Wnt Signaling Directs a Metabolic Program of Glycolysis and Angiogenesis in Colon Cancer

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Editor: Thomas Schwarz-Romond

1st Editorial Decision 24 June 2013

Thank you very much for submitting your study on a Wnt-driven metabolic program for consideration to The EMBO Journal editorial office. Having received two very consistent assessments, I am in a position to reach a final decision.

As you will recognize, both judge the major message as relevant and of potential interest for our general audience. They take however significant issues with the current amount of experimental support for this proposal, particularly in light of some conceptual similarity with the recent study on metabolic responses upon Wnt/Lrp signals triggered during osteoblast differentiation (Cell Metab. May 2013). The aim of significant further revisions should thus be to substantiate and therefore generalize this novel role of Wnts also in the context of CRCs.

From the editorial perspective, it would be crucial to:

- extend the analysis to additional colon cancer cells, with signal inhibition at multiple level in the cascade
- complement the dominant-negative approach with shRNA (or other means of) depletion
- corroborate the Wnt-dependent glycolytic gene signature also with expression data from actual
patient tumor samples
- mechanistically assess a potential engagement of mTOR-signaling respective directness of PDK1-
regulation by Tcf-family members
- complement the interesting imaging approach with a metabolic analysis on glycolysis/OXPHOS.
Conditioned on thus relatively significant amendments, we would be delighted to assess a
thoroughly revised study for publication in The EMBO Journal. I realize that these are demanding
and time-consuming experiments.

We are therefore ready to offer time beyond our usual three months revision period, if required.
Please do not hesitate to get in touch in case of further questions (preferably via E-mail).

Please me reminded that The EMBO Journal considers only one round of revisions and the ultimate
decision on publication will depend on the outline and strength of the revised manuscript.

I am very much looking forward to your revised dataset and remain with best regards.

REFEREE REPORTS:

Referee #1:

Pate et al. report that Wnt signaling apparently controls glycolysis and angiogenesis in colon cancer
cell lines and xenografts in nude mice. Initial gene expression analysis as well as previous work
from the same lab pointed that glycolysis (and genes encoding for metabolic enzymes of this
pathway) seems to correlate with Wnt activation (mainly using dominant negative LEF/TCF). The
main assay to assess the predominance of glycolysis vs oxidative phosphorylation was a
fluorescence technique (Fluorescence Life time Imaging) that is able to distinguish free and bound
NADH. This technique was used in cultured cells as well as in xenografts. The authors argue that
the downstream mediator of the observed glycolytic phenomenon is the PDH kinase PDK1, and this
was tested using pharmacological inhibition and overexpression systems. Overall, this manuscript
reports some interesting observations, however the data is too preliminary and would need to require
extensive work to support the conclusions raised by the authors.

1- The novelty of the work is somewhat touched by the recent manuscript in Cell Metabolism,
reporting the same phenomena in osteoblast differentiation. It might be that the mechanism is
different. Thus, the authors should address if in colon cancer cells if mTORC2 could also play a
role, or the mechanism is different. In addition, the authors have also previously report that Wnt
signaling correlates with glycolysis, though the mechanisms were not addressed.

2- The work is to some extent narrow in the number of colon cancer cells used. The authors should
use additional human colon cancer cell lines and inhibit Wnt signaling at different points. Most of
the work is based in the use of dominant negative, complementary analysis using shRNAs of several
components of the Wnt signaling should be applied.

3- Along the same lines the correlation between Wnt signaling and glycolytic gene expression
should also be analyzed from gene expression data from colon tumors, is any correlation between
these two pathways at the gene expression level. This is critically to show for the relevance of these
observations within the context of cancer biology.

4- The experiments to support that PDK1 mediates the effects of Wnt signaling and glycolysis is
problematic. First, the use of DCA should be tested when PDK1 is knock-down to assess the
specificity of DCA which is known to have many off-target effects
(http://jpet.aspetjournals.org/content/324/3/1163.full). Overexpression should be performed also
with inactive PDK1 enzymes. In the xenograft experiments the DCA data should be taken into
caution, and again shRNA inducible against PDK1 should be used using several colon cancer cell
lines.

5- Along the previous point, since the novelty of this manuscript is compromised, the authors should
include more insights into mechanisms by which Wnt controls PDK1. Is the mechanism through
direct promoter binding of TCF4? Chip analysis and reporter assays should be included.

6- Another critical point relates to the metabolic and energetic characterization. Most of this analysis
rely on the Fluorescence Life time Imaging, that would need to be complemented with more direct
analysis of glycolysis and oxidative phosphorylation. Thus, a complete metabolic analysis from the
cell lines and xenografts should be performed to assess direct respiration and oxygen consumption.
as well as glucose oxidation. Isotope labeling and metabolomic analysis should be also use to support their conclusions.

Referee #2:

Pate et al "Wnt Signaling Directs a Metabolic Program of Glycolysis and Angiogenesis in Colon Cancer"

In their manuscript the authors examine gene expression changes in response to β-catenin blockade, and investigate the significance of the regulation of genes involved in metabolism. The authors use an interesting pair of DN binding partners that can inhibit much of the transactivation of β-catenin without affecting the growth of the tumor cells (taking the growth rate out of the equation). They determine that the PDHK1 gene is downregulated during β-catenin blockade, and so infer that PDHK1 is induced upon Wnt signaling, making cells more glycolytic (it would be nice to see if this were the case). While this is an interesting finding and a reasonable series of experiments, it relies heavily upon the analysis of NADH autofluorescence. The metabolic analysis is therefore somewhat incomplete, as many things can change the redox balance within the cell. In addition, it is not clear what effects this metabolic change has on the behavior of tumor cells. The topic is interesting and the experiments well done as far as they go, but there are several gaps that need to be filled in.

Major points

1. What is the magnitude of the gene expression changes shown in figure 1? No quantitation is given, so it is difficult to evaluate how significant the changes are, or why the list of specific genes is shown.
2. Figure 2 is basically a negative result, and while it is important for the interpretation of the data could probably be in the supplemental figures.
3. The authors need a more thorough analysis of the metabolic changes that they describe. The changes in NADH autofluorescence is not a standard metabolic assay, so they need to confirm these findings with a more common method. Ideally, they should look at mitochondrial oxygen consumption because they are attributing these effects to changes in PDHK1 expression, which should be most evident in the glucose-dependent oxygen consumption.
4. The authors do not describe the media that there cells were grown and experimented in. High glucose DMEM is 25 mM glucose and 5x physiological levels. This has a profound effect on suppressing mitochondrial function (ie Crabtree effect). Mitochondrial OCR should be measured (as asked for in 3 above) in physiological 5 mM glucose, and in media without glutamine in order to determine the flux of glucose through the mitochondria, and how it changee with PDHK1 expression.
5. In figure 4 the changes in PDHK1 mRNA, protein, and activity (phosphoE1α of PDH) seem to be very modest. Again, analysis of mitochondrial OCR would be helpful in understanding the functional significance of these changes.
6. In figure 5 20mM DCA is toxic because it is a superphysiological dose. Pyruvate or lactate is toxic at these doses as well (reference 40, Stockwin et al Int J Cancer 2010), so it is difficult to attribute toxicity to inhibition of PDHK1.
7. What is the effect of PDHK1 overexpression in the dnTCF cells in terms of tumor growth? You have made these cells, so I assume that you have done the tumor growth experiments. You actually quantitate the vessel growth in these tumors in figure 8, indicating that you were able to grow the cells as tumors. I am suspicious that you have not shown the growth rate results (was there no effect?)
Summary:

In our revised manuscript we present new experiments that further strengthen the link between Wnt signaling and glycolysis in cancer. In the process of addressing the critiques we have initiated two new collaborations: Dr. Michael Teitell (UCLA; stem cell metabolomics) and Dr. Chad Garner (UC Irvine; cancer genetics and epidemiology). We have expanded the list of colon cancer cell lines examined, the depth of gene regulation of PDK1 by the Wnt pathway, the spectrum of methods by which we probe metabolism, and an initial foray into the bioinformatics of colon cancer cell metabolism. Collectively, the new data support our conclusions that Wnt regulates glycolysis and angiogenesis in colon cancer. Below we address each comment starting with the Editor’s summary and followed by responses to each reviewer.

