nature portfolio

Peer Review File

Cell Cycle Length Determines Oncogenic Transformation Capacity

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
In this paper, Chen et al attempt to answer a fundamental question about why oncogenic events involving specific oncogenes or tumor suppressors exhibit tissue and/or cell lineage specificity. They sought to identify hallmarks that could predict which cell lineage within a tissue would give rise to a tumor upon an oncogenic event. The authors began by deleting Rb and p107 in the retina, which induces tumors from amacrine cells but not other cell lineages in the retina. Their strategy was to make additional genetic perturbations that would rescue tumor formation and then measure various hallmarks of cancer to see which of these was also rescued, thus identifying which hallmarks were correlated with tumor susceptibility. In the case of the Rb/p107 DKO, genetically manipulating the Skp2-p27-CDK axis rescued tumor formation, but the only cancer hallmark that was rescued was cell cycle time. The cell cycle time of DKO amacrine cells was shorter than that of other cell lineages and was lengthened by genetic manipulations of the Skp2-p27-CDK axis. However, the cell cycle times of other cell lineages was not affected by genetic manipulations of the Skp2-p27-CDK axis. The authors conclude that the shorter cell cycle time of the amacrine cells upon Rb/p107 knockout compared to the other cell lineages in the retina is predictive of their tumor formation sensitivity.
The authors attempt to demonstrate this is a general principle of oncogenic events by finding a similar correlation of cell cycle time after Rb knockout with the cell-of-origin of pituitary tumors. They also show this with concurrent Rb and p53 knockout in lung NE cells and expression of oncogenic KRas in lung AT2 cells. Thus the authors propose that the cell-of-origin of a particular tissue given a specific oncogenic event can be predicted by the relative cell cycle time.
Overall, this paper tackles an interesting question. However, the results are largely correlational, and the study focuses too specifically on genetic manipulations of the cell cycle signaling pathways to the exclusion of other cancer-relevant signaling pathways. Thus, there is concern that the authors reached their conclusion that cell cycle time is the only factor that differentiates cancer susceptibility too quickly without sufficiently testing other hypotheses. The manuscript as currently written is also overly confusing and difficult to understand, with many terms not being well defined, which makes this paper less appealing to a general audience. Given these concerns, I do not feel that this study merits publication in a high profile journal.

Below are a list of specific concerns and comments.

Major Concerns:
1. The authors extrapolate their findings too broadly by stating cell cycle time is “a general hallmark of lineage-specific predisposition to cancer initiation”. A previous study from Vishva Dixit’s group (PMID: 31000662) found that sensitivity to apoptosis upon mutation of the tumor suppressor BAP1 explained the tissue specific predisposition to BAP1-mediated tumors. Thus, the claims made by the authors should be toned down to reflect just those tumor suppressor and oncogene manipulations they tested in the current study. Its likely that each tumor suppressor has different properties that determine their tissue specificity, likely linked to their normal function in the cell. For example, Rb plays a critical role in the proliferation-quiescence decision and the initiation of the cell cycle, therefore its reasonable that cell cycle length would be the hallmark most correlated with tumor susceptibility upon its deletion. But this may not be the case for other tumor suppressors or oncogenes.

2. The authors strategy to test their hypothesis is to introduce additional genetic changes that suppress tumor formation upon Rb/p107 knockout and then measure changes in cancer hallmarks. However, the authors only introduce a genetic manipulations that affect one very specific cell-cycle related pathway, the Skp2-p27-CDK axis. Thus, it is not surprising that

knocking out Skp2 phenocopies expression of a mutant p27 that cannot bind CDK or knocking out CDK2 itself. To make their conclusion that cell cycle time is the only universal hallmark of the cell-lineage of origin, the authors would need to test many other tumor suppressive mutations that are not in the Skp2-p27-CDK axis. Put another way, the authors knockout Skp2, a well-known cell cycle protein, and find that it only alters cell cycle timing and not other hallmarks, such as apoptosis. But the authors should introduce a tumor suppressive mutation in the apoptosis pathway and measure the same set of cancer hallmarks. Ideally this would also be done for genes in the senescence pathway, DNA damage pathway, etc. Had the authors included this important control, they may have observed changes in apoptosis as well as cell cycle timing. In other words, by solely focusing on cell cycle related genes (Skp2, p27, CDK2) the authors were biasing their results to find defects in the cell cycle rather than other hallmarks.

3. This study is largely correlational, finding that cell cycle time (Tc) is correlated with tumor susceptibility of different lineages within a tissue. In order to prove convincingly that short Tc is the reason specific cell-lineages are particularly cancer susceptible, which was the original question posed, the authors need to speed up the Tc in tumor resistant cell-lineages and show that they are now tumor sensitive. This is obviously a very difficult experiment, as any mutation that speeds up the Tc are also likely tumor suppressors or oncogenes. Another way to test for causation is to introduce two different oncogenic mutations in the same tissue and look at the relative changes in Tc and correlate that with the relative tumor susceptibility. The authors in fact already have this data in the current paper where they either knockout Rb and p53 or express oncogenic KRas in the lung. They find that in both cases, AT2 cells have a Tc of ~22hrs. However, AT2 cells only form tumors when oncogenic KRas is expressed and not when Rb/p53 are deleted. So despite shortening Tc by the same amount in the same cell-lineage, the different mutations have different effects on tumor susceptibility. This result shows that tumor susceptibility is in fact independent of Tc. The authors note that this result suggests “additional factors influence cancer susceptibility” (line 229), which seems to undermine the overall conclusions of the study.

4. All of the hallmarks tested through-out the manuscript are primarily measured at p8. There are several experiments that measure hallmarks at times up to p21, but given that the Tc and Ts measurements in Fig 4B-D are primarily measured at p8, this is the one time point universally shared with the rest of the experiments. However, as shown in Figure 1A, retinal tumors are not observed until day 125, indicating that transformation may not take place until long after p8. Have the authors measured the various hallmarks of cancer at times approaching p125-p150? Many of the hallmarks of cancer tested, such as disrupted apical polarity and immune infiltrate, may not manifest until much later. By testing for these hallmarks at such early timepoints, the authors may have missed any changes in the other hallmarks that may occur at later time points.

Minor Concerns:
1. The authors frequently switch between the terms “hallmark” and “feature” (eg Lines 15-21). This can be confusing. Please choose one term and stick with that throughout the manuscript.
2. In the introduction, the term “APC” needs to be defined as this acronym can stand for several different things.
3. It would help if Figure S2 had a legend. Parts of this diagram are certainly self-explanatory, but other parts would benefit from a legend.
4. There are several references to “TKO” cells in the text, but not in the figure legends. I find this confusing. I see that TKO is defined as Rb-/-,p107-/-, SKP2-/- at one point in the text, but it is easy to forget this as one is reading through the text. Particularly since the authors use another triple-knockout line, Rb-/-, p107-/-, CDK2-/-, that could be confused as TKO.
5. Throughout the manuscript, abbreviations, arrows and other annotations on the microscopy images are not defined. For example, ONL, INL, GCL in Fig. 2A or the arrows in Fig. 2f, but there are other instances as well.
6. In the abstract, the authors state “Cancer-prone retina exhibits defective apoptosis, senescence, immune-surveillance, angiogenesis, DNA-repair, apical polarity, and proliferation index.” Are the authors referring to a normal amacrine cell in the retina or one in which Rb and p107 have been knocked out? This is not clear as currently written.
7. The authors routinely use one-way ANOVA with Bonferroni correction for the multiple comparisons test, but given the small sample size (typically n=3) for most experiments, a non-parametric test such as the Kruskal-Wallis test is most appropriate.

Referee #2

(Remarks to the Author)
In this manuscript by Chen, Bremner, and colleagues, investigate how cancer drivers initiate tumors, with a focus on the role of length of the cell cycle length in the cancer cell of origin. The authors first use mouse genetics to investigate genes whose loss may block the initiation of tumors in the retina upon loss of Rb and p107. These experiments validate a Skp2-p27-Cdk1/2 axis. Whether tumors grew or not, depending on the combination of genetics mutations, the authors did not find differences in apoptotic cell death or senescence in amacrine cells (from which tumors arise), and no differences in immune infiltration. Similarly, no differences were observed in DNA damage, angiogenesis, or apical polarity. Single-cell RNA-seq analyses and lineage tracing experiments suggested that the main difference associated with tumor development is the cell cycle status of cells in the amacrine lineage. Triple labeling experiments (EdU/Brdu/Ki67) showed that the genetic alterations that suppressed tumor development also increased the overall length of the cell cycle (that the authors call “Tc”), not the duration of S phase. Additional staining for amacrine cells showed that these cells have the shortest Tc and that Tc in these cells increases when mutations that suppress cancer are present. Similar results were observed in a mouse model of retinoblastoma dependent on Rb loss for cancer initiation, in a mouse model of SCLC dependent on Rb and p53 loss for cancer initiation, and in a mouse model of lung adenocarcinoma driven by oncogenic K-RAS.

The authors performed an impressive amount of work using state-of-the-art genetically engineered mouse models of human cancer to identify overall cell cycle length as a fundamental feature of cancer initiation. The experiments in the retina are striking because multiple cell types are investigated within the developing retina, with multiple genetic alterations. The

experiments in the lungs are also important because they compare different lung cancer types originating in distinct cell types upon specific genetic alterations. Conceptually, it is well known that different drivers initiate cancer in different cell types but the underlying mechanisms remain unclear: the data presented in this manuscript highlight a role for the cell cycle. The experiments are well performed, well controlled, and well presented. The text is very clear. Statistical analyses are shown. Overall, this study will be of great interest to scientists in the cell cycle and cancer biology fields.

I have only three specific comments and some more general thoughts.

Point #1: Because amacrine cells are the cell of origin of retinal tumors, it may be important to focus all analyses on this cell lineage. For example, in Figures 1G and 2A-B, is it possible to specifically quantify senescence and DNA damage in Ap2a+ cells?

Point #2, related to the calculation of cell cycle length: The measure of Ts and Tc in Figure S5D-F is interesting but a bit difficult to understand. First, Edu+ cells may not just be cells that were in S-phase at the beginning of the experiment but may also include some cells in G1, because EdU in the circulation is likely to last some time - or maybe I am wrong? I understand that the previous papers using this formula did not take this into account, and I am not sure why.Also, some cells may have divided after the initial EdU injection (not enough dilution of the signal to distinguish cells that have divided and cells that have not, but I could be wrong again, maybe 2 hours is never enough to go through G2 and M?). Would that affect the numbers? Is the red line in S5D accurately representing the situation in vivo in mice. Finally, looking back at the manuscript that the authors refer to, the formula there is different (PMID: 29445897), shouldn't the numerator for Ts calculation include Edu+ BrdU+ double positive cells (cells that stayed in S phase)?

Point #3, related to the timing of cancer initiation: What may be a bit misleading in this study is that pediatric retinoblastoma arises from cells that are dividing or about to exit the cell cycle. But the three other cancer types that the authors examine normally occur in adults (mice and patients), at a time where pituitary cells, neuroendocrine cells, and AT2 cells are not dividing any more. However, the authors examine cell cycle kinetics at early time points for the lung cancer models (E18 for SCLC, and 4-week-old mice for lung adenocarcinoma), while tumors in these models are usually initiated when mice are between 8-12 weeks of age. Thus, their work may look at the cell of origin in the lung cancer models at the "wrong" time, and possibly the results would be different at later time point. For the K-RAS model, it should be straightforward to add tamoxifen a few weeks later and repeat some of the staining. For the RB/p53 model (and the K-RAS model), Adeno-CMV-Cre (or AAV-Cre) may be another option as it should infect cells in all the main lineages. It may not be required to repeat all the experiments at later time points, but some data at these later time points may be important.

General comments:

There is of course a bias in the way the authors conducted their study: the Skp2-p27-Cdk1/2 axis is related to cell cycle progression primarily. One could easily imagine that other alterations blocking cancer initiation would promote cell death for example, which may have changed some of the conclusions. It is also noteworthy that the authors have chosen models in which cell death in the cell of origin is not very prevalent, which may not be the case in other models.

The authors show a number of "negative" experiments but have the appropriate positive controls. Still, negative data are always difficult to interpret: what if the authors looked at P6 instead of P4 and P8 in the developing retina, maybe then cell death becomes more relevant? Of course, it is impossible to quantify all phenotypes at all time points.

Finally, it is worth noting that the study from the Cobrinik lab in human retinoblastoma (Nature, 2014) had identified a role for cell death inhibition in cancer initiation (via MDM2). Thus, one could argue that the identification of cell cycle length by the authors in this new study is an artefact of mouse models.

It is difficult to address this issue experimentally (how many models would be needed to disprove the model…). Overall, the authors can conclude that in the specific models they examined, a shorter cell cycle length correlates with greater cancer initiation, but they cannot exclude that other mechanisms may be at play in other contexts – something worth noting in the manuscript.

Referee #3

(Remarks to the Author)
In this work, Chen et al set out to address one of the fundamental open questions in cancer biology: why do oncogenic mutations induce tumours in some cell types and not others? To do this, they use a mouse model of retinoblastoma where Rb/p107 double knockout (DKO) in the retina induces tumours, previously shown to arise specifically from amacrine cells. Here the authors combine this DKO with a series of further mutations that perturb cell cycle entry (knocking out CDK1 or disrupting the p27/skp2 interaction). Intriguingly, these genotypes induce similar early effects on proliferation and tissue morphology as the DKO but they do not go on to form tumours. The authors then carry out a comprehensive characterisation of the differences between the tumour-causing DKO and six suppressive genotypes. The only consistent difference they find across all genotypes is the total length of the cell cycle (Tc), which is shorter in cancer-prone genotypes. They then go onto show that cell cycle length is also shorter in the amacrine cells-of-origin compared to other lineages in the retina and that the cells-of-origin also have shorter cell cycles in mouse models of two other Rb-driven tumours and a model of KRAS-driven lung cancer. This leads them to propose that cell cycle length is a new hallmark for cancer susceptibility.

The approach used in this work is original: it demonstrates that there is as much to be learned from cells that do not from tumours as those that do. To my knowledge, this is the first work to claim that total cell cycle length is a key determinant in cancer susceptibility. However, the claims of the universality of this mechanism are overstated in the manuscript. The authors claim that they have discovered a 'general hallmark of lineage-specific predisposition to cancer initiation'. This work lacks the data to support such general claims and would be better framed as a specific mechanism in Rb (see suggestions below). That said, even as a Rb-focused paper, this work is likely to be of wide interest to scientists in the cell cycle field and in cancer biology more generally. I leave it up to the editor to decide if a Rb-specific paper would be of general enough interest to merit publication in Nature or whether this work would be better suited to a more specialist journal.

The data presented is robust and high quality. Multiple complementary techniques are used to demonstrate key findings (eg. use of scRNAseq data and EdU/BrdU labelling for cell cycle analysis). Error bars and statistical tests are properly described and are appropriate. The figures are clear and well-presented and do a good job of clearly distilling a large amount of data. The manuscript is well written. Overall, this work is of the quality that would be expected for publication in Nature.

Suggestions for improvement:

1. Universality of claims: the authors claim to have revealed cell cycle length as 'a blueprint to identify any cell-of-origin' (abstract) and 'the first general hallmark of lineage-specific susceptibility to cancer initiation (line 248). However they haven't convincingly shown that this is a general mechanism. It needs to be made clear throughout the manuscript that the findings relate to a mouse model of retinoblastoma and that any wider extrapolation is purely speculative. I would also suggest adding 'in retinoblastoma' to the title. Given the specific role that Rb plays in the cell cycle in repressing E2F transcription and cell cycle entry in G1, it makes sense that knockout would shorten the cell cycle. Similarly the suppressive genotypes either involve depletion of Cdks or result in in p27 activation, both of which would be expected to slow cell cycle entry in G1 and therefore lengthen the cycle. However, it doesn't follow that other oncogenes or tumour suppressors would behave in the same way or that this would be a general hallmark for all cancers. The authors do show that cell cycle length is shorter in the cells-of-origin for KRAS-driven lung cancer (Fig 5 E,F) but the evidence for this is far weaker than the rest of the work and I have some issues about the quantification of cell cycle lengths in the lung (see point 2).

2. More detail should be given on how Tc is quantified. The central claim of this work relies on the calculation of cell cycle lengths in vivo (Fig 4 C, D), so it is of utmost importance that these methods are well described. Examples of the original images used for calculations in Fig 4 C & D should be shown in the supplement with examples of cells scored Ki67+ or EdU+BrdU- highlighted. The method for cell counting in the microscopy images should be better described – did it involve automated image analysis or was it manual? If manual, how was the accuracy and reproducibility of cell counting ensured? The n numbers given for these experiments refer to the number of animals, but it is important to state in the methods the number of images analysed per animal and also the total number of cells counted in different conditions. I worry that low cell numbers could lead to errors in Tc calculation. This is particularly the important in the lung when analysing rare cell types such as NE cells. For example, in figure S6D, there are very few NE cells present in the example image and only one that looks ki67 positive and would be included in the analysis. Also, Tc in NE cells in fig 5D is calculated to be 11 hours, which is unusually short for a mammalian cell cycle, given that replicating the genome usually takes at least 7-8 hours (and the cells still need time to carry out the transcriptional programmes associated with licensing in G1 and preparation for mitosis in G2.) I am concerned that the calculations do not hold up as well in rare cell types such as NE cells and would like to see proof of the robustness of the method.

3. The authors should comment further on the timing of oncogenic events. One limitation of this work is that the authors are examining early oncogenic events at P4-P21, while tumours develop at 100+ days. Some of the hallmarks examined, such as immune infiltration and angiogenesis would be more relevant at later stages of tumorigenesis. Later apoptosis or cell cycle arrest could also halt tumour development in suppressive genotypes. It would be interesting to get some information in the text and/or figures about when the oncogenic and suppressive genotypes start to diverge from one another phenotypically. The differences in cell cycle length (Fig 4, C & D) are measured at P8 only. Do these persist at later time points?

Minor comments

• The authors show that total cell cycle length but not DNA replication time is increased in the suppressive genotypes. Given the role of Rb in cell cycle entry it seems likely that the main difference would be in the length of G1. Fig 3D supports this idea as it shows an increased proportion of G1 cells in a suppressive genotype. Could it be that G1 length is the crucial factor rather than Tc? The authors should comment on this in the text.

• Figure S1C – it's not very clear how the numbers plotted in S1D relate to the images. For example, the p27KI/+ genotype apparently has 80% of amacrine cells that are positive for p27 but in the associated image in S1C, it does not look like 80% cells are both red and green. It would be helpful to show a greater zoom with colours separated.

• Figure 2f – is the control the same as in previous figures? In figure 1 and 2A-E, the non-tumorigenic control is p107KO but here it is labelled Rbf/f. Please can the authors clarify why this is different.

• Figure 5 – I found it confusing that the same colours are used for the graphs in the left and right panels when they are not comparing the same thing.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The revised manuscript by Chen et al. has been significantly strengthened through extensive revisions and the addition of new data, effectively addressing the previous major concerns. In particular, I think the Discussion is much improved and better explains the strengths of their findings and better defines the scope of their conclusions. I now believe the manuscript is poised for publication, pending a minor adjustment to the text to ensure clarity and precision.

In lines 49-50 in the Abstract, “Notably, every tumor-suppressive mutation increased Tc.” is too broadly stated and could be interpreted as every single tumor-suppressive mutation known, whereas I believe the intention of the authors is to say every mutation they tested in this study. More accurately, the sentence should be revised to “Notably, every tumor-suppressive mutation tested increased Tc.” or something similar.

Referee #2

(Remarks to the Author)
The authors did a great job answering the points raised by the Reviewers. The clarification that the study focuses on cells surviving the oncogenic insult makes sense.

I agree with the authors in response to the reviews that one may not expect members of the Skp2-p27-Cdk axis to have the same function in tumorigenesis (similar to the p16-CycD/Cdk4,6-RB axis – these pathways are not exactly linear). Their results that complete loss of SKP2 rescues apical polarity defects are a good example of this.

The new data with young adult mice in the SCLC model are nice and supportive of the general hypothesis, as are the new data with RAF.

The authors should be commended for their in-depth analysis of so many cellular phenotypes in multiple genetic backgrounds. Their work tackles a key question in cancer biology, starting with the retinoblastoma paradigm. The extension to other cancer models is really powerful. The work presented by the authors raises a lot of really interested questions, but these belong to follow up studies in my opinion.

Referee #3

(Remarks to the Author)
In this revision, Chen et al have put significant effort into addressing the reviewers’ comments including the addition of new data. The revised manuscript is improved, not only due to the additional data, but in writing style (the abstract in particular is improved), data presentation, figure labelling and descriptions of methodology. My specific comments have been comprehensively addressed in the revision. The description of Tc calculation and methodology are much improved, and the microscopy examples shown in Supplementary Figures 4-7 are clearer and more convincing. Including the raw cell cycle data in a supplementary table adds transparency and this should definitely be included in the final publication. The additional data in Supp Figure 8 also strengthens the methodical approach.

