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Photoactivation of Mutant Isocitrate Dehydrogenase 2 Reveals Rapid Cancer-Associated Metabolic and Epigenetic Changes

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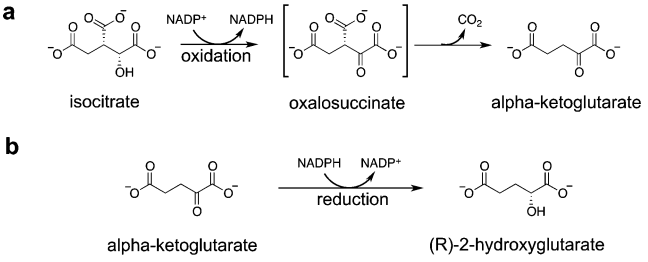
Supporting Information

ABSTRACT: Isocitrate dehydrogenase is mutated at a key active site arginine residue (Arg172 in IDH2) in many cancers, leading to the synthesis of the oncometabolite (*R*)-2-hydroxyglutarate (2HG). To investigate the early events following acquisition of this mutation in mammalian cells we created a photoactivatable version of IDH2(R172K), in which K172 is replaced with a photocaged lysine (PCK), via genetic code expansion. Illumination of cells expressing this mutant protein led to a rapid increase in the levels of 2HG, with 2HG levels reaching those measured in patient tumor samples, within 8 h. 2HG accumulation is closely followed by a global decrease in 5-hydroxymethylcytosine (5-hmC) in DNA, demonstrating that perturbations in epigenetic DNA base modifications are an early consequence of mutant IDH2 in cells. Our results provide a paradigm for rapidly and synchronously uncloaking diverse oncogenic mutations in live cells to reveal the sequence of events through which they may ultimately cause transformation.

The citric acid cycle enzyme isocitrate dehydrogenase (IDH)[1,2] catalyzes the $NADP^+$-dependent oxidative decarboxylation of isocitrate to alpha-ketoglutarate (KGA) (Scheme 1a).

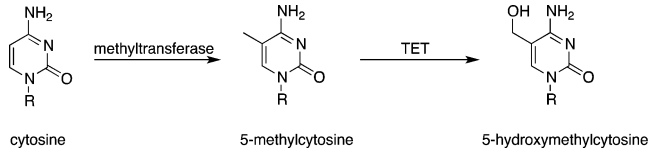
In a large proportion of cancers, including low grade gliomas, secondary glioblastoma multiforme, and acute myeloid leukemias, IDH is mutated at a key active site arginine residue. This arginine residue ordinarily forms hydrogen bond interactions with the alpha- and beta-carboxyl groups of isocitrate.[3] Mutant cytosolic and peroxisomal IDH1 contains an Arg132His mutation (IDH1 R132H), while mutant mitochondrial IDH2, contains the analogous Arg172Lys mutation (IDH2 R172K). These mutations may hinder the enzyme's interaction with isocitrate and increase its affinity for NADPH and KGA. The mutant forms of IDH catalyze an additional reaction: the NADPH-dependent reduction of KGA to the (*R*)-enantiomer of 2-hydroxyglutarate [(*R*)-2HG] (Scheme 1b).[4−6]

Scheme 1. Chemical Conversions Catalyzed by Wild-Type (a) and Mutant (b) IDH



(*R*)-2HG has been proposed to be an oncometabolite, which together with the mutant enzyme is capable of driving cancer-associated cellular transformations.[7,8] (*R*)-2HG is believed to drive transformation, in part, by competitively inhibiting KGA-dependent dioxygenase enzymes. Ten−eleven-translocation enzymes (TET) belong to this protein family and catalyze the conversion of the epigenetic DNA base 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) (Scheme 2).[9−11] A decrease in global 5-hydroxymethylcytosine levels is characteristic of some cancers, including those with mutations in IDH.[12,13] However, it has not been possible to temporally define the sequence of metabolic and epigenetic changes that immediately follow the acquisition of the potentially oncogenic mutations in IDH.

