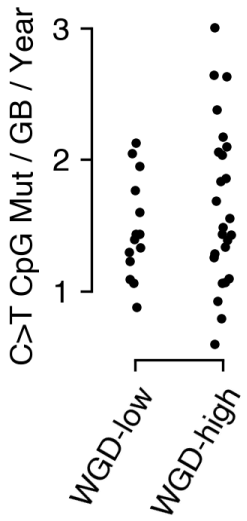
With respect to the residual 0xWGD cells, we agree that truncal WGD is incompatible with residual 0xWGD cells. We have thus revised the figures and text for more consistency and clarity. We now deliberately define patients as having a residual 0xWGD population (i.e., an extant population of 0xWGD cells that shares a pre-WGD MRCA with the WGD population) only if they have 0xWGD cells that are not classified as divergent (see **Supplementary Note** for reasoning). Residual 0xWGD cells were observed in OV-045, OV-075, OV-081, and OV-125. Both OV-045 and OV-075 have small populations (>1%) of 0xWGD cells, and these populations are clonally homogeneous with high similarity to a halved version of the 1xWGD population (**Extended Data Fig. 3A-B**). Thus we classify OV-045 as parallel WGD, and OV-075 as subclonal WGD. Patient OV-081 has a large population of 0xWGD cells and is classified as having a subclonal WGD. Patient OV-125 is excluded from doubleTime analysis due to low cell count. Other WGD-high tumors do not have any non-divergent 0xWGD cells and are classified as clonal WGD.

[C6]: Does the number of C>T mutations at CpG sites correlate with patient age? How confident can the authors be this truly represent a constant mutation rate?

[R6]: In general, we are relying on prior work which has shown the utility of using C>T mutations at CpG sites as an approximation of chronological time (Alexandrov et al. 2015; Baker et al. 2024; Gerstung et al. 2020). We do see a strong correlation between patient age and C>T mutations at CpG sites (**Rebuttal Fig. 8**). The distribution of patient age and per GB rate of C>T mutations at CpG sites matches with the ovarian cohort from the Gerstung et al. paper (Gerstung et al. 2020), as does the correlation between the two. Similar to Gerstung et al., we find that the C>T CpG mutation data can only be explained by an acceleration in mutation rate at some point in the evolutionary history of the tumor. Importantly, we do not have evidence this acceleration in rate depends on WGD (**Rebuttal Fig. 9**).



Rebuttal Figure 8: Patient age at time of surgery (x-axis) versus number of C>T mutations at CpG sites as mutations per GB (y-axis), plotted for both SPECTRUM and the PCAWG Ovarian cohorts. Only clonal mutations, those assigned to the root branch of the doubleTime tree, are shown for SPECTRUM. Normalization per GB takes into account changes in ploidy for patients with a WGD event on the root branch of the doubleTime Tree.

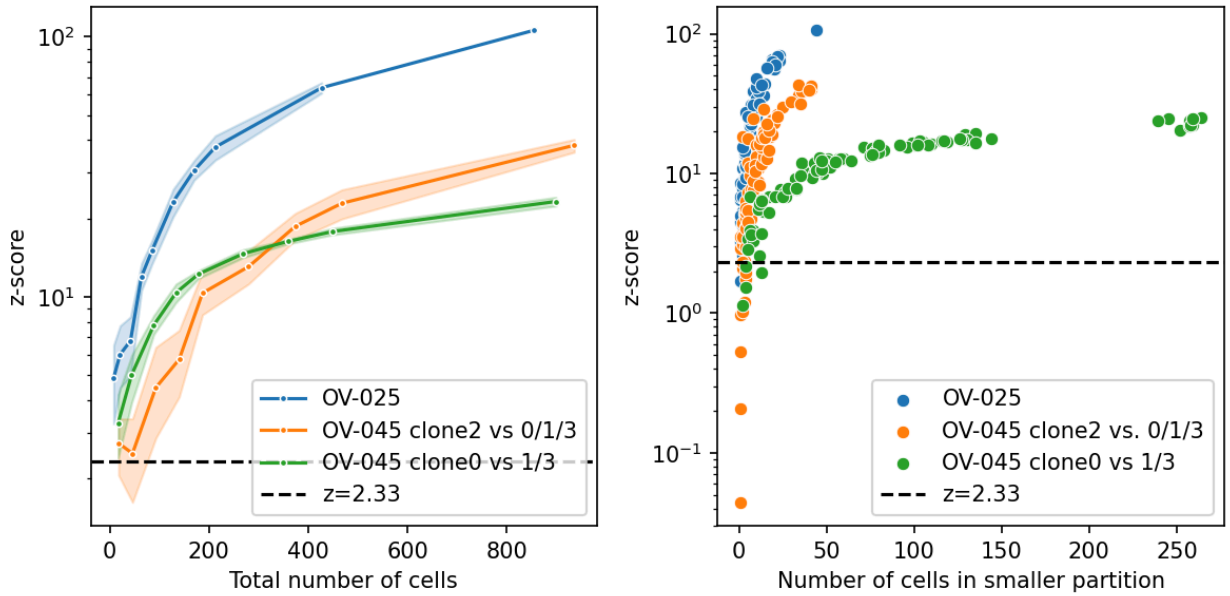


Rebuttal Figure 9: C>T mutations at CpG sites per GB normalized by patient age for WGD-low vs WGD-high. Comparison is not significant by Mann-Whitney U.

[C7]: The authors have different numbers of cancer cells sequenced for different patients. The number of cells sequenced may have an impact on the likelihood of detecting parallel WGD or cases with mixed WGD. It would be useful to evaluate how robust the results are to sampling bias?

[R7]: First, we note that in the main text, we identified a distinct WGD event in OV-045 clone 2 which contains only 59 cells. Second, we performed a simulation analysis to test the sensitivity of our parallel WGD test in **Supplementary Note** section 5.3, reproduced below.

> *To evaluate the sensitivity of our parallel WGD test to the number of sequenced cells, we subsampled cells from patients OV-025 and OV-045 and evaluated how many cells were required to arrive at a significant result. Specifically, for each bipartition, we randomly sampled 1%-50% of the cells, aggregated their SNV counts, and recomputed the log-likelihood ratio score and its p-value and z-score using the same permutation test described in Methods. We found that the statistical test consistently produced a significant result ($z > 2.33$, which corresponds to a p-value of 0.01 under the assumption that the sampling distribution is normal) with as few as 200 total cells and as few as 45 cells in the smaller partition (**Supplemental Fig. 25**).*



Supplemental Figure 25: Results from applying parallel WGD test on a subset of cells from each clone. The y-axis shows the z-score for the permutation test statistic, and the x-axis shows the total number of cells (left) and the number of cells in the smaller partition (right). The dashed black line indicates a z-score of 2.33 which would correspond to the 99th percentile of scores in a standard normal distribution. OV-045 clone 0 is the larger of the two clones with an isolated WGD event.

Finally, to help mitigate the differences in the number of cells sampled per patient, we generated additional scWGS data for 10 samples from 5 patients, totalling 1,699 high-quality malignant cells (**Extended Data Fig. 1B**).

[C8]: The authors state that for "23 out of 25 Prevalent WGD patients, a single ancestral WGD event was common to the dominant 1×WGD population of cells (Fig. 2B)" Does this mean in the prevalent WGD cases there is almost never a 0x WGD population? I think this should be made clearer – the previous paragraph implies that many previous bulk analyses were wrong, and potentially wrong with regards to overstating WGD as a truncal event. As an aside, given that if there is a truncal WGD all subsequent cells will necessarily inherit the WGD – if the authors are confident in the truncal WGD – they can assess their false negative rate of WGD calls in these tumours.

[R8]: We agree the sentence in question was confusing and have modified this analysis and test to improve the clarity. We have refined how we classify patients with respect to residual 0xWGD cells as described in [R5] above. We are also more deliberate about which patients have robustly defined populations of 0xWGD cells (see response above [R5]). We therefore no longer refer to patients with 0xWGD populations as having truncal or clonal WGD. In this revised analysis, only a minority of WGD-high tumors have 0xWGD populations (4/27 patients: OV-045, OV-075, OV-081, OV-125).

We assert that the single cell data is better suited to capture the continuum of WGD evolutionary histories, particularly the dynamics of emergent WGD as evoked by the patients with parallel subclonal and subclonal WGD expansion. However, we have tempered our criticism of bulk studies in the text in favour of highlighting the insights possible using single cell sequencing (**Discussion** Line 410-415):

> *Within the limitations of cohort size, sampling bias and genomic coverage, the single-cell analysis we present here enables several key under-studied measures in patient samples: i) detection of WGD and other rare copy-number profiles representing as few as 1% of cells, ii) cell-specific rates of CNA indicative of cellular genomic diversity and rates of missegregations, iii) and co-occurrence of multiple WGD states in the same tumor (including residual near diploid 0×WGD cells and 2×WGD cells in WGD-high tumors).*

Finally, although we think it would be interesting to use 0xWGD cells to assess false positive rate, we believe these 0xWGD cells to be a real and interesting phenomenon in some patients thus inappropriate for benchmarking. Instead, we tested the accuracy of our WGD calls by downsampling per-cell coverage in **Supplementary Note** section 3.4.

[C9]: Rather than define the tumours as Prevalent of Rare WGD, I would suggest defining the tumours by the mode of WGD` evolution; e.g. ancestral truncal WGD; parallel WGD, subclonal WGD etc. And then tumour may have multiple different types, and be grouped accordingly. Prevalent and Rare obscures key differences between tumours.

[R9]: We agree with the reviewer and have reconfigured Figure 2 with patients now grouped according to the mode of WGD evolution. We have defined the groups as follows:

- Truncal WGD: a single WGD ancestral to all cells (excluding divergent 0xWGD subpopulations)
- Parallel WGD: multiple WGD clones with different ancestral WGD events
- Subclonal WGD: a single WGD clone that is not ancestral to all cells, coexisting with a (non-divergent) 0xWGD subpopulation
- Unexpanded WGD: no WGD clones evident, although WGD cells are typically present (10/11 patients)

As stated above, these modes do not directly correspond with the WGD-high/low classes, as we observe Subclonal WGD in both WGD-high and WGD-low tumors.

[C10]: doubleTime will clearly depend a lot on the accuracy of Articull and the associated mutations. Given that we are not reviewing Articull it would be useful to demonstrate that the tool is reasonably robust.

[R10]: The ArtiCull preprint is publicly available at https://www.biorxiv.org/content/10.1101/2025.02.24.639589. We note in the response to Rev #1 [R2b] and Rebuttal Figs 4,5 the extensive benchmarking and evaluations we have performed on ArtiCull.

[C11]: I find the statement “In total, 5% of all tumour cells (n=1,481 cells), were part of non-dominant WGD states across the cohort (median of 2.6% of cells per patient Extended Data Fig. 2L)”, a little confusing. By non-dominant, do you mean <50%? I think this needs to be clearly defined in the main text.

[R11]: We have removed and replaced the word “dominant” to avoid confusion. The quoted sentence (Line 163-165) now reads:

> *In total, 4% of all tumor cells across the cohort (n=1,213 cells) represented non-majority WGD states (median of 2.5% of cells per patient; **Extended Data Fig. 2M**).*

[C12]: I'm not sure how much disagreement there is between the previous bulk analyses and the single cell data here. This is implied throughout the manuscript. But, a proper exploration of the benefits/disadvantages of single-cells vs. bulk is lacking. For instance, I'm not sure I understand why a difference would be expected in the case of clonal WGD? Likewise, the logic of timing in single cell and DLP+ pseudo-bulk from a clonal event should be similar – are the authors suggesting previous analysis was wrong due to lack of single-cell? Thus, while I appreciate the resolution should be better with single-cell, I think the authors should consider: 1. Avoiding over-stating differences (unless they're very confident this is significantly different from previous reports). 2. Directly evaluate what differences can be inferred with single cell compared to bulk.

