Inhibition of Mitochondrial Aconitase by Succination in Fumarate Hydratase Deficiency

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SUMMARY

The gene encoding the Krebs cycle enzyme fumarate hydratase (FH) is mutated in hereditary leiomyomatosis and renal cell cancer (HLRCC). Loss of FH activity causes accumulation of intracellular fumarate, which can directly modify cysteine residues to form 2-succinocysteine through succination. We undertook a proteomic-based screen in cells and renal cysts from Fh1 (murine FH)-deficient mice and identified 94 protein succination targets. Notably, we identified the succination of three cysteine residues in mitochondrial Aconitase2 (ACO2) crucial for iron-sulfur cluster binding. We show that fumarate exerts a dose-dependent inhibition of ACO2 activity, which correlates with increased succination as determined by mass spectrometry, possibly by interfering with iron chelation. Importantly, we show that aconitase activity is impaired in FH-deficient cells. Our data provide evidence that succination, resulting from FH deficiency, targets and potentially alters the function of multiple proteins and may contribute to the dysregulated metabolism observed in HLRCC.

INTRODUCTION

Altered metabolism is a key feature and hallmark of cancer cells (Hanahan and Weinberg, 2011). How this arises, and what steps link it to oncogenesis, still eludes us. One possible answer lies with “oncometabolites,” described as metabolites whose abnormal accumulation causes both metabolic and nonmetabolic (such as epigenetic) dysregulation and potential transformation to malignancy (Thompson, 2009). Fumarate hydratase (FH) has been identified as a tumor suppressor because germline loss-of-function mutations are associated with the development of hereditary leiomyomatosis and renal cell cancer (HLRCC) (Tomlinson et al., 2002). FH has roles in both the mitochondria and cytosol, catalyzing the hydration of fumarate to malate. In mitochondria, FH is a key component of the Krebs cycle, essential for cellular energy production and macromolecular biosynthesis, whereas in the cytoplasm, FH metabolizes fumarate generated from arginine synthesis and the purine nucleotide cycle (Salway, 1999; Shambaugh, 1977). Loss of FH activity results in accumulation of fumarate in cells. Elevated fumarate has been implicated in the development of FH-associated tumors through a number of pathways, e.g., by competitive inhibition of 2-oxoglutarate (2OG)-dependent oxygenases, including the hypoxia-inducible factor (HIF) hydroxylases, leading to stabilization of HIF and activation of oncogenic HIF-dependent pathways (O’Flaherty et al., 2010). However, there is increasing evidence that multiple independent pathways may have roles in FH-associated oncogenesis as a consequence of fumarate acting as an oncometabolite (Yang et al., 2012). In addition to being an allosteric inhibitor of the 2OG-dependent oxygenases similar to other oncometabolites, fumarate acts as an endogenous electrophile. It reacts spontaneously by a Michael addition reaction with free sulfhydryl groups to generate a thioether linkage with cysteine residues in proteins. This results in formation of S-(2-succino) cysteine (2SC), a process termed succination (Alderson et al., 2006). This mechanism is distinct from succinylation of cysteine in which a thioester would be formed (Zhang et al., 2011). Furthermore, 2SC immunohistochemistry is sufficiently sensitive and specific for use as a clinical biomarker of HLRCC (Bardella et al., 2011).

Significantly, succination of Kelch-like ECH-associated protein 1 (KEAP1) in FH-deficient cells leads to abrogation of its
interaction with the transcription factor Nuclear factor erythroid 2-related factor 2 (NRF2) and activation of the potentially oncogenic NRF2-mediated antioxidant defense pathway (Adam et al., 2011; Ooi et al., 2011). Furthermore, NRF2 activation has been shown recently to modulate cell metabolism possibly augmenting the cellular stress response (Mitsutani et al., 2012). Elucidation of the functional consequences of KEAP1 succination prompted us to search for other 2SC targets that may contribute to the pathogenesis of FH-associated disease. Hence, we conducted a proteomic screen for 2SC in an Fh1-deficient (knockout [KO]) mouse embryonic fibroblast (MEF) cell line (O’Flaherty et al., 2010) and in murine kidney tissue and fluid where Fh1 has been deleted from the kidney tubules (Pollard et al., 2007). We identified 94 succinated proteins, including some that are succinated on functional cysteine residues. In particular, we investigated the succination of three key cysteines in the Krebs cycle enzyme, mitochondrial aconitase hydratase (Aconitase2, ACO2). We show here that fumarate-mediated succination of ACO2 impairs its enzymatic activity in a dose-dependent manner and that Fh1KO cells exhibit reduced aconitase activity. Our findings further highlight succination as a significant event that could target multiple cellular pathways in FH-associated pathogenesis.

