



Poldip2 is an oxygen-sensitive protein that controls PDH and α KGDH lipoylation and activation to support metabolic adaptation in hypoxia and cancer

Felipe Paredes^a, Kely Sheldon^a, Bernard Lassègue^a, Holly C. Williams^a, Elizabeth A. Faidley^a, Gloria A. Benavides^b, Gloria Torres^a, Fernanda Sanhueza-Olivares^a, Samantha M. Yeligar^{c,d}, Kathy K. Griendling^a, Victor Darley-Usmar^b, and Alejandra San Martin^{a,1}

^aDivision of Cardiology, Department of Medicine, Emory University, Atlanta, GA 30322; ^bDepartment of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294; ^cDivision of Pulmonary, Allergy, Critical Care, and Sleep, Department of Medicine, Emory University, Atlanta, GA 30322; and ^dAtlanta Veterans Affairs Medical Center, Decatur, GA 30033

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Although the addition of the prosthetic 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 (α KGDH) 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 peptidase (Clp)-protease complex and degradation of the lipoate-activating enzyme Ac-CoA synthetase medium-chain family member 1 (ACSM1). ACSM1 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 1 α (HIF-1 α) by loss of substrate inhibition of prolyl-4-hydroxylases (PHDs). HIF-1 α -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 key during hypoxia and cancer cell metabolic adaptation.

Poldip2 | lipoylation | mitochondria | hypoxia | metabolism

The polymerase- δ interacting protein 2 (Poldip2; also known as PDIP38 and mitogenin 1), a ubiquitously expressed protein (1), was initially identified as a DNA polymerase- δ interacting protein and a binding partner for the proliferating cell nuclear antigen (2). Later studies showed that homozygous deletion of Poldip2 in mice results in reduced fetal weight and perinatal lethality of unknown cause (3). Adult heterozygous mice have no evident physiological phenotype, but more detailed characterization has shown that they display disrupted extracellular matrix organization with excessive and disorganized collagen deposition (4) and an impaired response to ischemia-induced collateral vessel formation (5). Interestingly, Poldip2 contains an N-terminal mitochondrial localization sequence and has been found to interact with components of the mitochondrial nucleoid (6), suggesting a mitochondrial role that remains unexplored. One of the aims of this study was to understand the function of Poldip2 in the mitochondria.

The α -(R)-lipoic acid (5-[(3R)-1,2-dithiolan-3-yl] pentanoic acid) is a universally conserved fundamental metabolite that serves as a

prosthetic group. It is required for the activity of two key catabolic enzymes: the pyruvate dehydrogenase (PDH) and the α -ketoglutarate dehydrogenase (α KGDH) complexes. Specifically, α -lipoic acid is covalently attached to dihydrolipoamide S-acetyltransferase (DLAT) and dihydrolipoamide S-succinyltransferase (DLST), the dihydrolipoyl transacetylase (or E2) subunits of the PDH and the α KGDH, respectively.

Lipoic acid synthesis and the mechanisms leading to protein lipoylation have been investigated most thoroughly in *Escherichia coli* and, recently, in *Saccharomyces cerevisiae*, but they are still poorly understood in mammals. Currently, two mammalian pathways are proposed. In the first, which is evolutionarily conserved, lipoic acid is synthesized in the mitochondria from an octanoyl-acyl carrier protein provided by the fatty acid synthesis pathway. The octanoyl moiety is attached to lipoate-dependent enzymes by the ligase LIPT2. Next, the [Fe-S] cluster-containing enzyme LIAS catalyzes the radical-mediated insertion of two sulfur atoms into the C-6 and C-8 positions of the octanoyl moiety bound to the lipoyl domains of lipoate-dependent enzymes. The second and less characterized is the salvage pathway, which uses exogenous lipoate that is taken up via the sodium-dependent multivitamin transporter (7, 8). Exogenously scavenged lipoic acid is then activated by addition of AMP and transferred to lipoate-containing enzymes. In contrast to bacteria and yeast,

Significance

The present work establishes that the addition of the prosthetic group lipoic acid to catabolic enzymes is a dynamically regulated posttranslational modification that increases metabolic plasticity under hypoxia and in cancer cells. We show that 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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The authors declare no conflict of interest.