[R12]: To address the reviewer’s specific inquiry: in principle, if a sample consisted entirely of a single WGD clone with no normal admixture, the results of a bulk analysis regarding WGD detection and timing could be similar. However, a tumor typically consists of multiple clones and for much of the cohort there are too few WGD cells for them to be detectable via bulk. Specifically, the <15% of WGD cells in WGD-low tumors would be very difficult to detect in bulk copy-number analysis, especially in admixed samples without 100% tumor purity. Also, calling purity and ploidy from bulk sequencing data is not straightforward: it typically involves manual review, and large consortia (e.g. TCGA/GDAN, PCAWG) typically apply several different copy-number inference methods and use a consensus approach to produce a final set of purity, ploidy, and copy number calls.

With single-cell data, estimating tumor purity and precisely identifying non-tumor cells is considerably easier. Therefore, estimating cell ploidy is much more direct – and can be directly corroborated by cell-level biophysical measurements using the optical components of DLP+. This is a wholly unique technical advance of our study: simultaneous single-cell optical and whole-genome measurements which we use to maximize the veracity of single-cell WGD calling. Our ploidy estimates are correlated with IMPACT ploidy (**Extended Data Fig. 2F**), bulk WGS ploidy (**Extended Data Fig. 2G**), and measurements of individual cells including cell size (**Extended Data Fig. 2J**), mtDNA copy number (**Extended Data Fig. 2K**) and average fraction of overlapping reads (**Extended Data Fig. 2L, Rebuttal Fig. 7**). We applied extensive filtering to remove doublets and ensure that WGD cells were well-supported (**Supplementary Note** Section 2).

In single-cell data, we can answer further questions that have not previously been studied using bulk data about the cellular heterogeneity in patient samples. With a single-cell approach, we claim the following advances where it is now possible to:

1. Measure multiple WGD states in the same tumor
2. Detect WGD subclones in predominantly diploid samples
3. Detect residual non-WGD cells in predominantly WGD samples, which rules out Truncal WGD
4. Identify clones in the same tumor that underwent distinct ancestral WGD events
5. Identify cells with rare copy-number profiles (<1-2% of cells)
6. Reason about WGD timing in individual clones (as well as the timing of clonal divergence) using SNVs
7. Use cell-specific copy-number changes to estimate rates of aneuploidy-driving processes such as whole-arm and whole-chromosome gains and losses
8. Rather than asking if a tumor is WGD, we can ask how much of the tumor is WGD, and interrogate those specific cells and their subsequent copy number evolution.
9. Map WGD clones in in vitro cell lines to compare expression programs of co-existing 0xWGD and 1xWGD cell populations

We have added text to the Discussion section to convey these advantages (Line 410-415):

> *Within the limitations of cohort size, sampling bias and genomic coverage, the single-cell analysis we present here enables several key under-studied measures in patient samples: i) detection of WGD and other rare copy-number profiles representing as few as 1% of cells, ii) cell-specific rates of CNA indicative of cellular genomic diversity and rates of missegregations, iii) and co-occurrence of multiple WGD states in the same tumor (including residual near diploid 0×WGD cells and 2×WGD cells in WGD-high tumors).*

[C13]: When the authors say 'late' WGD patients, do they mean late on the trunk? Late in evolutionary time? The term ‘late’, here, is subject to some confusion. The fact that extant populations with 0xWGD are observed suggests the WGD cannot be in the MRCA – this should be made clearer.

[R13]: We are now explicit about what we mean by ‘late’ WGD. We define ‘late’ WGD as either 50% of the way through the ancestral branch or trunk, or after the MRCA. We have amended the text (Line 204-207):

> *Although WGD events generally occurred early in tumor evolution, a long tail of later events were observed: in 8/25 WGD-high tumors the WGD event occurred more than 50% of the way through the tumor's ancestral branch or after the Most Recent Common Ancestor (MRCA).*

For clarifications regarding 0xWGD populations, please see the response to [C5] above. In addition we have annotated ‘early’ and ‘late’ with differently colored glyphs in **Figure 2B**.

[C14]: Differences in the karyotype landscape between WGD and non-WGD cells will reflect both the rate of chromosome missegregation events, but also tolerance of losses/gains following a missegregation rate. This needs to be acknowledged in Fig 3E and Ex4F. For instance, the observation that ploidy- adjusted chromosome losses were 4.1 times more abundant in Rare WGD 1×WGD cells compared to Rare WGD 0×WGD cells (p=5.6×10-4, Mann-Whitney U test), 2.3 times more abundant in Prevalent WGD 1×WGD cells compared to Rare WGD 0×WGD cells (p=5.6×10-4, Mann-Whitney U 258 test), and 1.8 times more abundant in Rare WGD 1×WGD cells compared to Prevalent WGD 1×WGD cells (p=0.016, Mann-Whitney U test), is likely not as simple as purely a difference in rate.

[R14]: The reviewer is correct that the observed rate of missegregation events is likely influenced by both mutation and selection. This is a limitation of the study that results from studying live cells, and we now mention this limitation in the **Discussion** (Line 416-419):

> *"That we observe cells with considerable homozygous deletions (or nullisomy) suggests we can capture some part of the non-viable population. Future studies in controlled systems that quantify and model cell death could further enhance our understanding of the relationship between WGD, cellular fitness, and clonal expansion."*

We believe our use of IF measurement helps mitigate this limitation as we are able to identify a similar relationship between CIN and WGD in this orthogonal data. In addition, we are able to find interpretable correlations between the scWGS-derived missegregation rates and phenotypic changes. The relationship between missegregation and STING protein expression is now corroborated by the IF data. Thus, despite the limitation raised by the reviewer, we are able to uncover and confirm key relationships between scWGS-derived rates, IF-derived rates, and cell-intrinsic signalling.

[C15]: In Figures 4B and C – would the authors expect whole genome doubled chromosomes/arms to have an increased probability of losing more DNA? It would be interesting to see if losses are more prevalent in/specific to certain genes/genomic regions post-WGD.

[R15]: We agree that looking at the distribution of post-WGD losses across the genome would be interesting. Our initial genome-wide analysis of this question did not yield interesting findings and we have thus left this question for future similar studies of larger patient cohorts. We now analyze the relative rates of losses vs gains for both cell-specific and ancestral branches (**Extended Data Fig. 4J**). We do not find a difference in loss/gain ratio between WGD-high and WGD-low, suggesting that WGD may not affect the rate of missegregation+selection that produces cell-specific losses versus gains. We note that while the genomic analyses did not uncover specific genes or pathways, the scRNA and IF analyses do illuminate phenotypic consequences that are elaborated on in **Fig. 5,6** and **Extended Data Fig. 6,7**.

[C16]: The final part of the analysis focuses on tumour cell phenotypes and microenvironment remodelling in the context of WGD. Here, I think it would be useful to attempt disentangle the extent to which the results being observed truly reflect WGD vs. increased chromosomal instability (potentially as a cause or consequence of WGD).

[R16]: Given that WGD is inherently a process of increased genomic instability, as evidenced by the increased rates of chromosome missegregation events in WGD samples in our analysis, we believe that it may not be possible to fully disentangle WGD from CIN. However, it is possible to interrogate how CIN rates relate to various phenotypic features within WGD and non-WGD subsets. We hypothesized that increasing CIN in an already genome-doubled cell manifests different phenotypic consequences as compared to a non-WGD cell. Indeed, we observed that chromosomal loss rates in WGD-low samples correlated with an increase in G1 and decrease in G2M specifically in WGD-low cases (**Fig 5F**).

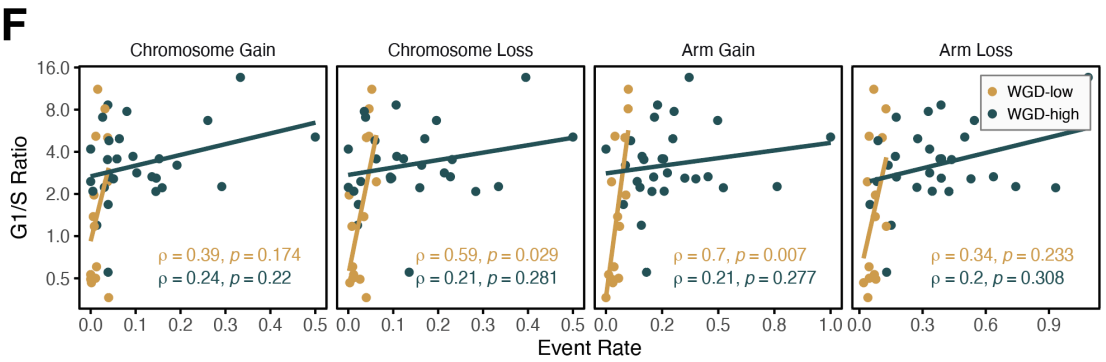


Figure 5F: Scatter plot of G1/S cell count ratios (y-axis) by rates (counts per cell) of large chromosomal changes (x-axis) split by WGD-low and WGD-high (color). Lines indicate the result of a linear regression within either WGD-high or WGD-low tumors. Regression coefficients and significance results are shown separately for WGD-low and WGD-high.

We have now expanded this analysis to other tumor-cell-intrinsic phenotypes, where a similar pattern emerges with increasing rates of CIN having oftentimes opposite relationships to tumor cell signalling pathways in the context of WGD (**Fig. 6B**).

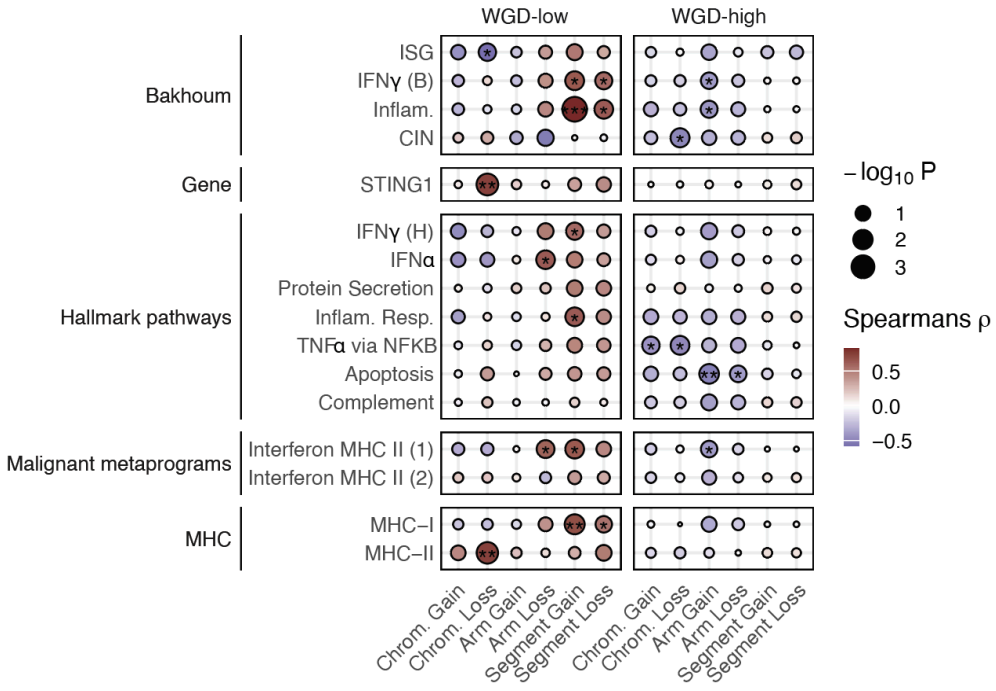


Figure 6B: Dotplot of correlations between missegregation rates derived from scWGS (column) and cancer-cell-intrinsic pathways from scRNA-seq in site-matched samples (row). Spearman's rho significance is annotated as 'ns': 5.0×10-2 < p <= 1, '*': 1.0×10-2 < p <= 5.0×10-2, '**': 1.0×10-3 < p <= 1.0×10-2, '***': 1.0×10-4 < p <= 1.0×10-3, '****': p <= 1.0×10-4.