RESULTS

Identification of 2SC Protein Targets

Previously using Fh1 MEFs, we confirmed by immunoblotting that accumulated intracellular furamate results in high levels of 2SC in Fh1KO, but not Fh1 wild-type (WT), MEFs (Bardella et al., 2011). To detect potential 2SC targets at low abundance, we performed mitochondrial and nuclear fractionations of Fh1KO MEFs (Figure S1A). To identify 2SC targets from biological tissue, we used cystic kidneys and aspirated kidney fluid from a 30-week-old Fh1KO mouse where Fh1 is conditionally deleted in the renal tubular epithelium causing the development of hyperplastic cysts (Pollard et al., 2007). Protein extracts from mitochondrial, nuclear, and cytosolic fractions of Fh1KO MEFs and Fh1KO kidneys were separated by SDS-PAGE analyses and subjected to in-gel trypsin digestion and liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses as described before (Adam et al., 2011). Combined proteomic analyses identified 4,095 proteins and 306,558 target peptide spectrum matches (PSMs) from Fh1KO MEFs (false discovery rate [FDR] 2.32%) and 3,569 proteins/226,606 PSMs from Fh1KO kidney tissue and fluid (FDR 1.96%). The MS/MS spectrum for each succination site was verified, and a total of 110 nonredundant 2SC sites were identified in 94 distinct proteins (Table 1). 2SC targets identified thus comprise proteins from diverse cellular pathways; but significantly, approximately 50% are metabolic processes (Figure S1B). Notably, ACO2, mitochondrial NDUFA1 iron-sulfur cluster scaffold homolog, Protein DJ-1, Peroxiredoxin-1, and Peroxiredoxin-3 are succinated on cysteine residues involved in their function (Andres-Mateos et al., 2007; Mirel et al., 1998; Tong et al., 2003; Yang et al., 2002). Also, the succination of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at C149 was confirmed as reported previously by Blatnik et al. (2008a, 2008b).

Endogenous ACO2 Is Succinated at Three Critical Cysteines in Fh1KO MEFs

To investigate the functional consequences of succination, we focused on ACO2 because of its role in the Krebs cycle, where it catalyzes the stereospecific isomerization of citrate to isocitrate via cis-aconitate. In particular, it is a mitochondrial oxidative stress sensor and requires an active Fe4S4 cluster, bound directly by three conserved cysteine residues, for catalysis (Lloyd et al., 1998). LC-MS/MS analyses of the tryptic peptide 379VGLIGS(2SC)TNSSYEDMGRR395 derived from endogenous ACO2 in Fh1KO MEFs assigned succination to C385 unambiguously (Figure 1A). The tryptic peptide spanning C448 and C451 was detected as a mixture of two isomers 438DLGGIVLANA(2SC)GP(2SC)IGQWDRK458 and 438DLGGIVLANA(2SC)GP(2SC)IGQWDR457 that are succinated at C448 and C451 (Figure 1B), respectively. Due to their identical mass and composition, the two succinated species could not be separated by LC-MS/MS, but measurement of resulting fragment ion masses in the MS/MS scan allowed identification of succination on both sites. Generally, we observed higher succination at C451 (~90%) compared to C448 (~10%) as determined by Mascot analysis.

Human ACO2 Is Succinated at Homologous Residues When Expressed Stably in Fh1KO MEFs

To determine if human ACO2 can be succinated on homologous cysteine residues, we transfected Fh1WT and Fh1KO MEFs with a V5-tagged ACO2 gene. LC-MS/MS analysis following V5 immunoprecipitation confirmed succination at all three cysteine residues (C385, C448, and C451) within the active site in ACO2 expressed in only Fh1KO MEFs. To complement the mouse data, we detected succination at C448 and C451 simultaneously from the same tryptic peptide, 438DLGGIVLANA(2SC)GP(2SC)IGQWDRK458 (Figure S1C).

