ABHD11 maintains 2-oxoglutarate metabolism by preserving functional lipoylation of the 2-oxoglutarate dehydrogenase complex

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2-oxoglutarate (2-OG or α-ketoglutarate) relates mitochondrial metabolism to cell function by modulating the activity of 2-OG dependent dioxygenases involved in the hypoxia response and DNA/histone modifications. However, metabolic pathways that regulate these oxygen and 2-OG sensitive enzymes remain poorly understood. Here, using CRISPR Cas9 genome-wide mutagenesis to screen for genetic determinants of 2-OG levels, we uncover a redox sensitive mitochondrial lipoylation pathway, dependent on the mitochondrial hydrolase ABHD11, that signals changes in mitochondrial 2-OG metabolism to 2-OG dependent dioxygenase function. ABHD11 loss or inhibition drives a rapid increase in 2-OG levels by impairing lipoylation of the 2-OG dehydrogenase complex (OGDHC)—the rate limiting step for mitochondrial 2-OG metabolism. Rather than facilitating lipoate conjugation, ABHD11 associates with the OGDHC and maintains catalytic activity of lipoyl domain by preventing the formation of lipoyl adducts, highlighting ABHD11 as a regulator of functional lipoylation and 2-OG metabolism.
he ability to sense and respond to nutrient abundance is a fundamental requirement for cell survival, and to achieve this, cells have evolved several strategies that link metabolic function to transcriptional adaptation. One such strategy is the coupling of 2-oxoglutarate (2-OG) metabolism to gene transcription, whereby 2-OG, a key component of TCA cycle, can facilitate cell function by modulating the activity of 2-OG dependent dioxygenases involved in the hypoxia inducible factor (HIF) response, DNA methylation, and histone modifications.

The relevance of 2-OG in modulating the activity of these dioxygenases is exemplified by changes in the relative abundance of cellular 2-OG. An increased 2-OG/succinate ratio promotes embryonic stem cell pluripotency, and antagonises the growth of solid organ tumours through increased hydroxymethylation of DNA (5hmC) and histone demethylation. Conversely, elevated cellular 2-OG can drive its own reduction to L-2-hydroxyglutarate (L-2-HG), which counterintuitively inhibits 2-OG dependent dioxygenases, leading to decreased DNA hydroxymethylation and histone demethylation, activation of the HIF response, altered T cell fate, and haematopoietic cell differentiation. Consequently, understanding how 2-OG metabolism is regulated has broad biological implications.

Central to maintaining cellular 2-OG homeostasis is the 2-oxoglutarate dehydrogenase complex (OGDHc, also known as the α-ketoglutarate dehydrogenase complex), the rate-limiting enzyme within the TCA cycle that oxidatively decarboxylates 2-oxoglutarate to succinyl-CoA. This evolutionarily conserved enzyme also requires lipoic acid, a redox sensitive cofactor that is synthesised within the mitochondria and conjugated to a single lysine within the OGDHc E2 subunit, dihydrolipoamide S-succinyltransferase (DLST)–10. The cyclical reduction and oxidation of the two thiols of conjugated lipoic acid (lipoamide to dihydrolipoamide) serves as a redox intermediate, coupling the formation of succinyl CoA to generation of NADH. The importance of DLST and its lipoylation is highlighted by the recent identification of genetic mutations leading to human disease. Patients with germline mutations in lipoic acid synthesis genes develop a severe variant of the neurological condition, Leigh syndrome, and loss of heterozygosity mutations in the OGDHc lead to angiogenic tumours (pheochromocytomas and paragangliomas), similar to other hereditary cancer syndromes activating the HIF pathway. However, how OGDHc function and 2-OG abundance is regulated is unclear.

Here, we use the sensitivity of the HIF pathway to 2-OG abundance to gain insights into how 2-OG availability and 2-OG metabolism is regulated. We first screened the effects of 2-OG on HIF reporter activity. The canonical HIF pathway involves cellular 2-OG-dependent dioxygenase activity of the prolyl hydroxylases (PHDs or EGLNs)4,13,15, which is controlled by the VHL E3 ligase-mediated degradation of HIF-α in hypoxic conditions (2% oxygen), thereby preventing oxygen availability limiting PHD function. We next asked if ABHD11 loss resulted in HIF-1α accumulation. Two genome-wide CRISPR sgRNA libraries were used to identify genes that when mutated activated the HIF reporter: the Brunello human genome-wide library (containing 76,441 sgRNA)16, and the Toronto genome-wide knockout library (containing 176,500 sgRNA)19. HeLa cells stably expressing the HRE-GFP OD D cells, using three different sgRNA, validated the findings from the screens (Fig. 1d, Supplementary Fig. 1e). Stabilisation of HIF-α was observed in multiple cell types (Fig. 1e, f, Supplementary Fig. 1f) and complementation of ABHD11 mixed knockout populations with overexpressed ABHD11 restored HIF-1α levels (Fig. 1g), confirming that ABHD11 loss resulted in HIF-1α accumulation.

We next asked if ABHD11 loss resulted in HIF-1α stabilisation through impaired 2-OG dependent dioxygenase activity. PHD function can be readily assessed by measuring HIF-1α prolyl hydroxylation using a HIF prolyl hydroxylation antibody. Mixed knockout populations of ABHD11 in HeLa cells stably expressing the HRE-GFP ODD cells, using three different sgRNA, validated the findings from the screens (Fig. 1d, Supplementary Fig. 1e).

Results

ABHD11 mediates activity of 2-OG dependent dioxygenases.

To find genes involved in 2-OG metabolism we utilised the sensitivity of the HIF response to 2-OG availability, and carried out CRISPR/Cas9 mutagenesis screens in human cells using a fluorescent HIF reporter we developed4,15. This reporter encodes the consensus HIF responsive element (HRE) in triplicate that drives the expression of GFP fused to the oxygen and 2-OG sensitive region of HIF-1α (Supplementary Fig. 1a). Therefore, reporter stability is dependent on 2-OG dependent dioxygenase activity of the prolyl hydroxylases (PHDs or EGLNs)4,13,15, which was confirmed with treatment with the PHD inhibitor dimethyl-allyloxylglycine (DMOG), cell permeable 2-OG (dimethyl-2-OG) or incubation in 1% oxygen (Supplementary Fig. 1b–d).4,15

Two genome-wide CRISPR sgRNA libraries were used to identify genes involved in the canonical pathway for HIF stability (VHL, EGLN1 (PHD2)) and 2-OG metabolism (OGDHc components, lipoic acid synthesis pathway), validating the approach (Fig. 1b, c). Other biological processes that were significantly enriched for sgRNA included intracellular iron metabolism, the mTOR pathway, and transcriptional regulation (Fig. 1b, c). The reliance of the HIF pathway on these processes is well substantiated and in line with our prior studies using gene-trap mutagenesis in haploid cells. In addition to these known pathways, we identified an uncharacterised α/β hydrolase, ABHD11, that was highly enriched for sgRNA in both screens (Fig. 1b, c).

