

## Supporting Information

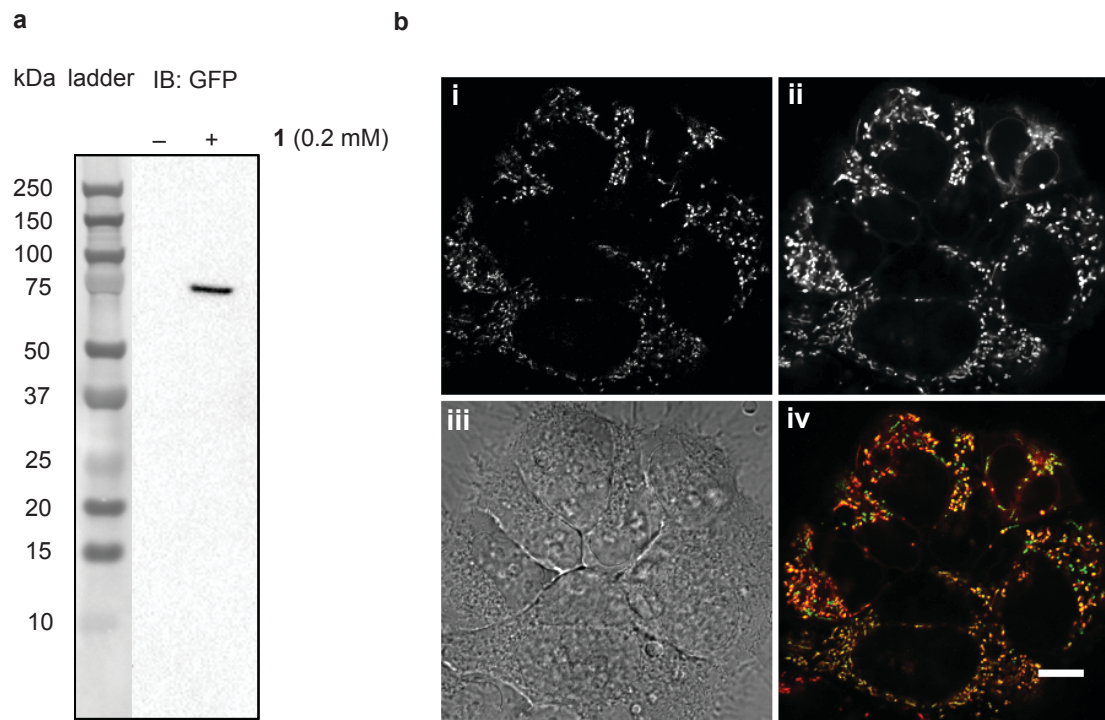
### **Photoactivation of mutant isocitrate dehydrogenase 2 reveals rapid cancer-associated metabolic and epigenetic changes**

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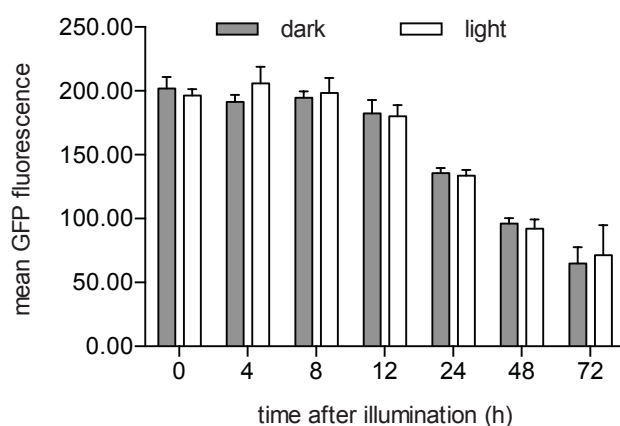
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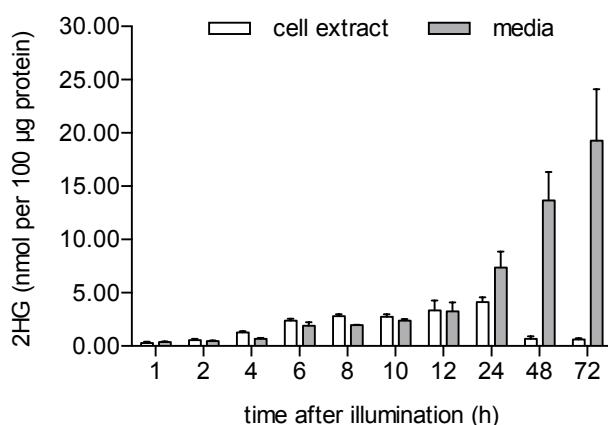
## Supplementary Figures



