Cited2, a Transcriptional Modulator Protein, Regulates Metabolism in Murine Embryonic Stem Cells*

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Background: The function of HIF-1, a master regulator of metabolism, is in part modulated by Cited2. The role of Cited2 in murine embryonic stem cell (mESC) glucose metabolism remains unknown.

Results: Deletion of Cited2 in mESCs results in impaired mitochondria morphology, reduced glucose oxidation, increased glycolysis, and defective mESC differentiation.

Conclusion: Cited2 coordinates glucose metabolism to regulate mESC differentiation.

Significance: Cited2 is a potential target for metabolic reprogramming in mESCs.

CREB-binding protein (CBP)/p300 interacting transactivator with glutamic acid (Glu) and aspartic acid (Asp)-tail 2 (Cited2) was recently shown to be essential for gluconeogenesis in the adult mouse. The metabolic function of Cited2 in mouse embryonic stem cells (mESCs) remains elusive. In the current study, the metabolism of glucose was investigated in mESCs, which contained a deletion in the gene for Cited2 (Cited2⁻/⁻). Compared with its parental wild type counterpart, Cited2⁺/⁺ ESCs have enhanced glycolysis, alternations in mitochondria morphology, reduced glucose oxidation, and decreased ATP content. Cited2 is recruited to the hexokinase 1 (HK1) gene promoter to regulate transcription of HK1, which coordinates glucose metabolism in wild type ESCs. Reduced glucose oxidation and enhanced glycolytic activity in Cited2⁻/⁻ ESCs correlates with defective differentiation during hypoxia, which is reflected in an increased expression of pluripotency marker (Oct4) and epiblast marker (Fgf5) and decreased expression of lineage specification markers (T, Gata-6, and Cdx2). Knockdown of hypoxia inducible factor-1α in Cited2⁻/⁻ ESCs re-initiates the expression of differentiation markers T and Gata-6. Taken together, a deletion of Cited2 in mESCs results in abnormal mitochondrial morphology and impaired glucose metabolism, which correlates with a defective cell fate decision. (Cited2) is a multifunctional protein essential for mouse embryogenesis (1–4). Embryos with a deletion of Cited2 have defects in multiple organs including the heart, liver, and adrenal glands and do not survive beyond E14.5 (1–4). A knock-out of Cited2 in mice causes defects in arterial and ventricular septum and outflow tract, which are responsible for embryonic lethality of a Cited2 gene deletion (1, 3, 4). By interacting with hepatocyte nuclear factor 4α, Cited2 regulates lipid and carbohydrate metabolism in embryos (2). Interestingly, embryos in which the gene for Cited2 has been deleted have no adrenal gland (1). In adult mice, hematopoietic stem cells are metabolically inactive, as evidenced by cell quiescence, whereas the maintenance of hematopoietic stem cells is sensitive to changes in the metabolism of glucose and fatty acids (5, 6). Importantly, we and others have shown that Cited2 regulates quiescence and apoptosis in adult hematopoietic stem cells via HIF-1 and p53-dependent mechanisms (7, 8). Recently, by adenovirus-mediated delivery of Cited2 small hairpin RNA (shRNA) to mouse hepatocytes, it was demonstrated that Cited2 regulates gluconeogenesis by interacting with histone acetyltransferase GCN5 to affect acetylation and activity of peroxisome proliferative-activated receptor γ co-activator 1α (PGC-1α) (9).

Although Cited2 has been suggested as a crucial player in the regulation of gluconeogenesis, the role of Cited2 in glucose metabolism, especially glycolysis and oxidation phosphorylation in murine embryonic stem cells (mESC) remains elusive. The gene for Cited2 is present on chromosome 6q.23, a chromosomal region that is associated with genes involved in the control of insulin concentrations and insulin resistance in Mexican-Americans (10, 11). Indeed, Cited2 mRNA was down-regulated embryoid body; LDH, lactate dehydrogenase; FCCP, p-trifluoromethoxy carbonyl cyanide phenylhydrazone; TCA, tricarboxylic acid; 2-DG, 2-deoxyglucose; TMRM, tetramethylrhodamine methyl ester; OCR, oxygen consumption; HK, hexokinase; PFK1, phosphofructokinase 1; CFU, colony formation unit.
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ulated by insulin in skeletal muscle of 17 healthy volunteers exposed to acute physiological hyperinsulinemia (10). In contrast, Cited2 mRNA was one of the top five up-regulated transcripts in 8 Type 2 diabetics, as compared with 8 non-diabetic subjects after low-dose insulin infusion (12). These studies strongly suggest that Cited2 plays an important role in insulin-mediated signaling pathways that regulate glucose metabolism in humans.

