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Although the addition of the progesthctic group lipoate is essential to the activity of critical mitochondrial catabolic enzymes, its regulation is unknown. Here, we show that lipoylation of the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (αKGDH) complexes is a dynamically regulated process that is inhibited under hypoxia and in cancer cells to restrain mitochondrial respiration. Mechanistically, we found that the polymerase-δ interacting protein 2 (Poldip2), a nuclear-encoded mitochondrial protein of unknown function, controls the lipoylation of the pyruvate and α-KDH dihydrolipoamide acetyltransferase subunits by a mechanism that involves regulation of the caseinolytic protease (Clp)-protease complex and degradation of the lipoyte-activating enzyme Ac-CoA synthetase medium-chain family member 1 (ACSMT1). ACSMT1 is required for the utilization of lipoic acid derived from a salvage pathway, an unacknowledged lipoylation mechanism. In Poldip2-deficient cells, reduced lipoylation represses mitochondrial function and induces the stabilization of hypoxia-inducible factor 1α (HIF-1α) by loss of substrate inhibition of prolyl-4-hydroxylases (PHDs). HIF-1α-mediated retrograde signaling results in a metabolic reprogramming that resembles hypoxic and cancer cell adaptation. Indeed, we observe that Poldip2 expression is down-regulated by hypoxia in a variety of cell types and basally repressed in triple-negative cancer cells, leading to inhibition of lipoylation of the pyruvate and α-KDH complexes and mitochondrial dysfunction. Increasing mitochondrial lipoylation by forced expression of Poldip2 increases respiration and reduces the growth rate of cancer cells. Our work unveils a regulatory mechanism of catabolic enzymes required for metabolic plasticity and highlights the role of Poldip2 as a key during hypoxia and cancer cell metabolic adaptation.

Author contributions: F.P. and A.S.M. conceived the project; F.P., B.L., K.S.G., V.D.-U., and A.S.M. performed research; A.S.M. contributed new reagents/analytic tools; F.P., B.L., G.A.B., G.T., V.D.-U., and A.S.M. analyzed data; and A.S.M. wrote the paper.

The present work establishes that the addition of the progesthctic group lipoate to catabolic enzymes is a dynamically regulated posttranslational modification that increases metabolic plasticity under hypoxia and in cancer cells. We show that the polymerase-δ interacting protein 2 (Poldip2) is an oxygen-sensitive protein that regulates the lipoylation and activation of the pyruvate and α-ketoglutarate dehydrogenase complexes. Additionally, our work reveals that mitochondrial peptidases participate in an integrated response needed for metabolic adaptation. This study positions Poldip2 as a key regulator of mitochondrial function and cell metabolism.

Significance

The thymine group lipoate is essential for the activity of critical mitochondrial catabolic enzymes, its regulation is unknown. Here, we show that lipoylation of the pyruvate dehydrogenase (PDH) and the α-ketoglutarate dehydrogenase (αKGDH) complexes is a dynamically regulated process that is inhibited under hypoxia and in cancer cells to restrain mitochondrial respiration. Mechanistically, we found that the polymerase-δ interacting protein 2 (Poldip2), a nuclear-encoded mitochondrial protein of unknown function, controls the lipoylation of the pyruvate and α-KDH dihydrolipoamide acetyltransferase subunits by a mechanism that involves regulation of the caseinolytic protease (Clp)-protease complex and degradation of the lipoyte-activating enzyme Ac-CoA synthetase medium-chain family member 1 (ACSMT1). ACSMT1 is required for the utilization of lipoic acid derived from a salvage pathway, an unacknowledged lipoylation mechanism. In Poldip2-deficient cells, reduced lipoylation represses mitochondrial function and induces the stabilization of hypoxia-inducible factor 1α (HIF-1α) by loss of substrate inhibition of prolyl-4-hydroxylases (PHDs). HIF-1α-mediated retrograde signaling results in a metabolic reprogramming that resembles hypoxic and cancer cell adaptation. Indeed, we observe that Poldip2 expression is down-regulated by hypoxia in a variety of cell types and basally repressed in triple-negative cancer cells, leading to inhibition of lipoylation of the pyruvate and α-KDH complexes and mitochondrial dysfunction. Increasing mitochondrial lipoylation by forced expression of Poldip2 increases respiration and reduces the growth rate of cancer cells. Our work unveils a regulatory mechanism of catabolic enzymes required for metabolic plasticity and highlights the role of Poldip2 as a key during hypoxia and cancer cell metabolic adaptation.


The authors declare no conflict of interest.