Editor requests:

“From the editorial perspective, it would be crucial to: “

1. “Extend the analysis to additional colon cancer cells, with signal inhibition at multiple level in the cascade.”

We have added the metabolic analyses of additional colon cancer cell lines (Fig. 3, Fig. S2, S3). In these analyses, we compared the metabolic profile of SW480, SW620, DLD-1, LS174T, HT29, and HCT116 colon cancer cell lines. These cell lines differ in their genetic profiles and in their levels of activated Wnt signaling. We demonstrate the difference in Wnt signaling between lines with a TOPflash luciferase assay (Fig. S2B). We show that strong Wnt signaling cell lines have disrupted glycolysis upon expression of dnLEF/dnTCFs, in contrast to cell lines that are not activated for Wnt signaling and do not demonstrate this metabolic shift.

These data highlight the specificity of dnLEF/dnTCF expression (no general off-target effects) and they confirm that disruption of bona fide oncogenic Wnt signaling causes a shift in metabolism with repression of glycolysis.

2. “complement the dominant-negative approach with shRNA (or other means of) depletion”

Multiple attempts to remove PDK1 via shRNA preceded submission of the original manuscript. Unfortunately, all attempts were only partially successful. This problem, coupled with the issue of PDK redundancy among the four PDK family members has complicated this aspect of the project. We address this issue and its specific challenges in response to Referee 1 (point #5, below). To remove all PDK activity in cells, we used the small molecule inhibitor dichloroacetate (DCA). We used this small molecule as a proxy method for removing PDK activity and as a positive control because DCA is a well known inhibitor and has been used widely for in vitro and in vivo applications in mice and humans. Interestingly, use of DCA in human cancer patients is well tolerated and its positive affects on tumor growth and tumor vasculature support the data presented in our study (Michelakis, E. D. et al. “Metabolic modulation of glioblastoma with dichloroacetate”. Science Translational Medicine 2, 31ra34 (2010)).
3. “corroborate the Wnt-dependent glycolytic gene signature also with expression data from actual patient tumor samples”

This request prompted a new collaboration with Dr. Chad Garner (UC Irvine, Dept. of Epidemiology). Dr. Garner used publically available data from The Cancer Genome Atlas (TCGA) Data Portal (https://tcga-data.nci.nih.gov/tcga/) to ask whether there was any correlation between PDK1 expression and signatures of overactive Wnt signaling. No correlation was identified, a finding consistent with a recent high-profile study by Dennis Vitkup, Lewis Cantley, Matthew Vander Heiden of a large tumor dataset (> 2,500) that found confounding heterogeneity for gene signatures of metabolism (Hu, J. et al. “Heterogeneity of tumor-induced gene expression changes in the human metabolic network” Nat Biotechnol. 31, 522-9 (2013)). Specifically, gene signatures for oxidative phosphorylation were not readily discernible while glycolysis expression signatures were somewhat upregulated. Very few glycolytic genes emerged from this published analysis. It could be that the heterogeneous environment of tumors modifies a simple genetic relationship between signal and gene target. It could also be that the heterogeneity in cellular composition, or the dynamic evolution of individual tumors masks any obvious connection when considered collectively with a whole host of tumors. In any case, a simple relationship between PDK1 and expression of classic Wnt target genes is not detectable. However, our analysis identifies a connection between Wnt signaling and glycolysis when a different bioinformatics approach is used. In Figure S10 we show that a simple comparison of the expression level of Wnt genes and glycolysis genes (as defined by KEGG) enables unsupervised clustering of 238 human tumors into nine groups (Fig. S10a). While each cluster has a subtle discernible difference in gene expression level amongst the two pathways, a single group of 21 patient tumors is strongly clustered. This same group of tumors emerges more dramatically when the relationship between the tumor gene expression signatures for Wnt and glycolysis within and between the nine groups are further analyzed. A heatmap is presented that shows the relative distance, i.e., correlation, between the gene expression signatures of each tumor with the cladograms identifying the gene expression clusters (Fig. S10b). In that analysis the same 21 tumor cluster was detected on the basis of its exceptional homogeneous Wnt and glycolysis gene expression signature (Fig. S10b), a signature that stands out from the remaining tumors and clusters. The distinguishing feature of this tumor cluster is the pattern of their strong correlation in gene signature among the 185 Wnt and glycolysis genes (both positive and negative (anti)-correlations). Importantly, PDK1 ranks as one of the most highly correlated genes in this cluster - showing statistically significant correlation coefficients greater than |0.70| with 25 Wnt and glycolysis genes. Examination of this list reveals that most correlations between PDK1 and Wnt are negative, whereas most correlations with glycolysis genes are positive. We submitted the list of these correlating and anti-correlating genes to STRING analysis, an open-access algorithm that maps genes onto known protein networks (Fig. S10c). We observe that the anti-correlating genes are antagonists of Wnt signaling or components of non-canonical Wnt signaling and many of them interact directly or indirectly with the signaling component Dishevelled. While these findings are interesting and compelling, we present the data as supplemental information because it does not alter the fundamental conclusions of our study and they represent a starting point for a longer, deeper, more thorough investigation beyond the scope of our current work. We note that any clustering of colon tumors on the basis of gene expression signatures is interesting. Unlike other cancers, such as breast cancer, it has not been easy to classify colon tumors into subtypes. Perhaps this initial study represents a promising way to probe differences between colon tumors.
This interesting study was published just as we were submitting our manuscript to EMBO and we thank the referees for pointing it out (Esen E, et al. “WNT-LRP5 signaling induces Warburg effect through mTORC2 activation during osteoblast differentiation”. Cell Metab. 17:745-55. (2013)). We have read through the study with great interest as the authors describe a novel signal transduction pathway through mTORC2 that triggers changes in glycolysis minutes after stimulation by Wnt. There are four fundamental differences between this report and our study, i) the cell system, ii) the time course of the signal, iii) the actual components of the signal transduction system, and iv) the lack of involvement of transcription regulation.

i. Esen et al identified changes in metabolism in normal, differentiating osteoblast cells where the resting level of Wnt signaling is very low. In our system, we are studying colon cancer cells, where the resting level of Wnt signaling is high.

