Control of intestinal stem cell function and proliferation by mitochondrial pyruvate metabolism

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Most differentiated cells convert glucose to pyruvate in the cytosol through glycolysis, followed by pyruvate oxidation in the mitochondria. These processes are linked by the mitochondrial pyruvate carrier (MPC), which is required for efficient mitochondrial pyruvate uptake. In contrast, proliferative cells, including many cancer and stem cells, perform glycolysis robustly but limit fractional mitochondrial pyruvate oxidation. We sought to understand the role this transition from glycolysis to pyruvate oxidation plays in stem cell maintenance and differentiation. Loss of the MPC in Lgr⁵-EGFP-positive stem cells, or treatment of intestinal organoids with an MPC inhibitor, increases proliferation and expands the stem cell compartment. Similarly, genetic deletion of the MPC in Drosophila intestinal stem cells also increases proliferation, whereas MPC overexpression suppresses stem cell proliferation. These data demonstrate that limiting mitochondrial pyruvate metabolism is necessary and sufficient to maintain the proliferation of intestinal stem cells.

It was first observed almost 100 years ago that, unlike differentiated cells, cancer cells tend to avidly consume glucose, but not fully oxidize the pyruvate that is generated from glycolysis.¹ This was originally proposed to be due to dysfunctional or absent mitochondria, but it has become increasingly clear that mitochondria remain functional and critical. Mitochondria are particularly important in proliferating cells because essential steps in the biosynthesis of amino acids, nucleotide and lipid occur therein.³,⁵ Most proliferating stem cell populations also exhibit a similar glycolytic metabolic program,⁴–⁹, which transitions to a program of mitochondrial carbohydrate oxidation during differentiation.¹⁰,¹¹ The first distinct step in carbohydrate oxidation is import of pyruvate into the mitochondrial matrix, where it gains access to the pyruvate dehydrogenase complex (PDH) and enters the tricarboxylic acid (TCA) cycle as acetyl-CoA. We, and others, recently discovered the two proteins that assemble to form the mitochondrial pyruvate carrier (MPC).¹²,¹³ This complex is necessary and sufficient for mitochondrial pyruvate import in yeast, flies and mammals, and thereby serves as the junction between cytoplasmic glycolysis and mitochondrial oxidative phosphorylation. We previously showed that decreased expression and activity of the MPC underlies the glycolytic program in colon cancer cells in vitro and that forced re-expression of the MPC subunits increased carbohydrate oxidation and impaired the ability of these cells to form colonies in vitro and tumours in vivo.¹⁴ This impairment of tumorigenicity was coincident with a loss of key markers and phenotypes associated with stem cells. This prompted us to examine whether glycolytic non-transformed stem cells might also exhibit low MPC expression and mitochondrial pyruvate oxidation, which must increase during differentiation.

RESULTS

MPC expression is low in intestinal stem cells and increases following differentiation

As a model system, we have studied LGR⁵⁺ intestinal stem cells (ISCs), which reside near the base of the intestinal crypt and continuously proliferate. Their progeny differentiate and populate the

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intestinal epithelium, which is completely renewed approximately every 5 days\textsuperscript{15,16}. Using EGFP fluorescence from an Lgr5-EGFP-IRES-CreERT2 allele, we single-cell-sorted intestinal crypt stem cells into low-, medium- and high-LGR5-expressing populations (Supplementary Fig. 1a). As expected Lgr5 messenger RNA, as well as that of other markers of stem cells, correlated with Lgr5-EGFP expression, while Krt20 and other markers of differentiation anti-correlated with EGFP (Fig. 1a,b and Supplementary Table 1). The pattern of Mpc1 and Mpc2 expression resembled that of differentiation genes, exhibiting lower expression in the more stem-like cells that increased with differentiation. In vitro organoids maintained in stem cell or differentiation-promoting conditions displayed a similar pattern. When grown in basal medium containing EGF and Noggin, organoids exhibit a largely differentiated gene expression pattern, which is progressively more stem-like when R-spondin 1 and Wnt3a are added to the medium (Fig. 1c,d and Supplementary Table 2). Expression of Mpc1 and, to a lesser extent, Mpc2 again correlate with the expression of differentiation genes. Both in vivo and in vitro, the expression of indicators of mitochondrial biogenesis such as Tfam and Nrf1 was higher in more stem-like cell populations (Fig. 1a–d) suggesting that the decreased MPC expression is not due to a global suppression of mitochondrial gene expression. Similarly, immunohistochemical analysis of the proximal small intestine (jejunum) revealed that MPC1 was nearly absent from the base of the crypt, the site of LGR5\textsuperscript{+} ISCs, but strongly expressed through the upper crypt and villus, whereas VDAC, a marker of total mitochondrial mass, was more abundant at the base of the crypt relative to the remainder of the intestinal epithelium in both mouse and human (Fig. 1e). Similar anti-correlation of MPC1 and LGR5 expression was observed by immunofluorescence staining of small intestine (Fig. 1f). This pattern of MPC1 and VDAC expression was consistent throughout the murine small intestine (jejunum and ileum) and NRF1, TFAM and PDK1 were also more abundant in the crypt cells in human intestine while the differentiation mark CK20 was less abundant\textsuperscript{17,18} (Supplementary Fig. 1b,c). Electron microscopy also showed high mitochondrial content in crypt stem cells, and isolated Lgr5-EGFP stem cells robustly stain with a dye dependent on mitochondrial membrane potential (Fig. 1g,h). These data are consistent with the hypothesis that crypt stem cells contain functional mitochondrial, but that they are geared toward biosynthetic activities or oxidation of fatty acids or other non-carbohydrate fuels.

**Drosophila** MPC regulates intestinal stem cell proliferation

These observations led us to hypothesize that the MPC plays an active and direct role in regulating ISC proliferation. To begin to test this hypothesis, we examined the role of the MPC in the ISCs of the fruit fly *Drosophila*, which share key aspects of their biology with mammalian ISCs\textsuperscript{19,20}. Both MPC1 and MPC2 are expressed in all four cell types of the intestine, with the lowest level of expression in the ISCs and the highest expression in the differentiated enteroendocrine cells\textsuperscript{21,22}. Confocal imaging of intestines dissected from dMPC1 mutants revealed that the epithelium exhibits multilayering unlike the normal single-cell layer seen in controls (Fig. 2a). This is a classic overgrowth phenotype that is associated with oncogene mutations in *Drosophila*\textsuperscript{26}. Accordingly, we used MARCM clonal analysis to determine whether a specific loss of the MPC in ISCs leads to an increase in their proliferation\textsuperscript{25}. On average, newly divided GFP-marked dMPC1 mutant clones are more than twofold larger than control clones, which were generated in parallel using a wild-type chromosome, indicating that the MPC is required in the ISC lineage to suppress proliferation (Fig. 2b and Supplementary Table 7). Because GFP-marked clones could include cells that differentiate into mature enterocytes or enteroendocrine cells, we also conducted clonal analysis in the absence of Notch, thereby blocking ISC differentiation\textsuperscript{24,25}. Under these conditions, we again observed an approximately twofold increase in the size of dMPC1 mutant ISC clones (Fig. 2c and Supplementary Table 7). To confirm and extend these results, we disrupted MPC function specifically in the ISCs by using the DI-GAL4 driver in combination with UAS-GFP, which facilitates stem cell identification. Once again, approximately twofold more GFP-marked stem cells were observed relative to controls when either dMPC1 or dMPC2 expression was disrupted by RNA-mediated interference (RNAi) along with increased ISC proliferation as detected by staining for phosphorylated histone H3 (pHH3) (Fig. 2d). Similar results were obtained when RNAi was targeted to the E1 or E2 subunits of PDH to specifically disrupt the next step in mitochondrial pyruvate oxidation (Fig. 2d and Supplementary Fig. 2a and Supplementary Table 7). Importantly, an opposite phenotype was seen when Ldh was reduced by RNAi in the ISCs or progenitor cells (Fig. 2e,f and Supplementary Fig. 2a and Supplementary Table 7). Ldh suppression is known to result in a significant increase in pyruvate levels, which can promote pyruvate oxidation\textsuperscript{27}. Taken together with our results with Pdh RNAi, these observations support the model that the MPC limits stem cell proliferation through promoting oxidative pyruvate metabolism in the mitochondria. It also appears to be sufficient as specific overexpression of MPC1 and MPC2 in ISCs or progenitors caused a reduction in stem cell proliferation, the opposite of the loss-of-function phenotype. This can be seen in either *Pseudomonas*-infected intestines (Fig. 2e and Supplementary Fig. 2a), which undergo rapid stem cell proliferation\textsuperscript{27}, or under basal conditions in aged animals (Fig. 2f). Consistent with this, MPC overexpression under basal conditions had no effects on intestinal morphology, while the intestines from infected flies displayed a fully penetrant size reduction, which is probably due to the inability of ISCs to maintain tissue homeostasis (Fig. 2g and Supplementary Figs 2b and 7c). Taken together, our results demonstrate that mitochondrial pyruvate uptake and metabolism is both necessary and sufficient in a stem cell autonomous manner to regulate ISC proliferation and maintain intestinal homeostasis in *Drosophila*.