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Significance

The present work establishes that the addition of the progesthctic group lipoate to catabolic enzymes is a dynamically regulated posttranslational modification that increases metabolic plasticity under hypoxia and in cancer cells. We show that the polymerase-δ interacting protein 2 (Poldip2) is an oxygen-sensitive protein that regulates the lipoylation and activation of the pyruvate and α-ketoglutarate dehydrogenase complexes. Additionally, our work reveals that mitochondrial peptidases participate in an integrated response needed for metabolic adaptation. This study positions Poldip2 as a key regulator of mitochondrial function and cell metabolism.


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where one enzyme executes both reactions, mammals require two separate enzymes for the activation and ligation reaction of lipoyte (9). Indeed, the isolation of a unique lipoic acid-activating enzyme activity (lipoic acid + NTP → lipoyl-NMP) from bovine liver was reported by Tsunoda and Yasunobu (10), and was subsequently identified as the product of the Ac-CoA synthetase medium-chain family member 1 (ACSM1) gene (11), a nuclear-encoded mitochondrial protein. The contribution of ACSM1 to protein lipoylation remains unexplored.

The caseinolytic peptidase (Clp) complex is a mitochondrial matrix protease from the ATPase associated with the diverse cellular activity (AAA+) superfamily (12). The Clp complex is a proteasome-like cylinder composed of the CLP proteolytic subunit (CLPP) and the ATP/chaperone component ClpX-like (CLPX), which is thought to be responsible for targeting specific substrates for degradation (13). CLPP and CLPX are evolutionarily conserved from bacteria to humans. Recently, the activity of the Clp-protease complex has been implicated in cellular metabolism (14), although its mechanism of regulation is unknown.

Here, we show that Poldip2 governs a critical mechanism linking Clp, ACSM1, and protein lipoylation, thus regulating mitochondrial function. Reduced Poldip2 expression triggers the Clp-protease complex-mediated degradation of ACSM1, which prevents PDH and αKGDH complex lipoylation, inhibiting their activity, and represses mitochondrial function. The inhibition of the tricarboxylic acid (TCA) cycle and oxidative respiration reduces the amount of α-ketoglutarate (α-KG), with resultant metabolic inhibition of prolyl-hydroxylases and hypoxia-inducible factor 1α (HIF-1α)-mediated retrograde signaling. We show that the addition of the prosthetic C group, lipoylic acid, to catabolic enzymes is a regulated process controlled through a particular mammalian salvage pathway of lipoylation. Additionally, our work reveals that Poldip2 is a regulator of cell metabolism and mitochondrial function that participates in metabolic adaptation.

**Fig. 1.** Mitochondrial protein Poldip2 is required for oxidative metabolism. (A) HASMC fractions showing Poldip2 mitochondrial localization. (B) Profile of mitochondrial function over time in siControl- and siPoldip2-treated HASMCs. Cytosol (C), mitochondria (M), and histones (N). Four basal OCR and ECAR measurements were made, and 1 μM oligomycin (O), 1 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (F), and 10 μM antimycin-A (A) were then sequentially injected. (C) Box plot summarizing the data obtained from the analysis of OCR/ECAR profiles (basal corresponds to oligomycin-A-inhibitable, ATP-linked corresponds to oligomycin-inhibitable, and maximal capacity corresponds to the increase induced by the uncoupler FCCP). (D) Energy map showing the metabolic reprogramming induced by Poldip2 deficiency. All data are presented as mean ± SE from three to six independent experiments. Seahorse experiments were performed three independent times with similar results.

**Results**

Poldip2 contains an N-terminal mitochondrial localization sequence that predicts it will be localized to the mitochondrion (15). Indeed, to begin to define its function, we investigated the subcellular distribution of Poldip2. As predicted from the primary sequence, we found that endogenous Poldip2 localizes almost exclusively to the mitochondria in a variety of cell types, including human aortic smooth muscle cells (HASMCs) (Fig. 1A), human mammary epithelial cells (HMECs), and mouse embryonic fibroblasts (MEFs) (Fig. S1). Poldip2 was detected at an apparent molecular mass of 37 kDa, which corresponds to the predicted molecular mass of the mitochondrial protease-processed form (16, 17).

