Peer Review File

Manuscript Title: Low glycemic diets alter lipid metabolism to impact tumor growth

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

Lien et al investigate caloric restriction (CR) versus ketogenic diet (KD) in LSL-53 Kras(G12D);Trp53fl/fl;Pdx1-Cre implanted pancreatic tumors. They find that only CR but not KD impairs tumor growth in this specific model. They find that one difference between CR and KD is that in CR lipid levels in the tumor interstitial fluid are reduced. In particular they find that lipid reduction in vitro increases palmitoleate levels and blocking SCD reduces proliferation of cancer cells. As a mechanism they propose the accumulation of toxic saturated FAs (palmitate, stearate). Based on this they argue that SCD downregulation might be important for the effect of CR diet.
The in vivo findings that CR versus KD have different effects on pancreatic tumors is very interesting. If indeed SCD expression is the determining difference this would be an exciting finding. However, the provided evidence that this is the important difference between KD and CR is currently missing in the manuscript.
The in vitro data are supported by previous work and lipotoxicity indeed has been shown to be an important mediator of the effects of SCD inhibition (e.g. Diabetologia. 2020 Feb;63(2):395-409. doi: 10.1007/s00125-019-05046-x.; J Gastroenterol Hepatol. 2009 May;24(5):703-6. doi: 10.1111/j.1440-1746.2009.05823.x.; Am J Physiol Endocrinol Metab. 2008 Aug;295(2):E339-49. doi: 10.1152/ajpendo.00022.2008.). To proof the claim that the levels of saturated FAs are more important than the ratio between saturated fatty acids and MUFAs/PUFAs more data would need to be provided.

Specific comments:
1) A westernblot similar to Figure 4c should be provided for KD versus control to support the interesting idea that SCD activity is important for the observed effect of CR versus KD on tumor growth.
2) To proof that the difference between CR and KD is SCD activity, mice should be treated with the SCD inhibitor on KD and control diet.
3) The authors should provide in addition to plasma and TIF lipid data (Figure 2e) also the levels of these FA in the tumor.
4) In figure 4g,h saturated FA levels (palmitate and stearate) should be provided (not the ratios) to support the claim that accumulation of saturated FAs rather than a decrease of MUFAs/PUFAs is responsible for the observed effect as claimed in the abstract.
5) The data provided in Figure 4a actually strongly argue against the claim of the authors that "Fatty acid desaturation is required to dispose of toxic saturated fatty acids, and not because monounsaturated fatty acids are specifically needed for proliferation." Because palmitate and stearate are not increased in CR compared to control, while MUFAs are decreased.
6) All FA levels and synthesis rates should be displayed in absolute values and not in relative values normalized to 1. Because all these fatty acids differ highly in abundance.
7) The claims need to be adapted to the data presented, i.e. only one specific cancer model is studied in vivo and in vitro only 4 cell lines are studied. Thus, the provided evidence might be highly specific to the tumor model and the provided cell line data. Similar, the claim that toxicity of saturated FA is more important than MUFAs needs to be refined to the specific cell lines and in vivo model the authors used. Thus, less generalization is required.
8) If indeed saturated FA toxicity is most important, response to SCD inhibitors should correlate with palmitate/stearate levels in cells. Is this the case?

Minor points:
1) The lipid labeling data look very unusual especially given that the labeling time was only 48h. For such data one would expect a distribution of MIDs around M+10 to M+14. See previous work of the authors (Nature. 2011 Nov 20;481(7381):380-4. doi: 10.1038/nature10602). But here all MIDs found are above M+12.
2) The graphs are often hard to comprehend because it is unclear what is measured and what is supplemented. I would suggest giving on the y-axis what is measured and work on the x-axis with +/- for the supplementation (Figure 3), and give the FA name if the FA is not supplemented but measured (Figure 4)
3) Ref 50 should be cited along with Ref 38, 39. Moreover, it seems based on Ref 50 that basal 16:1 n-10 levels are more important for SCD inhibitor sensitivity than the increase in 16:1 n-10 upon SCD inhibition. Is this the case for the here tested cell lines?
4) Why is only palmitoleate but not oleate increased in low lipid media in Figure 3b? SCD mediates both reactions.

Referee #2 (Remarks to the Author):

This is an important and elegant study by Lien and colleagues since it provides evidence that severe calorie restriction delays tumor growth by mechanisms that maybe in part independent of glucose and insulin levels and dependent on the alteration of lipid profile and specific enzymes catalyzing lipid-associated reactions.

The finding of a differential effect of the CR and KD on interstitial glucose levels suggesting reduced glucose availability is very interesting and important. Could this be caused or influenced by the death or dying or quiescence state of the cells exposed to CR? The authors should look at cell cycle and cell death under the 2 conditions.

The effect of CR on blood glucose levels is large but this is compressed when tumor interstitial fluid glucose is examined. However, how was the TIF collected? Does it represent the average glucose levels to which the different layers of cancer cells in tumor mass are exposed? If so, how is the growth rate of the external cells in the tumor mass compared to the internal ones? If the external ones grow much more rapidly, this could be in part driven by the much higher glucose concentration in the blood. In that case, the change of blood glucose levels from approx. 8 to 5mM should also be considered since it is possible that the combination of this glucose change with reduced lipid availability could affect tumor growth.

Line 118, the authors write: "In contrast, levels of almost all fatty acids are reduced by CR, particularly in the TIF". However, according to Fig. 2E, CR has a much stronger effect on the reduction of plasma rather than TIF levels of fatty acids

The role of SCD in the proliferation of various cancer cells is clear and impressive both in vitro and in vivo. However, the authors propose that "Fatty acid desaturation is required to dispose of toxic saturated fatty acids, and not because monounsaturated fatty acids are specifically needed for proliferation". Can this be tested by feeding mice 16:0 FA? Can A939572 be administered in vivo? If so, it would be very interesting to combine 16:0 feeding and A939572

The role of SCD in the reversal of the effects of CR on tumor growth are very convincing. However, if the reduction of 16:0 fatty acid by SCD is central in protecting cancer cells from death, how do the authors explain the reduced/low levels of 16:0 in the plasma and TIF of the CR mice and their high levels in KD mice? Wouldn't these levels predict protection of cancer cells in CR mice and toxicity to them in KD mice? (unless I'm not reading this correctly). How do the authors explain

the difference in 16:0 in control and CR mice in figure 2, in which 16:0 is much lower after CR (TIF) and figure 4, in which 16:0 is the same (tumors)?

The authors write: "and the decrease in SCD activity limits the ability of cancer cells within the tumor from adapting to a lower lipid environment (Fig. 4i)" Isn't the lack of MUFA, caused by a reduced SCD activity during CR a better explanation for the toxicity to cancer cells compared to the increased levels of the toxic 16:0 SFA? In fact, in the section described in Fig. 4, the authors do focus on reduced lipid availability as an explanation for the effects of CR, but this seems to be disconnected with the statement in the abstract and the previous focus on the toxicity of 16:0 accumulation.

Referee #3 (Remarks to the Author):

In this study by Lien et al., the authors identified that caloric restriction (CR), but not the ketogenic diet (KD), inhibits the growth of a mouse pancreatic ductal adenocarcinoma (PDAC) subcutaneous tumor allograft. Interestingly, by examining the metabolite profiles in the tumor interstitial fluid, the authors identified that mice fed the CR diet, unlike the KD, exhibit decreased environmental lipid availability, suggesting that the CR diet inhibits tumor growth through lipid limitation. Through mechanistic exploration using lipid-depleted in vitro cultures and metabolomics analysis of saturated, monounsaturated, and polyunsaturated fatty acids levels, the authors revealed that fatty acid desaturation, namely stearoyl-CoA desaturase (SCD), is necessary for cancer cell growth under lipid starvation, and specifically to inhibit the toxicity of saturated fatty acids.

Overall, I found this study to extremely well written and presented. It describes an exciting observation suggesting that diet-mediated metabolic changes can induce cancer cell adaptation to altered nutrient availability within the tumor microenvironment to enable tumor growth. The limitations in this manuscript include discrepancies in their mechanism when examined in vitro vs. in vivo, and an outstanding question as to generality of these potentially exciting findings due to the use of one type of cancer and only one cell line.

Major comments:
1. Through exhaustive efforts to identify the fatty acid composition in the tumor interstitial fluid, followed by more physiologically relevant in vitro studies to reflect the in vivo metabolite environment, the authors identified the necessity for SCD activity through the ratio of 16:1 (n-7)/16:0 (Fig. 3E-H). However, SCD-overexpressing tumors show a modest effect on desaturation (Fig. 4H), despite the lipid-deplete in vivo environment and tumor outgrowth under the CR diet. Given the known cellular heterogeneity of PDAC tumors, can the authors provide metabolic analysis of the fatty acid levels specifically in the cancer cells, instead of bulk tumors? The reviewer appreciates that this is a non-trivial request, but, to more conclusively assess the proposed mechanism, a more nuanced approach is required (e.g. cell fractionation followed by lipid analysis, tissue mass spectrometry). Additionally, can the authors provide western blots or histology of SCD in tumors from mice fed the CR, KD, and control diets? Together these data would help to clarify their mechanism and the seemingly opposing effect of SCD activity in the in vitro vs. in vivo experiments.
2. The authors primarily rely on one PDAC cell line for their in vivo studies. To further bolster their model, additional cell lines and tissue types should be tested, and optimally in orthotopic models. It would be curious, though not critical, to assess if the immune system is impacting the phenotype by running a parallel experiment in an immunodeficient background.

Minor Comments:

1. Can the authors provide western blots of SCD activity from in vivo tumors at end point vs. day 7, at which point 16:1 (n-7)/16:0 levels were examined (Fig. 4G and H).
2. This study suggests that CR may have dual roles on tumor metabolism, be it through nutrient availability and/or direct inhibition of SCD activity in cancer cells, can the authors comment on the potential therapeutic benefit of SCD inhibition?

Referee #4 (Remarks to the Author):

Summary
The current manuscript by Lien et al. explores the interconnection between caloric restriction and control of tumor growth. By focusing on pancreatic ductal adenocarcinoma (PDAC), the authors demonstrate that caloric restriction (CR) but not a ketogenic diet (KD) in mice led to reduced volume of sc transplanted tumors as compared with control diet. Plasma metabolites of the central carbon and amino acid metabolism remained largely unchanged between the experimental groups but CR was found to lower glucose levels in the tumor interstitial fluid (TIF), which might partly explain the growth inhibitory effects of CR as demonstrated by glucose exposure experiments in cultured PDAC cells. Further, CR lowered lipid availability in vivo, and lipid starvation in vitro led to a distinct induction of intracellular 16:1(n-7) monounsaturated fatty acids (MUFA) which was explained by induction of stearoyl-CoA desaturase (SCD) activity upon exogenous lipid deprivation. In turn, pharmacological SCD inhibition in lipid-deprived PDAC cultures slowed down tumor cell proliferation, the effects of which mostly depended on the accumulation and toxicity of 16:0 saturated fatty acids (SFA) upon SCD inhibition. In vivo, CR triggered a reduction in intra-tumoral SCD activity, and overexpression of SCD in PDAC cells partially reversed the beneficial impact of CR on sc tumors, however, without changing the ratios of MUFA/SFA in response to CR. Overall, the authors conclude that CR limits lipid availability and impairs tumor SCD activity, leading to tumor growth inhibition.