**MPC deletion in the Lgr5-EGFP cells expands the intestinal stem cells compartment and increases proliferation**

To test whether loss of the mammalian MPC might have similar effects on ISC homeostasis, we generated mice carrying the Lgr5-EGFP-IRES-CreERT2 allele together with homozygous floxed alleles of *Mpc1* (Supplementary Fig. 3a). Tamoxifen treatment resulted in a significant but incomplete (~30%) loss of MPC1 in the proximal small intestine at 30 days (Fig. 3a,f). Loss of MPC1 had no effect on body weight and caused no change in the morphology or height of intestinal crypts or villi (Fig. 3a and Supplementary Figs 3b–d). We also observed no change in alcian blue staining, which marks goblet cells or perturbation in the mature enterocyte marker CK20 (Fig. 3a and Supplementary Fig. 3c). These animals exhibited,
however, a significant increase in the number and zone size of actively proliferating cells in the crypt as measured both by BrdU incorporation and Ki67 staining (Fig. 3b,c and Supplementary Fig. 3f). In addition, Mpc1<sup>KO</sup> mice had an increased number of crypt stem cells as assessed by Olfm4, Lgr5 and LRIG1 expression as well as by expression of GFP from the Lgr5-EGFP allele (Fig. 3d,e and Supplementary Fig. 3g–i). Consistent with these data, we found that the zone of Mpc1-deficiency increased over time post-Mpc1 deletion, suggesting positive selection for Mpc1-deleted cells (Fig. 3f). These data support our results in <i>Drosophila</i> and demonstrate that Mpc1<sup>Lgr5-KO</sup> crypts display increased cell cycling and ISC expansion.

Figure 1 MPC expression is low in intestinal stem cells and increases following differentiation. (a) The indicated transcripts were quantified in Lgr5-EGFP cells sorted for low, mid and high EGFP intensity (n = 13, low and mid, n = 12 high). (b) Heat map of mRNA content from the Lgr5-EGFP populations in a. (c) The indicated transcripts were quantified from organoids transferred to differentiation conditions (EN: EGF and Noggin) compared with stem-maintaining conditions containing RSPO1 and Wnt3A (ENR) or RSPO1 and Wnt3A (ENR + Wnt) (n = 3 per treatment). (d) Heat map of mRNA content from the organoids in c. (e) Antibody stain of MPC1 and VDAC on crypts of proximal small intestine in mouse (top) and human (bottom). (f) Immunofluorescence images of mouse proximal small intestine staining for MPC1 (red) and EGFP for Lgr5 intestinal stem cells (green). (g) Electron micrographs of enterocytes (left) and crypt stem cells and surrounding Paneth cells (right) at low (top) and high (bottom) magnification. The yellow arrowheads indicate mitochondria. (h) Isolated live crypts imaged for Lgr5-EGFP and co-stained with MitoTracker Red CMXRos. Data are mean ± s.e.m. **P < 0.01, ***P < 0.001; NS, not significant. Scale bars, 50 μm (e, human; h) and 20 μm (e, mouse; f). P values for a,c were calculated by one-way ANOVA with correction for multiple comparisons.
Figure 2 Drosophila MPC regulates intestinal stem cell proliferation. (a) Intestines from controls and dMPC1 mutants stained with phalloidin (red) and DAPI (blue), (n=10 control, n=7 knockout). (b) MARCM dMPC1 mutant clones marked by GFP along with quantification of clone size (n=13 clones from control, n=12 clones from knockout, 15 animals assessed for each group). (c) MARCM dMPC1 mutant clones with Notch RNAi marked by GFP along with quantification (n=31 from control, n=38 from knockout). (d) Dl-GAL4 used to target RNAi as indicated, (control n=8, dMPC1 RNAi n=8, dMPC2 RNAi n=8, PDH-E1 n=7, PDH-E2 n=7 experimental replicates). Increased proliferation as detected by staining for phosphorylated histone H3 (pHH3) (pHH3 + cells counted from control n=8, dMPC1 RNAi n=8, PDH-E1 n=7). (e) Dl-GAL4 used to target RNAi as indicated or MPC overexpression (o/e) under infected conditions (control n=5 (no infection), control with infection n=10, dMPC o/e n=10, Ldh RNAi n=10, dMPC1 RNAi n=10, PDH RNAi n=10). (f) esg-GAL4 was used to direct MPC overexpression (o/e) or RNAi targeting Ldh or dMPC1 under uninfected basal conditions in 23-day-old adults maintained at 29°C (control n=10, MPC o/e n=10, Ldh RNAi n=10, dMPC1 RNAi n=10). (g) Intestine size is unaffected in animals overexpressing MPC under basal conditions in aged flies (left two panels) and reduced in infected animals (right two panels) (n=10 for control and MPC o/e). Data are mean ± s.e.m. ∗P < 0.05, **P < 0.01, ***P < 0.001. All P values were calculated using Student’s t-test. Pe, Pseudomonas entomophila.

In vitro loss of the MPC increases stem cell function and organoid formation

A dramatic increase in organoid-forming potential was observed after plating crypts isolated from Mpc1<sup>1kr5-KO</sup> mice, supporting the conclusion that these animals have an enlarged population of proliferating ISCs (Fig. 4a and Supplementary Table 7). Similar results were seen when organoids were established from single Lgr5<sup>+</sup> stem cells treated with tamoxifen <em>in vitro</em> to eliminate MPC1 (Fig. 4b and Supplementary Table 7), exhibiting more Lgr5-EGFP-positive branches (Fig. 4c). When cells from these organoids were dissociated and sorted, Mpc1<sup>1kr5-KO</sup> organoids maintained a higher fraction of GFP-positive cells (Fig. 4d and Supplementary Fig. 4a).
Figure 3 MPC deletion in the Lgr5-EGFP compartment increases intestinal stem cell maintenance and proliferation. (a) Images of haematoxylin and eosin (H&E), MPC1, CK20, Ki-67, alcian blue, VDAC, Lrig1 and BrdU staining in control and Mpc1Lgr5-KO proximal small intestine. (b) Representative images and quantification of BrdU stained crypt nuclei in control and Mpc1Lgr5-KO animals (n=9 control, n=10 knockout). (c) Representative images and quantification of Ki-67-positive nuclei in crypts of control and Mpc1Lgr5-KO animals (n=9 control, n=10 knockout). (d) Representative images of Olfm4 in situ hybridization of control and Mpc1Lgr5-KO small intestine with quantification of positive cells per crypt (right) (n=9 control and knockout). (e) Images of GFP-stained cells in control and Mpc1Lgr5-KO animals with quantification of GFP-positive cells per crypt (n=9 control and knockout). (f) Percentage of area of proximal small intestine without MPC1 by immunohistochemistry in animals 8, 30 and 160 days post-tamoxifen treatment (n=5 for 8 and 30 day, n=4 for 160 day). Data are mean ± s.e.m. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Scale bars, 100 μm (a) and 20 μm (b-e). All P values were calculated using Student’s t-test.

When cells from these wild-type and Mpc1Lgr5-KO organoids were dissociated, but not sorted for EGFP, those from Mpc1Lgr5-KO organoids maintained their increased organoid-forming ability (Fig. 4e) and were more likely to be Lgr5-EGFP-positive (Fig. 4f). This increase in organoid formation of single Mpc1Lgr5-KO cells was maintained over extended passaging over two months (Supplementary Fig. 4b). Moreover, Mpc1Lgr5-KO organoids transferred to basal medium lacking the drugs that maintain stemness rapidly lost EGFP fluorescence, while Mpc1Lgr5-KO organoids retained significant EGFP expression surrounding the autofluorescent lumen (Fig. 4g).

