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Epistasis, aneuploidy, and functional mutations underlie evolution of resistance to induced microtubule depolymerization

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st - Editorial Decision 26th Apr 2021

Thank you again for submitting your manuscript on resistance evolution against microtubule depolymerization for our editorial consideration. It has now been reviewed by three expert referees, whose comments are copied below. As you will see, all referees acknowledge the interest of your findings in principle, but also note several experimental and presentational aspects of the work that would need to be improved prior to eventual publication.

Should you be able to decisively address these various issues, in particular the main points of referee 3, we would be interested in pursuing a revised version of this work further for EMBO Journal publication. Since it is our policy to consider only a single round of major revision, it will however be crucial to comprehensively respond to all the points raised at the time of resubmission. In light of the present pandemic-related disturbances and their affect on lab work, I would be open to extending the revision period beyond the default three-months time frame if needed to carefully complete such revision; with our 'scooping protection' (meaning that competing work appearing elsewhere in the meantime will not affect our considerations of your study) remaining valid also during an extended revision period.

Further information on preparing and uploading a revised manuscript can be found below and in our Guide to Authors; should you have any additional questions or comments regarding the reports and this decision letter, please contact me for further feedback or discussion.

REFEREE REPORTS

Referee #1:

In this manuscript from corresponding author Andrea Ciliberto, the researchers conduct an evolution experiment on yeast with a temperature-sensitive tubulin mutant to determine how cells adapt to decreased tubulin polymerization. They find that cells adapt primarily through mutations in tubulin, gamma tubulin, and the kinesin Kip3. The cells also adapt through gaining an extra copy of chromosome 8. The authors show that either tubulin mutations, the kinesin mutations or the chromosome 8 disomy are sufficient to partially suppress the phenotype of the original mutant. They additionally show that there is clear temporal order to the events, with the gain of chromosome 8 coming first and a single point mutation arising later in the adaptation time line.

Combining different types of mutations typically has an additive effect, although two mutations in combination with aneuploidy resulted in decreased cellular fitness. Intriguingly, the authors find that there is a significant overlap between the residues that they find mutated in beta tubulin and those that had been previously identified in cancer patients.

The conclusions made in this study are generally quite well supported, as the mutations show a strong suppression and the phenotypes are very consistent. However, the genes that they identify mutations in are very similar to those found in previous screens for mutations that are resistant to the microtubule depolymerizing drug benomyl. The main differentiating factor from those screens in the suppression via chromosome 8 disomy and the observation that aneuploidy precedes the other mutations. Therefore, the advancements from a cell biology perspective are minimal and the connection to cancer treatment resistance mechanisms is tenuous. That being said, such a clear evolutionary timeline is rare and the subject of the screen is broadly important, making the study of potential interest to a large audience.

Major points:
1) The epistatic experiments are a bit confusing. The chromosome 8 disomy shows a decrease in growth under standard conditions and the Q219H mutant suppresses the tub2-401 mutant to near WT levels. Therefore, how does the combined mutant grow even better? Would the authors not predict the negative effects of the aneuploidy to have a stronger effect here? Are the aneuploidy-associated growth defects somehow suppressed by the Q219H mutation?

As a related question, what is the reason for the decreased growth in the triple kip3, Q219H, chr. 8 mutant? The discussion says that they are "hyperstabilizing" tubulin, but this is not addressed experimentally. Is the phenotype even stronger without the tub2-401 mutant or at the permissive temperature?

2) I don't understand the logic of this statement: "Deletion of tubulin genes are either inviable or decrease cellular viability. Hence, mutations of these genes that recover growth can be interpreted as gain-of-function." It is quite common to have compensatory loss-of-function (but not null) mutations in essential genes. This is typically seen in genes coding for proteins with opposing functions such as a kinase and a phosphatase or a plus-end motor and a minus-end motor.

3) The long spindles in figures 5E/S4C appear to be at a length consistent with anaphase. Are these length measurements an indirect assay for metaphase arrest duration? This is not clear in the text. How would this "confirm that the adaptive effect is due to the increased stability of microtubules"?

4) For the comparisons between the mutations identified in cancers and those identified in this study, why was this only done for beta tubulin and not alpha tubulin or gamma tubulin?

Referee #2:

general summary
The authors describe their experiments where they mutate a b-tubulin and study the evolutionary adaptation. They find that cells rapidly adapt to approach WT fitness in 150 generation and find the causative mutations.

I find this a well-written and well-executed interesting study. Especially the genetics are well done.

I have some mainly minor comments about it.
In figure 1b: what is the confidence interval? Do all 24 mutant lines behave similarly? Yhat is not what I would have expected and why does the fitness first drop for all?

I find stating that the cells approach WT fitness an overstatement since from an evolutionary perspective the fitness difference is still high. These mutants would still rapidly get lost in a population that also contains WT cells.

I find it surprising how fast in number of generations the suppressor point mutations are "found". What was the initial population size, what is the population size during the experiment and how large are the bottle necks that happen every dilution? Discussing this aspect of the study in the discussion part of the paper may be interesting to some readers.

the authors initially state: "Based on the data gathered from the movie, we interpreted the initial slow growth rate as a consequence of both prolonged activation of the mitotic checkpoint and cell death caused by massive chromosome missegregation."

(- detailed question: which movie?)