Scheme 2. TET Enzymes Catalyze the Conversion of 5-mC to 5-hmC and Further Oxidation Steps[9,11]



Strategies to photocage key residues within a target protein provide a powerful approach for creating inactive proteins that can be rapidly activated with a pulse of light. Amino acids have been targeted for photocaging by chemical ligation and introduced into cells by microinjection.[14,15] More recently photocaged amino acids have been site-specifically and cotranslationally incorporated into proteins via genetic code expansion. Photocaged lysine, tyrosine, serine, and cysteine have been site-specifically incorporated in place of key residues in proteins.[16−23]

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The approaches developed have allowed photocontrol of nuclear localization sequences,[18] and several enzymes, including kinases,[22] proteases,[23] inteins,[24,25] and cas9,[26] as well as photocontrol of the sites of post-translational modification.[16,19] These approaches have revealed the kinetics of complex signaling pathways with high spatial and temporal resolution[22] and provided approaches to spatially and temporally control tools for proteome and genome editing.[23,26]

We have previously evolved a PCKRS/tRNA$_{CUA}$ pair, which directs the incorporation of photocaged lysine (PCK, 1, Figure 1a) into target protein.[18] This pair is derived from the pyrrolysyl-

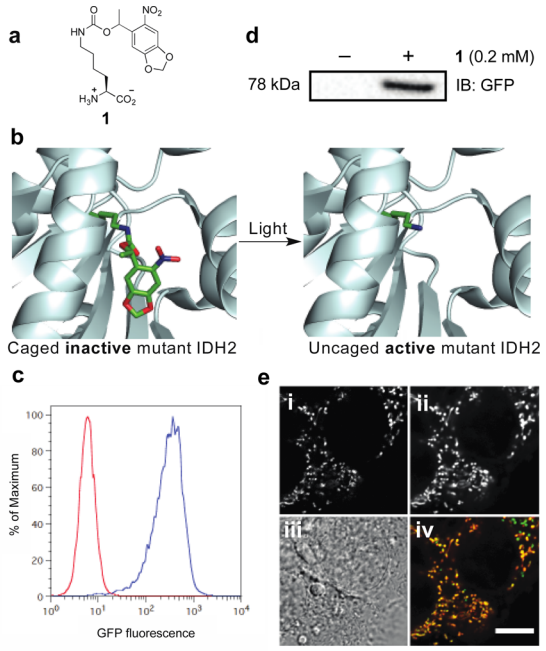

Figure 1. Site-specific incorporation of photocaged lysine at residue 172 of mitochondrial IDH2. (a) Photocaged lysine PCK, 1. (b) Caged lysine in the IDH2(R172PCK) active site is proposed to prevent the NADPH-dependent reduction of KGA to (*R*)-2HG. Illumination reveals the active mutant protein (the figure is a structural model created using Pymol, Phenix, and IDH2 structure PDB: 4JA8). (c) GFP fluorescence of HEK293 cells, stably expressing IDH2(R172TAG)-GFP and the PCKRS/tRNA$_{CUA}$ pair, in the presence (blue) and absence (red) of 0.2 mM PCK, 1. The data were collected by flow cytometry. (d) Immunoblot (IB) of cells from panel c with anti-GFP antibody confirms expression of IDH2-GFP fusion protein with a molecular weight of 78 kDa. Please see Supplementary Figure S1a for the full gel. (e) Mitochondrial colocalization of IDH2(R172TAG)-GFP protein was confirmed using MitoTracker Red CMXRos. (i) GFP, (ii) MitoTracker, (iii) DIC, and (iv) merge of GFP and MitoTracker. Scale bar is 10 μm. Colocalization was also evident in neighboring cells (Supplementary Figure S1b).

tRNA synthetase (PylRS)/tRNA$_{CUA}$ pair, which has been developed for the site-specific incorporation of diverse unnatural amino acids, via genetic code expansion, in *E. coli*, *S. cerevisiae*, *C. elegans*, *D. melanogaster*, and mammalian cells.[27] We reasoned that photocaging K172 in IDH2(R172K) may create an inactive protein, IDH2(R172PCK) that could be activated by a pulse of light (Figure 1b), allowing us to follow the immediate consequences of revealing IDH2(R172K) in the cell.