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¹To whom correspondence should be addressed. Email: asanmartin@emory.edu.

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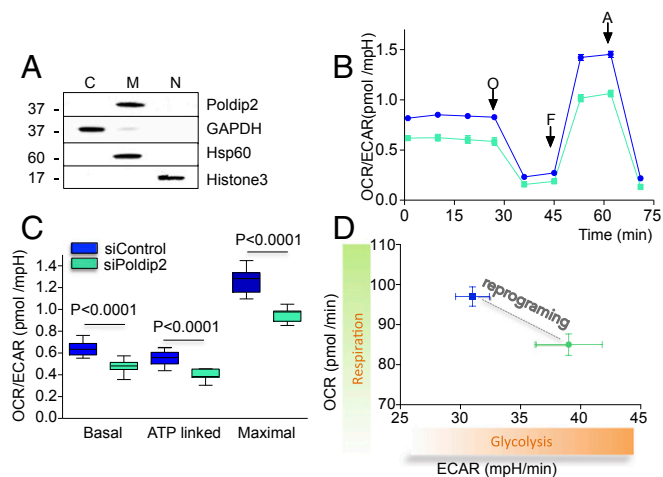


Fig. 1. Mitochondrial protein Poldip2 is required for oxidative metabolism. (A) HASMC fractions showing Poldip2 mitochondrial localization. (B) Profile of mitochondrial function over time in siControl- and siPoldip2-treated HASMCs. C, cytosol; M, mitochondria; N, nucleus. Four basal OCR and ECAR measurements were made, and 1 μ M oligomycin (O), 1 μ M carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (F), and 10 μ M antimycin-A (A) were then sequentially injected. (C) Box plot summarizing the data obtained from the analysis of OCR/ECAR profiles (basal corresponds to antimycin-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 \pm SE from three to six independent experiments. Seahorse experiments were performed three independent times with similar results.

where one enzyme executes both reactions, mammals require two separate enzymes for the activation and ligation reaction of lipolate (9). Indeed, the isolation of a unique lipic acid-activating enzyme activity (lipic acid + NTP \rightarrow 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 α KGDH 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 1 α (HIF-1 α)-mediated retrograde signaling. We show that the addition of the prosthetic group, lipic 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.

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 ($102 \pm 14\%$, $P = 0.9$ at 24 h; $94 \pm 7\%$, $P = 0.5$ 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/ μ g 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-1 α and induces expression of PDH kinase (PDK) and, consequently, the inhibitory phosphorylation of the PDHE1 α 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-1 α (Fig. S3). Normally, HIF-1 α has a

