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When Yeast Cells Change their Mind: Cell Cycle "Start" is Reversible under Starvation

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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.)

Thank you for submitting your manuscript on Start reversibility in the yeast cell cycle for our consideration. I sent it to three expert referees, who have now returned the below-copied comments. While at least two of these referees are supportive in principle, you will see that all three reports raise a variety of substantive concerns that, taken together, presently prevent publication in The EMBO Journal.

That said, given the potential importance of the key findings/conclusions of the study pointed out by referees 1 and 3, and the number of constructive criticisms and concrete suggestions, I realize that the study may well become a more compelling candidate for an EMBO Journal article if revised along these lines. I would therefore still like to give you an opportunity to address the reviewers' concerns by way of a formal revision. While it would not seem realistic to experimentally follow up on every single aspect raised and to fully work out all underlying mechanisms, key points would be to confirm that "Start in response to pheromones [as compared to nutrients] is quantitatively different in otherwise identical media and growth conditions" as asked by referee 1, and to follow-up on a potential mechanistic involvement of Swe1 as suggested by referees 1 and 3; as well as to further support the functional significance of Whi5 re-import into the nucleus.

Referee #1:

Background
Unidirectional cell cycle progression is thought to be ensured - at least in part - through irreversible cell cycle transitions or commitment points. These transition points are hubs for cellular networks to integrate external and internal information in order to decide whether to proceed or halt the cell cycle. The decision is generally followed by a switch-like progression into the next cell cycle phase mediated by positive or double negative feedback. One of these transition points is positioned ahead of DNA replication at the end of G1, referred to as 'Start' in budding yeast and 'Restriction point' in vertebrates. Historically, Start has been defined as the time when yeast cells become unresponsive to pheromone and instead continue to divide. However, other signals including nutrient availability can modulate progression through Start as well. In yeast, Start (as defined by hormone insensitivity) has been demonstrated to coincide with the export of about 50% of Whi5, a transcriptional repressor, from the nucleus and the triggering of a feedback involving Whi5, Cln2 and additional transcriptional regulators. The involved positive feedback was thought to render this transition irreversible. Generally, these cellular decision processes are of wide spread interest.

Manuscript summary
In this manuscript, Irvali and colleagues investigated the cell cycle-dependent response of budding yeast to carbon starvation. Intriguingly, using their microfluidic microscopy setup they show that even if Whi5 has left the nucleus - and therefore the positive feedback has presumably been triggered - some cells reverse this decision upon carbon starvation and reimport Whi5 into the nucleus. Although this phenomenon has been reported by other groups before (e.g. Wood et al. 2020, Liu et al. 2015), it had never been carefully investigated. Here, the authors go on to show that 'Start reversal' is highly likely if carbon starvation occurs within 15-20 min after Whi5 nuclear export - though a quantitatively distinct re-import can also be observed in a portion of cells after significantly longer time periods. The authors further show that these cells indeed reverse into a G1-like state, where they become again responsive to pheromones, repress the Cln2 promoter and probably dephosphorylate Whi5. This raises the interesting possibility that nutrient deprivation feeds into the Start network in a yet unknown way and can interfere with or even reverse the positive feedback loop. However, the authors fall short of providing a satisfying mechanistic explanation of their observation. They test a number of candidates, none of which impacts the reversal behavior significantly. They speculate about the contribution of other regulators (e.g. Cdc55 or APC/CCdh1) but do not test these rigorously.
Whereas more mechanistic insight would have been desirable and there are a number of improvements to be made to this manuscript (see below), the general in-depth description of Start reversal might be of interest to the large cell biology community and therefore warrant publication.

Major concerns:

- The Whi5 threshold for passing Start as defined by cells becoming insensitive to pheromones might be different in different media. To support the author's argument that there are two different commitment points for hormones and nutrients, the authors would need to demonstrate that indeed Start in response to pheromones is quantitatively different in otherwise identical media and growth conditions.
- In Figure 7B,C the authors argue that the Whi5 hyperphosphorylation associated with passing Start becomes reversed (either through inhibition of Cln-Cdk activity or activation of a phosphatase). However, Whi5 phosphorylation can also be observed during G1 progression, therefore some experimental evidence should be provided that a majority of cells at the time of the media switch had actually passed start.

- Throughout the manuscript there seem to be inconsistencies about whether cells mainly arrest in G1 and G2 in response to carbon starvation or at any cell cycle stage and the authors themselves argue that they cannot assess S phase progression after medium switch. Whether cells finish S phase and arrest in G2 therefore remains to be demonstrated. Furthermore, the authors should at least be able to detect increases in histone signal in cells ahead of the medium switch thereby strengthening their argument that indeed the population of cells that show a flat Whi5 re-import slope are the cells that had started DNA replication ahead of carbon starvation.
- On page 4 the authors say "...all analyzed cells activated the Cln2 promoter at Whi5 exit". What criteria did the authors use to define Cln2 promoter activation (e.g. a certain threshold)? How many cells were analyzed? How was the nucleus segmented in these strains (that seem not to carry an ambiguous nuclear marker)?
- Although the deletion of cdc55 alone is very sick, swe1 deletion rescues most of the pleiotropic phenotypes. Maybe the authors could use this genetic background to unequivocally demonstrate whether cdc55 plays a role? What about Rim15 or Igo1/2?

Minor concerns (related to the respective figure):
Figure 1:
- B: In the figure legend it says "Arrows indicate the times used for x and y axis in graph C", it would be helpful to indicate in the figure which arrow reflects the x and y axis in C.
- C: The dashed green lines are presumably depicting the two standard deviations. As such I would expect them to be symmetric to the mean, however, they don't seem to be symmetric in the figure.
- C: Ideally the color code (red, green, blue) is described in the figure and not only explained in the legend.
Figure 2:
- Out of the 59% arrested cells, how many re-imported Whi5?
- Scale bars in B and D are missing.
Figure 3:
- Could Whi5 synthesis in S/G2/M contribute to the observed slow nuclear accumulation?
- B: Typo in the labeling of the y axis.
- E: Scale bars missing.
Figure 4:
- A: Y-axis labeling should say AU/min not AU/time (also in the legend). Inset is missing the unit for the y axis.
- I would suggest to combine this figure with Figure 3.

Figure 5:
- No convincing Cln2 promoter activation ahead of starvation (maybe construct dependent?) for the one cell depicted in A. Why were these cells only starved for 5 h?
- How does the Cln2 promoter behave in cells which show a slope <200? Do they sustain the Cln2 promoter activity or is it repressed as well but does not show a later peak?
- A: Unit of promoter activity should be AU/min or similar (also in B and E)
- How was the Cln2 peak defined or called for the analysis in C?
- D: Scale bars missing.

S1:
- C: How many cells were analyzed?

Supplementary table 1:
- Strains do not seem to match figures, e.g. DI002 and DI028 refer to Figure 2.

Citations:
- Citation 27/28 seems a duplicate.

Methods:
- The methods state that data analysis was performed on 8 bit raw tiff images, is this correct? 8 bit seems somewhat low for a raw fluorescence image.

Referee #2:

General summary and opinion about the principle significance of the study, its questions and findings.

In this study by Irvali et al, the authors try to make the case that in yeast cells, "start" can be reversed upon starvation. The manuscript is built around the observation that upon starvation, many cells re-import whi5 into their nucleus. In its current form, this study is mainly phenomenological, it does not properly demonstrate the cell cycle reversibility and it does not provide insight into the molecular mechanism behind Whi5 re-localization. In fact, this study is very preliminary, critical controls are missing and many conclusions are overstated. That is why, in reviewer's opinion, it is clearly not suitable for a journal with a high impact such as EMBO Journal.