General comments
As a consequence of the obesity pandemic, metabolic etiologies have gained importance for a number of tumor entities, including e.g. liver and also pancreatic cancer (PDAC). This has also sparked the interest in the development of metabolic therapies for tumor diseases and substantial efforts are currently under way to identify metabolic tumor vulnerabilities. In this respect, the current manuscript by Lien et al. addresses an interesting and clinically relevant topic in biomedical research. The study employs state-of-the-art technologies and is well written and clearly structured. While SCD is a long-known and well established contributing factor to tumorigenesis in various tumor entities, its pro-tumorigenic role is mostly believed to rely on its regulatory function for intracellular MUFA levels. This study now expands this view by suggesting that the prevention of toxic SFA levels may contribute to this pro-carcinogenic function. However, the following main issues require additional attention by the authors:
a) The main conceptual advance of this study relates to the inhibitory impact of CR on SCD activity. However, the mechanistic explanation for this inhibitory effect by CR remains completely elusive, e.g. which endocrine/molecular pathways act upstream of SCD to confer the CR impact? It is also conceptually worrisome that the SCD overexpression/rescue experiment partially restores the growth inhibitory effects of CR but leaves fatty acid levels/ratios mostly unaffected which questions the proposed concept in general. Admittedly, technical limitations –as outlined by the authors- might be responsible for the absence of correlations between tumor growth and fatty acid ratios in vivo but still leave the study with a high degree of uncertainty with regards to causal links.
b) Given the significant differences between male and female energy homeostasis, it seems inappropriate to pool male and female mice into combined experimental groups as done in the initial tumor implantation experiments. This holds particularly true for the fact that male and female body weight development is quite different upon CR (Ex Fig 1), and the author's statement

that both CR and KD resulted in reduced body weights is only partly true. In fact, these in vivo experiments have to be repeated by appropriate numbers of male and female mice in separate cohorts to make proper conclusions about normalized tumor volume developments etc. In its current form, all conclusions about the metabolic status of experimental animals are flawed by the mixed male/female composition of the corresponding experimental cohorts (e.g. how do tumor curves, glucose levels lipid ratios etc look in the male KD cohort alone? How would the comparison between female CR vs KD look like?).
c) The study will benefit from the inclusion of some human data to strengthen the overall relevance for the human disease. One would expect that e.g. cachectic (i.e. calorically restricted) pancreatic cancer patients show diminished SCD activity as compared to non-cachectic counterparts.

Further specific comments are listed below:

Specific comments
1. The study should be strengthened by a second, independent tumor model. SCD has been shown to trigger liver cancer growth. Do you find the same lipidomic alterations in hepatic tumors upon CR?
2. Given the current interest in fasting regimes for disease prevention, does CR also prevent PDAC growth in vivo?
3. Fig. 1: please provide data on the overall body composition upon CR and KD in male and female mice.
4. Fig. 2c: It is generally difficult to translate in vivo parameters into appropriate cell culture conditions. Did you consider other fasting/CR mediators in your cell culture conditions, e.g. glucagon, glucocorticoids, FGF21 beyond the glucose? Why would the KD have a different impact on TIF glucose than CR? Please discuss.
5. Fig. 2d: How do the PDAC cells respond to b-OHB in vitro? Does enhanced b-OHB exposure alter the lipidomics profile within the cells?
6. Fig. Ex4a-e: Except for Panc1, pyruvate itself has no effect on tumor cell doubling times. How do you ensure that "functional" pyruvate levels have been used in these experiments? Conclusions are based on negative data.
7. Fig. 3m: Please show vehicle and inhibitor groups in separate bar graphs, respectively.
8. Fig. 3: The text jumps back and forth between the different figure panels which makes it difficult to follow. Please consider re-arrangement.
9. Fig. 3n, o: How does FASN inhibition by 18:1(n-9) occur? What is the proposed mechanism of interaction between the FAS and SCD pathways? Do SFA accumulate because SCD is inhibited or does SCD activity somehow depend on the activity of FAS and ACC? This part is somewhat confusing and should be clarified beyond the rather vague statement in the current text.
10. Fig. 4c: This WB is not convincing and also in this experiment, male and female mice seem to be mixed in the experimental groups (see general comments above). Please quantify WG and indicate male/female samples. Please also provide data on SCD expression levels in KD-exposed tumors.
11. Fig. 4e: Please provide data on food intake and split into male/female cohorts.
12. Fig. Ex7: It is difficult to use data from day 7 as a proof of SCD activity induction because at this stage there is still no difference in tumor volumes across groups. If SCD activity was not different after 20 days (Fig. Ex 7) but changed after 7 days (with no tumor size alterations yet), does this mean that SCD loses importance during tumor development, at least with respect to the proposed concept?

Author Rebuttals to Initial Comments:

Response to Referees' Comments

Referee #1 (Remarks to the Author):

Lien et al investigate caloric restriction (CR) versus ketogenic diet (KD) in LSL-53 Kras(G12D);Trp53fl/fl;Pdx1-Cre implanted pancreatic tumors. They find that only CR but not KD impairs tumor growth in this specific model. They find that one difference between CR and KD is that in CR lipid levels in the tumor interstitial fluid are reduced. In particular they find that lipid reduction in vitro increases palmitoleate levels and blocking SCD reduces proliferation of cancer cells. As a mechanism they propose the accumulation of toxic saturated FAs (palmitate, stearate). Based on this they argue that SCD downregulation might be important for the effect of CR diet.

The in vivo findings that CR versus KD have different effects on pancreatic tumors is very interesting. If indeed SCD expression is the determining difference this would be an exciting finding. However, the provided evidence that this is the important difference between KD and CR is currently missing in the manuscript.

The in vitro data are supported by previous work and lipotoxicity indeed has been shown to be an important mediator of the effects of SCD inhibition (e.g. Diabetologia. 2020 Feb;63(2):395-409. doi: 10.1007/s00125-019-05046-x.; J Gastroenterol Hepatol. 2009 May;24(5):703-6. doi: 10.1111/j.1440-1746.2009.05823.x.; Am J Physiol Endocrinol Metab. 2008 Aug;295(2):E339-49. doi: 10.1152/ajpendo.00022.2008.). To proof the claim that the levels of saturated FAs are more important than the ratio between saturated fatty acids and MUFAs/PUFAs more data would need to be provided.

We thank the Referee for the thoughtful comments, and appreciate that additional experiments would clarify and strengthen the claim that the differential effects we observe between CR and the KD can be explained by their distinct effects on lipid availability in the setting of reduced tumor SCD activity. In the revised manuscript, we provide extensive new data, including data from experiments where we manipulate fat content in both diets to test predictions of the model. These new data further support the claim that an imbalance in saturated versus unsaturated fatty acids in the setting of low SCD activity contribute to the anti-tumor effects of low glycemic diets. We note that the new data led us to temper the claim that toxicity from excess saturated fatty acids is the sole driver of this phenotype in vivo. In the context of CR, we now show that a shift in the ratio of unsaturated fatty acids to saturated fatty acids correlates best with diet-induced tumor growth inhibition. On the other hand, in the context of the KD, we find that altering the KD fat composition to increase saturated fatty acid delivery to the tumor can also reduce tumor growth. In both cases it is an imbalance in saturated versus unsaturated fatty acids in the setting of low SCD activity that tracks with diet-induced tumor growth inhibition.

Specific comments:

1) A western blot similar to Figure 4c should be provided for KD versus control to support the interesting idea that SCD activity is important for the observed effect of CR versus KD on tumor growth.

We thank the Referee for the suggestion to examine SCD expression in mice fed the KD, which also led us to assess fatty acid profiles in tumors from these mice. Importantly, the data from these experiments provided new insights into the metabolic mechanisms underlying both CR and the KD. Specifically, in Figure 4f and Extended Data Figure 11h-i of the revised manuscript, we show that KD tumors have reduced SCD protein levels relative to controls. As shown in Figure 4d-e and Extended Data Figure 11e-g of the revised manuscript, KD tumors also have lower levels of 16:1(n-7) and a lower 16:1(n-7)/16:0 ratio, arguing that like CR, the KD also inhibits tumor SCD activity. However, unlike CR, KD tumors maintain levels of 18:1(n-9) and the 18:1(n-9)/18:0 ratio. Interestingly, we postulated that this is because lard was the fat source used in the KD, which consists of 18:1(n-9) as the most abundant fatty acid (see Extended Data Table 2 in the revised manuscript). Indeed, our analysis of plasma and tumor interstitial fluid (TIF) fatty acid composition confirms that the KD raises levels of 18:1(n-9) in both compartments (see Figure 2e in the revised manuscript).

These new data suggest that both CR and the KD inhibit tumor SCD activity, and that the difference between these two diets is in how they affect lipid availability in the tumor microenvironment. CR lowers lipid availability in the tumor, which synergizes with SCD inhibition to contribute to tumor growth inhibition. In contrast, though the KD also inhibits tumor SCD activity, this is buffered by the KD raising lipid availability to the tumor, in particular 18:1(n-9), which prevents the SCD inhibition from impairing tumor growth. This model is explicitly illustrated in Extended Data Figure 16 of the revised manuscript and led to experiments involving further dietary manipulations to test this hypothesis as discussed in response to the other comments from the Referee.

2) To proof that the difference between CR and KD is SCD activity, mice should be treated with the SCD inhibitor on KD and control diet.

As discussed above in response to Point 1, our new data suggests that the difference between CR and the KD is not tumor SCD activity, since tumor SCD expression is low in animals fed either diet. Instead, the difference between CR and the KD is in how these diets affect tumor lipid availability in the setting of diet-induced SCD inhibition. That is, CR lowers lipid availability, while the KD raises it, and this suggested that further dietary manipulations of fat content would be a more direct test of the model.

First, we examined whether we could exploit KD-mediated SCD inhibition by manipulating the lipid composition of that diet. Specifically, based on findings from the in vitro studies suggesting that toxicity of SCD inhibition is greatest when accompanied by saturated fatty acid accumulation (Figure 3), we examined whether a KD enriched in saturated fatty acids might inhibit tumor growth. We formulated a KD based on palm oil (KD-Palm), which consists of 16:0 as the most abundant fatty acid and contains ~2x more 16:0 while maintaining similar levels of 18:1(n-9) as the lard-based KD (Extended Data Table 2). We confirmed that KD-Palm increased saturated fatty acid levels in

circulation and in the tumor (Extended Data Figure 15b,e). Importantly, and in support of the model, we also found KD-Palm inhibits tumor growth, and this growth inhibition is rescued by exogenous SCD expression in cancer cells (Figure 4k-l, Extended Data Figure 15a). Therefore, KD-mediated inhibition of tumor SCD activity can be exploited to slow tumor growth by manipulating the saturated fatty acid content of the diet. These results reinforce the overall model that fatty acid availability in the tumor in combination with diet-driven SCD inhibition reduces tumor growth.

Second, we asked whether a high fat CR (HFCR) diet could increase tumor lipid availability and rescue the growth inhibitory effects of CR. To do this, we formulated a HFCR diet by doubling the amount of fat at the expense of further lowering carbohydrate content relative to our standard CR diet (Extended Data Table 1). Our standard CR diet consists of soybean oil, so one of our HFCR diets was made with soybean oil (HFCR-Soybean). We also formulated a HFCR diet with palm oil (HFCR-Palm), which contains large amounts of both 16:0 and 18:1(n-9) fatty acids (Extended Data Table 2). Interestingly, we find that only HFCR-Palm restores levels of circulating 16:0 and 18:1(n-9) back to control diet levels (Extended Data Figure 14a), which subsequently also rescues tumor levels of 16:0, 18:1(n-9), and the 18:1(n-9)/18:0 ratio back to control levels (Extended Data Figure 14c-d). These effects are not observed with HFCR-Soybean, suggesting that distinct fat sources can have different effects on tumor fatty acid availability. Most importantly, and consistent with the model, only HFCR-Palm, which rescues fatty acid levels in the tumor, is able to rescue tumor growth relative to the standard CR diet (Figure 4i-j). Interestingly, this partial rescue by HFCR-Palm is not observed with the LGSP lung adenocarcinoma model (Extended Data Figure 14f-g), but again consistent with the model, the lack of rescue occurs in a setting where HFCR-Palm does not restore 18:1(n-9) and 18:1(n-9)/18:0 levels in those tumors (Extended Data Figure 14h-i). We suspect that compared to the AL1376 tumors, LGSP tumors may not as readily take up the increased fats in circulation from HFCR-Palm.

Together, these additional experiments show that we can influence the effects of both CR and the KD in a predictable manner by manipulating the dietary fat composition. This further supports the conclusion that how low glycemic diets affect tumor lipid availability in the setting of reduced SCD activity determines whether they slow tumor growth. This model is now explicitly illustrated in Extended Data Figure 16 of the revised manuscript.

3) The authors should provide in addition to plasma and TIF lipid data (Figure 2e) also the levels of these FA in the tumor.

The full fatty acid compositions of tumors from mice fed either CR or the KD are now reported in Figure 4a-b, Figure 4d-e, and Extended Data Figures 10-11. The full fatty acid composition of tumors from the new experiments described above with KD-Palm and the HFCR diets are presented in Extended Data Figures 14-15 in the revised manuscript.