Regarding the universality of the claims, the addition of new data showing the effect of different timings of KRAS activation and the BRAF experiments in the lung (Figure 5) do demonstrate that the association of shortest Tc with susceptibility to transformation holds true in other contexts and is not solely an Rb-specific effect. The lung experiments are important as three different oncogenic mutations are shown to have the same effect on Tc but in different cells of origin. Of course, this does not prove universality (which would not really be possible) but it does strengthen the claim that this is a general rather than Rb-specific finding. The authors have also edited the text to better reflect this. As the authors point out in the discussion, exploring how far these findings apply to other tumour settings and to human tissue is something that they (and the field in general) can follow up in coming years. Overall, this is now a very strong paper that presents a genuinely new idea in cancer biology.

Minor points
- The authors have added additional text (lines 108-114) to the introduction to detail the roles of Rb and Skp2/Cdks outside of cell cycle control. While I understand why they have added this in response to the reviewers’ comments, it disrupts the narrative of the introduction and would be better to be cut or moved to the discussion.
- The analogy to behavioural science (Ref 82 – lines 433-438) is a bit of a stretch and I find it unhelpful to equate cancer development to human choices. This discussion would fit better in a review/opinion piece and should be removed.

Detailed rebuttal, Chen *et al*: Cell cycle length predicts cancer susceptibility. Nature reviews.

Referee #1 (Remarks to the Author):

In this paper, Chen et al attempt to answer a fundamental question about why oncogenic events involving specific oncogenes or tumor suppressors exhibit tissue and/or cell lineage specificity. They sought to identify hallmarks that could predict which cell lineage within a tissue would give rise to a tumor upon an oncogenic event. The authors began by deleting Rb and p107 in the retina, which induces tumors from amacrine cells but not other cell lineages in the retina. Their strategy was to make additional genetic perturbations that would rescue tumor formation and then measure various hallmarks of cancer to see which of these was also rescued, thus identifying which hallmarks were correlated with tumor susceptibility. In the case of the Rb/p107 DKO, genetically manipulating the Skp2-p27-CDK axis rescued tumor formation, but the only cancer hallmark that was rescued was cell cycle time. The cell cycle time of DKO amacrine cells was shorter than that of other cell lineages and was lengthened by genetic manipulations of the Skp2-p27-CDK axis. However, the cell cycle times of other cell lineages was not affected by genetic manipulations of the Skp2-p27-CDK axis. The authors conclude that the shorter cell cycle time of the amacrine cells upon Rb/p107 knockout compared to the other cell lineages in the retina is predictive of their tumor formation sensitivity.

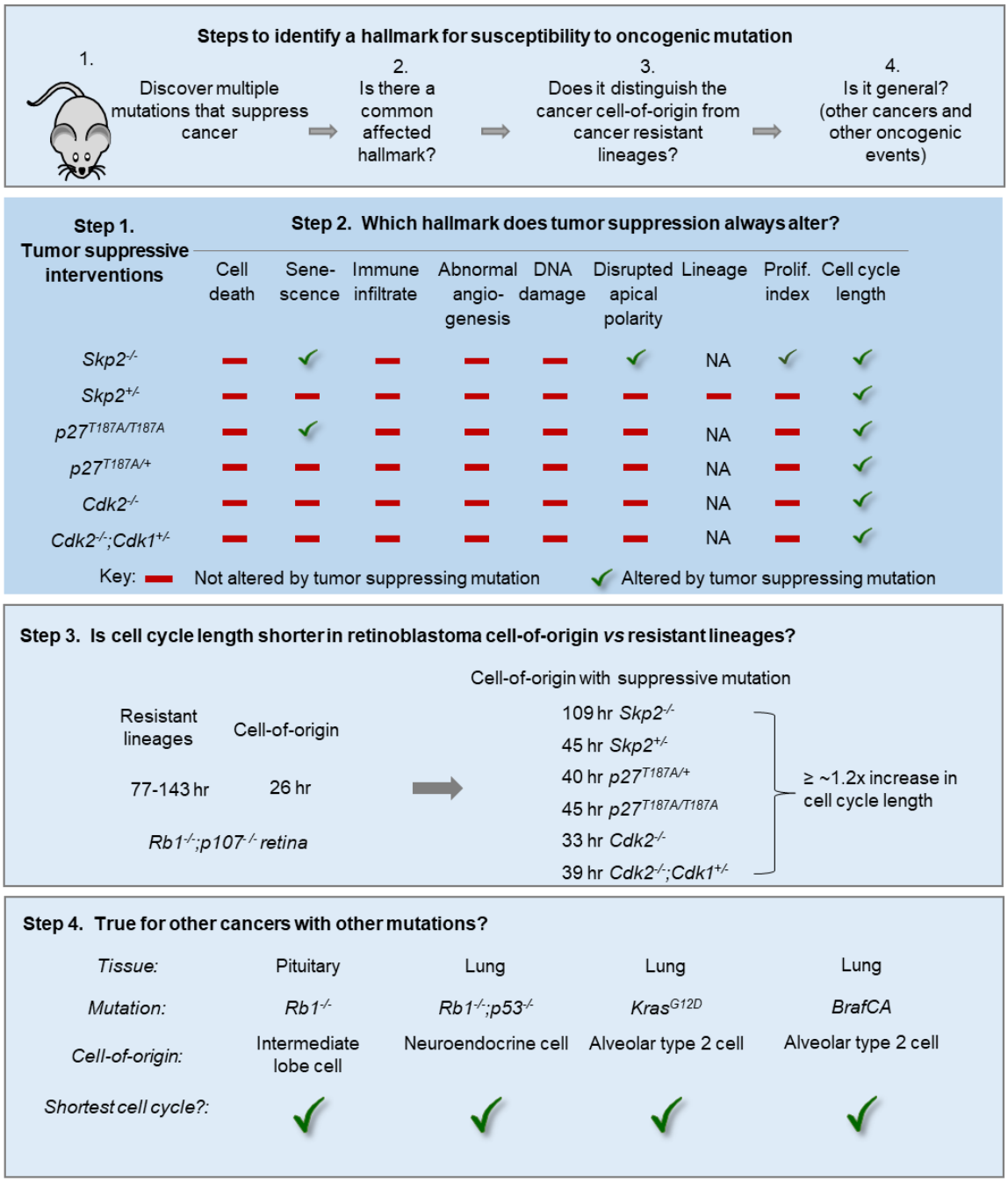
The authors attempt to demonstrate this is a general principle of oncogenic events by finding a similar correlation of cell cycle time after Rb knockout with the cell-of-origin of pituitary tumors. They also show this with concurrent Rb and p53 knockout in lung NE cells and expression of oncogenic Kras in lung AT2 cells. Thus, the authors propose that the cell-of-origin of a particular tissue given a specific oncogenic event can be predicted by the relative cell cycle time.

Overall, this paper tackles an interesting question. However, the results are largely correlational, and the study focuses too specifically on genetic manipulations of the cell cycle signaling pathways to the exclusion of other cancer-relevant signaling pathways. Thus, there is concern that the authors reached their conclusion that cell cycle time is the only factor that differentiates cancer susceptibility too quickly without sufficiently testing other hypotheses. The manuscript as currently written is also overly confusing and difficult to understand, with many terms not being well defined, which makes this paper less appealing to a general audience. Given these concerns, I do not feel that this study merits publication in a high-profile journal.

We thank the reviewer for noting that lineage-specific cancer susceptibility is a fundamental and interesting question, and we appreciate their thoughtful critique of our work. First, we respond briefly to the general comments above, then we provide a more comprehensive response to the referee's specific concerns and comments.

Re "largely correlational": Many fundamental scientific breakthroughs are correlational, such as high-profile studies in the past involving GWAS, transcriptomics or proteomics, or spatial transcriptomics in the current literature. The keys are the tightness of the association observed and the extent to which it redirects or stimulates the field to birth new hypotheses. Cancer research focuses on the transforming effects of oncogenic events, paying most heed to the tumor. However, the response to oncogenic mutations in most lineages is not cancer, but resistance, and this is rarely studied. There is even less focus on the fact that, despite some initial ectopic division in response to an oncogenic event, normal development, as opposed to say cell death, senescence, or immune clearance, is the common cancer resistance mechanism (refs and more detail below). A cancer initiation hallmark in surviving cells is exciting because it invigorates this area with a new model, opening a door to mechanism and preventive therapies.

Re the focus on "cell cycle signaling pathways": The cancer genes we used to initiate tumorigenesis (*Rb/p107*, *Rb*, *Rb/p53*, *Kras*, and now also *Braf*), like most major oncogenic lesions, regulate many cancer hallmarks, not just cell cycle. Historically, the best studied Rb function is proliferation, but one should not overlook its potent effects on other hallmarks (details below, and now added to manuscript). At the start of this work, it was unclear whether any hallmark would predict cancer susceptibility, and despite decades of research, no publication has described a hallmark that could distinguish $Rb^{-/-}$ cancer-prone lineages from $Rb^{-/-}$ cancer-resistant lineages. Indeed, the most probable outcome, if one had tried to predict, would be that there would be no consistent correlate. Even cell cycle seemed at first irrelevant because, while retinoblastoma suppression by homozygous *Skp2* loss reduced proliferation index, all the other tumor suppressing mutations did not (summarized in **Extended data Fig 2**). It was not until single cell data exposed only cell cycle genes that we revisited cell division, focussing on rate rather than index.



Extended Data Fig. 2. An *in vivo* strategy to identify a cancer cell-of-origin hallmark.
The top panel summarizes the four steps. Step 1 introduces mutations to suppress cancer (here, retinoblastoma). Step 2 assesses multiple (here, nine) cancer hallmarks to determine whether any are always altered upon tumor suppression; only cell cycle length (Tc) fulfilled this criterion. Step 3 asks whether Tc is shorter in the cancer-prone (here, amacrine cell) *vs.* cancer-resistant lineages (Müller glia, horizontal cells). Tc in cancer-prone or resistant lineages is indicated on the left, and the effect of multiple tumor-suppressing mutations on Tc is shown on the right. Step 4 compares Tc of cancer-prone *vs.* resistant lineages in multiple other contexts. Tc was always shortest in the cancer-prone lineage independent of oncogenic lesion or tissue (indicated by green check marks).

Moreover, our studies are not limited to Rb. Tc also predicted cancer susceptibility in response to *Kras*^{G12D} activation. Importantly, we have added new data showing that Tc is independent of when we inactive *Rb* or activate *Kras*, and we have added new analysis of the Ras pathway effector *Braf*^{V600E}. In every case, Tc singled out the cell-of-origin. Kras and Braf are not canonical cell cycle regulators, yet like Rb, and most of the major cancer genes, they perturb division and a host of other hallmarks (details added to manuscript). It's surprising that the same hallmark can predict lineage-specific susceptibility in response to Rb and Ras pathway defects.

Re the writing style: Reviewer #2: "The experiments are well performed, well controlled, and well presented. The text is very clear."; and Reviewer #3: "The figures are clear and well-presented and do a good job of clearly distilling a large amount of data. The manuscript is well written. Overall, this work is of the quality that would be expected for publication in Nature". We have carefully considered Reviewer 1's comments and modified the text to further improve clarity for a general audience (details below).

Below are a list of specific concerns and comments.

Major Concerns:

1. The authors extrapolate their findings too broadly by stating cell cycle time is "a general hallmark of lineage-specific predisposition to cancer initiation". A previous study from Vishva Dixit's group (PMID: 31000662) found that sensitivity to apoptosis upon mutation of the tumor suppressor BAP1 explained the tissue-specific predisposition to BAP1-mediated tumors. Thus, the claims made by the authors should be toned down to reflect just those tumor suppressor and oncogene manipulations they tested in the current study.

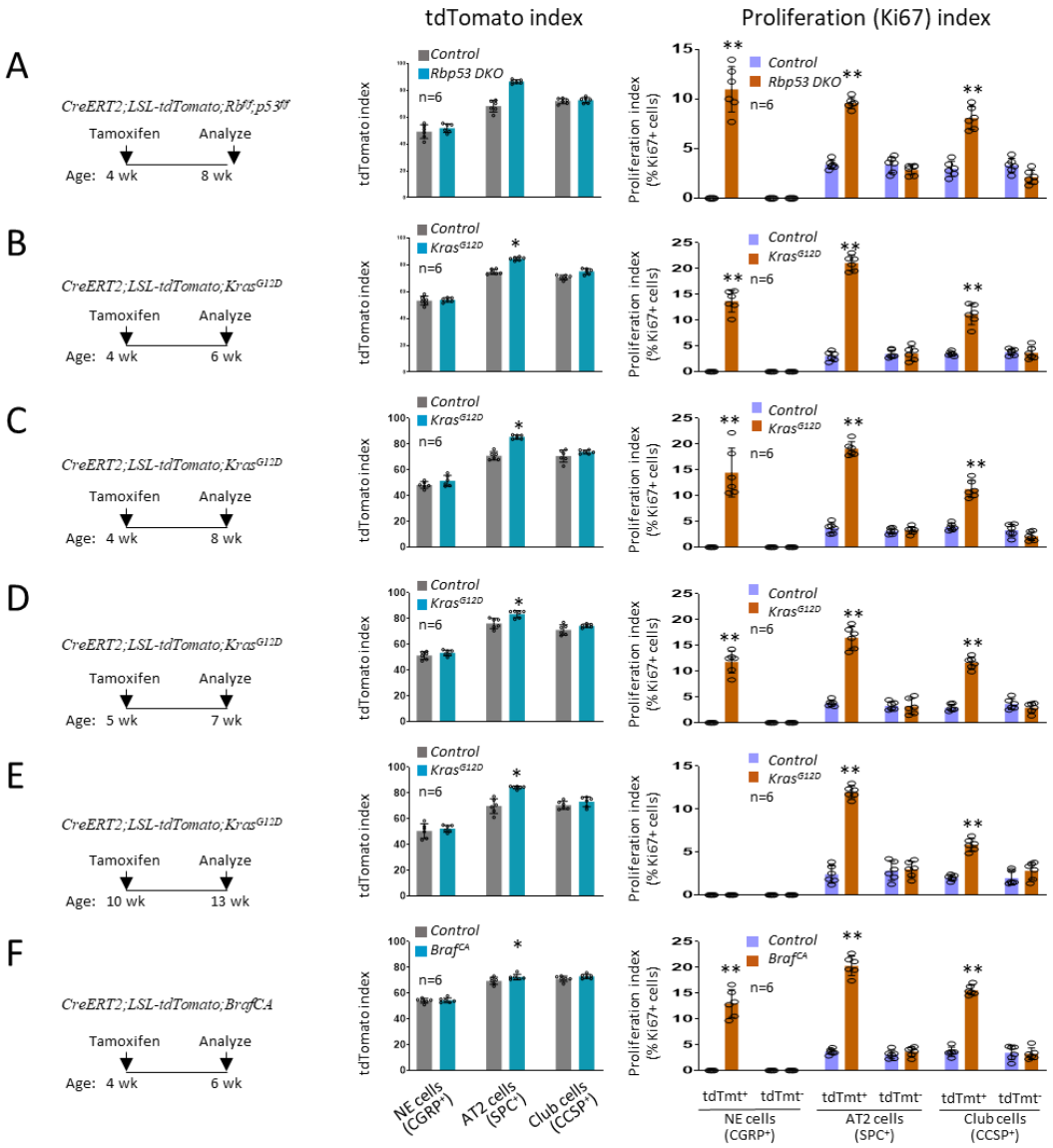
The reviewer is correct that cell death explains lineage-specific cancer resistance in some instances. However, humans carry millions of normal, functional cells with oncogenic mutations [1,2], thus normal cell development is a common cancer-escape mechanism. In chimeric mice, made by injecting *Rb*$^{-/-}$ ES cells into *Rb*$^{+/+}$ blastocysts, *Rb*$^{-/-}$ cells constitute 50% of nearly all adult tissues, yet only the pituitary develops tumors [3,4]. Trillions of other *Rb*$^{-/-}$ cells are alive, yet non-cancerous, an astonishing observation. Other chimeric studies made similar observations, e.g. with *Ink4a* (p16/Arf) loss, *Fos* overexpression, or a *Mll-AF9* gene fusion [5–7]. Moreover, *Kras* or *Braf* mutations do not kill neuroendocrine or club cells in the lung, yet these lineages resist transformation. It is unclear why these and so many other lineages resist cancer, despite their ability to survive oncogenic lesions. The modified opening paragraph in the introduction (lines 58-77) now includes the BAP1 paper and clarifies that our focus is on whether there is any way to distinguish cancer susceptibility *vs*. resistance in cells that *survive* an oncogenic event. Lineage-specific cancer susceptibility is not well understood, particularly for the typical scenario in which cells survive oncogenic insults. Our work exposes lengthened Tc as a fundamental driver of cancer evasion, without the need for apoptosis, senescence or immune surveillance.

To further test and expand the original claims, we added many new experiments. Additional replicates were performed for multiple *in vivo* experiments to increase n from 3 to 6 (**Fig 1D-G, Fig 1I, Fig 2B-E, Fig 4A-D, Fig 5A, Extended data Fig 5A, D, Extended data Fig 6A, B, Extended data Fig 7A**). We also performed n = 6 for new data sets added to the manuscript (**Fig 5B-D**, **Extended data Fig 7B-F, Supplementary Fig 2B, Supplementary Fig 8B**). New induction time points were assessed for *Rb/p53* deletion or *Kras*^{G12D} activation in the lung (**Fig 5B, C**), and another oncogenic event in the Ras pathway was studied (*Braf*^{V600E}; **Fig 5D)**. Details are as follows:

Previously we studied *Rb/p53* loss in the embryonic lung at E18 using *hSPC-rtTA;tetCre;Rb*$^{f/f}$*;p53*$^{f/f}$ mice, and now we also studied this oncogenic lesion in adult lung by generating *CreERT2;LSL-tdTomato;Rb*$^{f/f}$*;p53*$^{f/f}$ mice or control *CreERT2;LSL-tdTomato* littermates and inducing recombination with tamoxifen at 4wk of age and harvesting 4wk later. Recombination was efficient, and ectopic division was only observed following *Rb/p53* loss (**Extended data Fig 7A, Supplementary Fig 5E, F**). As in the embryonic lung, neuroendocrine (NE), alveolar type 2 (AT2) and club cells all showed ectopic division, but cancer-prone NE cells had a shorter Tc than cancer resistant AT2 or club lineages (**Fig 5Bii**). The new text accompanying this extra data is at lines 336-346.

Before, we studied *Kras*^{G12D} activation using *CreERT2;LSL-tdTomato;Kras*^{G12D} mice and control *CreERT2;LSL-tdTomato* mice treated with tamoxifen at 4wk of age and harvested at 6wk (**Fig 5Ci**). Now we also quantified lineage-specific Tc after treating with tamoxifen at 4wk and harvesting lung at 8wk (different post-initiation period) or adding tamoxifen at 5wk or 10wk of age and harvesting lung at 7wk or 13wk of age, respectively (different

initiation timepoints). Recombination was efficient and ectopic division was observed in NE, AT2 and Club cells upon induction at 4 or 5wk, while induction at 10wk triggered ectopic division of AT2 and Club but not NE cells,



Extended data Fig. 7. tdTomato (tdTmt) index and proliferation (Ki67) index of tamoxifen-induced *Rb/p53* DKO, *Kras^{G12S}* and *BrafCA* mouse lung cells. A, Control (*CreERT2;LSL-tdTomato*) and *Rb/p53* DKO animals (*CreERT2;LSL-tdTomato;Rb$^{f/f}$;p53$^{f/f}$*) injected with tamoxifen at 4 wk and assessed at 8 wk of age. **B-E,** Control (*CreERT2;LSL-tdTomato*) and *Kras* (*CreERT2;LSL-tdTomato;Kras^{G12D}*) mice injected with tamoxifen at: 4 wk and assessed at 6 wk of age (B); 4 wk and assessed at 8 wk of age (C); 5 wk and assessed at 7 wk of age (D); 10 wk and assessed at 13 wk of age (E). **F**, Control (*CreERT2;LSL-tdTomato*) and *BrafCA* animals (*CreERT2;BrafCA;LSL-tdTomato*) injected with tamoxifen at 4 wk and assessed at 6 wk of age. Schematics on the left summarize the experiments. Graphs in the center show tdTmt index of the indicated cell types (grey control, blue oncogenic lesion induced). Graphs on the right show Ki67 index of the indicated cell types for control tdTmt$^+$ or tdTmt$^-$ cells in *CreERT2;LSL-tdTomato* mice or control unrecombined tdTmt$^-$ cells in *CreERT2;LSL-tdTomato; Rb/p53, Kras^{G12D}, or Braf^{CA}* mice (blue bars), or induced tdTmt$^+$ cells in the latter (red bars). Error bars: SD (n = 6); asterisks: significant difference between control *vs.* mutated mice (* $p < 0.05$, ** $p < 0.01$. unpaired student's t-test).