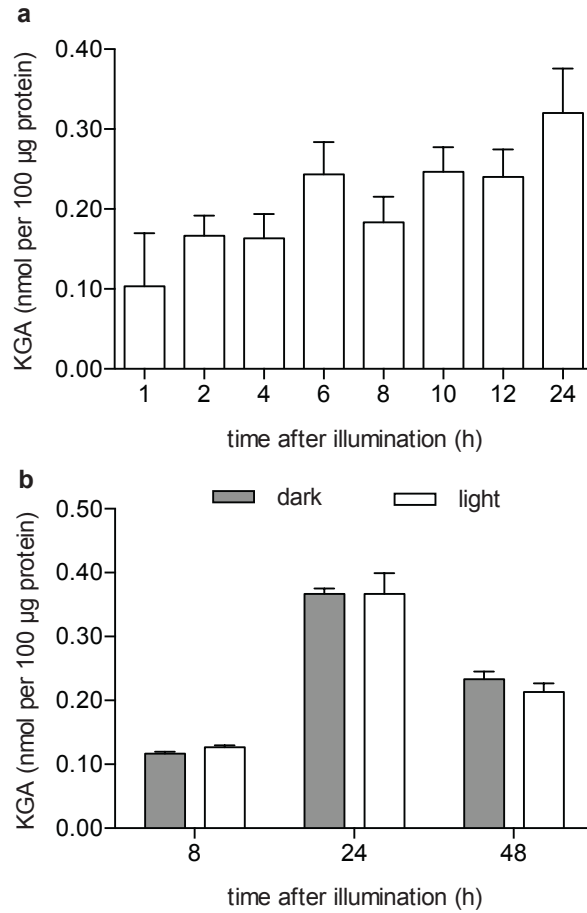
**Figure S1. Confirming the expression of IDH2(R172TAG)-GFP protein.** (a) Immunoblot (IB) of HEK293 cells, stably expressing IDH2(R172TAG)-GFP and the PCKRS/tRNA<sub>CUA</sub> pair, in the presence (+) and absence (-) of 0.2 mM PCK, **1**, using anti-GFP antibody. This shows the PCK-dependent expression of IDH2(R172TAG)-GFP protein, with a molecular weight of 78 kDa. (b) Mitochondrial co-localization of IDH2(R172TAG)-GFP protein was confirmed in several cells using MitoTracker Red CMXRos. i) GFP ii) MitoTracker iii) DIC iv) Merge of GFP and MitoTracker. Scale bar is 10  $\mu$ m.