4) In figure 4g,h saturated FA levels (palmitate and stearate) should be provided (not the ratios) to support the claim that accumulation of saturated FAs rather than a decrease of MUFAs/PUFAs is responsible for the observed effect as claimed in the abstract.

The levels of saturated fatty acids associated with the original Figure 4g-h are now provided in Extended Data Figure 13h of the revised manuscript. We discuss these data below in the response to Referee's comment 5.

5) The data provided in Figure 4a actually strongly argue against the claim of the authors that "Fatty acid desaturation is required to dispose of toxic saturated fatty acids, and not because monounsaturated fatty acids are specifically needed for proliferation." Because palmitate and stearate are not increased in CR compared to control, while MUFAs are decreased.

The Referee makes a very good point that while CR does inhibit tumor SCD activity, it does not necessarily lead to the accumulation of saturated fatty acids in any of the tumor models shown in Figure 4a, Extended Data Figure 10a-g, and Extended Data Figure 13h of the revised manuscript. To clarify, our in vitro analysis of SCD inhibition in cultured cells (Figure 3) is what suggested that the toxicity of SCD inhibition is maximized and leads to cell death when it is accompanied by the accumulation of saturated 16:0. Our initial statement that "fatty acid desaturation is required to dispose of toxic saturated fatty acids" referred to this in vitro result. We did not intend to claim that unsaturated fatty acids are not necessary for cell proliferation, but rather that the accumulation of the saturated fatty acid 16:0 from SCD inhibition may be important to cause cell death. Indeed, in Figure 3o, the fatty acid synthesis inhibitors GSK2194069 and PF-05175157 rescue cell death induced by the SCD inhibitor A939572, but do not restore maximal proliferation compared to vehicle control. This is consistent with cells still needing to acquire fatty acids de novo, including monounsaturated fatty acids, to optimally proliferate in lipid-limited environments.

We also note that the in vitro context in which we observe SCD inhibition leading to cell death through 16:0 accumulation is in lipid-depleted media, in which only trace levels of lipids remain (now quantified in Extended Data Figure 5a). While CR does lower TIF lipid levels (Figure 2e), it does not induce a completely lipid-starved tumor environment. As the Referee notes, CR does not necessarily lead to saturated fatty acid accumulation in tumors, but does decrease MUFAs and the ratio of MUFA/SFAs (Figure 4a-b, Extended Figure 10e-f). This finding is consistent with CR inhibiting tumor growth, rather than causing tumor regression (Figure 1a). Also consistent with CR impairing cancer cell proliferation rather than causing cell death in tumors, we find CR decreases Ki67 positivity, but does not affect cleaved caspase 3 staining (Extended Data Figure 3a-b). Moreover, as discussed in our response to Point 2 above, we also now demonstrate the dietary relevance of saturated fatty acid accumulation, as SCD inhibition in the context of KD-Palm also inhibits tumor growth (see Figure 4k-l).

In the revised manuscript, we have modified the text to more explicitly discuss our model that the ratio of saturated to unsaturated fatty acids best correlates with low glycemic diet-induced tumor growth inhibition observed in mice, and we are more precise in discussing the role of desaturases in preventing saturated fatty acid accumulation and balancing the ratio of UFA/SFAs.

6) All FA levels and synthesis rates should be displayed in absolute values and not in relative values normalized to 1. Because all these fatty acids differ highly in abundance.

The Referee raises a reasonable point, and while we appreciate that absolute quantification of metabolites can be informative, we also note that the method of fatty acid analysis used (fatty acid methyl esters by GC-MS) involves the measurement of the total fatty acid composition in cells, which includes both free fatty acids and fatty acids esterified to complex lipid species. Moreover, we note that the majority of experiments, particularly in Figure 3 and Extended Data Figures 5-9, involve comparisons of individual fatty acids between conditions, and our conclusions would not change with absolute quantification of these fatty acids. Nevertheless, we agree with the Referee that distinct fatty acids differ highly in abundance, and it is important to know if the major fatty acids of interest in this study are abundant in cells. Since our study is focused primarily on SCD, the most relevant fatty acids are the substrates and products of this reaction: 16:0, 18:0, 16:1(n-7) and 18:1(n-9). In Extended Data Figure 5f in the revised manuscript, we now show the absolute quantification of a panel of fatty acids from the AL1376 and LGSP cells used in the study. We find that the above fatty acids involved in the SCD reaction are among the most abundant fatty acid species in these cells. Therefore, the effects of manipulating SCD activity throughout our study indeed involve perturbations to fatty acids that are abundant in cells/tumors, consistent with their alterations correlating with changes in cell proliferation and tumor growth. We now also highlight this point in the text describing Extended Data Figure 5.

7) The claims need to be adapted to the data presented, i.e. only one specific cancer model is studied in vivo and in vitro only 4 cell lines are studied. Thus, the provided evidence might be highly specific to the tumor model and the provided cell line data. Similar, the claim that toxicity of saturated FA is more important than MUFAs needs to be refined to the specific cell lines and in vivo model the authors used. Thus, less generalization is required.

We apologize for any over-generalization of the findings, as the Referee is correct that our conclusions are based on the analysis of specific models. We have worked to correct this in the revised manuscript. Nevertheless, we have repeated most in vivo experiments using both a lung and pancreatic cancer model, and observe similar results despite these cancers arising in different tissues. Importantly, we discuss that future work will be necessary to identify the cellular characteristics that can dictate how cancer cells respond to CR and KD diets and to SCD inhibition in

other contexts, as the data illustrate that how different diets affect lipid availability and tumor SCD activity may predict their effects on tumor growth.

8) If indeed saturated FA toxicity is most important, response to SCD inhibitors should correlate with palmitate/stearate levels in cells. Is this the case?

We thank the Referee for suggesting this experiment. In Extended Data Figure 8a, we treat five cell lines with the same concentration of the SCD inhibitor A939572, and we find that these cells have varying sensitivities to SCD inhibition. 50 nM A939572 induces cell death in AL1376 and LGSP cells, whereas Panc1 and A549 cells are particularly resistant. In Extended Data Figure 8b-g, we show the extent to which 50 nM A939572 changes 16:0, 18:0, 16:1(n-7), 18:1(n-9), 16:1(n-7)/16:0, and 18:1(n-9)/19:0 in each of these cells. We find that the induction of 16:0 levels does indeed correlate with sensitivity to SCD inhibition (Extended Data Figure 8b), whereas an increase in 18:0 levels does not correlate (Extended Data Figure 8c). This correlation with 16:0 induction supports the conclusions drawn from Figure 3h-o that SCD inhibition in vitro leads to cell death when it is accompanied by the accumulation of 16:0. Interestingly, decreases in 18:1(n-9) levels also correlate with A939572 sensitivity (Extended Data Figure 8e). This is consistent with the discussion related to Point 5 above; while strong accumulation of 16:0 levels are necessary for induction of cell death by SCD inhibition, reductions in monounsaturated fatty acids levels such as 18:1(n-9) can still contribute to decreased cell proliferation.

Minor points:

1) The lipid labeling data look very unusual especially given that the labeling time was only 48h. For such data one would expect a distribution of MIDs around M+10 to M+14. See previous work of the authors (Nature. 2011 Nov 20;481(7381):380-4. doi: 10.1038/nature10602). But here all MIDs found are above M+12.

We appreciate the Referee noticing this detail. For ^{13}C fatty acid labeling, a left to right shift in MID represents the fraction of the lipogenic acetyl-CoA pool that is derived from the labeled nutrient. In the study that the Referee refers to, labeling of palmitate in cells is done with either ^{13}C-glucose or ^{13}C-glutamine, with the MID around M+10 to M+14. In this study, labeling is done with both ^{13}C-glucose and ^{13}C-glutamine simultaneously, which results in greater lipogenic acetyl-CoA labeling and leads to MIDs above M+12. We are now more explicit about how this experiment was done in the revised manuscript.

2) The graphs are often hard to comprehend because it is unclear what is measured and what is supplemented. I would suggest giving on the y-axis what is measured and work on the x-axis with +/- for the supplementation (Figure 3), and give the FA name if the FA is not supplemented but measured (Figure 4)

We thank the Referee for pointing this out, and we have modified the figures in the revised manuscript as suggested.

3) Ref 50 should be cited along with Ref 38, 39. Moreover, it seems based on Ref 50 that basal 16:1 n-10 levels are more important for SCD inhibitor sensitivity than the increase in 16:1 n-10 upon SCD inhibition. Is this the case for the here tested cell lines?

We now cite these references together as Refs. 48-50 in the revised manuscript. In Ref. 50 Figure 1c, it is shown that the basal 16:1(n-10)/16:0 ratio of cells correlates with SCD inhibitor sensitivity. In Extended Data Figure 8i, we show this ratio in all five cell lines studied under both basal and SCD inhibitor-treated conditions and do not observe a strong correlation between the basal 16:1(n-10)/16:0 ratio and SCD inhibitor sensitivity. We also observe that the change in total 16:1(n-10) levels does not correlate with SCD inhibitor sensitivity for the lines we tested (Extended Data Figure 8h). However, we do find that the change in the 16:1(n-10)/16:0 ratio upon SCD inhibition does correlate with SCD inhibitor sensitivity (Extended Data Figure 8j). The sensitive cells, in which SCD inhibition induces cell death (AL1376 and LGSP), exhibit a significant drop in this ratio, whereas the resistant cells (A549, Panc1) exhibit either an increased or unchanged ratio. Since the production of 16:1(n-10) through FADS2 is an alternative reaction from SCD that can also dispose of the toxic 16:0 saturated fatty acid (Ref. 50), this correlation is consistent with our conclusion that SCD inhibition leads to cell death when accompanied by the accumulation of 16:0. That is, cells that are less capable of disposing of 16:0, whether through SCD or FADS2, are more sensitive to SCD inhibition.

4) Why is only palmitoleate but not oleate increased in low lipid media in Figure 3b? SCD mediates both reactions.

We note that in Figure 3b, 18:1(n-9) oleate is increased in lipid-depleted media, although not to the same degree as palmitoleate. We also note that a robust increase in the 18:1(n-9)/18:0 ratio is observed in lipid-depleted media (Figure 3e), which is probably a better surrogate of SCD activity than individual MUFA levels, and is driven more by a decrease in 18:0 levels (Figure 3b). These observations are consistent with increased SCD activity that converts more 18:0 into 18:1(n-9), resulting in lower steady-state 18:0 levels and an increased 18:1(n-9)/18:0 ratio.

Referee #2 (Remarks to the Author):

This is an important and elegant study by Lien and colleagues since it provides evidence that severe calorie restriction delays tumor growth by mechanisms that maybe in part independent of glucose and insulin levels and dependent on the alteration of lipid profile and specific enzymes catalyzing lipid-associated reactions.

We appreciate the Referee's support for the novelty of our conclusions about the metabolic mechanisms underlying how caloric restriction and the ketogenic diet impact tumor growth beyond their effects on glucose and insulin. We hope the Referee agrees that our efforts to address their comments with additional experiments further strengthens the study.

The finding of a differential effect of the CR and KD on interstitial glucose levels suggesting reduced glucose availability is very interesting and important. Could this be caused or influenced by the death or dying or quiescence state of the cells exposed to CR? The authors should look at cell cycle and cell death under the 2 conditions.

We thank the Referee for suggesting this experiment. In Extended Data Figure 3, we now show IHC staining of Ki67 and cleaved caspase 3 (CC3) in tumor sections from mice fed control, CR, and KD diets. We find that CR leads to slightly decreased Ki67 staining in tumors, while CC3 staining is unchanged (Extended Data Figure 3a-b). This is consistent with CR inhibiting cancer cell proliferation to slow tumor growth, rather than CR inducing cell death (Figure 1a). In contrast, the KD does not affect Ki67 or CC3 positivity (Extended Data Figure 3c-d), consistent with this diet not affecting growth of the tumors examined (Figure 1b).

The effect of CR on blood glucose levels is large but this is compressed when tumor interstitial fluid glucose is examined. However, how was the TIF collected? Does it represent the average glucose levels to which the different layers of cancer cells in tumor mass are exposed? If so, how is the growth rate of the external cells in the tumor mass compared to the internal ones? If the external ones grow much more rapidly, this could be in part driven by the much higher glucose concentration in the blood. In that case, the change of blood glucose levels from approx. 8 to 5mM should also be considered since it is possible that the combination of this glucose change with reduced lipid availability could affect tumor growth.