Specific major concerns essential to be addressed to support the conclusions

No physiological or molecular insight are proposed. What is the significance of Whi5 re-entry into the nucleus? What does it cause? What is the signaling pathway involved?
The "reversibility" of the cell cycle, as claimed in the title, is NOT demonstrated. The authors try to make the case that cells that re-import Whi5 reverse into the cell cycle. But what is the argument for that? Re-localizing Whi5 into the nucleus is not a proof of cell cycle rewiring. It is just an observation. As a start the author should demonstrate, using microfluidic, that individual cells that will re-import Whi5 but have not done it yet (very early after starvation) are insensitive to pheromone, but will respond to it after Whi5 're-import' into the nucleus.
What would be the role of Whi5 when it goes back into the nucleus after starvation? The authors argue that "Whi5 re-associates with DNA, thus likely inhibiting expression of G1/S genes" but this is only speculative and no data supporting this claim is provided in the manuscript. To get some molecular insight, the authors should use a whi5 mutant that does not bind DNA or develop tricks to prevent Whi5 binding to Cln2 promoter, or optogenetic tools to prevent Whi5 re-entry into the nucleus etc....
To address the biological significance of their observation, the authors should discuss why cells that are deleted for Whi5 do enter quiescence properly as demonstrated in previous published studies? In addition, previous studies have shown that indeed cells can stop proliferating and enter quiescence in other cell cycle phases than G1 (see for example Wei et al, 1993, Laporte et al 2011, Arguello-Miranda et al, 2021. It is well established in yeast and mammals (and discussed in many reviews) that "start" is not a point after which cells are committed to finish a full cell cycle. The reviewer deeply regrets that the authors do not discuss their results in light of these previous published studies.
Additionally, what is the physiological rationale in discriminating cells that re-import Whi5 rapidly form cells that does it rather slowly? Do these cells have a distinct survival rate? The authors claim that cells that re-import Whi5 slowly do not show a second Cln2 burst. But is the total amount of Whi5 the same in fast and slow cells? Such a difference can explained the absence of Cln2 burst. In addition, the authors should investigate the potential relationship between a fast or slow re-entry of Whi5 and the high-Cdk1 level observed in some quiescent cells.
What is the signal that triggers Whi5 re-entry into the nucleus? Only negative results are shown. The authors have to develop a non-phosphorylable whi5 mutant to make their case and/or determine the precise phosphorylation Whi5 pattern upon starvation condition. If it is indeed Whi5 dephosphorylation that drives its re-entry into the nucleus, the authors should try to find the molecular link with nutrient sensing pathways. As a start, the authors may want to screen a library of phosphatase-dead strains (available in yeast).
Many statements are unclear or not supported by the data presented here. As an example, it is stated "while the initial decision to interrupt Start is probably not caused by these transcriptions factors, they likely contributes (directly or indirectly) to full start reversal" Where are the data supporting this point? It is stated that cells depicted in blue in Fig1C are cells that have "completed their cell cycle"? What does that mean?.
Finally, several figures are unnecessary or could be shown as Sup Data (3BC, 4B). Similar data are presented twice (Fig1B, 2A). Two figures present negative results (Fig 6 and 7). In many cases, critical controls are missing. Fig1C is hard to understand (shouldn't the red line be parallel to the X-axis ?). Some quantifications are mentioned but not shown. In many cases, only one cell is shown (Fig2, Fig6). Experimentally the study is poor and standard of the field are not reached (failure to quantify Histone-GFP in Fig4A, CHIP failure in Fig7; in Fig2, ad minima the distribution of whi5/cln2 fluorescence ratio should be shown...).

Referee #3:

The manuscript „When Yeast Cells Change their Mind: Cell Cycle "Start" is Reversible under Starvation" by Irvali, Ewald, and colleagues demonstrate that in S.cerevisiae upon starvation the commitment decision at cell cycle Start can be reversed.
The authors monitored Whi5, the cell cycle inhibitor whose export from the nucleus determines Start (or the point-of-no-return of the cell cycle), and found that cells that have passed Start can re-import Whi5 back into the nucleus. Cells that re-import Whi5 also behave like pre-Start cells as they become sensitive to mating pheromone, forming schmoo-like shapes. Becoming insensitive to pheromone is one hallmark of passing the Start. The re-entries were classified as fast and slow re-entries (before and after entering S phase). The authors observe a fast return of Whi5, which occurs when cells are faced with starvation up to 20 minutes after Start. In this case, the DNA replication had not started yet.
The findings are very interesting and can potentially change the definition of Start in budding yeast. The study shows that yeast commitment to Start could be a multi-step process. The manuscript narrows down the possible mechanisms by excluding the starvation-related transcriptional regulation and G1-cyclin synthesis as mechanistic reasons behind the re-import. The authors conclude that the mechanistic basis of re-import should be direct regulatory suppression of CDK activity. However, I would suggest that the authors make some further attempts to define the mechanism and explain how CDK activity could be suppressed in this situation.

Major points

1. The authors show that the cells became sensitive to mating pheromone after re-import of Whi5, and thus the cells behaved like pre-Start cells. Considering the general mechanistic conclusion that CDK activity is suppressed upon re-import, will the CDK inhibitor Far1 be also re-synthesized, or was it never degraded in cells with steep and early re-import?
2. The data on histone fluorescence intensity suggests that all cells with fast Whi5 re-imports are pre-replication cells. Does this mean that there is no free Clb/CDK activity, that would be required for initiation of DNA replication, and the whole CDK downregulation mechanism must materialize via Cln/CDK complexes?
3. As the other mechanisms were mostly excluded, it was proposed that an inhibitory post-translational modification of CDK or the cyclins, or the selective degradation of Start components could be the basis of Whi5 re-import mechanism. The authors could test if CDK inhibitor Swe1 is overexpressed or activated in these conditions. These experiments would be easy to perform using western blotting. It is also possible to follow the inhibitory phosphorylation of CDK using phospho-form specific antibodies. Although Wee1/Swe1 kinases are thought to function mostly at G2/M transitions, it is not known if in certain special conditions the inhibitory tyrosine phosphorylation of CDK kinase subunit may play functional roles also earlier in the cell cycle. For example, in mammalian cells WEE1 suppresses CDK1 and CDK2 kinase activities to regulate the G1/S transition after the origin licensing is complete: "WEE1 kinase inhibitor AZD1775 induces CDK1 kinase-dependent origin firing in unperturbed G1- and S-phase cells" by Moiseeva et al PNAS (2019).
4. Why the cells with tiny buds that have not yet started replicating, can reverse (and later re-activate) Cln1/2-CDK activity but do not shmoo upon pheromone addition?
5. The authors propose that the observed dephosphorylation of Whi5 CDK sites could be caused by the upregulation of a starvation-induced phosphatase, but they did not find any evidence pointing in that direction. To exclude this possibility more definitively, perhaps it would be good to provide a little more detailed literature analysis, whether such phosphatases may exist, and if so, is their specificity is directed to phosphorylated CDK consensus sites.

Minor points
1. Chromatin-immunoprecipitation figure citations in the text are missing (Figure 7D in Results section).
2. The figure citation (or „data not shown") is missing also for the sentence „The deletion mutant of Cdc55 is very sick with irregular cell cycle progression, and therefore Whi5 behavior could not be quantified reliably".
3. Yeastract should be with a capital letter in the beginning: (data summarized on yeastract [31]).