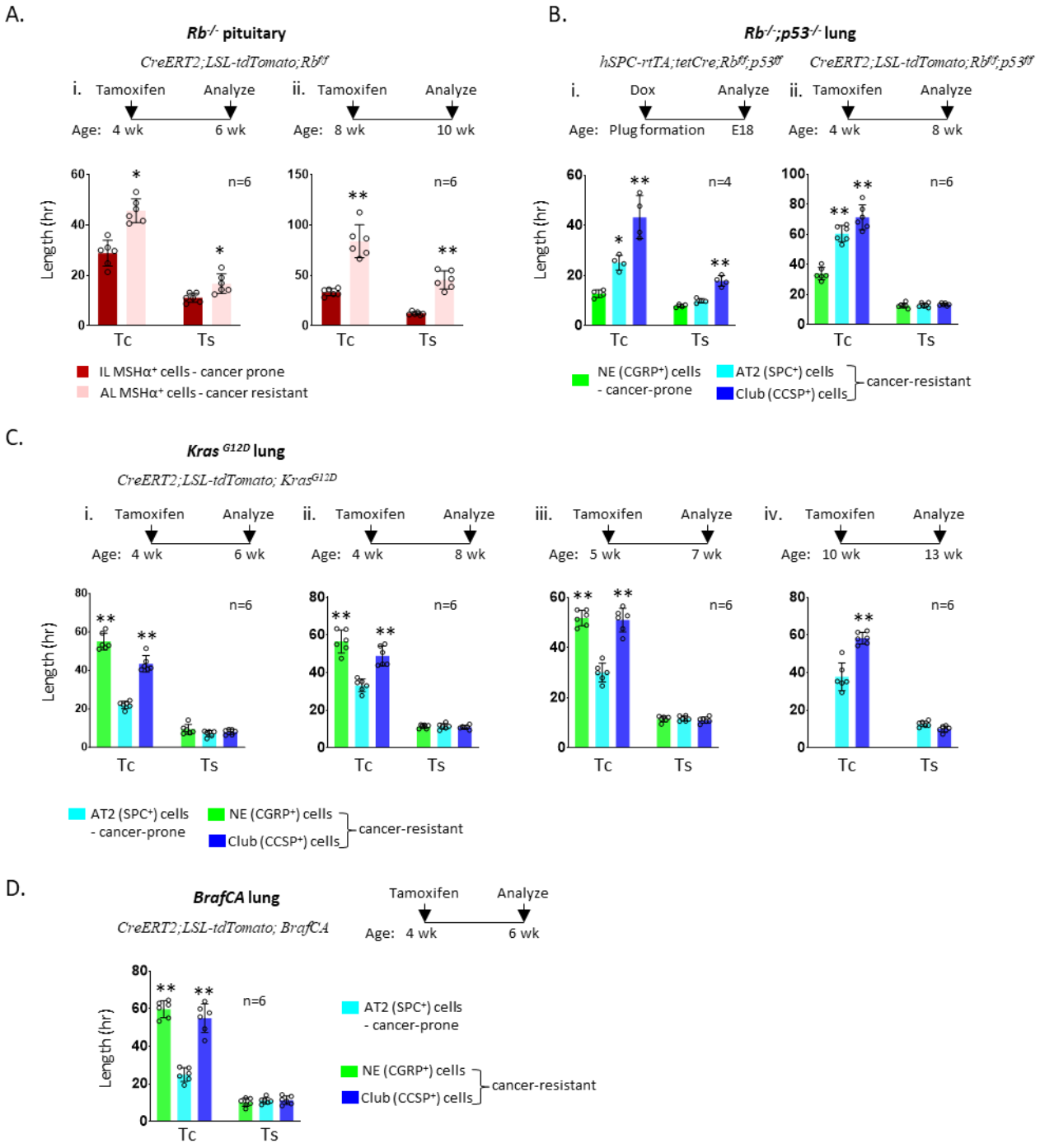


Fig. 5. Shortest Tc as a general hallmark of cancer susceptibility.

A. Tc and Ts of MSHα+ cells in the intermediate (IL) and anterior (AL) pituitary lobes of *Rb* null (*CreERT2;LSL-tdTomato;Rb$^{f/f}$*) mice injected with tamoxifen at 4 or 8 wk and assessed at 6 or 10 wk of age, respectively. **B.** Tc and Ts of lung CGRP+ neuroendocrine (NE), SPC+ Alveolar type 2 (AT2) and CCSP+ club cells of E18 *Rb/p53* DKO animals (*hSPC-rtTA;tetCre;Rb$^{f/f}$;p53$^{f/f}$*) injected with doxycycline at plug formation and assessed at E18 of age, and of P56 *Rb/p53* DKO animals (*CreERT2;LSL-tdTomato;Rb$^{f/f}$;p53$^{f/f}$*) injected with tamoxifen at 4 wk and assessed at 8 wk of age. **C.** Tc and Ts of lung NE, AT2 and club cells of *Kras^{G12D}* animals (*CreERT2;Kras^{G12D};LSL-tdTomato* mice) injected with tamoxifen at 4 wk, 4 wk, 5 wk, and 10 wk and assessed at 6 wk, 8 wk, 7 wk and 13 wk of age respectively. **D.** Tc and Ts of lung NE, AT2 and club cells of *BrafCA* (*CreERT2;BrafCA;LSL-tdTomato*) mice injected with tamoxifen at 4 wk and assessed at 6 wk, of age. Error bars: SD (n = 6 for A, Bii, C, D; n=4 for Bi); asterisks: significant difference between MSHa+ IL *vs.* AL cells (A), or NE *vs.* AT2/club cells (B), or AT2 *vs.* NE/club cells (C, D) (* $p < 0.05$, ** $p < 0.01$. one-way ANOVA, Bonferroni correction for D and F, unpaired t-test for A, B, C, and E).

and tumors developed as expected from AT2 cells (**Extended data Fig 7C-E, Supplementary Fig 6A-C**). In every case, Tc was shortest in the cancer-prone AT2 cells (**Fig 5Cii-iv**).

To study $Braf^{V600E}$ activation we generated *CreERT2;LSL-tdTomato;BrafCA* mice which were treated at 4wk and analyzed at 6wk of age (*BrafCA* = a constitutively active V600E mutation [8]). Tamoxifen treatment induced efficient recombination, and ectopic division to comparable levels in NE, AT2 and Club cells only in mice with the *BrafCA* allele, and tumors arose from AT2 cells as expected (**Fig 5D, Extended data Fig 7F, Supplementary Fig 7**). As with $Kras^{G12D}$ activation, the tumor-prone AT2 cells exhibited the shortest Tc (**Fig 5D**).

Previously, we had already studied induction of *Rb* loss in the pituitary at two distinct time points (**Fig 5A**). These extensive new data confirm emphatically that Tc is predictive independent of initiation event timing across different tissues (pituitary, lung) and oncogenic events (*Rb, Rb/p53, Kras*). The new *Braf* results indicate the general relevance to Ras pathway activation.

We agree that Tc may not predict cancer susceptible *vs*. resistant lineages in all tissues where cells survive an oncogenic lesion. Our work provides extensive assessment of the *Rb* and *Ras* pathways, all *in vivo*, addressing the fundamental, yet poorly understood issue of why normal development is such a common mechanism of cancer-escape in so many lineages. To avoid overstatement, the original comment in the **Abstract** about Tc being a "general hallmark" was removed, and we now note that the results apply "across multiple tumor models *in vivo*" or "in several settings" as follows, Lines 44-46: "Here, we show that total cell cycle length (Tc) predicts susceptibility to transformation across multiple tumor models *in vivo*."; and lines 54-56 to close the abstract: "Thus, relative Tc is an initiation hallmark that distinguishes cancer-prone from cancer-resistant lineages in several settings, explaining how mutated cells can escape transformation without inducing apoptosis, senescence or immune surveillance".

Also, at the end of the opening paragraph in the **Discussion** we now state directly that future studies are needed to further test generality, while also emphasizing the novelty of our discovery, Lines 408-411: "Further studies will determine the extent to which this correlation holds across additional tissues and oncogenic events, but our work provides a framework to understand how so many lineages escape cancer without deploying resistance mechanisms such as senescence, apoptosis or immune surveillance [1–7]".

Its likely that each tumor suppressor has different properties that determine their tissue specificity, likely linked to their normal function in the cell. For example, Rb plays a critical role in the proliferation-quiescence decision and the initiation of the cell cycle, therefore its reasonable that cell cycle length would be the hallmark most correlated with tumor susceptibility upon its deletion. But this may not be the case for other tumor suppressors or oncogenes.

Our work is not limited to Rb. We demonstrate that relative Tc also distinguishes cancer-prone *vs*. cancer-resistant lineages subject to Ras pathway mutations. *Rb/p53* loss as well as *Kras* or *Braf* activation in the lung trigger ectopic division of NE, AT2 and Club cells, but while *Rb/p53* loss triggers tumors from NE cells, *Kras* or *Braf* mutations induce AT2-derived tumors. Relative Tc predicts the distinct cell-of-origin in lung independent of the oncogenic event or the timing of that event (**Fig 5B-D**).

Even without the *Kras* and *Braf* analyses, the historical link between Rb and the cell cycle should not obscure the fact that it's loss can also affects DNA repair [9], apical polarity [10,11], angiogenesis [12], immunity [13], lineage plasticity [14,15], cell survival [16–18] and senescence [19,20] (summary at: https://cancer.sanger.ac.uk/cosmic/census-page/RB1). Indeed, it is the norm that major oncogenes and tumor suppressors regulate multiple cancer hallmarks. Historically, they were associated first with one aspect of biology: e.g. Rb with proliferation, Notch and Wnt with development, Ras with cytoplasmic signaling, etc.; but they affect multiple outputs. Cell cycle regulation was/is not the best-known Kras or Braf function, but they both alter proliferation, and like *Rb* loss, their manipulation disrupts numerous other cancer hallmarks (https://cancer.sanger.ac.uk/cosmic/census-page/KRAS). These perspectives are now highlighted in the Results (lines 95-98, and 347-350) and the opening paragraph of the Discussion (lines 387-411).

At the start of this project, it seemed unlikely that any hallmark, or any combination would consistently predict cancer susceptibility. Indeed, although Rb was functionally linked to proliferation over 30 years ago [21], no studies had linked Tc to susceptibility to *Rb* loss. At best we anticipated that different hallmarks would be predictive for distinct oncogenic events and/or in different tissues. It was surprising that Tc predicted cancer susceptibility independent of tissue (retina, pituitary, lung), independent of the timing of the oncogenic event (*Rb* in pituitary, *Rb/p53* in lung, *Kras* in lung), and independent of the initiating event (*Rb, Rb/p107, Rb/p53, Kras, Braf*). Whether it

is also the case for other oncogenes/tumor suppressors is beyond the scope of this manuscript, but our extensive *in vivo* data sets that stage.

2. The author's strategy to test their hypothesis is to introduce additional genetic changes that suppress tumor formation upon Rb/p107 knockout and then measure changes in cancer hallmarks. However, the authors only introduce a genetic manipulations that affect one very specific cell-cycle related pathway, the Skp2-p27-CDK axis. Thus, it is not surprising that knocking out Skp2 phenocopies expression of a mutant p27 that cannot bind CDK or knocking out CDK2 itself. To make their conclusion that cell cycle time is the only universal hallmark of the cell-lineage of origin, the authors would need to test many other tumor suppressive mutations that are not in the Skp2-p27-CDK axis. Put another way, the authors knockout Skp2, a well-known cell cycle protein, and find that it only alters cell cycle timing and not other hallmarks, such as apoptosis. But the authors should introduce a tumor suppressive mutation in the apoptosis pathway and measure the same set of cancer hallmarks. Ideally, this would also be done for genes in the senescence pathway, DNA damage pathway, etc. Had the authors included this important control, they may have observed changes in apoptosis as well as cell cycle timing. In other words, by solely focusing on cell cycle related genes (Skp2, p27, CDK2) the authors were biasing their results to find defects in the cell cycle rather than other hallmarks.

It is not the case that homozygous *Skp2* loss only affected cell cycle length, as it also induced senescence and reversed apical polarity defects (**Extended data Fig 1H, Fig 2G, H**, summarized in **Extended data Fig 2**). It is also not the case that *Skp2* knockout phenocopied constitutively active *p27* (*p27*^{T187A}) or *Cdk2* knockout: homozygous *p27*$^{T187A/T187A}$, like homozygous *Skp*2 loss, induced senescence, but it did not rescue apical polarity, and heterozygous *p27*^{T187} or Cdk2 or Cdk2/1 manipulations affected neither polarity nor senescence (**Extended data Fig 2**). Thus, manipulating Skp2 does not only affect proliferation and, surprisingly, manipulating p27 and Cdk2 does not phenocopy Skp2 loss, yet all these alterations suppress retinoblastoma.

The ability of homozygous *Skp2* loss to reduce proliferation index, reverse the apical polarity defect and induce senescence matched our initial, but ultimately incorrect, assumption that tumor suppression would affect several cancer hallmarks. We were shocked that even though removing only one *Skp2* allele suppressed retinoblastoma as effectively as removing both, heterozygosity had no effect on proliferation index, apical polarity or senescence. We were further puzzled that neither *Skp2* homozygosity nor heterozygosity altered multiple other hallmarks, including cell survival, DNA damage, angiogenesis, and immune infiltration (summarized in **Extended data Fig 2**). It is remarkable that a tissue can have so many cancer hallmarks yet remain cancer-free. That triggered deeper analysis of this pathway. Homozygous *p27*$^{T187A/T187A}$, like homozygous *Skp*2 loss, induced senescence, but it did not rescue polarity or affect other hallmarks, and none of the other p27 or Cdk2 or Cdk2/1 manipulations affected any of the hallmarks. Even proliferation index was unaltered, which initially distracted us from cell cycle. It was not until the scRNAseq results that a more thorough analysis of cell cycle rate was considered.

Even when we saw that every tumor suppressing mutation lengthened total cell cycle length (Tc), it remained possible that Tc would be the same in the cell-of-origin *vs*. the other two surviving but cancer-resistant retinal lineages. However, Tc was shorter in the cancer-prone lineage.

That striking result in retina raised the issue of generality: Is this phenomenon limited to mouse retinoblastoma, or does it have broader relevance? It would be interesting to suppress retinoblastoma in additional ways, but that would not test the general predictive power of relative Tc. Testing generality requires assessment of Tc in different scenarios where an oncogenic insult induces ectopic division in two or more lineages.

We could have observed that a shorter Tc in the cell-of-origin *vs*. other surviving but cancer-resistant lineages was limited to murine retinoblastoma. Instead, relative Tc predicted cancer susceptibility in multiple tissues, including retina, pituitary, and lung, and following different oncogenic events including *Rb, Rb/p107, or Rb/p53* loss, as well as *Kras* or *Braf* activation. As noted above, multiple new assays extend our initial findings to show that the correlation with relative Tc is independent of when we delete *Rb/p53* (fetal or adult lung) or activate *Kras* (4, 5, or 10 wks of age), and whether we activate the Ras pathway in lung through *Kras* or *Braf* activation (**Fig 5C, D**). Normal cell development is the typical response to oncogenic events [1–7]. Lineage-specific cancer susceptibility is not well understood, particularly for the typical scenario in which cells survive oncogenic insults. Our work exposes

lengthened Tc as a fundamental driver of cancer evasion, without the need for apoptosis, senescence or immune surveillance [1–7].

The text has been modified to highlight: **1.** That cancer genes drive multiple cancer hallmarks, not just those they were historically first associated with, and that it was not clear before this work whether any hallmark would be consistently associated with cancer-prone lineages (Results, lines 95-116; 347-350, Discussion, lines 387-411); **2.** The distinct effects (and lack thereof) of mutating Skp2-p27-Cdk components on cancer hallmarks in the cancer-prone *Rb/p107* null retina despite tumor suppression in every case, and the remarkable finding that a tissue with multiple cancer hallmarks can remain cancer-free (Abstract, lines 46-49; Results, lines 218-220; Discussion, lines 412-422); **3.** The value of new insight into normal cell development as a cancer-escape mechanism, which is widespread but understudied (Abstract, lines 54-56, Introduction, lines 58-77; Discussion, lines 387-411).

3. This study is largely correlational, finding that cell cycle time (Tc) is correlated with tumor susceptibility of different lineages within a tissue. In order to prove convincingly that short Tc is the reason specific cell-lineages are particularly cancer susceptible, which was the original question posed, the authors need to speed up the Tc in tumor resistant cell-lineages and show that they are now tumor sensitive. This is obviously a very difficult experiment, as any mutation that speeds up the Tc are also likely tumor suppressors or oncogenes. Another way to test for causation is to introduce two different oncogenic mutations in the same tissue and look at the relative changes in Tc and correlate that with the relative tumor susceptibility. The authors in fact already have this data in the current paper where they either knockout Rb and p53 or express oncogenic KRas in the lung. They find that in both cases, AT2 cells have a Tc of ~22hrs. However, AT2 cells only form tumors when oncogenic KRas is expressed and not when Rb/p53 are deleted. So despite shortening Tc by the same amount in the same cell-lineage, the different mutations have different effects on tumor susceptibility. This result shows that tumor susceptibility is in fact independent of Tc. The authors note that this result suggests "additional factors influence cancer susceptibility" (line 229), which seems to undermine the overall conclusions of the study.

As noted in response to the opening general comments, many high-profile studies show correlations which are of broad interest because of their implications for the field. Normal development is a common mechanism of cancer-escape, but we have little insight into what distinguishes lineages that utilize this protection versus those that succumb to transformation. Our discovery connects lengthened Tc to cancer evasion in multiple scenarios, offering an explanation as to how so many lineages can resist oncogenic mutations without the need for apoptosis, senescence or immune surveillance [1–7]. The text has been modified to underscore this key point: Abstract, lines 54-56, Introduction, lines 58-77; Discussion, lines 387-411.

It would be informative, but challenging, to try to speed up the cell cycle in cancer-resistant lineages, however, we did, exhaustively, show that slowing the cell cycle in multiple ways in a cancer-prone cell always inhibits tumorigenesis. Thus, introducing *Skp2*$^{-/-}$, *Skp2*$^{+/-}$; *p27*$^{KI/+}$; *p27*$^{KI/KI}$; *Cdk2*$^{-/-}$; *or Cdk2*$^{-/-}$;*Cdk1*$^{+/-}$ alleles all lengthen Tc in *Rb/p107 DKO* amacrine cells, and all supress retinoblastoma (**Fig 1A-C**, **Fig 4C, D**). Moreover, the degree to which Tc is lengthened in the cell-of origin correlates with the degree of tumor suppression (**Fig 1C**, **Fig 4D**). The latter was mentioned in lines 289-293 and has been emphasized in the updated discussion (lines 398-402).

In addition, as the reviewer noted, we also already tested distinct oncogenic events in the same tissue that transform distinct lineages, and relative Tc was predictive in each case. Thus, in the *Rb/p53* null lung, Tc was shortest in cancer-prone NE cells relative to cancer-resistant AT2 cells or club cells, whereas in the *Kras* mutated lung, Tc was shortest in cancer-prone AT2 cells compared to NE or club cells. As detailed above, we extended these studies considerably with multiple extra initiation times points, and we also demonstrated that the rule holds for a second Ras pathway driver, *Braf* (**Fig 5**). Text explaining these new experiments has been added: Abstract, lines 51-54; Results, lines 336-385: Discussion, lines 387-411.

The reviewer notes correctly that absolute Tc is not sufficient to predict cancer susceptibility. Indeed, that is expected, because normal mammalian cell types can have a very short Tc, such as in the murine E6.5-7.5 embryo epiblast where Tc is 5-7h, and < 3h for cells in the proliferative zone [22]. Other hallmarks, in various degrees and combinations, will cooperate with Tc and influence the absolute Tc threshold needed for transformation. Our data show that for one initiation event in one tissue Tc is predictive, but not between different initiation events or different tissues. Irrespective of the circumstances, Tc identifies the cancer-prone lineage in each single context. To

use a behavioural analogy, Tc is akin to a self-control score: the absolute score alone cannot predict when a child will spiral out of control, because that also depends on circumstances, but it can predict who among two or more children in a single set of circumstances is likely to lose it first. The text has been altered to emphasize the contextual relevance of Tc as a predictive biomarker: Discussion, lines 422-438.

On a molecular level it is interesting to speculate why relative Tc predicts cancer-susceptibility. Addressing this issue is beyond the scope of the current work, but in the Discussion (lines 439-454) we note that a shorter Tc may be a proxy for higher Cdk2 activity in early G1 (immediately after M phase), which Spencer *et al* showed correlates with whether cells in culture re-enter S-phase [23]. The Cdk2 activity required to favor re-entry will vary depending on other inputs ("circumstances"), such as the strength of pro-differentiation signals. Of note, Cdk2 directly inhibits various pro-differentiation transcription factors [24,25]. This balance will influence the timing of re-entry and, therefore, Tc. Tc needs to drop below some threshold to aid transformation, but the absolute threshold in each case will depend on other circumstances. The complexity underlying neoplastic transformation underscores how surprising it is that a simple correlate like Tc is predictive in so many *in vivo* settings (*Rb/107*-retina, *Rb*-pituitary, *Rb/p53*-lung, *Kras*-lung, *Braf*-lung).