ii. The mTOR signal is a short-term signal triggered by addition of Wnt3a. Changes in mTORC2 and glycolytic enzymes are detectable within 10-20 minutes with decay after 24 hours. We tested whether dnLEF/dnTCF or treatment with the Wnt signaling inhibitor XAV939 (Huang, S.M. et al. “Tankyrase inhibition stabilizes axin and antagonizes Wnt signaling”. Nature 461:614-20. (2009)), had effects on mTORC2 on this time scale in two different colon cancer cell lines. We used the same method of monitoring mTORC2 activity as Esen et al., which is the specific phosphorylation of ser473 on Akt, the protein kinase substrate of mTORC2. These data are presented in Figure S9 as a time course for up to three hours (XAV939) and three days (dnLEF-1). We did not observe any changes in mTORC2 activity, suggesting that our connection between Wnt and glycolysis is different.

iii. Esen et al describe a Wnt3a-Lrp5-Rac1-mTORC2-Akt signal transduction pathway. The authors take great care to show how this signal is independent of β-catenin. This is a fundamental difference between our study and their report as we show that Wnt uses β-catenin/LEF/TCF complexes to change gene transcription of the PDK1 gene locus. Colon cancer cells (particularly SW480 and DLD-1 cells) have stabilized β-catenin protein and our inducible expression of dnLEF-1, or lentiviral introduction of dnLEF/dnTCFs specifically disrupts β-catenin actions in the nucleus. In this revision we also use XAV939, a small molecule inhibitor of Tankyrase that reduces b-catenin by stabilizing the axin-dependent destruction complex. XAV939 induces the same effects on metabolism as dnLEF-1 or dnTCF-1E confirming that a β-catenin component is essential.

iv. Esen et al. report that short-term Wnt signals work through mTORC2 to increase protein levels of PDK1, HK2, LDHA, and other enzymes involved in glycolysis. These changes are not evident at the mRNA level. The authors speculate that either there are changes in the stability of these proteins, or alterations in their production at the level of translation. Our findings are very different. We find that PDK1 levels are increased due to increased rates of transcription of the PDK1 gene. In this revision we use chromatin immunoprecipitation, 4-thiouridine mRNA labeling and transient transfection analysis to define at least two regulatory regions surrounding the PDK1 locus that are occupied by TCFs and that are Wnt regulated. We also find that the PDK1 promoter itself is sensitive to dnLEF/dnTCF (Fig. 4, S4).

Having described these four fundamental differences, we propose that the two modes of Wnt signaling are connected. In the discussion of Esen et al. there is a brief mention that prolonged treatment (>24 hr) of osteoblasts with Wnt3a caused changes in PDK1 mRNA. Perhaps Wnt signals
trigger a short-term, β-catenin-independent burst of glycolysis in osteoblasts, but they also induce a long-term, β-catenin-dependent change in transcription of the glycolytic gene program. Our study discovers the latter connection – a link particularly important for aerobic glycolysis in cancer and in development where cells are exposed to stable Wnt morphogen gradients.

5. “complement the interesting imaging approach with a metabolic analysis on glycolysis/OXPHOS”

Our original manuscript reported metabolic changes with lactate production, ATP production and Fluorescence Lifetime Imaging of NADH in living cells and living tumors. As will be detailed below in response to Reviewer #1 and #2, we now complement these studies with additional measures. These include glucose consumption, oxygen consumption rates (OCR; Seahorse), and extracellular acidification rates (ECAR; Seahorse). We have also analyzed an additional four colon cancer cell lines – chosen on the basis of their low/high, chronic levels of endogenous Wnt signaling. These additional measures greatly strengthen our conclusion that Wnt signaling drives glycolysis in colon cancer. We are especially grateful to Dr. Michael Teitell (UCLA) and his associate Tara TeSlaa for collaboration on the Seahorse experiments as their extensive expertise with metabolism and stem cell biology greatly enhanced this aspect of the study.

Referee #1:

1- The novelty of the work is somewhat touched by the recent manuscript in Cell Metabolism, reporting the same phenomena in osteoblast differentiation. It might be that the mechanism is different. Thus, the authors should address if in colon cancer cells if mTORC2 could also play a role, or the mechanism is different. In addition, the authors have also previously report that Wnt signaling correlates with glycolysis, though the mechanisms were not addressed.

We have addressed the connection between this interesting study and our own via the approach described above in our response to the Editor request (#4). In brief, Esen et al., describe a signal of Wnt3a-Lrp5-Rac1-mTORC2-Akt that happens on a short timescale of minutes to hours. They monitor mTORC2 activity via western blot detection of phospho-Akt (ser473). We use this same antibody to test for changes in mTORC2 activity in our system where we disrupt Wnt signaling by either dnLEF-1 or XAV939, the small molecule inhibitor that reduces β-catenin levels. Both of these methods reduce PDK1 protein and mRNA in our colon cancer cells, but neither treatment affects the activity of mTORC2. Thus, our signaling system which utilizes β-catenin to change PDK1 transcription in the nucleus is quite different from the β-catenin-independent signal that changes protein levels in the cytoplasm. We identify four key differences between our mechanisms and describe them in detail above in the answer to the Editor’s point #4.

2- The work is to some extent narrow in the number of colon cancer cells used. The authors should use additional human colon cancer cell lines and inhibit Wnt signaling at different points. Most of the work is based on the use of dominant negative, complementary analysis using shRNAs of several components of the Wnt signaling should be applied.

The reviewer makes several important points and we have addressed them in several ways:

i. We have expanded the use of colon cancer cells:
In addition to the original 2 colon cancer cell lines (SW480 and 2 individual clones of DLD-1 stable cells), we have extended our analysis to 4 additional colon cancer cell lines: SW620, LS174T, HCT116, and HT29. We used lentiviral transduction to express dnLEF1 and dnTCF1Emut in each of these cell lines. While the overall transduction efficiency was lower for some of the cell cultures
and Wnt inhibition was milder than in the DLD1 and SW480 cells (Fig. S2), the effects on metabolism, as measured by lactate production, glucose consumption, and FLIM, were consistent (Fig. S2 and S3). Importantly, the level of Wnt signaling correlated with the degree of change in metabolism when disrupted. The cell line with the highest levels of Wnt signaling, SW480, also showed the largest reduction in the glycolytic phenotype with Wnt signaling disruption, and so on.

ii. Knockdown of Wnt signaling components:

Using shRNA knockdown strategies to complement the dnLEF/dnTCF approach is complicated in colon cancer cell systems because β-catenin protein is stabilized via loss of APC and the destruction complex that mediates its degradation. Knockdown of other Wnt signaling components such as receptors or cytoplasmic mediators will have no consequence. Thus, to interfere with Wnt signaling it is necessary to knockdown β-catenin itself or signaling components that it interacts with in the nucleus. We did not use shRNAs for β-catenin (which would be lethal for cells) and we did not use shRNAs for LEF/TCFs since three of the four family members are expressed in colon cancer cells. Simultaneous knockdown of three separate LEF/TCFs would require a mixture of shRNAs and viruses raising the chances of off-target effects. Knockdown of transcription co-regulators (CDK8, PYGO, RNF14, etc) or overexpression of co-repressors (Groucho/TLEs) would significantly affect regulation of other gene regulatory networks since these co-factors are not dedicated strictly to Wnt signaling. Perhaps the most important complication with an shRNA strategy is that knockdown of each of the proteins listed above would have strong negative effects on proliferation. Our entire study is predicated on the ability to interfere partially with oncogenic Wnt signaling without affecting proliferation.