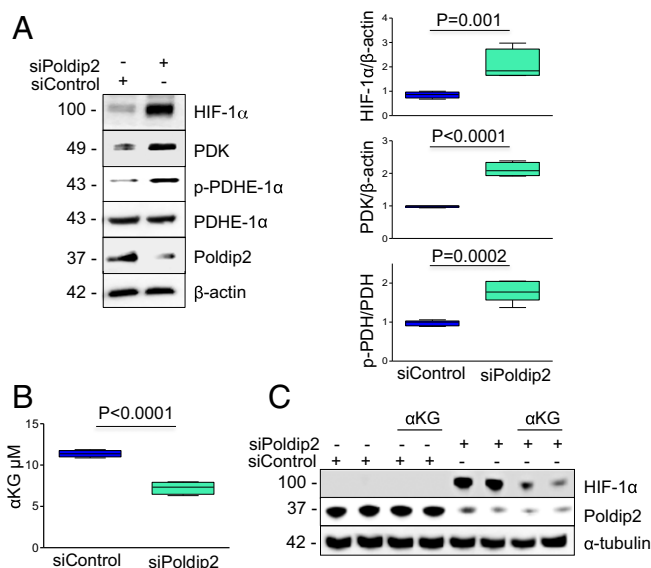


Fig. 2. Poldip2 deficiency induces the expression of metabolic shift markers. (A, Left) Western blot of metabolic shift markers in HASMCs treated with siControl or siPoldip2. (A, Right) Box plots represent mean \pm SE from five independent experiments. The P value indicated was calculated by an independent t test. (B) Total intracellular α -KG concentrations (C) HIF-1 α stabilization induced by Poldip2 deficiency is reversed by incubation with a cell-permeable α -KG analog (octyl- α -KG, 1 mM). The blot is representative of four independent results.

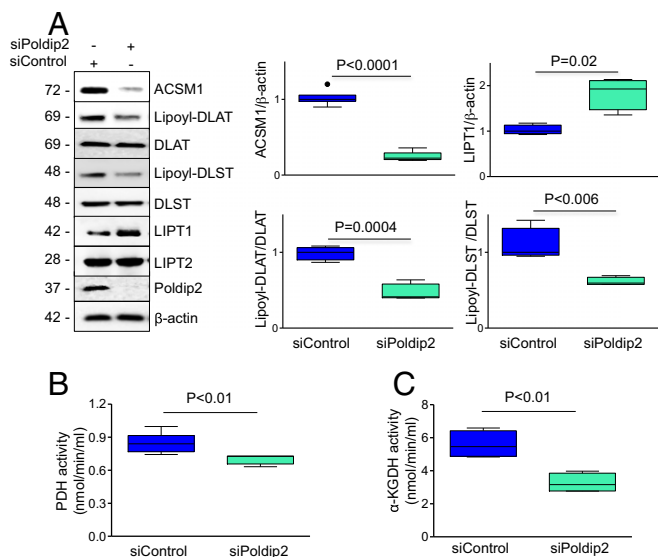


Fig. 3. Poldip2 deficiency induces loss of DLAT and DLST lipoylation and degradation of ACSM1. (A, Left) Western blot showing the expression of proteins of the lipoylation pathway in siControl- and siPoldip2-treated cells. (A, Right) Box plots represent mean \pm SE from six independent experiments. (B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

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 μ M, PHD activity is inhibited and HIF-1 α 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-1 α (21). Since Poldip2 down-regulation stabilizes HIF-1 α under normoxia, we hypothesized 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-1 α 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 lipoyl-dependent enzymes, two of which participate in oxidative catabolism: the PDH and α KGDH complexes. The lipoylation occurs in the E2 subunit DLAT of PDH and DLST of α KGDH. 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 unrelated siRNA sequences against Poldip2 and in Poldip2^{-/-} MEFs, Fig. S4 A–C). Consistent with the reduction in the addition of the prosthetic group lipoate, the activities of PDH and α KGDH 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 produces a deficit in *LIPT1* substrate, we observed an almost complete loss of ACSM1, the proposed lipoyl 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. S4 A–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