CRISPR/Cas9 mixed knockout populations of ABHD11 in HeLa cells stably expressing the HRE-GFP ODD cells, using three different sgRNA, validated the findings from the screens (Fig. 1d, Supplementary Fig. 1e). Stabilisation of HIF-1α was observed in multiple cell types (Fig. 1e, f, Supplementary Fig. 1f) and complementation of ABHD11 mixed knockout populations with overexpressed ABHD11 restored HIF-1α levels (Fig. 1g), confirming that ABHD11 loss resulted in HIF-1α accumulation.

We next asked if ABHD11 loss resulted in HIF-1α stabilisation through impaired 2-OG dependent dioxygenase activity. PHD function can be readily assessed by measuring HIF-1α prolyl hydroxylation using a HIF prolyl hydroxylation-specific antibody. Mixed knockout populations of ABHD11 stabilised HIF-1α in a non-hydroxylated form, similar to the HIF-1α stabilisation with DMOG (Supplementary Fig. 1g). In contrast, inhibition of the VHL E3 ligase with VH29820, which stabilises HIF-1α by preventing ubiquitination and proteasome-mediated degradation showed high levels of hydroxylated HIF-1α (Supplementary Fig. 1g). To verify that the increased prolyl hydroxylation was due to impaired PHD activity, we directly measured prolyl hydroxylation of a recombinant HIF-1α protein in control or ABHD11 deficient lysates. Rapid prolyl hydroxylation was observed with a HeLa control lysate but this was markedly reduced in the ABHD11 depleted cells, similarly to loss of OGDHc function (Supplementary Fig. 1h, i). This PHD inhibition activated a transcriptional HIF response, promoting activation of HIF-1α target genes, VEGF and carbonic anhydrase 9, similarly to loss of VHL or OGDHc (Fig. 1h, i).
We also explored whether ABHD11 loss altered the activity of other 2-OG dependent dioxygenases involved in transcription. ABHD11 KO cells showed a marked decrease in total DNA 5-hydroxymethylcytosine (5hmC) levels, similar to those observed when OGDHc function is impaired\(^4\) (Fig. 1j, Supplementary Fig. 1j), indicating that Ten-eleven translocation (TET) activity was impaired. However, the steady state levels of selected histone marks were not altered by ABHD11 depletion (Supplementary Fig. 1k). As levels of methylation depend on transferase activity, demethylation and nucleosome turnover, lysine demethylases (KDM) may still be affected by ABHD11 loss. Despite these differences between TET and KDM activity, these studies suggested that ABHD11 loss had broader implications for 2-OG dependent dioxygenase function, aside from PHDs.

**ABHD11 is required for OGDHc function.** Impaired 2-OG dependent dioxygenase activity under aerobic conditions suggested
that ABHD11 may be involved in 2-OG metabolism. Therefore, we first examined the consequences of ABHD11 loss on 2-OG levels and other TCA cycle intermediates. HeLa cells were depleted of ABHD11 and small molecule metabolites traced by incubating cells with uniformly $^{13}$C labelled ([U-$^{13}$C$_5$]) glutamine, followed by liquid chromatography mass spectrometry (LC-MS) (Fig. 2a). Cells deficient in OGDH were used as a control to measure perturbations of 2-OG metabolism. ABHD11 depletion resulted in 2-OG accumulation, similarly to OGDH loss (Fig. 2b). This increase in 2-OG was not due to activation of the HIF response, as we previously demonstrated that PHD2 deficiency does not perturb 2-OG levels alone. $^{13}$C tracing confirmed that ABHD11 depletion impaired OGDHc function, as TCA cycle metabolites downstream of the OGDHc were decreased (succinate, fumarate and malate) (Fig. 2c–e), and cells adapted by showing a shift from oxidative metabolism to reductive carboxylation, with a relative decrease in citrate, and an increase in m + 4 and m + 2 citrate, and an increase in m + 5 and m + 3 citrate isotopologues (Fig. 2f).

To substantiate that ABHD11 levels altered OGDHc function, we measured OGDHc enzymatic activity in isolated mitochondria, using a colorimetric assay which detects oxidation of exogenous 2-OG with a redox sensitive probe (Fig. 2g). OGDHc activity was decreased in ABHD11 deficient mitochondria, similarly to levels observed with depletion of the OGDH subunit (Fig. 2g). Loss of OGDHc function was not due to HIF stabilisation, as VHL depletion had no effect on OGDHc activity (Fig. 2g). Bioenergetic profiling also showed that ABHD11 depletion impaired oxygen consumption rates (Fig. 2h, i), consistent with a major defect in the TCA cycle and oxidative phosphorylation.

2-OG accumulation can impair 2-OG dependent dioxygenase activity through the formation of L-2-HG (Fig. 2a)$.^{4,6,7}$ Consistent with this, we observed an accumulation in 2-OG levels following ABHD11 depletion, similarly to OGDH loss (Fig. 2j). 2-HG predominantly accumulated in its L enantiomeric form, although a small increase in D-2-HG was also observed (Fig. 2k). Stable isotope tracing with [U-$^{13}$C$_5$]glutamine confirmed L-2-HG was derived directly from 2-OG in both the ABHD11 and OGDH deficient cells (m + 5 isotopologues) (Fig. 2k).