Thus, the advantage of inducing expression of moderate, somewhat physiological levels of dnLEF/TCFs is that we can control their expression to partially interfere with the oncogenic Wnt pathway and thus reveal other gene programs such as metabolism – programs that are masked when the cells stall in the cell cycle or when they are dying (as happens when Wnt signaling is completely blocked). We do, however, agree with the referee that our study should not solely rely on the dnLEF/dnTCF strategy. Therefore, in this revision we present how we have used the XAV939 small molecule inhibitor to respond to this criticism. XAV939 partially interferes with Wnt signaling because it stabilizes activity of the defective axin-dependent destruction complex for β-catenin. This action reduces, but does not eliminate, β-catenin protein levels and it is not cell lethal. Feng Cong and associates report that XAV939 does not affect cell proliferation in vitro to any great extent (Huang, S.M. et al. “Tankyrase inhibition stabilizes axin and antagonizes Wnt signaling”. Nature 461:614-20. (2009)), but they find strong effects on tumor growth in vivo (consistent with our results). Thus, we are confident that like dnLEF/TCFs, XAV939 treatment partially interferes with β-catenin and can therefore reveal other functions of the Wnt signaling system in cancer cells.

Using XAV939 we examined lactate production, glucose consumption, ECAR/OCR (using Seahorse), and a FLIM profile of cells treated with XAV939 (Fig. 2 and 3). We observe the same results as with dnLEF/TCF expression. That is, we observe a decrease in the ratio of glycolysis:oxidative phosphorylation with reduced Wnt signaling.

3- Along the same lines the correlation between Wnt signaling and glycolytic gene expression should also be analyzed from gene expression data from colon tumors, is any correlation between these two pathways at the gene expression level. This is critically to show for the relevance of these observations within the context of cancer biology.

To respond to this request, we initiated a collaboration with Dr. Chad Garner (UC Irvine, Department of Epidemiology). Our findings are described above in greater detail to the Editor’s request.
(point #3). In brief, our test was performed with 238 primary human patient tumors and Illumina Hi-seq data downloaded from The Cancer Genome Atlas. We do not find a simple stand-out correlation between PDK1 mRNA levels and Wnt signaling, consistent with what other groups have reported for heterogeneity in tumors (Hu, J. et al. "Heterogeneity of tumor-induced gene expression changes in the human metabolic network" Nat Biotechnol. 31, 522-9 (2013)). However, we did uncover intriguing connections between the expression of Wnt signaling components and glycolytic genes. This comparison enabled clustering of tumor subsets with one subset (21 tumors) exhibiting a very high correlation between glycolysis and Wnt signaling pathways, with PDK1 expression being a leading correlated component. While the results are intriguing, they do not directly inform on the main findings of our study. Rather, they represent an exciting new direction requiring extensive follow-up – a full study in its own right. For this reason we prefer to present the approach and the data in a new Supplemental Figure S10, with a succinct description in the Discussion section.

4- The experiments to support that PDK1 mediate the effects of Wnt signaling and glycolysis is problematic. First, the use of DCA should be tested when PDK1 is knock-down to assess the specificity of DCA which is known to have many off-target effects (http://jpet.aspetjournals.org/content/324/3/1163.full). Overexpression should be performed also with inactive PDK1 enzymes. In the xenograft experiments the DCA data should be taken into caution, and again shRNA inducible against PDK1 should be used using several colon cancer cell lines.

We agree with the referee that DCA can have off-target effects in cells as well as myriad effects when given to whole animals (particularly through catabolism by MAAI/GSTz-1, a liver enzyme that converts DCA to other compounds – an activity described in the reference provided by the referee). We nevertheless attempted to address this criticism via knockdown of PDK1, as this is a good idea to test for specificity. We were not successful with three different siRNA designs. Also, we note that PDK4 is expressed in the colon cancer cell lines and a double knockdown strategy would have also likely been necessary – with increased risk of a new set of off-target effects with infection by two lentiviruses. Other groups have been more successful with PDK knockdown strategies and they have compared these knockdowns with DCA. Each study finds good concordance where DCA treatment mimicked the effects of knockdown (PDK2; Bonnet, et al 2007), (PDK2; Sutentra, et al 2012), (PDK1; Hitosugi, 2011), (siPDK1 McFate 2008), (siPDK1 Velpula 2013), (siPDK1 Fujiwara 2013). We also note the referee’s caution about using DCA in xenografts, however, we have never used this drug in our xenograft experiments. The only xenograft tumors we have analyzed are tumors from cells transduced with mock, dnLEF-1 or dnTCF-1Emut – expressing lentiviruses.

We are unaware of a mammalian PDK1 catalytic mutant that has been designed and tested (only one in Arabidopsis); thus, we were not able to efficiently respond to this request by constructing and in vitro testing multiple PDK1 mutant proteins by overexpression in colon cancer cells. Overall we note that the referee is concerned about off-target effects from the use of high doses of DCA, a concern also raised by Referee #2. To respond to this valid concern, we performed Seahorse experiments with lower doses of DCA (10mM, Fig. 5). We find that DCA treatment produces similar effects on oxygen consumption and the extracellular acidification rates (OCR and ECAR) that we observe with dnLEF-1 or dnTCF-1Emut expression (Fig. 5C). High dose DCA treatment also matches the metabolic changes we detect with FLIM (Fig. 5B). Finally, we note here that DCA treatment of colon cancer cells at 20mM had only a slight effect on proliferation over the course of 10 days (Fig. 5D).

5- Along the previous point, since the novelty of this manuscript is compromised, the authors should include more insights into mechanisms by which Wnt controls PDK1. Is the mechanism through direct promoter binding of TCF4? Chip analysis and reporter assays should be included.

This was an excellent suggestion. To respond to the referee’s request we used data from an in-house ChIP-seq study that we are preparing for publication. We identified two regions of TCF-1
occupancy surrounding the PDK1 locus. These sites are 28kb and 30kb upstream and downstream of the locus respectively. We subcloned these genomic regions and tested them in two different luciferase reporter plasmids for responsiveness to Wnt signaling and β-catenin in colon cancer cells. We found that both regions are responsive (that is, dnLEF-1 or dnTCF-1Emut downregulated their activity in colon cancer cells), and that the endogenous PDK1 promoter is itself responsive to β-catenin and dnLEF/dnTCFs. We also used a 30 minute treatment of 4-thiouridine to label nascently transcribed RNAs in DLD-1 colon cancer cells. 4-thiouridine transcripts were isolated after a 30 minute pulse, then biotinylated for purification via streptavidin beads. In this way we were able to ask specifically whether a two hour induction of dnLEF-1 affected PDK1 mRNA transcription. The new data are presented in Figure 4, and they show that dnLEF-1 reduces the rate of PDK1 transcription by half within two hours of induction. Taken together these data show that PDK1 is a direct target of Wnt/b-catenin signaling and that the promoter and two distal regions contribute to this regulation (Fig. 4 and S4).

6- Another critical point relates to the metabolic and energetic characterization. Most of this analysis rely on the Fluorescence Life time Imaging, that would need to be complemented with more direct analysis of glycolysis and oxidative phosphorylation. Thus, a complete metabolic analysis from the cell lines and xenografts should be performed to assess direct respiration and oxygen consumption as well as glucose oxidation. Isotope labeling and metabolomic analysis should be also use to support their conclusions.