Fumarate-Mediated Succination Reduces ACO2 Activity In Vitro

The three cysteine residues C385, 448, and 451 are crucial for iron-sulfur cluster binding in ACO2 (Figure 2A). To investigate if succination of ACO2 impairs its enzymatic activity, we preincubated pig heart ACO2 with furamate and assayed its activity in vitro. One hour pre-exposure of ACO2 to increasing concentrations of sodium furamate at pH 7.4 resulted in dose-dependent inhibition of its activity in the range of 1–50 mM furamate, which parallels detection of 2SC by immunoblotting (Figure 2B). We then performed LC-MS/MS analyses of trypsin-digested ACO2 derived from assay mixtures. Succination was detected at five cysteine residues (C126, C385, C410, C451, and C592), and further, the levels of succination of the C385- and C451-containing peptides increased with increasing fumarate concentration (Figures 2C, 2D, and S2A). We correlated ACO2 activity with succination in the range of 5–50 mM furamate and obtained a negative linear correlation with succinated peptides containing 2SC385 and 2SC451/448 (Figure S2B). To relate the in vitro data to pathophysiological settings, we measured fumarate concentrations by capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) (Soga et al., 2003) in FH-deficient mouse kidneys and HLRCC tumors; these were estimated to contain 1.7 ± 0.4 mM and 3.4 ± 1.2 mM fumarate, respectively.
| Swiss-Prot Accession No. | Gene Symbol | Protein Name | Succination Site(s) | Source | PSMs | Sequence Coverage | 2SC Peptide Instances |
|--------------------------|-------------|--------------|---------------------|--------|------|------------------|-----------------------|
| Q8BGQ7                   | Aars        | alanine-tRNA ligase, cytoplasmic | C403 | M(c) | 302 | 49.9%            | 3                     |
| Q99K0                    | Aco2        | aconitate hydratase, mitochondrial | C385, C448, C451 | M(m) | 1,340 | 66.7%            | C385(30), C451(3)     |
| Q9R0X4                   | Acot9       | acyl-coenzyme A thioesterase 9, mitochondrial | C154 | K    | 121 | 57.9%            | 13                    |
| Q9NB1                    | Acss1       | acetyl-coenzyme A synthetase 2-like, mitochondrial | C41  | K    | 86  | 47.8%            | 6                     |
| P00329                   | Adh1        | alcohol dehydrogenase 1 | C83  | K    | 508 | 63.7%            | 25                    |
| Q9WTP6                   | Ak2         | adenylate kinase 2, mitochondrial | C208 | K    | 51  | 53.9%            | 13                    |
| Q9WTP7                   | Ak3         | GTP:AMP phosphotransferase, mitochondrial | C85  | K    | 33  | 70.9%            | 4                     |
| P07724                   | Alb         | serum albumin | C471 | K    | 26,069 | 89.6%     | 501                   |
| Q92110                   | Aldh18a1    | ß-1-pyruvoly-5-carboxylate synthase | C88, C612 | M(n) | 1,185 | 77.9% | C88(10), C612(27) |
| P10107                   | Anxa1       | annexin A1 | C189, C324 | M(n,c) | 407 | 73.4% | C189(5), C324(7) |
| P07356                   | Anxa2       | annexin A2 | C223 | M(n,c),K | 1,001 | 77.0% | 6                     |
| Q8K0Q5                   | Arhgap18    | Rho GTPase-activating protein 18 | C637 | K    | 8   | 5.0%             | 5                     |