Furthermore, several pieces of evidence from our multi-modal dataset implicate WGD as a modulator of cell-intrinsic signaling. The IF data show STING protein downregulation in WGD-high tumors despite elevated cGAS-inferred MN rates, and the DLP and scRNA results show correlation of CIN and STING1 mRNA expression in the WGD-low and not in the WGD-high, as discussed in lines 351-359 (**Fig 6C-G**). Taken together, these point to a de-coupling of CIN-induced cell intrinsic signaling as a function of WGD. Additionally, the new results comparing WGD-low and WGD-high tumors within the HRD-Dup subset show similar cell cycle, inflammatory, and immunosuppressive patterns as the entire cohort. In total, this evidence suggests that WGD stimulates additional phenotypic consequences over and above the CIN that is expected in HRD tumors.

[C17]: Overall, the flow of the manuscript is a bit jagged. There are often long sentences, spanning multiple lines of text, with few punctuations, making it difficult to distinguish key points from the abundance of results presented. For example, confusing terminology across lines 284-285, "significantly higher number of low-CCF high level amplifications per cell", required several rereads to understand. I would be inclined to make such statements clearer.

[R17]: We appreciate this comment and acknowledge that the manuscript flow could be improved. Accordingly, we have edited the text to improve the narrative flow. We have avoided run-on sentences, cut down long sentences, used clearly defined terminology, removed some figures that were not salient to the core claims, and improved punctuation throughout the text.

Minor points:

[C18]: Extended Data Figure 2J – p-values would be useful.

[R18]: We applied a Wilcoxon paired one-sided test and annotated the plot with significance indicators.

[C19]: Figure 2A – Schematic could be more representative of the actual methodology described in the methods section (the synopsis in the main text is also a bit confusing). It is also unclear what “T” and “S” stand for in the rightmost panel.

[R19]: We have updated the rightmost panel in **Fig. 2A** to show a probabilistic graphical model representation of doubleTime. We have also updated the description in the main Results section for clarity.

[C20]: Figure 2D – I am a bit confused about the lack of orange colouring, denoting WGD, in the phylogenies track of the figure because the track underneath suggests the presence of WGD events

[R20]: The upper track of barplots in **Fig. 2B** (formerly **Fig. 2B-D**) are shown in logarithmic scale to highlight WGD events in small subpopulations. Unlike Truncal WGD or Parallel WGD tumors, Unexpanded WGD tumors (and some Subclonal WGD tumors) each only contain a small number of WGD cells which cannot be accurately placed on the clone phylogenies.

[C21]: Figure 3I – Only one p-value is significant, which along with the other p-values, probably would not hold after multiple-test correction. Describing it as a “modest correlation” seems to be overselling the association.

[R21]: We have removed this figure from the paper.

[C22]: Extended Data Figure 4D – Might be better to facet by rare vs prevalent WGD to show significance of divergent cells

[R22]: We agree and have updated the figure.

[C23]: Extended Data Figure 4I

[C23a]: Wrongly referenced on line 318 as “Extended Data Figure 4J”

[R23a]: The callout now refers to **Extended Data Fig. 4K** and has now been fixed in the text.

[C23b]: The authors state that “In clonal WGDs, the number of chromosome losses and arm gains and losses were significantly correlated with the age of the WGD as estimated using mutations”. However, looking at the figure panels, only the correlations with the losses seem to be significant. Also, stating the R2 values might be helpful for better interpretation of the actual correlation.

[R23b]: We have adjusted the text and have added the Spearman r values to the plot.

[C24]: Sample processing (Methods) – in point 2, the authors state scRNA-seq was performed on samples from 32 patients, but in point 3, “for each specimen with scRNA-seq”, this increases to 37

[R24]: We have corrected the number of patients and specimens with scRNA-seq in the **Methods** to be consistent across all sections (123 sites from 32 patients).

Referee #2 (Remarks on code availability):

I appreciate the effort for making the code available.

Referee #3

Summary
In the manuscript ‘Ongoing genome doubling promotes evolvability and immune dysregulation in ovarian cancer’, the authors present a new bioinformatics tool, doubleTime, and the findings from applying this method to single cell whole genome sequencing (scWGS) data from 65 primary tumor samples from 40 high grade serous ovarian cancer (HGSC) patients that are part of the MSK SPECTRUM cohort. The authors combine this data with immunofluorescence data to examine DNA sensing / micronuclei formation, and previously generated single cell RNA sequencing (scRNAseq) and to a lesser extent bulk WGS data and MSK-IMPACT clinical sequencing data. The key findings of the study include:
(1) Through analysis of single cell genomes the evolutionary history of copy number aberrations (CNA) and whole genome doubling (WGD) can be determined. Multiple WGD states are frequently identified within individuals, and through analysis of the timing of CNA events compared to WGD distinct evolutionary patterns are discernable.
(2) Cases with WGD have higher numbers of ruptured micronuclei, as determined by cGAS immunofluorescence than cases with rare WGD, these cases also had higher numbers of subclonal CNA that are not detected in bulk WGS.
(3) Cases with high prevalence of WGD cells have a higher proportion of cancer cells in the G1 phase of the cell cycle compared to cases with rare WGD, and similar findings to recent work that HGSC cases with WGD have reduced expression of immune signalling pathways.

While there are no technical or experimental flaws that preclude publication, there are a number of key points that should be addressed:

We thank the reviewer for spending the time to thoroughly evaluate and critique the manuscript. We have addressed each comment point-by-point to the best of our ability. As a result, we think the manuscript is markedly improved for clarity and rigor.

[C1]: While I appreciate that scWGS is not a straightforward and cost-effective method to use, I have concerns around the inclusion of the 8 patients with fewer than 200 cells when examining clonality and heterogeneity, or a lack of discussion of the limitations in making conclusions regarding these patients.

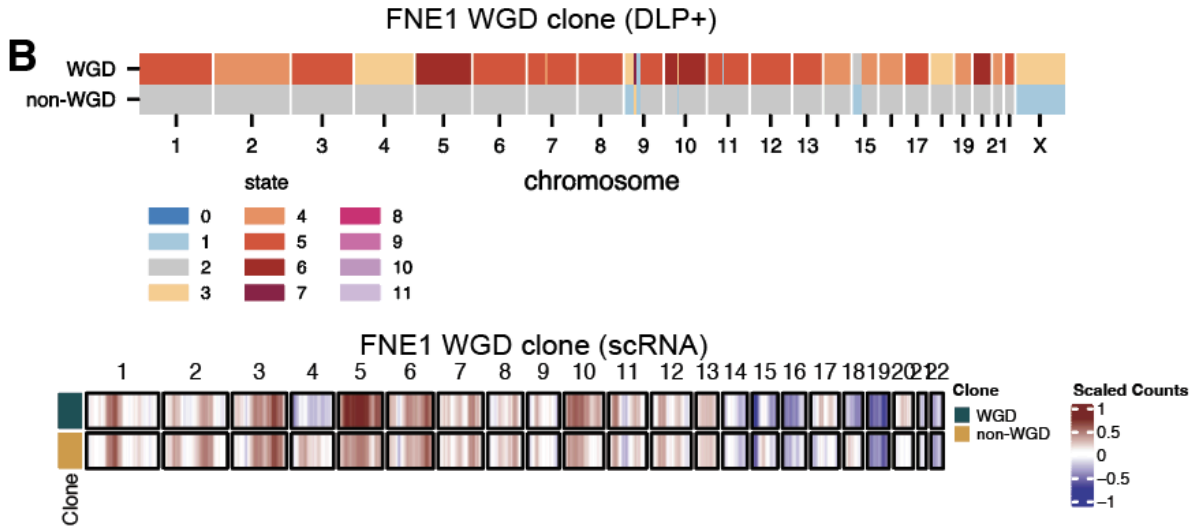
[R1]: We appreciate the reviewer's concern and have added text to the **Discussion** describing the limitations of the scWGS dataset with respect to our conclusions (Line 410-415):

> *"Within the limitations of cohort size, sampling bias and genomic coverage, the single-cell analysis we present here enables several key under-studied measures in patient samples: i) detection of WGD and other rare copy-number profiles representing as few as 1% of cells, ii) cell-specific rates of CNA indicative of cellular genomic diversity and rates of missegregations, iii) and co-occurrence of multiple WGD states in the same tumor (including residual near diploid 0×WGD cells and 2×WGD cells in WGD-high tumors)."*

To supplement the scWGS cohort, we have sequenced 10 additional samples from 5 patients (including 1 new patient) for a total of 1,699 additional filter-passing tumor cells. We exclude any SBMClone clone with fewer than 20 cells from doubleTime analysis, which includes all clones for patient OV-125. As part of these revisions, we have performed several simulation analyses to test the sensitivity of WGD calling and mixed WGD classification to coverage (**Supplementary Note** section 3.4) and the sensitivity of parallel WGD calling to the number of cells (**Supplementary Note** section 5.3).

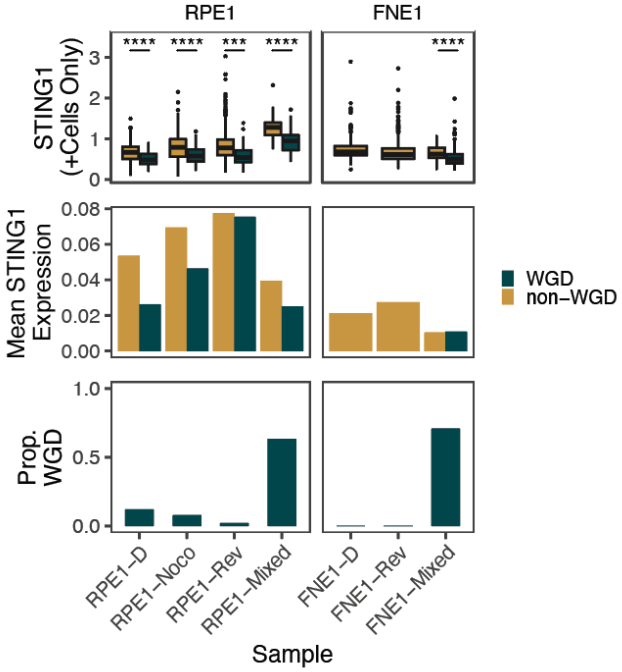
[C2]: Although only a small component of the study, the in vitro work only utilises a single cell line limiting the confidence in the results from this experiment. It is also unclear why retinal pigment epithelial cells were chosen rather than a more biologically relevant model such as untransformed fallopian tube cell lines or even HGSC lines without WGD.

[R2]: We have now replicated the in-vitro experiments using the immortalized fallopian tube epithelial cell line FNE1. In brief, we generated *TP53-/-* cells and subjected cells to reversine to induce chromosomal missegregation, sequencing both DMSO control and reversine-treated cells using 10x scRNA and DLP+. In addition, we propagated the TP53-/- FNE1 cells and monitored for the existence of WGD clonal outgrowth by DNA FISH, sequencing the resulting mixed population using 10x scRNA and DLP+. As in the RPE1 data, we found a 1xWGD clone in DLP+ with characteristic copy-number aberrations which we were able to detect in scRNA-seq (**Extended Data Fig. 7B,** reproduced below)



Extended Data Fig. 7B: Clone copy number inferred from DLP (top) and scRNA-seq (bottom) for FNE1 cells. Two clones were identified in both modalities: one WGD and one non-WGD.