Three enzymes are implicated in the formation of L-2-HG from 2-OG: lactate dehydrogenase A (LDHA), malate dehydrogenase 1 and malate dehydrogenase 2$.^{6,8}$ Reductive carboxylation and an acidic environment potentiate the reduction of 2-OG to L-2-HG and inhibition of LDHA alone is sufficient to prevent L-2-HG formation$.^{4,7}$ Therefore, to confirm that L-2-HG was responsible for decreased 2-OG dependent dioxygenase activity, we treated cells with sodium oxamate, which inhibits LDHA as well as decreasing 2-OG formation from glutamine$,^{4,7}$ or the selective LDHA inhibitor GSK-2837808A, and measured HIF-1α levels by immunoblot (Fig. 2m). Both treatments restored HIF-1α turnover in ABHD11 deficient HeLa cells. Together, these confirmed that impaired OGDHc function and L-2-HG accumulation was responsible for the decreased PHD activity and activation of the HIF response.

**ABHD11 is a mitochondrial hydrolase.** ABHD11 is a member of the alpha-beta hydrolase family, which contains 19 known genes, and encodes an $\alpha/\beta$ hydrolase fold (Supplementary Fig. 2a), typical of many proteases and lipases$.^{22}$ Unlike most alpha-beta hydrolase family members, ABHD11 is predicted to localise to the mitochondria through a classical mitochondrial targeting sequence (Fig. 3a, Supplementary Fig. 2a). Therefore, we used immunofluorescence microscopy to determine whether ABHD11 resided within mitochondria. Endogenous ABHD11 could not be readily detected by immunofluorescence, but exogenously expressed ABHD11 fused to GFP (ABHD11-GFP), which still retained function (see Fig. 4f), colocalised with MitoTracker DeepRed (Fig. 3b, c).

We biochemically confirmed ABHD11’s endogenous localisation using isolated mitochondria and a Proteinase K protection assay. Cytoskeletal and outer membrane proteins were rapidly lost with the addition of Proteinase K (30 min at 37°C), but ABHD11 levels were unaffected, suggesting localisation inside of the outer membrane (Fig. 3d). Furthermore, ABHD11 was still retained in mitoplasts, irrespective of proteinase K treatment, consistent with its localisation to the mitochondrial matrix (Fig. 3d). As the stable isotope tracing demonstrated that ABHD11 loss altered OGDHc activity, we determined if ABHD11 associated with components of the complex. Both OGDH and DLST immunoprecipitated with HA conjugated ABHD11 (Fig. 3e), and ABHD11-GFP colocalised with the OGDH (Fig. 3f). We also subjected immunoprecipitated ABHD11-HA to mass spectrometry, which confirmed the association with OGDH and DLST (Supplementary Table 1). Furthermore, our findings were consistent with a prior unbiased mass spectrometry analysis of interactions between mitochondrial proteins, which identified that ABHD11 associated with OGDH with high confidence$.^{23}$

We next examined if ABHD11 enzymatic activity was required for its effect on HIF-1α stability. Structural modelling of ABHD11 predicted a typical $\alpha/\beta$ hydrolase fold with two catalytic motifs (Fig. 3a, g). Hydrolase activity is predicted to arise from the serine nucleophile motif (GXSXG), but ABHD11 also encodes a putative
acyltransferase motif (HXXXXD) found in several other α/β hydrolases22 (Fig. 3a, g, Supplementary Fig. 2b). Reconstituting ABHD11 deficient cells with the putative ABHD11 nucleophile mutant (S141A) or acyltransferase mutant (H296A) did not restore HIF-1α to basal levels, suggesting that ABHD11’s hydrolase activity was required (Fig. 3h). Importantly, this was not due to mis-localisation of the ABHD11 mutants, as both ABHD11 S141A and ABHD11 H296A were visualised within mitochondria (Supplementary Fig. 3a, b).

To confirm that these mutations were altering ABHD11 enzymatic activity, we purified wildtype and S141A ABHD11 and measured hydrolysis of p-nitrophenyl ester, a substrate validated for generic α/β hydrolase activity 22,24. Wildtype ABHD11 protein and the S141A mutant were isolated by Oxidative metabolism

Reductive metabolism

2-hydroxyglutarate

Acetyl-CoA

Oxaloacetate

Aspartate

2-Oxoglutarate

Glutamate

Fumarate

Succinate

Succinyl-CoA

2-oxoglutarate

31.5

1.5

1.0

0.5

0.0

0

100

200

300

400

50

100

OGDHc activity in isolated mitochondria

Basal OCR

(timepoint 1)
expression in HEK293T cells and FLAG tag affinity purification (Supplementary Fig. 4). ABHD11 predominantly migrated as a single species but a slower migrating form was apparent in the cell extract and purified protein, consistent with an immature form prior to mitochondrial insertion (Supplementary Fig. 4a). Mass spectrometry analysis confirmed ABHD11’s identity and demonstrated that the mitochondrial targeting sequence was lost in the predominantly expressed form (Supplementary Fig. 4b) (the slower migrating species was of too low abundance). Size exclusion chromatography identified two peaks but full length ABHD11 was only detected in the second peak, at an elution volume consistent with a monomeric species (Supplementary Fig. 4c, d). Hydrolysis of the p-nitrophenyl ester confirmed ABHD11 enzymatic activity, but this was lost with the S141A mutant and following heat treatment (Fig. 3i). Thus, ABHD11 is a mitochondrial hydrolase that associates with the OGDHc, and loss of its enzymatic activity leads to HIF-1α accumulation.

**ABHD11 loss impairs lipoylation of the OGDHc.** Conversion of 2-OG to succinyl-CoA by the OGDHc requires decarboxylation and the formation of succinyl intermediate (succinyl-dihydrolipoate), dependent on the cyclical reduction and oxidation of the lipoylated DLST subunit (Fig. 4a). Therefore, to understand how ABHD11 is required for OGDHc function, we first examined whether protein levels of core OGDHc components or its lipoylation were altered. ABHD11 depletion did not alter total levels of the OGDHc subunits (OGDH, DLST or DLD) in HeLa cells (Fig. 4a, b). However, using a specific anti-lipoate antibody that detects conjugated lipoamide, we observed a reproducible loss of the faster migrating lipoylated protein species, attributed to the lipoylated DLST subunit of the OGDHc (Fig. 4b, c). Immunoprecipitation of endogenous DLST confirmed loss of lipoylation following ABHD11 depletion, without altering total DLST levels (Supplementary Fig. 5a), and this decreased DLST lipoylation was observed in several cell types (Fig. 4d, e). Furthermore, in contrast to complete disruption of lipoic acid synthesis by LIAS depletion, ABHD11 loss preferentially decreased DLST lipoylation, without altering the other abundantly lipoylated protein within the mitochondria, the DLAT (dihydrolipoamide acetyltransferase) subunit of the pyruvate dehydrogenase complex (PDHC) (Fig. 4b–e, Supplementary Fig. 5b). Indeed, PDHC function, as measured by [U-13C5] glucose stable isotope tracing, was not impaired in the ABHD11 deficient HeLa cells (Supplementary Fig. 6a–g), and lactate production was not increased compared to control HeLa cells (Supplementary Fig. 6h).