As described in point #5 to the Editor’s requests, we have now collaborated with Dr. Michael Teitell and associates (UCLA) to use the Seahorse system to measure oxygen consumption and extracellular acidification rates (OCR and ECAR), which provide reproducible measures of mitochondrial activity and lactate production in living cell cultures. We also measured the content of lactate in xenograft tumors (Figure 7F), glucose consumption in 6 colon cancer cell lines expressing dnLEF/TCF, glucose consumption in SW480 cells treated with XAV939, and Seahorse analysis of SW480 cells expressing dnLEF/TCF and SW480 cells treated with XAV939. We note that the lactate content of xenograft tumors matches the pattern of metabolic shifts that we measure with FLIM analysis. We also emphasize that FLIM was performed on living tumors that were still intact in the animal and actively perfused by a vessel system delivering oxygen and nutrients. Such studies are not possible with Seahorse or other standard metabolomics approaches.

Referee #2

1. What is the magnitude of the gene expression changes shown in figure 1? No quatitation is given, so it is difficult to evaluate how significant the changes are, or why the list of specific genes is shown.

The criteria for the lists shown in Figure 1 is now provided in the figure legend, we apologize for that omission. The changes in gene expression range from -1.3 to -6.0 and the cut-off p-value = 0.05. The microarray data are derived from a previously published report from our group (Hoverter et al. 2012, Mol. Cell Biol.) and the data are deposited in the GEO public database.

2. Figure 2 is basically a negative result, and while it is important for the interpretation of the data could probably be in the supplemental figures.

We agree with the Referee and have now placed the data in supplemental Figure S1.
3. The authors need a more thorough analysis of the metabolic changes that they describe. The changes in NADH autofluorescence is not a standard metabolic assay, so they need to confirm these findings with a more common method. Ideally, they should look at mitochondrial oxygen consumption because they are attributing these effects to changes in PDHK1 expression, which should be most evident in the glucose-dependent oxygen consumption.

We agree with the Referee and note that this was a request in common with Referee #1. We have addressed this issue by adding additional metabolism analyses, most importantly with the Seahorse system as described above (Referee #1, comment 6). The referee predicts that mitochondrial oxygen consumption should be a responsive change since PDK1 levels are reduced. We find however that OCR was not the most responsive parameter. In fact, ECAR (extracellular acidification rates) showed the greater change (Figure 2H), which interestingly, is the same finding reported by Esen et al., in their study of short term, β-catenin-independent effects of Wnt ligands on glycolysis.

4. The authors do not describe the media that there cells were grown and experimented in. High glucose DMEM is 25 mM glucose and 5x physiological levels. This has a profound effect on suppressing mitochondrial function (ie Crabtree effect). Mitochondrial OCR should be measured (as asked for in 3 above) in physiological 5 mM glucose, and in media without glutamine in order to determine the flux of glucose through the mitochondria, and how it changes with PDHK1 expression.

We agree with the omission of our media formulation in the methods. That was an oversight that is now corrected. We agree with the Referee that this formulation is important for evaluating the results of the metabolism assays. To summarize here, DMEM media has 25mM glucose and RPMI has 11mM glucose. To address the concern of non-physiological glucose being used, we performed the Seahorse assays with 5.5mM glucose, and the glucose consumption measurements were also performed using 5.5mM glucose in the media. As for the request to perform experiments without glutamine, our media formulation includes 2mM glutamine which will certainly affect mitochondrial activities. Since effects on proliferation is a condition that we avoid in our studies, we tested the growth of our colon cancer cells in the absence of glutamine under low and high glucose conditions. In both cases we found that glutamine is necessary for proliferation. Elimination of glutamine stalls the growth of the cells, which then die within 48 hours. Of course this would profoundly alter the metabolism of the cells and we therefore did not proceed with these suggested conditions.

5. In figure 4 the changes in PDH1 mRNA, protein, and activity (phosphoE1a of PDH) seem to be very modest. Again, analysis of mitochondrial OCR would be helpful in understanding the functional significance of these changes.

We agree with the Referee that the changes in PDK1 expression are partial – protein and mRNA are down by 50%. We think this reflects the fact that PDK1 is a different type of Wnt target gene, not like the “classic” LGR5, AXIN2, SP5, LEF1 where disruption of signaling strongly reduces their expression level to almost nothing. PDK1 is largely ubiquitously expressed and it is regulated by a whole host of factors (including MYC and HIF1alpha). Its transcription is therefore likely to be subject to complex regulation. We read our data to say that Wnt elevates PDK1 expression more than the usual basal level of mRNA. Do these changes have meaningful effects on metabolism? Our FLIM data certainly shows dramatic effects, but this is a sensitive measure, and therefore the new metabolism data with the Seahorse system and more traditional measures of lactate production, glucose consumption, ATP production, and others show that the changes in metabolism are significant. This is
particularly evident in the ECAR data (Fig. 2H). We compared our changes in metabolic measures with those published in Esen et al (short-term Wnt stimulation) and find that our changes are larger in magnitude, but always in the same trend.

6. In figure 5 20mM DCA is toxic because it is a superphysiological dose. Pyruvate or lactate is toxic at these doses as well (reference 40, Stockwin et al Int J Cancer 2010), so it is difficult to attribute toxicity to inhibition of PDHK1.

We agree with the Referee that the DCA dose is substantial. The study referenced by the Referee shows that DCA treatment exhibits IC50 toxicities on cell viability of a range 17mM to 70mM depending on the cancer cell line, (although colon cancer cells were not tested in that study). For our own study, we tested 20mM DCA on colon cancer cell proliferation (Fig. 5D). Ten days of DCA treatment had only a modest effect on proliferation unless combined with Irinotecan. In that figure we show that dnLEF-1 had a similar profile – that is – no effect on cell proliferation unless combined with a dose of Irinotecan that on its own also has no effect on proliferation. These data suggest that we are using doses of DCA that are appropriate. Nevertheless, in response to the Referee’s concern, we used an even lower dose of DCA in the Seahorse experiments (Fig. 5C). Consistent with the shift in the FLIM phasor plot, the OCR/ECAR ratio increased significantly, mainly due to a substantial reduction in ECAR.

7. What is the effect of PDHK1 overexpression in the dnTCF cells in terms of tumor growth? You have made these cells, so I assume that you have done the tumor growth experiments. You actually quantitate the vessel growth in these tumors in figure 8, indicating that you were able to grow the cells as tumors. I am suspicious that you have not shown the growth rate results (was there no effect?)

We have indeed measured tumor mass in our rescue experiments; we certainly do not want to make readers suspicious! We observed variable results with PDK1 rescue of tumor mass. Some tumors were not larger than the dnLEF/dnTCF tumors while a few had up to a 40% increase in mass. Overall (over 20 tumor sets), changes in mass averaged approximately 20% but this was not statistically significant. Thus, it is unlikely that PDK1 does much to increase tumor size. Since it was a negative result we did not show the data but described it in the text (pg 15-16, Discussion section). We apologize that it was not very noticeable and have modified text to highlight this more.

In principle we would find it surprising that replacing a single gene could rescue everything in dnLEF/dnTCF expressing tumors. Dominant negative dnLEF-1 downregulates ~70 genes, and dnTCF-1E downregulates hundreds of genes. The fact that PDK1 rescue restored metabolism and so much vessel growth is a striking and exciting finding, but it would be even more amazing were this single gene able to fully restore all aspects of tumorigenesis. We think this shows that there are other essential Wnt-regulated gene programs that must coordinate with glycolysis for a full rescue of the proliferation index and development of the tumor. Two examples of other important target genes that remain repressed in the PDK1 rescue tumors are presented in the manuscript: the lactate transporter MCT1, and VEGF (Fig. 7E and S8). Therefore, even if glycolysis is at least partially restored, the produced lactate is still not transported properly outside cells. There are likely to be many other downregulated genes that contribute to a full tumor mass as seen for the MOCK condition.
Thank you very much for revised study that has now been assessed by the original referees.