Response to reviewers for Irvali et al

When Yeast Cells Change their Mind: Cell Cycle "Start" is Reversible under Starvation

We thank the editor and the reviewers for their thoughtful comments and critical feedback. Please find the point-by-point replies integrated into the reviewers' text below. We have highlighted major changes in green directly in the main text.

In addition to the direct responses to the reviews, we would like to make one general remark:

Sudden starvation leads to drastic changes in the cell. This includes a change in pH, a change in redox and a change in the fluidity of the cytoplasm (see e.g. work by Liam Holt's lab). All these things strongly affect the physics and chemistry of (fluorescent) proteins, for example maturation kinetics and photon yields; or can lead to oligomerization of fluorophores that are normally considered monomeric. This makes quantifying amounts of proteins at the transition to and under starvation, especially those being just synthesized, extremely noisy and artefact-prone. This is why we have such trouble to quantify histone synthesis, promoter activities, and basically any change in protein concentration at the onset of starvation. We have discussed this with several experts in the yeast imaging field and there is not really much we can do about it. We have tried to use work-arounds such as the dPSTR constructs wherever sensible.

Referee #1:

Background
Unidirectional cell cycle progression is thought to be ensured - at least in part - through irreversible cell cycle transitions or commitment points. These transition points are hubs for cellular networks to integrate external and internal information in order to decide whether to proceed or halt the cell cycle. The decision is generally followed by a switch-like progression into the next cell cycle phase mediated by positive or double negative feedback. One of these transition points is positioned ahead of DNA replication at the end of G1, referred to as 'Start' in budding yeast and 'Restriction point' in vertebrates. Historically, Start has been defined as the time when yeast cells become unresponsive to pheromone and instead continue to divide. However, other signals including nutrient availability can modulate progression through Start as well. In yeast, Start (as defined by hormone insensitivity) has been demonstrated to coincide with the export of about 50% of Whi5, a transcriptional repressor, from the nucleus and the triggering of a feedback involving Whi5, Cln2 and additional transcriptional regulators. The involved positive feedback was thought to render this transition irreversible. Generally, these cellular decision processes are of wide spread interest.

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al. 2020, Liu et al. 2015), it had never been carefully investigated. Here, the authors go on to show that 'Start reversal' is highly likely if carbon starvation occurs within 15-20 min after Whi5 nuclear export - though a quantitatively distinct re-import can also be observed in a portion of cells after significantly longer time periods. The authors further show that these cells indeed reverse into a G1-like state, where they become again responsive to pheromones, repress the Cln2 promoter and probably dephosphorylate Whi5. This raises the interesting possibility that nutrient deprivation feeds into the Start network in a yet unknown way and can interfere with or even reverse the positive feedback loop. However, the authors fall short of providing a satisfying mechanistic explanation of their observation. They test a number of candidates, none of which impacts the reversal behavior significantly. They speculate about the contribution of other regulators (e.g. Cdc55 or APC/CCdh1) but do not test these rigorously.
Whereas more mechanistic insight would have been desirable and there are a number of improvements to be made to this manuscript (see below), the general in-depth description of Start reversal might be of interest to the large cell biology community and therefore warrant publication.

Major concerns:
- The Whi5 threshold for passing Start as defined by cells becoming insensitive to pheromones might be different in different media. To support the author's argument that there are two different commitment points for hormones and nutrients, the authors would need to demonstrate that indeed Start in response to pheromones is quantitatively different in otherwise identical media and growth conditions.

- We have addressed this concern in two ways:

Firstly, we have performed mating commitment assays similar to Doncic et al 2011. Our strain was grown in the same medium as the pre-starvation medium in the other experiments and then subjected to 1µM alpha-factor (with unperturbed nutrient supply). We monitored cells that were at or close to Whi5 exit and scored whether they immediately arrested in G1 or completed one round of division before arresting. A logistic regression of this data confirms that the commitment point to the cell cycle is somewhere between 30 and 70% Whi5 exit. (Given the small uncertainty in the timing, noise in the data etc., we do not want to claim a more exact value.) We report this experiment in Figure EV1.We thus went with the previous definition by Doncic 2011 that Start is at 50% Whi5 exit.

Secondly, we did another control experiment with mating factor, which we report in Figure 2F. We subjected cells to even higher alpha factor concentrations (10µM) and checked whether we would see any re-entries of Whi5 as in the starvation experiments. We looked at traces of more than 500 cells and did not see such a phenomenon. In Figure 2, for illustration, we show traces of 50 example cells that passed Start 0-30 minutes before the switch to mating factor, and compare them to 50 example cells that had passed Start 0-30 minutes before facing starvation.

- In Figure 7B,C the authors argue that the Whi5 hyperphosphorylation associated with passing Start becomes reversed (either through inhibition of Cln-Cdk activity or activation of a phosphatase). However, Whi5 phosphorylation can also be observed during G1 progression,

therefore some experimental evidence should be provided that a majority of cells at the time of the media switch had actually passed start.

- We repeated this bulk experiment using Whi5-GFP in the cell cycle inducible strain background. We took images and quantified the distribution of the nuclear/cytoplasmic GFP at the sampling time points. This quantification clearly shows a shift towards less Whi5 in the nucleus after release, which changes back after starvation. We show example images, the quantification as well as the bud count of these experiments in Figure 6 and Figure EV5.

- Throughout the manuscript there seem to be inconsistencies about whether cells mainly arrest in G1 and G2 in response to carbon starvation or at any cell cycle stage and the authors themselves argue that they cannot assess S phase progression after medium switch. Whether cells finish S phase and arrest in G2 therefore remains to be demonstrated.

- Indeed we agree with the reviewer that at this point we cannot really say whether the cells actually initiate or complete replication. This is mostly our hypothesis and we have phrased this more carefully.

-Furthermore, the authors should at least be able to detect increases in histone signal in cells ahead of the medium switch thereby strengthening their argument that indeed the population of cells that show a flat Whi5 re-import slope are the cells that had started DNA replication ahead of carbon starvation.

- We tried very hard to get useful data out of the histone-TFP (and also other fluorophores) signal. In unperturbed cells this is not a problem, but in starved cells the data is too noisy, see general comment above. We have now added an Appendix Figure S5 to illustrate this: Even in cells that are clearly in G1 when starvation starts, the Htb2-TFP signal increases over time more than it would in a normal S-phase. We tried subtracting the average increase in G1 cells from the increase in post-Start cells, but the cell to cell variability is too big. Due to maturation of the fluorophore and noise in the data our “blind spot” increases until 10-20 minutes *before* the actual starvation switch. Whether the increase is purely a fluorescent artefact due to starvation or whether there is some additional biology behind it (e.g. starvation-induced chromatin condensation) we cannot say at this point. Other tagged proteins also give us strange signals upon switch to starvation, but not in this magnitude. We considered engineering a dPSTR for the histone promoter (needs adjustments in the dynamic range etc. compared to Cln2). But this would only give us the onset of histone transcription. While histone synthesis is clearly linked to S-phase, the onset of transcription and the firing of the first origin are not mechanistically coupled. We are thus working on getting good readouts for actual replication, rather than further trying to get information out of histones. However, this will take much more time.

- On page 4 the authors say "...all analyzed cells activated the Cln2 promoter at Whi5 exit". What criteria did the authors use to define Cln2 promoter activation (e.g. a certain threshold)? How many cells were analyzed?