In vitro loss of the MPC alters protein expression and metabolism
To further define the molecular effects of MPC loss, we isolated wild-type and Mpc1Lgr5-KO organoid populations and subjected them...
Mpc1

organoids and wild-type organoids treated with UK-5099, which is consistent with a failure of cytoplasmic pyruvate to enter the mitochondrial TCA cycle (Fig. 5c) and Supplementary Fig. 4f). Mpc1<sup>14r5-KO</sup> organoids also showed a significant impairment in flux of 13C-glucose into the TCA cycle as measured by M+0 loss and M+2 accrual in the citrate pool (Fig. 5d and Supplementary Table 7). Oxygen consumption was also markedly impaired under both basal and FCCP-stimulated (maximal) conditions in both Mpc1<sup>14r5-KO</sup> organoids and wild-type organoids treated with UK-5099, which is a specific and efficacious MPC inhibitor<sup>12,13,31</sup> (Fig. 5e). No further decrease in oxygen consumption was observed when Mpc1<sup>14r5-KO</sup> organoids were treated with the inhibitor. This led us to explore whether fatty acid oxidation might be increased to compensate for loss of Mpc1-dependent carbohydrate oxidation. In fact, we found that etomoxir-sensitive respiration (that is, fatty acid oxidation) was profoundly increased by either inhibition or genetic deletion of MPC function (Fig. 5f).

**In vitro inhibition of MPC activity increases organoid formation**

In addition to genetically MPC-deficient organoids, we next utilized the MPC inhibitor UK-5099 to examine the effects of acute MPC loss. Similar to Mpc1<sup>14r5-KO</sup> organoids, we found that treatment of crypt-derived organoids with UK-5099 led to enhanced maintenance of EGFP expression in the epithelial lining (Fig. 6a) and a larger number of crypt-like branches per organoid, indicative of increased stem cell activity (Fig. 6b and Supplementary Table 7). We also observed that UK-5099 treatment caused an approximately fourfold increase in the efficiency of organoid formation from proximal small intestine (Fig. 6c and Supplementary Table 7). When treated organoids were dissociated to protein analysis. As expected, Mpc1-deleted organoids had an essentially complete loss of MPC1 protein, and a significant reduction in Mpc2, which is dependent on MPC1 for its stability (Fig. 5a and Supplementary Figs 4c and 7a,d)<sup>14,28–30</sup>. Both the LGR5 and beta-catenin stem cell markers were elevated in Mpc1<sup>14r5-KO</sup> organoids (Fig. 5a and Supplementary Fig. 7), consistent with effects on the mRNAs encoding stem and differentiation marks (Supplementary Fig. 4d and Supplementary Table 3). We also observed that histone acetylation, and specifically H3K27 and H3K4 acetylation marks, were decreased in Mpc1-deleted organoids (Fig. 5b and Supplementary Figs 4e and 7b). As expected, Mpc1<sup>14r5-KO</sup> organoids exhibited a steady-state increase in pyruvate and a decrease in citrate, malate and alpha-ketoglutarate, consistent with a failure of cytoplasmic pyruvate to enter the mitochondrial TCA cycle (Fig. 5c and Supplementary Fig. 4f). Mpc1<sup>14r5-KO</sup> organoids also showed a significant impairment in flux of 13C-glucose into the TCA cycle as measured by M+0 loss and M+2 accrual in the citrate pool (Fig. 5d and Supplementary Table 7). Oxygen consumption was also markedly impaired under both basal and FCCP-stimulated (maximal) conditions in both Mpc1<sup>14r5-KO</sup> organoids and wild-type organoids treated with UK-5099, which is a specific and efficacious MPC inhibitor<sup>12,13,31</sup> (Fig. 5e). No further decrease in oxygen consumption was observed when Mpc1<sup>14r5-KO</sup> organoids were treated with the inhibitor. This led us to explore whether fatty acid oxidation might be increased to compensate for loss of Mpc1-dependent carbohydrate oxidation. In fact, we found that etomoxir-sensitive respiration (that is, fatty acid oxidation) was profoundly increased by either inhibition or genetic deletion of MPC function (Fig. 5f).

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and assessed by flow, UK-5099 treatment significantly increased the percentage of GFP-positive cells (Fig. 6d and Supplementary Table 7). MPC inhibition enhanced organoid formation from all regions of the small intestine and from the colon to a degree similar to that allowing passive differentiation, UK-5099 delayed the emergence of highly autofluorescent organoid lumens, suggesting fewer or lower activity of differentiated secretory cells (Supplementary Fig. 5c). We also examined the effect of MPC inhibition in comparison with the HDAC inhibitor valproic acid and/or GSK-3β inhibitor CHIR99021, both of which promote stem cell maintenance independent of the Paneth cell niche (Fig. 6e and Supplementary Table 7) with each drug being used at its optimal concentration and combination with other drugs being used at their optimal concentration.

 Interestingly, organoids could be maintained for at least one year of continuous UK-5099 treatment and they continued to display an increase in crypt domains per organoid (Fig. 6f). The addition of UK-5099 also improved passaging efficiency of early crypts (Supplementary Fig. 5b). When organoids were transferred to differentiation conditions, treatment with UK-5099 resulted in maintenance of Lgr5-EGFP expression in the crypt domains (Fig. 6g). Similarly, when removing stem-cell-maintaining factors and conditions were maintained with all other combinations. We conclude that MPC inhibition has profound effects on stem cell maintenance that are at least partially mechanistically distinct from those caused by HDAC or GSK-3β inhibition.
GSK-3beta inhibition. Given the previous suggestion that valproic acid might inhibit the MPC,\textsuperscript{33,34} we examined \textsuperscript{13}C-glucose flux with and without valproic acid and found that valproic acid had no effect on the kinetics of citrate labelling, demonstrating that, at the concentrations used, this drug does not inhibit MPC activity (Supplementary Fig. 6a).

These observations of enhanced stem cell function and previously described metabolic effects (Fig. 5d-f) of UK-5099 treatment were

- GSK-3beta inhibition
- Enhanced stem cell function
- Described metabolic effects

Figure 6 In vitro inhibition of MPC activity increases organoid formation. (a) Images of organoids from crypts with and without UK-5099. The blue arrowheads point to \textit{Lgr5-EGFP}\textsuperscript{+} crypt domain and the purple asterisks indicate autofluorescent lumens. (b) Quantification of crypt domains per organoid with and without UK-5099 (\(n=6\) independent organoids). (c) Quantification of organoid formation from crypts of proximal small intestine with and without UK-5099 (\(n=16\) wENR, \(n=14\) UK-5099 experimental replicates). (d) Flow cytometry for the cells in (c) showing the percentage of GFP-positive (\(n=3\) replicates per condition). (e) Human colon tissue with wENR basal medium (set to 1) or medium containing wENR UK-5099 for 12 months (wENR n = 46 organoids from 2 separate wells, UK-5099 n = 26 organoids from 2 separate wells). (g) Images of organoids treated with IWP-2 and DAPT to promote differentiation with and without UK-5099. The blue arrowheads point to crypt domains and the yellow asterisks indicate autofluorescent lumens. (h) Organoid formation from crypts treated with the indicated single, double and triple drug combinations (\(n=10\) experimental replicates, \(P\) values are for each treatment compared with wENR). (i) Transcript abundance in organoids showing the ratio of UK-5099-treated to control (\(n=3\) experimental replicates, \(P\) values calculated for each transcript compared with wENR). (j) Heat map for additional transcripts from (i). (k) Probability of cells infected with virus containing empty vector or both MPC1-iRFP and MPC2-mCherry also being GFP-positive (\(n=4\) independent organoids with at least 15 infected cells counted). Data are mean ± s.e.m. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\); NS, not significant. Scale bars, 50 \(\mu\)m (a) and 100 \(\mu\)m (f,g). \(P\) values for 6 h were calculated by one-way ANOVA with correction for multiple comparisons. All other \(P\) values were calculated using Student's t-test.
accompanied by changes in the expression of molecular markers of stemness. UK-5099 treatment in mixed organoid populations led to a robust increase in several stem cell markers, including Lgr5, Ascl2, Cdh44 and Myc, along with a substantial decrease in the abundance of the Krt20, Villin1, Chga and Fubi2 differentiation markers (Fig. 6i) and Supplementary Table 4). These data demonstrate that loss of mitochondrial pyruvate import leads to enhanced maintenance of molecular and functional markers of ISC (and decreased markers of differentiation) to a degree similar to well-described stem-cell-maintaining agents.