Complementation studies were used to determine whether the enzymatic activity of ABHD11 was required for lipoylation of the OGDHc. Exogenous wildtype or mutant ABHD11 were expressed in mixed ABHD11 KO populations and lipoylation levels measured by immunoblot. DLST lipoylation was restored with the wildtype ABHD11 but not with the S141A or H296A mutants (Fig. 4f). HIF-1α levels were only reduced to basal levels by reconstituting with wildtype ABHD11 but not the nucleophile mutants (Fig. 4f), as previously shown.

Lastly, to confirm the enzymatic requirement of ABHD11 in OGDHc lipoylation, we used a highly selective covalent inhibitor of ABHD11, ML226, that was initially developed as a tool for screening serine hydrolases. ML226 treatment inhibited ABHD11 p-nitrophenyl ester hydrolysis in vitro (Fig. 4g) and decreased OGDHc lipoylation in HeLa cells (Fig. 4h) and cultured myoblasts (C2C12) (Fig. 4i). In addition, DLST lipoylation recovered after ML226 washout in cells, indicating OGDHc lipoylation can be restored (Fig. 4j). Thus, ABHD11 hydrolase activity is required for OGDHc lipoylation.

**ABHD11 maintains functional lipoylation of DLST.** The finding that ABHD11 loss showed a selective loss of DLST lipoylation was unexpected, as prior genetic studies of lipoate conjugation had not shown a requirement for an additional enzyme. Furthermore, we confirmed that ABHD11 loss differed to depletion of other components of the lipoic acid synthesis pathway by generating CRISPR/Cas9 mixed KO populations of the key enzymes involved (Supplementary Fig. 7a, b). Lipoyl(octanoyl)transferase 2 (LIPT2), LIAS, and lipoyltransferase 1 (LIPT1) all had not shown a requirement for an additional enzyme. Therefore, we used a highly selective covalent inhibitor of ABHD11, ML226, that was initially developed as a tool for screening serine hydrolases. ML226 treatment inhibited ABHD11 p-nitrophenyl ester hydrolysis in vitro (Fig. 4g) and decreased OGDHc lipoylation in HeLa cells (Fig. 4h) and cultured myoblasts (C2C12) (Fig. 4i). In addition, DLST lipoylation recovered after ML226 washout in cells, indicating OGDHc lipoylation can be restored (Fig. 4j). Thus, ABHD11 hydrolase activity is required for OGDHc lipoylation.

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levels (Supplementary Fig. 8b). Small changes in mitochondrial ROS were observed with ABHD11 loss, using MitoSOX Red, similarly to OGDH or LIAS depletion (Supplementary Fig. 8c, d). However, ML226 treatment, showed no change in MitoSOX Red levels, and importantly, Antimycin A, which increased mitochondrial ROS to higher levels than ABHD11 inhibition, was not sufficient to activate the HIF reporter (Supplementary Fig. 8e). Thus, alterations in mitochondrial ROS were unlikely to account for the HIF stabilisation or altered lipoylation following ABHD11 loss or inactivation of the OGDHc.

To explore further how ABHD11 activity altered DLST lipoylation we used mass spectrometry analysis of the lipoate

levels.
moiety. Immunoprecipitated DLST was treated with a reducing agent and then incubated with N-ethylmaleimide (NEM), forming a NEM-lipoyl conjugate, which had previously been shown to aid detection of the lipoyte moiety29 (Fig. 5a, Supplementary Fig. 9a). Interestingly, NEM treatment prevented detection of immunoprecipitated lipoylated DLST by immunoblot (Supplementary Fig. 9a), demonstrating that the anti-lipoate antibody only detected the functional lipoate and not the NEM-modified form, suggesting that the apparent loss of DLST lipoylation in ABHD11 deficient cells may be due to modification of the lipoyte moiety. We next measured levels of DLST lipoylation (NEM-lipoyl) by label-free quantification on immunoprecipitated DLST from wildtype HeLa cells or those deficient in LIAS or ABHD11 (Fig. 5a, b, Supplementary Fig. 9b). To account for potential differences in DLST protein abundance around the lipoylated region (DK*TSVQVSPSA), we normalised these peptides to the sum of all DLST peptide abundance. Therefore, a modification of the lipoyte moiety on immunoprecipitated DLST, but these modifications did not account for the unassigned mass of the DLST peptide (Supplementary Dataset 2). However, the complex nature of lipid based adducts of undefined and variable lengths may preclude their detection.

While the exact nature of the lipoyl adduct formed in ABHD11 deficient cells was unclear, we examined whether exogenous treatment with 4-HNE could alter DLST lipoylation, similarly to ABHD11 depletion. 4-HNE treatment of cell lysates preferentially decreased detection of DLST lipoylation by immunoblot (Fig. 5d), consistent with the formation of lipoyl adducts preventing binding to the antibody. DLAT lipoylation was only affected at high concentrations (5 mM) of 4-HNE (Fig. 5d), suggesting that DLAT may be more resistant to lipoyl adduct formation than DLST, and consistent with our findings that ABHD11 loss preferentially effects the OGDHc.