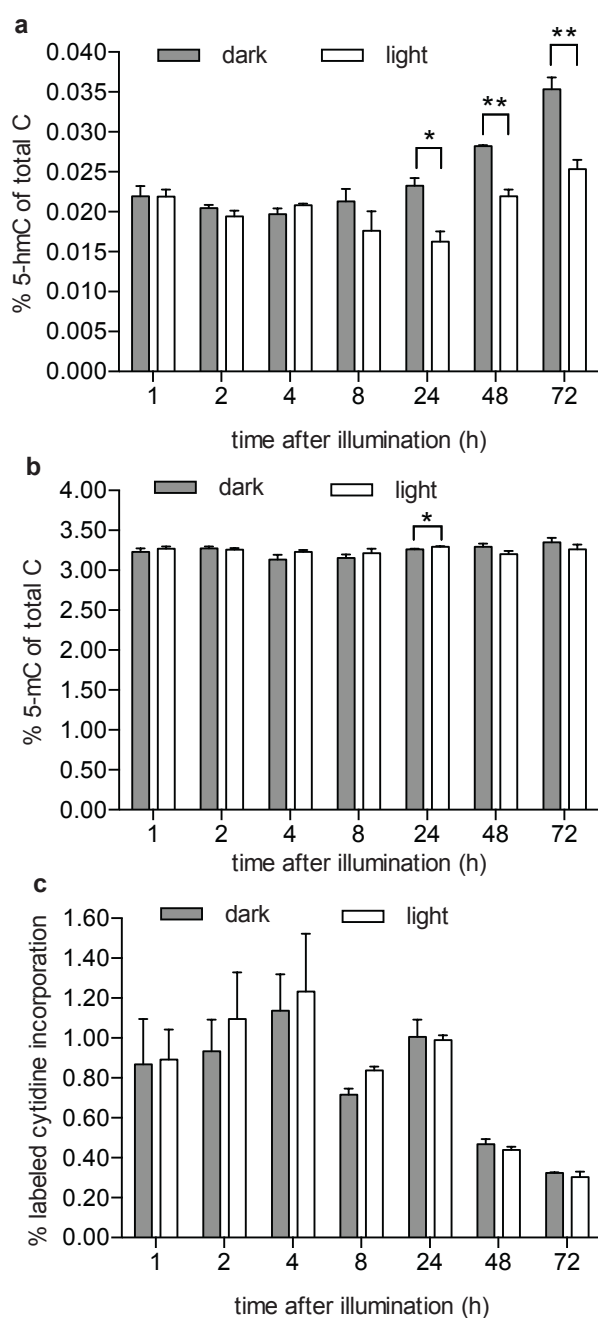
**Figure S2. Measuring the half-life of the IDH2(R172PCK)-GFP protein.** Mean GFP expression was quantified by flow cytometry at various time points after lysine 1 withdrawal at 0 h, preventing further photocaged protein synthesis. The half-life of the mutant protein was reached at approximately 48 h. Error bars represent the standard deviation of the mean of at least three biological replicates.



**Figure S3. Measuring cellular and media 2HG at later time points.** Cellular 2HG values for 0–24 h (from Figure 2b) were re-plotted with media 2HG levels, but this time including measurements for 48 h and 72 h. There is a drop in cellular 2HG at 48 h and 72 h to 0.69 and 0.61 nmol per 100 µg of protein respectively. This can, at least in part, be explained by the accompanied increase in media 2HG levels. The lag in media 2HG over the first 10 h suggests that the translocation of the metabolite out of the cell only occurs significantly once cellular 2HG concentrations above 4.14 nmol per 100 µg protein are reached at 24 h. All values were quantified by LC-MS/MS in HEK 293 cells stably expressing IDH2(R172TAG)-GFP and the PCKRS/tRNA<sub>CUA</sub> pair, normalizing to total cellular protein content. Error bars represent the standard deviation of the mean of at least three biological replicates.



**Figure S4. KGA levels do not change significantly as a function of illumination.** (a) KGA appears to increase over time on illuminating cells stably expressing IDH2(R172TAG)-GFP and the PCKRS/tRNA<sub>CUA</sub> pair. However, direct comparison of dark and light-treated samples at three time points in (b) confirms that there are no significant differences in KGA values between samples left in the dark and those illuminated at 365 nm for 60 s at any time point: 8 h ( $P=0.10$ ), 24 h ( $P=0.99$ ) or 48 h ( $P=0.33$ ), by Student's *t*-test. All values were normalized to total cellular protein content. Error bars represent the standard deviation (a) or standard error (b) of the mean of at least three biological replicates.



**Figure S5. Exploring the effect of mutant IDH2 activation on global DNA base modification levels.** Global 5-hmC and 5-mC was measured by LC-MS/MS in HEK 293 cells stably expressing IDH2(R172TAG)-GFP and the PCKRS/tRNA<sub>CUA</sub> pair at various time points after illumination at 365 nm. (a) There is a significant decrease in the percentage of 5-hmC of total cytosine in cells illuminated at 365 nm for 60 s in comparison to those left in the dark, at 24 h ( $P=0.012$ ), 48 h ( $P=0.002$ ) and 72 h ( $P=0.006$ ), Student's t-test. (b) The percentage of 5-mC of total cytosine in cells illuminated at 365 nm does not change significantly in comparison to those left in the dark for the majority of time points. (c) The percentage incorporation of an isotopically-labelled nucleoside analogue into nascent DNA, as a measure of cell proliferation. The cytidine molecule, 1',2',3',4',5'-<sup>13</sup>C<sub>5</sub>-5,6-D<sub>2</sub>-1,3-<sup>15</sup>N<sub>2</sub>-cytidine, was used, because of its availability in the lab. The label was supplied in the media one