4. All of the hallmarks tested through-out the manuscript are primarily measured at p8. There are several experiments that measure hallmarks at times up to p21, but given that the Tc and Ts measurements in Fig 4B-D are primarily measured at p8, this is the one time point universally shared with the rest of the experiments. However, as shown in Figure 1A, retinal tumors are not observed until day 125, indicating that transformation may not take place until long after p8. Have the authors measured the various hallmarks of cancer at times approaching p125-p150? Many of the hallmarks of cancer tested, such as disrupted apical polarity and immune infiltrate, may not manifest until much later. By testing for these hallmarks at such early timepoints, the authors may have missed any changes in the other hallmarks that may occur at later time points.

It is not the case that retinoblastoma appears at day 125. The data in **Fig 1A** refers to the detection of large tumors visible upon inspection with the naked eye. Emerging nascent tumors, visible microscopically in sections, appear around P8 [16], now clarified at lines 92-94 and 143-148. As the goal was to search for a hallmark that distinguishes the cancer cell-of-origin from cancer-resistant lineages, it was critical to assess effects at the beginning of tumorigenesis, not months later when resistant cells have long since escaped transformation. Thus, we focused on effects at or around P8 in the retina. In several cases, hallmark analysis covered multiple time points e.g. retinal thickness, cell death and senescence, were all measured at P4, P8, P10 and P21 (**Fig 1E**, **Extended data Fig 1E, H**). To clarify these issues for the retinoblastoma work we modified the text where we first introduce murine retinoblastoma in the results (lines 92-94) "Tumors in this model become evident microscopically at P8 [26], and become visible to the naked eye beyond ~100 days (**Fig 1A**)". Then, at the point where we start describing the effect of tumor suppressing mutations (lines 143-148) we added: "Tumors in the *Rb/p107* DKO model emerge microscopically around P8, many cancer hallmarks are evident then, and while both cancer-prone amacrine cells and cancer-resistant horizontal and Müller glia undergo ectopic division during this window, the latter two lineages gradually exit the cell cycle such that by ~P30 only the amacrine-cell derived tumors continue to proliferate [16]. Thus, we focussed on P4-P21 to assess how tumor suppression affects cancer hallmarks in the *Rb/p107* DKO retina".

More importantly, though, the retinoblastoma suppression studies in the first part of the manuscript only pointed to the notion that relative Tc might predict cancer susceptibility. It was possible that Tc would not be different in the cell-of-origin *vs*. cancer-resistant lineages. Follow-up studies in the retina revealed that Tc distinguished the cancer-prone amacrine lineage from cancer-resistant glial or horizontal cell lineages. Measuring retinoblastoma suppression at more time points would not address whether Tc can predict lineage-specific cancer susceptibility in different tissues or for other initiation events. The critical step was to examine Tc in other tissues and with other oncogenic events, which revealed a consistent relationship between relative Tc and lineage-specific cancer susceptibility. Notably, and we feel more importantly, we tested whether relative Tc was predictive at multiple initiation time points, and indeed, in every case, that proved to be the case (**Fig 5**).

Minor Concerns:

1. The authors frequently switch between the terms "hallmark" and "feature" (eg Lines 15-21). This can be confusing. Please choose one term and stick with that throughout the manuscript.

To simplify the updated text, we prioritized the use of "hallmark" but tried to avoid repetition in the same sentence, thus in some cases used "hallmark" first and "feature" second.

2. In the introduction, the term "APC" needs to be defined as this acronym can stand for several different things.
The reviewer is correct and indeed we had APC in the introduction and methods with entirely distinct meanings! "Adenomatous polyposis coli (APC)" has been added to the introduction, and "allophycocyanin (APC)-conjugated anti-B220" to the methods.

3. It would help if Figure S2 had a legend. Parts of this diagram are certainly self-explanatory, but other parts would benefit from a legend.
We added the following legend: **Extended data Fig 2. An *in vivo* strategy to identify a cancer cell-of-origin hallmark**. The top panel summarizes the four steps. Step 1 introduces mutations to suppress cancer (here, retinoblastoma). Step 2 assesses multiple (here, nine) cancer hallmarks to determine whether any are always altered upon tumor suppression; only cell cycle length (Tc) fulfilled this criterion. Step 3 asks whether Tc is shorter in the cancer-prone (here, amacrine cell) *vs*. cancer-resistant lineages (Müller glia, horizontal cells). Tc in cancer-prone or resistant lineages is indicated on the left, and the effect of multiple tumor-suppressing mutations on Tc is shown on the right. Step 4 compares Tc of cancer-prone *vs*. resistant lineages in multiple other contexts. Tc was always shortest in the cancer-prone lineage independent of oncogenic lesion or tissue (indicated by green check marks).

4. There are several references to "TKO" cells in the text, but not in the figure legends. I find this confusing. I see that TKO is defined as Rb-/-,p107-/-, SKP2-/- at one point in the text, but it is easy to forget this as one is reading through the text. Particularly since the authors use another triple-knockout line, Rb-/-, p107-/-, CDK2-/-, that could be confused as TKO.
The reviewer is correct, and it was also problematic because we had referred to the *Rb*$^{-/-}$*;p107*$^{-/-}$*;Skp*$^{-/-}$ genotype as *DKO-Skp2*$^{-/-}$ in the figures, but TKO in the text, whereas the *Rb*$^{-/-}$*;p107*$^{-/-}$*;Skp2*$^{+/-}$ genotype was referred to as *DKO-Skp2*$^{+/-}$ in both text and figures. To be consistent throughout, we now refer to the *Rb*$^{-/-}$*;p107*$^{-/-}$*;Skp*$^{-/-}$ genotype as *DKO-Skp2*$^{-/-}$, matching the figures and figure legends, and harmonizing with the *DKO-Skp2*$^{+/-}$ annotation, which also matches all the annotation for other mutations added to the *Rb/p107* DKO background (e.g. *DKO-p27*$^{KI/KI}$, *DKO-Cdk2*$^{-/-}$, etc).

5. Throughout the manuscript, abbreviations, arrows and other annotations on the microscopy images are not defined. For example, ONL, INL, GCL in Fig. 2A or the arrows in Fig. 2f, but there are other instances as well.
Abbreviations and arrows are now explained in the legends.

6. In the abstract, the authors state "Cancer-prone retina exhibits defective apoptosis, senescence, immune-surveillance, angiogenesis, DNA-repair, apical polarity, and proliferation index." Are the authors referring to a normal amacrine cell in the retina or one in which Rb and p107 have been knocked out? This is not clear as currently written.
We assumed, incorrectly, that "cancer-prone retina" would indicate that it is the *Rb/p107* DKO context, as the normal retina does not exhibit any cancer hallmarks. To avoid confusion the new abstract states: "Cancer-prone *Rb/p107* DKO retina exhibits..."

7. The authors routinely use one-way ANOVA with Bonferroni correction for the multiple comparisons test, but given the small sample size (typically n=3) for most experiments, a non-parametric test such as the Kruskal-Wallis test is most appropriate.

Following a power analysis, as detailed below, 4-6 animals were needed for each experiment to detect impact with 80% -90% power. We increased the sample size to 6 in most experiments ((**Fig 1D-G, Fig 1I, Fig 2B-E, Fig 4A-D, Fig 5A-D, Extended Fig 5A, D, Extended data Fig 6A, B, Extended data Fig 7A-F**, **Supplementary Fig 2B, Supplementary Fig 8B**). One-way analysis of variance (ANOVA) followed by Bonferroni correction was used for multiple comparisons, as the reviewer suggested.

Methods have been altered thus (lines 811-824): "Sample sizes (n) were estimated by the formula n = [2(Zα+Z1–β)2]/SES2. Type I error (α) is set as 0.05, type II error (β) is set as 10–20% and is a two-sided effect. The SES (or Cohen's d) is the standardized effect size, equal to the ES (effect size) divided by the pooled SD (standard deviation), so it is the magnitude of the difference between the means of two groups in units of SDs [27]. For animal experiments, SES of 1.1, 1.5, and 2.0 SDs are suggested to represent small, moderate, and significant responses,

respectively [27]. According to the formula above, 4-7 animals are required to detect impact with 80% power, or 6-10 animals are needed to detect effects with 90% power. So, 4-6 animals are needed for each experiment to detect impact with 80%- 90% power. All data are presented as mean ± SD. Kaplan–Meier survival curves and statistical analysis were performed using the GraphPad Prism software (GraphPad Prism Software, Inc., San Diego, CA, USA). An unpaired student's t-test was used to compare the two groups. One-way analysis of variance (ANOVA) followed by Bonferroni correction was used for multiple comparisons. The p values of KM curves were calculated using the log-rank (Mantel-Cox) test".

Referee #2 (Remarks to the Author):

In this manuscript by Chen, Bremner, and colleagues, investigate how cancer drivers initiate tumors, with a focus on the role of length of the cell cycle length in the cancer cell of origin. The authors first use mouse genetics to investigate genes whose loss may block the initiation of tumors in the retina upon loss of Rb and p107. These experiments validate a Skp2-p27-Cdk1/2 axis. Whether tumors grew or not, depending on the combination of genetic mutations, the authors did not find differences in apoptotic cell death or senescence in amacrine cells (from which tumors arise), and no differences in immune infiltration. Similarly, no differences were observed in DNA damage, angiogenesis, or apical polarity. Single-cell RNA-seq analyses and lineage tracing experiments suggested that the main difference associated with tumor development is the cell cycle status of cells in the amacrine lineage. Triple labeling experiments (EdU/Brdu/Ki67) showed that the genetic alterations that suppressed tumor development also increased the overall length of the cell cycle (that the authors call "Tc"), not the duration of S phase. Additional staining for amacrine cells showed that these cells have the shortest Tc and that Tc in these cells increases when mutations that suppress cancer are present. Similar results were observed in a mouse model of retinoblastoma dependent on Rb loss for cancer initiation, in a mouse model of SCLC dependent on Rb and p53 loss for cancer initiation, and in a mouse model of lung adenocarcinoma driven by oncogenic K-RAS.

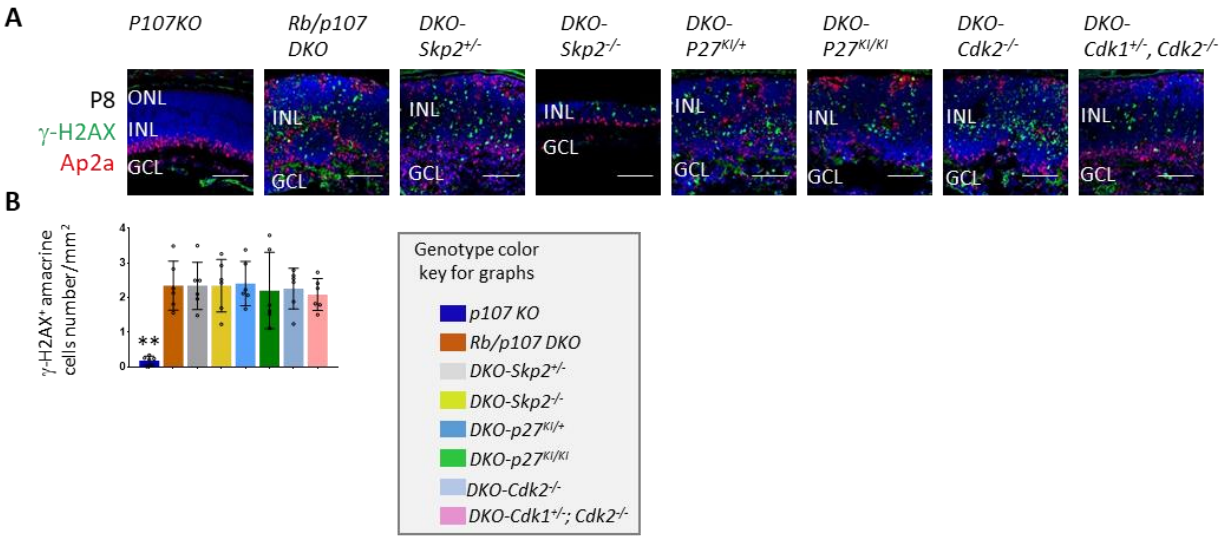
The authors performed an impressive amount of work using state-of-the-art genetically engineered mouse models of human cancer to identify overall cell cycle length as a fundamental feature of cancer initiation. The experiments in the retina are striking because multiple cell types are investigated within the developing retina, with multiple genetic alterations. The experiments in the lungs are also important because they compare different lung cancer types originating in distinct cell types upon specific genetic alterations. Conceptually, it is well known that different drivers initiate cancer in different cell types but the underlying mechanisms remain unclear: the data presented in this manuscript highlight a role for the cell cycle. The experiments are well performed, well controlled, and well presented. The text is very clear. Statistical analyses are shown. Overall, this study will be of great interest to scientists in the cell cycle and cancer biology fields.

We thank the reviewer for their encouraging comments on the striking nature of the results and significance of the breakthrough to the field.

I have only three specific comments and some more general thoughts.

Point #1: Because amacrine cells are the cell of origin of retinal tumors, it may be important to focus all analyses on this cell lineage. For example, in Figures 1G and 2A-B, is it possible to specifically quantify senescence and DNA damage in Ap2a+ cells?

In addition to Tc (**Figure 4D**), we quantified apoptosis (**Figure 1F**), and proliferation index (**Extended data Figure 5**) specifically in the amacrine lineage. Regarding senescence, whole retina analyses showed that multiple tumor-suppressing mutations had zero senescent cells at all time points assessed (P4, P8, P10, P21, **Figure 1G**), with example images provided (**Extended data Fig 1H**). As there are no senescent cells at all, it is not necessary to specifically assess amacrine or other cell types. To these data, as the reviewer requested, we have now added quantification of DNA damage in $Ap2a^{+}$ amacrine cells (**new Supplementary Fig 2**). None of the six tumor suppressing mutations altered the proportion of amacrine cells with DNA damage.

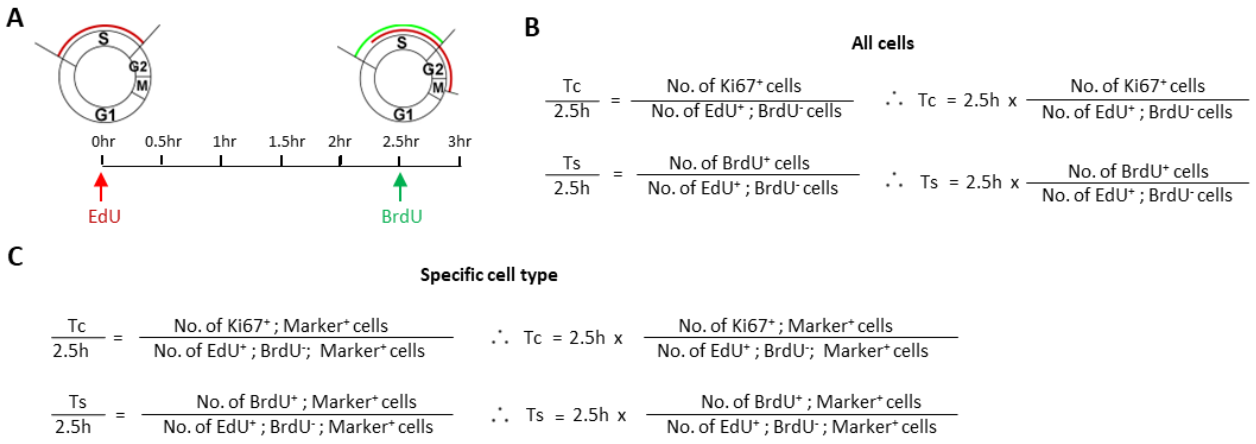


Supplementary Figure 2. Tumor suppression without altering DNA damage in the cell-of-origin.
A. P8 horizontal retinal sections of indicated genotypes were stained for nuclei (DAPI, blue), DNA damage (γ-H2AX, green) and amacrine cells (Ap2a, red). **B.** γ-H2AX$^+$ Ap2a$^+$cells/mm^2 of retina from (A). Error bars: SD (n=6); Asterisks: significant difference between Rb/p107 DKO and indicated genotypes (*, $p < 0.05$; **$p < 0.01$; one-way ANOVA, Bonferroni correction). Scale bar: 50μm. ONL: outer nuclear layer. INL: inner nuclear layer. GCL: ganglion cell layer.

Point #2, related to the calculation of cell cycle length: The measure of Ts and Tc in Figure S5D-F is interesting but a bit difficult to understand. First, Edu+ cells may not just be cells that were in S-phase at the beginning of the experiment but may also include some cells in G1, because EdU in the circulation is likely to last some time - or maybe I am wrong? I understand that the previous papers using this formula did not take this into account, and I am not sure why. Also, some cells may have divided after the initial EdU injection (not enough dilution of the signal to distinguish cells that have divided and cells that have not, but I could be wrong again, maybe 2 hours is never enough to go through G2 and M?). Would that affect the numbers? Is the red line in S5D accurately representing the situation in vivo in mice. Finally, looking back at the manuscript that the authors refer to, the formula there is different (PMID: 29445897), shouldn't the numerator for Ts calculation include Edu+ BrdU+ double positive cells (cells that stayed in S phase)?

To better explain the assay and results, we added: **1.** A detailed explanation of the basic principle and the calculations (**Supplementary Fig 3**); **2.** Many additional images of the multi-labeled tissue sections (**Supplementary Fig 4-7). 3.** All the raw data for the cell cycle calculations (**Supplementary Table 1**); and **4.** New data confirming that EdU-labeled cells do not divide in the 3h window used for Tc calculations (**Supplementary Fig 8**). Details as follows:

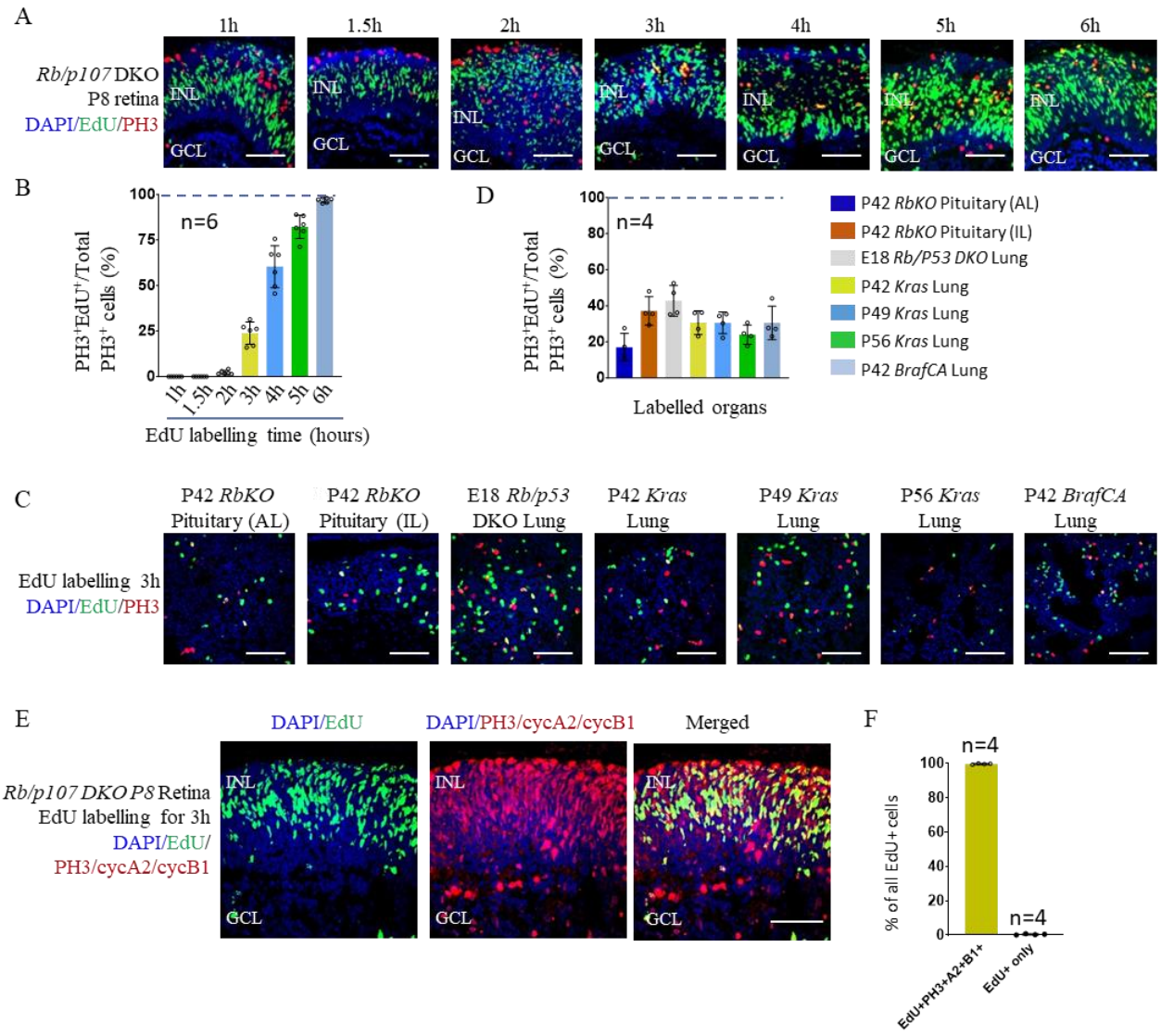
Re whether EdU persists so that cells that were in G1 at the start of the assay become EdU-labeled when they enter S-phase later: The half-life of IP-injected EdU (30μg/g body weight) is only ~ 15 min [28]. Cells in S-phase close to the time of injection are labeled, but because circulating EdU disappears rapidly, cells that were in G1 at the start of the experiment that subsequently enter S-phase are not labeled. Even if such G1->S cells were labeled it would not affect the calculation because the purpose of EdU labeling is to mark a 2.5h long EdU-only (BrdU-negative) population that exits S and enters G2/M. If EdU was long-lasting and G1->S cells became labeled, that EdU$^+$ population would also be BrdU$^+$ and would not be counted as EdU-only cells. The EdU-only cells allows us to calculate Tc: Tc/2.5h = No. of Ki67$^+$ cells/No. of EdU$^+$;BrdU$^-$ cells, which is then solved for Tc (**Supplementary Fig 3**).