| Q9D0L7                   | Armc10      | isoform 2 of Armadillo repeat-containing protein 10 | C275 | M(n) | 38  | 57.2%            | 4                     |
| Q55143                   | Atp2a2      | sarcoplasmic/endooplasmic reticulum calcium ATPase 2 | C998 | M(n) | 380 | 47.9%            | 8                     |
| Q9YN19                   | Bag2        | BAG family molecular chaperone regulator 2 | C16  | M(n) | 53  | 70.0%            | 2                     |
| P12658                   | Calb1       | calbindin | C187 | K    | 143 | 67.4%            | 2                     |
| Q62Q38                   | Caid1       | cullin-associated NEDD8-dissociated protein 1 | C942 | M(n) | 299 | 55.3%            | 6                     |
| Q8K354                   | Cbr3        | carbonyl reductase (NADPH) 3 | C160 | K    | 128 | 83.0%            | 3                     |
| P80314                   | Cct2        | T complex protein 1 subunit j | C535 | M(n,c) | 443 | 82.4%            | 5                     |
| Q9CQ85                   | Cisd2       | CDGSH iron-sulfur domain-containing protein 2 | C92  | M(m) | 26  | 62.2%            | 3                     |
| Q8BKM4                   | Ckap4       | cytoskeleton-associated protein 4 | C79  | M(n,m), K | 813 | 82.8% | 4                     |
| P30725                   | Ckmt1       | creatine kinase U-type, mitochondrial | C317 | K    | 110 | 54.8%            | 8                     |
| Q8FD5                   | Clict       | clathrin heavy-chain 1 | C870 | M(n,c) | 1,297 | 65.6% | 16                    |
| A6H584                   | Colla5      | collagen α-5(VI) chain | C1974 | K    | 267 | 45.8% | 15                    |
| Q61665                   | Ddx5        | probable ATP-dependent RNA helicase DDX5 | C200 | M(n) | 346 | 62.5% | 19                    |
| Q5016-1                   | Ddx17      | isoform 1 of Probable ATP-dependent RNA helicase DDX17 | C191, C198 | M(n) | 144 | 56.5% | C191(1), C198(19) |
| Q9RP5                   | Dstrn       | dextrin | C23  | M(c) | 64  | 55.8%            | 3                     |
| Q9QC43                   | Dut         | deoxyuridine triphosphatase | C3   | M(c) | 65  | 63.6%            | 10                    |
| Q9JHU4                   | Dynch1h1    | cytoplasmic dynein 1 heavy-chain 1 | C4284 | M(n) | 1,054 | 57.6% | 1                     |
| Q8QV3                   | Eci1        | Dci enoyl-CoA ß isomerase 1, mitochondrial | C87 | K    | 91  | 63.0%            | 25                    |
| P58252                   | Eef2        | elongation factor 2 | C41  | M(c,n) | 1,746 | 71.2% | 33                    |
| Q8BG9                   | Eif4b       | eukaryotic translation initiation factor 4B | C457, C543 | M(n) | 231 | 56.0% | C457(4), C543(2) |
| Q8CX96-2                  | Epc1        | isoform 2 of Enhancer of polycomb homolog 1 | C515 | M(n) | 3  | 41.1% | 3                     |
| Q99M71-1                  | Epdr1       | isoform 1 of Mammalian ependymin-related protein 1 | C88  | M(n) | 14  | 37.5% | 1                     |
| Q8CG7                  | Eprs        | bifunctional aminoacyl-tRNA synthetase | C744 | M(c) | 503 | 49.5% | 8                     |
| Q8BTM8                  | Flna        | filamin-A | C8, C574 | M(n) | 2,440 | 78.8% | C8(5), C574(27) |