You will recognize that both commend you on the provided amendments. However, they also maintain the original concerns on the directness of PDK1-regulation by Wnt-signals and their differentiation from Wnt/mTOR-activity (and thus the previously published conceptual indication).

Please note that I fully recognize and appreciate the distinctions, superior clinical relevance of the CRC-system, the experimental attempts/difficulties that may arise from redundancies/complementary citations that indicate DCA-effects mimicking PDK Lof-approaches.

However, both referees still request more definitive characterization/differentiation before being able to offer their crucial support for further consideration at The EMBO Journal.

Once again, I am fully aware of the inherent general interest of your findings. However, my initial letter flagged the necessity to solve these issue upfront. Given the experimental concerns that are seemingly difficult to overcome AND the new patient data analyses questioning the generality of a correlative Wnt-target/PDK relationship, I am afraid the only conclusion I can reach at this point is to return the manuscript to at least enable rapid proceedings at an alternative venue.

Please understand that I do not take this decision lightly and we remain interested if you were to find a way to address at least the molecular concerns satisfactorily and within a reasonable timeframe.

As the study currently stands however, I am truly sorry that I do see no other way than hoping it will find a suitable venue rapidly.

REFeree REPORTS:

Referee #1:

The manuscript by Pate et al. has improved in this new revised version. There are, however, two major points that, although the authors provided some explanations, would need to be addressed with new experiments.

1. The authors provide a long explanation related to the mTOR publication that linked the two pathways (Wnt and glycolysis); direct experiments manipulating mTOR would be needed to clarify the novelty of the present study.

2. As indicated in the previous review, the use of DCA is not specific for PDK and conclusions that the authors raised would not be supported by the data. If the authors claim (see abstract) a critical role for PDK1, they should deplete this enzyme in their cellular system. Related to depletions, a long list of arguments are provided to avoid knock-down of the components of the Wnt pathway; new experiments should address at least that manipulating one component (through depletion) provides a similar phenotype.
Referee #2:

In their resubmission Pate et al have added new experimental results to the manuscript. While these new findings support the main contentions from the original manuscript, I do not feel that they have adequately addressed 2-3 of the 5 points raised by the editors as necessary for acceptance.

Specifically,

Point 1, Additional cell lines were added. Perhaps a positive effect where Wnt addition to the cells stimulates PDK1 expression/promoter activity would help make this point.

Point 2, Knockdown of PDK1 was not added. Several publications have shown good PDK1 knockdown (McFate et al JBC 2008 Aug 15;283(33):22700-8) DCA is not a good inhibitor of the PDKs. It is a pyruvate mimetic that the authors are using at levels higher than endogenous pyruvate concentrations. In addition to off target effects, DCA is an inhibitor (to greater or lesser degree) of all four of the PDKs. This makes it hard to attribute the DCA effect to PDK1. What is the effect of DCA on the other PDKs? Is it the total PDK activity that is important?

Point 3, The authors have admitted that there is not a clear relationship between classical Wnt target genes and PDK1 in clinical data sets. They do make a heroic statistical argument that in a subset of tumors some relationship holds, however, this may be just due to patient selection.

Point 4, The relationship between Wnt and mTOR signaling is addressed to some degree. It is true that Wnt could be signaling through multiple pathways in different cell types (Esen et al looked exclusively at osteoblast differentiation). They have produced some data that there are distant genomic elements that may stimulate PDK1 in a Wnt-dependent manner. However, if it is a direct effect, why is the relationship between Wnt target genes and PDK1 in the clinical samples so weak?

Point 5, Additional metabolic analysis has been added that supports the original findings with the FLIM technology.

In addition, the lack of a statistical effect on tumor growth when PDK1 is expressed in the dnTCF cells further raises questions about the significance of the findings. The authors make a big point that PDK1 is essential the the metabolic effects of Wnt signaling in CRC cancer, then claim that it is just one facet of a multi gene effect.

Additional Author Correspondence 09 December 2013

I received your letter and the reviewer responses to our manuscript and have spent some time reflecting and discussing with the co-authors and collaborators involved. I agree with the reviewers that knockdown of PDK1 is the direct test that this kinase mediates the push to glycolysis in cancer cells with oncogenic Wnt/beta-catenin signaling. In fact my group has expended intensive efforts to use siRNAs to knockdown PDK1 via multiple strategies (single siRNAs, cocktails). Unfortunately, we did not observe reductions in PDK1 mRNA and protein and therefore could not fully test the model. However, we have not tried the siRNA that the referee suggests in the latest review. Also, we have recently obtained five shRNA lentiviruses directed against PDK1. We can immediately use the siRNA suggestion as well as the shRNA viruses for FLIM and Seahorse assays in cultured colon cancer cells. If these strategies enable successful knockdown of PDK1, we can carry out the test the reviewers are requesting, which is to ask whether knockdown causes the same shift in colon cancer metabolism that we observe when we interfere with beta-catenin (dnLEF/dnTCFs, Tankyrase...
inhibition).

If PDK1 knockdown supports our model, then we trust that you will reconsider the decision on our manuscript**. If however, knockdown of PDK1 is compensated for by other PDK family members or is lethal to cells as we previously commented, we will not be able to fully test this aspect of our model and this will not satisfy the reviewers. In this case, we propose to re-format the manuscript and our model to focus on the discovery that oncogenic Wnt signaling uses beta-catenin (as shown by dnLEF/dnTCF and XAV939) to enhance glycolysis in colon cancer without claiming that PDK1 is the key target. Fundamentally, the novelty of our study stands on our discovery of the link between overactive, carcinogenic Wnt signaling (beta-catenin) and the Warburg form of glycolysis, and the influence that this has on the tumor microenvironment. I also emphasize that another exciting and entirely novel aspect of our study is our development of Fluorescence Lifetime Imaging (FLIM) to monitor metabolism in living tumors actively perfused with oxygen and nutrients. This development enabled the discovery of the link between oncogenic Wnt/beta-catenin signaling and the angiogenesis response in the tumor microenvironment.

Additional Editorial Correspondence 11 December 2013

Thank you very much for your very constructive message that I read with great excitement and much appreciate your problem-solving ad forward-looking attitude.

I would be delighted to offer a chance for reconsideration in case the specific experiments to address PDK1 engagements were to fully test the suggested model. I am also quite certain that your remarks re further-reaching mTORC2 manipulation should be appreciated/understood by the referee if a new version with hopefully positive results on direct PDK1-involvement could be presented in a relatively timely manner.

If however the proposal would not stand the test, I would prefer to discuss a consolidated/focused study (as you indicated) with our sister journal EMBOreports for the possibility to move ahead their quickly (e.g. taking advantage of the already existing comments and thus not having to enter a new and tedious cycle of peer-review).

I hope you understand the rational of such a suggestion, as The EMBO Journal is relatively molecular-mechanistic focused and with the definitive molecular link in the absence of the PDK1 (or an alternative route) missing, the observations remain striking, but molecularly not sufficiently substantiated to justify presentation in The EMBO Journal.