I find this cell biological observation interesting and the hypothesis would be that cell would adapt by reducing the missegregation rate and as such increase their population growth rate, because less cells die. However the authors do not come back anymore to this point but mostly discuss the causative mutations in the light of their molecular effects. I think it would be interesting if the authors can discuss their results more in what the causative mutations mean for the important cellular function of microtubules: properly segregating chromosomes.
And if possible, make a similar quantification that is now in figures S1Bb for the mutants at the final time point.

Referee #3:

Drugs that target microtubules play a major role in cancer therapy. However, cancer cells sometimes develop resistance towards microtubule drugs, causing a significant clinical problem. Several mechanisms, including mutations in tubulins, changes in the expression level of tubulins, and prevention of drug accumulation in the cell (multidrug resistance) contribute to this phenomenon in cell lines, but the adaptive events in patients are much less clear. In this manuscript, to mimic the adaptation of microtubule-drug-treated cells without the influence of the multidrug resistance mechanism, Pavani et al., performed an evolution experiment using a yeast strain carrying a defective tubulin Tub2-401. Evolved cells appear to partially regain the ability to polymerize microtubules. The authors then identified and characterized recurrent mutations and genomic alterations in the evolved strains. The authors found a predominant gain of chromosome

VIII that precedes any adaptive mutations in all evolving strains. Adaptive mutations acquired later during the evolution include various mutations in tubulins and Kip3, a kinesin that depolymerizes microtubules. The authors showed that disomy of Chromosome VIII and these adaptive mutations could partially enhance microtubule polymerization and growth of tub2-401 cells.
This is an interesting and important topic and the experiments are in general well done. If the authors can address the points below, I would support publication of the paper in EMBO.
Main point: The main data that support the epistasis model (Fig. 6C) comes from additive effects of VIII disomy with other adaptive mutations (Fig. 6A). My concern is whether the growth effects are really explained by improvement in microtubule function. There is some data to support a general improvement of microtubule function (response to nocodazole and Benomyl), but the only direct data are measurements of spindle length (Fig. 5E) seems inconsistent with the model. In contrast to the growth data, the individual mutants look the same (double mutants were not analyzed) raising the question of whether kip3Δ for example would really be an advantage on top of the VIII gain. The authors should address this by more direct measurements of either microtubule dynamics (if possible) and/or astral microtubule length measurement (single and double mutants). Functional assays for microtubule function such as chromosome loss rates or spindle orientation would be additional/alternative approaches.
The authors also need to address the fact that in Figure 1b, there seems to be no improvement of the growth rate for the Gf sample (mutation + VIII gain) relative to the Gr sample (VIII gain alone). The authors should assess microtubule function more directly comparing Gf and Gr.
Minor points:
1. The authors should clarify how they chose the Gr time point in Fig 1B.
2. It has been reported previously that fitness effects of aneuploidy are usually not driven by a few dosage-sensitive genes (Bonney et al., Genes & Dev, 2015). When discussing the fitness changes associated with the disomy of Chromosome VIII (Page 20, "Clearly, there are other unknown genes whose copy number increase contributes to the adaptive effect…."), the authors should add this notion and cite the reference above.

We thank the reviewers for their generally positive assessment of our work. Some of their comments are overlapping (especially on the characterization of evolved cells), and thus some of our answers are inevitably redundant. We apologize for that.

Referee #1:

In this manuscript from corresponding author Andrea Ciliberto, the researchers conduct an evolution experiment on yeast with a temperature-sensitive tubulin mutant to determine how cells adapt to decreased tubulin polymerization. They find that cells adapt primarily through mutations in tubulin, gamma tubulin, and the kinesin Kip3. The cells also adapt through gaining an extra copy of chromosome 8. The authors show that either tubulin mutations, the kinesin mutations or the chromosome 8 disomy are sufficient to partially suppress the phenotype of the original mutant. They additionally show that there is clear temporal order to the events, with the gain of chromosome 8 coming first and a single point mutation arising later in the adaptation time line. Combining different types of mutations typically has an additive effect, although two mutations in combination with aneuploidy resulted in decreased cellular fitness. Intriguingly, the authors find that there is a significant overlap between the residues that they find mutated in beta tubulin and those that had been previously identified in cancer patients.

The conclusions made in this study are generally quite well supported, as the mutations show a strong suppression and the phenotypes are very consistent. However, the genes that they identify mutations in are very similar to those found in previous screens for mutations that are resistant to the microtubule depolymerizing drug benomyl. The main differentiating factor from those screens in the suppression via chromosome 8 disomy and the observation that aneuploidy precedes the other mutations. Therefore, the advancements from a cell biology perspective are minimal and the connection to cancer treatment resistance mechanisms is tenuous. That being said, such a clear evolutionary timeline is rare and the subject of the screen is broadly important, making the study of potential interest to a large audience.

We thank the reviewer for commenting in positive terms our work.

Major points:
1) The epistatic experiments are a bit confusing. The chromosome 8 disomy shows a decrease in growth under standard conditions and the Q219H mutant suppresses the tub2-401 mutant to near WT levels. Therefore, how does the combined mutant grow even better? Would the authors not predict the negative effects of the aneuploidy to have a stronger effect here? Are the aneuploidy-associated growth defects somehow suppressed by the Q219H mutation?