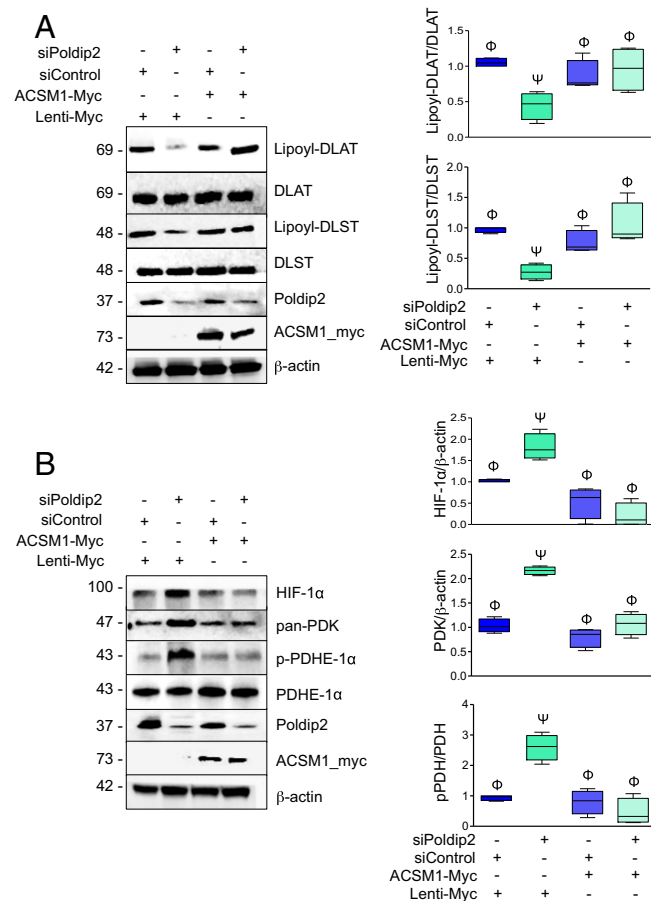


Fig. 4. Forced expression of ACSM1 reverses the phenotype of Poldip2-deficient cells. (A, Left) Western blot showing the effect of ACSM1 forced expression in siControl- and siPoldip2-treated cells on the lipoylation of DLAT and DLST. (A, Right) Box plots represent mean \pm SE from four to six independent 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 \pm 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.

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-1 α 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

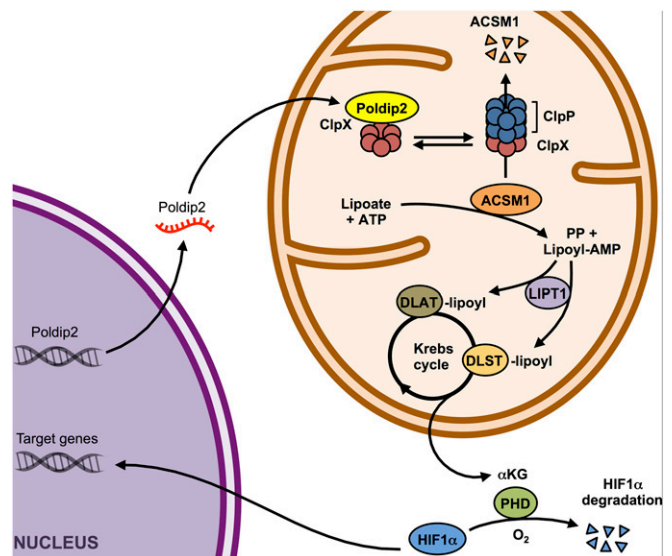


Fig. 6. Model. Decreased Poldip2 expression and binding to CLPX permit activation of the Clp-protease and degradation of the lipoic acid-activating enzyme ACSM1. As a result, no lipoyl-AMP is synthesized and LIP1 has no substrate for lipoylation of DLAT and DLST. Lipoylation deficiency inhibits the TCA cycle, inducing PHD2 metabolic inhibition, HIF-1 α stabilization, and signaling. PP, pyrophosphate.