The role of Poldip2 in the mitochondria is unknown. Therefore, to investigate its distinct contribution to mitochondrial function, we performed a series of experiments manipulating Poldip2 expression. Using extracellular flux analysis to determine cellular bioenergetics as a function of time (Fig. 1B), we found that Poldip2-deficient cells display a lower ratio of basal oxygen consumption rate to extracellular acidification rate (OCR/ECAR), lower OCR/ECAR associated with ATP synthesis, and lower OCR/ECAR maximal capacity (Fig. 1C). Despite the fact that Poldip2 deficiency represses mitochondrial function, we did not observe a reduction in mitochondrial biogenesis (102 ± 14%, P = 0.9 at 24 h; 94 ± 7%, P = 0.5 at 72 h), and even though Poldip2-deficient cells produce less ATP by oxidative respiration, total cellular ATP levels were preserved (4.4 ± 0.8 vs. 4.8 ± 0.6 cd/μg of protein, P = 0.7). These data suggest a potential cellular reprogramming with up-regulation of glycolysis that was confirmed by an increased ECAR compared with OCR, as shown in the energy map (Fig. 1D). In fact, we found that Poldip2 down-regulation stabilizes HIF-1α and induces expression of PDH kinase (PDK) and, consequently, the inhibitory phosphorylation of the PDHε1a subunit of the PDH complex in HASMCs (Fig. 2A). Similar results were found in MEFs from Poldip2−/− mice as well as with two unrelated siRNAs against Poldip2 (Fig. S2). As expected, the induction of PDK and phospho-PDH induced by Poldip2 deficiency was reversed by down-regulation of HIF-1α (Fig. S3). Normally, HIF-1α has a
half-life of minutes, as it is constantly hydroxylated by a member of
the prolyl-4-hydroxylase (PHD) family, and is then recognized
by an E3 ubiquitin ligase complex, polyubiquitinated, and
degraded by the proteasome (18, 19). PHDs use molecular oxygen
and α-KG as cosubstrates for their catalytic reaction. It is known
that under oxygen concentrations below their K_m of about
250 μM, PHD activity is inhibited and HIF-1α is stabilized (20).
Similarly, PHD activity is dependent on the availability of
α-KG even in the presence of oxygen. For example, disruption or
deregulation of TCA cycle enzymes inhibits PHDs, with subsequent
metabolic-mediated stabilization of HIF-1α (21). Since Poldip2
down-regulation stabilizes HIF-1α under normoxia, we hypothe-
sized that this was a consequence of metabolic inhibition of
PHD2. In support of this idea, we found that Poldip2-deficient
cells have significantly lower concentrations of α-KG (Fig. 2B)
and that HIF-1α stabilization was reversed when the cell-permeable
α-KG derivative α-KG octyl ester (1 mM) was added to the
culture media (Fig. 2C). Because α-KG is a key intermediate
metabolite in the TCA cycle, we investigated whether Poldip2
regulates the activity of the TCA cycle enzymes. Gene expression
data in Caenorhabditis elegans predicts a functional association
between the products of Tag-307 and gip-2 (22). Interestingly,
the putative homologs of Tag-307 and gip-2 in humans are the genes
Poldip2 and lipoyltransferase 1 (LIPT1), respectively. LIPT1 is
a nuclear-encoded mitochondrial protein proposed to participate
in a functional mammalian salvage pathway of lipoylation, that
is, to catalyze the transfer of the lipoyl group from lipoyl-AMP to
the specific lysine residue of lipoyl domains of lipoate-dependent
enzymes, two of which participate in oxidative catalysis: the
PDH and αKGDH complexes. The lipoylation occurs in the
E2 subunit DLAT of PDH and DLST of αKGDH. Therefore, we
evaluated if LIPT1 expression and protein lipoylation were
affected in Poldip2-deficient cells. We found that lack of
Poldip2 significantly reduces lipoyl-DLAT and lipoyl-DLST in
HASMCs (Fig. 3A). Similar results were obtained with two un-
related siRNA sequences against Poldip2 and in Poldip2−/−
MEFs, Fig. S4A–C). Consistent with the reduction in the
addition of the prosthetic group lipoate, the activities of PDH and
αKGDH were significantly inhibited in Poldip2-deficient cells
(Fig. 3B and C). Interestingly, despite defective lipoylation,
LIPT1 expression was increased (Fig. 3A), suggesting an
ineffective compensatory mechanism presumably induced by a
shortage in the substrate for the LIPT1-catalyzed reaction.
Consistent with the notion that Poldip2 down-regulation pro-
duces a deficit in LIPT1 substrate, we observed an almost
complete loss of ACSM1, the proposed lipoic acid-activating
enzyme, in Poldip2-deficient HASMCs (Fig. 3A). Similar results
were obtained with two unrelated siRNA sequences against
Poldip2 and in Poldip2−/− MEFS, Fig. S4A–C). Interestingly,
11.5 d post coitum (dpc) Poldip2−/− embryos exhibit a similar
reduction of ACSM1 levels (Fig. S4D). Indeed, ACSM1 down-
regulation produces a similar reduction in DLAT and DLST
lipoylation (Fig. S5). More importantly, forced expression of
ACSM1 in Poldip2-deficient cells was sufficient to rescue
lipoylation levels (Fig. 4A) and to reverse metabolic shift (Fig.
4B). These data indicate that loss of ACSM1 is the primary
mechanism by which Poldip2 deficiency inhibits lipoylation.
To understand the mechanism by which Poldip2 controls ACSM1
levels, we examined its potential binding partners within the
mitochondria. Immunoprecipitated protein complexes containing
myc-tagged Poldip2 were subjected to liquid chromatography-
tandem mass spectrometry (LC-MS/MS). The ATP-dependent
Clp-protease ATP-binding subunit CLPX was the top-ranked
molecule associated with Poldip2 (Fig. 5A). This interaction was