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| Swiss-Prot Accession No. | Gene Symbol | Protein Name | Succination Site(s) | Source | PSMs | Sequence Coverage | 2SC Peptide Instances |
|-------------------------|-------------|--------------|---------------------|--------|------|------------------|---------------------|
| Q80X90                  | Flnb        | filamin-B    | C1434, C2501        | M(n), K | 2,264 | 82.1%            | C1434(25), C2501(9) |
| P97494                  | Gclc        | glutamate-cysteine ligase catalytic subunit | C501 | K | 246 | 63.3% | 6 |
| P16858                  | Gapdh       | glyceraldehyde-3-phosphate dehydrogenase | C22, C150 | M(c,n) | 137 | 62.0% | C22(13), C150 (42) |
| P53702                  | Hccs        | cytochrome c-type heme lyase | C39 | M(m) | 117 | 65.4% | 11 |
| P70333                  | Hrmp2       | heterogeneous nuclear ribonucleoprotein H2 | C267 | M(n) | 177 | 49.2% | 15 |
| P61979-2                | Hrmpk       | isoform 2 of Heterogeneous nuclear ribonucleoprotein K | C132 | M(n) | 700 | 62.9% | 16 |
| Q8R081                  | Hrmp1       | heterogeneous nuclear ribonucleoprotein L | C469 | M(n) | 333 | 79.9% | 8 |
| Q9D0E1-1                | Hrmpm       | isoform 1 of Heterogeneous nuclear ribonucleoprotein M | C26, C652 | M(n) | 416 | 80.5% | C26(3), C652(4) |
| P47879                  | Igtbp4      | insulin-like growth factor-binding protein 4 | C211 | K | 2 | 7.1% | 1 |
| Q8CA08-2                | Immt        | isoform 2 of Mitochondrial inner membrane protein | C172 | M(m), K | 14 | 81.8% | 27 |
| Q0GNC1-1                | Int2        | isoform 1 of Inverted formin-2 | C284 | M(n) | 67 | 35.6% | 9 |
| Q60749                  | Khdrbs1     | KH domain-containing, RNA-binding, signal transduction-associated protein 1 | C19 | M(n), K | 53 | 26.0% | 7 |
| P06151                  | Ldha        | L-lactate dehydrogenase A chain | C84 | M(c) | 822 | 72.6% | 21 |
| P48678                  | Lmna        | isoform A of Prelamin-A/C | C572 | M(n), K | 201 | 60.6% | 4 |
| Q3UMR5                  | Mcu         | calcium uniporter protein, mitochondrial | C190 | K | 16 | 28.3% | 4 |
| Q9CQ65                  | Mtap        | S-methyl-5'-thioadenosine phosphorylase | C130 | M(c) | 106 | 69.6% | 9 |
| Q791V5                  | Mtch2       | mitochondrial carrier homolog 2 | C296 | K | 45 | 36.6% | 1 |
| Q3V3R1                  | Mthfd1      | monofunctional C1-tetrahydrofolate synthase, mitochondrial | C129 | M(m) | 1,248 | 82.8% | 40 |
| Q8VDD5                  | Myh9        | myosin-9 | C988 | K | 3,408 | 67.4% | 54 |
| Q8KZ2J-4                | Ncapd2      | isoform 2 of Condensin complex subunit 1 | C439 | M(n) | 176 | 42.8% | 12 |
| Q3UYV9                  | Ncbp1       | nuclear cap-binding protein subunit 1 | C44 | M(n) | 64 | 31.5% | 9 |
| Q9QZ23                  | Nfu1        | NFU1 iron-sulfur cluster scaffold homolog, mitochondrial | C213 | M(m) | 47 | 43.5% | 3 |
| Q9CRB2                  | Nhp2        | HACA ribonucleoprotein complex subunit 2 | C18 | M(n) | 22 | 66.0% | 7 |
| Q99LX0                  | Park7       | protein DJ-1 | C106 | K | 50 | 89.9% | 2 |
| Q88KZ9                  | Pdhx        | pyruvate dehydrogenase X component, mitochondrial | C170 | M(m) | 25 | 31.5% | 1 |
| Q5SUR0                  | Pfas        | phosphoribosylformylglycinamide synthase | C1055 | M(c) | 128 | 32.8% | 5 |
| Q9ESW8                  | Pg pep1     | pyroglutamyl-peptidase 1 | C108 | K | 11 | 34.4% | 6 |
| Q80UU9                  | Pgmrcc2     | membrane-associated progesterone receptor component 2 | C75 | M(n,m) | 34 | 38.2% | 5 |
| Q61753                  | Phgdh       | D-3-phosphoglycerate dehydrogenase | C369 | M(n) | 191 | 45.6% | 35 |
| Q92076                  | Pkdrej      | polycystic kidney disease and receptor for egg jelly-related protein | C136 | M(c) | 3 | 1.3% | 1 |
| Q99K51                  | Pls3        | plastin-3 | C104 | K | 407 | 75.1% | 5 |
| P35700                  | Prdx1       | peroxiredoxin-1 | C173 | K | 236 | 88.4% | 26 |
| P20108                  | Prdx3       | thioredoxin-dependent peroxide reductase, mitochondrial | C230 | K | 47 | 64.6% | 7 |
| P99029-1                | Prdx5       | peroxiredoxin-5, mitochondrial | C96 | M(m) | 215 | 74.1% | 21 |
| Q9R0G7                  | Ptges3      | prostaglandin E synthase 3 | C58 | M(c) | 54 | 44.3% | 13 |