ESCs represent a unique cell population that are characterized by their small size, rapid proliferation, and resistance to senescence, which provides a robust platform for metabolic studies (13). In the undifferentiated state, the majority of glucose (80%) enters the glycolytic pathway to maintain ESC proliferation and self-renewal (14). At mid-to-late differentiation, ESCs undergo a metabolic switch from oxidative phosphorylation to glycolysis to provide sufficient ATP for differentiation (19, 20). A metabolic switch from oxidative phosphorylation to glycolysis would thus favor reprogramming of terminally differentiated mouse fibroblasts to induced pluripotent stem cells (21).

Despite these metabolic fluctuations, there are different time points of ESC differentiation. In the early stages of differentiation into epiblast stem cells, 98% of glucose is consumed for lactate production (14). At mid-to-late differentiation, ESCs undergo a metabolic switch from glycolysis to mitochondrial oxidative phosphorylation to provide sufficient ATP for differentiation (19, 20). A metabolic switch from oxidative phosphorylation to glycolysis would thus favor reprogramming of terminally differentiated mouse fibroblasts to induced pluripotent stem cells (21). Although the metabolic dynamics is critical for proper ESC differentiation, little is known about how ESC pro-differentiation factors modulate glucose homeostasis during this process.

Cited2 was recently characterized as a pro-differentiation factor in the mouse ESCs (22, 23). In this study, we report that mESCs with a deletion in the gene for Cited2 (Cited2Δ/−) have swollen mitochondria, increased mitochondrial number, and reduced mitochondrial function as reflected by a reduced rate of glucose oxidation. Impaired glucose oxidation and increased glycolytic activity in Cited2Δ/− ESCs are associated with defective differentiation during hypoxia. In particular, Cited2Δ/− ESCs display increased mRNA expression of pluripluripotency marker (Oct4) and epiblast marker (Fgf5), and defective mRNA induction of lineage markers (T, Gata-6, and Cdx2). Knockdown of hypoxia inducible factor (HIF)-1α in Cited2Δ/− ESCs re-initiates expression of lineage markers T and Gata-6. Furthermore, Cited2 is recruited to the HK1 gene promoter to directly modulate the expression of HK1, a key enzyme in glycolysis. Thus, Cited2 coordinates glucose metabolism, pluripotency, differentiation, and cell proliferation and is a potential target for metabolic reprogramming in ESCs.

**EXPERIMENTAL PROCEDURES**

**ES Cell Culture and Induced Differentiation—**Cited2Flox/+ (hereafter referred to as wild type, WT) and Cited2Δ/− ESC (clone number 3) were utilized for most of the experiments performed in this study. To maintain ESCs under undifferentiated status in feeder-free culture system, WT and Cited2Δ/− ESCs stably transfected with Oct4-IRES-GFP-PuroR reporter plasmid were grown on gelatin-coated plates in complete ESC media (DMEM (Invitrogen) containing 25 mm glucose, 15% ES-qualified FBS (Germini), 2.5 mm Glutamax (Invitrogen), 1× non-essential amino acids (Invitrogen), 55 μM 2-mercaptoethanol (Invitrogen), and 1,000 IU/ml of ESGRO® leukemia inhibitory factor (Millipore)). ESCs were periodically selected with puromycin to maintain a population of undifferentiated ESC. ESCs were induced to differentiate via a methylcellulose-based method as described previously (22). For hypoxia experiments, ESCs were cultured or induced to differentiate in an incubator with an adjustable oxygen concentration, as described elsewhere (24).