As seen in the RPE-1 context, expression of STING1 was decreased in the 1xWGD population **(Extended Data Fig. 7G** (right)**,** reproduced below).



Extended Data Fig. 7G: STING1 expression in STING1 positive cells (top), mean STING1 expression (middle), and proportion of WGD cells (bottom) for non-WGD and WGD RPE1 (left) and FNE1 (right) cells by treatment condition.

I also think that there are a number of missed opportunities in the manuscript as currently presented, and I would encourage the authors to consider including some additional analysis or discussion of the following areas.

[C3]: As most of the analyses have combined the data to the patient level there is limited examination of heterogeneity across tumor sites within individual patients, despite recent papers finding genetic heterogeneity in HGSC patients (eg Cunnea et al Cell Reports Medicine, Burdett et al Nature Genetics).

[R3]: We increased the number of patients with multi-site scWGS data during the revisions, with 21/41 patients having more than 1 site with scWGS. Mixed WGD states across sites are common, and this is now also highlighted in a new panel that shows within-patient variation in WGD states between tumor sites.

Supplemental Figure 20. Absolute (top bar plot) and relative (middle bar plot) compositions of malignant scWGS cells in terms of the number of ancestral WGD events, divided by site. Samples are separated by patient and ordered by the proportion of cells with at least 1 WGD out of all cancer cells.

We also added the following section analyzing multi-site variability in WGD to the Supplementary Note.

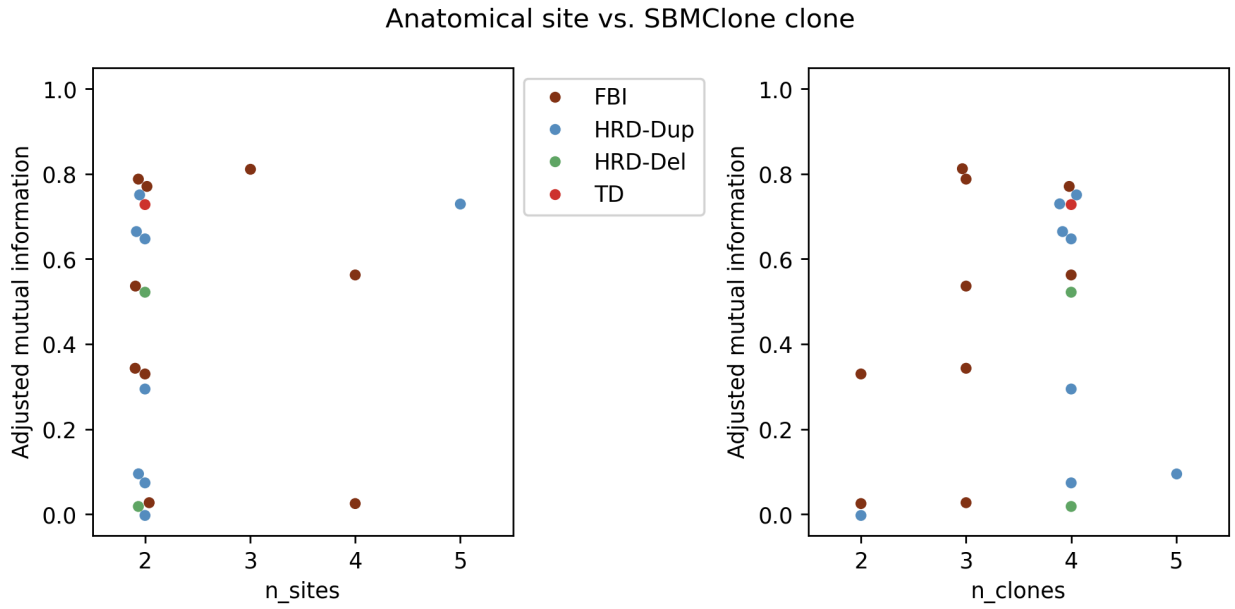
> *"The patients in our cohort exhibit varying levels of WGD heterogeneity across sites. 21/41 patients have more than 1 site with filter-passing scWGS tumor cells, and all of these patients have mixed WGD states (**Supplemental Fig. 20**). 16/21 patients with mixed WGD states have mixed WGD represented in more than 1 site, and 15/21 patients have mixed WGD represented in all sites (although 12/15 of these patients have only 2 sites). 11/21 patients have 3 WGD states represented, and 4/11 of these patients have all 3 WGD states represented in more than 1 site. Overall, mixed WGD states tend to be common across multiple sites, consistent with ongoing WGD. Interestingly, the only patient whose WGD classification would vary if sites were processed independently is OV-081: the infracolic omentum sample has 93% ≥1 WGD cells which would be*

classified as WGD-high, whereas the left adnexa sample has 99.5% nWGD cells which would be classified as WGD-low."

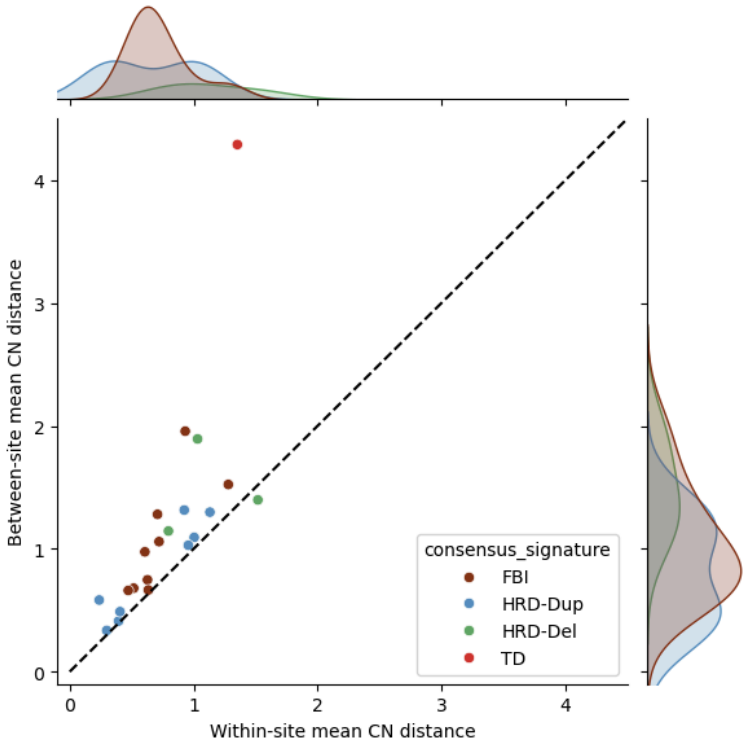
We added text reflecting this analysis to **Results** (Line 165-167):

*Of the 21 patients where we sequenced cells from multiple sites with DLP+, mixed WGD states were observed in multiple sites for 16 patients, consistent with WGD as an ongoing process (**Supplementary Note**).*

We also examined the SNV-based and copy-number-based heterogeneity across anatomical sites for the 21 patients with multiple anatomical sites sequenced. We found that there was a persistent but weak relationship between SBMClone clones and anatomical sites as measured by adjusted mutual information and ARI (**Rebuttal Fig. 10**), and that copy number distances (average mean squared distance between all pairs copy number profiles) tended to be higher between samples than within samples, especially for FBI patients (**Rebuttal Fig. 11**). Note that these findings are limited by the small number of patients with multiple sites for each signature, and may be biased by the specific anatomical sites that were sequenced – for example, we would expect more heterogeneity between more distant sites.



Rebuttal Figure 10: SBMClone heterogeneity across anatomical sites as measured by adjusted mutual information (y-axis) compared to the number of sites (x-axis, left) and the number of SBMClone clones (x-axis, right). Each patient is colored by mutational signature.



Rebuttal Figure 11: Average pairwise copy-number distance among cells in the same anatomical site (x-axis) compared to cells in different anatomical sites (y-axis) colored by mutational signature.

[C4a]: There was limited discussion of the genes involved in the CNA or mutations in relation to events prior to WGD – can the authors explain which events may be causing WGD to occur?

[R4a]: Clearly TP53 mutation is one factor that enables WGD. This is well-documented in the literature, and in our own in-vitro experiments for which WGD clonal outgrowth will eventually occur in subsequent passages following TP53 knockout. Nevertheless, WGD outgrowth does not immediately succeed TP53 knockout in-vitro nor in the patient setting so we agree there may be other adaptations that take place. This may include clustering of centrosomes (Acilan and Saunders 2008), downregulation of STING to allow immune escape (Xia et al. 2016; Konno et al. 2018; Xia, Konno, and Barber 2016; Kitajima et al. 2019), or other mechanisms that allow adaptation to the increasing levels of CIN. While we think the reviewer's question is certainly interesting, providing a comprehensive answer would be a considerable undertaking and we believe it will require years of further research.

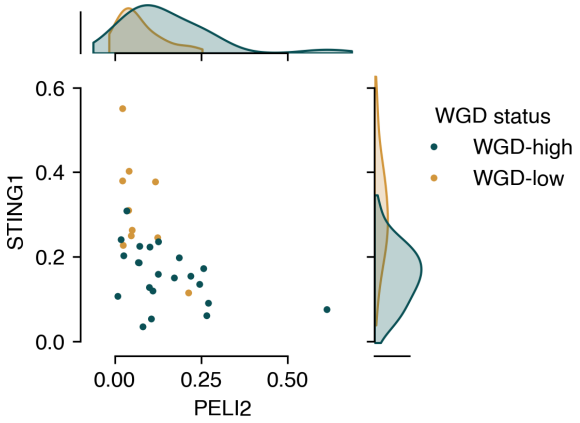
[C4b]: It would also be interesting to consider how the WGD timing relates to p53 signature and STIC formation and discuss how the findings relate to those made by Cheng et al (J Path 2024).

We added a sentence in the **Discussion** (Line 455-458) to raise this important point:

"Studying WGD and cGAS-STING in the context of serous tubal epithelial carcinoma (STIC) precursor lesions (Cheng et al. 2024; Kader et al. 2024) could yield important insights into how WGD and cGAS-STING modulation contributes to tumorigenesis in HGSOC."

[C5]: The decreased STING expression in the prevalent WGD cases despite the high prevalence of cGAS staining at the MN is an interesting finding that could have been explored further, for example are there mutations or decreased expression of upstream regulators of STING?

[R5]: We added additional *in vitro* validation in FNE1 cells reproducing STING1 downregulation in WGD cells (**Extended Data Fig 7G**) seen in tumors and in RPE1 cells. Furthermore, we have added the protein-level correlation from IF data indicating that STING1 protein abundance is lower in WGD-high tumors (**Fig 6F**). In addition, we investigated a number of mechanisms that could explain the decreased STING expression. One patient (OV-014) has amplification of USP35, which has been found to regulate STING (J. Zhang et al. 2021). Another patient (OV-068) was found to have a high impact nonsense STING mutation. In addition, the PELI2 gene, a negative regulator of STING (Ritchie and Li 2024), was found to have higher expression in WGD-high tumors and a pattern of mutual exclusivity with STING (**Rebuttal Figure 12**). Promoter methylation is a known mechanism that could be responsible for decreased STING expression in our cohort (Konno et al. 2018). For further discussion of the literature surrounding cGAS/STING, we encourage the reviewer to refer to R5b response to reviewer 1. As these were not recurrent events across the cohort, we leave as potential avenues of future study a more comprehensive look at the various mechanisms of STING deactivation in HGSOC.



Rebuttal Figure 12: Mean patient expression of STING1 and PELI2 in tumor cells colored by WGD status.

In addition, we have added a passage on this topic in the Discussion (Line 449-455):

"*While WGD was associated with increased cGAS+ ruptured micronuclei, as expected given the higher levels of CIN, WGD-high tumors showed decreased cell-intrinsic and cell-extrinsic interferon signaling, consistent with previous findings on chronic-CIN induced immunosuppression. The disrupted correlation between CIN and STING1 in WGD-high tumors implicates STING1 transcriptional repression as a prerequisite for clonal expansion of WGD. Given the very early timing of WGD in some patients, our*

results also prompt further investigation of STING1 repression as an early event in the evolutionary history of some HGSOC tumors which may precede WGD."