Finally, to explore whether ABHD11 protected against the formation lipoyl adducts, such as those formed by 4-HNE, we measured if ABHD11 loss or inhibition made the OGDHc more susceptible to lipid peroxidation damage. Control or ABHD11 depleted HeLa cells or lysates were treated with 4-HNE, and lipoylation detected by immunoblot. 4-HNE decreased the detection of DLST preferentially to DLAT within cell lysates (Fig. 5d), consistent with lipoyl adducts preventing detection of the lipoyte moiety by immunoblot. 4-HNE treatment of cells also decreased DLST functional lipoylation preferentially to DLAT, and ABHD11 deficient cells were more susceptible to 4-HNE treatment compared to the control cells (Fig. 5e). Overexpression of ABHD11 inactive mutants competed with endogenous ABHD11 to also show an increase lipoyl-adduct formation following 4-HNE treatment (Supplementary Fig. 9c). ABHD11 overexpression did not increase DLST lipoylation compared to control cells (Supplementary Fig. 9c) but this finding is consistent with exogenous ABHD11 not increasing total lipoylation levels and reflect that OGDHc lipoylation is tightly regulated, with only 50% of DLST modified by lipoylation. While these studies demonstrated that 4-HNE could disrupt functional lipoylation, we were concerned that the concentrations required were higher than prior reports33,34, and considered that this may be due to the presence of L-cysteine within media. Therefore, we repeated these assays using lower concentrations of 4-HNE in media without L-cysteine (Fig. 5f). We now observed impaired DLST lipoylation with low concentrations of 4-HNE (40 µM) in control HeLa cells,
with complete loss of DLST in ABHD11 deficient cells at 20 µM 4-HNE (Fig. 5f). Furthermore, while 4-HNE preferentially altered DLST, DLAT lipoylation was also decreased in the ABHD11 null cells compared to the controls (Fig. 5f). Similar findings were observed with ABHD11 inhibition, consistent with a requirement for ABHD11 to maintain functional lipoylation in the context of lipid peroxidation products. In conclusion, while the nature of adducts formed on lipoylated DLST remain to be fully determined, these studies demonstrate that ABHD11 is required for functional lipoylation of the OGDHc, and may protect against the formation of lipoyl adducts, such as those formed by 4-HNE (Fig. 6).

**Discussion**

This study identifies ABHD11 as a mitochondrial enzyme required for OGDHc function, and to our knowledge, is the first example of a mitochondrial pathway that maintains TCA cycle integrity by preserving functional OGDHc lipoylation (Fig. 6).
Moreover, we demonstrate that ABHD11 inhibition allows 2-OG metabolism to be modulated in multiple cells and in a reversible manner, with potential broad implications for altering cell fate-decisions and manipulating 2-OG abundance in tumours.

The selective loss of lipoylated DLST following ABHD11 depletion initially suggested that it may be necessary for OGDHc lipoate conjugation. However, a requirement for ABHD11 in lipoate synthesis had not been previously observed13,26,35, and LIPT1 deletion or human loss of function mutations prevent PDHc and OGDHc lipoylation26–28. It was possible that ABHD11 transfers lipoate moieties between 2-oxoacid dehydrogenases, but our mass spectrometry findings argued against this. If ABHD11 was required for lipoate transfer, the unmodified DLST peptide should accumulate in ABHD11 depleted cells. Instead, we found an absence of both the modified and unmodified lipoylated region of DLST by mass spectrometry (Fig. 5b, c). Similar coverage of DLST peptides upstream and downstream of the lipoylated region confirmed that there was no change in total DLST levels following ABHD11 loss. Therefore, a peptide of undefined mass must account for the apparent loss of this region, indicating a post-translational modification other than lipoylation or the formation of a lipoyl adduct.

Common post-translational modifications (e.g. ubiquitination, phosphorylation or acetylation), combinations of modifications, or known DLST intermediates (e.g. succinyl-dihydrolipoamide, acyl-dihydrolipoamide or S-glutathionylation) (Supplementary Dataset 2) did not account for the peptide loss of the lipoylated DLST region, suggesting that this DLST peptide was not uniformly modified. Lipid peroxidation products (hydroxyalkenals, such as 4-HNE), arise from free radical propagation through phospholipids31, and can easily react with thiol groups, inactivating 2-oxoacid dehydrogenases by forming lipoil adducts32. We did not observe 4-HNE lipoil adducts on immunoprecipitated DLST, but the hydrophobicity and complex nature of these adducts may preclude their detection by mass spectrometry, accounting for the apparent loss of DLST IK*TSVQVPSA peptide that we observed (Fig. 5b). We also required high concentrations of 4-HNE to decrease detectable lipoylation in immunoprecipitated DLST, and 0.1 mM 4-HNE in cells incubated in serum-free media. High levels or prolonged treatment of 4HNE may lead to depletion of cellular antioxidant levels and apoptosis36,37, but we did not observe changes in DLST lipoylation at 0.1 mM consistent with a selective effect on DLST, as previously observed38. Furthermore, treatment of HeLa cells with 4-HNE in media without L-cysteine resulted in selective loss of DLST lipoylation at lower, biologically relevant 4-HNE concentrations33,34.

The apparent specificity of ABHD11 for DLST may relate to the preponderance of lipoil adducts formed by the OGDHc compared to other lipoylated proteins, or selectively binding to the OGDHc, rather than all 2-oxoacid dehydrogenases. It is possible that ABHD11 may regulate DLST indirectly, but this is unlikely to be due to altered mitochondrial ROS (Supplementary Fig. 8). The association with DLST and OGDH (Fig. 3e), and marked accumulation of 2-OG (Fig. 2) are also consistent with a direct role on OGDHc function. These findings are also supported by prior mass spectrometry interactome studies, showing an association of ABHD11 with OGDH23. Whether ABHD11 interacts with other 2-oxoacid dehydrogenases will be of interest to explore further. We observed that ABHD11 can interact with DLAT by mass spectrometry (Supplementary Table 1), and a small decrease in DLAT lipoylation following ABHD11 loss was observed. ABHD11 deficient cells were also more susceptible to impaired lipoylation following 4-HNE treatment. However, ABHD11 loss did not increase pyruvate levels in HeLa cells (Supplementary Fig. 5), which would be expected if PDHc activity was significantly impaired.

ABHD11 is one of a family of alpha-beta hydrolase domain-containing enzymes, which as a group are poorly characterised. Of these, only ABHD10 and ABHD11 are known to be mitochondrial39. ABHD10 is has recently been shown to be an acyl protein thioesterase, with S-depalmitoylase activity against the anti-oxidant protein peroxiredoxin-540. These findings would be consistent with our observations for ABHD11 regulating the thiol-containing lipoate moiety on DLST. However, ABHD11 has less sequence identity with ABHD10 than other ABHD family members (Supplementary Fig. 2), and is not inhibited by ML22625.