As a related question, what is the reason for the decreased growth in the triple kip3, Q219H, chr. 8 mutant? The discussion says that they are "hyperstabilizing" tubulin, but this is not addressed experimentally. Is the phenotype even stronger without the tub2-401 mutant or at the permissive temperature?

We address the two questions separately. First: if *Q219H* is like *TUB2*, why the addition of Chr VIII does not impair growth of *Q219H*?

One first point to consider is that in fact *Q219H* is close to but less fit than WT (Figure 5A). Yet, it is also true that we do not see an improvement comparing *Q219H* and *chrVIII 2X Q219H* (Figure 6, columns 4 and 6). A possible explanation for this discrepancy is that our growth assay is not sensitive enough to identify such small differences. A third explanation is offered by the reviewer, namely that *Q219H* rescues the aneuploidy-associated growth defects. The suggested experiments with either *TUB2* or *tub2-401* at 30 °C, when disomy impairs growth, are well suited to test whether some mutations suppress growth defects coming with aneuploidy. Thus, we grew cells at 30 °C and asked how *Q219H* impacts on the growth of *chrVIII 2X* (Figure for rebuttal 1 -- R1). Interestingly, we measured positive epistasis for *Q219H chrVIII 2X*, while *kip3Δ chrVIII 2X* had negative epistasis, showing that improving microtubule polymerization *per se* does not explain the result. Thus, these data seem to confirm the reviewer's idea that *Q219H* may suppress the aneuploidy-associated growth defects of the disomy. Based on the data at 18 °C, part of the growth defects come from the duplication of *SPC97* (*TUB2 ChrVIII 2X spc97Δ* grows better than *TUB2 ChrVIII 2X* -- Figure 5D columns 2 and 4). Possibly, *Q219H* rescues defects of microtubule polymerization coming with the extra copy of *SPC97* when polymerization is not impaired. However, more experiments would be required to fully understand this effect.

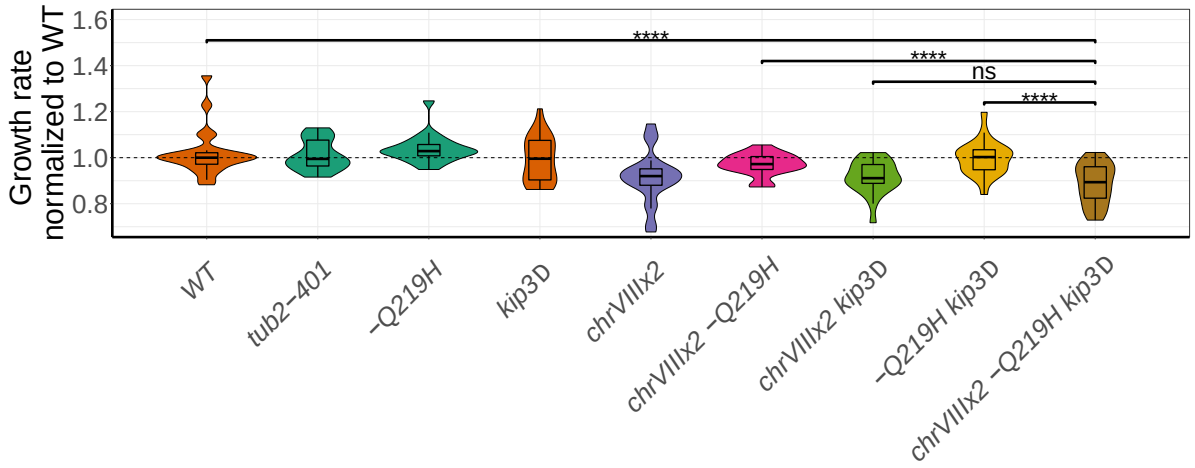

Figure R1. Growth rates of different mutants at 30 °C. Cells were grown at 30 °C, and growth rate in exponential phase was measured based on OD sampling every 10 minutes.

In summary, to answer the reviewer's point, we propose that in *Q219H chrVIII 2X* we do not see detrimental effects of the disomy for different reasons: (i) in absolute terms, *Q219H* is not as good as the wild type *TUB2*; (ii) our growth assays may not be sensitive enough to detect small differences in fitness; (iii) *Q219H* rescues partially growth defects coming with the disomy of chrVIII. Concerning the latter point, we feel that a thorough analysis of this effect would require many experiments, and is not central to our story. We added in the text the concept that *Q219H* may rescue the defect coming with disomy, but we did not include the growth assay at 30 C in the manuscript.

The second point raised by the reviewer concerns the decreased growth observed in the triple mutant. Indeed, in the text, we propose this may be due to microtubule hyperstabilization. In the new experiment at 30 °C the triple mutant shows the worst growth rate (Figure R1). However, we find it hard to conclude this being due to hyperstabilization of microtubules, especially due to the unclear role of microtubule dynamics on the detrimental effect at 30 °C

of disomy of chrVIII. We looked by IF at spindles in triple mutants and they do not have any obvious morphological defect. More sophisticated experiments would be needed to properly analyze their stability. Since data do not suggest a clear interpretation for the reduced growth of triple mutants, we removed any mention to hyperstabilization from the text.