Resubmission 27 March 2014
With our revised manuscript we offer the following responses to the reviewer’s comments. Prompted by their questions and critique, we performed additional experiments to address two outstanding questions: the specific role of PDK1 to glycolysis in colon cancer cells, and the effect of mTORC2 on PDK1 mRNA levels. We appreciate the reviews, because they have led to a stronger study – particularly with regard to the dominant role of PDK1 and its role in the Wnt-targeted gene program for metabolism and the distinction between β-catenin signaling and mTORC2. Below we respond to each reviewer point-by-point and describe these new insights.

Referee #1:

The manuscript by Pate et al. has improved in this new revised version. There are, however, two major points that, although the authors provided some explanations, would need to be addressed with new experiments.

1. The authors provide a long explanation related to the mTOR publication that linked the two pathways (Wnt and glycolysis); direct experiments manipulating mTOR would be needed to clarify the novelty of the present study.

To further address the issue of mTORC2 complexes in colon cancer cells, we have used the ATP-site competitive inhibitor PP242 that specifically blocks mTOR activity. If the mTOR pathway regulates PDK1 transcription, then complete inhibition of this kinase should register a change in PDK1 mRNA levels. We find that even after 24 hours of mTOR inhibition, PDK1 mRNA levels are not altered. This differs from the Esen et al. study which showed transient, increased levels of PDK1 protein within 30 minutes of Wnt3a treatment (and no changes in mRNA up to 24 hours). This is in contrast to significant and consistent downregulation of PDK1 transcription by XAV939 or dominant negative LEF1 or TCF1 (dnLEF1, dnTCF1), treatments that interfere with canonical, β-catenin-dependent Wnt signaling and last for weeks in cells grown as xenograft tumors in mice. We add this data to Supplemental Figure S9 (see panel C). Figure S9 already shows the converse experiment, which is that interference with Wnt/β-catenin signaling via dnLEF1 or XAV939 treatments has no effect on mTORC2 activity (Fig. S9A, S9B). Overall we conclude that the canonical Wnt signal that regulates transcription of PDK1 and other glycolysis-connected target genes is different than the short-term, noncanonical, β-catenin-independent signal operating in the cytoplasm of primary, differentiating osteoblasts. We imagine that there may be cross-talk between the two arms of Wnt signaling (an interesting hypothesis that deserves a full study beyond the scope of this manuscript), but the mTORC2 signal does not appear to be dominant in colon cancer.

2. As indicated in the previous review, the use of DCA is not specific for PDK and conclusions that the authors raised would not be supported by the data. If the authors claim (see abstract) a critical role for PDK1, they should deplete this enzyme in their cellular system. Related to depletions, a long list of arguments are provided to avoid knock-down of the components of the Wnt pathway; new experiments should address at least that manipulating one component (through depletion) provides a similar phenotype.

In our previous response we explained how we had used multiple PDK1 knockdown experiments using an array of different siRNA reagents. We gave this approach its due diligence and tried multiple siRNAs for knockdown. However, all of these knockdown attempts were unsuccessful. Responding to the suggestion by Reviewer #2 that we try an shRNA approach, we screened shRNA sequences for knockdown efficacy. We have now identified four shRNAs in a lentiviral infection/selection system that works. In this revised manuscript, we show that each of the four shRNAs can reduce PDK1 protein levels in colon cancer cells. Drug selection ensured that the bulk of the culture had reduced PDK1 protein and activity – a definite advantage over the transient siRNA transfection approach. We tested these knockdown cultures for effects on metabolism using
Fluorescence Lifetime Imaging (FLIM; Figure S5). Compared to an uninfected culture, or a culture expressing a scrambled shRNA, we observe a highly significant and definitive shift of the FLIM NADH signature away from free NADH (indicative of glycolysis), to bound NADH (indicative of oxidative phosphorylation). These results are directly consistent with the changes in metabolism that occur when we interfere with Wnt/β-catenin by dnLEF1 or dnTCF1 expression. The data are therefore confirmatory that PDK1 is an important, direct Wnt target gene for glycolysis.

Interestingly, we also observe that the effect of PDK1 knockdown on metabolism is temporary. We used FLIM to follow metabolism of the culture for multiple days. Unlike the stable shifts in metabolism established by dnLEF1 or dnTCF1 expression, singular knockdown of PDK1 produces an oxidative phosphorylation signature that is lost in 48-72 hours. Western blot analysis of lysates from these older cultures show that other members of the Pyruvate Dehydrogenase Kinase Family (PDK2, 3, and 4) must be involved in the compensation because phosphorylation of mitochondrial Pyruvate Dehydrogenase, the best known validated substrate for PDKs, is fully recovered (data not shown). Thus, while PDK1 is a key target gene in the Wnt-regulated program for metabolism, it alone is not sufficient for specifying stable modes of glycolysis. We conclude that PDK1 is one target gene among an entire gene program directed by Wnt/β-catenin. We have already noted in our manuscript that PDK4, another PDK family member, is regulated in our system, and that PDK2 and PDK3 are gene loci occupied by LEF/TCFs in multiple cell types (Fig. 4 and Fig. S8). It is possible that the entire PDK family is a target of Wnt signaling. Finally, other glycolysis-connected genes such as a lactate transporter and other nutrient transporters are regulated by oncogenic Wnt signaling (Fig. 1 and Fig. S8 and Supp. Table S1). To reflect this new discovery, we have altered text in the abstract to qualify the status of PDK1 as a key target gene:

“We identify Pyruvate dehydrogenase kinase 1 (PDK1) as an important direct target within a larger gene program for metabolism.”

Referee #2:

In their resubmission Pate et al have added new experimental results to the manuscript. While these new findings support the main contentions from the original manuscript, I do not feel that they have adequately addressed 2-3 of the 5 points raised by the editors as necessary for acceptance. Specifically, Point 1, Additional cell lines were added. Perhaps a positive effect where Wnt addition to the cells stimulates PDK1 expression/promoter activity would help make this point.

Our study focuses on oncogenic Wnt signaling in colon cancer, not in normal, untransformed cells. In the colon cancer setting, β-catenin is most often stabilized via genetic mutation/inactivation of APC. For this reason, activation of Wnt signaling by ligand:receptor binding on the plasma membrane does not have as much significance. In the multiple colon cancer cells that we used, we show that overactive Wnt signaling (stabilized β-catenin levels) is intrinsically connected to glycolysis. We show that this is true in vitro and in vivo in a living tumor that grows and develops over four weeks. We also show that β-catenin/TCF complexes directly regulate PDK1 transcription via two regions surrounding the gene locus. Interference with this oncogenic Wnt signal has marked effects on lower PDK1 expression and shifts away from glycolysis and it has strong, negative effects on tumor progression and proliferation. Our overall finding is therefore important for the disease of colon cancer. We agree that a study of this regulatory network in a normal setting is interesting. In Figure 8 we show that PDK protein is elevated in the stem cell compartment of normal intestinal crypts. We have also previously published a FLIM study of normal mouse intestine showing that glycolysis is a strong metabolic signature in the crypt base where stem cells and Paneth cells reside (Stringari et a. 2012, Sci. Rep.). In the future we intend to test whether Wnt signaling is responsible for this signature in primary colon epithelial cells, an
effort that will utilize organoid cultures, mouse models, and further modifications to the FLIM methods for detection of metabolism in living mice. However, the report here demonstrates the power of FLIM for measuring cancer metabolism in living systems and it pinpoints a molecular mechanism for linking oncogenic Wnt signaling to glycolysis to angiogenesis in the tumor microenvironment.