Supplementary Figure 3. Calculation of Tc and Ts.
A. Schematic summarizing the sequential EdU then BrdU labeling protocol which, coupled with Ki67 labeling to label all dividing cells, can be used to calculate cell cycle length. 30μg/g body weight EdU is injected IP at 0h, followed by BrdU at 2.5h, and tissue is harvested at 3h. The half-life of IP-injected EdU is ~ 15 min [119], so circulating EdU disappears rapidly. After 3h the EdU$^+$ cells that labeled S-phase at the start of the assay have moved to mid/late S phase and G2/M (red EdU line), whereas new early S-phase cells are EdU-negative. The 30 min pulse with BrdU marks cells in S-phase at the end of the experiment (2.5-3hr; green BrdU line). EdU$^+$;BrdU$^-$ cells are those that exited S-phase and moved into G2/M in the 2.5 hr period prior to BrdU injection (red EdU line but no green BrdU line). The number of these EdU$^+$;BrdU$^-$ cells is proportional to the 2.5h period in which they were generated. Ki67 stains all dividing cells, and the number of these cells is proportional to total cell cycle length (Tc). Similarly, the total number of BrdU$^+$ cells is proportional to S phase length (Ts). These values are used to calculate Tc or Ts as detailed in B and C. **B.** Top row: formula for total cell cycle time (Tc). The ratio of Tc over 2.5h equals the ratio of the number of Ki67$^+$ cells over the number of EdU$^+$;BrdU$^-$ cells generated in the 2.5h window (left hand side). With three known variables (2.5h; # of Ki67$^+$ cells; # of EdU$^+$;BrdU$^-$ cells), one can solve for Tc (right hand side). Bottom row: formula to calculate S-phase time (Ts). The ratio of Ts over 2.5h equals the ratio of cells in S-phase (# of BrdU$^+$ cells) over the number of EdU$^+$;BrdU$^-$ cells generated in the 2.5h window (left hand side). With three known variables (2.5h; # of BrdU$^+$ cells; # of EdU$^+$;BrdU$^-$ cells), one can solve for Ts (right hand side). Note that some authors have used double-labeled EdU$^+$;BrdU$^+$ cells for the number of S-phase cells, but in those cases the total labeling time was much shorter than the 3h used in our assay. With a 3h labeling window, many early S-phase cells are EdU-negative, so counting EdU$^+$;BrdU$^+$ cells would underestimate S-phase cell number. **C.** The formulae to calculate Tc or Ts in a specific cell type are as in (B) except that counts are limited to cells also labeled with a marker for the cell type of interest. Raw counts from the assays are provided in **Supplementary Table 1**.

Re whether some EdU-labeled cells divide during the 3h labeling period: The number of EdU$^+$;BrdU$^-$ cells form the denominator of Tc and Ts calculations (**Supplementary Fig 3**), thus if some of these cells divide this denominator would increase, artificially decreasing Tc and Ts. Prior publications assume that no or very few EdU-labeled cells divide, and that the EdU-only cells have reached G2 & M but not beyond. We performed additional assays to examine this assumption. The experiments are detailed below, and the results are in the new **Supplementary Fig 8** (also shown below):

First, we labeled P8 *Rb/p107* DKO mice with EdU for 1h, 1.5h, 2h, 3h, 4h, 5h, or 6h, then stained retinal sections for EdU, phospho-histone H3 (PH3) and DAPI (**Supplementary Fig 8A**). H3 phosphorylation begins in late G2 with chromosomal condensation, but is most prominent in early-mid M phase, and H3 dephosphorylation commences in late anaphase/early telophase [29], thus PH3 is widely used as a late G2/mitotic marker. If G2/M was <3h, all cells in S-phase at the time of EdU labeling would have reached M-phase by then, and the EdU$^+$ cells that were labeled in early-mid S-phase would continue to enter G2/M at later times (S-phase in *Rb/p107* null P8 retina is ~25hr, **Fig 4C**),



Supplementary Figure 8. EdU-labelled cells do not divide during the 3h labelling period.
A. P8 *Rb/p107 DKO* mice were labelled with EdU for 1h, 1.5h, 2h, 3h, 4h, 5h and 6h, retinal sections were stained for EdU (green), phospho-histone H3 (PH3, red) and DAPI (blue). **B.** The % of $PH3^+EdU^+$ double-labeled cells over all $PH3^+$ cells in A. **C.** Mice of the indicated genotypes and ages were labelled with EdU for 3h, and sections of the indicated tissues were stained for EdU (green), PH3 (red) and DAPI (blue). **D.** The % of $PH3^+EdU^+$ double-labeled cells over all $PH3^+$ cells in C. **E.** P8 *Rb/p107 DKO* mice were labelled with EdU for 3h, retinal sections were stained for EdU (green), a cocktail of rabbit antibodies against PH3 (lateG2/M)), cyclin A2 (S/G2)), cyclin B1 (G2) (red) and DAPI (blue). **F.** The % of $EdU^+PH3^+cycA2^+cycB1^+$ cells, or EdU^+ only cells over all EdU^+ cells. Error bars: SD (n = 6 for B, and n=4 for D and F); Scale bar in A, C, E is 50μm.

ensuring a long plateau of 100% $EdU^+;Ph3^+$ double-labeled cells beyond 3h. In contrast, if G2/M is > 3h only a fraction of $PH3^+$ cells would be also EdU^+, which would continue to rise with time, eventually reaching the 100% plateau. As expected, the fraction of PH3 cells that were also EdU^+ increased over time, confirming the movement of prior S-phase cells through G2 into M (**Supplementary Fig 8B**). No double-labeled cells were detected at 1h or 1.5h, and a tiny fraction was seen at 2h, which continued to increase, only reaching 100% at 6h. At 3h, the timepoint used in our Tc measurement studies, only 23.9% of $PH3^+$ cells were EdU^+ (**Supplementary Fig 8B**). These data suggest that 3h is insufficient time for EdU-labeled cells in the P8 *Rb/p107* DKO retina to divide.

Second, we also performed EdU/PH3/DAPI triple labeling in sections from *Rb*-pituitary, *Rb/p53*-lung, *Kras*-lung, and *Braf*-lung cancer models used in the paper (**Supplementary Fig 8C**). The fraction of M-phase cells that was EdU^+ after 3 h was ~30-50%, similar in range to the *Rb/p107* DKO retina, well below the 100% expected if EdU^+ cells could complete mitosis in 3h (**Supplementary Fig 8D**). Thus, 3 hr also appears insufficient for EdU-labeled cells to divide in these cancer models.

Third, as an additional test in retina, P8 *Rb/p107* DKO mice were exposed to EdU at 0h, then BrdU from 2.5-3h and retinal sections were stained for EdU, and a cocktail of three rabbit antibodies targeting cyclin A2, cyclin B1, and PH3 to label most S, all G2 and all M-phase cells (**Supplementary Fig 8E**). Any EdU-only cells that traverse M into G1 would be EdU^+, but negative for all the other markers (Cyclin A2 = mid/late S-phase and G2, Cyclin B1 = G2 and early M phase, PH3 = lateG2+M phase). Notably, all the EdU^+ cells co-labelled with the A2/B1/PH3 antibody mix, and no cells were positive only for EdU (**Supplementary Fig 8F**). This result confirms that EdU-labeled cells do not divide in a 3h window.

The new data confirming that EdU-labeled cells do not divide is presented in **Supplementary Fig 8** and is explained in the Methods section (lines 661-695).

Re whether $BrdU^+$;EdU^+ double-labeled cells, instead of $BrdU^+$ cells, can be used to determine S-phase cell numbers. In the calculation of Ts, we use $BrdU^+$ cells to quantify the number of S-phase cells. The short half-life of circulating unincorporated EdU means that by 2.5h, when we add BrdU, many cells that enter S-phase after circulating, EdU disappears will be EdU-negative (In the schematic in **Supplementary Figure 3A** these are the S-phase cells with the green line but no red line). Thus, EdU^+;$BrdU^+$ cells represent mid-late but not early S-phase, which is not useful for Ts calculations. To determine the entire S-phase population at the end of the assay it is critical to count the $BrdU^+$ population. Some authors have indeed used double-labeled cells in their Ts calculations, but the labeling period in those studies was much shorter; e.g. in the paper PMID: 29445897 (ref [30]), which the reviewer highlighted, the authors only labeled for a total of 1.5h (EdU added at 0h, BrdU added at 1h, harvest at 1.5h). Even there, it would be more accurate to rely on $BrdU^+$ cells, although their much shorter labeling time reduces any error. The legend to the updated explanatory figure now explains why counting $BrdU^+$ cells is best to calculate Ts (**Supplementary Fig 3**).

Point #3, related to the timing of cancer initiation: What may be a bit misleading in this study is that pediatric retinoblastoma arises from cells that are dividing or about to exit the cell cycle. But the three other cancer types that the authors examine normally occur in adults (mice and patients), at a time where pituitary cells, neuroendocrine cells, and AT2 cells are not dividing anymore. However, the authors examine cell cycle kinetics at early time points for the lung cancer models (E18 for SCLC, and 4-week-old mice for lung adenocarcinoma), while tumors in these models are usually initiated when mice are between 8-12 weeks of age. Thus, their work may look at the cell of origin in the lung cancer models at the "wrong" time, and possibly the results would be different at later time point. For the K-RAS model, it should be straightforward to add tamoxifen a few weeks later and repeat some of the staining. For the RB/p53 model (and the K-RAS model), Adeno-CMV-Cre (or AAV-Cre) may be another option as it should infect cells in all the main lineages. It may not be required to repeat all the experiments at later time points, but some data at these later time points may be important.

The time points at which we originally activated *Kras* or inactivated *Rb/p53* all cause cancer, so these are relevant contexts in which to ask whether cancer-prone lineages have a shorter Tc than cancer-resistant lineages. Nevertheless, we have now added several new initiation and analysis time points, as detailed below.

In the original manuscript, we deleted *Rb* at two separate time points for the pituitary study. Tamoxifen was added at 4 wk of age and assessed at 6 wk, and it was also injected at 8 wk of age and assessed at 10 wk. In both cases, Tc was shorter in the cancer-prone intermediate lobe (IL) than in the cancer-resistant anterior lobe (AL) (**Fig 5A**). We have now added new times for *Rb/p53* deletion and *Kras* activation in the lung (**Fig 5B, C**). In addition, to expand the Ras pathway analysis, we assessed Braf activation in lung (**Fig 5D**).

Rb/p53 lung: The original data was in embryonic lung using *hSPC-rtTA;tetCre;*$Rb^{f/f}$*;*$p53^{f/f}$ mice. To assess adult lung, we crossed the *Rb* and *p53* floxed alleles along with the *LSL-tdTomato* indicator locus onto the *CreERT2*

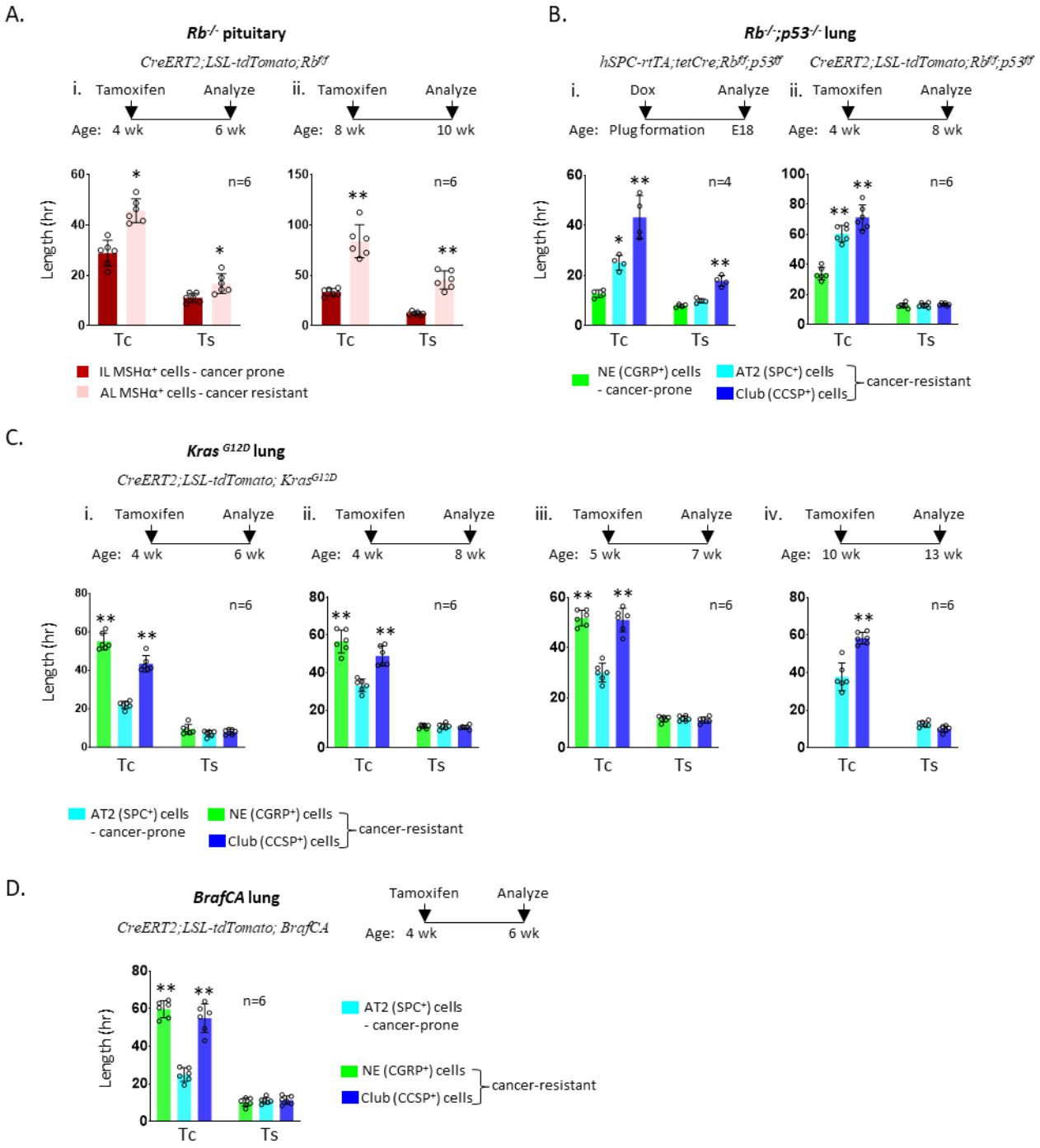


Fig. 5. Shortest Tc as a general hallmark of cancer susceptibility.

A. Tc and Ts of MSHα+ cells in the intermediate (IL) and anterior (AL) pituitary lobes of *Rb* null (*CreERT2;LSL-tdTomato;Rb$^{f/f}$*) mice injected with tamoxifen at 4 or 8 wk and assessed at 6 or 10 wk of age, respectively. **B.** Tc and Ts of lung CGRP+ neuroendocrine (NE), SPC+ Alveolar type 2 (AT2) and CCSP+ club cells of E18 *Rb/p53* DKO animals (*hSPC-rtTA;tetCre;Rb$^{f/f}$;p53$^{f/f}$*) injected with doxycycline at plug formation and assessed at E18 of age, and of P56 *Rb/p53* DKO animals (*CreERT2;LSL-tdTomato;Rb$^{f/f}$;p53$^{f/f}$*) injected with tamoxifen at 4 wk and assessed at 8 wk of age. **C.** Tc and Ts of lung NE, AT2 and club cells of *Kras^{G12D}* animals (*CreERT2;Kras^{G12D};LSL-tdTomato* mice) injected with tamoxifen at 4 wk, 4 wk, 5 wk, and 10 wk and assessed at 6 wk, 8 wk, 7 wk and 13 wk of age respectively. **D.** Tc and Ts of lung NE, AT2 and club cells of *BrafCA* (*CreERT2;BrafCA;LSL-tdTomato*) mice injected with tamoxifen at 4 wk and assessed at 6 wk, of age. Error bars: SD (n = 6 for A, Bii, C, D; n=4 for Bi); asterisks: significant difference between MSHa+ IL *vs.* AL cells (A), or NE *vs.* AT2/club cells (B), or AT2 *vs.* NE/club cells (C, D) (* $p < 0.05$, ** $p < 0.01$. one-way ANOVA, Bonferroni correction for D and F, unpaired t-test for A, B, C, and E).

background, treated mice with tamoxifen at 4 weeks of age and assessed cell cycle timing at 8 weeks. As in fetal lung (**Fig 5Bi**), the neuroendocrine cells exhibited a shorter Tc than cancer-resistant alveolar type 2 (AT2) or club cells (**Fig 5Bii**).

Kras lung: In the Ras model, we originally induced the oncogenic insult at 4 weeks of age and analyzed cell cycle kinetics at 6 weeks, which revealed that the cancer-prone AT2 cells had a shorter Tc than cancer-resistant neuroendocrine or club cells (**Fig 5Ci**). In the additional dataset, we induced *Kras* at 4, 5, or 10 weeks of age and assessed cell cycle at 8, 7, or 13 weeks, respectively. We detected ectopic division in all three lineages following oncogene activation at 4 or 5 wk, and in both AT2 and Club cell for the 10 -> 13wk assay. In all cases, AT2 cells had the shortest relative Tc (**Fig 5Cii-iv**). Thus, independent of when the oncogenic insult is applied, Tc predicts the cancer-prone population in different tissues (pituitary or lung) or with different initiating events (*Rb*, *Rb/p53*, *Kras* activation).

Braf lung: To expand Ras pathway analysis, we mutated Braf, a downstream Ras effector kinase that also induces lung tumors from AT2 cells [8]. For this, we generated *CreERT2, LSL-dtTomato, and BrafCA* mice, in which recombination replaces the wild-type *Braf* allele with a constitutively active (CA) $Braf^{V600E}$ allele [8]. Addition of tamoxifen at 4 wk of age induced ectopic division in AT2, NE and Club cells and, as predicted, AT2 cells exhibited the shortest Tc (**Fig 5D**). Thus, independent of when or how the RAS pathway is activated, the tumor-prone lineage exhibits a shorter Tc than tumor-resistant lineages.

These new data are explained in the Results section, lines 336-385. The overall results are summarized in lines 381-385: "Altogether, our results reveal that among different surviving lineages in a tissue the shortest Tc earmarks the most cancer-prone cell type in all tested cancer models independent of tissue (retina, pituitary, lung), oncogenic driver (*Rb/p107*, *Rb*, *Rb/p53* inactivation, *Kras* or *Braf* activation), or initiation event timing, and even for different cancers that arise from distinct cells-of-origin in the same tissue (SCLC-*Rb/p53*-NE cells; NSCLC-*Kras* or *Braf*-AT2 cells)".

General comments:

There is of course a bias in the way the authors conducted their study: the Skp2-p27-Cdk1/2 axis is related to cell cycle progression primarily. One could easily imagine that other alterations blocking cancer initiation would promote cell death for example, which may have changed some of the conclusions. It is also noteworthy that the authors have chosen models in which cell death in the cell of origin is not very prevalent, which may not be the case in other models.