Finally, we performed the converse of prior experiments and tested the hypothesis that forced expression of Mpc1 and Mpc2 in Lgr5+ ISC causes a loss of stemness. Indeed, using a previously validated system for overexpression14, we found that those cells expressing exogenous Mpc1 and Mpc2, which are both required for MPC function, were much less likely to maintain Lgr5 expression, as indicated by EGFP fluorescence (Fig. 6k and Supplementary Fig. 6b and Supplementary Table 7). This is consistent with work suggesting that stimulating glucose oxidation with the small molecule dichloroacetate (DCA) or by substitution of glucose with galactose promotes differentiation10.

**DISCUSSION**

Our studies in *Drosophila*, intestinal organoids and mice provide strong evidence that the MPC is necessary and sufficient in a cell autonomous manner to suppress stem cell proliferation. Consistent with this, we have demonstrated that ISC maintain low expression of the subunits that comprise the MPC, which enforces a mode of carbohydrate metabolism wherein glucose is metabolized in the cytosol to pyruvate and other biosynthetic intermediates. This glycolytic metabolic program appears to be sufficient to drive robust and continuous stem cell proliferation25. We also observed high mitochondrial content in ISC, which must be geared primarily toward biosynthetic functions and/or oxidation of other substrates such as fatty acids. Increased fatty acids, the metabolism of which is enhanced in MPC-deficient and MPC-inhibited organoids, have been shown to promote ISC expansion and proliferation via enhanced beta-catenin signalling and increasing tumour-initiating capacity36. MPC expression increases following differentiation, consistent with the shift in demand from macromolecule biosynthesis to ATP production in support of post-mitotic differentiated cell function. A similar switch in MPC expression can be seen following differentiation of embryonic stem cells, haematopoietic stem cells and trophoblast stem cells37–41. Conversely, MPC expression is reduced after reprogramming fibroblasts to induced pluripotent stem cells42–45. This suggests that the effects of altering pyruvate flux that we observe herein might not be restricted to ISC, but instead be representative of similar effects on multiple stem cell populations (see also the accompanying manuscript on hair follicle stem cells46). Interestingly, Myc is known to drive a metabolic program that is similar to that observed following MPC loss, characterized by increased glycolysis and reliance on glutamine and fatty acid oxidation with reduced glucose oxidation47–49. This suggests that Myc may play a role in repressing the MPC in stem cells, possibly acting downstream of Wnt/beta-catenin signalling50. Consistent with this, Myc and its repressive co-factors localize to the Mpc1 promoter and Myc expression is strongly anti-correlated with Mpc1 expression (Supplementary Fig. 6c)14,51–54.

Taken together, our studies demonstrate that changes in the MPC and mitochondrial pyruvate metabolism are required to properly orchestrate the proliferation and homeostasis of intestinal stem cells. Importantly, this metabolic program—mediated at least partially by the MPC—appears to be instructive for, rather than a downstream consequence of, cell fate. Future work will define the extent to which the results presented herein relate to those showing that diet quality and quantity can modulate ISC behaviour. It is tempting to speculate that ISC metabolism is used as a signal for increased or decreased demand for intestinal epithelium. Perhaps of most importance will be to define the mechanisms whereby altered partitioning of pyruvate metabolism affects stem cell proliferation and fate. We speculate that the robust changes that we observed in fatty acid oxidation and histone acetylation, which are probably downstream of altered metabolic utilization for acetyl-CoA production, play an important role56–57.

While the mechanisms are not as yet defined, these studies establish a paradigm wherein mitochondrial metabolism does not merely provide a permissive context for proliferation or differentiation, but rather plays a direct and instructive role in controlling stem cell fate.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

*Note: Supplementary Information is available in the online version of this paper.*

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**AUTHOR CONTRIBUTIONS**

Conceptualization, I.C.S., D.R.W., C.S.T. and J.R.; methodology, J.C.S., D.R.W., C.B., H.Z., P.W., J.T., A.E., J.M., L.K.S., C.S.E., K.A.O., D.D., P.K., M.P.B., D.Y.L., J.E.C., H.R.C., W.E.L., C.S.T. and J.R.; investigation, J.C.S., D.R.W., C.B., H.Z., P.W., J.T., A.E., J.M., L.K.S., C.S.E., R.M., D.D. and P.K.; formal analysis, J.C.S., D.R.W., C.B., P.W., T.C.W., R.M., L.J., R.J.D. and I.E.C.; writing—original draft, review and editing.
METHODS

Mice. Heterozygous Lgr5-EGFP-IRE-creERT2 mice were obtained from Jackson Labs®. MPCI fl/fl mice were generated as previously described. Control animals consisted of Lgr5-EGFP-IRE-creERT2 × MPCI WT/WT or MPCI fl/fl mice (negative control). For the knockdown experiments, these were backcrossed six times to C57BL/6J background and 2.5 mM EDTA was injected intraperitoneally with 100 μl of 20 mg ml⁻¹ tamoxifen (Sigma Aldrich T5648) dissolved in peanut oil (Sigma Aldrich P2144) for three successive days and euthanized 8, 30 or 160 days later. BrdU (Invitrogen b23151) was dissolved in PBS and injected intraperitoneally 4 h later euthanasia to label dividing cells. Injections were carried out in the morning (7:00 and 11:00) and tissue was collected 4 h later to 10 mg ml⁻¹ of cells. BrdU was used to detect replication at the ISCs and enteroblasts, and Use Committee.

randomization was performed for animal studies, animals were used as they became available, paired with littermate controls where possible, there was no preference for male or female animals, and both genders were included in each analysis. All IACUC guidelines were followed and the protocol for animal experiments carried out in this study was submitted to the University of Utah Institutional Animal Care and Use Committee.

Drosophila stocks. Drosophila stocks were maintained on standard food containing 3% sucrose, 6% glucose, 8% yeast and 1% agar in a 25 °C incubator. For dMPC1 and dMPC2 control flies were homogenized for a precise excision of the P[XP][CG14290][d00809] P-element insertion, and mutant flies were trans-heterozygous for the two deletion alleles, dMPC1 and dMPC2, as described previously. D. GALA (a gift from B. Edgar) was used to target RNAi to the ISC and esg-GAL4 (a gift from B. Edgar) was used to target RNAi to the ISCs and enteroblasts. RNAi transgenic stocks are as follows: UAS-dMPC1 RNAi and UAS-dMPC2 RNAi, UAS-PDH-E1 (VDRC101856), UAS-PDH-E2 (VDRC102893) and UAS-Notch RNAi (a gift from C. Mitchell). Temperature-sensitive Tub-GAL80ts was utilized to restrict RNAi to adults. For temperature-shift experiments, animals were raised at 18 °C and 4–5-day-old adults were transferred to 29 °C for five days after which intestines were dissected for analysis. Pseudomonas infections were performed as described previously. The Pseudomonas strain was a gift from B. Edgar. Overexpression of the MPC was achieved by inserting the open reading frames for dMPC1 and dMPC2 downstream from the UAS promoter in the pUAST-attB vector, with the coding sequence for the P2A peptide joining the two MPC coding regions. This was inserted into the attP40 site on the second chromosome using standard methods, and expression was achieved using GALA drivers specific to the ISCs or progenitor cells. All Drosophila studies were performed following standard ethical guidelines for working with this organism. As is standard in studies of Drosophila intestinal stem cells, all experiments were performed in females, which have a higher rate of basal cell proliferation than males. Experiments were conducted in adults aged four days to three weeks after eclosion.

Mosaic analysis. The MARCM system was used to generate wild-type and dMPC1 mutant lineages as previously described. Four- to five-day-old adult female flies of the following genotypes were subjected to two 30 °C heat pulses for 30–40 min: y, w, hs-FLP, UAS-GFP / w; +; Tub-GAL4, FRT82B, tub-GAL80 / FRT82B (control). Analyses were performed for five days after injection. The number of nuclei in GFP-expressing cells was used for quantification. For the dMPC1 MARCM studies in the presence of Notch RNAi, intestines were analysed five days after clone induction. Intestines were stained with antibodies to detect GFP and Prospero, with DAPI to mark nuclei. Animals of the following genotype were used: y, w, hs-FLP, UAS-GFP / w; UAS-Notch RNAi; Tub-GAL4, FRT82B, tub-GAL80 / FRT82B (control, Notch RNAi only), y, w; hs-FLP; UAS-GFP / w; UAS-Notch RNAi; Tub-GAL4, FRT82B, tub-GAL80 / FRT82B dMPC1 (Notch RNAi and dMPC1 mutant clones). Only clones that were negative for enteroendocrine cells (Prospero negative) and enterocytes (larger nuclei) were included in the analysis.