2) I don't understand the logic of this statement: "Deletion of tubulin genes are either inviable or decrease cellular viability. Hence, mutations of these genes that recover growth can be interpreted as gain-of-function." It is quite common to have compensatory loss-of-function (but not null) mutations in essential genes. This is typically seen in genes coding for proteins with opposing functions such as a kinase and a phosphatase or a plus-end motor and a minus-end motor.

We agree that the sentence was not correct. What we meant is that these were not null mutations. The comment of the reviewer made us realize that the term gain-of-function was not really appropriate in our context. We did not formally test that these mutations lead to a gain of function if not in the context of the *tub2-401* mutations. The more striking difference with other published evolution repair experiments resides in the very few null loss-of-function mutations coming with nonsense or frameshift mutations. As we elaborate in the Discussion, what we identify are rather 'recovery-of-function' mutations, that make use of the altered Tub2-401 beta-tubulin. Following this argument, in the revised version we removed explicit references to the fact that what we find are proper 'gain-of-function' mutations.

3) The long spindles in figures 5E/S4C appear to be at a length consistent with anaphase. Are these length measurements an indirect assay for metaphase arrest duration? This is not clear in the text. How would this "confirm that the adaptive effect is due to the increased stability of microtubules"?

Indeed, we took length measurements and the observation that Gf cells are less sensitive to nocodazole than ancestors as an indication of increased microtubule stability in evolved cells. As noticed by the reviewer, these data were taken as indirect assays for metaphase arrest duration. In other words, we proposed that the mitotic checkpoint was less often invoked in cells at the end of the experiment.

These results have been further confirmed in the revised manuscript. As a proof of shorter metaphase arrest, we synchronized cells in G1 and released evolved populations at 18 °C to express the *tub2-401* allele. FACS analysis showed that Gf cells spend less time than ancestor with 2C DNA content (Figure S1E).

This is in agreement with the hypothesis that the mitotic checkpoint is less actively engaged during a regular cycle in Gf cells. To confirm this hypothesis, we deleted *MAD2* (required for the mitotic checkpoint) in three engineered strains that carry mutations representative of Gf: t*ub2-401 chrVIII 2X kip3Δ, tub2-401 chrVIII 2X Q219H* and *tub2-401 chrVIII 2X tub1*^{D246Y}. We observed that they are less sensitive to the loss of *MAD2* than the ancestral *tub2-401* (Figure 7A), suggesting that they have improved ability to assemble a functional spindle. Likewise, we deleted *BUB2* (required for the spindle position checkpoint or SPOC). Again, we found that engineered Gf cells are less sensitive to the loss of SPOC (Figure 7A), implying that they recovered the ability to orient the mitotic spindle.

The results are consistent with the hypothesis that Gf cells segregate chromosomes more efficiently. We confirmed this point in the three engineered strains carrying mutations typical

of Gf, where we tagged chrV with GFP. Our data show that they improve chromosome segregation compared to ancestors *tub2-401* (Figure 7C).

In summary, we presented new data showing that evolved cells undergo a shorter metaphase arrest than ancestors, caused by reduced SAC and SPOC activation and increased ability to segregate chromosomes. All these results point to restored microtubule functions in evolved cells. The new data are included in Figure 7 and S6 of the revised manuscript, and discussed in the text.

4) For the comparisons between the mutations identified in cancers and those identified in this study, why was this only done for beta tubulin and not alpha tubulin or gamma tubulin?

Our choice was due to the lack of mutations identified in patients for alpha and gamma tubulin in the database. Typically, in the available literature researchers did not perform unbiased next generation sequencing, but rather focused on specific genes, beta-tubulin being by far the most common target.

Referee #2:

general summary
The authors describe their experiments where they mutate a b-tubulin and study the evolutionary adaptation. They find that cells rapidly adapt to approach WT fitness in 150 generation and find the causative mutations.

I find this a well-written and well-executed interesting study. Especially the genetics are well done.

I have some mainly minor comments about it.

We thank the reviewer for appreciating our work.

In figure 1b: what is the confidence interval? Do all 24 mutant lines behave similarly? That is not what I would have expected and why does the fitness first drop for all?

What we plot in Figure 1b is the median (thick line) and the interquartile range (shadowed area), as we now explain in the figure's legend. All populations behave quite similarly: they display a sudden drop, followed by a recovery that typically slows down as the growth rate increases. For explaining the drop, we propose that a combination of cell death and reduced cell growth caused by missegregation contribute to it. We hypothesize that a subpopulation (or possibly many) of cells carrying the disomy of chrVIII emerges quite early, divides faster, dies less, and brings to an increase of the average fitness.

In fact, it is not trivial that this explanation suffices to produce a minimum of fitness. Hence, we rationalized the fitness drop with a simple model, which we present hereafter. Arguments derived from the model have been added to the discussion.

The model is based on the following points: (i) euploid cells carrying *tub2-401* decrease their net growth (as they divide slower and die in large number, although no population extinctions

are observed); (ii) these cells have a high chance to missegregate, an event which can produce either cells with an even higher growth impairment (fitness cost $-\sigma_d < 0$ due to the gain of any extra chromosome but VIII), and cells that grow better than *tub2-401* (fitness benefit σ_b>0 coming with the gain of extra chrVIII). (iii) Cells that inherit an extra chromosome VIII reduce their death rate, divide faster, and show reduced missegregation rate.