- For the Cln2 dPSTR reporter constructs we call a peak, if the peak-to-trough of the Cln2 signal after Whi5 exit is at least 50% of the peak in the last unperturbed cell cycle. We write this now

more clearly in the methods and in the figure legends. For the dPSTR construct we quantified this for 102 cells (now reported in the text).

How was the nucleus segmented in these strains (that seem not to carry an ambiguous nuclear marker)?

-For many strains, we do not include a nuclear marker. Since labelled histones are so bright, you often get bleed through when trying to do 3-color imaging. We do nuclear detection as previously detailed in the method paper by Doncic et al 2013. In brief, we detect the brightest connected pixels and do a Gaussian fit with size constraints to determine the nucleus. For our initial Whi5 re-entry results, we included a labelled histone to confirm that nuclear Whi5 is correctly identified and that the Whi5 exits and re-entries were not falsely classified due to focus issues.

- Although the deletion of cdc55 alone is very sick, swe1 deletion rescues most of the pleiotropic phenotypes. Maybe the authors could use this genetic background to unequivocally demonstrate whether cdc55 plays a role? What about Rim15 or Igo1/2?

- We analyzed a Rim15 deletion and included the data in Figure 5A, which again shows no relevant phenotype. In combination with the Cdc55 results, this makes it very unlikely that the Rim15-Igo-Cdc55 axis is the key player. We nevertheless looked briefly at the Igo1/2 double deletion. This has a growth defect and some cells have a weird G1/S transition even under glucose conditions so we did not further quantify it carefully. We did see Whi5 re-entries under starvation.

- We analyzed a *swe1* deletion mutant to address reviewer 3's comments. The swe1 mutant has a lot of defects with G2/M regulation under starvation (see below), but it does not seem to impact the response of cells at the G1/S transition; here we still see Whi5 re-entries with a similar frequency as the wildtype. We thus made the swe1/cdc55 double deletion. This indeed has much less morphological defects in unperturbed conditions. But the G1 control still seems pretty abnormal. In many cells G1 is so short, that we do not see any nuclear Whi5 at all during growth on glucose. We did however find a few "normally" cycling cells; in these, we could also detect Whi5 re-entries when cells were starved early after passing Start. We did not attempt to quantify these diverse phenotypes, but we show a few example traces in Appendix Figure S12 (and uploaded the movies to Bioimage Archive). We thus conclude that Cdc55 is important for proper G1/S control, but not essential for Whi5 re-entries after starvation. It is not clear yet whether this is simply due to redundancy and "background" phosphatase activity, or if there is a specific other phosphatase responsible.

- Minor concerns (related to the respective figure):
  Figure 1:
  ▪ B: In the figure legend it says "Arrows indicate the times used for x and y axis in graph C", it would be helpful to indicate in the figure which arrow reflects the x and y axis in C.
- Good idea, thank you, we labelled these.

  - C: The dashed green lines are presumably depicting the two standard deviations. As such I

would expect them to be symmetric to the mean, however, they don't seem to be symmetric in the figure.

- The reviewer is correct. This must have happened during the last steps of figure formatting, the bottom line moved accidentally, we have fixed it.

- C: Ideally the color code (red, green, blue) is described in the figure and not only explained in the legend.

- Done.

Figure 2:
- Out of the 59% arrested cells, how many re-imported Whi5?

- The 59% refers to all the cells in Fig1, regardless of their time of starvation. Most of the arrested cells re-import Whi5, except those very late in the cell cycle. We now show a version of Figure 1C, where we color-code all Whi5 re-entries, in Appendix Figure S1 to address this.

- Scale bars in B and D are missing.

- We have added scale bars

Figure 3:
- Could Whi5 synthesis in S/G2/M contribute to the observed slow nuclear accumulation?

- This is an interesting point. Several labs showed that Whi5 is synthesized mainly in S/G2. In cell cycles of unperturbed cells, we can confirm this in our data. In unperturbed cells, we see an increase in total Whi5, but not much in nuclear Whi5 during S/G2 as reported.
  In our starvation experiments, this is a bit more tricky to address since we are again dealing with intensity changes that may be partially fluorescent artefacts. Additionally, we think that Whi5 transcription is induced in all cells as a response to starvation. While the Tang lab showed that Whi5 synthesis rate in steady state is independent of the environment, expression studies (summary from SGD) and transcription factor binding data (summary from Yeastract) suggest that Whi5 transcription is (probably transiently) stress-induced. In our datasets, averaged over many (asynchronous) cells, the total cellular Whi5-fluorescence upon starvation increases by 10-20%. This is much smaller than the several-fold change we see in nuclear fluorescence during Whi5 re-entry, but it is enough to distort estimates of total Whi5 synthesis. In Appendix Figures S3 and 4, we now show exemplary cells with raw data and scaled traces so the reader gets an impression of this.
  To address if the increase in nuclear Whi5 is driven *mainly* by synthesis or by directed import/nuclear enrichment we looked at the nuclear to cytoplasmic ratio. We compare the slope of the nuclear Whi5 intensity (as reported in all the main figures) with the slope of the increase in the nuclear to cytoplasmic ratio. There is a strong correlation between the two. This indicates that Whi5 increase in the nucleus is mainly caused by directed nuclear import, likely with some contribution by an overall increase in Whi5 concentration.

- B: Typo in the labeling of the y axis.

Fixed

- E: Scale bars missing.

- Fixed

Figure 4:
- A: Y-axis labeling should say AU/min not AU/time (also in the legend). Inset is missing the unit for the y axis.
- Fixed

- I would suggest to combine this figure with Figure 3.
- We have re-arranged figures 2/3/4 to accommodate the heat map and combine 3&4

- Figure 5:
  - No convincing Cln2 promoter activation ahead of starvation (maybe construct dependent?) for the one cell depicted in A. Why were these cells only starved for 5 h?
- Regarding the promoter activation: This is indeed a technical problem as detailed in EV 2 (delay from maturation time/no proper maturation under starvation). That is why we used the dPSTR constructs instead to monitor the pre-starvation Cln2. However, we have some cells that are already 10-15 minutes past Start where we do see a pre-starvation Cln2 peak also with this construct. We now use such a cell as example cell in Figure 4, we show additional example cells in Appendix Figure S7.
- The example cell we previously showed was indeed only starved for 5 hours. We used this strain to test for bleaching and phototoxicity and conducted experiments with 5,6, and 10 hours of starvation, as well as frame rates of 2,3, and 5 minutes. As we did not detect any relevant differences, we pooled this data for Figure 4B and C. The new example cell is from a 10 hour starvation experiment.
  - How does the Cln2 promoter behave in cells which show a slope <200? Do they sustain the Cln2 promoter activity or is it repressed as well but does not show a later peak?
- These cells have mostly passed the peak of Cln2 promoter activity. At the point of Whi5 re-entry in these cells the Cln2 promoter peak is declining or already over and there is no detectable firing during starvation. When cells continue their cycle after starvation, there is no Cln2 peak. We now illustrate this by showing example cells in Appendix Figure S7.

- A: Unit of promoter activity should be AU/min or similar (also in B and E)

- Agreed. We have changed this.

  - How was the Cln2 peak defined or called for the analysis in C?
- Slope of fluorescence increase (=promoter activity) was at least 50 % of the slope in the last unperturbed cell cycle. We explain this now more clearly in the figure legends and methods.