The Skp2-p27-Cdk pathway is best known for its role in cell cycle regulation. However, despite being less well studied, the components of this pathway can be referred to as cell survival regulators, senescence regulators, polarity regulators, etc. It is not common, due to literature history, but scientifically it is just as accurate. The influence of these regulators on many functions other than cell cycle is highlighted in the updated Results, lines 108-116: "For example, Skp2-mediated ubiquitylation regulates DNA repair, autophagy, apoptosis, adhesion, and epithelial mesenchyme transition, and, independent of E3 ligase activity, Skp2 regulates transcription, apoptosis and migration genes [30]; p27, independent of Cdks, regulates stemness, migration, and microtubule dynamics [31]; Cdk2 and Cdk1 regulate differentiation and epigenetics [32,33] and Cdk1 also regulates apoptosis, Golgi organization, nuclear/cytosolic transport, and vesicle mediated transport [34]. As Skp2, p27 and Cdks influence so many processes, we expected tumor suppression would alter multiple cancer hallmarks. First, we studied the effect of these genes on tumorigenesis, then the accompanying effects on cancer hallmarks."

In view of the ability of this pathway to regulate so many hallmarks, we were surprised to observe that most of the retinoblastoma-suppressing mutations targeting Skp2, p27 or Cdks had zero effect on cancer hallmarks, including proliferation index, cell survival, senescence, DNA damage, the immune landscape, aberrant angiogenesis, apical polarity, or differentiation (summary in **Extended data Fig 2**), so it was not obvious that total cell cycle length (Tc) would be the answer. Even when we saw that Tc was always lengthened when retinoblastoma was suppressed, it remained possible that Tc would be the same in the cell-of-origin *vs*. the other two surviving but cancer-resistant retinal lineages. However, Tc was shorter in the retinoblastoma-prone lineage.

That result raised the issue of generality: Is this phenomenon limited to mouse retinoblastoma, or does it have broader relevance? It would be interesting to suppress retinoblastoma in additional ways (e.g. cell-of-origin apoptosis) but that would not test the general predictive power of relative Tc among cancer-prone *vs*. cancer-resistant lineages that survive an oncogenic lesion (a very common scenario [1–7]). Instead, the priority was to ask whether Tc also differentiates cancer-prone and cancer-resistant lineages in additional contexts. We could have observed that a shorter Tc in the cell-of-origin *vs*. other surviving but cancer-resistant lineages was limited to murine retinoblastoma. Instead, relative Tc predicted cancer susceptibility in multiple tissues, including retina, pituitary, and lung, and following different oncogenic events including *Rb, Rb/p107, or Rb/p53* loss, as well Ras pathway activation. New data extends our findings by showing that the correlation with relative Tc is independent of when we delete *Rb/p53* (fetal or adult) or activate *Kras* (4, 5, or 10 wk of age). We also extended Ras pathway analysis to show that relative Tc also predicts the cancer-prone lineage following *Braf* activation in lung (**Fig 5D**). Normal cell development is a remarkably common response to oncogenic events [1–7]. Lineage-specific cancer susceptibility is not well understood, particularly for the typical scenario in which cells survive oncogenic insults. Our work exposes lengthened Tc as a fundamental driver of cancer evasion, without the need for apoptosis, senescence or immune surveillance.

We have clarified the importance of cell death as a selective mechanism in the modified introduction (lines 63-65). However, the focus of our work is on how *surviving* lineages resist cancer, now better delineated in the Abstract (lines 40-56), the Introduction (lines 58-77) and Discussion (lines 387-411). The high frequency of oncogenic lesions in normal human tissues [1,2], and the 50% contribution of *Rb*$^{-/-}$ ES cells to tumor-free adult chimeric tissues [3,4] (with similar findings for other chimeras made using ES cells with e.g. *Ink4a* (p16/Arf) loss, *Fos* overexpression, or a *Mll-AF9* gene fusion [5–7]) are striking examples of how normal development, not death, prevents cancer. Cell death, senescence, and, more recently, immune clearance are well-studied cancer escape mechanisms, but normal development is not. Thus, it is unclear why an oncogenic lesion that initially causes ectopic division in multiple lineages is transforming in one but harmless in most. Our work reveals an initiation hallmark that differentiates lineages that develop normally and thus resist cancer versus those that are susceptible to neoplastic transformation.

The authors show a number of "negative" experiments but have the appropriate positive controls. Still, negative data are always difficult to interpret: what if the authors looked at P6 instead of P4 and P8 in the developing retina, maybe then cell death becomes more relevant? Of course, it is impossible to quantify all phenotypes at all time points.

We assessed many of the hallmarks at multiple time points, but as detailed above, the purpose of the initial tumor suppression experiments was to search for a hallmark in retinoblastoma that always changes upon tumor suppression. That exploratory work exposed relative Tc, and so, rather than studying tumor suppression at more time points, the vital step was to test the hypothesis in multiple different contexts.

Finally, it is worth noting that the study from the Cobrinik lab in human retinoblastoma (Nature, 2014) had identified a role for cell death inhibition in cancer initiation (via MDM2). Thus, one could argue that the identification of cell cycle length by the authors in this new study is an artefact of mouse models. It is difficult to address this issue experimentally (how many models would be needed to disprove the model…). Overall, the authors can conclude that in the specific models they examined, a shorter cell cycle length correlates with greater cancer initiation, but they cannot exclude that other mechanisms may be at play in other contexts – something worth noting in the manuscript.

The relevance of cell death as a protective mechanism for retinoblastoma is not limited to the human retina. Apoptosis also protects multiple lineages in the murine retina, as four of the seven major retinal cell types that undergo ectopic division upon *Rb/p107* loss die (rods, cones, bipolar cells, ganglion cells) [16]. Thus, cell death is an essential mechanism by which some lineages resist transformation, whether in mice or humans. In contrast, the amacrine cell resists apoptosis, mimicking the resistance of the human cell-of-origin (cones) to apoptosis.

However, that does not explain why different ectopically dividing lineages that *survive* the oncogenic insult are either cancer-prone (amacrine) or cancer-resistant (glial and horizontal cells). Survival and normal development are a common outcome of "oncogenic" events, as discussed above [1–7]. Our work shows that relative Tc predicts which dividing, **surviving** lineages are cancer-prone or cancer-resistant, at least for the oncogenic insults and tissues tested

in our study. In the original manuscript, we showed predictive power in three different tissues (retina, pituitary, lung) and for different oncogenic events, (*Rb/p107*, *Rb*, or *Rb/p53* loss and *Kras* activation). Now, as detailed above, we added new contexts that further support the hypothesis, including extra time points when we inactivate *Rb/p53* or activate *Kras*, and another oncogene (*Braf*).

We acknowledge, as the reviewer notes, that other mechanisms may operate in other contexts. Nevertheless, relative Tc was predictive in multiple scenarios, all demonstrated *in vivo*. Our work presents a potentially broadly relevant paradigm for lineage-specific cancer susceptibility, and Tc may underlie the ability of many cells to resist cancer through normal development. We hope these results will stimulate the field to test this idea in other cancer initiation contexts.

To avoid overstatement, the original comment in the **Abstract** about Tc being a "general hallmark" was removed, and we now note that the results apply "across multiple tumor models *in vivo*" or "in several settings" as follows, Lines 44-46: "Here, we show that total cell cycle length (Tc) predicts susceptibility to transformation across multiple tumor models *in vivo*."; and lines 54-56 to close the abstract: "Thus, relative Tc is an initiation hallmark that distinguishes cancer-prone from cancer-resistant lineages in several settings, offering insight into how mutated cells can escape transformation without inducing apoptosis, senescence or immune surveillance".

Also, at the end of the opening paragraph in the **Discussion** we now state directly that future studies are needed to further test generality, while also emphasizing the novelty of our discovery, Lines 408-411: "Further studies will determine the extent to which this correlation holds across additional tissues and oncogenic events, but our work provides a framework to understand how so many lineages escape cancer without deploying resistance mechanisms such as senescence, apoptosis or immune surveillance [1–7]".

Referee #3 (Remarks to the Author):

In this work, Chen et al set out to address one of the fundamental open questions in cancer biology: why do oncogenic mutations induce tumours in some cell types and not others? To do this, they use a mouse model of retinoblastoma where Rb/p107 double knockout (DKO) in the retina induces tumours, previously shown to arise specifically from amacrine cells. Here the authors combine this DKO with a series of further mutations that perturb cell cycle entry (knocking out CDK1 or disrupting the p27/skp2 interaction). Intriguingly, these genotypes induce similar early effects on proliferation and tissue morphology as the DKO but they do not go on to form tumours. The authors then carry out a comprehensive characterisation of the differences between the tumour-causing DKO and six suppressive genotypes. The only consistent difference they find across all genotypes is the total length of the cell cycle (Tc), which is shorter in cancer-prone genotypes. They then go onto show that cell cycle length is also shorter in the amacrine cells-of-origin compared to other lineages in the retina and that the cells-of-origin also have shorter cell cycles in mouse models of two other Rb-driven tumours and a model of KRAS-driven lung cancer. This leads them to propose that cell cycle length is a new hallmark for cancer susceptibility.

The approach used in this work is original: it demonstrates that there is as much to be learned from cells that do not from tumours as those that do. To my knowledge, this is the first work to claim that total cell cycle length is a key determinant in cancer susceptibility. However, the claims of the universality of this mechanism are overstated in the manuscript. The authors claim that they have discovered a 'general hallmark of lineage-specific predisposition to cancer initiation'. This work lacks the data to support such general claims and would be better framed as a specific mechanism in Rb (see suggestions below). That said, even as a Rb-focused paper, this work is likely to be of wide interest to scientists in the cell cycle field and in cancer biology more generally. I leave it up to the editor to decide if a Rb-specific paper would be of general enough interest to merit publication in Nature or whether this work would be better suited to a more specialist journal.

The data presented is robust and high quality. Multiple complementary techniques are used to demonstrate key findings (eg. use of scRNAseq data and EdU/BrdU labelling for cell cycle analysis). Error bars and statistical tests are properly described and are appropriate. The figures are clear and well-presented and do a good job of clearly distilling a large amount of data. The manuscript is well written. Overall, this work is of the quality that would be expected for publication in Nature.

We thank the reviewer for their positive comments highlighting the importance of our work, its wide interest, the high quality of the data and writing, and particularly for noting that cells that do not form tumors provide insight that is as valuable as those that do, as it is this focus on cancer-resistant cells that underpins the novelty in our study. We also thank the reviewer for their detailed critique which we address below.

Suggestions for improvement:

1. Universality of claims: the authors claim to have revealed cell cycle length as 'a blueprint to identify any cell-of-origin' (abstract) and 'the first general hallmark of lineage-specific susceptibility to cancer initiation (line 248). However they haven't convincingly shown that this is a general mechanism. It needs to be made clear throughout the manuscript that the findings relate to a mouse model of retinoblastoma and that any wider extrapolation is purely speculative. I would also suggest adding 'in retinoblastoma' to the title. Given the specific role that Rb plays in the cell cycle in repressing E2F transcription and cell cycle entry in G1, it makes sense that knockout would shorten the cell cycle. Similarly the suppressive genotypes either involve depletion of Cdks or result in in p27 activation, both of which would be expected to slow cell cycle entry in G1 and therefore lengthen the cycle. However, it doesn't follow that other oncogenes or tumour suppressors would behave in the same way or that this would be a general hallmark for all cancers. The authors do show that cell cycle length is shorter in the cells-of-origin for KRAS-driven lung cancer (Fig 5 E,F) but the evidence for this is far weaker than the rest of the work and I have some issues about the quantification of cell cycle lengths in the lung (see point 2).

To avoid over-stating claims we have modified the manuscript appropriately. However, as discussed below, and expanded upon with new data, the impact is broader than retinoblastoma.

Re "the findings relate to a mouse model of retinoblastoma…". The tumor suppressor data was on retinoblastoma, but that served specifically to highlight Tc as a potential indicator of cancer susceptibility among lineages that survive an oncogenic lesion. Even though all tumor-suppressing mutations affected this hallmark, it remained possible that Tc in the cancer-prone retina would not be different in the cell-of-origin *vs*. cancer-resistant lineages. However, retinal amacrine cells did indeed have a shorter Tc. That raised the issue of generality, which is the

reviewer's main concern: Is this phenomenon limited to mouse retinoblastoma, or is it broader? We could have observed that a shorter Tc in the cell-of-origin *vs*. other surviving but cancer-resistant lineages was limited to murine retinoblastoma. Instead, relative Tc predicted cancer susceptibility in multiple tissues, including retina, pituitary, and lung, and following different oncogenic events including *Rb, Rb/p107, or Rb/p53* loss, as well Ras pathway activation. The correlation held when the timing of the oncogenic event was altered. Thus, whether we inactivated *Rb* at 4 or 8 weeks in pituitary, or, in new data, deleted *Rb/p53* in the embryonic or adult lung Tc was predictive (**Fig 5A, B**).

In further new data we address the reviewer's concern regarding the veracity of our Ras data. Previously, we activated *Kras* at 4wk and studied Tc at 6wk of age, but now we also performed 4wk -> 8wk, 5wk -> 7wk, and 10wk -> 13wk studies, and irrespective of the timing of the oncogenic insult the cancer-prone lineage always exhibited the shortest Tc *vs*. other ectopically dividing lineages (**Fig 5C**). To further address the reviewer's concern, we also activated the downstream Ras effector kinase, Braf. In this additional context the tumor-prone lineage also had the shortest Tc (**Fig 5D**). It is striking that although *Rb/p53* loss and Ras pathway activation ($Kras^{G12D}$ or $Braf^{V600E}$) cause tumorigenesis in distinct lineages (neuroendocrine or alveolar type 2 cells, respectively), Tc is predictive in both contexts.

These extensive additional studies are summarized in the modified Abstract (lines 51-54), the new *Rb/p53* adult lung data are explained in lines 336-346 of the Results, the new *Kras* lung data are outlined in lines 347-350 and 363-370, the new Braf data are detailed in lines 371-385, and the latter includes a summary of all the Tc data at the end of the Results (lines 381-385): "Altogether, our results reveal that among different surviving lineages in a tissue the shortest Tc earmarks the most cancer-prone cell type in all tested cancer models independent of tissue (retina, pituitary, lung), oncogenic driver (*Rb/p107*, *Rb*, *Rb/p53* inactivation, *Kras* or *Braf* activation), or initiation event timing, and even for different cancers that arise from distinct cells-of-origin in the same tissue (SCLC-*Rb/p53*-NE cells; NSCLC-*Kras* or *Braf*-AT2 cells).". The overall findings and novelty of the work are also summarized in the modified opening paragraph of the Discussion (lines 387-411).

Re Rb-cell cycle link: Although Rb is best known for regulating division, and this link was established 34 years ago [21], the connection between Tc and cancer-prone *vs*. cancer-resistant *Rb*-deficient lineages has never been made. Moreover, although the first historical link was division [21], Rb regulates many other cancer hallmarks, including DNA repair [9], apical polarity [10,11], angiogenesis [12], immunity [13], lineage plasticity [14,15], cell survival [16–18] and senescence [19,20] (summary at: https://cancer.sanger.ac.uk/cosmic/census-page/RB1). Other major oncogenic events, like Ras pathway activation, also affect many hallmarks (https://cancer.sanger.ac.uk/cosmic/census-page/KRAS). Each oncogenic event is associated, due to historical bias, with a particular "favorite" function (Rb-cell cycle, Notch/Wnt-development, Ras-cytoplasmic signaling, etc.) but typically they affect many hallmarks, explaining why they are so commonly mutated in cancer. We modified the Results to underscore the pleotropic effects of *Rb* loss (lines 95-98) or *Ras* activation (lines 347-350), as well as the Discussion (lines 387-411).

Similarly, Skp2, p27 and Cdks and cell cycle have many functions in addition to cell cycle (highlighted in more detail now in lines 108-116), but these were just used in the first part of the paper to find a hallmark that was commonly altered upon suppressing tumor suppression. Even though Tc, and not other hallmarks, was always changed upon suppressing retinoblastoma, it was not obvious that Tc would differentiate the cell-of-origin, either in retinoblastoma, or in other *Rb*-initiated tumors (pituitary and lung), or in Ras pathway tumors (lung, $Kras^{G12D}$ or $Braf^{V600E}$). Given the complexity of cancer initiation, it seemed unlikely that Tc would predict cancer susceptibility across many tissues and these very different oncogenic events. However, our data show that relative Tc is predictive in settings beyond just retinoblastoma and beyond Rb. There are many more tissues and oncogenic events that we have not tested, but our extensive *in vivo* data set the stage. To avoid overstatement, the original comment in the **Abstract** about Tc being a "general hallmark" was removed, and we now note that the results apply "across multiple tumor models *in vivo*" or "in several settings" as follows, Lines 44-46: "Here, we show that total cell cycle length (Tc) predicts susceptibility to transformation across multiple tumor models *in vivo*."; and lines 54-56 to close the abstract: "Thus, relative Tc is an initiation hallmark that distinguishes cancer-prone from cancer-resistant lineages in several settings, offering insight into how mutated cells can escape transformation without inducing apoptosis, senescence or immune surveillance". Also, at the end of the opening paragraph in the **Discussion** we now state directly that future studies are needed to further test generality, while also emphasizing the novelty of our discovery, Lines 408-411: "Further studies will determine the extent to which this correlation holds across additional tissues and oncogenic events, but our work provides a framework to understand how so many lineages escape cancer without deploying resistance mechanisms such as senescence, apoptosis or immune surveillance [1–7]".

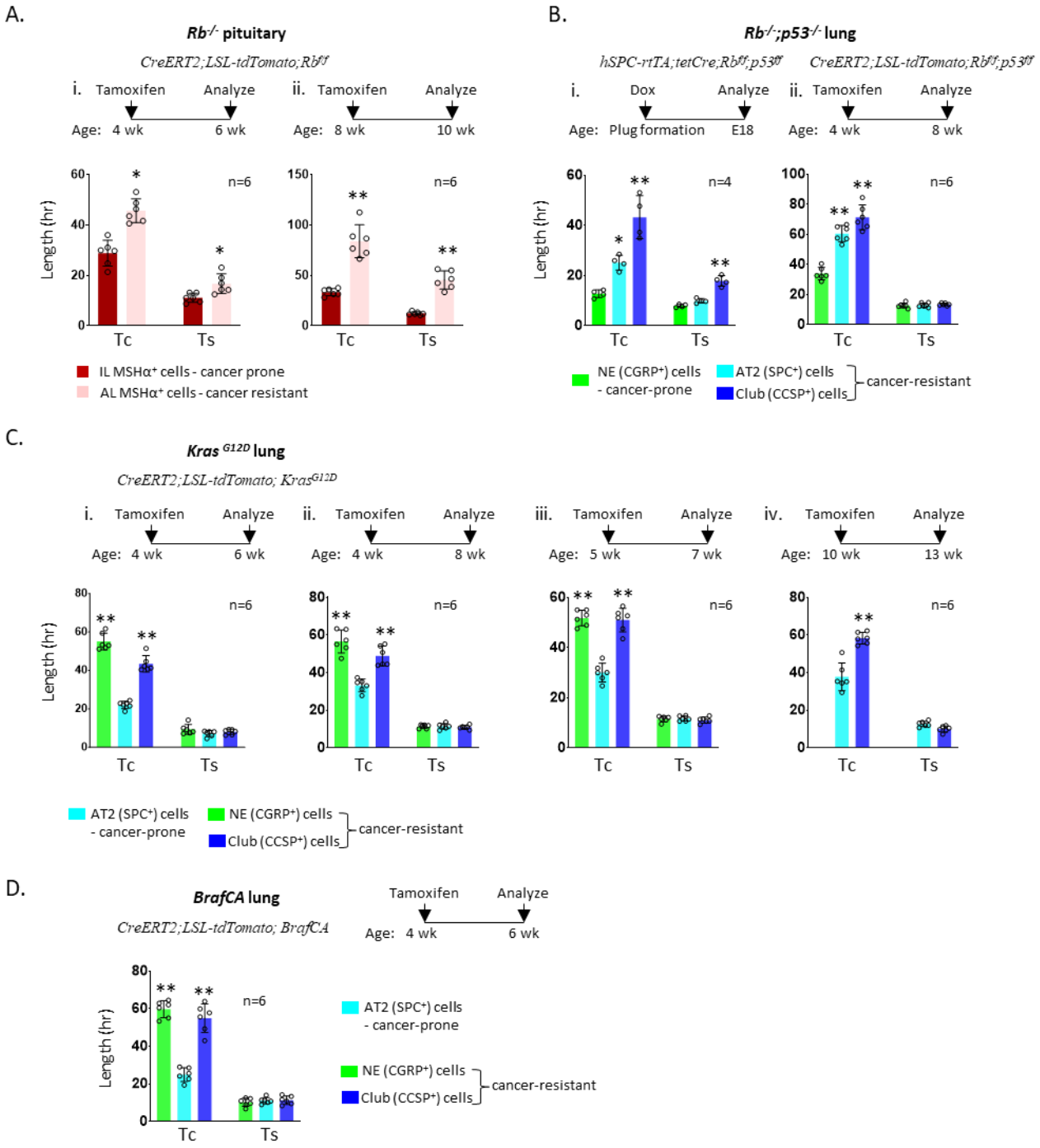


Fig. 5. Shortest Tc as a general hallmark of cancer susceptibility.