The TIF was collected as described in Ref. 3 of the revised manuscript (PMID 30990168) and undoubtedly represents an average of glucose levels throughout the entire tumor. The Referee raises a good point that some cancer cells closer to blood vessels may experience higher glucose concentrations. We did not observe any clear differences in Ki67 staining across tumor sections, but it is hard to relate areas of Ki67 positivity with respect to vascularization, as this is a complex study in and of itself. Nevertheless, as suggested by the Referee, in Figure 2c we now assess whether glucose concentrations of 8 mM and 5 mM limit cell proliferation, either in lipid-replete or lipid-depleted conditions. Of note, we find cell proliferation remains unaffected until glucose falls to 0.8 mM,

where a slight defect in proliferation is observed. This result is consistent with our original conclusion that changes in the availability of other nutrients can contribute to the tumor growth inhibitory effects of CR.

Line 118, the authors write: "In contrast, levels of almost all fatty acids are reduced by CR, particularly in the TIF". However, according to Fig. 2E, CR has a much stronger effect on the reduction of plasma rather than TIF levels of fatty acids

We agree with the Referee that CR has a stronger effect on plasma fatty acids levels than it does on TIF fatty acid levels, and we have amended the "particularly in the TIF" statement accordingly.

The role of SCD in the proliferation of various cancer cells is clear and impressive both in vitro and in vivo. However, the authors propose that "Fatty acid desaturation is required to dispose of toxic saturated fatty acids, and not because monounsaturated fatty acids are specifically needed for proliferation". Can this be tested by feeding mice 16:0 FA? Can A939572 be administered in vivo? If so, it would be very interesting to combine 16:0 feeding and A939572

The Referee makes an excellent suggestion. Since our in vitro data in Figure 3 illustrates that the toxicity of SCD inhibition is maximized when accompanied by the accumulation of toxic 16:0, we agree that it would be particularly interesting to combine 16:0 dietary feeding with SCD inhibition. Administration of A939572, and SCD inhibitors in general, in mice is hindered by poor compound solubility, poor bioavailability, and most critically, major adverse eye and skin effects (see PMID 24295027). However, we were able to test the Referee's suggestion by using the KD, which we now show in Figure 4d-f also impairs tumor SCD activity. Of note, we observe that KD tumors have lower levels of 16:1(n-7) (Figure 4d) and a lower 16:1(n-7)/16:0 ratio (Figure 4e), which is consistent with decreased SCD activity. Interestingly, unlike CR, KD tumors maintain levels of 18:1(n-9) and the 18:1(n-9)/18:0 ratio (Figure 4d-e), likely because the fat source in the KD used is lard, which contains 18:1(n-9) as the most abundant fatty acid (see Extended Data Table 2). Indeed, our analysis of the fatty acid composition in plasma and TIF in Figure 2e confirms that the KD raises levels of 18:1(n-9) in both plasma and TIF. Together, these data argue that the KD inhibits tumor SCD activity, but the fat composition of the diet (lard) raises 18:1(n-9) levels in the tumor to buffer the growth inhibitory effects of SCD inhibition. These results led us to question whether replacing the fat source in the KD with a 16:0-rich fat would synergize with KD-mediated SCD inhibition to impair tumor growth (as the Referee suggests). To accomplish this, we formulated a KD with palm oil (KD-Palm), which consists of 16:0 as the most abundant fatty acid (Extended Data Table 2). Relative to the lard-based KD, KD-Palm contains ~2x more 16:0 while maintaining similar levels of 18:1(n-9) (Extended Data Table 2). Accordingly, KD-Palm increases circulating levels of saturated fatty acids (Extended Data Figure 15b) and increases saturated fatty acid levels in tumors (Extended Data Figure 15e). Importantly, and consistent with the model, KD-Palm inhibits tumor growth, and this is rescued by SCD expression in cancer cells (Figure 4k-l). These data demonstrate that KD-mediated inhibition of tumor SCD activity can be exploited to inhibit tumor growth if saturated fatty acids are delivered to the tumor. These results also support a model where a mismatch between which fatty acids are available in the tumor

cooperates with diet-induced reductions in SCD activity to slow tumor growth.

The role of SCD in the reversal of the effects of CR on tumor growth are very convincing. However, if the reduction of 16:0 fatty acid by SCD is central in protecting cancer cells from death, how do the authors explain the reduced/low levels of 16:0 in the plasma and TIF of the CR mice and their high levels in KD mice? Wouldn't these levels predict protection of cancer cells in CR mice and toxicity to them in KD mice? (unless I'm not reading this correctly). How do the authors explain the difference in 16:0 in control and CR mice in figure 2, in which 16:0 is much lower after CR (TIF) and figure 4, in which 16:0 is the same (tumors)?

The Referee makes a good point that intracellular fatty acid levels do not always match extracellular fatty acid levels. This is likely because steady-state intracellular fatty acid levels are set by a combination of uptake from the environment, intracellular synthesis, and activity of pathways that consume fatty acids or sequester those fatty acids in lipid droplets (PMID 30184495). For example, in Extended Data Figure 5a, we show that lipid-depleted media contains drastically reduced levels of all fatty acids. Nevertheless, cells cultured in lipid-depleted media maintain levels of most fatty acids (Figure 3b, Extended Data Figure 5c) because they increase de novo synthesis (Figure 3c, Extended Data Figure 5d). Similarly, while CR may reduce 16:0 levels in TIF (Figure 2e), tumor 16:0 levels can be maintained through intracellular synthesis and/or inhibition of 16:0 utilizing pathways such as SCD, which we observe is impaired in tumors (Figure 4a-c). Likewise, while the KD raises 16:0 levels in the TIF (Figure 2e), this does not lead to accumulation of 16:0 within the tumor (Figure 4d). As discussed above, when the fat in the KD is replaced with palm oil, which is enriched in 16:0, this diet further increases circulating levels of saturated fatty acids (Extended Data Figure 15b), which in turn raises levels of 16:0 and other saturated fatty acids in tumors (Extended Data Figure 15e) and inhibits tumor growth (Figure 4k-l). We have modified the text to discuss these points. We have also more explicitly stated that while accumulation of 16:0 leads to cell death upon loss of SCD activity in settings of extreme lipid deprivation, shifts in the ratio of saturated to unsaturated fatty acids correlate best with tumor growth inhibition observed with CR in vivo. This is likely because complete deprivation of lipids in tumors by diet is impossible, and is consistent with the observed reduction in Ki67 but not increased cell death in tumors whose growth is affected by CR (Extended Data Figure 3a-b).

The authors write: "and the decrease in SCD activity limits the ability of cancer cells within the tumor from adapting to a lower lipid environment (Fig. 4i)" Isn't the lack of MUFA, caused by a reduced SCD activity during CR a better explanation for the toxicity to cancer cells compared to the increased levels of the toxic 16:0 SFA? In fact, in the section described in Fig. 4, the authors do focus on reduced lipid availability as an explanation for the effects of CR, but this seems to be disconnected with the statement in the abstract and the previous focus on the toxicity of 16:0 accumulation.

The Referee makes another very good point that while CR does inhibit SCD, it does not lead to the accumulation of 16:0 in tumors. Rather, it reduces tumor MUFA levels and the MUFA/SFA ratio (Figure 4a-b). To clarify, our in vitro analysis of SCD inhibition in cultured cells (Figure 3) is what

demonstrates that SCD inhibitor toxicity is maximized and leads to cell death when it is accompanied by 16:0 accumulation. Our initial statement that "fatty acid desaturation is required to dispose of toxic saturated fatty acids" was therefore intended to describe this result. Moreover, we did not intend to claim that unsaturated fatty acids are not necessary for cell proliferation, but rather that SCD inhibition does not lead to cell death unless it is accompanied by 16:0 accumulation. Indeed, as shown in Figure 3o, the fatty acid synthesis inhibitors GSK2194069 and PF-05175157 rescue cell death induced by the SCD inhibitor A939572, but do not restore maximal proliferation compared to vehicle. This is consistent with cells still needing to acquire fatty acids de novo, including unsaturated fatty acids, to optimally proliferate in lipid-limited environments. We also note that the in vitro context in which we observe SCD inhibition leading to cell death through 16:0 accumulation is lipid-depleted media, in which only trace levels of lipids remain (now quantified in Extended Data Figure 5a). While CR does lower TIF lipid levels (Figure 2e), it does not induce a completely lipid-starved environment and we agree that a decrease in MUFAs and the UFA/SFA ratio (Figure 4a-b) is a better explanation for how CR slows tumor growth. Of note, this interpretation is also consistent with CR mediating tumor growth inhibition rather than regression (Figure 1a), and CR decreasing Ki67 positivity without affecting cleaved caspase 3 staining (Extended Data Figure 3a-b).

Related to this issue, we have added an additional experiment to show that CR inhibits tumor growth in part through decreasing tumor MUFAs and the UFA/SFA ratio. We hypothesized that a high fat CR (HFCR) diet could potentially rescue the tumor growth inhibitory effects of CR by increasing lipid availability to the tumor. To test this, we formulated HFCR diets by doubling the amount of fat at the expense of further lowering carbohydrate content relative to our standard CR diet (Extended Data Table 1). Our standard CR diet consists of soybean oil, so one of our HFCR diets was made with soybean oil (HFCR-Soybean). We also formulated an HFCR diet with palm oil (HFCR-Palm), which contains large amounts of 16:0 and 18:1(n-9) (Extended Data Table 2). Interestingly, we find that only HFCR-Palm restores the levels of circulating 18:1(n-9) back to control diet levels (Extended Data Figure 14a), and subsequently also rescues tumor levels of 18:1(n-9) and the 18:1(n-9)/18:0 ratio back to control levels (Extended Data Figure 14c-d). As a result, only HFCR-Palm partially rescues tumor growth relative to the standard CR diet (Figure 4i-j). These effects are not observed with HFCR-Soybean, suggesting that distinct fat sources can have different effects on fatty acid availability to tumors. We note that these diets have even lower carbohydrate content than the standard CR diet, yet they rescue the effects of CR (or not) based solely on manipulation of lipid content. Thus, these data further support a model where shifts in the UFA/SFA ratios are driving the tumor growth inhibition observed with low glycemic diets. Lending even more support to this model, we observe that the partial rescue of tumor growth inhibition by HFCR-Palm is not observed with the LGSP lung adenocarcinoma cancer cell-derived tumors (Extended Data Figure 14f-g). In this setting, HFCR-Palm does not restore tumor levels of 18:1(n-9) and 18:1(n-9)/18:0 (Extended Data Figure 14h-i), suggesting that compared to AL1376 tumors, LGSP tumors may not be as capable at taking up relevant fatty acids (in particular 18:1(n-9)) from circulation when mice are fed HFCR-Palm.

We thank the Referee again for raising this issue, and have modified the abstract and main text to better relay these conclusions that we think are best supported by the data.

nature portfolio

Referee #3 (Remarks to the Author):

In this study by Lien et al., the authors identified that caloric restriction (CR), but not the ketogenic diet (KD), inhibits the growth of a mouse pancreatic ductal adenocarcinoma (PDAC) subcutaneous tumor allograft. Interestingly, by examining the metabolite profiles in the tumor interstitial fluid, the authors identified that mice fed the CR diet, unlike the KD, exhibit decreased environmental lipid availability, suggesting that the CR diet inhibits tumor growth through lipid limitation. Through mechanistic exploration using lipid-depleted in vitro cultures and metabolomics analysis of saturated, monounsaturated, and polyunsaturated fatty acids levels, the authors revealed that fatty acid desaturation, namely stearoyl-CoA desaturase (SCD), is necessary for cancer cell growth under lipid starvation, and specifically to inhibit the toxicity of saturated fatty acids.

Overall, I found this study to extremely well written and presented. It describes an exciting observation suggesting that diet-mediated metabolic changes can induce cancer cell adaptation to altered nutrient availability within the tumor microenvironment to enable tumor growth. The limitations in this manuscript include discrepancies in their mechanism when examined in vitro vs. in vivo, and an outstanding question as to generality of these potentially exciting findings due to the use of one type of cancer and only one cell line.