Haploid cells are assumed to generate aneuploid cells with a given missegregation rate. This rate is higher than in wild-type *TUB2* yeast populations because of the *tub2-401* mutation. In each missegregation event, the beneficial karyotype (extra chrVIII) emerges with probability p.

Our minimal model displays a drop of the mean fitness, as observed in the experimental condition, under the condition

$$(1-p)\sigma_d > p\ \sigma_b$$

which is satisfied by realistic values of the model parameters of our experimental setup, for which we have $\sigma_d > 1$ (cells with the deleterious karyotype are dying out), $\sigma_b \simeq 0.1$ (see Fig. 6A, the difference between *tub2-401* and *tub2-401 chrVIII 2X*, i.e., first and second column) and $p < 1/2$ (a conservative estimate: certainly, given the high (~50%) measured missegregation rate, in a realistic setting less than a half of the mis-segregations will end up with only the duplication of chrVIII).

I find stating that the cells approach WT fitness an overstatement since from an evolutionary perspective the fitness difference is still high. These mutants would still rapidly get lost in a population that also contains WT cells.

We agree with the reviewer, we changed the text accordingly.

I find it surprising how fast in number of generations the suppressor point mutations are "found".What was the initial population size, what is the population size during the experiment and how large are the bottle necks that happen every dilution? Discussing this aspect of the study in the discussion part of the paper may be interesting to some readers.

The experiment was performed with a population size between 10^6 and 10^7 individuals. Dilution factors for *tub2-401* cells was 1:6 - 1:7 at Gf. These are not extreme numbers, in line with what reported in other experiments (eg, Lenski reports a dilution factor 1:10 --American Naturalist, 1991 -- Fumasoni and Murray 1:1000 -- eLife 2020), and thus we do not expect the dilutions to have caused bottlenecks so strong to have an impact on the fixation dynamics. Indeed, the duration in generations of the dynamics leading to the fixation of the recurrent point mutations is roughly compatible with those observed in similar yeast evolutionary experiments (Levy...Sherlock, Nature 2015; Laan...Murray, eLife 2015). In our experiment, fixations of beneficial mutations roughly take place in about 80 to 120 generations, and roughly similar events were observed after ~ 100 generations (eg, Levy...Sherlock, Nature 2015) and ~ 200 generations (eg, Laan...Murray, eLife 2015).

To further support this point, we showed that a standard evolutionary model with a constant population size, the Wright-Fisher model with mutation and selection, can explain the

observed times to fixations of the recurrent mutations. This argument is now included in the discussion, and the Wright-Fisher model is introduced as Supplementary Text. Finally, in the revised paper we mention explicitly the population numbers and dilution factors.

the authors initially state: "Based on the data gathered from the movie, we interpreted the initial slow growth rate as a consequence of both prolonged activation of the mitotic checkpoint and cell death caused by massive chromosome missegregation."

(- detailed question: which movie?)

We apologize, indeed, the movie was missing. Samples of the movie have been introduced as Supplementary Movie.

I find this cell biological observation interesting and the hypothesis would be that cell would adapt by reducing the missegregation rate and as such increase their population growth rate, because less cells die. However the authors do not come back anymore to this point but mostly discuss the causative mutations in the light of their molecular effects. I think it would be interesting if the authors can discuss their results more in what the causative mutations mean for the important cellular function of microtubules: properly segregating chromosomes.

We agree with the reviewer. In the revised manuscript there are several additional experiments (improved segregation; reduced activation of the mitotic and spindle positioning checkpoint; increased spindle length; decreased missegregation -- see new Figure 7 and the answer to the next point) in line with what emphasized by the reviewer. Based also on this new evidence, we extend the session in the Discussion where we discuss the improved ability of cells to properly segregate chromosomes.

And if possible, make a similar quantification that is now in figures S1Bb for the mutants at the final time point.

In Figure S1B, we kept track of chromosome segregation using chrV-GFP. Since we did not have the GFP-tagged chrV in the ancestor, the evolved populations lack this construct. Unfortunately, this problem can hardly be circumvented. In principle, we could tag cells at Gf, but these are mixed populations. In principle, we could have derived clones, but they would not be representative of the whole population. What we did, instead, was to analyze chromosome segregation in engineered strains that carry mutations typical of Gf (disomy of chrVIII plus either *kip3Δ*, *Q219H* or *tub1*^{D246Y}). As in Figure S1B, we tagged chrV with GFP and followed its segregation. Cellasic chambers are limited to 4 channels, and here we planned to follow 6 strains in parallel. Thus, we did not make a movie, instead we fixed cells at different time-points from G1-release. The results show that cells expressing mutations typical of Gf reduce missegregation rates compared to *tub2-401*. This is shown in Figure 7C.

Referee #3:

Drugs that target microtubules play a major role in cancer therapy. However, cancer cells sometimes develop resistance towards microtubule drugs, causing a significant clinical

problem. Several mechanisms, including mutations in tubulins, changes in the expression level of tubulins, and prevention of drug accumulation in the cell (multidrug resistance) contribute to this phenomenon in cell lines, but the adaptive events in patients are much less clear. In this manuscript, to mimic the adaptation of microtubule-drug-treated cells without the influence of the multidrug resistance mechanism, Pavani et al., performed an evolution experiment using a yeast strain carrying a defective tubulin Tub2-401. Evolved cells appear to partially regain the ability to polymerize microtubules. The authors then identified and characterized recurrent mutations and genomic alterations in the evolved strains. The authors found a predominant gain of chromosome VIII that precedes any adaptive mutations in all evolving strains. Adaptive mutations acquired later during the evolution include various mutations in tubulins and Kip3, a kinesin that depolymerizes microtubules. The authors showed that disomy of Chromosome VIII and these adaptive mutations could partially enhance microtubule polymerization and growth of tub2-401 cells.