-

▪ D: Scale bars missing.
Fixed

S1:
- C: How many cells were analyzed?
- now reported in the figure legend

Supplementary table 1:
▪ Strains do not seem to match figures, e.g. DI002 and DI028 refer to Figure 2.

- We have double-checked and updated the entries in the table according to the new figure layouts. For some figures, related but different strains were used as biological replicates to make sure an observation is not specific to a certain clone. We have many strains containing Whi5-mCherry in combination with e.g. labelled Sic1 or Cln2-Pr constructs. It was reassuring to see that the re-entries were reproducibly seen in all strains and also the re-entry slopes were quantitatively consistent in all of these wild type strains. Thus there are several instances of different strains referring to the same figure.

Citations:
▪ Citation 27/28 seems a duplicate.
-Thank you for pointing it out, fixed.

Methods:
▪ The methods state that data analysis was performed on 8 bit raw tiff images, is this correct? 8 bit seems somewhat low for a raw fluorescence image.

- Sorry, this was misleading. The raw camera recordings are 12-bit in the vendor's image format. A given protein-fluorophore never requires the full dynamic intensity range of the camera. Therefore, images are then exported and converted to 8-bit tiffs before analyzing. With 'raw' we were contrasting to the de-noised pictures in the movies and figures (which is obviously a non-linear image conversion and should therefore not be used for quantitative analysis). We wrote this more clearly in the methods.

Referee#2:

General summary and opinion about the principle significance of the study, its questions and findings.

In this study by Irvali et al, the authors try to make the case that in yeast cells, "start" can be reversed upon starvation. The manuscript is built around the observation that upon starvation, many cells re-import whi5 into their nucleus. In its current form, this study is mainly phenomenological, it does not properly demonstrate the cell cycle reversibility and it does not provide insight into the molecular mechanism behind Whi5 re-localization. In fact, this study is very preliminary, critical controls are missing and many conclusions are overstated. That is

why, in reviewer's opinion, it is clearly not suitable for a journal with a high impact such as EMBO Journal.

Specific major concerns essential to be addressed to support the conclusions

No physiological or molecular insight are proposed. What is the significance of Whi5 re-entry into the nucleus? What does it cause? What is the signaling pathway involved? The "reversibility" of the cell cycle, as claimed in the title, is NOT demonstrated.

- We do not claim that the entire cell cycle is running backwards, just that the Start decision - as currently defined in the textbooks - can be reversed. Given that the current definition of Start is the sensitivity to mating pheromone (confirmed for our strain in Figure EV1), we think we do demonstrate a functional reversal (see Figure 4).

- The authors try to make the case that cells that re-import Whi5 reverse into the cell cycle. But what is the argument for that? Re-localizing Whi5 into the nucleus is not a proof of cell cycle rewiring. It is just an observation. As a start the author should demonstrate, using microfluidic, that individual cells that will re-import Whi5 but have not done it yet (very early after starvation) are insensitive to pheromone, but will respond to it after Whi5 're-import' into the nucleus.

- Many studies have shown that cells are insensitive to mating pheromone after Whi5 exit, which is the current definition of Start. To confirm that also in our strain Whi5 exit corresponds to insensitivity to mating pheromone, we do two mating experiments, now reported in EV Fig 1 and in the heatmap in Figure 2.

- The suggested experiment is an interesting Gedankenexperiment, but is physically impossible to do. For example, starved cells cannot properly respond to mating pheromone since they do not have resources to make a shmoo (directly confirmed by our Far1 experiments, see below). We do show in figure 4 that cells that have exported and re-imported Whi5 become sensitive to mating pheromone again, which is the closest we can get to the suggested experiment.

- What would be the role of Whi5 when it goes back into the nucleus after starvation? The authors argue that "Whi5 re-associates with DNA, thus likely inhibiting expression of G1/S genes" but this is only speculative and no data supporting this claim is provided in the manuscript. To get some molecular insight, the authors should use a whi5 mutant that does not bind DNA or develop tricks to prevent Whi5 binding to Cln2 promoter, or optogenetic tools to prevent Whi5 re-entry into the nucleus etc....

- Our ChIP data strongly suggests that Whi5 re-binds DNA. We ran additional 2 replicates of this starvation experiment to strengthen the data (p-value=0.0059). Given that there is a second Cln2 peak after starvation, presumably Whi5 suppresses Cln1/2 (and other) transcription again. However, the most important claim we want to make in this manuscript is not about Whi5 per se, but that the positive feedback loop determining Start can be interrupted.

To address the biological significance of their observation, the authors should discuss why cells that are deleted for Whi5 do enter quiescence properly as demonstrated in previous published studies?

- Quiescence (as the term is used in the yeast field) is a metabolic state characterized for example by high storage levels. We do not directly look at this anywhere in our studies or make specific claims on whether our starved cells fulfill the classical definition of quiescence.
- Whi5 deletion mutants are also perfectly viable under unperturbed conditions, and yet many studies agree that Whi5 is an important regulator of Start.
- Our current argument is that nutrient signaling is targeting CDK-Cln1/2 directly (see discussion, now with more emphasis). Therefore, Whi5 re-entry is likely more of a "symptom", than the cause. So even in absence of Whi5, whatever is inhibiting CDK activity can still do this.

In addition, previous studies have shown that indeed cells can stop proliferating and enter quiescence in other cell cycle phases than G1 (see for example Wei et al, 1993, Laporte et al 2011, Arguello-Miranda et al, 2021. It is well established in yeast and mammals (and discussed in many reviews) that "start" is not a point after which cells are committed to finish a full cell cycle. The reviewer deeply regrets that the authors do not discuss their results in light of these previous published studies.

- We would like to cite our introduction: "Evidence from our work and others suggests that cells can respond to nutrients in all phases of the cell cycle. …. Wood et al. found in single cell experiments that cells can enter a quiescence-like state outside of G1 when responding to acute starvation, in agreement with earlier population-based studies showing that even budded cells can enter quiescence **[->Laport 2011]**. A recent report also demonstrated that cells can arrest their cell cycle in a "high CDK-state" **[->Arguello-Miranda 2021]** when facing nutrient perturbations." Therefore, we do not agree that we failed to discuss these studies.
- We have now included Wei 1993 in our references, although this paper makes a slightly different point. Since they use cell cycle arrested ts mutants, they look at the effect of cell cycle on nutrient responses and not the other way around like we do.
- There are several papers/reviews on potentially different commitment points in mammals (many of which we cite in the discussion). We did not find one mention of this in yeast in any review. In fact, it was argued during an informal discussion at a cell cycle conference that this may be one of the key differences between the mammalian and the yeast G1/S transition. It was rather satisfying for us to see that the yeast model may be able to help us understand the principle of a multi-step commitment in more complex systems.

- Additionally, what is the physiological rationale in discriminating cells that re-import Whi5 rapidly form cells that does it rather slowly? Do these cells have a distinct survival rate? The authors claim that cells that re-import Whi5 slowly do not show a second Cln2 burst. But is the total amount of Whi5 the same in fast and slow cells? Such a difference can explained the absence of Cln2 burst.

- We do not understand this comment. Not necessarily the outcome, but the starting point in these cells is likely what is different. But we now comment on the total Whi5 synthesis as detailed for Rev 1 above.

- In addition, the authors should investigate the potential relationship between a fast or slow re-entry of Whi5 and the high-Cdk1 level observed in some quiescent cells.
- We emphasize this study even more now in our discussion.

- What is the signal that triggers Whi5 re-entry into the nucleus? Only negative results are shown.