hour prior to harvesting cells for nucleoside analysis at each time point. Percentage label incorporation was calculated as the ratio of labeled cytidine to the sum of labeled- and unlabeled cytidine. All measurements were made by LC-MS/MS. There is a decrease in label incorporation at 48 h and 72 h, suggesting that cells have reached confluency. This drop in cell proliferation helps explain the increase in global 5-hmC levels at 48 h and 72 h seen in (a): 5-hmC has been shown to be a predominantly stable modification and is formed from 5-mC which is 10–25 h old.<sup>1</sup> As cells reach confluency and proliferation drops, TET activity persists, resulting in the accumulation of 5-hmC at 48 h and 72 h. The increase is less pronounced in cells expressing photoactivated IDH2 (light), suggesting that 5-hmC production is being inhibited. In addition, the drop in cell proliferation will hamper the replication-dependent dilution of old 5-hmC and could therefore delay the recovery of 5-hmC levels. This could help explain why 5-hmC depletion persists in light-activated samples at 48 h–72 h despite the drop in cellular 2-HG levels at these later time points. Error bars represent the standard deviation (a,b) or standard error (c) of the mean of at least three biological replicates.

## Materials and Methods

### Reagents

Photocaged lysine **1** was prepared as reported previously.<sup>2</sup> MitoTracker Red CMXRos was purchased from Invitrogen. Western Blot analysis was performed using an antibody against turboGFP (abcam 513). turboGFP is referred to as GFP throughout.

For LC-MS/MS, 2-hydroxyglutaric acid (2HG) and  $\alpha$ -ketoglutaric acid (KGA) reference standards were obtained from Sigma. Internal isotopically labeled standards (SIL) for 2-hydroxyglutaric acid (2,3,3-D<sub>3</sub>; OD) and  $\alpha$ -ketoglutaric acid (1,2,3,4-<sup>13</sup>C<sub>4</sub>) were purchased from Cambridge Isotope Laboratories. 1',2',3',4',5'-<sup>13</sup>C<sub>5</sub>-5,6-D<sub>2</sub>-1,3-<sup>15</sup>N<sub>2</sub>-cytidine was synthesized using published procedures.<sup>3</sup>

### DNA constructs

One shot stbl3 chemically competent *Escherichia coli* (Invitrogen) were used for cloning steps. IDH2(R172TAG)-GFP and the PCKRS/tRNA<sub>CUA</sub> pair were cloned into PiggyBac vectors using a similar strategy as described previously.<sup>4,5</sup>

The TAG codon was introduced into the IDH2-GFP construct (Origene, RG201152) by site-directed mutagenesis using primers R172TAG\_2f/R172TAG\_2r and AccuPrime Pfx Supermix (Life Technologies). The region coding IDH2-172TAG-GFP was amplified using primers IDH2\_BamHI\_15f/tGFP\_BamHI\_15r. The PCR fragment was inserted into the BamHI site of a PiggyBac targeting vector (PB533A, System Biosciences)

using the In-Fusion HD cloning kit (Clontech) resulting in pPB-IDH2-172TAG-GFP. PB533A carries an EF1 promoter and Neo selection marker expressed from an IRES.

The plasmid allowing the stable integration of the orthogonal PCKRS/tRNA<sub>CUA</sub> pair, pPB-4xU6-pyIT-PCKRS, was derived from a PiggyBac targeting vector (PB510B1, System Biosciences). PCKRS was cloned into a BamHI site after the CMV promoter. Four copies of a U6-PyIT cassette, flanked by SpeI and AvrII restriction sites, were sequentially cloned into the SpeI site of PB510B1. PB510B1 also carries a Puro selection marker driven by an EF1 promoter. pPB-IDH2-172TAG-GFP and pPB-4xU6-pyIT-PCKRS were used to generate stable cell lines, as outlined below.