Additional points for clarification

We provide concise responses to each point below.

[C6]: Title: I would recommend that the title be reconsidered, as 'ongoing genome doubling' suggests that the focus of the manuscript is the 2nd WGD which it is not. Consider removing the word ongoing from the title or rearrange to Genome doubling promotes ongoing evolvability and immune dysregulation in ovarian cancer. Also consider being more specific with regards to the disease as this study only examines high grade serous ovarian cancers and not all ovarian cancers.

[R6]: There have been multiple studies in the literature utilizing the term "ongoing chromosomal instability" to refer to active and persisting processes (e.g. chromosome missegregation, chromothripsis) only observed in rare populations of cells (e.g. Valle-Inclán et al, Cell 2025). Per the histology specificity, we are constrained by editorial guidelines on title length and do not have the space to include the fully-described high grade serous term, although we agree with the point in principle. Accordingly, we stress in the **Abstract** (Line 66), **Introduction** (Line 96-97), throughout the **Results** (Line 113, 115, 134, 145, 180, …), and in the **Discussion** (Line 409) that this study is on HGSOC patients.

[C7]: The abstract and introduction mention quiescence in the prevalent WGD cases but this term is not used in the results or discussion, for simplicity for the reader please be consistent in the terminology used throughout the manuscript.

[R7]: We have removed the term "quiescent" from the manuscript to avoid confusing readers, and instead refer more precisely to the cell cycle dysregulation results.

[C8]: The introduction has limited discussion about HGSC, and in particular does not describe the actual frequency of WGD in HGSC (either at primary disease or during disease recurrence/end stage), nor recent papers that have looked at WGD in HGSC (eg Cheng et al 2024 J Path, Burdett et at 2023 Nature Genetics). Additionally, the second paragraph of the introduction has a lot of detail regarding the results that are not appropriate for the introduction.

[R8]: We now cite several papers in the introduction reporting the frequency of WGD in HGSC (Burdett et at 2023 Nature Genetics, Cheng et al 2024 J Path).

[C9]: Methods

[C9a]: Some sections of the results are written in present tense and others in past tense, please update so it is all in past tense.

[R9a]: We have updated the Results statements to be in past tense.

[C9b]: With regards to the flow-sorting of low purity samples, how was purity determined and what purity cutoff was used?

[R9b]: Tumor purity was estimated based on site-matched scRNA-seq data previously generated, derived from identical aliquots of single-cell suspensions. Cell type classification of malignant, immune, and stromal cells in CD45-sorted samples was used to estimate the fraction of cancer cells in $CD45^-$ samples. As an added layer of filtering for malignant cells in resulting DLP+, we used analytical properties of aneuploidy to filter out normal cells (see **Methods** section *Cell filtering - Removal of normal cells* and **Supplementary Note** section 2).

[C9c]: In the sample processing section is a reference to Supp Tab 2 – I could not find a table with metrics for numbers of sequenced cells.

[R9c]: We have added a new table (**Supplementary Table 3**) which includes sample-level sequencing metrics for scWGS datasets (e.g. numbers of sequenced cells). The previous table callout in the **Methods** section (*Sample processing*) was incorrect, and we have updated the callout accordingly.

[C9d]: More details of the sequencing itself for the scWGS would be helpful – for example what read length, single or paired end.

[R9d]: We have included additional details in the **Methods** section (*Library preparation and sequencing*), specifying that scWGS was performed at the MSKCC Integrated Genomics Core on Illumina NovaSeq 6000 using paired-end 150-bp reads.

[C9e]: The paragraph about the spontaneous RPE1 subclone with WGD in the methods (lines 765-769) may be more appropriate in the results section of the manuscript.

[R9e]: We removed some interpretive text from this **Methods** section, although we retained several details that we feel are too technical for the main **Results** section.

[C9f]: Some of the bioinformatics tools used do not have citations and the version used is not described (eg bwa-mem, Picard).

[R9f]: We have updated this **Methods** section (*Alignment*) to cite missing publications describing each software tool. We have also indicated the relevant software versions (bwa v0.7.17, Picard v2.27.4).

[C9g]: In the alignment section, the authors mention that local indel realignment can be performed if required. Was it required and if so for which samples?

[R9g]: This was not required and as such, we removed this section from the manuscript.

[C9h]: With regards to the haplotype-specific copy number section, more detail is needed for this section to be reproducible, for example is only a single matched normal used or one for each patient and is that bulk WGS data?

[R9h]: A matched bulk normal WGS sample for each patient was used to infer haplotype-specific copy numbers. The **Methods** section *Haplotype-specific copy number* now begins: *"In a bulk WGS matched normal sample for each patient, we measure…"* The initial methodology for allele-specific analysis is fully described in Funnell et al Nature 2022 (ref#7) in the SIGNALS algorithm.

[C9i]: If possible consider having the cell filtering steps in the methods and the supplementary note in the same order.

[R9i]: We updated the order of the cell filtering steps in the **Methods** to match the **Supplementary Note**, which is also the order in which they were performed.

[C9j]: With regards to the filtering of the S-phase cells – as custom thresholds have been utilised for each patient without the thresholds being provided this section is currently not reproducible.

[R9j]: The table of S-phase thresholds used to filter cells for this publication has been added as **Supplementary Table 4.**

[C9k]: Are the results comparing the bulk WGS copy number inferred by FACETs to the scWGS provided? If not please include.

[R9k]: Yes, we have now included comparisons between scWGS-derived mean ploidy and compared to bulk WGS in **Extended Data Fig. 2G**. Similarly to the comparison between scWGS and MSK-IMPACT **Extended Data Fig. 2F** that was already included, the values are highly concordant. Unsurprisingly, the bulk WGS concordance to scWGS is much higher than that of FACETs-inferred ploidy estimates from MSK-IMPACT, which are expected to be less precise due to the hybrid-capture and panel-based approach of MSK-IMPACT.

[C9l]: In the section on detecting WGD in single cells is a reference to Ext Data Fig 3H,I – is this correct or meant to be Ext Data Fig 2?

[R9l]: Yes, this **Methods** section refers to **Extended Data Fig. 2H,I**. The figure reference has now been corrected in the text.

[C9m]: In the patient level WGD classifications what is meant by cells classified as "1WGD>1"?

[R9m]: This was a typo that has been corrected. The related text now reads: *"Patients were classified as WGD-high if the fraction of cells with at least 1 WGD…"*

[C9n]: For subclonal WGD classification – please clarify what fraction of cells was used as the cutoff for comprising 'the majority population', and is this different to the 25% mentioned for patients 081 and 125 with 0x WGD?

[R9n]: Instead of introducing new definitions for this classification, we now apply the patient-level evolutionary categories to the classification of clones: Truncal WGD clones are those clones in Truncal WGD patients (i.e., WGD clones representing all tumor cells), and Subclonal WGD clones are those clones in Subclonal WGD patients (i.e., WGD clones that coexist with 0xWGD cells). Please refer to the newly added Glossary of Terms (**Supplementary Note** section 1**)**.

[C9o]: In the SNV calling section please clarify what is mean by "variant loci that are detected by either caller" when only the Mutect2 tool is described as being run.

[C9o]: We ran Mutect2 for SNV variant calling, which we favored over a consensus variant calling approach with MutationSeq and Strelka. We have updated the text in the **Methods** section to reflect that only Mutect2 is used in our analyses.

[C9p]: In the section Enumerating events on ancestral branches please confirm if this sentence is correct as written as the pre-WGD and post-WGD text appears to be the same. "Events were classified as non-WGD if they were predicted to occur on the root branch of a Rare WGD patient, pre-WGD if they were predicted to occur prior to the WGD event on the root branch of a Prevalent WGD patient, and post-WGD if they were predicted to occur prior to the WGD event on the root branch of a Prevalent WGD patient."

[R9p]: Thank you for catching this. The original text was incorrect, we modified the post-WGD text to clarify that events were classified as *"post-WGD if they were predicted to occur after the WGD event on the root branch".*

[C9q]: In the section on WGD classification of the single-cell RNA sequencing, please provide the data showing that the copy number profiles are highly concordant to the scWGS as Extended Data Fig 5A is a UMAP of the scRNAseq data and does not appear to refer to copy number profiles. If this is meant to be Ext Data Fig 6A please consider also providing additional information – either showing some copy number plots or a heatmap for each patient. Also, is the reference to Extended Data Fig 5B in this section correct?

[R9q]: We have added the inferCNV copy-number heatmaps from the scRNA-seq data for each patient to an html visualization showing the scWGS copy number, MEDICC2 trees, SBMClone results, and doubleTime results. See supplementary file spectrum-tree.html.

The figure callout in this section was referring to what is now **Extended Data Fig. 7A**, which has been corrected in the text.

[C9r]: In the Immunofluorescence section, please provide more detail on how the ROIs were defined – ie how were regions of tumor foci identified? Will all of the raw images and the segmentation model for identifying the micronuclei be made available?

[R9r]: We have substantially improved our approach to define ROIs. ROIs are defined as tumor-rich regions. In our initial submission, ROIs were identified based on histological patterns and nuclear morphology, reflected in the DAPI signal in 2-color IF images (DAPI, cGAS) and the IF-adjacent H&E image. In our revised manuscript, we have reacquired 6-color IF images (DAPI, cGAS, STING1, panCK, CD8, p53). These include panCK as an epithelial marker and p53 as a tumor-specific marker in addition to DAPI. We have provided additional details in the **Methods** as suggested by the reviewer (Line 1304-1313). The raw images and segmentation model will be made available prior to publication. We have also added a new table (**Supplementary Table 5**) that includes the MN rates per slide, the MN rates per ROI, as well as summary statistics of nuclear morphology and marker intensity per slide and per ROI.

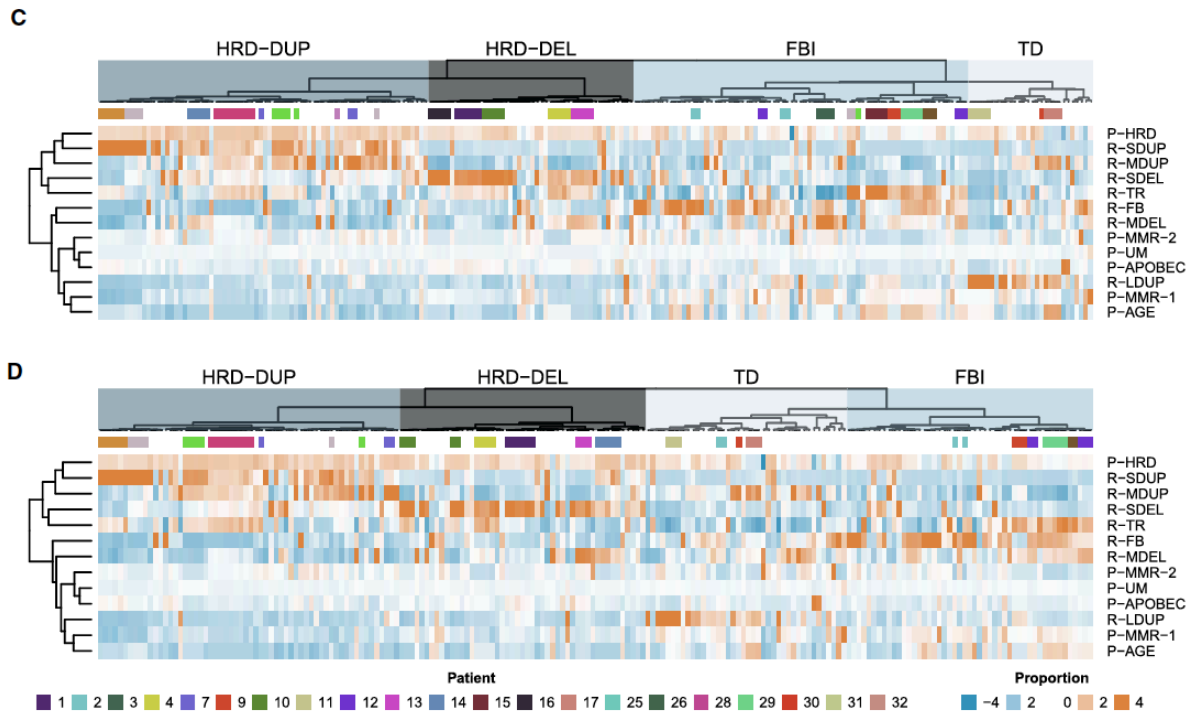
[C9s]: Some of the information in the section on the identification of WGD subclones in the RPE1 line may be more appropriate in the results than the methods.