This is an interesting and important topic and the experiments are in general well done. If the authors can address the points below, I would support publication of the paper in EMBO.

We thank the Reviewer for the nice words about our work.

Main point: The main data that support the epistasis model (Fig. 6C) comes from additive effects of VIII disomy with other adaptive mutations (Fig. 6A). My concern is whether the growth effects are really explained by improvement in microtubule function. There is some data to support a general improvement of microtubule function (response to nocodazole and Benomyl), but the only direct data are measurements of spindle length (Fig. 5E) seems inconsistent with the model. In contrast to the growth data, the individual mutants look the same (double mutants were not analyzed) raising the question of whether kip3Δ for example would really be an advantage on top of the VIII gain. The authors should address this by more direct measurements of either microtubule dynamics (if possible) and/or astral microtubule length measurement (single and double mutants). Functional assays for microtubule function such as chromosome loss rates or spindle orientation would be additional/alternative approaches.

The authors also need to address the fact that in Figure 1b, there seems to be no improvement of the growth rate for the Gf sample (mutation + VIII gain) relative to the Gr sample (VIII gain alone). The authors should assess microtubule function more directly comparing Gf and Gr.

The reviewer asks whether the growth improvement that we observe throughout the evolution experiment is actually due to improved microtubule function. Following the two points raised by the reviewer, we distinguish two different types of improvements: (i) 'overall', between ancestor and final generation (Gf); and (ii) 'incremental', between generation recovery (Gr) and Gf. Both nocodazole treatment (Fig 1E) and spindle measurements of Gf (Fig 1D) were in support of the overall improvement. However, the lack of data concerning Gr cells (eg, spindle measurements in the double mutant cited by the reviewer) did not allow us to draw strong conclusions concerning the incremental improvement.

Hence, we performed new experiments aimed at testing the differences in terms of microtubule function for both incremental (Gf>Gr) and overall improvement (Gf>Anc). Experiments were either performed with a subset of populations evolved during the

experiment (Gr evo and Gf evo), or with engineered strains that carry mutations typical of Gr (Gr engineered: *chrVIII 2X tub2-401*) or typical of Gf (Gf engineered: *chrVIII 2X tub2-401* plus one among *kip3Δ*, *Q219H* and *tub1*^{D246Y}.)

Inactivation of the spindle assembly checkpoint (SAC) or mitotic checkpoint

tub2-401 cells can grow at 23 °C, but at this temperature the cell cycle relies on the mitotic checkpoint (Figure S1B). Partial destabilization of microtubules requires the ability of cells to correct attachment errors. We reasoned that if cells at Gr and Gf improved microtubule functions, their cell cycle should rely less on the mitotic checkpoint. Thus, as a test for microtubule function, we deleted *MAD2* in engineered Gr and Gf, and observed cellular viability by dilution assays at 23 °C. The results confirmed overall improvement (ie, engineered Gf and also Gr grow better than the ancestor). For both the addition of *Q219H* and *tub1*^{D246Y}, we noticed also an improvement over *chrVIII 2X*. This was not the case for *kip3Δ*: growth of *tub2-401 chrVIII 2X kip3Δ mad2Δ* was comparable to that of *tub2-401 chrVIII 2X mad2Δ*. Apparently, the deletion of *KIP3* does not improve much kinetochore/microtubule attachment.

Inactivation of the spindle positioning checkpoint (SPOC)

The reviewer mentions astral microtubules, which are greatly affected in *tub2-401* mutants (Sullivan and and Huffaker, 1992). Astral microtubules are involved in spindle positioning and spindle orientation. Thus, the reviewer proposes to verify whether spindle orientation is improved in evolved cells. Measurement of astral microtubules or spindle orientation at low temperature are technically quite challenging, and we run in several technical problems. An alternative approach is to impair cells' ability to detect the improper orientation of mitotic spindles. This can be accomplished by inactivating the spindle position checkpoint (SPOC). To this aim, we deleted the essential SPOC gene *BUB2* in the ancestor and in engineered cells representative of Gr and Gf. We observed that *bub2Δ* cells grow poorly at 23 °C (Figure S1B and 7A), similar to *mad2Δ* cells. Importantly, deletion of *BUB2* in engineered Gf and Gr strains had a much milder effect (Figure 7A), showing that spindle positioning is improved in these cells. Differently from *mad2Δ*, Gf cells carrying *kip3Δ* cells have improved growth compared to engineered Gr (*tub2-401 chrVIII 2X*).

Taken together, the results obtained with *mad2Δ* and *bub2Δ* show that: i) duplication of chrVIII recovers, but only partially, spindle orientation and microtubule/kinetochore attachment; ii) the addition of *tub1*^{D246Y} and *Q219H* largely rescue both functions; iii) the deletion of *KIP3* primarily rescues spindle orientation and less effectively microtubule/kinetochore attachment. These are relevant new results, especially those related with spindle positioning, for which we thank the reviewer.