## **Cell Culture and transfection**

Human embryonic kidney 293 cells (Flp-In<sup>TM</sup>– 293, Life Technologies) were grown in Dulbecco's modified Eagle's medium (DMEM) + GlutaMAX (Gibco) with 10% fetal bovine serum (HyClone) at 37°C in a 5% CO<sub>2</sub> atmosphere.

Cell lines were generated following the PiggyBac Transposon System protocol (System Biosciences). 48 h after transfection of the constructs pPB-IDH2-172TAG-GFP, pPB-4xU6-pyIT-PCKRS and the PiggyBac Transposase expression vector (System Biosciences) using TransIT-293 (Mirus Bio), stable clones were selected for 3 days with the antibiotics G418 (100 µg/ml) and Puromycin (5 µg/ml) (InvivoGen). 0.2 mM photocaged lysine was added to antibiotic-selected cells for 48 h and single GFP positive stable clones were sorted by flow cytometry and propagated.

For photoactivation experiments, cells were grown in media containing 0.2 mM photocaged lysine **1** for 72 h. One hour prior to light-activation, the media was replaced with fresh DMEM+GlutaMAX+fetal bovine serum without amino acid **1**. Photoactivation was carried out for 60 s using a 365 nm LED array positioned under the cell culture plate with a LED power of 9.5 mW per cm<sup>2</sup>.

## **Flow cytometry**

Cells were washed with PBS, detached with 1X trypsin-edta (Gibco) and resuspended in PBS. The GFP fluorescence of samples was analyzed on an Eclipse Flow Cytometry Analyser (iCyt, Sony) equipped with three co-linear lasers (405 nm, 488 nm and 642 nm). GFP fluorescence was measured using the 488 nm laser and 525/50 nm optical filter. Single live cells were gated using SSC/FSC. For single cell sorting during stable cell line generation, a MoFlo Legacy cell sorter (Beckman Coulter) was used. GFP was excited using a 488 nm laser and emission was collected using a 530/40 nm band pass optical filter. Data was processed using FlowJo software.

## **Immunoblotting**

Cells were washed with phosphate buffered saline (PBS) and lysed with RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Roche). Samples were resolved by SDS-PAGE and analyzed by immunoblotting with anti-turboGFP (Evrogen ab513) after transfer to a nitrocellulose membrane. Image Lab software (Biorad) was used.

## **Microscopy**

Mitochondrial co-localization experiments: cells were grown in Lab-Tek chambered coverglass (Thermo Scientific), in media supplemented with 0.2 mM photocaged lysine **1** for 72 h. They were then incubated with MitoTracker Red CMXRos (Invitrogen) for 30 min, following the manufacturer's protocol. Cells were visualized on a Zeiss LSM 780 inverted confocal microscope with a 40x magnification. Microscope settings: scan resolution 1024x1024, averaging 2, scanning speed 30.98. Images were processed using ImageJ software.

## **Liquid Chromatography**

LC-MS/MS methods were developed by PK/Bioanalytics core facility, Cancer Research UK Cambridge Institute.

### Metabolite measurements

#### *Sample preparation*

After having washed cells with PBS, cellular metabolites were extracted by addition of 80% aqueous methanol directly to the culture well.<sup>6</sup> Methanol-suspended cells were placed at -80°C for a minimum of an hour, thawed, sonicated for one minute and centrifuged. The resulting supernatant was dried and reconstituted in 100 µL of 0.1% formic acid in water, followed by the addition of 50 µL of internal standard working solution. Samples were injected into the LC-MS/MS for quantitative analysis. Calibration standards and isotopically-labeled internal standards (SIL) were included (details given above in reagents section).

#### *Preparation of calibration standards*

10 mM stock solutions of 2HG or KGA were prepared in 0.1% formic acid in water. These were diluted in 0.1% formic acid in water to form calibration and quality control standards. Calibration standards ranged from 0.125 to 500 µM.