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Table 1. Continued

| Swiss-Prot Accession No. | Gene Symbol | Protein Name | Succination Site(s) | Source | PSMs | Sequence Coverage | 2SC Peptide Instances |
|--------------------------|-------------|--------------|---------------------|--------|------|------------------|----------------------|
| Q8VI36                   | Pxn         | paxillin     | C535, C538          | K      | 64   | 61.9%            | 5                    |
| Q64012                   | Raly        | RNA-binding protein Raly | C255 | M(n), K | 58   | 64.2%            | 11                   |
| Q60973                   | Rbbp7       | histone-binding protein RBBP7 | C116 | M(n) | 69   | 74.8%            | 2                    |
| Q8BG51-3                 | Rho1        | isoform 3 of Mitochondrial Rho GTPase 1 | C535 | K | 17   | 14.3%            | 1                    |
| P62717                   | Rpl18a      | 60S ribosomal protein L18a | C22, C109 | M(n,m,c) | 287 | 58.0% | C22(10), C109(30) |
| P47955                   | Rplp1       | 60S acidic ribosomal protein P1 | C61 | M(n,m,c) | 111 | 67.5% | 30                    |
| Q91YQ5                   | Rpn1        | dolichyl-diphosphooligosaccharide protein glycosyltransferase subunit 1 | C478 | M(m,n) | 605 | 68.8% | 11                    |
| D3YXK2                   | Safb        | scaffold attachment factor B | C81 | M(n) | 82   | 35.5%            | 4                    |
| O70456                   | Sfn         | 14-3-3 protein α | C38 | K | 29   | 61.3%            | 2                    |
| Q8VEM8                   | Slc25a3     | phosphate carrier protein, mitochondrial | C71 | K | 288 | 61.1% | 12                    |
| Q9CYN2                   | Spcs2       | signal peptidase complex subunit 2 | C26 | M(m,n), K | 143 | 61.1% | 73                    |
| Q62266                   | Sprr1a      | cornifin-A   | C41, C120          | K      | 9    | 43.8%            | C41(2), C120(4)     |
| Q64674                   | Smr         | spermidine synthase | C89 | M(c) | 58   | 44.0%            | 3                    |
| Q921F2                   | Tarbp       | TAR DNA-binding protein 43 | C50 | M(n) | 16   | 63.8%            | 8                    |
| Q9R099                   | Tbl2        | transducin β-like protein 2 | C43 | M(n) | 78   | 59.5%            | 3                    |
| Q08784                   | Tcof1       | treacle protein | C580 | M(n,c) | 67   | 29.5%            | 12                   |
| Q61029-1                 | Trmpo       | isoform i of Lamina-associated polypeptide 2, isoforms β\|γ\|γ' | C362 | M(n) | 11   | 61.5%            | 13                   |
| P17751                   | Tpi1        | triosephosphate isomerase | C71, C77 | K | 233 | 81.5% | 13                    |
| P21107-2                 | Tpm3        | isoform 2 of Tropomyosin α-3 chain | C233 | M(n) | 30   | 71.8%            | 11                   |
| Q921Q9                   | Vars        | valyl-tRNA synthetase | C41 | M(n), K | 328 | 53.4% | 2                    |
| Q60930                   | Vdac2       | voltage-dependent anion-selective channel protein 2 | C77, C228 | M(m), K | 489 | 65.7% | C77(2), C228(1)     |
| Q60931                   | Vdac3       | voltage-dependent anion-selective channel protein 3 | C8, C65 | M(m), K | 365 | 66.1% | C8(3), C65(13)      |
| Q62468                   | Vilt1       | villin-1     | C134 | K | 268 | 60.2% | 23                   |