This work provides insights into the functional role of ABHD11, for which no physiological substrate or role has been identified previously. ABHD11 is one of ~26 genes included in the 7q11.23 hemizygous deletion of Williams-Beuren syndrome, a rare multisystem disorder often characterised by developmental and cardiac abnormalities41. While the phenotype of cardiac and soft tissue disease is felt largely due to loss of Tropoelastin 1 (ELN)41, a functional understanding of ABHD11 may offer some insights into aspects of this syndrome. It will also be of interest to explore further the biological consequences of ABHD11 loss compared to other lipoate enzymes. ABHD11 loss disrupts the TCA cycle, impairing oxidative phosphorylation and promoting reductive metabolism, but ABHD11 has a distinct metabolic phenotype compared to loss of other lipoic acid pathway enzymes. Germline mutations in LIAS, LIPT2 and LIPT1 result in impaired PDHc and OGDHc activity13,27,42,43.
but ABHD11 loss did not significantly alter DLAT lipoylation in several cancer lines. PDHc activity is required for lipogenesis, by providing acetyl-CoA, and LIPT1 mutations result in defective lipid synthesis.44 As ABHD11 acts predominantly on the OGDHc, it is possible that the preserved activity of PDHc fuels lipogenesis, and cell survival, and it will be important to determine whether ABHD11 activity, and the resulting HIF activation, feeds back on lipid synthesis and the mitochondrial fatty acid pathway.

This study and prior reports demonstrate that human mutations in lipoylation enzymes increase 2-OG levels and promote L-2-HG formation.42 However, how L-2-HG inhibits 2-OG sensitive enzymes when 2-OG is abundant remains to be fully resolved. It is possible that L-2-HG may allosterically inhibit the enzymes, or that additional factors aside from L-2-HG result in decreased dioxygenase activity. The reasons for the propensity to form L-2-HG under certain conditions of 2-OG accumulation also remains to be determined, as in other cellular responses, such as embryonic stem cell pluripotency,2 2-OG treatment does not result in inhibition of 2-OG dependent dioxygenases. These discrepancies may relate to the reductive environment that occurs when OGDHc activity is impaired (which is likely to occur with

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**Fig. 5 ABHD11 prevents the formation of lipoyl adducts by lipid peroxidation products.**

**a, b** Mass spectrometry analysis of DLST lipoylation. DLST was immunoprecipitated from HeLa control, ABHD11 deficient or LIAS deficient cells, and treated with NEM to modify the free thiols and maintain the lipoyl moiety in a reduced state. After SDS-PAGE, protein samples were digested with Asp-N protease and analysed by LC-MS/MS. **b** Normalised level of lipoylated DLST peptide compared to DLST reference peptide. Relative levels of the unmodified, NEM-dihydrolipoamide, and lipoamide DLST peptide are shown. n = 3, Mean ± SEM. **c** Schematic of normal cyclical reduction and oxidation of the lipoyl moiety on DLST (left). The reduced dihydrolipoamide can react with lipid peroxidation products (e.g., 4-HNE) to form lipoyl adducts through the free thiols, which may also propagate (right). **d–f** Effect of 4-HNE treatment on lipoylation. HeLa cells lysates were treated with 4-HNE at the indicated concentrations for 60 min (50 °C) and immunoblotted for lipoylated proteins or total DLST (**d**). Control or ABHD11 deficient HeLa cells were treated with 4-HNE in serum-free Optimem (**e**) or serum and L-cysteine free DMEM (**f**) for 90 min (37 °C), lysed and immunoblotted for lipoylation and total DLST.
ABHD11 loss), or the metabolic phenotype of the cell (e.g. reliance on oxidative phosphorylation versus glycolysis).

Lipoic acid has been traditionally described as an essential cofactor for 2-oxoacid dehydrogenases, but only 50% of DLST in HeLa cells was observed to be lipoylated in resting cells (Fig. 5b), and OGDHc lipoylation was rapidly stored after washout of 2-OG (Fig. 4j). These findings show that lipoylation is a dynamic modification that must be maintained, which is further supported by recent observations that SIRT4 acts as a lipoyamidase, altering PDHc function and that increased lipoylation is a dynamic modification that must be maintained, to a functional lipoylation and allowing the conversion of 2-OG to succinyl CoA.

Methods

Cell lines and reagents. HeLa, MCF-7, C2C12 and HEK293T cells were maintained in DMEM (Sigma D6429), and THP-1 cells maintained in RPMI-1640 (Sigma R8758), both supplemented with 10% foetal calf serum (Sigma P4333) and 100 units/ml penicillin with 100 µg/ml streptomycin, in a 5% CO2 incubator at 37 °C. HeLa HRE-GFPODD cells were produced by lentiviral transduction of the I.M.A.G.E. cDNA clone (IRATp970F0688D, Source Bioscience), cloned into the pHR3SIN pSFFV backbone with pGK-LoxP Blasticidin resistance (a gift from Paul Lehner), using NEBuilder HiFi (NEB). Prior to assembly, silent mutations were introduced inside the sequence targeted by ABHD11 sgRNA 2, using PCR primers detailed in Supplementary Table 3. Mutations of catalytic residues serine 141 to alanine (S141A) and histidine 296 to alanine (H296A) of ABHD11 were created using NEBuilder HiFi with primers detailed in Supplementary Table 3. Lentiviral vectors encoding eGFP, mCherry, or eGFP/mCherry were constructed using lentiviral expression vectors (pHRSIN) and pMD.G (VSVG)48. Viral supernatant was treated was treated with 2 µg DNA (DNA was mixed in a 3:2:4 ratio of the relevant expression vector pKLV-U6gRNA(BbsI)-PGKpuro2ABFP46. All sgRNA sequences are detailed in Supplementary Table 3.

Preparation of lentivirus and transductions. HEK293T cells were transfected using TransIT-293 (Mirus) according to the manufacturer’s protocol. For small scale experiments, lentivirus was produced in 6-well plates containing 2 ml media, using 2 µg DNA (DNA was mixed in a 3:2:4 ratio of the relevant expression plasmid, pCMV-dR8.91 (gag/pol) and pMD.G (VSVG)48. Viral supernatant was harvested after 48 h, passed through a 0.45 µm filter, and frozen at −80 °C. Cells were transduced with lentivirus by adding an appropriate volume of thawed viral supernatant. In the case of single-gene knockdown in HeLa cells,
250 µl of virus with 5 × 10^4 cells in a 24-well plate made up to 1 ml media. For the screens, a titration of increasing volumes of virus was used, with 10^6 HeLa cells in a 6-well plate (triplicates). Plates were centrifuged for 1 h at 37 °C at 750 x g immediately after addition.