Histology and immunostaining. Adult flies were dissected in 1×PBS and the gastrointestinal tract was fixed in 4% formaldehyde (Polysciences, EM grade) overnight at 4 °C. Tissues were washed four times with 0.1% Triton, 1×PBS (PBST) and incubated with PBST and primary antibodies overnight, washed again, and then incubated for 3–4 h with secondary antibodies in PBST. Samples were mounted using Vectashield (Vector) with DAPI. Antibody information is provided in Supplementary Table 5. Images were acquired using an Olympus FV1000 confocal microscope and assembled into Z-stack projections for the figures. For the RNAi experiments, GFP-positive cells were quantified per ×40 frame in the mid-R2 region of the intestine. For the number of dividing cells, the average number of PHH3-positive cells for the entire midgut was reported.

Cryopreservation and single-cell isolation. Small intestinal crypts and single GFP-positive cells were isolated as described. The entire small intestine was harvested, opened longitudinally and washed with PBS. Mucus and villi were scraped and discarded using a thin glass coverslip and tissue was cut into small (approximately 2 cm) sections. These were washed twice in 2.5 mM EDTA diluted in PBS for 1 h with gentle agitation at 4 °C. Crypts were liberated and collected by centrifugation (400g for 5 min at 4 °C) followed by washing with PBS to remove EDTA. Crypts in PBS were passed through a 70-μm cell strainer and centrifuged as before. This pellet was resuspended in 50% cell culture medium (described below) and 50% Corning Growth Factor-Reduced Matrigel (Fisher Scientific no. 356231) for culture or dissociated for single cells. Single-cell isolation was carried out as described previously by resuspending crypts in TrypLE Express (Invitrogen no. 1260413) with DNase I (Gold Biotechnology no. D-300-1) for 10 min at 37 °C. Dissociated cells were washed in culture medium without growth factors, centrifuged at 700g, resuspended and passed through a 35-μm strainer (Fisher Scientific no. 08-771-23) and analysed by FACs. Negative staining was carried out with DAPI and single live cells were collected, pelleted and snap-frozen for analysis or resuspended for culture.

Culture of crypts and single cells. Crypts or single cells were maintained as previously described with minimal modification. For regional specific assays of organoid formation the intestine was divided up as follows: the duodenum was classified as the first 4–5 cm, jejunum was classified as the proximal half, and ileum the distal half. The entire colon, excluding the caecum and rectum were pooled and crypts isolated. Medium consisted of advanced DMEM/F12 (Invitrogen no. 12630428) medium prepared with HEPE (Invitrogen no. 15630-080), Glutamax (Invitrogen no. 35050061), and Penicillin–Streptomycin (Invitrogen no. 15140122) with N-2 supplement (Invitrogen no. 17502048). B27 serum-free supplement (Invitrogen no. 17504044) N-acetyl-1-cysteine (1 mM) containing all growth factors (Wnt3A to final concentration 2.5 ng ml⁻¹; Peprotech no. 315-20, Noggin to final concentration 100 ng ml⁻¹; Peprotech no. 250-38, EGF to final concentration 50 ng ml⁻¹; Gold Biotechnology no. 1350-04-500, mBSPO1-FC conditioned medium from 293T cells provided by C. Kuo at Stanford University, USA, as previously described and 50% Matrigel (Fisher Scientific no. 356321). Crypts were resuspended in medium and Matrigel was spotted in the centre of wells in a 48-well plate and allowed to polymerize at 37 °C prior to overlaying with medium. Inhibitors CHIR99021 (3 μM, R & D Systems no. 4423), valproic acid (1 mM made fresh each day, R & D Systems no. 28-151-100) and UK-5099 (10 μM, R & D Systems no. 4186) used to compare organoid formation from crypts were added to the cry Matrigel mixture as well as to the medium overplay and changed every 2–3 days. These drug concentrations were identified as standard from previous studies (CHIR99021: 3 μM, Valproic Acid: 1 mM, and UK-5099 10 μM) but were titrated for efficacy at dosages of 0.5×, 1×, 3× and 9×. Total organoid number and number of crypt domains per organoid were counted after 9 days. For single cells, CHIR99021 and valproic acid were added to maintain Lgr5 stem cells as previously described along with Rho kinase inhibitor Y-27631 (10 μM Fisher Scientific no. 125410), J成语-1 (1 μM AnaSpec, Inc no. AS-61298).

Organoid knockout. Single Lgr5-EGFP-positive cells from MPCI fl/fl × Lgr5-EGFP-IRE-creERT2 were sorted and plated as above. Control genetically matched single cells were cultured under identical conditions (described below) and 50% Corning Growth Factor-Reduced Matrigel (Fisher Scientific no. 200-2040) with Wnt3A (200 nM, Fisher Scientific no. 34-1210). Organoids grown from these were re-sorted for live EGFP-positive cells and plated as above or collected for mRNA analysis. Prior to use of knockout organoid culture in further experiments, loss of MPC1 was verified by western blot where we also looked for a significant destabilization of MPC2. If we could still detect MPC1 protein, cultures were treated with (Z)-4-hydroxytamoxifen (200 mM, Fisher Scientific no. 34-1210). Organoids grown from these were re-sorted for live EGFP-positive cells and plated as above or collected for mRNA analysis. Secondary organoids. Organoids were passaged to single cells as above. Cell viability was determined by exclusion of 0.2% trypan blue (Sigma Aldrich) and both cell number and viability counted using the Cellometer Auto T4 (Nexcelom). Organoid formation and percentage of EGFP positivity from single live cells was measured once mature organoids had formed (7–9 days).

Organoid differentiation. Organoids grown for differentiation were passaged to single cells and allowed to establish for 6–8 days in standard medium. Following this, medium was replaced with medium lacking Wnt3A and Rspo1-conditioned medium (EN) lacking Wnt3A (ENR), or medium supplemented with 9 ng ml⁻¹ Wnt3A (ENR). Organoids were maintained in this medium for 48 h with fresh medium added after 24 h. To promote robust differentiation, IWP-2 (2 μM Fisher Scientific no. 3343-31) and DAPT (10 μM Fisher Scientific no. 26-341-0) were added to organoid medium along with vehicle (dimethylsulfoxide) or UK-5999
described above with TrypLE and DNase followed by resuspension, filtering and described with modifications MPC overexpression.

MPC overexpression. Viral infection of Lgr5 stem cells was carried out as previously described with TrypLE and DNase followed by resuspension, filtering and described with modifications MPC overexpression.

(a) immunofluorescence staining of mouse small intestine. All tissue processing, staining and analysis was done in a blinded manner. Paran sections (34 μm) were blocked/permeablized by treating for 1 h in a humid dark box in 3% BSA, 0.02% azide, PBS. For staining: anti-Lgr5 (1:1000), anti-Cldn2 (1:1000) or anti-Cldn3 (1:1000) was incubated at room temperature for 3 h. Slides were then washed twice with PBS as above and mounted using 100 μg/ml 1.5% DABCO in 100% ethanol, and air-dried. Slides were blocked with 5% NBT/BCIP substrate (Thermo). Slides were then fixed in 10% neutral buffered formalin for 20 min.

Immunohistochemistry. All tissue processing, staining and analysis was done in a blinded manner. Paraffin sections (3–4 μm for immunohistochemistry, 7 μm for in situ hybridization) were cut on positively charged slides (BSC), placed at 60 °C for 30 min, and placed on an automated slide stainer (BenchMark, Discovery, Ventana Medical Systems) for processing. Slides were de-paraffinized with EZ Prep solution, pre-treated with Cell Conditioner 1, pH 8.5, and stained with primary antibody for 1 h at 37 °C (Supplementary Table 5). Secondary antibody (rabbit 1:100 for VDAC1 and MPC1, rat 1:100 for BsdU) was applied and detection performed using iView DAB detection kit followed by haematoxylin counterstaining. Slides were scanned using an automated slide scanner (Zeiss Axio Scan.Z1). For analysis of Olfr4 and Lgr5 staining, images of Swiss-rolled proximal small intestine were used. Areas with a high density of crypts in cross-section that were continuous with villi were used for counting the number of stained cells. Multiple crypts from at least three different areas were counted and the average cell number was calculated per mouse. At least nine control and nine knockout animals were used and images were taken only if the section did not have any cracks or air bubbles. Staining orientation was inadequate for counting. For histological analysis all small intestinal tissue was processed, stained and imaged blinded. Animals were excluded from analysis if tissue orientation, staining or acquisition failed and precluded analysis and for no other reason. For analysis, images of the proximal small intestine were selected that contained areas with crypts in cross-section continuous with villi. Three separate areas and a minimum of ten crypts were analysed and averaged to give a value for that animal used for statistics. IRB for histology of human intestine: U of UT IRB 91019.