![Fig. 3. Poldip2 deficiency induces loss of DLAT and DLST lipoylation and
degradation of ACSM1. (A, Left) Western blot showing the expression of proteins of the lipoylation pathway in siControl- and siPoldip2-treated cells. (A, Right) Box plots represent mean ± SE from six independent experiments. (B and C) PDH and αKGDH activities in isolated mitochondria from Poldip2-deficient cells. All data are presented as mean ± SE from three to six independent experiments.

![Fig. 4. Forced expression of ACSM1 reverses the phenotype of Poldip2-deficient cells. (A, Left) Western blot showing the effect of ACSM1 forced expression in siControl- and siPoldip2-treated cells on the lipoylation of DLAT and DLST. (A, Right) Box plots represent mean ± SE from four to six independent experiments. (B, Left) Western blot showing the effect of ACSM1 forced expression in siControl- and siPoldip2-treated cells on the lipoylation of DLAT and DLST. (B, Right) Box plots represent mean ± SE from four to six independent experiments. Symbols above boxes indicate statistical significance. Pairs of bars that do not share any symbol are significantly different from each other.](image-url)
confirmed by coimmunoprecipitation of CLPX with the overexpressed and endogenous Poldip2 protein (Fig. 5B). As noted, CLPX is a chaperone ATPase that binds to the caseinolytic peptidase CLPP to form the Clp-protease complex. CLPX acts as an energy-dependent unfoldase required to allow the entrance of proteins to the CLPP-composed barrel-shaped chamber that, otherwise, is too small for the access of native proteins (23). CLPX is also thought to be responsible for substrate specificity of the Clp-protease complex (13).

The specific binding between Poldip2 and CLPX suggests to us that Poldip2 may inhibit Clp-protease complex activity by sequestering CLPX and that the Poldip2 deficiency is sufficient to activate the Clp-protease complex and degrade ACSM1. In fact, in CLPP-deficient cells, Poldip2 down-regulation fails to induce the degradation of ACSM1. Consequently, the DLAT and DLST lipoylation is preserved (Fig. 5C). PHD2 is not inhibited, and the HIF-1α stabilization is absent (Fig. 5C). These data demonstrate that Poldip2 governs a mitochondrial pathway responsible for the Clp-protease complex-mediated degradation of ACSM1, which impairs lipoylation of the PDH and αKGDH complexes, limits mitochondrial respiration, and induces HIF-1α retrograde signaling (Fig. 6).

The cellular changes induced by Poldip2 deficiency are similar to those observed during cellular adaptation to hypoxic conditions. Therefore, we evaluated if oxygen tension regulates its expression. Indeed, Poldip2 expression is dramatically repressed under hypoxic conditions in HASMCs (Fig. 7A), human cardiac ventricular fibroblasts (HVF), HMECs, and MEFs (Fig. S6). Since oxygen tension regulates Poldip2, we evaluated the relevance of this mechanism under hypoxia. As shown in Fig. 7B, hypoxia significantly reduces ACSM1 protein amount and the level of DLST and DLAT lipoylation at the same time that it induces stabilization of HIF-1α. Exogenous Poldip2 expression under hypoxia was sufficient to restore the levels of ACSM1, lipoyl-DLST, and lipoyl-DLAT.