A. Tc and Ts of MSHα+ cells in the intermediate (IL) and anterior (AL) pituitary lobes of *Rb* null (*CreERT2;LSL-tdTomato;Rb$^{f/f}$*) mice injected with tamoxifen at 4 or 8 wk and assessed at 6 or 10 wk of age, respectively. **B.** Tc and Ts of lung CGRP+ neuroendocrine (NE), SPC+ Alveolar type 2 (AT2) and CCSP+ club cells of E18 *Rb/p53* DKO animals (*hSPC-rtTA;tetCre;Rb$^{f/f}$;p53$^{f/f}$*) injected with doxycycline at plug formation and assessed at E18 of age, and of P56 *Rb/p53* DKO animals (*CreERT2;LSL-tdTomato;Rb$^{f/f}$;p53$^{f/f}$*) injected with tamoxifen at 4 wk and assessed at 8 wk of age. **C.** Tc and Ts of lung NE, AT2 and club cells of *Kras^{G12D}* animals (*CreERT2;Kras^{G12D};LSL-tdTomato* mice) injected with tamoxifen at 4 wk, 4 wk, 5 wk, and 10 wk and assessed at 6 wk, 8 wk, 7 wk and 13 wk of age respectively. **D.** Tc and Ts of lung NE, AT2 and club cells of *BrafCA* (*CreERT2;BrafCA;LSL-tdTomato*) mice injected with tamoxifen at 4 wk and assessed at 6 wk, of age. Error bars: SD (n = 6 for A, Bii, C, D; n=4 for Bi); asterisks: significant difference between MSHa+ IL *vs.* AL cells (A), or NE *vs.* AT2/club cells (B), or AT2 *vs.* NE/club cells (C, D) (* $p < 0.05$, ** $p < 0.01$. one-way ANOVA, Bonferroni correction for D and F, unpaired t-test for A, B, C, and E).

2. More detail should be given on how Tc is quantified. The central claim of this work relies on the calculation of cell cycle lengths in vivo (Fig 4 C, D), so it is of utmost importance that these methods are well described. Examples of the original images used for calculations in Fig 4 C & D should be shown in the supplement with examples of cells scored Ki67+ or EdU+BrdU- highlighted. The method for cell counting in the microscopy images should be better described – did it involve automated image analysis or was it manual? If manual, how was the accuracy and reproducibility of cell counting ensured? The n numbers given for these experiments refer to the number of animals, but it is important to state in the methods the number of images analysed per animal and also the total number of cells counted in different conditions. I worry that low cell numbers could lead to errors in Tc calculation. This is particularly the important in the lung when analysing rare cell types such as NE cells. For example, in figure S6D, there are very few NE cells present in the example image and only one that looks ki67 positive and would be included in the analysis. Also, Tc in NE cells in fig 5D is calculated to be 11 hours, which is unusually short for a mammalian cell cycle, given that replicating the genome usually takes at least 7-8 hours (and the cells still need time to carry out the transcriptional programmes associated with licensing in G1 and preparation for mitosis in G2.) I am concerned that the calculations do not hold up as well in rare cell types such as NE cells and would like to see proof of the robustness of the method

Re need for well described Tc calculations: To better explain the assay and results, we added: **1.** A detailed explanation of the basic principle and the calculations (**Supplementary Figure 3**); **2.** More detailed methods for the imaging and counting (Materials and Methods, lines 650-695) and many examples of original images used for the cell cycle calculations in retina, pituitary and lung (**Supplementary Figure 4-7**); **3.** A new table with all the raw counts used in Tc and Ts calculations **(Supplementary Table 1**).

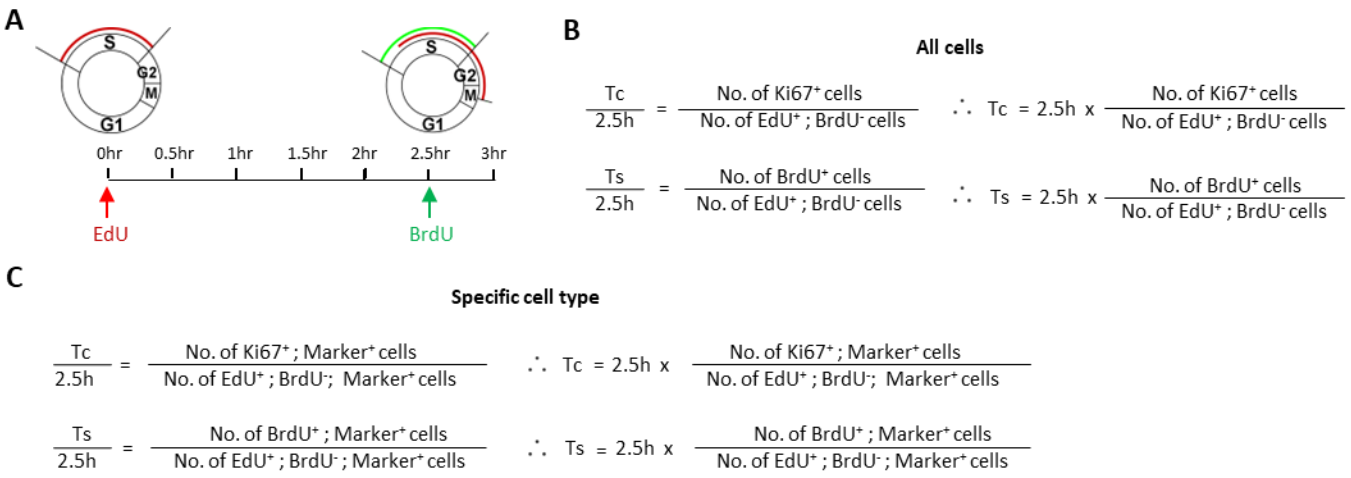
The new **Supplementary Figure 3** and its legend provides a detailed explanation of the methodology and calculations:

Re more detailed methods and example images: The following text was added to provide more detail as to how cells were counted (lines 650-660): "Image J (Fuji) was used to manually count EdU/BrdU labelled cells in 60X images using the image\color\channels tool. For cell-specific cell cycle data, we first counted the number of a cell type of interest (e.g. $CGRP^+$ NE cells). Next (or first in cases where no cell type specific marker was used), we added the Ki67 channel to count dividing cells ($CGRP^+$; $Ki67^+$), then we added the EdU channel to count how many dividing cells were EdU-positive ($CGRP^+$; $Ki67^+$; EdU^+). Next, we added the BrdU channel to count how many dividing cells were BrdU-positive ($CGRP^+$; $Ki67^+$; $BrdU^+$). After that, we combined EdU and BrdU data with Marker/Ki67 to count $Marker^+$; $Ki67^+$; $BrdU^-$; EdU^+ cells. Counting of each image was performed three times to ensure accuracy. Functions used to calculate Tc and Ts are illustrated and explained in detail in **Supplementary Fig 3**. All the raw counts used in Tc and Ts calculations are provided in **Supplementary Table 1.** Examples of images used for counts are provided in **Supplementary Figures 4-7"**.

Supplementary Fig 4 - images from *Rb/p107* null retina
Supplementary Fig 5 – images from *Rb/p53* null lung
Supplementary Fig 6 – images from $Kras^{G12D}$ lung (including high magnification images of NE clusters)
Supplementary Fig 7 – images from $Braf^{V600E}$ lung.

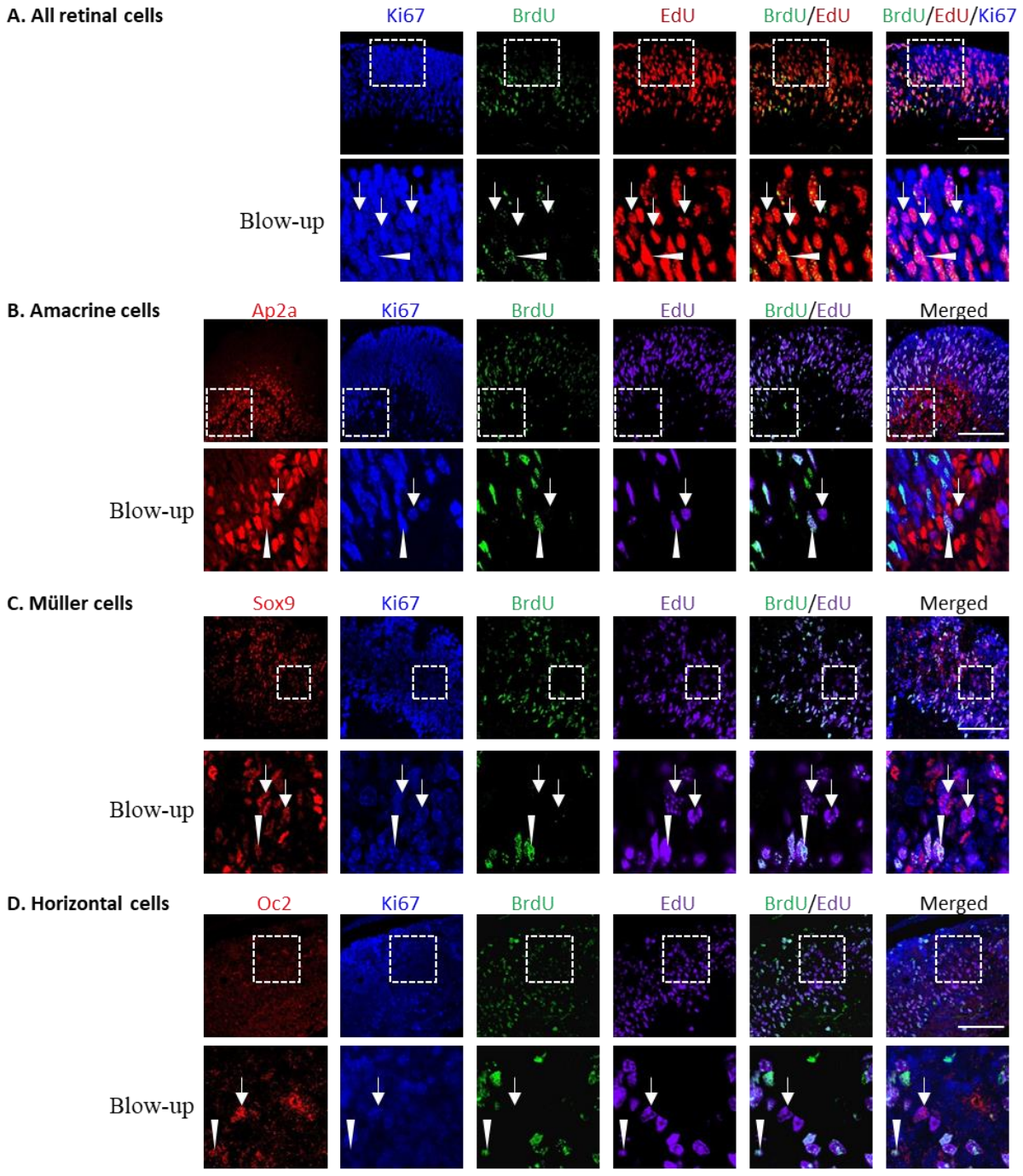
Re the notion that mammalian cells can't divide in 11 hours. That is not the case. In the murine E6.5-7.5 embryo the Tc of epiblasts is 5-7 hours, and for cells in the proliferative zone it is < 3 hrs [22], while for E14 rat retinal progenitors it is 14 hr [31] which is only 3h longer than the Tc we observe in embryonic *Rb/p53* null lung NE cells. We added comparisons in the second paragraph of the Discussion.

Re counting NE cells, which are rare relative to other cell types. NE cells are indeed not trivial to count, but irrespective we assessed many: for the E18 *Rb/p53* null lung we counted 102 images across four mice (23-30 images per animal) and 1552 NE cells (311-483 cells per mouse) of which 408 were $Ki67^+$ (75-136 cells per mouse). Tc was longer in the adult *Rb/p53* null lung (33.8h) but, as for all other contexts, it was shorter than in cancer resistant AT2 (60.4h) or Club (71.3h) cells (**Fig 5B**). Specifically for NE cells in the $Kras^{G12D}$ lung model, we also added high magnification images **(Supplementary Fig 7C**). Overall, we assessed many NE cells in lung (and across many ages), and relative Tc predicted cancer susceptibility in all cases. The shorter Tc in *Rb/p53* null NE cells contrasted $Kras^{G12D}$ or $Braf^{V600E}$ lungs where AT2 cells had the shortest relative Tc (**Fig 5C, D**).

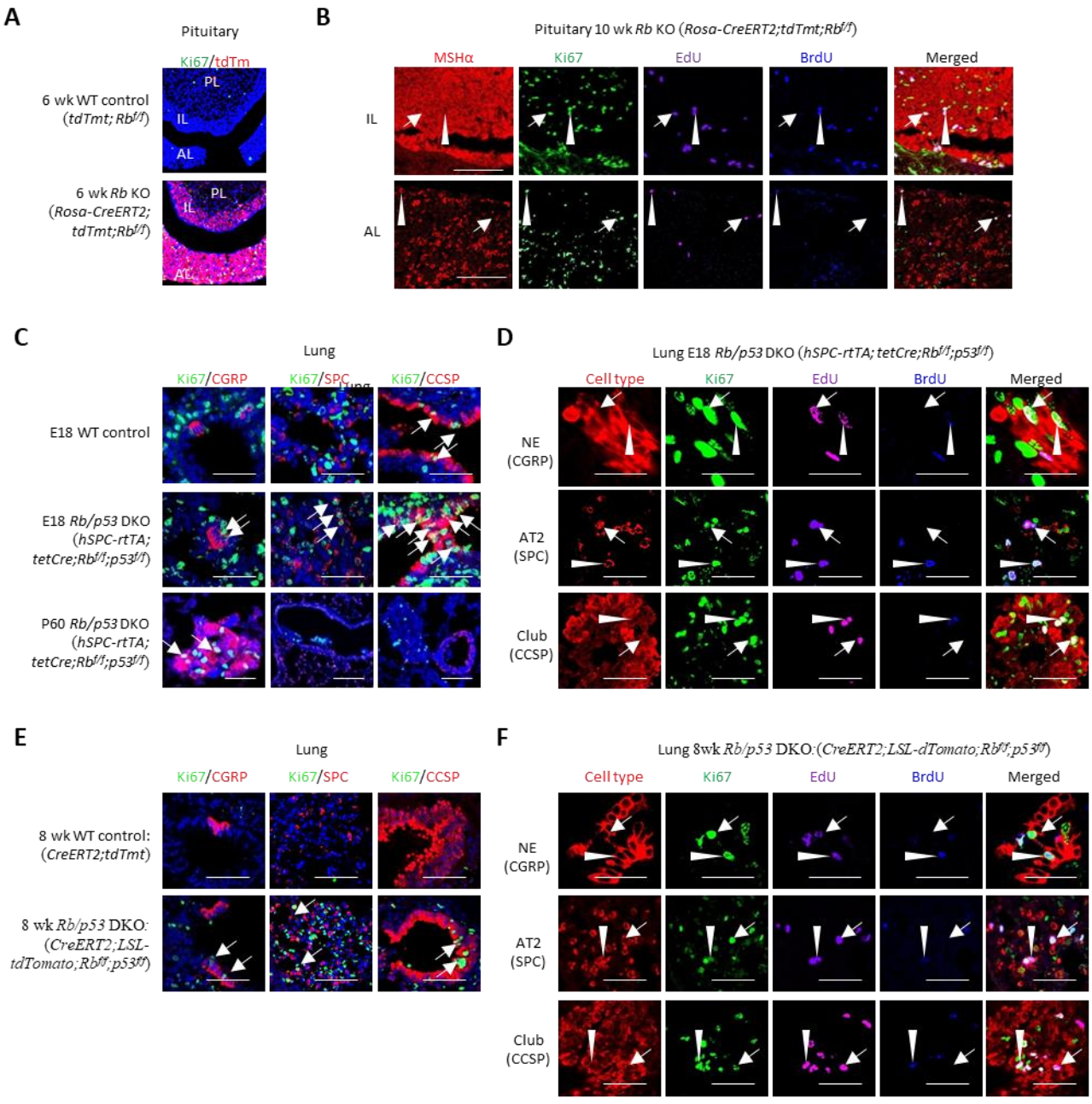


Supplementary Figure 3. Calculation of Tc and Ts.

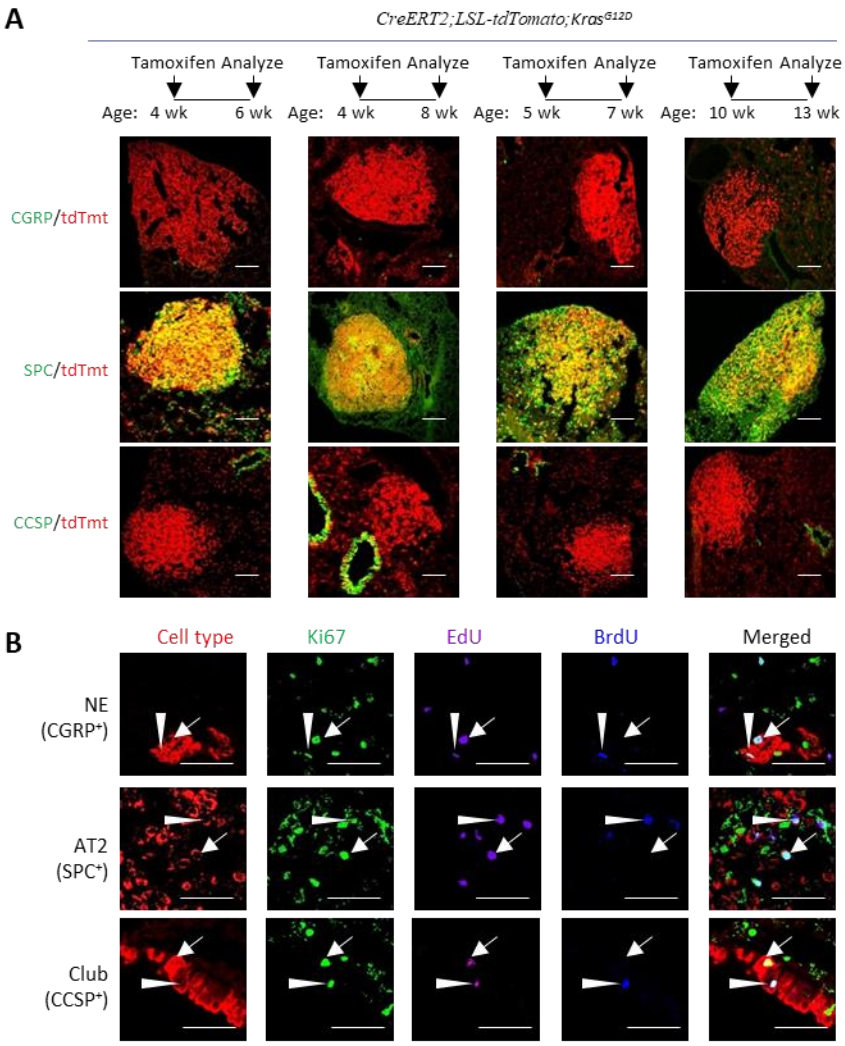
A. Schematic summarizing the sequential EdU then BrdU labeling protocol which, coupled with Ki67 labeling to label all dividing cells, can be used to calculate cell cycle length. 30μg/g body weight EdU is injected IP at 0h, followed by BrdU at 2.5h, and tissue is harvested at 3h. The half-life of IP-injected EdU is ~ 15 min [119], so circulating EdU disappears rapidly. After 3h the EdU^+ cells that labeled S-phase at the start of the assay have moved to mid/late S phase and G2/M (red EdU line), whereas new early S-phase cells are EdU-negative. The 30 min pulse with BrdU marks cells in S-phase at the end of the experiment (2.5-3hr; green BrdU line). EdU^+;$BrdU^-$ cells are those that exited S-phase and moved into G2/M in the 2.5 hr period prior to BrdU injection (red EdU line but no green BrdU line). The number of these EdU^+;$BrdU^-$ cells is proportional to the 2.5h period in which they were generated. Ki67 stains all dividing cells, and the number of these cells is proportional to total cell cycle length (Tc). Similarly, the total number of $BrdU^+$ cells is proportional to S phase length (Ts). These values are used to calculate Tc or Ts as detailed in B and C. **B.** Top row: formula for total cell cycle time (Tc). The ratio of Tc over 2.5h equals the ratio of the number of $Ki67^+$ cells over the number of EdU^+;$BrdU^-$ cells generated in the 2.5h window (left hand side). With three known variables (2.5h; # of $Ki67^+$ cells; # of EdU^+;$BrdU^-$ cells), one can solve for Tc (right hand side). Bottom row: formula to calculate S-phase time (Ts). The ratio of Ts over 2.5h equals the ratio of cells in S-phase (# of $BrdU^+$ cells) over the number of EdU^+;$BrdU^-$ cells generated in the 2.5h window (left hand side). With three known variables (2.5h; # of $BrdU^+$ cells; # of EdU^+;$BrdU^-$ cells), one can solve for Ts (right hand side). Note that some authors have used double-labeled EdU^+;$BrdU^+$ cells for the number of S-phase cells, but in those cases the total labeling time was much shorter than the 3h used in our assay. With a 3h labeling window, many early S-phase cells are EdU-negative, so counting EdU^+;$BrdU^+$ cells would underestimate S-phase cell number. **C.** The formulae to calculate Tc or Ts in a specific cell type are as in (B) except that counts are limited to cells also labeled with a marker for the cell type of interest. Raw counts from the assays are provided in **Supplementary Table 1**.