Chromosome missegregation

Both SAC and SPOC supervise proper chromosome segregation. Given the previous results, and as suggested by the reviewer, we aimed at confirming an improvement in chromosome segregation in cells carrying mutations typical of Gf and, to a lesser extent, of Gr. In engineered strains we followed tagged chromosome V in fixed cells at different time points after synchronization at 18 °C. Our results show that both engineered Gr and Gf segregate chromosomes better than *tub2-401* (Figure 7C). Gf engineered cells missegregate less than Gr engineered cells, which perform slightly better than ancestors.

Metaphase arrest

Evolved cells have re-established microtubule function, segregate chromosome efficiently and do not rely so heavily as their ancestors on the SAC and SPOC. Thus, one can expect that they experience reduced metaphase arrest during a regular cell cycle in restrictive conditions. To test this prediction, we measured cell cycle dynamics on evolved populations at 18 °C. The results show that indeed evolved Gf are less delayed with 2C DNA content than ancestors (Figure S1E). We also observed a small but consistent difference between Gf and Gr, the latter being also less delayed with 2C DNA content than ancestors (Figure R2A).

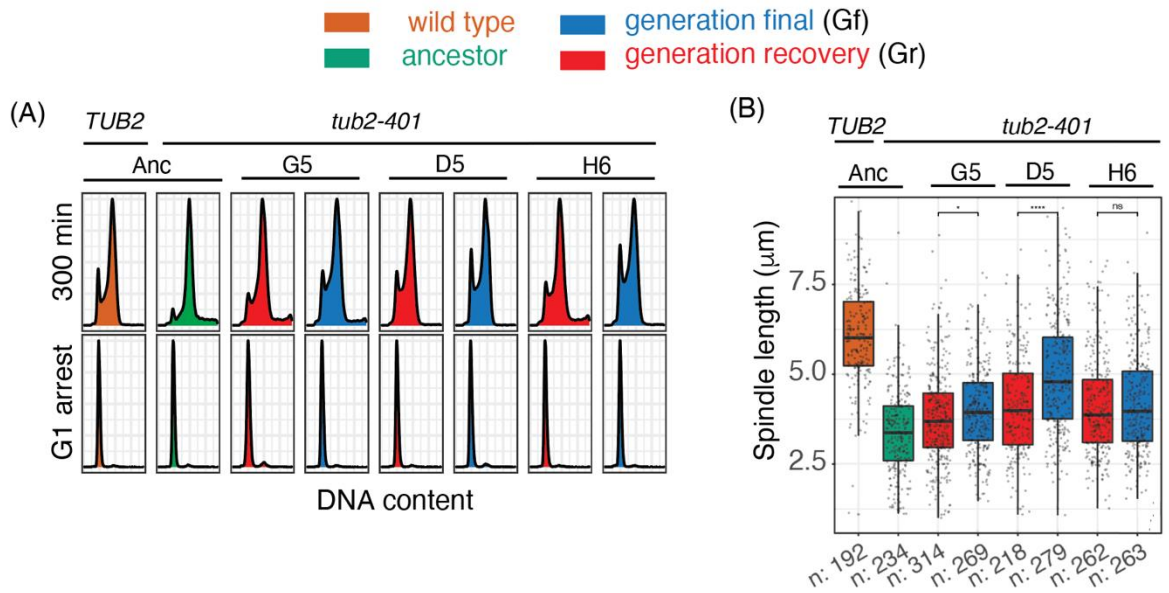

Figure R2. Comparison among chosen populations of evolved Gf and Gr in terms of spindle length and cell cycle dynamics. Cells were grown at 30C, arrested in G1 by alpha-factor, and released at 18C. In brown *TUB2*, in green *tub2-401*, in red Gr and in blue Gf. We compared the behavior of three populations: G5, D5 and H6.

Spindle length

Our last readout for improved microtubule function is mitotic spindle length. Upon reviewer's input, we (i) compared evolved Gf and Gr (Figure R2B); and (ii) measured spindle length in ancestors, engineered disomic strains and in the double mutant *tub2-401 chrVIII 2X kip3Δ* (ie, engineered Gr and one engineered Gf, respectively) (Figure 7B). In both cases, we observed increased spindle length in Gf cells. The difference between Gf and Gr was larger in engineered than in evolved cells, were it was not always statistically significant, a result we mention in the revised manuscript.

All new experiments aimed at testing microtubule function are summarized in the grey cells of Table R1. A bird's eye view of the Table shows that both Gr and Gf, either engineered or evolved, have improved microtubule-dependent functions compared to the ancestors. In terms of overall improvement, one major concern of the reviewer, the results are very solid and consistent. The improvement between Gf and Gr (a second point of concern) is also evident. The extent of improvement depends on the experimental system, with the largest increase being observed in engineered strains (spindle length, growth rate, missegregation, *bub2Δ* and *mad2Δ*). Differences between Gf and Gr in evolved populations are milder (growth rates, mitotic delay and spindle length). Here, the results are in agreement with the concept of diminishing return epistasis among beneficial mutations, which results in the decrease of rate

of fitness increase often observed in lab evolution experiments [Elena, Lenski, Nat Rev Gen, 2003].
The difference between engineered and evolved Gf and Gr may be due either to an underestimation of growth rate at Gr, or an overestimation of growth rate at Gf, or both. The first effect, could be due to the non recurrent, but possibly compensatory, mutations that are present at Gr. The second effect, may originate by the fact that recurrent mutations are present at 100% frequency in the engineered strains, and at a lower frequency in most of the evolved populations.