- We totally agree that it would have been nice to find the actual mechanism. We made dozens of mutants, the most plausible/obvious/intriguing ones are shown in figure 5, but none of these seem to be responsible for inhibiting CDK activity. We suggest several additional mechanisms in the discussion, which we have now further expanded. Publishing our results now will allow a community effort to trace down the exact mechanism.

- The authors have to develop a non-phosphorylable whi5 mutant to make their case and/or determine the precise phosphorylation Whi5 pattern upon starvation condition.

- In previous Figure 7A, now Figure EV5, we do report on a non-phosphorylatable mutant where the known non-CDK sites are mutated to alanine. Mutating individual or combination of CDK sites, leads to either no phenotype or Start/ nuclear export is messed up already in unperturbed cells (published e.g. by the de Bruin and Igual labs). Whi5 has 19 known phosphorylation sites. Several groups have published entire papers to maps these sites.

- If it is indeed Whi5 dephosphorylation that drives its re-entry into the nucleus, the authors should try to find the molecular link with nutrient sensing pathways. As a start, the authors may want to screen a library of phosphatase-dead strains (available in yeast).
- Screening a phosphatase library could be interesting if evidence leads us further in this direction, which it is currently not. Screening a library (on the single cell level!) “as a start” to testing a hypothesis we do not believe in is something that a normal lab of a young investigator cannot do. Apart from this, many phosphatases have very pleiotropic phenotypes such as now illustrated for Cdc55 in Figure S12. Analyzing the effects of nutrient perturbations on cell cycle control in these strains is not necessarily meaningful if the baseline is already completely different than wildtype.

  Many statements are unclear or not supported by the data presented here. As an example, it is stated "while the initial decision to interrupt Start is probably not caused by these transcriptions factors, they likely contribute (directly or indirectly) to full start reversal"
- There are three sentences before detailing why we think this is the case: We see Whi5 re-entries in these cells, but the timing and steepness of the slopes seem to be slightly different. This is a side note and the phrasing is very clear that this is our speculation and it is only a side note not directly related to the main story.

- It is stated that cells depicted in blue in Fig1C are cells that have "completed their cell cycle"? What does that mean?.
- We have re-phrased it as “cells completed their cell cycle (i.e. completed S/G2/M phases as evidenced by Whi5 re-entering mother and daughter cell nucleus)”

-Finally, several figures are unnecessary or could be shown as Sup Data (3BC, 4B)

-We indeed moved 3BC to the Suppl as we reorganized figs 2-4. Figure 4B (now Figure 3D) we consider very relevant information and have left it in the main figures.

-Similar data are presented twice (Fig1B, 2A).

- These cells show two very different phenotypes that we would like to specifically emphasize.

- Two figures present negative results (Fig 6 and 7).
- Only figure 6 (now figure 5) shows “negative” results. And these results are important to exclude many plausible mechanisms. Showing these negative results may save others many months of work trying to test these hypotheses.

- In many cases, critical controls are missing.
- We are sorry that we could not address this due to the unspecific nature of the comment.

- Fig1C is hard to understand (shouldn't the red line be parallel to the X-axis ?).
- The red line depicts cells that are arrested until the end of the starvation period. The starvation period is 10 hours, therefore the line is 10 hours plus x-axis and thus has a slope. For example, a cell that had passed Start 1 hour before starvation and then arrests until the end of the starvation phase spent a total of 11 hours in S/G2/M.

- Some quantifications are mentioned but not shown. In many cases, only one cell is shown (Fig2, Fig6).
- Figure 2 shows an example cell for the reader to understand the phenomenon. We have now included a heatmap showing the quantification of 50 such cells in figure 2 F. In figure 3, we quantify over 800 such cells. Figure 2 is also backed up by a movie of a (different) example cell.
- Figure 6B shows an example cell of a Sic1 trace to make the point that Sic1 is likely irrelevant for the observed re-entry. The quantification of many such cells is presented in the Appendix S11.
- We do not understand which quantifications are not shown.
- Additionally, raw data is uploaded to a repository for anyone to re-analyze.

- Experimentally the study is poor and standard of the field are not reached (failure to quantify Histone-GFP in Fig4A, CHIP failure in Fig7; in Fig2, ad minima the distribution of whi5/cln2 fluorescence ratio should be shown...).
- These authors deeply regret that this reviewer seems to define the standards of the field in a way that these authors cannot reach.

- We comment on the histone data above. This is not a failure on our side, but the biology of fluorescent proteins under starvation.
- The ChIP worked perfectly fine, six replicates confirm that Whi5 unbinds DNA after Start, but rebinds it upon starvation. What did not work reliably, was a qPCR to determine which promoters get re-bound first. This will require further optimization but is not relevant for the conclusions of this study.
- Unfortunately, we did not understand what the reviewer means with "the distribution of whi5/cln2 fluorescence ratio". The Cln2 data refers to promoter activity, not the concentration of the Cln2 protein. Therefore, this ratio does not make any biological sense.

Referee #3:

The manuscript „When Yeast Cells Change their Mind: Cell Cycle "Start" is Reversible under Starvation" by Irvali, Ewald, and colleagues demonstrate that in S.cerevisiae upon starvation the commitment decision at cell cycle Start can be reversed.
The authors monitored Whi5, the cell cycle inhibitor whose export from the nucleus determines Start (or the point-of-no-return of the cell cycle), and found that cells that have passed Start can re-import Whi5 back into the nucleus. Cells that re-import Whi5 also behave like pre-Start cells as they become sensitive to mating pheromone, forming schmoo-like shapes. Becoming insensitive to pheromone is one hallmark of passing the Start. The re-entries were classified as fast and slow re-entries (before and after entering S phase). The authors observe a fast return of Whi5, which occurs when cells are faced with starvation up to 20 minutes after Start. In this case, the DNA replication had not started yet.
The findings are very interesting and can potentially change the definition of Start in budding yeast. The study shows that yeast commitment to Start could be a multi-step process. The manuscript narrows down the possible mechanisms by excluding the starvation-related transcriptional regulation and G1-cyclin synthesis as mechanistic reasons behind the re-import. The authors conclude that the mechanistic basis of re-import should be direct regulatory suppression of CDK activity. However, I would suggest that the authors make some further attempts to define the mechanism and explain how CDK activity could be suppressed in this situation.

Major points
1. The authors show that the cells became sensitive to mating pheromone after re-import of Whi5, and thus the cells behaved like pre-Start cells. Considering the general mechanistic conclusion that CDK activity is suppressed upon re-import, will the CDK inhibitor Far1 be also re-synthesized, or was it never degraded in cells with steep and early re-import?

- This is an interesting point. We constructed strains with Whi5-mCherry plus Far1-Neongreen (additional control Far1-sfGFP) und repeated the glucose-starve-glucose+alpha-factor experiments. We were quite intrigued by the outcome. It looks like there is an interesting cross-talk between mating signaling and nutrient signaling. We first looked at cells that had just completed mitosis and were in a "normal" G1 at the onset of starvation. Far1 was readily detected as cells entered G1, but was completely lost within 30 minutes of starvation (see figure below). The Far1 (nuclear or total) signal was undetectable until glucose returned. This is true also for the "Whi5 re-entry cells". The ones with fast re-entries still have some

Far1 when they face starvation, but it is completely degraded and then re-synthesized. Arguello-Miranda 2022 actually report a similar finding (Sup Figure 1F) in a slightly different context. Though the dynamics are a little slower they show that Far1 is degraded in G1 cells during starvation.
If you consider that starvation triggers sporulation in diploid cells, it actually makes complete sense that haploids would avoid mating under starvation conditions and actively shut down the mating pathway when they sense nutrient depletion.