[R9s]: The main results from the RPE1 cell line analysis are a) the relationship between experimental conditions and chromosomal instability, and b) analysis of WGD vs. non-WGD scRNA cell populations. The **Methods** section describes technical details that facilitate this analysis (identification of CNAs that characterize cells as non-WGD, and a description of how this enabled us to separate cell populations in scRNA analysis) but do not add to the claims presented in **Results**. In our opinion, the text describing the details of the RPE1 analysis would likely be less interesting to readers than much of the remaining text. We note in this section we have now added the FNE-1 *in vitro* results which we think further increases the need to succinctly describe these results in the main text.

[C10]: Results

[C10a]: Ovarian cancer patient cohort section – consider clarifying whether the mutations and mutational signatures shown in Ext Data Fig 1B are seen in all tumour sites from a patient or if any limited to a single site.

[R10a]: The reviewer raises a key question. We have previously studied mutational signatures using WGS across multiple sites in HGSOC and found that major subtype assignments remain stable between sites from within each patient (**Rebuttal Figure 13**) (Zhang et al., *Cell*, 2018).



Rebuttal Figure 13. Fig S6 from (A. W. Zhang et al. 2018) above indicates conservation of signature subtype using the MMCTM on multisite WGS. **C)** Standardized proportions of each mutation signature for multisite HGSC, OV-AU, and HGSC Wang et al. (2017) samples, showing clustering of samples from the same patient. **D)** Standardized proportions of each mutation signature for multisite HGSC, OV-AU, and HGSC Wang et al. (2017) samples, where only non-ancestral mutations were considered for the multisite HGSC cohort. Heatmap-values were clipped between -4 and 4.

[C10b]: A figure or table comprising the number of cells per sample before and after filtering, and the scWGS coverage range per sample would aid in interpreting the results, particularly with regards to the patients that had >200 cells. This table could also include the number of cells per sample of each WGD state which is hard to interpret from Fig 1G.

[R10b]: We have added a new table (**Supplementary Table 3**) which includes sample-level metrics for scWGS datasets, including the suggested metrics (numbers of cells before and after filtering, and the number of cells in each WGD state).

[C10c]: Considering that cell size correlated with WGD in the DLP+, did the authors examine if nuclei size in the IF correlated with the WGD status from the scWGS?

[R10c]: Yes, tumor nuclei sizes were significantly larger for WGD-high tumors. We now include this result as **Fig. 3H** (reproduced below).

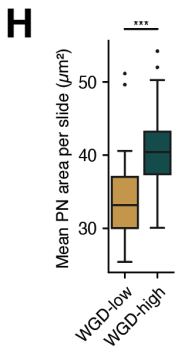


Figure 3. H. Mean nuclear area of each slide, comparing slides for WGD-high vs WGD-low tumors. Significance of WGD-high vs. low calculated using a GEE model with patients as groups is annotated as per **F**.

[C10d]: Please consider adding a rationale for classifying cases as having 'prevalent WGD' when 50% of cells have a WGD. Did the authors consider classifying samples as high, intermediate or rare WGD rather than the 2 categories?

[R10d]: As shown in **Figure 1G** (uppermost bar plot below the scatterplot), the cohort is bimodal: patients have either very few WGD cells (<15%) or consist mostly of WGD cells (>95%) with the exception of two patients each with an expanded WGD clone (OV-081 and OV-125). In our opinion, the binary classification between patients with and without a large WGD clone is a better description of the biology of the cohort than a ternary classification that separates two patients into an additional class. To make the distinction clearer, we added this reasoning and changed the text to use 15% and 50% (**Results** Line 167-171):

> *As 39/41 patients' tumors were dominated by a single WGD state (>85% of cells), we dichotomized each tumor as either WGD-high: harboring >50% ≥1×WGD cells (27/41 patients); or WGD-low: with <15% ≥1×WGD cells (14/41 patients). The 2 tumors with intermediate (50-85%) ≥1×WGD cells were grouped with WGD-high as they had large WGD clones."*

[C10e]: The terminology around classification of tumors or patients as having WGD as currently written is confusing (lines 161 – 164). Initially the text states that each tumor was dichotomized, then only referring to the number of patients with prevalent WGD (ie 40 patients not 65 tumors). On lines 163 and 164 – the authors state that 26/40 patients have been classified as prevalent WGD, then state 60% are classified as having prevalent WGD, and on line 176 23 out of 25 prevalent WGD patients are mentioned – which is correct?

[R10e]: Lines 163-164 in the original manuscript were correct in stating that 26/40 patients are classified as WGD-high (formerly Prevalent WGD). With the newly added WGD-high tumor 087, bringing the total to 27/41, we have corrected the text to indicate that this corresponds to 66% of patients. On line 176 where we mentioned 23 out of 25 WGD-high tumors, this was referring to the 25 WGD-high tumors for which we were able to run doubleTime. We were unable to run doubleTime on OV-024 which has a clonal 2xWGD, though we apologize for not making this

clear in the manuscript. We have added a note in **Results** (Line 187-188) which we elaborate on in **Methods** (Line 1031-1033):

> *Patient OV-024 was excluded because its clones are predominantly 2×WGD, which is not supported. Patient OV-125 was excluded due to low cell counts (no SBMClone clone with at least 20 cells).*

Throughout the manuscript, we use the term “tumor” as shorthand for a patient’s cancer rather than a specific anatomical site, in line with common usage in the field.

[C10f]: Were there any cases with multiple tumors profiled where sites would have been classified differently if the classification was done at a per sample rather than per patient level?

[R10f]: The only patient whose samples would be classified different from each other if they were each processed independently is OV-081: the infracolic omentum sample has 93% ≥1 WGD cells which would be classified as WGD-high, whereas the left adnexa sample has 99.5% nWGD cells which would be classified as WGD-low (**Supplemental Fig. 20**). This is now mentioned in Supplemental Note section 3.2.

[C10g]: The use of two terminologies regarding the WGD is not clear – do all prevalent cases have clonal WGD and rare cases have subclonal WGD? Please consider either expanding the definition of and the differences between clonal/subclonal and rare/prevalent or using only one terminology.

[R10g]: We appreciate the reviewer’s criticism and we have updated the terminology we use to describe both WGD prevalence and WGD evolution in the manuscript. Both of these classifications are necessary, as the two do not co-segregate in our cohort. However, we no longer use the additional terms “dominant” and “clonal” which were previously analysis-specific classifications.

- We refer to patients as either “WGD-low” or “WGD-high” depending on the prevalence of WGD cells among their tumor cells (**Fig. 1** and related text).
- We refer to patients as Truncal WGD, Parallel WGD, Subclonal WGD, or Unexpanded WGD depending on the number and size of WGD clones detected (**Fig. 2** and related text).

To clarify the differences between these classifications, we include a Glossary of Terms (**Supplementary Note** section 1).

[C10h]: Was there statistics performed for Ext Data Figure 2M-P? Please include in text or figure.

[R10h]: These panels are now **Extended Data Fig. N-Q**. We have added the p-values for independent t-tests to **Extended Data Figure 2N-O**.

[C10i]: On line 169 please consider stating which cases did not have multiple WGD states, as it is very difficult to determine from Fig 1G.

[R10i]: This was not precisely described in the text previously. We observe at least 2 co-existing WGD states in 40/41 patients. We have updated the statement in the text accordingly and included a callout to **Fig. 1G** (Line 162-163):

> *"Surprisingly, tumors with co-existing WGD states were present in 40/41 patients (**Fig. 1G**, see **Supplementary Note** for further description of WGD state heterogeneity)."*

[C10j]: For the section on the evolutionary histories, please clarify if doubleTime can time the second WGD and if so why there is only a single orange triangle shown in Fig 2B even in cases with 2 WGD. Please also clarify how the cases are split into Fig 2B, C and D in the figure legend. If no orange arrows are shown in Fig 2D because there are not sufficient cells with the WGD to time the event, please consider describing this in more detail in the methods or the limitations section of the discussion.

[R10j]: To clarify, doubleTime does not infer timing for successive WGD events as they typically lack sufficient cell numbers. The trees previously presented in **Fig. 2B-D** are now all presented in **Fig. 2B** and are grouped and labeled by evolutionary category as described in the text (Line 187-200). WGD events are labeled with orange arrows, including multiple WGD events for Parallel WGD patients and within-clone WGD events for some Subclonal WGD patients with WGD clones that were too small to be detected by SBMClone/doubleTime. The only patients that lack an annotated WGD event in **Fig. 2** are those for which we could not identify a WGD clone (i.e., Unexpanded WGD patients).

[C10k]: Are theVAF plots shown in Fig 2C available for the other patients?

[R10k]: We have added the VAF plots for all WGD-high tumors with sufficient data as **Supplementary File 1**.

[C10l]: On line 180 the authors refer to Ext Data Fig 3A-B after mentioning patients 025 and 045 – however the figure shows patients 045 and 075. Please clarify in the text or figure legends why certain cases are discussed/shown in the Figures or have the figures match the text.

[R10l]: This was a typo. We had meant to say patients 045 and 075 in the text. We have corrected the error.

[C10m]: Lines 192 – 194 require some clarification: I'm not sure that the Gerstung paper found all ovarian tumors had early WGD, and a recent Nature Communications paper (Burdett et al) described 24 of 36 HGSC cases in the Gerstung paper as having 'late' WGD. Additionally, the authors state that "later WGD clonal expansions … were common, with 7/25 WGD events" – 7 out of 25 is less than 30% so is this really common? Furthermore, consider indicating in the text or a figure which are the 7 cases with late WGD as it is not easy to determine which cases they are from Fig 2B.

[R10m]: We agree with the reviewer that our previous framing of the results did not correctly describe how our results improve upon those of previous studies. We now emphasize the ability of single cell techniques to capture the full continuum of WGD evolution. This includes resolution of mixtures of WGD subpopulations and the identification and timing of WGD subclones within a tumor. Both of these aspects of our results would be difficult if not intractable with bulk data. We have adjusted the text to more accurately describe how our results improve upon previous WGS studies. In addition, we now indicate late and early WGD events with specific markers in **Figure 2.**

In Burdett et al., the principal classification of patients (Fig. 1 of their paper) is between WGD (50/79) and non-WGD patients (29/79), similarly to the Gerstung paper (118 WGD and 48 non-WGD). They proceed to separate WGD patients into early and late WGD. This analysis has the same limitation as a binary indicator of WGD, as it cannot capture the features of ongoing WGD: individual WGD cells, small WGD clones, and the general co-existence of cells with different WGD states. Additionally, we are able to further characterize the cell-to-cell variation within different mixed WGD contexts.