Immunofluorescence staining of mouse small intestine. Tissue for immunofluorescence was prepared as follows: the jejunum from LGR5 Cre+ mice was cut and fixed at room temperature in 4% PFA for 2.5 h. This was followed by washing in PBS and equilibrating in OCT on ice then transferred to a chilled mould that was frozen on dry ice and stored at −80 °C. From this block, 10 μm sections were cut and transferred to slides, which were cured for 30 min at room temperature in the dark. OCT mounting media was washed twice, 5 min each using PBS at 37 °C. Sections were blocked/permeabized by treating for 1 h in a humid dark box in 1× PBS+C+Ca+Mg, 0.1% saponin, 10% donkey serum, 3% BSA. For staining: anti-MPC1 Sigma cat. no. HPA045119 in 1× PBS+1% normal donkey serum, 3% BSA-no saponin at 1:100, incubated in the dark overnight at 4 °C. The next morning, slides were washed twice with PBS for 2 min each. Secondary antibody (1:100 (10 μg/ml−1 donkey anti-rabbit IgG Alexa 594 conjugate) +5 μg/ml−1 DAPI in 1× PBS+3% BSA) was incubated at room temperature for 3 h. Slides were then washed twice with PBS as above and mounted using 100 μg/ml−1 PVP mounting medium (Tris buffer +0.02% azide + 5% DABCO +100 μg/ml−1 polyvinylpyrrolidone +1/25 volume glycerol) and cured for 2 h at room temperature.

Human colon crypt isolation and growth. Human colon biopsy samples for organoid cultures were collected under IRB 00551140, Molecular markers of sporadic hyperplastic colon polyps. Patients with a history of inflammatory bowel disease or patients with a personal or family history of familial colon cancer were excluded from the study. Briefly, study patients underwent screening colonoscopy as part of their regular clinical care. Participants scheduled for colonoscopy were not recruited for the study alone. Informed consent was obtained prior to any sedation. The study was approved by the IRB at the University of Utah and VA Medical Center. All samples were labelled with code numbers to protect confidentiality. All procedures were clinically indicated and recommended by the patients’ physician or consultant. As per US preventative services task force (USPSTF), colon cancer screening is recommended for subjects who are 50 years and older for detection and management of colon polyps and colon cancer.

Removing organoid cells from contact with colon tissue is a standard procedure during colonoscopy. The biopsy forceps used to obtain colon biopsies are 2 to 3 mm in diameter. Endoscopic biopsies are commonly taken as part of regular clinical care to diagnose problems and evaluate suspicious tissues. The risks associated with this are minor; patients do not feel the biopsies because the colon does not have pain receptors (just stretch receptors), and bleeding is self-limited. In all situations, minimizing any potential risk to the participants was a priority. If in the clinical judgement of the physician, biopsies should not be taken, they are not. In addition, if taking biopsies will make the endoscopic procedure too long, they will not be taken.

Cryp imaging. Intestinal crypts were isolated as above and transferred to basal medium lacking growth factors. MitofTracker Red CMXROS (Invitrogen no. M7512) was prepared according to the manufacturer’s instructions and added to suspended crypts in basal medium at a final concentration of 50 nM and incubated at 37 °C for 20 min.

Organoid imaging. Live organoids were imaged on the Axio Observer Z1 imaging system (Carl Zeiss) fitted with a heated humidity-controlled stage (LiveCell 05-11-0032 Rev B) and analysed using Zen 2 software (Carl Zeiss). The laser intensity was kept constant when making comparisons of organoids and adjustments for brightness and contrast were made to the entire image equally across groups being compared.

In situ hybridization. For detection of Lgr5 and Olfn4 mRNA, we designed riboprobes targeting the coding sequence to the 3’UTR region of these genes (Supplementary Table 5). Targeted probe region DNAs were amplified using standard PCR, with the amplicon length of about 500 bp. These DNA amplicons were used to generate DIG-labelled riboprobes using T7 RNA polymerase. For staining, formalin-fixed paraffin-embedded tissue sections were de-paraffinized with Citrisolv, washed in 100% ethanol, and air-dried. Slides were blocked with Boehringer Blocking Reagent (Roche) and yeast RNA at 68 °C for 1 h. Probes (0.5 mg ml−1) were hybridized to sections at 68 °C overnight. After washing, sections were again blocked with Boehringer Blocking Reagent and 10% sheep serum at room temperature for 1 h. Alkaline-phosphatase-labelled sheep anti-DIG antibody (Roche) was incubated with sections at 1:2,000 dilution at 4 °C overnight. After washing, positive signals were developed using NBT/BCIP substrate (Thermo). Slides were then fixed in 10% neutral buffered formalin and mounted with aqueous mounting medium.

RNA extraction and analysis. Lgr5-GFP sorted cells were directly lysed in buffer RLT (Qiagen no. 79216) containing 2-mercaptoethanol (Sigma Aldrich no. M6250) and total lysate was assayed for transcript count as per the manufacturer’s instructions. Total RNA was purified from organoids using the RNeasy Mini Kit (Qiagen) with 100 ng used for the assay.

mRNA analysis via NanoString. Transcript abundance was compared using the NanoString Elements platform. A custom gene set was created containing intestinal stem cell and differentiation genes including five housekeeping genes (Actb, B2m, Clc, Gusb, Rpl19, Tbp and Ywhaz). nSolver analysis software was used for data analysis with raw counts normalized to positive control probes and housekeeping genes. Only samples passing quality control were used for normalization and analysis. Data were visualized with Java Treeview and graphed using Prism 7 (GraphPad). Source data for generation of heat maps in the transcript are provided in Supplementary Tables 1–4. Sequences for probe design and source data for the generation of heat maps are provided in Supplementary Table 6.

Immunoblot analysis. Biological replicate organoids from independent single-cell isolations were harvested, washed in cold PBS and snap-frozen. Pellets were resuspended in tissue lysis buffer containing protease and phosphatase inhibitors as previously described. Samples were disrupted by sonication, cleared by centrifugation at 10,000g for 5 min, and protein concentration was determined by BCA assay (Thermo Scientific) and diluted to allow for loading equal volumes. For histone acetylation assays, organoids were collected and nuclei were purified using
For fatty acid oxidation, substrate-limited medium was prepared as adapted from the stem cell recipe (all concentrations were kept the same except B27 supplement was 66.6 µl, t-glucose 0.5 mM, 1 mM glutamine and carnitine 0.5 mM per 5 ml of 4x medium). The following drugs were added to substrate-limited medium: 3 mM CHIR99021, 1 mM valproic acid, 2.0 mM MgSO₄, 2.0 mM Na₂HPO₄, + 2.5 mM t-glucose, 0.5 mM t-carnitine, and 5 mM HEPES buffer (pH 7.4). The following drugs were added to 1x: KHB; 3 mM CHIR99021, 1 mM valproic acid, ±10 µM UK-5099. Following medium change into 1x KHB, the plate was maintained at 37°C in a non-CO₂ incubator. At 30 min post media change, 40 µM final Etomoxir (R & D Systems cat. no. 45393) or vehicle was added; and at 45 min post media change, BSA or palmitate (Agilent cat. no. 102720-100) was added according to the manufacturer’s protocol. Following the addition of the BSA or palmitate substrates, the assay was run in the XF96e Analyzer for the Mito Stress Test. The assay protocol was adapted as follows (three measurements per phase, acute injection followed by 3 min mixing, 3 min waiting, 3 min measuring) with the final concentrations of drugs as follows (oligomycin 2 µM, FCCP 2 µM, rotenone 0.5 µM and antimycin A 0.5 µM). Normalization to cellular protein was quantified by Pierce BCA assay (Fisher Scientific cat. no. 23227) and read on a photometric plate reader (Varioskan Flash). Results were analysed in WAVE software and processed through the XF Mito Stress Test Report and Glycolysis Stress Test Generators.