Since these data only open yet another "Pandora's box" without contributing to the storyline, we did not include these in the new version of the manuscript. However, we can do so if requested.

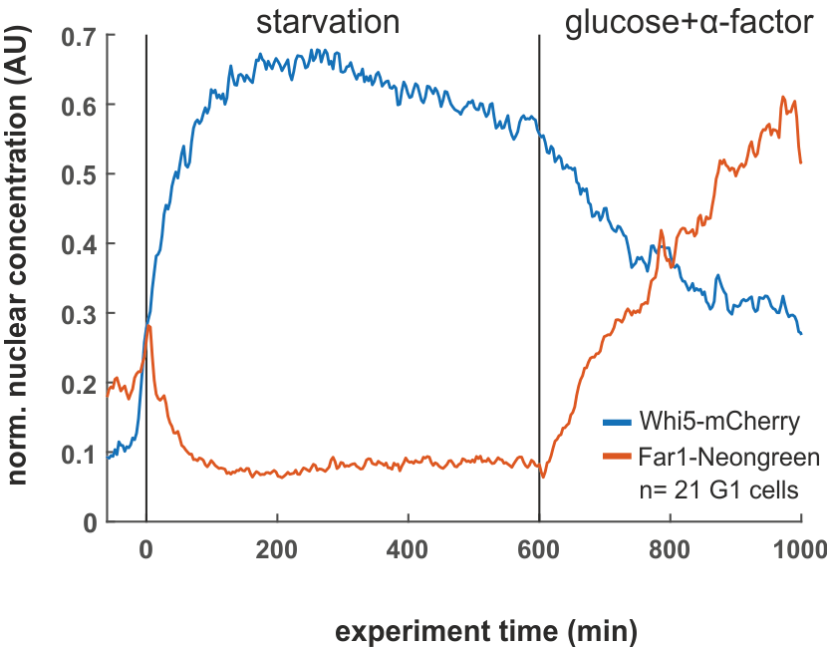

Figure: We looked for cells that had completed mitosis and entered G1 just before starvation. We averaged nuclear Whi5 and Far1 (total Far1 looks similar) from 21 such cells. In these and all cells that we observed Far1 was degraded upon starvation.

2. The data on histone fluorescence intensity suggests that all cells with fast Whi5 re-imports are pre-replication cells. Does this mean that there is no free Clb/CDK activity, that would be required for initiation of DNA replication, and the whole CDK downregulation mechanism must materialize via Cln/CDK complexes?

- This is indeed what we think. Our hypothesis is that the Cln1/2-CDK complexes are strongly inhibited by starvation signaling and Clb5/6-CDK complexes are not/not so much. So the final commitment could be related to the point where CDK-Clb activity is self-sustaining and /or is strong enough to trigger origin firing (but interestingly the CDK-Clb inhibitor Sic1 does not seem so important here). We tried hard to look at Clb5/6 expression, but here again we have the problem that (in the interesting cells) these are being transcribed at the switch to

starvation and we have to deal with sudden jumps in fluorescence. The dPSTR system is (at least in its current form) in the wrong dynamic range.
We could however monitor Clb2 expression. There is no Clb2 peak before starvation, nor is Clb2 induced in any of the Whi5 re-entry cells, indicating cells arrest before the G2/M transition.

3. As the other mechanisms were mostly excluded, it was proposed that an inhibitory post-translational modification of CDK or the cyclins, or the selective degradation of Start components could be the basis of Whi5 re-import mechanism. The authors could test if CDK inhibitor Swe1 is overexpressed or activated in these conditions. These experiments would be easy to perform using western blotting. It is also possible to follow the inhibitory phosphorylation of CDK using phospho-form specific antibodies. Although Wee1/Swe1 kinases are thought to function mostly at G2/M transitions, it is not known if in certain special conditions the inhibitory tyrosine phosphorylation of CDK kinase subunit may play functional roles also earlier in the cell cycle. For example, in mammalian cells WEE1 suppresses CDK1 and CDK2 kinase activities to regulate the G1/S transition after the origin licensing is complete: "WEE1 kinase inhibitor AZD1775 induces CDK1 kinase-dependent origin firing in unperturbed G1- and S-phase cells" by Moiseeva et al PNAS (2019).

- This is a great point, which we addressed in two ways:
- We have generated *swe1* deletion cells and monitored the Whi5 response. Swe1 KOs have a problem properly controlling G2/M after starvation. We see a fairly high fraction of binucleated cells and cells that seem to “abort” the bud. However, this does not seem to play a role for those cells that have just passed Start. Cells within the first ~20 minutes after passing Start can still re-import Whi5 and do not seem to have any defects in consecutive cell cycles after glucose replenishment. We report this in Figure EV5A and in Appendix Figure S12 for the swe1cdc55 double KO.

- We also looked at the Swe1-mediated CDK-Y19 phosphorylation in our bulk release-starvation experiments using a commercially available phospho-specific antibody. We only got very weak bands when inducing starvation at 45 min after release from G1. As a control, when we looked at cells 90 minutes after release, these gave a clear signal. After starvation, this further increased. We thus conclude that Swe1 likely responds to starvation, but this does not play a (major) role for the G1/S transition. We report this in Figure EV5B.

-

4. Why the cells with tiny buds that have not yet started replicating, can reverse (and later re-activate) Cln1/2-CDK activity but do not shmoo upon pheromone addition?

- This is likely related to the points above. Budding is linked to Cln1/2 activity, while replication is triggered mainly by Clb activity. There seems to be a time window (which seems to be rather noisy in duration) when Cln activity is high enough to trigger budding and “override” the mating pathway, but Clb activity has not risen yet sufficiently to trigger replication. It seems likely that in these cells, Start activation is reversed, but Cdc24/42

activation for polarization is maintained. This will be subject to future investigations but, as discussed with the editor, goes beyond the scope of this work.

- 5. The authors propose that the observed dephosphorylation of Whi5 CDK sites could be caused by the upregulation of a starvation-induced phosphatase, but they did not find any evidence pointing in that direction. To exclude this possibility more definitively, perhaps it would be good to provide a little more detailed literature analysis, whether such phosphatases may exist, and if so, is their specificity is directed to phosphorylated CDK consensus sites.

- We have screened pretty much every paper written on Whi5 to find a link to phosphatases. There are two phosphatases experimentally linked to Whi5: Cdc14 and PP2A-Cdc55. Cdc14 is a mitotic kinase with no (to the best of our knowledge) known link to G1/S. There have been a few studies linking Cdc14 to Tor1 activity (all from Ushimaru lab), but not to glucose signaling. In fact, Feng et al (https://doi.org/10.1083/jcb.202107151, Supp Fig 1G) specifically looked at Cdc14 localization upon glucose starvation and did not see Cdc14 release in quiescent, non-meiotic cells. We thus focused on Cdc55, which is known to counteract CDK1 in G1. We performed the *swe1 cdc55* double KO experiment as suggested by reviewer 1. These cells have abnormal G1/S and trouble dealing with starvation, but there are still cells that can re-import Whi5 directly after Start (see above and Appendix Figure12). We didn't include the data, but we also briefly looked at the other subunit of PP2A, Rts1, which again has no relevant phenotype. We also dug into several large scale phosphoproteomics screens (such as Bodenmiller 2010) but did not find any further evidence of anything specifically targeting Whi5. So likely Cdc55 contributes to dephosphorylating Whi5 in the wild type, but other phosphatases can do the job, so there is probably not much specificity on the level of phosphatases in this situation. But of course we cannot conclusively claim this until the actual mechanism breaking the positive feedback loop has been found.