Comparison / Experiment	Gf vs Anc		Gr vs Anc		Gf vs Gr	
mitotic spindles length	*eng* Gf (*kip3Δ*) Fig 7B	*evo* Gf Fig 1D	*eng* Gr Fig 5E	*evo* Gr Fig R3B	*eng* Gf (*kip3Δ*) Fig 7B	*evo* ~ (Gf) Fig. R3B
missegregation	*eng* Gf Fig 7C		*eng* ~ (Gr) Fig 7C		*eng* Gf Fig 7C	
mad2Δ	*eng* Gf Fig. 7A		*eng* Gr Fig. 7A		*eng* Gf Q219H, Gf D246Y ~ (Gf *kip3Δ*) Fig 7A	
bub2Δ	*eng* Gf Fig 7A		*eng* Gr Fig 7A		*eng* Gf Fig 7A	
mitotic delay	*evo* Gf Fig S1E		*evo* Gr Fig R3A		*evo* ~ (Gf) Fig R3A	

Table R1. Experiments aimed at identifying phenotypic differences between ancestor (Anc), evolved cells at Gr and Gf (evo), and engineered strains representatives of Gr and Gf (eng). The ancestor (Anc) is a strain carrying *tub2-401*. Gr and Gf evo are evolved populations (G5, D5, H6, D4, B4 and E5). The engineered Gr is *tub2-401 chrVIII 2X* while the engineered Gf are *tub2-401 chr VIII 2X* with one among *kip3Δ/tub2*^{Q219H}*/tub1*^{D246Y}. In gray, experiments produced for the revised manuscript. In each column, we compare two strains; in green we show the prevailing one. If the two strains are comparable we use the symbol '~' and in parenthesis the better strain.

To conclude, in the manuscript, we introduce a new Figure 7 to characterize evolved cells. The figure includes *mad2Δ* and *bub2Δ*, the improved spindle length in double mutant *tub2-401 chrVIII kip3Δ*, and the reduced missegregation rate of engineered evolved cells. We emphasize the strong overall improvement in microtubule function, which is more limited between evolved Gr and Gf.

Minor points:
1. The authors should clarify how they chose the Gr time point in Fig 1B.

We chose the Gr as the first point where growth rate approaches the wild type. Our rationale was to avoid analyzing earlier events that most likely carried mixed populations with very low mutation frequencies or highly different karyotypes. We now state this explicitly in the text.

2. It has been reported previously that fitness effects of aneuploidy are usually not driven by a few dosage-sensitive genes (Bonney et al., Genes & Dev, 2015). When discussing the fitness changes associated with the disomy of Chromosome VIII (Page 20, "Clearly, there are other

unknown genes whose copy number increase contributes to the adaptive effect...."), the authors should add this notion and cite the reference above.

We thank the reviewer and added this relevant citation.

Thank you for submitting your revised manuscript to The EMBO Journal. Two of the original referees have now once more looked at it, and found the previously-raised points satisfactorily addressed. We shall therefore be happy to accept the study for publication in our journal, pending incorporation of the remaining minor referee comments (below) and the following editorial points.

REFEREE REPORTS

Referee #1:

The revised manuscript thoroughly addresses all of my critiques and questions. I recommend its publication.

A couple of corrections:

"missegregate less chromosomes" page 19. I think this should read "fewer chromosomes"

"Why so few complete loss-of-function in our evolution experiment?" page 23. This is not a full sentence.

Referee #3:

The authors have addressed my concerns in an exemplary fashion. In the revised manuscript, the authors demonstrate that the evolved strains had improved microtubule function by three criteria: longer spindles, reduced chromosome mis-segregation, and lower sensitivity to deletion of

checkpoint proteins. Direct comparison of engineered strains representing Gr and Gf cells also adds to the general conclusion that adaptive mutations in tubulins or kip3 further restore the function of microtubules after the gain of chr VIII. I enthusiastically support the publication of the revised manuscript without further modiciation.

we submit the revised version of our manuscript "Epistasis, aneuploidy and functional mutations underlie the evolution of resistance to induced microtubule depolymerization" (2021-108225).

We are very pleased with the Reviewers' appreciation of our work.

The authors have made all requested editorial changes.

Accepted **14th Sep 2021**

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Andrea Ciliberto
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2021-108225

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was not chosen a priori.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We excluded data based on controls' behavior.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	All analyses were performed automatically by scripts.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	When required (less than 200 observations), we checked normality either using Shapiro-Wilk or by visual inspection of QQ plots of the residuals.
Is there an estimate of variation within each group of data?	Yes, the interquartile range.

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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	FITC-conjugated anti-rat antibody from Jackson ImmunoResearch Laboratories - https://www.jacksonimmuno.com/catalog/products/112-095-003 anti-Tub1 primary antibody YOL1/34 Biorad https://www.bio-rad-antibodies.com/monoclonal/yeast-tubulin-alpha-antibody-yol1-34-mca78.html?f=purified
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	A data availability section is included for the NGS data.
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20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	We have no human clinical data in the manuscript.
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G- Dual use research of concern

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