**ENCODE Chip-seq for MPCI promoter.** This track shows Chip-seq data from the Myers Lab at the Hudson Alpha Institute for Biotechnology and by the laboratories of M. Snyder, M. Gerstein and S. Weissman at Yale University, P. Farnham at the University of Southern California, K. Struhl at Harvard, K. White at the University of Chicago, and V. Iyer at the University of Texas, Austin. These data were processed into uniform peak calls by the ENCODE Analysis Working Group pipeline developed by A. Kundaje. The clustering of the uniform peaks was performed by UCSC (https://genome.ucsc.edu/cgi-bin/hgGateway). The Factorbook motif identifications and localizations (and valuable assistance with interpretation) were provided by J. Wang, B. H. Kim and J. Zhuang of the Zlab (Weng Lab) at UMass Medical School.

**Statistics and reproducibility.** No pre-specified effect size was calculated, and no statistical method was used to predetermine sample size. For comparisons of multiple groups, an ordinary one-way ANOVA with Dunnett or Holm–Sidak correction for multiple comparisons (Fig. 1a groups compared with low, 1c groups compared with EN, 4f groups compared with wENR) was used. Statistical tests were appropriate for comparisons being made; for all t-tests and ANOVA, data meet assumptions of the tests; assessment of variation was carried out but not included. For mouse studies, an n of 10 was chosen for control (equal Lgr5-EGFP heterozygous × WT/WT and Lgr5-EGFP wild-type × Mpc1 fl/fl) and knockout (Lgr5-EGFP heterozygous × Mpc1 fl/fl) comprised of both males and females. No statistical method was used to predetermine sample size. Experiments were not randomized. Investigators were not blinded to allocation during experiments and outcome assessment. Reproducibility: Fig. 2a: representative of two experiments; Fig. 2b: representative of two experiments; Fig. 2c: representative of three experiments (pooled); Fig. 2d: representative of three experiments: Fig. 2e: representative of two experiments; Fig. 2f: representative of two experiments; Fig. 2g: representative of two experiments; Fig. 2h: representative of two experiments; Fig. 2i: representative of two experiments; Fig. 3a: representative of two experiments; Fig. 3b: representative of two experiments; Fig. 3c: representative of two experiments; Fig. 3d: representative of two experiments; Fig. 3e: representative of two experiments; Fig. 3f: representative of two experiments; Fig. 3g: representative of two experiments; Fig. 3h: representative of two experiments; Fig. 3i: representative of two experiments; Fig. 3j: representative of two experiments; Fig. 3k: representative of two experiments; Fig. 3l: representative of two experiments; Fig. 3m: representative of two experiments; Fig. 3n: representative of two experiments; Fig. 3o: representative of two experiments; Fig. 3p: representative of two experiments; Fig. 3q: representative of two experiments; Fig. 3r: representative of two experiments; Fig. 3s: representative of two experiments; Fig. 3t: representative of two experiments; Fig. 3u: representative of two experiments; Fig. 3v: representative of two experiments; Fig. 3w: representative of two experiments; Fig. 3x: representative of two experiments; Fig. 3y: representative of two experiments; Fig. 3z: representative of two experiments; Fig. 4a: representative of two experiments; Fig. 4b: representative of two experiments; Fig. 4c: representative of two experiments; Fig. 4d: representative of two experiments; Fig. 4e: representative of two experiments; Fig. 4f: representative of two experiments; Fig. 4g: representative of two experiments; Fig. 5a: representative of two experiments; Fig. 5b: representative of two experiments; Fig. 5c: representative of two experiments; Fig. 5d: representative of two experiments; Fig. 5e: representative of two experiments; Fig. 5f: representative of two experiments; Fig. 6a: representative of two experiments; Fig. 6b: independent infections, data pooled and Supplementary Fig. 2a: representative of three experiments; Supplementary Fig. 4b: representative of three experiments; Supplementary Fig. 6a: representative of two experiments and Supplementary Fig. 6b: two independent infections, data pooled (as in Fig. 6d).

**Code availability.** The software tools used for ENCODE are freely available at: https://genome.ucsc.edu/ENCODE/encodeTools.html.

**Data availability.** Previously published ENCODE Chip-Seq data for Myc that were re-analysed here are available under UCSC accession codes: wgEncodeEH000545, wgEncodeEH000670, wgEncodeEH002800, wgEncodeEH000536 and wgEncodeEH001133. Data corresponding to mRNA transcript abundance have been deposited at GEO under the accession numbers GSE108830 and GSE108831.
Metabolomics data have been deposited in Figshare and can be accessed under http://dx.doi.org/10.6084/m9.figshare.5117269.v1.

Source data for the figures have been provided as Supplementary Table 7. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Supplementary Figure 1 Additional images of flow gating and IHC in mouse and human.

a, Gating strategy for sorting of Lgr5-EGFP positive stem cells for transcript abundance. Top showing final gating for collection of GFP-low, GFP-mid, and GFP-high cells for transcript abundance. Bottom showing full gating strategy exclusion of cell debris and purification of singlet cells.

b, IHC of VDAC1 and MPC1 throughout the mouse small intestine from proximal to distal showing staining of crypt base and differentiating transient amplifying cells. (scale bar = 20 um).

c, Human Protein Atlas (http://www.proteinatlas.org) images of duodenum, small intestine, and colon stained for TFAM, NRF1, PDK1, and CK20 (scale bar = 20 um (b), 100 um (c)).
Supplementary Figure 2  Drosophila MPC regulates stem cell proliferation. 

a, esg-GAL4 used to target RNAi as indicated or MPC overexpression (o/e) under infected conditions (scale bar = 50 μm). pH3+ cells was quantified per intestine. (Control n=10 (no infection) Control (with infection) n=10, MPC O/E n=10, LDH RNAi n=10 MPC RNAi n= 10 PDH RNAi n=10) b, Western blot analysis to detect dMPC1 protein in control animals, dMPC1 mutants, HSP70-GAL4 controls, and HSP70-UAS-dMPC1-dMPC2 transgenic animals following a 30-minute heat treatment and four hour recovery. Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. All p values were calculated using Student’s t-test. Unprocessed western blots are provided in Supplementary Figure 7.
**Supplementary Figure 3** MPC1 allele and additional quantification of in vivo phenotype

**a**, Schematic of MPC1 allele used for inducible knockout. **b**, Close-up images of villi tip from MPC1 knockout stained for H&E, Alcian Blue, MPC1, VDAC and CK20 (scale bar = 20 μm). **c**, Body weight for control and MPC1 knockout animals 30 days post-treatment. (n = 5 male control and knockout, n = 6 female control, n = 3 knockout) **d**, Total crypt+villi height and crypt height from individual regions of the small intestine. (n = 9 control, n = 10 knockout for crypt+villi, n = 10 control, n = 9 knockout for crypt height from each region) **e**, Quantification of Alcian Blue positive cells per area of the proximal small intestine. (n = 10 control and knockout) **f**, Quantification for BrdU positive cells per crypt of proximal small intestine. (n = 9 control, n = 10 knockout) **g**, Images of Olfm4 and Lgr5 in situ in control and MPC1 knockout. (scale bar = 50 μm) **h**, Additional quantification of in situ staining. (n = 9 control, n = 10 knockout) **i**, Quantification of Lrig1 IHC staining in control and MPC1 knockout proximal small intestine. (n = 9 control, n = 10 knockout) Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. All p values were calculated using Student’s t-test.
**Supplementary Figure 4** Additional characterization of in vivo Mpc1 Lgr5-KO organoids showing stem and differentiation marks (n = 3). a, Flow cytometry profile for organoids from control and MPC1 knockout cultures showing GFP positivity. b, Long-term cultures retain increased growth ability throughout extended passaging (over ~2 months) (n = 15 control and knockout, scale bar = 200 um). c, Control and MPC1 knockout organoid western blots for MPC1 and MPC2 and pyruvate dehydrogenase (phospho 293 and total). d, Transcript abundance of MPC1 knockout organoids showing stem and differentiation marks (n = 3). e, Quantification of specific Acetyl Histone H3 marks in control and knockout organoids (paired control set to 1.0) (n = 8 organoids). f, Steady state metabolite abundance for control and MPC1 knockout organoids (n = 6 control and knockout). Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. All p values were calculated using Student’s t-test. Unprocessed western blots are provided in Supplementary Figure 7.
Supplementary Figure 5 Effects of UK-5099 in maintaining organoids. 
a, Organoid formation from different regions of small intestine and colon with treatment conditions (n = 6 per condition, p values by t-test with respect to wENR for each location). 
b, Loss of passaging efficiency in crypts maintained in wENR compared to UK-5099. When identical cell numbers are passaged normally, wENR treated are more likely to fail to passage. Images are of multiple matrigel/organoid spots of identical size (5uL) on a 6 well plate tiled to make one image (organoids from n = 3 wells were counted and number per well used for quantification, scale bar = 2000 um). 
c, Formation of hyperintense lumens in crypt organoids grown in wENR compared to UK-5099 3 days after changing media to remove CHIR99021 and Valproic acid (n = 69 total organoids from 6 individual wells). 
d, Dose curves for individual drugs in supporting organoid formation from crypts (n = 8 wENR, all other conditions n = 6, p value by t-test with respect to wENR). Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. All p values were calculated using Student’s t-test.
Supplementary Figure 6  Effects of UK-5099 and MPC over expression

(a) Flux profiling of U-C13 Glucose incorporation into Citrate for tMEF and C2C12 cell lines treated with UK-5099 or Valproic Acid over 4 hours (n = 3 per condition per timepoint).  