We appreciate the Referee's support for the study and thank them for their suggestions. We think that addressing their comments with additional experiments has improved the manuscript.

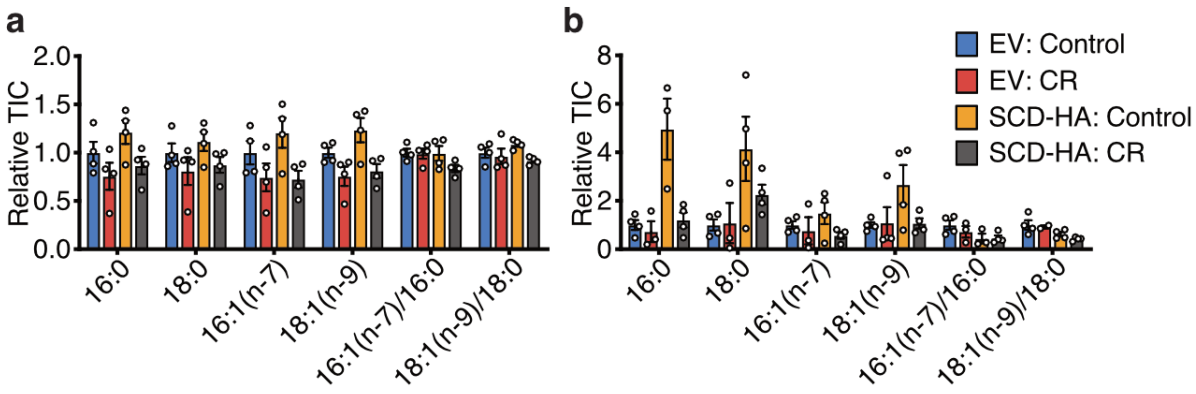
Major comments:

1. Through exhaustive efforts to identify the fatty acid composition in the tumor interstitial fluid, followed by more physiologically relevant in vitro studies to reflect the in vivo metabolite environment, the authors identified the necessity for SCD activity through the ratio of 16:1 (n-7)/16:0 (Fig. 3E-H). However, SCD-overexpressing tumors show a modest effect on desaturation (Fig. 4H), despite the lipid-deplete in vivo environment and tumor outgrowth under the CR diet. Given the known cellular heterogeneity of PDAC tumors, can the authors provide metabolic analysis of the fatty acid levels specifically in the cancer cells, instead of bulk tumors? The reviewer appreciates that this is a non-trivial request, but, to more conclusively assess the proposed mechanism, a more nuanced approach is required (e.g. cell fractionation followed by lipid analysis, tissue mass spectrometry). Additionally, can the authors provide western blots or histology of SCD in tumors from mice fed the CR, KD, and control diets? Together these data would help to clarify their mechanism and the seemingly opposing effect of SCD activity in the in vitro vs. in vivo experiments.

We agree with the Referee that it would be ideal to analyze fatty acid levels specifically in cancer cells instead of in bulk tumors, and also appreciate the Referee understanding the difficulty in carrying out this analysis. We recently published a study confirming that analysis of biomass from sorted cancer cells is feasible (PMID 32648540), and we attempted to analyze saponified fatty acids from cancer cells expressing empty vector (EV) or SCD that were sorted from AL1376 PDAC tumors in

mice fed a control or CR diet. For these experiments, the implanted cancer cells were tdTomato+ such that flow cytometry could be used to sort cancer cells (tdTomato+) from non-cancer cells (tdTomato-) and analyze the fatty acid compositions of each cell population by GC-MS.

In Referee Figure 1 below, we show levels of 16:0, 18:0, 16:1(n-7), 18:1(n-9), 16:1(n-7)/16:0, and 18:1(n-9)/18:0 measured in both the sorted cancer cells and non-cancer cells from these experiments. In bulk tumors, we previously found that CR decreases tumor levels of 16:1(n-7), 18:1(n-9), 16:1(n-7)/16:0, and 18:1(n-9)/18:0 (Figure 4a-b). Unfortunately, we do not see these differences recapitulated in either the sorted cancer cells or non-cancer cells; however, several technical issues make these fatty acid measurements different from those we present from bulk tumors in the manuscript.



Referee Figure 1. Sorted cancer and non-cancer cells from CR tumors do not exhibit robust changes in MUFA and MUFA/SFA levels. a, **b**, The indicated fatty acid levels in tdTomato^{+}CD45^{-} cancer cells (**a**) and tdTomato^{-}CD45^{-} non-cancer cells (**b**) sorted from AL1376 allograft tumors expressing empty vector (EV) or SCD-HA from C57BL/6J mice exposed to a control or CR diet. n = 4 per group; Data are presented as mean ± SEM.

First, unlike bulk tumors that are flash frozen within minutes of euthanizing a mouse, harvesting and digesting tumors into single cells followed by staining with necessary antibodies/dyes (including a live/dead stain and CD45 staining to sort out hematopoietic cells) and then sorting takes 5-6 hours. It is possible that the fatty acid composition of cells in the tumor may change over this time, during which tumors are being dissociated with various digestive enzymes, cells undergo repeated centrifugation and wash steps, and isolated single cells sit on ice in suspension while detached from a matrix. In addition, the process of sorting cells can also change metabolite levels, posing a challenge for making reliable measurements of metabolites in sorted cells (see PMID 32648540, DOI 10.7554/eLife.61980). Critically, in a recent study that systematically assesses the reliability of metabolomic profiling in sorted cells (DOI 10.7554/eLife.61980), the authors found that many lipid

species in cells change after sorting, including the fatty acids of interest in our study (16:0, 18:0, 16:1, and 18:1).

Second, 20-30 minutes of sort time is sufficient to isolate 150-250k cancer/stromal cells. While detecting fatty acids by GC-MS in this number of cells is possible, the mass spectrometry signals are weak, the peak shapes are not ideal, and the measurement is near the lower limit of detection. We also gated on live cells at the end of the sorting process, and this may eliminate the cells most stressed by the dietary intervention if they die during the isolation procedure.

Third, as we discuss in the manuscript, the partial rescue of both MUFA levels and the MUFA/SFA ratios by exogenous SCD-HA expression is not observed in large tumors (Extended Data Figure 13c-e), but it is observed in smaller tumors collected 7 days after diet initiation (Extended Data Figure 13f-h). We discuss that in addition to tumor heterogeneity, tumors that are large have variable necrotic and hypoxic regions, which may affect the oxygen-requiring SCD reaction even in control tumors and thereby affect MUFA levels. To obtain enough cells for analysis of sorted populations, we had to allow the tumors to grow to a larger size in order to have enough material given that the mass spectrometry signals were already approaching the lower limit of detection. Therefore, we could not assess fatty acid levels in sorted cells at the same time point they were assessed in bulk tumors of the same small size (7 days after diet initiation) when we observe exogenous SCD expression having an effect on bulk tumor fatty acid composition (Extended Data Figure 13f-h).

Given the technical challenges and caveats of measuring fatty acid levels specifically in cancer cells, we acknowledge that our data in Extended Data Figure 13f-h showing that SCD expression partially rescues both MUFA levels and the MUFA/SFA ratios in bulk tumors from mice fed the CR diet is currently our best evidence that SCD expression in cancer cells maintains SCD activity in these tumors. In the text, we now discuss this caveat.

Regarding the second part of the Referee's comment, we now provide Western blots for SCD in tumors from mice fed the control, CR, and KD diets in Figure 4c, Figure 4f, Extended Data Figure 10h-i, and Extended Data Figure 11h-i of the revised manuscript. As the Referee surmised, these data have further clarified the metabolic mechanisms underlying the effects of CR and the KD. We now show that both CR and the KD impair tumor SCD activity, and this result led to additional experiments showing that changing the lipid composition of these diets can alter their effects on tumor growth. Importantly, changes in tumor growth in all cases correlate with their effects on relative levels of MUFAs and SFAs in the tumor (see response to Reviewer 1 Point 2, as well as new data in Figure 4, Extended Data Figures 14-15).

2. The authors primarily rely on one PDAC cell line for their in vivo studies. To further bolster their model, additional cell lines and tissue types should be tested, and optimally in orthotopic models.

It would be curious, though not critical, to assess if the immune system is impacting the phenotype by running a parallel experiment in an immunodeficient background.

We agree with the Referee that the number of models considered in this study is limited. In the revised manuscript, we include data from repeating almost all in vivo experiments in an additional model: tumors derived from LGSP Kras(G12D)/p53-null mouse lung adenocarcinoma cells. Of note, even though this is a model of cancer that arose in a different tissue, we observe similar results as those obtained with the AL1376 PDAC model. While it is challenging to discern whether the observed effects of diet apply to all cancers, the fact that our findings are robust across two disparate models increases confidence that the mechanisms reported are not unique to a specific context. Nevertheless, we have modified the text to include less generalization. We also discuss the importance of future work to identify the characteristics that dictate whether cancer cells respond to either CR or KD diets and to SCD inhibition. Indeed, our work illustrates that tumor SCD activity can affect response to CR and the KD, and it will be interesting to explore how variation in SCD expression and regulation across different tumor models affects tumor responses to these diets.

Regarding assessment of orthotopic pancreatic cancer models, we explicitly avoided this because we observed a caloric restriction-like effect from pancreatic exocrine insufficiency that is driven by even small lesions in the pancreas (PMID 29925948), and we now discuss that tumor location may affect lipid availability and tumor responses to diet.

Finally, while we agree with the Referee that assessing whether the immune system impacts the phenotypes observed in this study would be interesting, we note that several previous studies examining how CR affects tumor growth in the literature have been conducted in immunodeficient mice and show similar anti-tumor effects (e.g. PMID 19279572, 24804677, 6278189, 27651895). We now discuss and cite this work in the revised manuscript, and would be happy to reproduce these results if the Referee and Editor feel strongly. However, we note that if any phenotypes differ in immunodeficient mice, understanding those differences would require a new line of investigation to dissect the interactions between diet and the immune system.

Minor Comments:

1. Can the authors provide western blots of SCD activity from in vivo tumors at end point vs. day 7, at which point 16:1 (n-7)/16:0 levels were examined (Fig. 4G and H).

In Extended Data Figure 13a-b, we now provide Western blots of mouse SCD protein levels from tumors at endpoint versus Day 7 that relate to the original Fig. 4g-h (now Extended Data Figure 13f-g in the revised manuscript).

2. This study suggests that CR may have dual roles on tumor metabolism, be it through nutrient

availability and/or direct inhibition of SCD activity in cancer cells, can the authors comment on the potential therapeutic benefit of SCD inhibition?

We agree with the Referee that this study suggests SCD inhibition may have a therapeutic benefit in some settings. We now include a discussion of this in the revised manuscript. In particular, we highlight two points. First, we show in Figure 3o that inhibitors of ACC or FASN rescue the toxic effects of SCD inhibition by preventing the accumulation of de novo synthesized 16:0, which may suggest that ACC/FASN inhibitors should not be combined with SCD inhibitors. This illustrates that inhibition of fatty acid metabolism at different nodes may antagonize each other, and understanding how distinct lipid metabolism pathways interact will be important for the development of cancer therapies that target lipid metabolism. Second, we recognize that CR and the KD are extreme diets and may not be feasible for some patients. Nevertheless, uncovering the metabolic mechanisms underlying these diets has revealed therapeutic strategies that may be applied through orthogonal methods. Our CR and KD-Palm experiments show that the tumor growth inhibitory effects of SCD inhibition are exacerbated by either a low-lipid environment or when accompanied by increased delivery of saturated fatty acids to the tumor microenvironment. Therefore, other diets or interventions that induce these conditions in the tumor microenvironment might be expected to improve the efficacy of SCD inhibitor-based therapy.