2SC targets identified from Rh1KO MEFs and mouse kidney tissue and fluid. Succinated proteins are listed alphabetically by gene symbol, with succinated cysteine residues indicated. PSM, peptide spectrum matches; M, MEFs; c, cytosolic fraction; m, mitochondrial fraction; n, nuclear fraction; K, kidney tissue or fluid. See also Figure S1.
ACO2 Activity Is Impaired in Fh1KO MEFs
When whole-cell lysates of Fh1WT and Fh1KO MEFs were compared to determine if succination impairs endogenous ACO2 activity, Fh1KO MEFs displayed significantly reduced aconitase activity (Figure 3A). To differentiate mitochondrial and cytosolic aconitase activity (ACO2 and ACO1, respectively), we utilized two cell lines derived from the Fh1KO MEFs, reconstituted with either full-length FH (Fh1KO+FH), or FH restricted to the cytosol by deleting the mitochondrial-targeting sequence (Fh1KO+FH\(^{-}\)). Comparison of whole-cell lysates from the four MEF cell lines showed that aconitase activity is completely restored in Fh1KO+FH cells and only partially restored in Fh1KO+FH\(^{-}\), relative to that in Fh1KO cells (Figure 3A).

Previously, we showed that despite having significantly reduced total cellular fumarate compared to Fh1KO, Fh1KO+FH\(^{+}\) MEFs (as measured by \(^{1}\)H-nuclear magnetic resonance spectrometry) retain abnormal mitochondria morphology and impaired respiration (O’Flaherty et al., 2010). We reetermined fumarate targets in FH deficiency. We describe the succination of three cysteine residues crucial for iron-sulfur cluster binding in the aconitase are potentially inactive in Fh1KO MEFs (Figure 3F).

DISCUSSION
Here, we report the results of a proteomic screen to identify 2SC targets in FH deficiency. We describe the succination of three cysteine residues crucial for iron-sulfur cluster binding in the active site of the Krebs cycle enzyme mitochondrial Aconitase2 (ACO2) in Fh1KO cells, which exhibit reduced aconitase activity compared to Fh1WT cells. We have demonstrated that in vitro...
The inhibition of ACO2 is a direct consequence of dose-dependent fumarate-mediated succination, particularly at $\geq 5$ mM fumarate, equivalent to concentrations measured in FH-deficient tissues. Because tissue samples are a heterogeneous mix of both control and FH-deleted cells, precise determination of intracellular fumarate is difficult, and the actual concentrations in these FH-deficient cells could be significantly underestimated. Fumarate concentrations in subcellular compartments, e.g., mitochondria versus cytosol, could also be variable and differentially affect local protein succination. Additionally, compared to...
the relatively simple in vitro situation, the catalytic activity of aconitase in cells could be influenced by multiple components such as cosubstrate and cofactor availability. Interestingly, we did identify succination of proteins involved in iron-sulfur cluster assembly in our proteomic screen, potentially further hindering aconitase activity. Stable isotope tracer studies showed that FH1KO MEFs do not utilize the reductive carboxylation mechanism for citrate synthesis, which may be a consequence of fumarate-dependent succination of ACO2, adding a further layer of complexity to the disruption of mitochondrial metabolism caused by FH deficiency.

Cytosolic Aconitase1 (ACO1) also contains three iron-sulfur-binding cysteine residues and is a bifunctional enzyme that acts either as an iron response element (IRE)-binding protein to regulate iron uptake, sequestration, and utilization or as the cytosolic aconitase, depending on iron availability (Philpott et al., 1994). Whether succination affects the IRE-binding ability of ACO1 and, by inference, iron homeostasis in FH-deficient cells is an interesting question that warrants future investigation.

2SC has been described in aging and diabetes, and its functional consequences have been reported for GAPDH and adiponectin in addition to the KEAP1/NRF2 pathway (Adam et al., 2011; Frizzell et al., 2009; Thomas et al., 2012). Our proteomic screen for 2SC targets aims to expand our current knowledge of the extent of this modification and its cellular impacts. Despite the fact that our screen is biased toward abundant proteins, it is significant that whereas proteins encompassing diverse cellular pathways are targets for succination, around half are involved in metabolism. A few proteins including the iron-sulfur cluster assembly protein NFU1 and the thioredoxin-dependent peroxide reductases are succinated on critical cysteine residues, suggesting that succination may adversely affect function in these targets. The thioredoxin adduct generated by fumarate modifications occurs nonenzymatically and is believed to be irreversible (Alderson et al., 2006; Frizzell et al., 2011, 2012). However, it is conceivable that 2SC may influence signal transduction by targeting proteins that are cellular stress sensors such as KEAP1. Alternatively, 2SC may compete with other cysteine modifications such as S-nitrosylation and oxidation to sulfenic acid to indirectly target other cellular signaling events. Although the effects on individual proteins require closer investigations, our data provide evidence that succination is a significant posttranslational modification in FH deficiency and a potential key mechanism linking multiple pathways that may cause dysregulation of cell metabolism and contribute to oncogenesis.