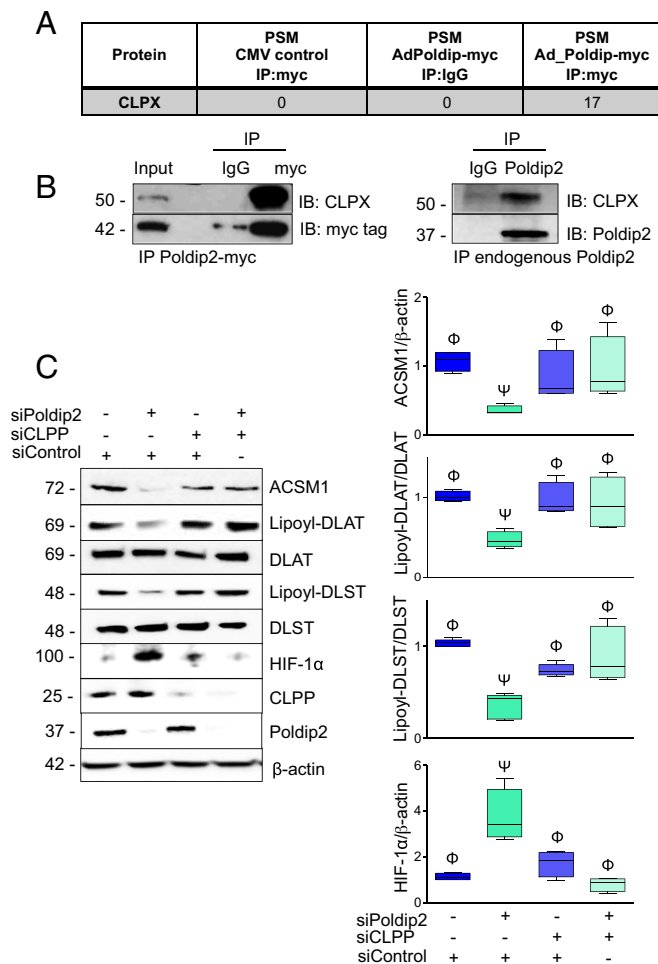


Fig. 5. Poldip2 regulates DLAT and DLST lipoylation by Clp-protease complex-mediated degradation of ACSM1. (A) LC-MS/MS's peptide-spectrum match scores for CLPX binding to myc-tagged Poldip2. IP, immunoprecipitation; PSM, peptide-spectrum match. (B) Coimmunoprecipitation of overexpressed tagged Poldip2 and endogenous Poldip2 shows specific binding to CLPX. IB, immunoblot. (C, Left) Western blot showing the expression of proteins of the salvage lipoylation pathway in siControl-, siPoldip2-, siCLPP-, and siPoldip2 and siCLPP combined-treated HASMCs. (A, Right) Box plots represent mean \pm 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.

lipoylation of the PDH and α KGDH complexes, limits mitochondrial respiration, and induces HIF-1 α 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-1 α . 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 cells 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/mpH) and have an unfavorable clinical prognosis compared with estrogen receptor ER⁺ (in vitro OCR/ECAR = 15–20 pmol/mpH) (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 α KGDH complexes, and reduce the stabilization of HIF-1 α under normoxia. Consistent with these biochemical changes,

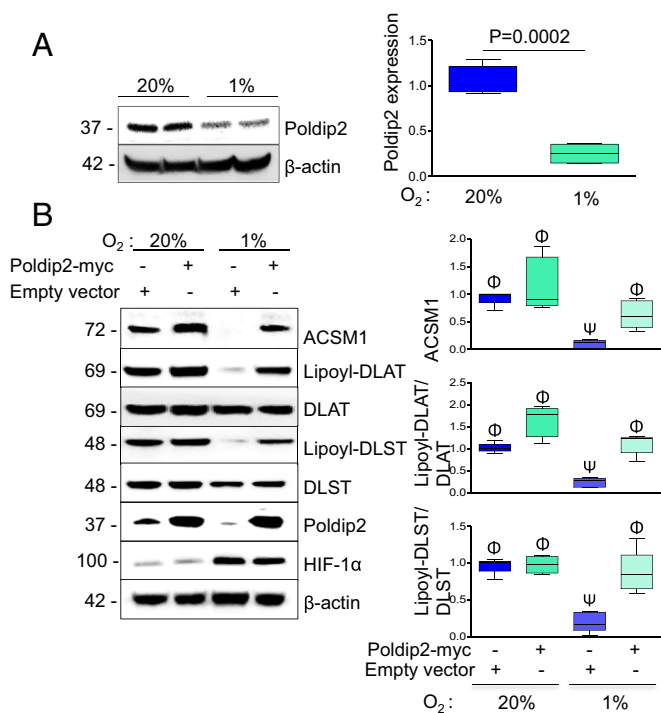


Fig. 7. Poldip2 is an oxygen-sensitive protein that controls protein lipoylation under hypoxia. (A, Left) Poldip2 in HASMCs cultured under normoxia or hypoxia for 48 h. (A, Right) Box plots represent the mean \pm SE from four to six independent experiments. (B, Left) Western blot of ACSM1 expression and protein lipoylation in cells infected with control adenovirus or AdPoldip2 after being maintained under normoxia or hypoxia for 48 h. (B, Right) Box plots represent the mean \pm 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.