Referee #4 (Remarks to the Author):

Summary

The current manuscript by Lien et al. explores the interconnection between caloric restriction and control of tumor growth. By focusing on pancreatic ductal adenocarcinoma (PDAC), the authors demonstrate that caloric restriction (CR) but not a ketogenic diet (KD) in mice led to reduced volume of sc transplanted tumors as compared with control diet. Plasma metabolites of the central carbon and amino acid metabolism remained largely unchanged between the experimental groups but CR was found to lower glucose levels in the tumor interstitial fluid (TIF), which might partly explain the growth inhibitory effects of CR as demonstrated by glucose exposure experiments in cultured PDAC cells. Further, CR lowered lipid availability in vivo, and lipid starvation in vitro led to a distinct induction of intracellular 16:1(n-7) monounsaturated fatty acids (MUFA) which was explained by induction of stearoyl-CoA desaturase (SCD) activity upon exogenous lipid deprivation. In turn, pharmacological SCD inhibition in lipid-deprived PDAC cultures slowed down tumor cell proliferation, the effects of which mostly depended on the accumulation and toxicity of 16:0 saturated fatty acids (SFA) upon SCD inhibition. In vivo, CR triggered a reduction in intra-tumoral SCD activity, and overexpression of SCD in PDAC cells partially reversed the beneficial impact of CR on sc tumors, however, without changing the ratios of MUFA/SFA in response to CR. Overall, the authors conclude that CR limits lipid availability and impairs tumor SCD activity, leading to tumor growth inhibition.

General comments

As a consequence of the obesity pandemic, metabolic etiologies have gained importance for a number of tumor entities, including e.g. liver and also pancreatic cancer (PDAC). This has also sparked the interest in the development of metabolic therapies for tumor diseases and substantial efforts are currently under way to identify metabolic tumor vulnerabilities. In this respect, the current manuscript by Lien et al. addresses an interesting and clinically relevant topic in biomedical research. The study employs state-of-the-art technologies and is well written and clearly structured. While SCD is a long-known and well established contributing factor to tumorigenesis in various tumor entities, its pro-tumorigenic role is mostly believed to rely on its regulatory function for intracellular MUFA levels. This study now expands this view by suggesting that the prevention of toxic SFA levels may contribute to this pro-carcinogenic function. However, the following main issues require additional attention by the authors:

We thank the Referee for the positive comments and suggestions to improve the work. In our revised manuscript, we provide additional experiments that we hope address the Referee's major concerns and further clarify the metabolic mechanisms that contribute to how low glycemic diets impair tumor growth.

a) The main conceptual advance of this study relates to the inhibitory impact of CR on SCD activity.

However, the mechanistic explanation for this inhibitory effect by CR remains completely elusive, e.g. which endocrine/molecular pathways act upstream of SCD to confer the CR impact? It is also conceptually worrisome that the SCD overexpression/rescue experiment partially restores the growth inhibitory effects of CR but leaves fatty acid levels/ratios mostly unaffected which questions the proposed concept in general. Admittedly, technical limitations –as outlined by the authors- might be responsible for the absence of correlations between tumor growth and fatty acid ratios in vivo but still leave the study with a high degree of uncertainty with regards to causal links.

We agree with the Referee that it would be interesting to understand the mechanism by which CR impairs tumor SCD activity and have gained further insight by examining whether the KD also affects SCD levels. Specifically, we find that like CR, the KD leads to decreased tumor SCD expression (Figure 4f, Extended Data Figure 11h-i). In addition, in Figure 4d-e we show that KD tumors have lower levels of 16:1(n-7) and a reduced 16:1(n-7)/16:0 ratio, consistent with lower SCD activity. Thus, these new findings suggest that tumor SCD activity is reduced by both CR and the KD, and the two low glycemic diets may alter SCD expression through a common endocrine/molecular pathway. Of note, insulin is known to be a strong activator of SCD expression (PMID 20713121), and insulin is lowered by both CR and the KD (Figure 1g-h). Consistent with this, we confirm that insulin stimulation increases SCD levels in both the AL1376 and LGSP cancer cells evaluated in this study (Extended Data Figure 11j). Therefore, we propose that CR- and KD-mediated reduction in circulating insulin is what leads to decreased SCD expression in tumors. This hypothesis is consistent with a prior study demonstrating that tumors with PI3K pathway mutations are more resistant to the effects of CR (PMID 19279572), as PI3K mediates the signaling response downstream of insulin, and inhibition of PI3K or mTORC1 has been shown to decrease expression of SREBP target genes including SCD (PMID 20670887, 20876744).

Further consideration of why the KD does not slow tumor growth despite reduced SCD activity led to experiments that strengthen the conclusion that shifts in lipid availability in the setting of reduced SCD activity contribute to low glycemic diet-driven tumor growth inhibition. We hypothesized that KD tumors are able to grow despite impairment of SCD activity not just because the diet increases fatty acid levels in the tumor, but also because we used lard to formulate the KD, and lard consists of 18:1(n-9) as the most abundant fatty acid (see Extended Data Table 2). Indeed, the KD raises levels of 18:1(n-9) in both plasma and tumor interstitial fluid (TIF) (Figure 2e), and KD tumors maintain levels of 18:1(n-9) and the 18:1(n-9)/18:0 ratio despite reduced SCD activity (Figure 4d-e). Importantly, similar changes in fatty acid profiles suggests SCD inhibition also occurs in LGSP lung adenocarcinoma tumors in animals fed a KD, yet these tumors are also able to maintain levels of 18:1(n-9) and the 18:1(n-9)/18:0 ratio (Extended Data Figure 11e-g, i). These findings support a hypothesis where an imbalance in saturated versus unsaturated fatty acids in the setting of low tumor SCD activity tracks with whether low glycemic diets slow tumor growth. These observations also led to additional experiments that further support the model in which we manipulate both SCD activity and fatty acid delivery to tumors in animals fed either CR or the KD.

First, we now show that a high fat CR (HFCR) diet can partially rescue CR tumor growth by increasing circulating lipids and raising monounsaturated fatty acid levels in tumors. Specifically, we tested whether a HFCR diet could increase lipid availability to the tumor. To do this, we formulated a HFCR diet by doubling the amount of fat at the expense of further lowering carbohydrate content relative to our standard CR diet (Extended Data Table 1). Our standard CR diet consists of soybean oil, so one of our HFCR diets was made with soybean oil (HFCR-Soybean). We also formulated a HFCR diet with palm oil (HFCR-Palm), which contains large amounts of both 16:0 and 18:1(n-9) fatty acids (Extended Data Table 2). Interestingly, we find that only HFCR-Palm is able to restore the levels of circulating 16:0 and 18:1(n-9) back to the control diet levels (Extended Data Figure 14a), which subsequently also rescues tumor levels of 16:0, 18:1(n-9), and the 18:1(n-9)/18:0 ratio back to control levels (Extended Data Figure 14c-d). Despite the HFCR diets having even lower levels of carbohydrates, HFCR-Palm rescues tumor growth relative to the standard CR diet (Figure 4i-j), which is consistent with the model that the MUFA/SFA ratios track with the effects of the studied diets on tumor growth. Importantly, these effects are not observed with HFCR-Soybean, which does not rescue 18:1(n-9) levels or the 18:1(n-9)/18:0 ratio, and illustrates how different dietary fat sources can have distinct effects on fatty acid availability in tumors to influence responses to CR.

Second, as described above, the KD also impairs tumor SCD activity; however, this is buffered by the KD raising 18:1(n-9) lipid availability to maintain the 18:1(n-9)/18:0 ratio in tumors. While lowering fat content is not possible with the KD, given our in vitro results that the toxicity of SCD inhibition is maximized when accompanied by the accumulation of toxic saturated fatty acids, we next asked whether increasing saturated fatty acid content in the KD would synergize with KD-mediated tumor SCD inhibition to impair tumor growth. To accomplish this, we formulated a KD with palm oil (KD-Palm), which consists of 16:0 as the most abundant fatty acid (Extended Data Table 2). Relative to the lard-based KD, KD-Palm contains ~2x more 16:0 while maintaining similar levels of 18:1(n-9) (Extended Data Table 2). Accordingly, KD-Palm increases circulating levels of saturated fatty acids (Extended Data Figure 15b) and increases saturated fatty acid levels in tumors (Extended Data Figure 15e). Importantly, and again consistent with the model, KD-Palm inhibits tumor growth, and this is rescued by exogenous SCD expression in cancer cells (Figure 4k-l).

With respect to the data mentioned by the Referee where SCD expression partially rescues the growth inhibitory effects of CR, we agree that technical limitations as well as biological factors likely contribute to why MUFA levels and the MUFA/SFA ratios in SCD expressing tumors are similar to control tumors at late time points when the harvested tumors are large (Extended Data Figure 13c-e). We remind the Referee that the data presented in Extended Data Figure 13f-h shows that SCD expression partially rescues both MUFA levels and the MUFA/SFA ratios in smaller CR tumors. Importantly, we note that SCD expression most robustly rescues 18:1(n-9) levels and the 18:1(n-9)/18:0 ratio in these CR tumors (Extended Data Figure 13f-g), reinforcing the model that the low glycemic diets that maintain tumor levels of 18:1(n-9) and 18:1(n-9)/18:0, including KD-Lard and HFCR-Palm described above, are not as effective at slowing tumor growth. Nevertheless, in the revised manuscript, we discuss in detail the potential reasons for why the partial rescue of MUFA/SFA ratios and MUFA levels is only observed in smaller tumors. Large tumors are well

described to have variable hypoxic/necrotic regions, which impairs the oxygen-requiring SCD reaction and makes cells dependent on exogenous lipid uptake (PMID 23671091, 30184495, 23699409). Therefore, exogenously expressed SCD may be better able to maintain activity in smaller tumors to promote resistance to CR, while this differential effect compared to the empty vector control is lost in large tumors where other factors such as oxygen limitation may also inhibit SCD activity. This interpretation is consistent with the observed partial rescue of MUFA/SFA ratios and MUFA levels in smaller, but not larger, tumors expressing exogenous SCD (Extended Data Figure 13).

Taken together, we think that the new data discussed above strengthens the conclusion that a mismatch between tumor fatty acid desaturation activity and the availability of specific fatty acid species in the tumor microenvironment is a mechanism by which low glycemic diets impair tumor growth. Importantly, we can influence the effects of both CR and the KD in a predictable way via manipulating either tumor SCD expression or the dietary fat composition. This lends confidence to a causal link between how the diets affect lipid availability in the tumor in the setting of reduced SCD activity as an explanation for why low glycemic diets do or do not decrease tumor growth. We explicitly illustrate this model in Extended Data Figure 16 of the revised manuscript.

b) Given the significant differences between male and female energy homeostasis, it seems inappropriate to pool male and female mice into combined experimental groups as done in the initial tumor implantation experiments. This holds particularly true for the fact that male and female body weight development is quite different upon CR (Ex Fig 1), and the author's statement that both CR and KD resulted in reduced body weights is only partly true. In fact, these in vivo experiments have to be repeated by appropriate numbers of male and female mice in separate cohorts to make proper conclusions about normalized tumor volume developments etc. In its current form, all conclusions about the metabolic status of experimental animals are flawed by the mixed male/female composition of the corresponding experimental cohorts (e.g. how do tumor curves, glucose levels lipid ratios etc look in the male KD cohort alone? How would the comparison between female CR vs KD look like?).

The Referee raises a valid point that differences between male and female energy homeostasis in mice may lead to sex-specific differences in tumor responses to CR and the KD. In consideration of this, we now present data in male and female cohorts separately throughout the revised manuscript. In Figure 1, Extended Data Figure 1, and Extended Data Figure 2, we carry out an extensive comparison of dietary effects in male versus female mice across two different tumor models. In all cases, we do not find sex-specific differences in tumor responses to CR or the KD. In both male and female mice, CR inhibits subcutaneous tumor allograft growth, while tumor growth is unaffected by the KD (Figure 1, Extended Data Figure 1). In Extended Data Figure 2, we show that both male and female mice lose weight under CR, whereas under the KD both male and female mice maintain their body weights over time. We conclude that even though differences in energy homeostasis between male and female mice do exist, we do not observe sex-dependent differences in tumor growth in response to CR or KD in the context of the diets and tumor models examined in this study.

c) The study will benefit from the inclusion of some human data to strengthen the overall relevance for the human disease. One would expect that e.g. cachectic (i.e. calorically restricted) pancreatic cancer patients show diminished SCD activity as compared to non-cachectic counterparts.