Point 2, Knockdown of PDK1 was not added. Several publications have shown good PDK1 knockdown (McFate et al JBC 2008 Aug 15;283(33):22700-8) DCA is not a good inhibitor of the PDKs. It is a pyruvate mimic that the authors are using at levels higher than endogenous pyruvate concentrations. In addition to off target effects, DCA is an inhibitor (to greater or lesser degree) of all four of the PDKs. This makes it hard to attribute the DCA effect to PDK1. What is the effect of DCA on the other PDKs? Is it the total PDK activity that is important?

We are thankful for reviewer #2’s suggestion of testing shRNA knockdown as this enabled us to observe a loss of glycolysis and confirm the importance of PDK1 with independent shRNAs (Figure S9C). However, as we explain above in comments by Reviewer #1, we observed compensatory reaction to PDK1 shRNA knockdown via recovered phosphorylation of the mitochondrial substrate pyruvate dehydrogenase within several days (phospho-PDH). Therefore this experiment further revealed that while PDK1 is important, it alone is not sufficient to stably alter glycolysis like we observe with DCA, (or dnLEF1, dnTCF1, or XAV939). Just as kinase activities rapidly adjust to compensate for single-node changes in a signaling network, metabolomes and their closely integrated kinases likely do this as well. We have revised the text in the abstract to reflect that PDK1 is an important target, but also part of an entire Wnt-regulated gene program for glycolysis.

As for DCA and its specificity: We agree that DCA is not a truly selective inhibitor of PDKs, but like the ß-catenin signaling inhibitor XAV939, or dnLEF1/dnTCF1 expression, DCA can force a stable decrease in total PDK activity, in part because it likely prevents compensatory actions by PDK2, 3, and 4. We also note in the discussion that DCA has been tested in human glioma patients with promising results including a reduction of the vessel density within the tumor – a strikingly similar result in the angiogenic vasculature that we observe in our xenograft experiments (Michelakis, E. D. et al. 2010. Science translational medicine).

Point 3, The authors have admitted that there is not a clear relationship between classical Wnt target genes and PDK1 in clinical data sets. They do make a heroic statistical argument that in a subset of tumors some relationship holds, however, this may be just due to patient selection.

To address the original reviewer’s request that we analyze the expression of PDK1 in primary tumors, we used publicly available RNA-seq data generated on the Illumina HiSeq platform for 238 colon cancer cases downloaded from The Cancer Genome Atlas (TCGA) data portal. It is not entirely clear from the annotation for the datasets whether the 238 patients were subjected to some sort of selection; there is incomplete clinical information connected to these tumors. Nevertheless the intriguing finding that a subset of tumors show a high correlation between Wnt and metabolism most definitely argue for an extensive follow-up study – an effort that is in the planning and grant-writing stages. The unique subset represented about 10% of the sequenced tumors and cross validation provided preliminary validation for the findings. It is also intriguing that this subset of tumors shows a negative correlation between Wnt antagonists and glycolysis. In other words, glycolysis signatures are highest when the expression of Wnt signaling antagonists is lowest. A possible explanation for this anti-correlation is that there are multiple signaling mechanisms for Wnt regulation of glycolysis and that therefore, no specific pattern of signal transduction components should emerge as much as the class of negative regulators that prevent all forms of Wnt signaling. It is significant that much of the negative
regulators of Wnt signaling connect to Dishevelled, a cytoplasmic signaling component involved in both canonical and non-canonical Wnt signaling.

Point 4, The relationship between Wnt and mTOR signaling is addressed to some degree. It is true that Wnt could be signaling through multiple pathways in different cell types (Esen et al looked exclusively at osteoblast differentiation). They have produced some data that there are distant genomic elements that may stimulate PDK1 in a Wnt-dependent manner. However, if it is a direct effect, why is the relationship between Wnt target genes and PDK1 in the clinical samples so weak?

PDK1 is a tightly regulated gene targeted by multiple pathways. There are six annotated HIF1alpha regulatory elements in the first intron of the PDK1 locus. Also, MYC is a known regulator and there are others. It is perhaps not surprising that a kinase with an important and powerful effect on metabolism should be subject to multiple levels of regulation. As we also emphasize in the manuscript, metabolic signatures are overall quite heterogeneous in tumors and there is only weak correlation with any one, specific metabolic metric such as glycolysis. This finding emerged from a large meta-analysis performed by Dennis Vitkup, Lew Cantley, Matthew van der Heiden and others, a study large enough that patient selection should not have been an issue (>2,500 tumors; Hu, J. et al. 2013. Heterogeneity of tumor-induced gene expression changes in the human metabolic network. Nature biotechnology 31, 522–9). The lack of definitive metabolic signatures could derive from the tremendous intra- and inter-tumor heterogeneity of metabolism as the authors of this study suggest.

Point 5, Additional metabolic analysis has been added that supports the original findings with the FLIM technology. In addition, the lack of a statistical effect on tumor growth when PDK1 is expressed in the dnTCF cells further raises questions about the significance of the findings. The authors make a big point that PDK1 is essential to the metabolic effects of Wnt signaling in CRC cancer, then claim that it is just one facet of a multi gene effect.

Our study highlights a connection between Wnt signaling to glycolysis to angiogenesis. When Wnt signaling is repressed (via dnLEF1 or dnTCF1 expression), vessel density and tumor size is dramatically reduced. Restoration of glycolysis by lentiviral expression of PDK1 fully rescues the vascular phenotype, but does not fully rescue the growth phenotype (although tumors recover at least 20% of their size). It would be surprising that a single Wnt target gene, PDK1, could restore every facet of tumorigenesis. There are many downregulated Wnt target genes in these tumors that contribute bona fide functions to cellular proliferation, survival, invasion, etc., including other metabolic genes we have identified, such as the lactate transporter, MCT1. What is surprising is that PDK1 rescue-expression alone seems to restore the vasculature. Of course, we do not have much information about the functionalities of these vessels; they appear somewhat tortuous and misshapen. However, the striking finding is that the tumor microenvironment is dramatically altered in response to elevated glycolysis inside the colon cancer cell. In other words, metabolism is not just a cell autonomous feature – it has powerful cell non-autonomous effects in the tumor. To underscore the significance of this concept and the novelty of the Wnt-glycolysis-angiogenesis link, we have modified the last sentence of the abstract to read:

“Thus we identify an important mechanism by which Wnt-driven Warburg metabolism directs the use of glucose for cancer cell proliferation and links it to vessel delivery of oxygen and nutrients.”
Thank you very much for the revised study that has been carefully assessed with the notion that the critical comments on causality from the previous referees have been experimentally addressed.

I am thus in a position to initiate formal acceptance/production of your paper.

For this, please note the following further requirements:

- The EMBO Journal encourages the publication of source data, particularly for electrophoretic gels/blots, with the aim to make primary data more accessible and transparent to the reader. This entails presentation of un-cropped/unprocessed scans. We would be grateful for one PDF-file per figure for which this applies.

- Please provide a minimal 2 up to 4 'bullet point' synopsis, that highlights the major novelty/advance provided by your study.

- We are also in a position to graphically feature your work. I would thus be delighted about an integrating figure in the format of 550 x 150 (max 400) pixel.

I am very much looking forward to receive these items and take the liberty to already congratulate you to this very interesting study.