mitochondrial respiration was significantly increased by Poldip2 expression (Fig. 8C). Importantly, increased lipoylation and mitochondrial function was accompanied by a significant inhibition of cell growth (Fig. 8D).

Discussion

Regulation of mitochondrial respiration is essential for the metabolic plasticity needed to adapt to different physiological conditions. Herein, we demonstrate that the regulation of the lipoylation of two key enzymes of the TCA cycle contributes to this plasticity. We found that this mechanism involves nuclear control of Poldip2 expression, which regulates the degradation of the lipoyl acid-activating enzyme ACSM1.

The activation of lipoyl acid and its subsequent transfer to lipoyl-containing enzymes was initially postulated by Reed (25). Later studies confirmed that such activity was present in bovine liver (10), and, more recently, it was identified as the product of the ACSM1 gene (11). However, the contribution of ACSM1 to protein lipoylation has remained unclear. Our work demonstrates that ACSM1 is required for lipoylation in mammalian cells and highlights the relevance of the salvage pathway.

In mammals, subunits of at least four mitochondrial enzymatic complexes require lipoyl acid for catalysis: (i) lipoylated DLAT from the PDH complex; (ii) DLST from α KGDH, which, in addition to its role in the TCA, participates in L-lysine degradation via the saccharopine pathway; (iii) glycine cleavage system protein H (GCSH), which participates in the degradation of glycine; and (iv) dihydrolipoamide branched chain transacylase, which is part of the branched-chain α -keto acid dehydrogenase complex that participates in the degradation of the branched-chain amino acids leucine, valine, and isoleucine. Interestingly, we observed that the degradation of

ACSM1 and lack of LIPT1-mediated lipoylation only affects the lipoylation of PDH and α KGDH. This is in agreement with the fact that, in contrast to mutations in genes from the lipoyl acid biosynthesis pathway that affect all lipoyl-dependent enzymes (26–28), two cases of LIPT1 mutations in humans showed low lipoylation of the PDH and α KGDH complexes but no alteration in glycine metabolism or accumulation of branched-chain amino acids in the urine (29, 30). This suggests that the ACSM1/LIPT1-mediated lipoylation pathway may preferentially target substrates from oxidative catabolism.

Homozygous deletion of Poldip2 results in fetal growth inhibition and perinatal lethality (3) of unknown cause. The dramatic reduction of ACSM1 in Poldip2^{-/-} embryos suggests that lactic acidosis may account for the subsequent perinatal lethality (31), as has been observed in a human case of lipoamide dehydrogenase deficiency (32), in which the reduced dihydrolipoamide is not oxidized to restore the electrophile lipoamide, and therefore cannot be used for catalysis.

The mechanism of Poldip2-mediated ACSM1 degradation involves Clp-protease complex activation. Of mostly unknown function in humans, CLPP and CLPX have been extensively studied in prokaryotes, where they act mostly as a quality control mechanism. Importantly, our work also reveals that mitochondrial peptidases are regulated by oxygen and participate in an integrated hypoxic response needed for metabolic adaptation.

It is well established that HIF-1 α depresses mitochondrial respiration by inhibiting the oxidation of pyruvate by the mitochondria due to transcriptional up-regulation of PDK and a subsequent increase in the inhibitory phosphorylation of PDH. Poldip2 deficiency negatively impacts PDH activity by increasing PDK-mediated phosphorylation and inhibiting LIPT1-mediated lipoylation. However, additional studies are required to define the relative contributions of these posttranslational modifications on PDH activity.