We agree with the Referee that human data would speak to the translatability of these findings. Although examining cachexia is an intriguing idea, we note that cachectic patients are not necessarily calorically restricted because there are many factors (both endocrine and dietary) that can contribute to the cachectic phenotype, and the contributions of these factors may differ across different cancer contexts. However, we now include some relevant human data that may speak to some findings of our study. By analyzing associations between dietary patterns and survival time among 1,165 pancreatic cancer patients in two large prospective cohorts (the Nurses' Health Study and Health Professionals Follow-up Study), we find that consumption of a dietary pattern lower in carbohydrates and higher in fat and protein was associated with longer survival time (Extended Data Table 3). Interestingly, this association was modestly stronger for low-carbohydrate dietary patterns in which the fat and protein components were plant-based instead of animal-based (Extended Data Table 3). Moreover, diets consisting of more fat, as opposed to protein, were also associated with longer survival times, and this association was again more pronounced for plant-based fats rather than animal fats (Extended Data Table 4). A major difference between plant- and animal-based fat is the degree of fatty acid saturation, with plant-based diets containing more unsaturated fats (PMID 10479224). Thus, although these data are correlations between dietary patterns and pancreatic cancer survival, the associations are consistent with the conclusions of our study that low carbohydrate diets can affect tumor growth by impacting lipid metabolism, and changing the fat content of these diets may have different effects.

Further specific comments are listed below:

Specific comments

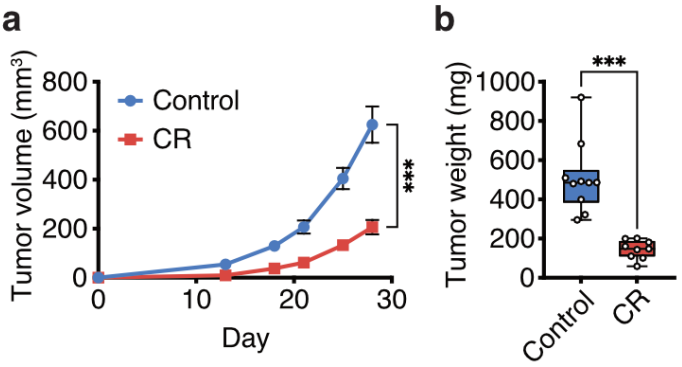
1. The study should be strengthened by a second, independent tumor model. SCD has been shown to trigger liver cancer growth. Do you find the same lipidomic alterations in hepatic tumors upon CR?

We agree with the Referee that the study would be strengthened by a second independent tumor model, and we appreciate that SCD has been shown to be important in multiple cancer types, including liver cancer. However, SCD is also important in lung cancers (e.g. PMID 18813799, 28368399), and we had ready access to a syngeneic mouse allograft lung cancer model involving Kras(G12D) and p53-null LGSP lung adenocarcinoma cells. Thus, we focused on this model and repeated almost all in vivo experiments using the LGSP model. Of note, we observe similar results across most diets tested, with no data contradicting the model suggested by our experiments involving PDAC tumors (Extended Data Figure 1,10-15).

We appreciate that including an additional model does not speak to all cancers, and thus have also modified the text to avoid overgeneralization. Finally, we discuss the importance of future work to identify the characteristics that can dictate how cancer cells respond both to CR and KD diets and to SCD inhibition. Indeed, our work illustrates that tumor SCD activity can affect response to CR and the KD, and it will be interesting to explore how variation in SCD expression and regulation across different tumor models, including hepatic tumors, affects tumor responses to these diets.

2. Given the current interest in fasting regimes for disease prevention, does CR also prevent PDAC growth in vivo?

We agree with the Referee that there is currently interest in fasting regimes for cancer prevention, and indeed many prior studies that have demonstrated tumor growth inhibition by CR have initiated the diet either prior to, or at the time of, tumor implantation (e.g. see Refs. 10, 13, 82, 84 of the revised manuscript). In fact, past work from our group has argued that CR from pancreatic exocrine insufficiency can delay progression of precancerous lesions in mouse PDAC models (PMID 29925948). In this current study, we intentionally initiated the diets after palpable tumors formed (~10 days after tumor implantation) in order to study the effects of these diets on tumor growth (as opposed to initiation). Nevertheless, we show below in Referee Figure 2 that mice exposed to a CR diet 7 days prior to tumor implantation can still form PDAC tumors, but tumor growth is similarly inhibited. These data are consistent with prior studies, so may not be appropriate to include here. Nevertheless, we are happy to include these data in a revised manuscript if the Referee or Editor feel strongly.



Referee Figure 2. Caloric restriction initiated before tumor implantation inhibits the growth of pancreatic allograft tumors. a, **b**, Tumor volumes (**a**) and tumor weights (**b**) of subcutaneous AL1376 pancreatic ductal adenocarcinoma allografts implanted in female C57BL/6J mice that were exposed to a control or CR diet for 7 days prior to tumor implantation. Control n = 5 mice, CR n = 5 mice. Data are presented as mean ± SEM (**a**) or as box-and-whisker plots displaying mean and interquartile ranges (**b**). Comparisons were made using two-way repeated measures analysis of variance (ANOVA) (**a**) or a two-tailed Student's t-test (**b**). ***P < 0.001.

3. Fig. 1: please provide data on the overall body composition upon CR and KD in male and female mice.

In Extended Data Figure 2d,k, we now report changes to gastrocnemius muscle and white adipose tissue (WAT) weight in male and female mice fed a CR or KD diet compared to control. The weights of these tissues are presented normalized to the mouse body weight, and neither the CR diet nor the KD used leads to significant changes in gastrocnemius muscle weight. In contrast, CR strongly reduces WAT weight, while WAT weights are maintained in mice fed the KD. This observation is consistent with our conclusion from Figure 2e that unlike the KD, CR lowers circulating lipid availability. This is also consistent with other studies of CR in aging, which show loss of WAT as a prominent phenotype (e.g. PMID 22414572, 27568549).

4. Fig. 2c: It is generally difficult to translate in vivo parameters into appropriate cell culture conditions. Did you consider other fasting/CR mediators in your cell culture conditions, e.g. glucagon, glucocorticoids, FGF21 beyond the glucose? Why would the KD have a different impact on TIF glucose than CR? Please discuss.

We agree with the Referee that hormone signaling can play an important role in the effects of CR and the KD on tumor growth, as has been previously explored (e.g. PMID 30051890, 19279572, 24804677). We took care to acknowledge this in the text, and now also show the effects of CR and the KD on plasma glucagon, FGF21, and corticosterone levels in Extended Data Figure 2e-g, l-n, in addition to confirming the effects of these diets on insulin in Figure 1g-h. Indeed, there are CR-specific effects on FGF21 and corticosterone; however, we note that the focus of this study is to explore how these diets change metabolite levels in the tumor microenvironment to influence tumor metabolism and growth. This does not rule out that changes in hormone signaling induced by these diets might also contribute to differential effects, and we now discuss this more explicitly in the revised manuscript.

Regarding the question about differing effects of the diets on glucose, it is not entirely clear why the KD has a different impact on TIF glucose compared to CR (Figure 2a-b), although we note in Figure 2a that there is a fair amount of variability in TIF glucose concentrations in mice fed a control diet. We speculate that these differences could be due to differences in tumor vascularization (e.g. PMID 12085212) or in tumor cell glucose uptake between CR versus the KD, and we now discuss these possibilities in the revised manuscript.

5. Fig. 2d: How do the PDAC cells respond to b-OHB in vitro? Does enhanced b-OHB exposure alter the lipidomics profile within the cells?

We agree with the Referee that β-OHB metabolism of cancer cells is an interesting area, especially since work by others has suggested that the metabolism of ketones may contribute to why the KD may not inhibit tumor growth in some cancer models (PMID 29414764). To consider this point further, we now show in Extended Data Figure 4 how β-OHB affects the PDAC cells used in the study. First, we report absolute quantification of β-OHB levels in plasma and TIF in mice fed the CR or KD diets (Extended Data Figure 4a). As also shown in Figure 2d, we find that while both CR and the KD raise β-OHB levels in the TIF, the KD is more effective at doing so. Next, we cultured AL1376 PDAC cells with β-OHB and find that levels found in tumors do not affect cell proliferation (Extended Data Figure 4b). Interestingly, culturing PDAC cells with [U-^{13}C]-β-OHB results in labeling of TCA cycle metabolites (Extended Data Figure 4c), consistent with the idea that some cancer cells can metabolize β-OHB, and this could support tumor growth under the KD. Since β-OHB can be a source of lipogenic acetyl-CoA, these cells also incorporate β-OHB carbon into fatty acids (Extended Data Figure 4d), and we find β-OHB exposure can alter the fatty acid composition of the PDAC cells (Extended Data Figure 4e-f). Interestingly, we find that β-OHB decreases the levels of 16:1(n-7) and the 16:1(n-7)/16:0 ratio, which is similar to the effects of the KD on tumor fatty acid composition (Figure 4d-e). Together, this suggests that β-OHB metabolism may influence the effects of the KD on tumor metabolism and growth as suggested by other work.

6. Fig. Ex4a-e: Except for Panc1, pyruvate itself has no effect on tumor cell doubling times. How do you ensure that "functional" pyruvate levels have been used in these experiments? Conclusions are based on negative data.

We agree with the Referee that a positive control for pyruvate supplementation would be useful for interpreting Extended Data Figure 7. We note that in a previous study (PMID 27746050), 1 mM pyruvate is sufficient to rescue mitochondrial inhibition across multiple cell lines. We now show in Extended Data Figure 7a that 1 mM pyruvate rescues mitochondrial complex I inhibition by rotenone in all of the cells used in this study.

7. Fig. 3m: Please show vehicle and inhibitor groups in separate bar graphs, respectively.

We have changed Figure 3m to show vehicle and inhibitor groups in separate bar graphs.

8. Fig. 3: The text jumps back and forth between the different figure panels which makes it difficult to follow. Please consider re-arrangement.

We have reformatted Figure 3i-m to make it more clear which fatty acids are being supplemented in the media, and which fatty acids are being measured in each graph. In the text, we describe Figure 3i-m as a block, describing the results in the order of each fatty acid being supplemented in the media. We hope that these rearrangements make it easier to follow the panels in Figure 3.

9. Fig. 3n, o: How does FASN inhibition by 18:1(n-9) occur? What is the proposed mechanism of interaction between the FAS and SCD pathways? Do SFA accumulate because SCD is inhibited or does SCD activity somehow depend on the activity of FAS and ACC? This part is somewhat confusing and should be clarified beyond the rather vague statement in the current text.

We propose in the text that exogenous 18:1(n-9) and 18:2(n-6) addition may block SREBP activation when cells are grown in lipid-depleted media, resulting in the downregulation of de novo fatty acid synthesis. In Extended Data Figure 5e, we now assess levels of the mature active form of SREBP1 (m-SREBP1). As expected, cells growing in lipid-depleted media have higher levels of m-SREBP1, and this is impaired by the addition of either 18:1(n-9) or 18:2(n-6) to the media. Notably, levels of m-SREBP1 remain high in lipid-depleted media even when cells are treated with an SCD inhibitor (Extended Data Figure 5e). This is consistent with the description in the text that 16:0 accumulates because SCD is inhibited by A939572 while de novo fatty acid synthesis remains active, and we more clearly describe these results in the revised manuscript.

10. Fig. 4c: This WB is not convincing and also in this experiment, male and female mice seem to be mixed in the experimental groups (see general comments above). Please quantify WG and indicate male/female samples. Please also provide data on SCD expression levels in KD-exposed tumors.

For the control versus CR experiments, we now provide Western blots for SCD, accompanied with quantification, for tumors from male mice (Figure 4c) and female mice (Extended Data Figure 10h). We also provide SCD expression data from control versus CR LGSP tumors from male mice in Extended Data Figure 10i. For control versus KD experiments, we also provide Western blots of SCD expression in tumors from male mice (Figure 4f), female mice (Extended Data Figure 11h), and LGSP tumors from male mice (Extended Data Figure 11i). Although there is some heterogeneity from tumor to tumor across these conditions, we consistently observe decreased SCD protein levels in the majority of tumors analyzed from mice exposed to CR or the KD.

11. Fig. 4e: Please provide data on food intake and split into male/female cohorts.

We now provide the data from the original Figure 4e in male and female cohorts in Figure 4g-h and Extended Data Figure 12a-f of the revised manuscript. We also provide data on food intake in Extended Data Figure 12g-h.

12. Fig. Ex7: It is difficult to use data from day 7 as a proof of SCD activity induction because at this stage there is still no difference in tumor volumes across groups. If SCD activity was not different

after 20 days (Fig. Ex 7) but changed after 7 days (with no tumor size alterations yet), does this mean that SCD loses importance during tumor development, at least with respect to the proposed concept?