EXPERIMENTAL PROCEDURES

See also Extended Experimental Procedures.

Aconitase Assay

The aconitase assay is based on the protocol described in the Aconitase Assay Kit (Cayman Chemical) with modifications. NADPH production was followed by fluorescence (excitation 340 nm; emission of 465 nm) over 45 min at 37°C. Activation of pig heart aconitase and preparation of MEF cell lysates followed the manufacturer’s protocol.

Proteomics and Mass Spectrometry

Cell fractionations were performed using Qproteome Mitochondria Isolation Kit (Qiagen), or as previously described (Adam et al., 2011). Kidney samples were homogenized and sonicated in Urea-SDS buffer (O’Flaherty et al., 2010). Protein extracts were separated by SDS-PAGE and processed for trypsin digestion and LC-MS/MS analyses as previously described (Adam et al., 2011). Database searches were performed against SwissProt (06/2011) or International Protein Index (09/2012) database using Mascot (Perkins et al., 1999) or CPPP 1.3.0 (Trudgian et al., 2010). For label-free quantitation of succinated peptides, samples were analyzed in three technical replicates. Relative quantitation was performed using Progenesis LC-MS v.4.0. Correlation analysis was performed using GraphPad Prism v.5 assuming Gaussian populations (Pearson) calculating two-tailed p values with a confidence interval of 95%. Tissue and cell samples for metabolite analysis by CE-TOFMS were prepared as described before (Adam et al., 2011; Soga et al., 2006, 2009).

Mice and Human Tissue Samples

All procedures were conducted in line with American Association for Cancer Research guidelines and performed under UK Home Office regulations after approval by the Local Ethical Review Process at Oxford University. Anonymized human tumors and normal samples were collected with full ethical approval (MREC 05/Q1605/66) as approved by the Oxford Centre for Histopathology Research.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and Extended Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.02.013.

LICENSING INFORMATION

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Figure 3. Aconitase Activity Is Reduced in Fh1KO MEFs

(A) Aconitase activities of Fh1WT, Fh1KO, Fh1KO+F, and Fh1KO+FHmom MEF cell lysates normalized to cell number. The aconitase inhibitor oxalomalate was used as a negative control.

(B) CE-TOFMS analyses of fumarate concentration in the four MEF cell lines.

(C) Extent of succination of ACO2 at C385 and C448/451 in the four MEF cell lines determined by measuring abundance of the relevant peptides by LC-MS/MS.

(D) CE-TOFMS analyses confirmed significant differences between Fh1WT and KO MEFs in the levels of the key Krebs cycle metabolites fumarate, succinate, malate, 2OG, isocitrate, and citrate.

(E) Mass isotopomer analysis of key Krebs cycle metabolites in Fh1WT and Fh1KO MEFs cultured with [d5]glutamine for 24 hr.

(F) Schematic of glutamine metabolism by the Krebs cycle in Fh1KO MEFs. Abbreviations are as follows: Ac-CoA, acetyl coenzyme A; ACL, ATP citrate lyase; ACO1 and ACO2, Aconitases 1 and 2; FH, fumarate hydratase; FUM, fumarate; Gln, glutamine; Glu, glutamate; IDH, isocitrate dehydrogenase; Mal, malate; OAA, oxaloacetate; Succ, succinate; Succ-CoA, succinyl coenzyme A; SDH, succinate dehydrogenase; 2OG, 2-oxoglutarate; αKGDH, α-ketoglutarate dehydrogenase.

All error bars indicate SEM. See also Figure S3.
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