Hypoxia-induced metabolic adaptation is directly connected to oncogenic signaling. Indeed, impaired mitochondrial functions and increased glycolysis offer cancer cells an advantage to better produce biomass to proliferate, survive, and become invasive in the tumor microenvironment. In particular, subtypes of breast cancers lacking the estrogen receptor, the progesterone receptors, and the human epidermal growth factor receptor-2 [triple-negative breast cancer (TNBC)] are highly glycolytic (in vitro OCR/ECAR = 2–5 pmol/mHpH) and have an unfavorable clinical prognosis compared with estrogen receptor ER+ (in vitro OCR/ECAR = 15–20 pmol/mHpH) (24). We hypothesized that Poldip2 deficiency may contribute to the highly glycolytic phenotype of TNBC. To test this idea, we compared three lines of breast cancer cells: the highly oxidative T47D and the TNBC highly and glycolytic lines MDA-MB-231 and BT549. Fig. 8A shows that, consistent with our hypothesis, TNBCs have significantly lower Poldip2 expression and completely inhibited DLAT and DLST lipoylation, suggesting that the inhibition of Poldip2 expression may contribute to the metabolic shift observed in this cell line. To further test the role of Poldip2 in the mitochondrial dysfunction observed in TNBC, we expressed Poldip2 in BT549 cells and evaluated the impact on mitochondrial function and protein lipoylation. As shown in Fig. 8B, overexpression of Poldip2 was sufficient to stabilize ACSM1, dramatically increase the lipoylation of the PDH and αKGDH complexes, and reduce the stabilization of HIF-1α under normoxia. Consistent with these biochemical changes,
Poldip2 down-regulation participates in BT549 metabolic reprogramming. (A) Highly glycolytic TNBCs exhibit low Poldip2 expression and inhibition of DLAT and DLST lipoylation. (B) Triple-negative cancer cell line BT549 was stably transfected with empty or Poldip2-expressing vectors. Poldip2 expression increases ACSM1 stabilization, increases DLAT and DLST lipoylation, and reduces HIF-1α. Western blots are representative of two experiments from cell lines; samples after were taken 4 wk and 8 wk after transfection. (C) Basal mitochondrial respiration in BT549 compared with BT549 expressing Poldip2. (D) Growth curves of BT549 compared with BT549 expressing Poldip2. Experiments were done in triplicate. The seahorse experiment was performed once with 10 replicates. Data and statistics shown were calculated by the wave software from one 96-well plate.
excacerate HIF-1α stabilization by metabolic inhibition of PHDs. Additionally, and more importantly, Poldip2 deficiency may specifically impact the fate and availability of Ac-CoA produced by nonglycolytic pathways such as fatty acid oxidation. Since, the TCA cycle is no longer functional, Ac-CoA would be redirected to cysolic and, most likely, biosynthetic pathways that may represent an alternative source of pyruvate or hydroxytryptophan. Further investigation will be necessary to elucidate the interplay between Poldip2 and HIF-1α at different oxygen concentrations and to investigate further the regulation of Poldip2 expression during normal physiology.

**Materials and Methods**

**Animals.** Poldip2 gene trap mice on a C57BL/6 background were produced by the Texas A&M Institute for Genomic Medicine. A gene trap construct was inserted into the first intron of Poldip2 in mouse embryonic stem cells. Characterization of these mice has been published previously (4). Embryos between 11.5 and 12.5 dpc were isolated as described below, fixed in paraformaldehyde, and sliced for immunohistochemistry.

**Cell Culture and Transfection.** HASMCs and HMECs were obtained from Thermo Fisher Scientific. HVFs were obtained from Lonza. Human epithelial cells from invasive ductal carcinoma (T47D and BT549) and adenocarcinoma (MDA-MB231) were obtained from the American Type Culture Collection. Cells were grown as recommended by the vendor in base media with the addition of growth supplements. After cells reached ~80% confluence, growth supplements were removed 24 h prior to experiments. MEFs were prepared from embryonic day 13.5 embryos as previously described (3). Hypoxia experiments were performed using a HypOxystation H35. Cells were transfected using Lipofectamine RNAiMAX.

**Cell Fractionation.** Cells were washed with PBS, and scraped in 1x buffer A provided by the cell fractionation kit (ab109719; Abcam) according to the manufacturer’s protocol. For mitochondrial biogenesis, we used the Human Mitochondrial DNA Monitoring Primer Set (catalog no. 7246; Clontech/Takara) in nuclear and mitochondrial DNA extracted with a PureLink Genomic DNA Kit (catalog no. K1820-01; Invitrogen) as recommended by the manufacturers.

**Statistical Analysis.** Data are presented as mean ± SEM. Significance was determined using a t test for unpaired samples and one- or two-way ANOVA followed by Bonferroni’s post hoc test for multiple comparisons. Prism6 (GraphPad) or SPSS was used for statistical analysis.

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