We created IDH2(R172TAG)-GFP, in which the codon for Arg 172 in an IDH2-green fluorescent protein fusion is converted to the amber codon. We introduced an IDH2(R172TAG)-GFP construct and an optimized PCKRS/tRNA$_{CUA}$ pair, for efficient incorporation of PCK in response to the TAG codon,[28] into HEK293 cells via PiggyBac integration,[29] and selected integrated clones using antibiotic selection. We used fluorescence activated cell sorting to identify single clones with high and homogeneous levels of GFP expression upon the addition of PCK to cells. Isolated clones exhibited clear PCK dependent read through of the amber stop codon in IDH2-(R172TAG)-GFP, as judged by flow cytometry and western blot (Figure 1c,d), consistent with the synthesis of IDH2(R172PCK)-GFP. IDH2(R172PCK)-GFP was mitochondrial, as judged by colocalization of GFP fluorescence with MitoTracker staining (Figure 1e). The half-life of IDH2(R172PCK)-GFP is approximately 48 h, providing an upper limit on the length of time after photoactivation for which measurements will result from a consistent level of mutant protein (Supplementary Figure S2).

To determine whether cells expressing IDH2(R172PCK)-GFP synthesize 2HG, we extracted metabolites from cells[5] and quantified cellular 2HG levels by liquid chromatography, coupled to tandem mass spectrometry (LC–MS/MS), using an isotopically labeled internal standard. We found that 2HG levels were below the detectable threshold, demonstrating that IDH2(R172PCK)-GFP does not appreciably catalyze the NADPH-dependent reduction of KGA to 2HG.

Illumination (365 nm, 60 s, 9.5 mW/cm^2) of cells expressing IDH2(R172PCK)-GFP led to the accumulation of 2.37 nmol of 2HG per 100 μg of protein, 8 h after illumination (Figure 2a).

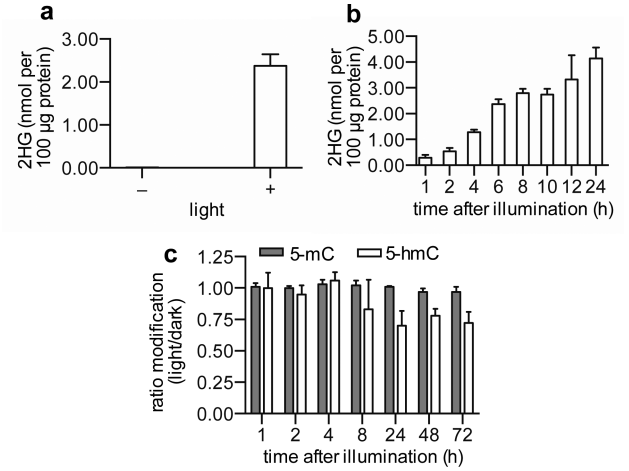

Figure 2. Metabolic and epigenetic effects of mutant IDH2 photoactivation, quantified by LC–MS/MS in HEK 293 cells stably expressing IDH2(R172TAG)-GFP and the PCKRS/tRNA$_{CUA}$ pair. (a) 2HG production is light-dependent. Metabolites were extracted 8 h after illumination at 365 nm (60 s, 9.5 mW/cm^2). (b) Cellular 2HG accumulates following illumination of cells expressing IDH2-(R172PCK)-GFP. (c) Cellular 5-hmC decreases following illumination of cells expressing IDH2(R172PCK)-GFP. The percent global level of 5-mC or 5-hmC of total cytosine, at each time point, is normalized to the corresponding value for nonilluminated sample. Error bars represent the standard deviation of the mean of at least three biological replicates.