We found that Poldip2 expression is down-regulated in hypoxia and cancer cells. During these conditions, loss of Poldip2 may

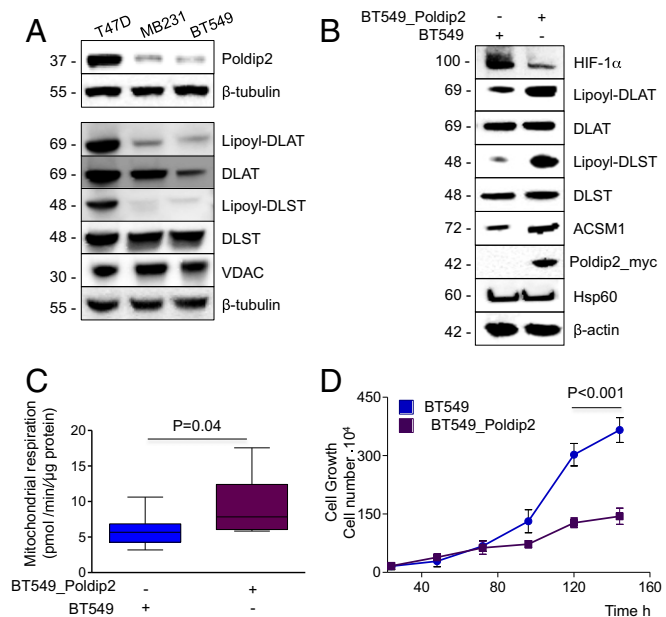


Fig. 8. Poldip2 down-regulation participates in BT549 metabolic reprogramming. (A) Highly glycolytic TNBCs exhibit low Poldip2 expression and inhibition of DLAT and DLST lipoylation. (B) Triple-negative cancer cell line BT549 was stably transfected with empty or Poldip2-expressing vectors. Poldip2 expression increases ACSM1 stabilization, increases DLAT and DLST lipoylation, and reduces HIF-1 α . 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.

exacerbate HIF-1 α 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 cytosolic and, most likely, biosynthetic pathways that may represent an advantage for cell proliferation or hypertrophy. Further investigation will be necessary to elucidate the interplay between Poldip2 and HIF-1 α 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. HVEFs 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 1 \times 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.

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Viral Expression of Poldip2 and ACSM1. The human Poldip2 cDNA (NP_056399) plus a C-terminal myc tag (EQKLISEEDL) were cloned into the pAdTrack-CMV vector. Human ACSM1cDNA (NP_001305819.1) containing lentivirus was obtained from Vigene.

LC-MS/MS Analysis. HASMCs expressing Ad_myc-tagged_Poldip2 were immunoprecipitated using a myc-tag antibody and subjected to MS. Spectra were searched using Proteome Discoverer 2.1 against the human UniProt database (90,300 target sequences). Percolator was used to filter the peptide spectrum matches to a false discovery rate of 1%.

Assessment of Bioenergetics. HASMCs or BT549 cells were plated (20,000–35,000 cells per well) on Seahorse Extracellular Analyzer XF96 plates in culture media; after 5 h, cells were transfected with small interfering control (siControl) or siPoldip2. Measurements of the OCR and ECAR were performed as previously described (33) by a XF96e extracellular flux analyzer (Agilent-Seahorse XF Technology).

Quantification of Total Cellular ATP. Total ATP was quantified using the ATP CellTiter-Glo Luminescent Cell Viability Assay kit following the manufacturer's recommendations.

α -KG Quantification and PDH and α -KGDH Enzymatic Activity Assays. Intracellular α -KGDH concentration was measured using a commercial kit (ab83431; Abcam). PDH and α -KGDH activities were determined in isolated mitochondria (ab110171; Abcam) using colorimetric enzymatic activity assays (MAK183 and MAK189, respectively; Sigma).

Statistical Analysis. Data are presented as mean \pm 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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