Minor points

1. Chromatin-immunoprecipitation figure citations in the text are missing (Figure 7D in Results section).

- Fixed.

2. The figure citation (or „data not shown") is missing also for the sentence „The deletion mutant of Cdc55 is very sick with irregular cell cycle progression, and therefore Whi5 behavior could not be quantified reliably".

- Now reported in more detail in the Appendix.

- 3. Yeastract should be with a capital letter in the beginning: (data summarized on yeastract [31]).
- Fixed.

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by original referees 1 and 3, whose comments are copied below. Since both are generally satisfied with your revision and only retain a few specific presentational concerns, we shall be happy to accept the study, once these remaining referee points have been incorporated during a final round of minor revision.

Referee #1:

The authors have addressed most of my technical concerns (except for the histone measurements, which they encountered unsurmountable technical problems with) and provide additional convincing evidence that indeed Start as defined by Whi5 nuclear export can be reversed (as judged by Whi5 nuclear re-import, pheromone sensitivity, and Cln2 promoter activity) for some time after Whi5 export in response to glucose starvation. Therefore, in budding yeast - as in mammalian cells - cell cycle commitment in G1 might not be as hard-wired and irreversible as presumed. Albeit noted in other contexts before, this is an intriguing observation and the authors provide now ample convincing evidence. The authors also go through great length to identify the mechanistic basis and while not successful in identifying the underlying mechanism exclude several others. Therefore, despite the lack of mechanism per se, I think this manuscript warrants publication.

Few minor comments:

(1) It seemed to me that there is some discrepancy between the data shown in Figure 2E (a significant number of re-entries observed after 60 min) and the one in S1 (no re-entries observed after 60 min). Is there an explanation for this?

(2) The data in EV1 looks significantly less switch-like than in the original paper. Is that due to the different media?

(3) I still find the manuscript rather difficult to follow and the figures often not very intuitive. Maybe the authors could have another critical look at it. For example, in different Figures the y axis describing Whi5 fluorescence in the nucleus is called "norm. nuclear intensity", "norm. nuclear concentration (AU)", "norm. nuclear Whi5 concentration (AU)" and "norm Whi5 nuclear concentration". If these all describe the same type of data and underlying measurements, it would be advisable to call them all the same (similarly for the description of the Whi5 slope). If they don't, it would be important to emphasize why they are different and why different ways for analysis where chosen.

Referee #3:

In the rebuttal letter the authors state: "Far1 was readily
detected as cells entered G1, but was completely lost within 30 minutes of starvation (see figure below). The Far1 (nuclear or total) signal was undetectable until glucose returned.
This is true also for the "Whi5 re-entry cells".
They should include an interpretation of this to the discussion and propose a mechanism explaining how the sensitivity to pheromone is re-established after the re-entry if one of the key players of the pheromone pathway, the Far1, is downregulated due to starvation.

We thank the reviewers and the editor for their support. Please find our answers below.

Referee #1:

The authors have addressed most of my technical concerns (except for the histone measurements, which they encountered unsurmountable technical problems with) and provide additional convincing evidence that indeed Start as defined by Whi5 nuclear export can be reversed (as judged by Whi5 nuclear re-import, pheromone sensitivity, and Cln2 promoter activity) for some time after Whi5 export in response to glucose starvation. Therefore, in budding yeast - as in mammalian cells - cell cycle commitment in G1 might not be as hard-wired and irreversible as presumed. Albeit noted in other contexts before, this is an intriguing observation and the authors provide now ample convincing evidence. The authors also go through great length to identify the mechanistic basis and while not successful in identifying the underlying mechanism exclude several others. Therefore, despite the lack of mechanism per se, I think this manuscript warrants publication.

Few minor comments:
(1) It seemed to me that there is some discrepancy between the data shown in Figure 2E (a significant number of re-entries observed after 60 min) and the one in S1 (no re-entries observed after 60 min). Is there an explanation for this?
Thank you very much for catching this! This was a mistake, we only highlighted the “fast” re-entries in the data. This was obviously not the point of the figure, so we have now highlighted all re-entries. You will now find that Appendix Fig S1 is in good agreement with main figures 2 and 3.

(2) The data in EV1 looks significantly less switch-like than in the original paper. Is that due to the different media?
We cannot exclude that the media is the reason for this, but we think it is unlikely. The biggest biological difference is that the Donic 2011 strains were Δbar1, while our strains have a functional Bar1. Bar1 is the protease that degrades alpha-factor. Especially in denser colonies, this could lead to the cells perceiving less alpha-factor or perceiving it more slowly. Additionally, there is probably a statistical effect – since we were only recapitulating a published result, we analyzed much fewer cells in our logistic regression than in the original Donicic 2011 paper.

(3) I still find the manuscript rather difficult to follow and the figures often not very intuitive. Maybe the authors could have another critical look at it. For example, in different Figures the y axis describing Whi5 fluorescence in the nucleus is called "norm. nuclear intensity", "norm. nuclear concentration (AU)", "norm. nuclear Whi5 concentration (AU)" and "norm Whi5 nuclear concentration". If these all describe the same type of data and underlying measurements, it would be advisable to call them all the same (similarly for the description of the Whi5 slope). If they don't, it would be important to emphasize why they are different and why different ways for analysis where chosen.
We agree that the axis labelling was not good and now have consistent labelling on all graphs.

We have also tried to add a few visual cues to help connect subfigures. And we have made a few changes in the text to hopefully enhance reading flow.

Referee #3:

In the rebuttal letter the authors state: "Far1 was readily detected as cells entered G1, but was completely lost within 30 minutes of starvation (see figure below). The Far1 (nuclear or total) signal was undetectable until glucose returned. This is true also for the "Whi5 re-entry cells". They should include an interpretation of this to the discussion and propose a mechanism explaining how the sensitivity to pheromone is re-established after the re-entry if one of the key players of the pheromone pathway, the Far1, is downregulated due to starvation.
We have added a paragraph to the discussion. We have found a paper specifically analyzing the nutrient-dependent expression of Far1 and showing a severalfold increase in glucose vs ethanol, so rich vs poor carbon conditions (Alberghina 2004). Consistently, if you analyze all the expression profiles in the yeast genome database, you will find that some of the biggest expression changes in Far1 were reported for nutrient switches (though nobody specifically commented on Far1 in these datasets). It makes sense that mating should be coupled to nutrient sensing, in the opposite way as sporulation. We therefore assume that as we replenish glucose, there is some Far1 expression being induced by nutrient sensing pathways, which then allows sensing and responding to alpha-factor as observed. There is of course a lot more to explore in this context, but this goes beyond the scope of this study.

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 Author Checklist

Corresponding Author Name: Jennifer C. Ewald	**USEFUL LINKS FOR COMPLETING THIS FORM**
Journal Submitted to: EMBO J	The EMBO Journal - Author Guidelines
Manuscript Number: EMBOJ-2021-110321	EMBO Reports - Author Guidelines
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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 $\chi 2$ 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods, Appendix Strain Table. No restrictions.

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods.

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods, Appendix Supplementary Table

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends, main text
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends, main text

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Statement .
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	