(b) Representative images of infected Lgr5-EGFP (green) organoids with either Empty Vector-mCherry (in red) or MPC1-iRFP (in blue) and MPC2 mCherry (in red). Traces below represent intensity of each color over the length of the line outlined by white dotted line in the image. (n = 4 organoids with at least 15 infected cells per organoid counted).  

(c) ENCODE track for Myc and associated transcription factors localized to the Mpc1 promoter.  

(d) Model where crypt homeostasis involves low pyruvate oxidation at the base which increases during differentiation. MPC1 loss leads to reduced pyruvate oxidation, increased glycolysis, increased proliferation, and an expansion of the stem cell compartment within the crypt. Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. All p values were calculated using Student’s t-test.
Supplementary Figure 7: Unprocessed Westerns

1, 2, 5, 6, 13, 14, 15, 16, 17, 20, 21, 28, 29: Marker
3, 18: Villi
4, 19: Crypt
7, 22: Control 1
8, 23: MPC1 Lgr5-KO 1
9, 24: Control 2
10, 25: MPC1 Lgr5-KO 2
11, 26: Mutated Line B control
12, 27: Mutated Line B MPC1 KO

Membrane 1, 2: Secondary (rabbit) 680nm channel
Membrane 1, 2: Secondary (mouse) 800nm channel

Supplementary Figure 7
- Unprocessed western blot membranes.
  a, Unprocessed western blot images to accompany Figure 5a.
  b, Unprocessed western blot images to accompany Figure 5b.
  c, Unprocessed western blot images to accompany Supplemental Figure 4e.
  d, Unprocessed western blot images to accompany Supplemental Figure 4c.
Supplementary Figure 7 Continued
Supplementary Figure 7 Continued

1. Lane Marker
2. Control Organoid 1
3. Knockout Organoid 1
4. Control Organoid 2
5. Knockout Organoid 2
6. Control Organoid 3
7. Knockout Organoid 3
8. Lane Marker
Supplementary Table Legends

**Supplementary Table 1** Single cell sort based on EGFP low, mid, and high z-scores for heat map in Figure 1b.

**Supplementary Table 2** Transcript z-scores for organoids grown in EN, ENR, and ENR +Wnt3a used to generate heatmap in Figure 1d.

**Supplementary Table 3** Transcript z-scores for Lgr5-EGFP Control vs. MPC1-KO organoids used to generate heat map in Supplemental Figure 4d.

**Supplementary Table 4** Transcript z-scores for organoids grown in wENR or UK-5099 used to generate the heat map in Figure 6j.

**Supplementary Table 5** Information on antibodies used in this work and probe sequences for in situ hybridization.

**Supplementary Table 6** Nanostring primer design information.

**Supplementary Table 7** Statistics Source Data
Experimental design

1. Sample size
   Describe how sample size was determined.

   No sample-size calculation was performed. An n of 10 was used for in vivo studies due to the high degree of variability. For in vitro studies a minimum of an n of 3 was used where sample was limiting and if possible an n of 6 was used.

2. Data exclusions
   Describe any data exclusions.

   No data was excluded from analysis for any reason other than being unusable. For histology this encompasses a lack or heterogeneity of stain or improper section orientation that precluded analysis. For in vitro experiments an unusable sample would be if a sample was lost or not detected in the case of metabolomics.

3. Replication
   Describe whether the experimental findings were reliably reproduced.

   No attempts at replication of experiments in the paper failed. Data in this paper was reproducible: numbers of experiments and source data has been provided.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.

   Animals and in vitro cultures were paired and distributed into control and experimental groups for side-by-side comparison. No formal allocation/randomization was done; animals were taken as they became available.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   For experiments involving histology animals were allocated into control and experimental. Throughout collection of tissue, sectioning, staining, and analysis researchers were blinded and unblinded only after results had been tabulated. For in vitro cultures, treated organoids were analyzed and quantification of was done before unblinding. Analysis for metabolomics, western blot, flow cytometry was also carried out blinded.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ❌ | ✓ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ❌ | ✓ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ❌ | ✓ A statement indicating how many times each experiment was replicated |
| ❌ | ✓ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
| ❌ | ✓ A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ❌ | ✓ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted |
| ❌ | ✓ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ❌ | ✓ Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.

7. Software

Policy information about availability of computer code

Describe the software used to analyze the data in this study.

Graphpad Prism 6 was used to analyze and graph data for this work. Types of graphs and tests have been described for all data.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials are available from the authors upon request or purchased commercially (vendor and catalog numbers provided).

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

All antibody catalog numbers and dilutions have been provided in Supplementary Table 5. For histology, immunofluorescence, and western blotting positive and negative control samples were used to confirm specificity and validated in the species being tested.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

b. Describe the method of cell line authentication used.

c. Report whether the cell lines were tested for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

Eukaryotic cell lines

- tMEFs were provided by Dean Tantin and C2C12 cells were purchased from ATCC.
- Cell lines were not authenticated.
- Cell lines were tested for mycoplasma contamination and found to be negative upon receipt but not tested immediately prior to the experiments in this work.
- No cell lines used in this work are listed as commonly misidentified by ICLAC. Cell lines used were for basic studies on the ability of Valproic Acid and UK-5099 to block mitochondrial pyruvate metabolism.
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Mice used in this study were purchased from Jackson Labs (Lgr5-EGFP) or generated from ES cells as previously published (Mpc1 fl/fl). For in vivo knockout experiments 6- to 8-week-old mice were used. No formal randomization was performed for animal studies, animals were used as they came available, paired with littermate controls where possible, there was no preference for male or female animals, both genders were included in each analysis. For Drosophila studies, mutant dMPC11 and dMPC12 flies have been previously described (reference 70); RNAi transgenic stocks are as follows: UAS-dMPC1 RNAi and UAS-dMPC2-RNAi, UAS-PDH-E1 (VDRC101856), UAS-PDH-E2 (VDRC 102893), and UAS-Notch-RNAi. All Drosophila studies were done following standard ethical guidelines for working with this organism. As is standard in studies of Drosophila intestinal stem cells, all experiments were performed in females, which have a higher rate of basal stem cell proliferation than males. Experiments were conducted in adults aged four days to three weeks after eclosion.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

During colonoscopy a biopsy or tissue was collected from the Ascending Colon. This was used to liberate crypts as previously described for in vitro growth assays in different drug treatment conditions. Biopsies were collected from healthy female patients undergoing routine colonoscopy between the ages of 51-60.
## Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

### Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

### Methodological details

5. Describe the sample preparation.

   Crypts were isolated from mice as described or organoids were dissociated from matrigel. Single cells were purified by TrypLE and DNase treatment, filtered, and resuspended in basal media. DAPI was used as an additional live/dead stain or gating was based on cell size to exclude debris and exclude doublets.

6. Identify the instrument used for data collection.

   BD Canto and BD FACS Aria

7. Describe the software used to collect and analyze the flow cytometry data.

   BD FACSDIVA and FlowJo v.10

8. Describe the abundance of the relevant cell populations within post-sort fractions.

   Populations of GFP positive cells (low mid and high) resulting from mice were between 0.5 and 5%. GFP positive cells from in vitro organoids ranged from 3.9 to 40% depending on experimental condition.

9. Describe the gating strategy used.

   Side Scatter and forward scatter area were used as an initial gate for cell debris and aggregates. Side scatter height by side scatter width followed by forward scatter height by forward scatter width were used to isolate singlets. Gating for GFP positive cells was set in reference to a negative wild-type control culture. GFP high, mid, and low population gates were set to allocate approximately 1/3 of the positive cells in each group for collection. An exemplar of this gating strategy is shown in Supplemental Figure 1a.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☑️