This is equivalent to an approximate cellular 2HG concentration of 2.9 mM and is within the range of 2HG levels reported in both cells transfected with the mutant protein and patient tumor samples.[4,30] This result demonstrates that photoactivation of IDH2(R172PCK)-GFP reveals IDH2(R172K)-GFP, which rapidly produces relevant levels of the oncometabolite (*R*)-2HG. The timecourse of (*R*)-2HG production following

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illumination of cells expressing IDH2(R172PCK)-GFP was followed (Figure 2b). The metabolite's levels increased steadily during the first 8 h after photoactivation, reaching 4.14 nmol per 100 μg of protein at 24 h. At longer time points the total level of 2HG continues to increase, but a larger fraction is found in the growth medium outside the cell (Supplementary Figure S3). In contrast KGA levels were not altered as a function of illumination (Supplementary Figure S4).

2HG inhibits the TET family of enzymes, which catalyze the conversion of 5-mC to 5-hmC and further oxidation reactions (Scheme 2).[8] A decrease in 5-hmC has been reported in melanomas and gliomas, as well as in cells expressing mutant IDH.[12,13] To investigate whether a change in 5-hmC could be detected hours after illumination of cells expressing IDH2-(R172PCK)-GFP, we measured global 5-hmC and 5-mC levels using an LC−MS/MS nucleoside method.[31] The percent of 5-hmC (or 5-mC) of total cytosine in samples illuminated was normalized to the nonilluminated control samples (Figure 2c). Direct comparison of global 5-hmC values measured in illuminated and nonilluminated samples demonstrated a significant drop in 5-hmC levels at 24 h after illumination (P = 0.012), 48 h (P = 0.002), and 72 h (P = 0.006) (Supplementary Figure S5a). These data demonstrate that global 5-hmC depletion is an early consequence of mutant IDH2 activity. There was no significant change in global 5-mC over the majority of time points (Supplementary Figure S5b). Since 5-mC is approximately 100 times more abundant than 5-hmC, any change in 5-mC levels are predicted to be a much smaller fraction of the total 5-mC levels and would be challenging to measure.

Following illumination, there is a lag in measurable 5-hmC depletion (Figure 2c) with respect to the rapid accumulation of 2HG in cells (Figure 2b, Supplementary Figure S3), and 5-hmC levels remain depressed at 48 and 72 h after illumination, when intracellular levels of 2HG have decreased (Figure 2c, Supplementary Figure S3). These observations are consistent with (i) the slow formation of 5-hmC from 5-mC, and (ii) the stability of 5-hmC.[31] At early time points, most of the 5-hmC measured is present before illumination, and it takes time for a measurable fraction of total 5-hmC to result from postillumination synthesis, and for total 5-hmC levels to respond to 2HG levels. At longer time points, when intracellular 2HG levels become depressed (Supplementary Figure S3), there will be a lag in re-establishing 5-hmC levels because of (i) the slow formation of 5-hmC from 5-mC, (ii) partial TET enzyme inhibition, even at low cellular 2HG, and (iii) other factors that cause a delay in 5-hmC recovery following its depletion (Supplementary Figure S5c).

In summary, we have generated a system to rapidly light-activate the cancer-associated mutant protein IDH2 in mammalian cells, providing insight into the very early effects of this mutation. In contrast to doxycycline-inducible systems, light control allows direct activation of the protein in minutes, circumventing the initiation of transcriptional and translational processes. We demonstrate that substantial 2-hydroxyglutarate levels are detectable within 1 h of revealing the mutation, and this is followed, after a lag, by a drop in global 5-hmC levels.

Our data demonstrate that 5-hmC depletion is an early consequence of IDH2 mutations and suggest that antagonizing metabolic and epigenetic imbalances could reverse the early effects of this mutation. There are more than 60 dioxygenase enzymes in mammalian cells,[32,33] many of which may be affected by the mutations in IDH that lead to the generation of 2HG. It will be interesting to investigate the immediate consequences of IDH mutation on the cellular pathways that these dioxygenases influence using the powerful approach we have reported. More broadly, we note that extensions of our approach will provide a strategy to rapidly and synchronously uncloak oncogenic mutations in diverse proteins in live cells, in culture or within animals.[34,35] By coupling this strategy to increasingly powerful analytical methods, we anticipate that it will be possible to reveal the early events in transformation by diverse oncogenes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b07627.

Supplementary Figures S1−S5 and methods and materials (PDF)

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Notes

The authors declare no competing financial interest.

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