While tumors from Day 7 are smaller, at this stage there is still the expected difference in tumor volumes across groups (please zoom in to see Day 7 data points in Figure 4g, also see Day 11 data points in Figure 4g): the EV CR tumors are slightly smaller than the EV control tumors, whereas there is no difference between the SCD-HA control and CR tumors. Even though this difference in absolute tumor volumes may be muted compared to the later times, we note that the rate of tumor growth at Day 7 would still be different across groups to result in the phenotype that is more clearly observed as the tumors grow larger. We propose that these differences in tumor growth rates over the entire course of the experiment is due in part to the enforced expression of SCD activity, which we captured at one time point on Day 7.

With respect to whether SCD loses importance during tumor development as tumors get larger, we acknowledge this possibility. In particular, we note that large tumors are well described to have variable hypoxic/necrotic regions, which impairs the oxygen-requiring SCD reaction and inhibits MUFA production in those poorly vascularized regions of the tumor (PMID 23671091, 30184495, 23699409). In fact, in Figure 4g and Extended Data Figure 12c, we observe that SCD-HA CR tumors do not begin to diverge from the SCD-HA control tumors until ~Day 15, whereas EV CR tumors begin to diverge from the EV control tumors as early as Day 7. It is therefore possible that before ~Day 15, exogenously expressed SCD is able to better maintain activity in smaller tumors to promote resistance to CR, and this effect is weakened in the larger tumors when other factors such as oxygen limitation may inhibit SCD activity. This is consistent with the observed partial rescue of MUFA/SFA ratios and MUFA levels in smaller tumors expressing exogenous SCD harvested on Day 7, as opposed to the lack of rescue in large tumors harvested on Day 18. We discuss these possibilities in the revised manuscript, and we note that while other factors such as tumor size, necrosis, and hypoxia may affect SCD activity, the data obtained from two models and multiple dietary interventions are all consistent with diet-mediated impairment of SCD combined with altered lipid availability in the tumor microenvironment contributing to how low glycemic diets inhibit tumor growth.

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

The authors have added significant amounts of new data. In my opinion their in vivo data on the different diets are very important and should be published. However, I am not convinced that the in vitro determined mechanism is one to one translatable to the in vivo situation. For example, in vitro 18:2(n-6) supplementation rescues SCD inhibition (line 237ff), while in vivo increasing 18:2(n-6) through soybean diet did not rescue tumor proliferation in CR condition (line 377ff). Thus, while the fatty acid levels enforced by the diet may certainly be important for their effects on

tumor growth the mechanism proposed through SCD regulation, SREBP1 feedback on FASN and palmitate induced lipotoxicity may not or only to a certain extent be relevant in vivo. It is also not clear to me how the human data from the Nurse study are mechanistically supportive of this study. Finally, studies suggesting negative effects of SFA (such as induction of pro-metastatic CD36 or negative effects on coronary diseases) should be mentioned to avoid a misunderstanding of the here shown data by the lay public.

Specific comments

Line 48: "and low glycemic diets are often presumed to inhibit tumor growth in part by lowering blood glucose levels7–9,11." Please add the link to insulin to avoid the common misunderstanding that tumors can be starved of glucose because of glucose restriction (blood glucose is tightly maintained at the lower bound due to gluconeogenesis).

Line 250 "16:0 has no effect on MUFA levels or the MUFA/SFA ratios, while still leading to increased levels of 16:0 (Fig. 3i-m, Extended Data Fig. 9c-g)." This statement is confusing because if MUFA don't change and MUFA/SFA don't change how can palmitate change?

For Figure ED4D please also show uneven MIDs

Line 296 the "that" is doubled ("activity in tumors, and consistently we find that that the majority")

Referee #2 (Remarks to the Author):

I have now had a chance to go through the responses to my concerns and questions and I'm satisfied with the additional experiments performed and the changes in the text.

Referee #3 (Remarks to the Author):

In this revised manuscript by Lien, et al., the authors build upon their earlier findings and present a technically intensive mechanistic study on the role of fatty acid metabolism and diet in cancer therapy. The mechanistic aspects of the study are extremely well done and provide novel insights into the regulation of fatty acid biosynthesis/availability, and these have demonstrated relevance to diet and tumor growth, an area of considerable growing interest. I would like to congratulate the authors on a thorough study, their exciting findings, and the clear presentation of this work.

My previous major concerns were first on discrepancies in the mechanistic aspects of the work and second on the generality of these findings. Regarding the former, I am impressed and pleased to see how this aspect of the work evolved. The new data support a role of fatty acid availability, insulin-mediated regulation of SCD expression, and desaturation as major mediators of tumor growth in vivo. They describe a new axis and demonstrate therapeutic vulnerabilities that could form the basis of future work. I was disappointed to find that my suggestion to profile tumor cell type-specific lipid profiles was unsuccessful, expecting that lipids would be more "stable" during the sorting/analysis. I appreciate the considerable efforts by the authors and suggest that these data be included in the Extended Data, as like me, other scientists may have the same misconceptions.

Second, as to the generality, the inclusion of a KP lung cell line, and the consistency between

models, is a welcome addition. I would have certainly preferred orthotopic analyses, but I appreciate the counter-points made by the authors. Perhaps a breast or brain cancer model would have been more appropriate / easier to model? The point with regard to immunocompromised studies is also well taken.

Finally, given the limitations noted above, I also thought the authors did a good job of tempering their findings in the abstract and discussion. I applaud their restraint.

Referee #4 (Remarks to the Author):

The authors have added a significant amount of new data and substantially improved the manuscript. All previous main concerns have been addressed.

Author Rebuttals to First Revision:

Response to Referee #1 Comments

Referee #1 (Remarks to the Author):

The authors have added significant amounts of new data. In my opinion their in vivo data on the different diets are very important and should be published.

We thank the Referee for the positive comments and for their appreciation of the new data that was added to the revised manuscript.

However, I am not convinced that the in vitro determined mechanism is one to one translatable to the in vivo situation. For example, in vitro 18:2(n-6) supplementation rescues SCD inhibition (line 237ff), while in vivo increasing 18:2(n-6) through soybean diet did not rescue tumor proliferation in CR condition (line 377ff). Thus, while the fatty acid levels enforced by the diet may certainly be important for their effects on tumor growth the mechanism proposed through SCD regulation, SREBP1 feedback on FASN and palmitate induced lipotoxicity may not or only to a certain extent be relevant in vivo.

The comment that the mechanisms associated with SCD inhibition in lipid-depleted tissue culture media do not all translate exactly to explain the full effects of each diet on tumors is a point well taken, and we appreciate the Referee's assertion that we should be more clear about how conclusions from the in vitro experiments helped us understand the effects of diet on tumors. Lipid-depleted tissue culture media is an admittedly imperfect model because dietary interventions cannot induce a completely lipid-starved environment in tissues. Nevertheless, modeling the extreme case to investigate how cancer cells adapt to low lipid environments helped reveal an imbalance in tumor levels of saturated versus unsaturated fatty acids as a contributor to the effects of low glycemic diets on tumor growth.

We agree with the Referee that palmitate-induced lipotoxicity per se does not entirely explain the effects of CR on tumors. The new data presented in the revised manuscript argued instead for a model where diet-induced changes in lipid availability in the setting of low SCD expression disrupts the balance of saturated versus unsaturated fatty acids when low glycemic diets slow tumor growth. In a revised manuscript, we will more explicitly discuss how the in vitro findings informed possible mechanisms that were tested in the in vivo experiments. This includes summarizing the main conclusions drawn from our in vitro experiments: (1) When deprived of extracellular lipids, up-regulation of SCD activity is important to maintain a balanced ratio of unsaturated to saturated fatty acids and support cell proliferation; and (2) the toxicity of SCD inhibition is maximized and leads to cell death when it is accompanied by the accumulation of toxic saturated fatty acids. Since CR does not induce SFA accumulation in tumors, we agree completely that palmitate-induced lipotoxicity does not explain why CR slows tumor growth. However, whether a CR diet induces an imbalance in the ratio of unsaturated to saturated fatty acids within tumors does correlate with whether that diet affects tumor growth in all cases studied. In the case of the KD, we also only observe

tumor growth inhibition with versions of the diet that perturb relative levels of saturated and unsaturated fatty acids in tumors. Of note, our in vitro observations motivated testing of the SFA-enriched KD-Palm Oil diet to leverage the effects of KD-mediated SCD inhibition and elicit tumor growth inhibition, where an increase in SFA might be more relevant.

We also agree with the Referee's specific example that although 18:2(n-6) rescues SCD inhibition in vitro, the HFCR-Soybean diet does not rescue CR-mediated tumor growth inhibition. However, we note that while the HFCR-Soybean diet is enriched in 18:2(n-6) (ED Table 2), it does not raise levels of 18:2(n-6) in circulation (ED Figure 14b), and circulating MUFA levels are lower in mice exposed to the HFCR-Soybean diet (ED Figure 14a). In contrast, the HFCR-Palm diet does raise 18:1(n-9) MUFA levels and rescues CR-mediated tumor growth despite having even lower levels of carbohydrates. These data are consistent with a model where HFCR-Soybean is unable to rescue tumor growth because unlike HFCR-Palm, HFCR-Soybean cannot restore the balance of unsaturated (either MUFAs or PUFAs) to saturated fatty acids in tumors. That 18:2(n-6)-enriched HFCR-Soybean does not raise circulating 18:2(n-6) levels highlights the complexity of diet studies by showing that raising levels of a nutrient in the diet may not lead to increases in systemic availability. It also argues for the value of future studies to better understand the mechanisms by which different dietary fat compositions do or do not change systemic or tumor fatty acid availability, and we will make these points more explicitly in a revised manuscript.

It is also not clear to me how the human data from the Nurse study are mechanistically supportive of this study.

We agree with the Referee that the human cohort data are suggestive, but do not prove a mechanism by which low glycemic diets affect tumor growth in patients. Nevertheless, these data were added because the correlative human data do support the overall idea that low glycemic diets can mediate their effects on tumor growth by altering lipid metabolism. A major difference between plant- and animal-based fats in low glycemic diets is saturated versus unsaturated fatty acid content. We also hope that these findings will inspire better collection of dietary data from patients. In the revised manuscript, we will expand the discussion to stress that future investigation is needed to understand how different dietary fat compositions affect systemic lipid availability, tumor lipid metabolism, and tumor growth in patients.

Finally, studies suggesting negative effects of SFA (such as induction of pro-metastatic CD36 or negative effects on coronary diseases) should be mentioned to avoid a misunderstanding of the here shown data by the lay public.

The Referee makes an excellent point, and we agree that citation and discussion of these studies is warranted to avoid misunderstanding by the lay public. We will include this discussion in a revised manuscript.

Specific comments

Line 48: “and low glycemic diets are often presumed to inhibit tumor growth in part by lowering blood glucose levels7–9,11.” Please add the link to insulin to avoid the common misunderstanding that tumors can be starved of glucose because of glucose restriction (blood glucose is tightly maintained at the lower bound due to gluconeogenesis).

We agree that the idea tumors can be starved of glucose is a common misunderstanding for the reasons stated by the Referee. We will better discuss the link to insulin along with appropriate references in a revised manuscript.

Line 250 “16:0 has no effect on MUFA levels or the MUFA/SFA ratios, while still leading to increased levels of 16:0 (Fig. 3i-m, Extended Data Fig. 9c-g).” This statement is confusing because if MUFA don’t change and MUFA/SFA don’t change how can palmitate change?

We thank the Referee for asking us to be more precise with this statement. We will modify the sentence to read: “16:0 supplementation does not prevent decreases in MUFA levels, decreases in the MUFA/SFA ratios, or the accumulation of 16:0 that is observed with SCD inhibition.”

For Figure ED4D please also show uneven MIDs

We will show the uneven MIDs in ED Figure 4D in a revised manuscript.

Line 296 the “that” is doubled (“activity in tumors, and consistently we find that that the majority”)

We will remove the extra “that”, and thank the Referee for catching this typo.

Reviewer Reports on the Second Revision:

Referee #1 (Remarks to the Author):

If the authors revise their manuscript in accordance with the plans explained in the response letter, I am supportive of publishing this manuscript in Nature.