**Whole-genome CRISPR/Cas9 forward genetic screens.** HeLa HRE-GFP^O3D cells were transduced with Streptococcus pyogenes Cas9 (pHR5SN-FLAG-NLS-CAS9-NLS-pGK-Hygro) and selected for Cas9 expression using hygromycin. 5 × 10^6 (Brunelli) or 10^6 (TKO) HeLa HRE-GFP^O3D cells were transduced with the appropriate volume of pooled sgRNA virus (multiplicity of infection (MOI) of ~0.3) maintaining at least 150-fold sgRNA coverage. After 30 h, cells were treated with puromycin 1 µg/ml for 5 days. Representation was maintained throughout the screen such that no selection event occurred where the library was cultured at fewer than 200 times the number of sgRNA sequences in the library. The library was pooled immediately before selection event. FACS was performed by harvesting 10^6 cells, washing the cells in PBS, and then resuspending them in PBS containing 2% foetal calf serum and 10 mM HEPES (Sigma #H8987). Cells were sorted using an Influx cell sorter (BD); GFP-high cells were chosen in a gate set at one log10 unit above the mode of the untreated population.

**Genomic DNA was extracted using a Gentra Puregene Core kit (Qiagen).** Lentiviral sgRNA inserts were amplified in a two-step PCR (with Illumina adapters added on the second PCR), as previously described. For the TOKO screen, the forward inner PCR and sequencing primers were modified (Supplementary Table 3). Sequencing analysis was performed by first extracting the raw sequencing reads, trimming the first 20 bp (FASTX-toolkit), and aligning against the appropriate sgRNA library using Bowtie5.10 Read counts for each sgRNA were compared between conditions, and Benjamini-Hochberg false discovery rates for each gene calculated, using MAGeCK51 (Supplementary Dataset 1). The analysis presented compares DNA extracted following the second sort to an unsorted DNA library taken at the same timepoint.

**Flow cytometry.** HeLa HRE-GFP^O3D cells were washed in PBS and fixed in PBS 1% formaldehyde prior to analysis. For MitosOX Red staining HeLa cells were plated in 24-well plates. After 24 h cells were treated with or without 10 µM Antimycin A for 37 °C for 30 min, washed with PBS or Hank's Balanced Salt Solution (HBSS), and then stained with 5× MitosOX Red in PBS or HBSS for 10 min at 37 °C. Cells were harvested and resuspended in PBS for analysis by flow cytometry (BD Fortessa; software: FACSDiva 8.0). Flow cytometry gating strategy is shown in Supplementary Fig. 10. Images were presented using FlowJo v10 (BD).

**Immuno blotting and immunoprecipitation.** Cells were lysed in an SDS lysis buffer containing 2% SDS, 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol, and 1:200 protease nucleosome (Sigma), for 15 min at room temperature, then heated at 90 °C for 5 min. Proteins were separated with SDSPAGE electrophoresis, transferred to a PVDF membrane, and probed using appropriate primary antibodies and a secondary with HRP conjugate. Densitometry measurements were made using ImageJ52. To identify protein interactions with ABHD11, HeLa cells lentivirally transduced with ABHD11 with a C-terminal HA tag were lysed in a buffer containing 100 mM Tris pH 8.0, 140 mM NaCl, 1% IGEPA CA-630 (Sigma), 1 mM PMSF (Sigma P7626) and Coomplete Protease Inhibitor Cocktail (Roche). After centrifugation at 17,000 x g, the supernatant was pre-cleared using Sepharose CL-4B (GE Healthcare) and incubated overnight with FLAG M2 antibody conjugated beads (Sigma). Following five washes with PBS, ABHD11-FLAG was eluted using 100 µg/ml 3xFLAG peptide (Sigma F7499), filtered using a 0.22 µm PVDF filter and separated using a Superdex 75 10/300 column on an Äkta-Pure liquid chromatography system (software: Unicorn 6.3). 500 µl fractions were collected and protein content visualised by SDS-PAGE and Coomassie staining. Protein identity was confirmed by LC-MS/MS.

**Liquid chromatography mass spectrometry.** Samples were reduced, alkylated and digested in-i-cell using either trypsin, GluC or AspN. The resulting peptides were analysed by LC-MS/MS, using an Orbitrap Fusion Lumos coupled to an Ultimate 3000 RSLC nano UHPLC equipped with a 100 µm ID x 2 cm Acclaim PepMap Precolumn (Thermo Fisher Scientific) and a 75 µm ID x 50 cm, 2 µm particle Atlantis HPTC-RpC18 (5 µm) column. Loading solvent A contained 3% acetonitrile, loading solvent B contained 0.1% formic acid and B: 80% acetonitrile + 0.1% formic acid. Samples were loaded at 5 µl/min loading solvent for 5 min before beginning the analytical gradient. The analytical gradient was 3-40% B over 42 min rising to 95% B and for 5 min followed by a 42 min wash at 95% B and equilibration at 3% solvent B for 10 min. Columns were held at 40 °C. Data were acquired in a DDA fashion with the following settings: MS1: 75-1500 Th, 120,000 resolution, 5 × 10^4 AGC target, 100 ms maximum injection time. MS2: Quadrupole isolation at an isolation width of m/z 1.6, HCD fragmentation (NCE 30) with fragment ions scanning in the Orbitrap from m/z 110, 5 × 10^4 AGC target, 100 ms maximum injection time. Dynamic exclusion was set to +/− 10 ppm for 60 s. MS2 fragmentation was triggered on precursors 5 x 10^4 counts and above.

Raw files were processed using PEAKS Studio (version 8.0, Bioinformatics Solutions Inc.). Searches were performed with either trypsin, GluC or AspN against a Homo sapiens database (Uniprot reference proteome downloaded 26/01/2018). Consensus spectra (58,131 sequences) and an additional contaminant database (containing 246 common contaminants). Variable modifications at PEAKS DB stage included oxidation (M) and carbamidomethylation with 479 built in modifications included at PEAKS PTM stage.