[C10n]: For the three evolutionary modes – do they relate to panels Fig 2 B, C and D? If not could an annotation be added to the figure as to which mode each patient fits in? Did the authors examine if there were any clinical or molecular characteristics of the patients that are associated with the WGD evolution mode?

[R10n]: Yes, the three evolutionary modes relate to **Fig. 2B** (formerly **Fig. 2B-D**), and the revised version of **Fig. 2B** includes annotations of the patient evolutionary categories to match the descriptions in the text. Unfortunately associations between WGD evolutionary mode and clinical and molecular characteristics are difficult due to limited numbers. We have thus focused our analysis of the broadest categorization, WGD-high vs WGD-low. However, we do use the WGD timing to understand the relationship between WGD age and both divergent cell fraction and amount of loss post-WGD. In addition, we contrast the amount of post-WGD loss between truncal and subclonal WGD in order to understand the timing of post-WGD losses.

With respect to clinical outcomes, we have executed another study in a subset of these patients with longitudinal clonal tracking in timeseries cfDNA. The results of that study are out of scope of this manuscript, but will be linked to this paper for the benefit of the readership. An initial preprint of that study is available here: https://www.biorxiv.org/content/10.1101/2024.08.21.609031v1.

[C10o]: Are the 8 patients with highly divergent cells (line 224) listed or depicted anywhere? Is it possible to comment on whether the highly divergent cells had one or two WGD, or were there any associations with the number of divergent cells and clinical or molecular features? Can the highly divergent cells be identified in the scRNAseq data?

[R10o]: We now include information about the number of divergent cells in each patient in **Supplementary Table 3**. As the reviewer suggests, we find that divergent cells are more likely to be classified as additional-WGD and have added this observation to the manuscript (see **Extended Data Fig. 4G**). We now also comment towards the end of that paragraph (Line 241-242) on the relationship between fraction of divergent cells and the age of WGD events in the WGD-high tumors:

> *"Furthermore, the fraction of divergent cells was highest in late-WGD tumors and decreased with age of the WGD event(s) (**Extended Data Fig. 4G**)."*

With regard to identification of these cells in scRNA, we think this is a very interesting future area of exploration, as understanding the transcriptional profile of highly divergent cells may help uncover mechanisms of adaptation to genomic stress, or vulnerabilities of cells in these distinct states. However, given the high level of noise in scRNA-derived copy-number profiles (due to both technical limitations and variability in gene expression) combined with the sparsity of these cells, we feel this presents a large technical hurdle that goes beyond the scope of our current analysis. Future methods that can produce high quality WGS and RNASeq from the same cell at scale will enable this type of analysis.

[C10p]: Why has the non-ploidy adjusted copy-number change plot been shown in Fig 3F and the adjusted data in Ext Data Fig 4G when most of the text regarding these results focus on the ploidy adjusted data?

[R10p]: We now show only the ploidy-adjusted data in **Figure 3** and have removed the non-ploidy adjusted rate figure. The main point we wish to illustrate with **Figure 3F** is that even correcting for ploidy, copy-number events occur at higher rates in WGD cells, indicating a change in chromosomal instability that is beyond what is expected given higher ploidy. When we correlate cell-specific copy-number changes with scRNA measurements, we use the non-ploidy-adjusted rate because any mis-segregation may impact cellular phenotype.

[C10q]: With regards to the comment on line 287 that the mutational processes that generate the HL amps might be increased in prevalent WGD cases – is it possible to examine the abundance of copy number signatures such as those described by Macintyre and colleagues (Nature 2018) in these cases in the scWGS or bulk WGS to provide further evidence for this statement?

[R10q]: We have removed results pertaining to HL amps in favor of the new data we have added such as the improved IF, the FNE-1 analysis and an overall streamlining of the text. We are preparing a separate manuscript with a highly detailed pan-cancer exploration of the evolution of HL amps from DLP+ (to be submitted).

[C10r]: With regards to categorising the CNA as pre- or post-WGD (lines 298-300) please consider explaining why no pre-WGD events were sought in the prevalent WGD cases, especially in cases with late but prevalent WGD.

[R10r]: Lines 291-294 (previously 297-300) included a typo – the sentence now reads:

> *"These events were divided into those inferred to occur (i) after WGD in the ancestral branches of WGD-high tumors (post-WGD) (ii) before WGD in ancestral branches of WGD-high tumors (pre-WGD) or (iii) on the ancestral branches of WGD-low tumors (non-WGD)."*

[C10s]: It is unclear why the focus of the discussion of the result in Fig 4B (lines 303-305) is on the gains when the losses are also significantly different but with higher counts than the gains.

[R10s]: We have reworked this section and now focus on gains, losses, and the ratio between the two.

[C10t]: Why are different thresholds used for calling events clonal – line 278 describes >90% for HL amps, line 309 states >95% for post-WGD events.

[R10t]: We have removed the section analyzing HL Amps from the **Results**, leaving this topic for more thorough study in future work.

[C10u]: Figure 3 shows that cells with WGD in the rare WGD cases do have CNA etc, so perhaps consider rephrasing the statement in lines 289-292 as it does not appear that it is only the cases with prevalent WGD that have genomic diversification.

[R10u]: We respectfully disagree with the reviewer. We believe that this sentence accurately describes the fact that we observe increased rates of several measures of genomic diversification in WGD-high tumors. This statement does not preclude genomic diversification in WGD-low tumors.

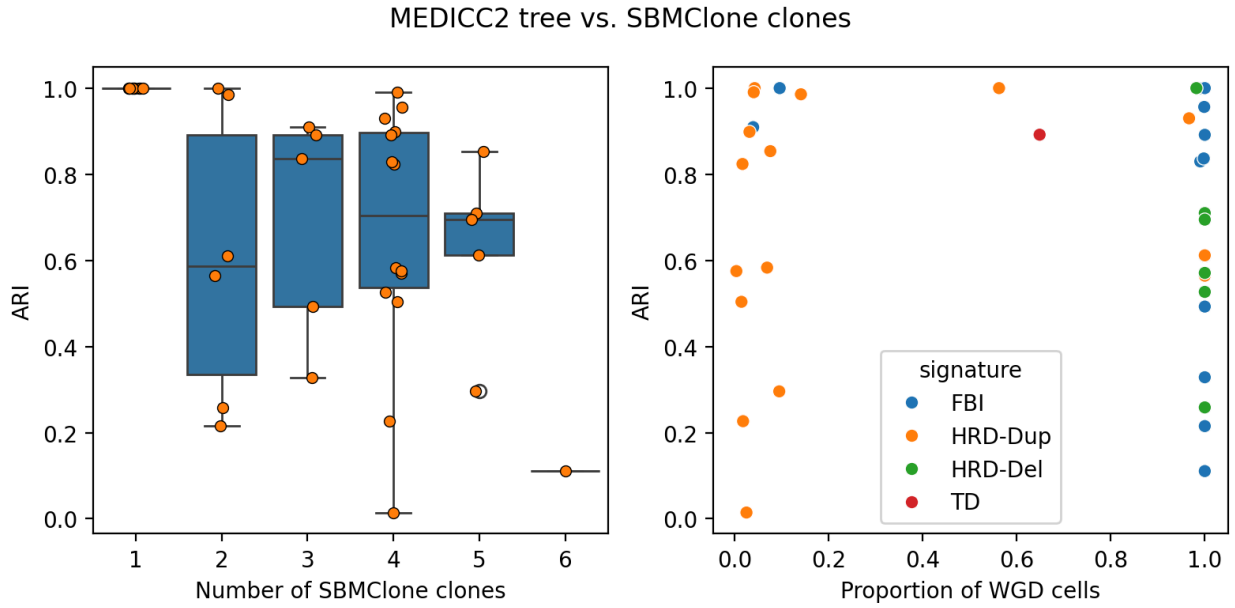
[C10v]: Line 318 refers to Ext Data Fig 4J – is this a mistake, as the version of Ext Data Fig 4 that I received only goes up to panel I.

[R10v]: The panel that this text refers to is now **Extended Data Fig. 4K** (not 4J). The figure reference has now been corrected in the text.

[C10w]: Do MEDICC2 phylogenetic trees look similar to the doubleTime trees?

[R10w]: The doubleTime trees are on SBMClone clones, while the MEDICC2 trees are on individual cells. Broadly speaking, the SBMClone clones closely match the MEDICC2 trees for some patients while for others they show little relationship. This is likely due to the uncertainty in the MEDICC2 CNA-based phylogeny for patients with little clonal structure and high cell-to-cell variability in copy number. To quantify this, we quantitatively compared the MEDICC2 trees to the SBMClone clustering. We used a heuristic algorithm to find the ordering of the children of each node in the MEDICC2 tree that maximizes the agreement between the resulting ordering of leaves and the sorted SBMClone clustering: at each node, we computed the average cluster ID of each subtree and moved the subtree with a lower average cluster ID to the left-hand side.

This approach aims to produce the sorting of nodes allowed by the tree with the most ordered clone assignment. We found that among patients with >1 SBMClone clone, the Adjusted Rand Index (ARI) between the sorted tree and sorted SBMClone clustering had a mean of 0.64 with a standard deviation of 0.29, indicating a moderate consistency between the two objects (**Rebuttal Fig. 15A**). We found no meaningful relationship between ARI and the number of SBMClone clones, mutational signature, or the proportion of WGD cells (**Rebuttal Fig. 15B**).



Rebuttal Figure 15: Comparison between sorted MEDICC2 trees and SBMClone clones via ARI. Both y-axes show ARI between the ordered tree leaves and the sorted SBMClone clustering, left x-axis shows the number of SBMClone clones, and the right x-axis shows the overall proportion of WGD cells.

[C10x]: A substantial amount is made regarding the findings of a single cell with a distinct copy number profile in one patient (lines 319 – 324, Fig 4E). Please consider moderating the language of the conclusions made regarding this cell/case. And also provide further information regarding the case shown in Fig 4F – for example how many cells have these different events?

[R10x]: We have removed this figure in order to streamline the paper.

[C10y]: There do not appear to be statistical tests performed for many of the statements and figure panels of the single cell RNAseq – please add.

[R10y]: We note that our initial submission contained statistical tests for all panels in **Fig. 5** and **Fig. 6** where warranted, however we made additional references to these tests in the relevant figure callouts to make this clearer. **Fig. 5B-C** contains qualitative data that did not warrant statistical testing, and **Fig. 6E** is paired with statistical testing in **Fig. 6F**.

[C10z]: Line 361 refers to Ext Data Fig G – is this 6G? And line 382 refers to Ext Data Fig 5C – should this be 6C?

[R10z]: These callouts have now been corrected to refer to **Extended Data Fig. 7E** and **Extended Data Fig. 7G** in the revised manuscript.

[C10aa]: Please consider adding additional relevant references regarding immune evasion associated with WGD in HGSC on line 371 – the Burdett et al Nature Communications paper seems particularly relevant but there may be others too.

[R10aa]: We have incorporated additional inline references in lines 351-353, highlighting concordant observations regarding immune evasion properties linked to WGD in HGSOC, such as MHC-II downregulation observed by Burdett et al. in cancer cells of non-WGD cases.

[C11]: Discussion

[C11a]: Lines 396-397 – it is unclear if this is referring specifically to the first WGD or any WGD – but there did not appear to be evidence presented regarding the timing of the second WGD. Considering that the second WGD seem to be subclonal doesn't this suggest that there are co-existing clones with WGD that developed at different times?

[R11a]: We realize that the language in lines 396-397 of the original manuscript was vague, and have revised this statement in the updated **Discussion** section (Line 435-437):

> "Intriguingly, in tumors where we observed parallel WGD events, these events occurred at approximately the same time in the evolutionary history of the tumor."