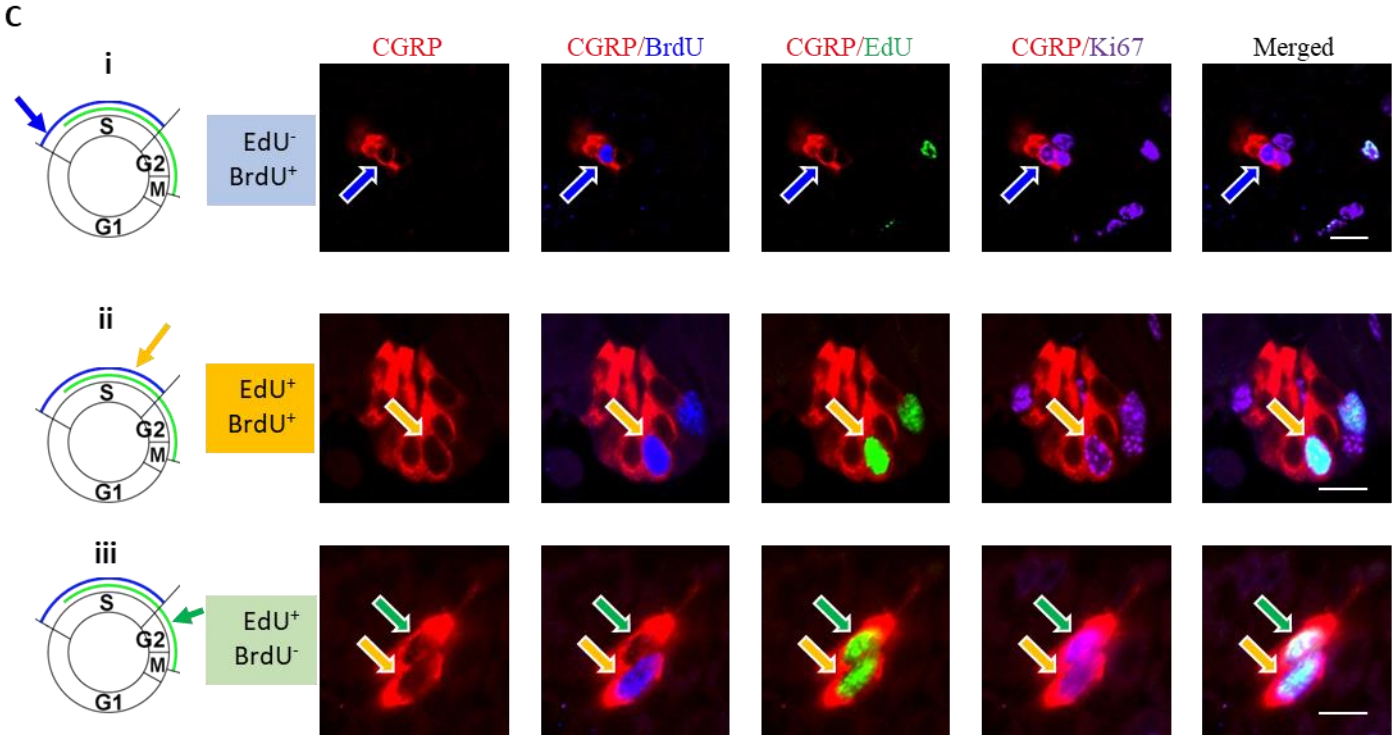
Supplementary Figure 4. Examples of immunostaining to calculate Tc and Ts in retina (related to Fig 4C, D). P8 *Rb/p107* DKO mice were exposed to EdU at 0 hr, BrdU at 2.5 hr, retinas harvested at 3 hr, and sections stained for the indicated markers to assess average total cell cycle time (Tc) or S phase time (Ts) in the whole retina (**A**) or in the indicated cell types (**B**-**D**). The formulae to calculate Tc or Ts and detailed explanations are provided in **Supplementary Fig 3**. Tc calculation requires the number of dividing ($Ki67^+$) cells and the number of EdU-labeled cells that exited S-phase and are thus BrdU-negative (EdU^+;$BrdU^-$ G2/M cells). Ts calculation also utilizes the latter statistic, but instead of all dividing cells, it utilizes the number of cells in S-phase at the time of harvest ($BrdU^+$ cells). For the average cell cycle across the entire retina, Ki67, EdU and BrdU labeling suffice (**A**), but for cell-type specific cell cycle calculations these three markers are combined with Ap2a, Sox9, or Oc2 staining to label amacrine (**B**), Müller (**C**) or horizontal (**D**) cells, respectively. Arrows in (A) indicate examples of $Ki67^+$;EdU^+;$BrdU^-$ G2/M cells, while arrowheads indicate examples of $Ki67^+$;$BrdU^+$ S-phase cells (the EdU status is unimportant and can be positive or negative). Arrows in B-D indicate examples of cell-specific-marker$^+$;$Ki67^+$;EdU^+;$BrdU^-$ cells, while arrowheads indicate examples of cell-specific-marker$^+$;$Ki67^+$;$BrdU^+$ S-phase cells. Scale bar is 100μm.

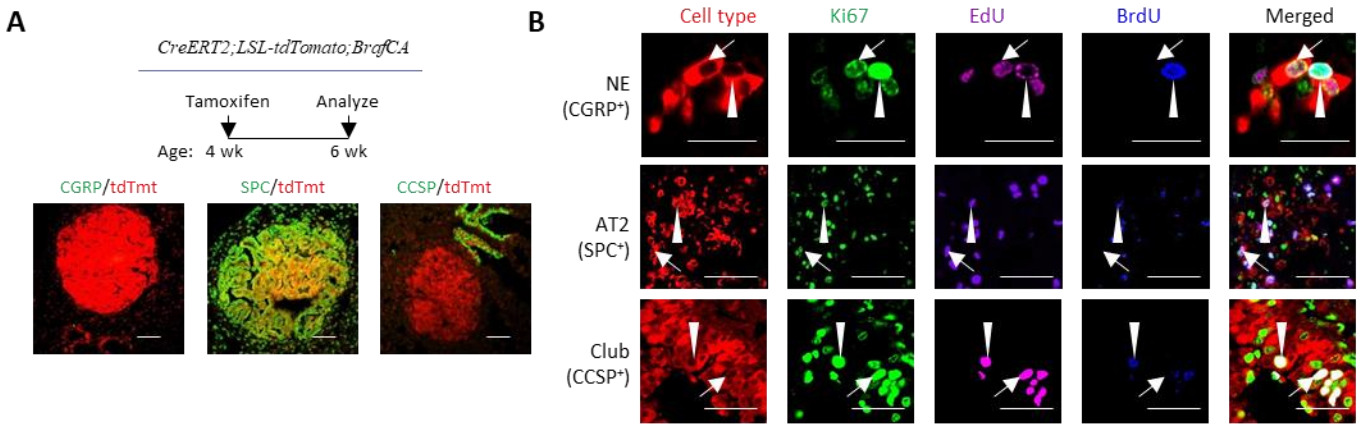


Supplementary Figure 5. Examples of immunostaining to calculate Tc and Ts in *Rb* null pituitary, and *Rb/p53* null embryonic or adult lung (related to Fig 5A, B). **A.** Control (*Cre*-less) and test (*CreERT2*) *tdTmt;Rb*$^{f/f}$ mice exposed to tamoxifen and stained for Ki67 (green) and tdTmt (red). There is near complete recombination (tdTmt$^+$) in test intermediate lobe (IL) and anterior lobe (AL) of pituitary, but the posterior lobe (PL) has a very low level of recombination. **B.** Quadruple labeling to assess Tc and Ts in MSHα$^+$ cells of IL (top) and AL (bottom). **C.** E18 WT (top), E18 Rb/p53 DKO (middle) and P60 Rb/p53 DKO lung (bottom) stained with the indicated markers to quantify proliferation index in the indicated cell types. The P60 images show a typical SCLC CGRP$^+$ lesion, but no lesions with other cell type markers. **D.** Quadruple labeling to assess Tc and Ts in the indicated E18 Rb/p53 null lung cell types. **E.** The indicated genotypes were treated with tamoxifen at 4 wk and harvested at 8wk of age. The lung stained with the indicated markers to quantify proliferation index in the indicated cell types. **F.** Quadruple labeling to assess Tc and Ts in the indicated Rb/p53 null lung cell types. Scale bar is 50μm. Arrows indicate examples of Ki67$^+$;EdU$^+$;BrdU$^-$ G2/M cells, while arrowheads indicate Ki67$^+$;BrdU$^+$ S-phase cells.



Supplementary Figure 6. Examples of immunostaining to calculate Tc and Ts of *Kras*^{G12D} lung (related to Fig 5C).
A. The indicated genotypes were treated with tamoxifen at indicated age (4-10 wk) and harvested at indicated ages (6-13 wk). Recombination (tdTomato$^+$) is evident in both strains, and all formed tumors, positive for the AT2 marker SPC, but not for club cells (CCSP) or neuroendocrine cells (CGRP). **B.** Quadruple labeling to assess Tc and Ts of *Kras*^{G12D} mice in the indicated lung cell types. Arrows indicate examples of Ki67$^+$;EdU$^+$;BrdU$^-$ G2/M cells, while arrowheads indicate Ki67$^+$;BrdU$^+$ S-phase cells. **C.** Higher magnification examples of immunostaining to calculate Tc and Ts of neuroendocrine (CGRP$^+$) cells of KrasG12D lung. 6 wk old *CreERT2;LSL-tdTomato;Kras*^{G12D} mice that had been treated with tamoxifen at 4 wk of age were exposed to EdU at 0 hr, BrdU at 2.5 hr, lungs harvested at 3 hr, and sections stained for the indicated markers to assess average total cell cycle time (Tc) or S phase time (Ts) in CGRP$^+$ neuroendocrine (NE) cells. **i.** Blue-arrow indicates a NE cell in the early S phase (EdU$^-$, BrdU$^+$). **ii.** Brown arrow indicates a NE cell in mid-late S phase (EdU$^+$, BrdU$^+$). **iii.** Brown arrow indicates a NE cell in the mid-late S phase (EdU$^+$, BrdU$^+$), green arrow indicates a NE cell in the G2/M phase (EdU$^+$, BrdU$^-$). The number of EdU-only (i.e. EdU$^+$;BrdU$^-$) cells was used for the calculation of Tc and Ts using the formula explained in **Supplementary Figure 3**. Scale bar is 200μm (A), 50 μm (B), 10μm (Ci) or 20 μm (Cii-iii).





Supplementary Figure 7. Examples of immunostaining to calculate Tc and Ts of *Braf*^{CA} lung (related to Fig 5D). **A.** The indicated genotypes was treated with tamoxifen at 4 wk and harvested at 6 wk. Recombination (tdTomato$^+$) is evident in the lung, and the lung formed tumors, positive for the AT2 marker SPC, but not for club cells (CCSP) or neuroendocrine cells (CGRP). **B.** Quadruple labeling to assess Tc and Ts of *BrafCA* mice in the indicated lung cell types. Arrows indicate examples of Ki67$^+$;EdU$^+$;BrdU$^-$ G2/M cells, while arrowheads indicate Ki67$^+$;BrdU$^+$ S-phase cells.

3. The authors should comment further on the timing of oncogenic events. One limitation of this work is that the authors are examining early oncogenic events at P4-P21, while tumours develop at 100+ days. Some of the hallmarks examined, such as immune infiltration and angiogenesis would be more relevant at later stages of tumorigenesis. Later apoptosis or cell cycle arrest could also halt tumour development in suppressive genotypes. It would be interesting to get some information in the text and/or figures about when the oncogenic and suppressive genotypes start to diverge from one another phenotypically. The differences in cell cycle length (Fig 4, C & D) are measured at P8 only. Do these persist at later time points?

Re timing of retinoblastoma: It is not the case that retinoblastoma tumors appear at 100+ days. The data in **Figure 1A** refers to the detection of large tumors visible upon inspection with the naked eye. Emerging nascent tumors, visible microscopically in sections, appear around P8 [16]. Moreover, ectopic division of cancer-resistant Muller and horizontal lineages gradually diminishes and all but ceases by P30 [16], and without ectopic division there can be no tumorigenesis. As the goal was to search for a hallmark that distinguishes the cancer cell-of-origin from cancer-resistant lineages, it was critical to assess effects at the beginning of tumorigenesis, not months later when the resistant cells have long since escaped transformation. Thus, we focused on effects at or around P8 in the retina. To clarify these issues for the retinoblastoma work we modified the text where we first introduce murine retinoblastoma in the results (lines 92-94) "Tumors in this model become evident microscopically at P8 [26], and become visible to the naked eye beyond ~100 days (**Fig 1A**)". Then, at the point where we start describing the effect of tumor suppressing mutations (lines 142-148) we added: "Tumors in the *Rb/p107 DKO* model emerge microscopically around P8, many cancer hallmarks are evident then, and while both cancer-prone amacrine cells and cancer-resistant horizontal and Müller glia undergo ectopic division during this window, the latter two lineages gradually exit the cell cycle such that by ~P30 only the amacrine-cell derived tumors continue to proliferate [16]. Thus, we focussed on P4-P21 to assess how tumor suppression affects cancer hallmarks in the *Rb/p107 DKO* retina".

We compared Tc in cancer-prone *vs*. cancer-resistant lineages at one time point in *Rb/p107* null retina, but we assessed two time points in *Rb* null pituitary (**Fig 5A**), and we have now also added multiple time points in the lung for both *Rb/p53* loss and *Kras* activation (**Fig 5B, C**). We inactivated *Rb* at 4 or 8 weeks in pituitary, deleted *Rb/p53* in the embryonic or adult lung, activated *Kras* at 4wk, 5wk, or 10wks of age and, to expand Ras pathway analysis, activated *Braf* at 4wk. In every case the cancer-prone lineage exhibited the shortest Tc *vs*. other ectopically dividing lineages (**Fig 5A-C**). Thus, relevance goes beyond retinoblastoma and beyond *Rb* loss.

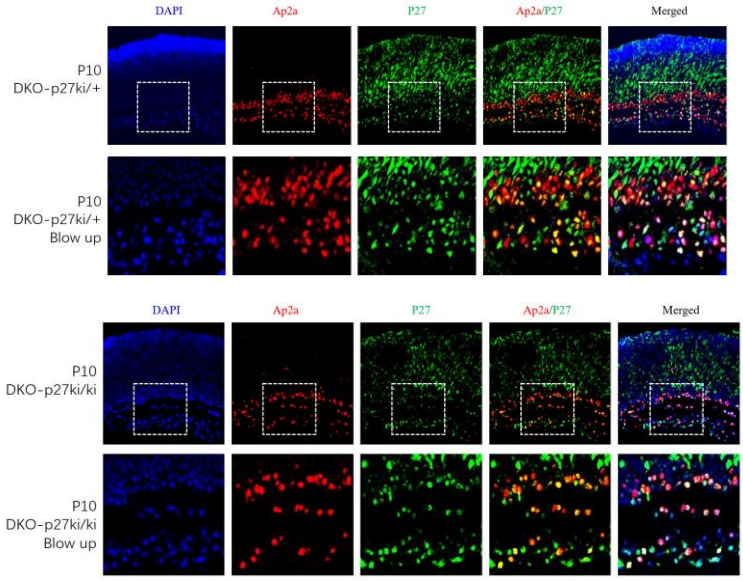
Minor comments

• The authors show that total cell cycle length but not DNA replication time is increased in the suppressive genotypes. Given the role of Rb in cell cycle entry it seems likely that the main difference would be in the length of G1. Fig 3D supports this idea as it shows an increased proportion of G1 cells in a suppressive genotype. Could it be that G1 length is the crucial factor rather than Tc? The authors should comment on this in the text.

As that G1 data shows proportions not time, we did not speculate on which phase(s) might explain differences in Tc. However, as it is an interesting question, we added this note in the discussion (lines 446-448): "Unlike Tc, S-phase did not predict cancer-prone lineages in multiple contexts, thus future work is required to delineate whether effects on G1, G2 and/or M determine Tc.".

• Figure S1C – it's not very clear how the numbers plotted in S1D relate to the images. For example, the p27KI/+ genotype apparently has 80% of amacrine cells that are positive for p27 but in the associated image in S1C, it does not look like 80% cells are both red and green. It would be helpful to show a greater zoom with colours separated.

We replaced the data in former **Fig S1C** with clearer images (new **Extended data Fig 1C**). For the reviewer, separated colours and zooms are provided below to show the high fraction of Ap2a+ cells that are also labeled for p27. There are already many Extended data and Supplementary figures, but we can add these to the paper if the reviewer/editor feel it is needed.



• Figure 2f – is the control the same as in previous figures? In figure 1 and 2A-E, the non-tumorigenic control is p107KO but here it is labelled Rbf/f. Please can the authors clarify why this is different.

Apical polarity defects are absent in *wt, p107*$^{-/-}$ or *αCre;Rb*$^{f/f}$ retinas (**Fig 2F, Extended data Fig 3D**), which are all tumor-free, and only appear in the tumor-prone *αCre;Rb*$^{f/f}$*;p107*$^{-/-}$ retina, so any of the former three can act as a control for normal apical F-actin distribution. To clarify we added the following text (lines 208-209): "Like wt or *Rb*$^{-/-}$ retina, apical polarity is normal in *p107*$^{-/-}$ retina (**Extended data Fig 3D**), which we used as a control in these whole mount assays (**Fig 2G**)."

• Figure 5 – I found it confusing that the same colours are used for the graphs in the left and right panels when they are not comparing the same thing.

We changed the colours to improve clarity. Further improving clarity, **Figure 5** now only contains the cell cycle length graphs (the most important data), with supporting graphs on Ki67 index and tdTomato index placed in additional files (**Extended data Fig 6, 7**). The data has expanded considerably due to the addition of several new time points for induction of *Rb/p53* loss or *Kras* activation, and the addition of *Braf* activation studies.

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Point-by-point response to referee comments:

We were pleased to read the positive remarks on our revisions: Referee #1: “significantly strengthened through extensive revisions”; “the Discussion is much improved”. #2: “authors did a great job answering the points raised by the Reviewers”; “should be commended for their in-depth analysis”; “tackles a key question in cancer biology”; “really powerful”; “raises a lot of really interested (sic) questions”. #3: “My specific comments have been comprehensively addressed”; “new data … demonstrate that the association of shortest Tc with susceptibility to transformation holds true in other contexts “; “this is now a very strong paper”.

Two referees had minor textual suggestions:

Referee #1 suggested we modify the Abstract from: “every tumor-suppressive mutation increased Tc”, to: “every tumor-suppressive mutation tested increased Tc”. We have made the requested change.

Referee #2 had no additional suggestions.

Referee #3 suggested we remove or move the description of non-cell-cycle Skp2, p27 and Cdk activities (they noted it was in the Introduction, but it is part of the initial Results section). We feel, however, that it is important to list these activities early in the manuscript because there is a historical, but unscientific bias that these factors only regulate proliferation. Indeed, referee #2 noted: “I agree with the authors in response to the reviews that one may not expect members of the Skp2-p27-Cdk axis to have the same function in tumorigenesis (similar to the p16-CycD/Cdk4,6-RB axis – these pathways are not exactly linear). Their results that complete loss of SKP2 rescues apical polarity defects are a good example of this”.

Referee #3 suggested we remove the analogy in the Discussion comparing relative cell cycle length (Tc) to a behavioral score. We would like to keep this text because in their reading of the first submission, Reviewer #1 commented that because relative but not absolute Tc is predictive it: “seems to undermine the overall conclusions of the study” (end of point #3 in their original review). Tc is predictive, but in a context-dependent manner, mimicking behavioral scores. We are open to editorial suggestions for a different analogy, but in view of Reviewer #1’s initial misinterpretation, we feel it is important to include one to ensure other readers do not misinterpret the findings.