#### *Chromatographic Conditions*

The LC system used consisted of an Agilent 1290 pump with an autoinjector, sample cooler and column heater. The sample cooler and column temperatures were 4 °C and 40 °C respectively. Analyte separation was

performed on a Waters ACQUITY UPLC™ HSS T3 1.7  $\mu\text{m}$  100 x 2 mm ID column. The mobile phases were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) delivered at 0.3 ml/min. The gradient program started with 97% A for 2.5 min, decreased to 5% A over 0.5 min, remained at 5% A for 0.5 min and then increased to 97% A for 0.01 min. Finally, it was held at 97% A for 1.49 min to give a total run time of 6 min.

#### *MS conditions*

An ABSciex 6500 fitted with an electrospray (ESI) probe, operating in negative mode with a source temperature of 350 °C, was used. Compounds were manually optimized utilizing Analyst software by infusing reference standards of 2HG/KGA and internal standards. The most abundant and selective transitions were found to be the following:

Analyte	Precursor ion (m/z)	Product ion (m/z)
2HG	147	129
KGA	145	101
2HG SIL	150	132
KGA SIL	149	105

#### *Calculations*

Metabolite data was normalized to the total protein content of that particular sample. This was measured by Pierce BCA protein assay on methanol-extracted cell pellets, which were resuspended in 1% SDS (Thermo Scientific). Units in nmol were converted into approximate cellular concentrations, using the following parameters: approximate cell volume of 2425  $\mu\text{m}^3$  (Bionumbers) and calculated cell protein content of 300 pg.<sup>7</sup>

#### Nucleoside measurements

Global 5-methylcytosine and 5-hydroxymethylcytosine levels were quantified by LC-MS/MS as described previously.<sup>1</sup>

#### **Primers**

Name	Sequence
R172TAG_2f	CTGGTCGCCATGGGCGTGCTAGCCAATGGTGATGGGCTTG
R172TAG_2r	CAAGCCCATCACCATTGGCTAGCACGCCCATGGCGACCAG
IDH2BamHI_15f	AATTTAAATCGGATCCGCCACCATGGCCGGCTACCTGCGGG
tGFPBamHI_15r	GCGCGGCCGCGGATCTTAAACTCTTTCTTCACCGGCATCTGCATCC

## Supplementary references

- (1) Bachman, M.; Uribe-Lewis, S.; Yang, X.; Williams, M.; Murrell, A.; Balasubramanian, S. *Nat. Chem.* **2014**, *6*, 1049.
- (2) Gautier, A.; Nguyen, D. P.; Lusic, H.; An, W.; Deiters, A.; Chin, J. W. *J. Am. Chem. Soc.* **2010**, *132*, 4086.
- (3) Amantea, A.; Henz, M.; Strazewski, P. *Helv. Chim. Acta* **1996**, *79*, 244. Kawashima, E.; Umabe, K.; Sekine, T. *J. Org. Chem.* **2002**, *67*, 5142. Rangwala, H. S.; Giraldes, J. W.; Gurvich, V. J. *J. Labelled Compd. Radiopharmaceut.* **2011**, *54*, 340. Schiesser, S.; Hackner, B.; Pfaffeneder, T.; Müller, M.; Hagemeyer, C.; Truss, M.; Carell, T. *Angew. Chem. Int. Ed.* **2012**, *51*, 6516.
- (4) Schmied, W. H.; Elsässer, S. J.; Uttamapinant, C.; Chin, J. W. *J. Am. Chem. Soc.* **2014**, *136*, 15577.
- (5) Elsässer, S. E.; Ernst, R. J.; Walker, O. S.; Chin, J. W. *Nat. Methods* **2015**, In press.
- (6) Ward, P. S.; Patel, J.; Wise, D. R.; Abdel-Wahab, O.; Bennett, B. D.; Collier, H. A.; Cross, J. R.; Fantin, V. R.; Hedvat, C. V.; Perl, A. E.; Rabinowitz, J. D.; Carroll, M.; Su, S. M.; Sharp, K. A.; Levine, R. L.; Thompson, C. B. *Cancer Cell* **2010**, *17*, 225.
- (7) Milo, R.; Jorgensen, P.; Moran, U.; Weber, G.; Springer, M. *Nucleic Acids Res.* **2010**, *38*, D750. Zhao, L.; Kroenke, C. D.; Song, J.; Piwnicka-Worms, D.; Ackerman, J. J. H.; Neil, J. J. *NMR Biomed.* **2008**, *21*, 159.