To elaborate: this text, as in the rest of the manuscript, primarily draws a distinction between cells with 1 WGD and cells without WGD. Analysis of cell-specific copy number suggests that the first clonally expanded WGD event is associated with a significant shift in chromosomal instability. When we dichotomize tumors into those that contain at least 1 expanded WGD clone (WGD-high) versus those that do not (WGD-low) we see significant microenvironmental and cell-intrinsic differences (**Figure 5-6** and related text). Thus we hypothesize that the first WGD clone is a significant event in the evolutionary histories of these tumors, and that once a tumor has transitioned to a WGD-favourable selective landscape, additional WGD clones are possible but long term persistence of a non-WGD population is less unlikely.

[C11b]: Please consider comparing your results around CNA following WGD with the Baker et al GRITIC paper.

[R11b]: We have added a comparison to the Baker et al. results to **Results** (Line L294-296):

> *We found that gains of chromosomes and arms were rare in general, though chromosome gains were significantly more numerous post-WGD compared to pre-WGD, similar to previous results (Baker et al. 2023) (**Fig. 4B**).*

[C12]: Figures

[C12a]: Figure 1) Have the copy number and BAF plots in panels D,E been swapped - the 1xWGD plot looks to have more copy 2 regions than in panel D for 0xWGD. The Fraction

Subclonal WGD at the bottom of panel G has not been described in the legend – is this the first or second WGD?

[R12a]: Yes, the 0xWGD and 1xWGD labels and their corresponding panels in **Fig. 1D and 1E** were flipped. This has now been corrected.

[C12b]: Figure 2) The legend for panels B-D states that “Each patient is annotated with mutation signature and age at diagnosis” – where is this shown?

[R12b]: The legend for **Fig. 2B-D** was outdated and referred to labels that are not used in the figure. We have updated the figure legend to reflect this.

[C12c]: Figure 4) In panels B and D – how can the count of CNA be less than 1 and what do the error bars represent?

[R12c]: The figure shows the distribution of counts for branches in each class and for many branches the count is 0 leading to a mean count less than 1. This has been clarified in the figure legend.

[C12d]: Figure 6) Please add a colour legend for panels C and D and a description of the line in the plot, for the x-axis of panel F is the direction of the differences the same as in panel A? In panel E please add the direction of the fold change ie is red higher in prevalent WGD?

[R12d]: We have added a description of the colors and regression lines to the figure legend for **Fig. 6C-D**, as well as additional text within the figure to denote the direction of the differences in panels **H** and **I** (formerly E and F), which indeed are consistent with the direction of the differences in **panel A**.

[C12e]: Extended Data Figure 1) What is meant in panel B legend for mutational signature undetermined as there do not appear to be any grey boxes in the oncoprint?

[R12e]: The mutational signature category “Undetermined” is unused, as we were able to successfully infer a mutational signature from bulk WGS and/or scWGS for each tumor. We have removed this category from the legend in **Extended Data Fig. 1B**.

[C12f]: Extended Data Figure 2) Please consider splitting/coloring the bars in panel A by sample not just patient. Please add details regarding the line and shading to the legend for panels F and G, and also add the correlation and p-values. What is meant in the legend for panel P about the “Ovarian Metacohort”? Consider changing the colors in panels N and P for non-WGD and WGD so they are distinct from prevalent and rare WGD. Does panel L only show the 2xWGD cells in patients with predominantly 1xWGD cells or does it also include the 1xWGD cells in patients with predominantly 0xWGD cells?

[R12f]: We now show a stacked bar plot for **Extended Data Fig. 2A** that splits counts by sample as suggested.

For **Extended Data Fig. 2F**,**G**, we now explain in the legend that the dashed line denotes the linear fit and the shading indicates the confidence intervals. We have also added Spearman's rank correlation coefficients and p-values to the plots.

The Ovarian Metacohort is a collection of WGS sequenced HGSOC as previously described in Funnell et al., (Funnell et al. 2019) and Funnell et al (Funnell et al. 2022). We have added the first citation in the figure legend.

We have updated the colors for panels N and P to differentiate non-WGD and WGD (bulk measurements) from WGD-low and WGD-high.

Extended Data Fig. 2M shows subclonal WGD cell fractions defined as the fraction of 1xWGD in patients with predominantly 0xWGD cells, and the fraction of 2xWGD cells in patients with predominantly 1xWGD cells.

[C12g]: Extended Data Figure 3) The legend for panels A-C states "0xWGD subpopulations" are shown but the y-axis of these graphs suggests both non-WGD and WGD clones are shown. Please provide the color legend for the CN and BAF plots as well as the tumor site.

[R12g]: We have updated the figure legend for **Extended Data Fig. 3A-B** (only 2 patients are shown in the revised version of the figure) and added color legends.

[C12h]: Extended Data Figure 5) Please clarify why there are cells of undetermined WGD class in panel A – if they are from patients where no scWGS data is available why are they shown? Panel F does not appear to be referred to in the text, it would also be interesting to know if the different patterns are associated with the proportion of cells with WGD or the number of WGD detected. It is unclear what the data points in panel G represent – are the points each patient?

[R12h]: There are tumors for which we were able to generate scRNA-seq data but no scWGS (OV-042, OV-053, OV-112, OV-116). These scRNA-seq datasets were previously reported in Vázquez-García et al, *Nature* (2022) together with cases that do have both scRNA-seq and scWGS.

Each individual data point in **Extended Data Fig. 5G** represents a tumors sample. This has now been described accordingly in the panel legend.

[C13]: Data availability – the Synapse site does not mention the scWGS or IF data described for the first time in this manuscript.

[R13]: The scWGS and IF datasets are now referenced on the Synapse site. At the present time the links provided are placeholders. Data will be made available prior to publication.

[C14]: The code availability statement only refers to tools, but does not include code for analysing the IF data or generating any of the figures.

[R14]: We have uploaded the code used to generate the figures used in the paper to a public repository: https://github.com/shahcompbio/spectrum_wgd_paper.

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Response to reviewers

Referee #1 (Remarks to the Author):

In their revised manuscript, the authors addressed most of the comments. This reviewer has only one remaining issue regarding the response to [C1].

[C1] The authors try to show the independent effect of WGD and mutational signature using linear models. However, this reviewer is not fully convinced the conclusion. Regardless of the methods/models used, there is a limitation in the statistical power for such a small number of cases (27 WGD-high tumors and 14 FBI tumors), for which these models might not be reliably used to separate the effects of WGD and FBI. So it would be safe to attenuate the statements regarding the effects of WGD-high.

We share the reviewers' concern and believe we have been sufficiently circumspect in our statements. For instance on line 289 we state: “The decrease was statistically significant for the cohort as a whole (**Fig. 5a**) and for the HRD-Dup subset (**Extended Data Fig. 7a**) with similar trends for the FBI subset (**Extended Data Fig. 7b**), suggesting that the impact of WGD on cell intrinsic immuno-phenotypic signaling may be independent of mutation signature.”

Referee #2 (Remarks to the Author):

The authors have done an excellent job of addressing my comments and improving the manuscript. I have included a few additional comments:

- I appreciate the simulations to show that the WGD heuristic is likely accurate. Although I think a more accurate simulation should include lots of losses before any WGD (apologies if I missed this). This is where things can be a bit trickier.

The simulations that we used to validate estimation of missegregation rates, on which the WGD heuristic showed an accuracy of 100%, indeed allow for both gains and losses before and after WGD events. Additionally, the omission of losses from the simple model of chromosomal gains was intended to err on conservative side, as in that model losses would only increase the number of independent copy-number events required before the WGD heuristic would falsely identify a false positive WGD cell.

- I agree that in most cases all chromosomes suggest a similar timing of WGD. However, there are a few notable exceptions (e.g. in OV-044 and OV-052). Does this reflect earlier copy number events, or suggest that it isn't really a single whole genome doubling event?

For most patients, apparent outliers in chromosome-specific WGD timing are on chromosomes with very few SNVs in which the bootstrap approach underestimated the variance, e.g., OV-003

chromosome 6 with 6 SNVs, OV-044 chromosome 11 with 12 SNVs, and OV-083 chromosome 14 with 12 SNVs. The only clear exception is OV-052 chromosome 18 which has an unusual enrichment of VAF=1 SNVs on the chromosome 18. For all tumor cells, most of this chromosome is LOH (i.e., minor copy number 0) and with major copy number 2. DoubleTime assumes that this results from a pre-WGD state of 1 major copy which is then doubled. However, the abundance of VAF=1 SNVs suggests that this chromosome was affected by post-WGD copy-number events much *later* than the WGD which affected the rest of the genome – for example, one copy of the major haplotype may have been lost and the other duplicated, resulting in a large number of VAF=1 SNVs.

Few additional comments:
WGD-high tumors comprised 66% of the cohort, were in older patients, and were enriched for FBI and HRD-Del mutation signatures, consistent with previous bulk genome sequencing studies10,18 173 (Extended Data Fig. 2N-Q). --> worth defining what 'older' is here.

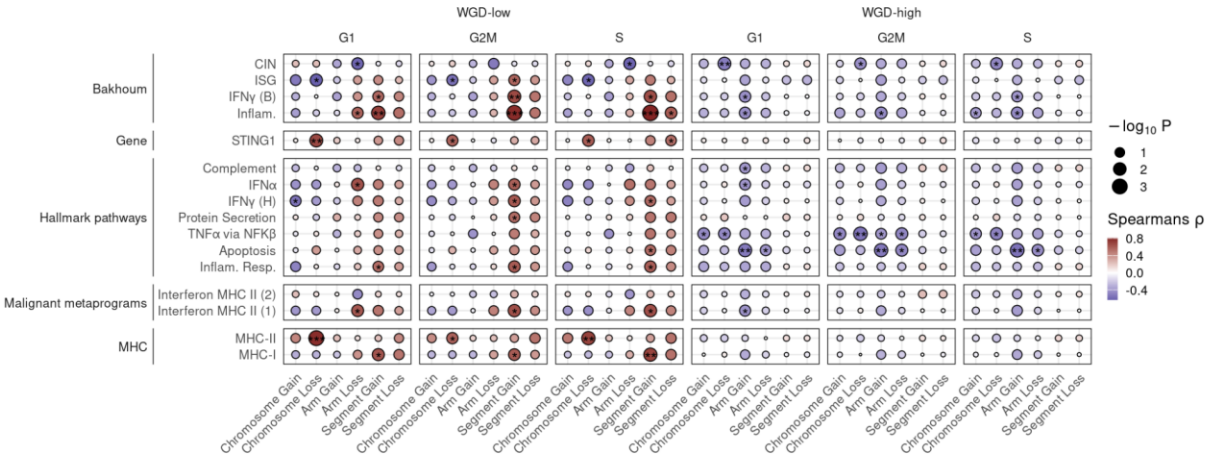
We have updated the text and now describe WGD patients as ‘significantly older’.

Was there any association with the treatment regime and likelihood of observing WGD (and type of WGD?)

This is a great question, and we are also interested in studying the relationship between WGD and treatment failure. We are presently accruing post-treatment samples to achieve a sufficiently large cohort size to study this question in future follow up work. The only answer we can provide at present is that since WGD-low tumors are more frequent in HRD-Dup in our cohort, WGD-low tumors are more likely to be treated by Parp inhibitors.

Can the authors further disentangle whether the decoupling of CIN and immune related programmes is entirely independent of the association with cell-cycle?

We partitioned the G2M, S and G1 cells identified in scRNA and repeated the correlations between scWGS derived CIN rates (at the whole sample level) and immune related signaling subset by cell cycle phase. Overall, the observed correlations, and in particular the decoupling of cell intrinsic immune signaling and CIN rates in WGD-high versus WGD-low tumors, were highly consistent across cell cycle phases.



Referee #2 (Remarks on code availability):

I have no issues with the code.

Referee #3 (Remarks to the Author):

The authors have provided a very comprehensive response to all of the reviewer comments which has improved the manuscript and I have no further comments.