**p-Nitrophenyl ester hydrolysis assay.** Hydrolytic activity of ABHD11-FLAG (or a heat-inactivated control made by incubation at 90 °C for 5 min) was assayed by incubating 50 µl in an assay buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% bovine serum albumin, 1.4% methanol, and 500 µM p-nitrophenyl acetate (Sigma N8130). 1.5 µg enzyme was added to 200 µl assay buffer, and the formation of p-nitrophenol was monitored using a Clariostar plate reader (BMG Labtech), recording absorbance at 405 nm while incubating at 37 °C for 40 min. The rate of formation of p-nitrophenol was calculated from the slope of the absorbance curve, subtracting the slope of a blank containing only assay buffer and substrate, and calibrated against a standard curve of p-nitrophenol (software: Microsoft Excel for Mac).
OGDH activity assay. OGDH activity was measured in whole cell lysates using a Biovision ketoglutarate dehydrogenase activity assay (Biovision K678), according to the manufacturer’s protocol. HeLa cells were seeded in 24-well plates 27 h prior to metabolite extraction, with a sixth well per condition used for the Mitochondrial bioenergetics assay. HeLa cells were seeded 24 h beforehand at 1.5 × 10^4 cells/well, and assayed using the manufacturer’s Mito Stress Test protocol.

Stable isotope tracing by LC-MS. HeLa cells were lysed by freeze-thaw cycles followed by passing 10 times through a 26-gauge needle. Activity was subtracted from a background control containing cell lysate but no substrate.

Mitochondrial bioenergetics assay. Dynamic measurements of oxygen consumption rate and extracellular acidification were recorded using a Seahorse XF24 (Agilent; software: Wave 2.3). HeLa cells were seeded 24 h beforehand at 1.5 × 10^4 cells/well, and assayed using the manufacturer’s Mito Stress Test protocol.

Immunoprecipitation and mass spectrometry detection of lipoylation. HeLa cells were lysed on ice for 30 min in a HEPES buffer (150 mM NaCl, 50 mM HEPES pH 7.0, 1% IGEPAL CA-630, 1 mM nicotinamide (Sigma), 5 mM tri(2-carboxyethyl)phosphine hydrochloride (Sigma), 1X Complete Protease Inhibitor Cocktail (Roche), and 1 mM PMSE). Lysates were centrifuged at 16,900 × g for 10 min. For LC-MS experiments, free thiols were blocked by addition of 10 mM N-ethyl maleimide (Sigma) and incubated on a rotator at 4 °C for 1 h. Lysates were immunoprecipitated by incubation with protein G beads (GE 17-0168-01), firstly for 1 h to pre-clear, and then overnight with the DLST or DLAT antibodies (Supplementary Table 2). Resins were washed four times with Tris-buffered saline containing 0.1% IGEPAL CA-630, followed by three washes with Tris-buffered saline. Protein was eluted using 2% SDS, 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM dithiothreitol and 10% glycerol, and incubated at 90 °C for 5 min. The proteins were then separated using SDS PAGE (Thermo NP0335), and visualised using SimplyBlue SafeStain (Invitrogen).

For mass spectrometry analysis, in-gel AspN digest and sample analysis were performed as previously described. To identify possible modifications of DLST K110, raw files were processed using PEAKS Studio (version 8.0, PEAKS Solutions Inc.) with the following parameters: AspN digestion; Human database (UniProt reference proteome downloaded 18 Dec 2018 containing 21,066 proteins) with additional contaminant database (containing 246 common contaminants); oxidation and carbamidomethylation as variable modifications at the PEAKS DB stage. The data were processed twice, with different variable modifications searched at the PEAKS PTM stage, either with 485 PEAKS built-in modifications listed in Supplementary Dataset 2 (sheet A) and 34 custom modifications listed in Supplementary Dataset 2 (sheet B).

XIC were obtained from Thermo Xcablibur Qual Browser (4.0.27.13). Mass ranges were limited to 579–564.82 m/z and 784–784.88 m/z for the unmodified, lipoylated and 2X NEM lipoylated peptides respectively. Label-free quantification values were obtained by processing raw files with MaxQuant (version 1.6.6.0) with the following parameters: specific AspN digestion; Human database (UniProt reference proteome downloaded 18 Dec 2018 containing 21,066 proteins); oxidation, lipoylation, 2X NEM lipoylation. N terminal acetylation as variable modifications; carbamidomethylation as a fixed modification; label-free quantification enabled. Label-free quantification values were normalised to the sum of DLST peptides label-free quantification values.

ABHD11 structural prediction. A structural model of ABHD11 was obtained using the NCBI reference sequence for ABHD11 transcript variant 1 (NP_683710.1), modelled with Phyre2 against a template of murine epoxide hydrolase (PDB: 1crf) and visualised using PyMOL 2.3 (Schrödinger, LLC). The mitochondrial targeting sequence was mapped with the MitoFates prediction tool. Phylogenetic analysis and multiple sequence alignment of ABHD11 family members was performed with protein sequences obtained from Uniprot (canonical transcript variant) and aligned using Clustal Omega (EBI).

Statistics and reproducibility. Statistical analysis of the screens was performed using MAGeCK version 0.5.521, testing the sgRNA read counts obtained following the second sort against sgRNA read counts obtained from unsorted cells lysed at the same timepoint. Quantification and data analysis of other experiments are expressed as mean ± SEM and P values were calculated using two-tailed Student’s t-test for pairwise comparisons, unless otherwise stated, and were considered significant using Graphpad Prism version 8. Metabolomic samples were blinded and randomised prior to their evaluation. Qualitative experiments were repeated independently to confirm accuracy. Specifically, Figs. 1e, f, g, 3e, h; 4f, i, j; 5d, f; Supplementary Figs. 1e, g, 5a, b; 9e were repeated twice with similar findings. Figs. 2l, m, 3d, 4d, h; 5c, Supplementary Figs. 1a, d; 7b and 9a were performed independently at least 3 times. Representative data are shown in the figures. Uncropped original scans of all immunoblots are displayed in Supplementary Fig. 11.

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Data availability
SgRNA read count tables from CRISPR/Cas9 genetic screens are shown in Supplementary Dataset 1. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE23 partner repository with the dataset identifier PXD020128 and the results and analysis are also available in Supplementary Dataset 2. LC-MS metabolic profiling has been deposited in MetaboLights36 MTBLS1875. Source data are provided with this paper (Source Data File). Source data are provided with this paper.

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