**Measurement of Glucose, Lactate, Glycolytic Activity, and Cell Proliferation—**Undifferentiated ES cells were cultured in 0.1% gelatin-coated dishes and media was collected at the indicated time points for measurement of glucose and lactate, as described elsewhere (25–27). For primary embryoid bodies (EB) that were differentiating in methylcellulose-based semi-solid media, the EBs were re-suspended at 37 °C in PBS and centrifuged at 300 × g for 5 min. The supernatant was diluted 10-fold for the measurement of glucose and lactate via spectrophotometric methods. For glucose measurement, the sample was mixed with trithionolzime buffer containing MgSO4, ATP/NADP, and Glc-6-PDH to record initial absorbance at 340 nm. Hexokinase solution was then added and the final absorbance was read when the absorbance was stabilized for 15 s. The absorbance difference was used for the calculation of glucose concentration. For lactate measurement, the sample was mixed with hydrazine/glycine buffer and NAD solution in a test tube and extinction E1 at 340 nm was recorded. After adding lactate dehydrogenase (LDH), the mixture was incubated at 37 °C for 30 min and the extinction E2 was determined. Extinction difference between E1 and E2 was then used to calculate lactate concentration. The relative percentage of glucose metabolized to lactate was utilized for the assessment of glycolytic activity (relative glucose conversion to lactate = [lactate production (μmol)/[2× glucose consumption (μmol)] × 100%). Cell proliferation was assessed by a CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega).

**Oxidation of Glucose, Acetate, and Glutamate to Carbon Dioxide—**The oxidation of [U-14C]-glucose, [2-14C]acetate, and [U-14C]glutamate to 14CO2 was determined as previously described (28). In brief, 1–6 × 106 cells were resuspended in 2 ml of DMEM (11966-025, Invitrogen) in a flask that contained [U-14C]glucose (5 mm, 0.5 μCi), [2-14C]acetate (1 mm, 0.5 μCi), or [U-14C]glutamate (1 mm, 0.5 μCi) and incubated at 37 °C for 4 h. The experimental procedures and device used for release of 14CO2 trapping were described elsewhere (28).

**Electron Microscopy (EM)—**Undifferentiated ESCs (5 × 104 cells) were seeded on gelatin-coated, 12-well plates and cultured for 2 days. Cells were fixed immediately with a triple aldehydemethyl sulfoxide fixative and processed by the Case Western Reserve University School of Medicine Electron Microscopy Core Facility.

**ATP Measurement—**The total ATP content of ESCs was determined by using an adenosine 5’-triphosphate bioluminescent somatic cell assay kit (Sigma) as described elsewhere (29).

**Oxygen Consumption Assay—**ESC oxygen consumption was determined using the Seahorse® XF-24 Bioanalyzer and Oxgraph-2K®-based platform. For the Seahorse XF-24-based assay, 120,000 undifferentiated ESCs were seeded on Cel-Tek®
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Enhanced Aerobic Glycolysis and Reduced Glucose Oxidation in Undifferentiated Cited2Δ−/− ESCs—Media in the three independent Cited2Δ−/− ESC clones previously generated via homologous recombination was noted to become acidic during EB-induced differentiation, as compared with the WT counterpart, suggesting that deletion of Cited2 may affect the rate of glucose conversion to lactate in ESCs. To gain insights into differential glucose metabolism between WT and Cited2Δ−/− ESCs, glucose consumption and the conversion of glucose to lactate and carbon dioxide were measured. During 3 h of incubation, ESCs with Cited2 deletion consumed ~20% more of glucose (Fig. 1A) and consequently produced ~30% more lactate (Fig. 1B). Besides conversion to lactate by LDH, glucose-derived pyruvate can be oxidized in the mitochondria to acetyl-CoA by the pyruvate dehydrogenase complex, which can then be oxidized to CO₂ in the tricarboxylic acid (TCA) cycle. Compared with WT ESCs, undifferentiated Cited2Δ−/− ESCs oxidized 75% less glucose to carbon dioxide (Fig. 1C). Unlike HEK293 cells (positive control), WT and Cited2Δ−/− ESCs had a minimal capacity to oxidize [2-14C]acetate to carbon dioxide (Fig. 1D). [U-14C]Glutamate also used a substrate to determine the rate of oxidation of 5-carbon intermediates in the TCA cycle. When [U-14C]glutamate was provided in glucose-free DMEM, glutamate oxidation was barely detectable in WT and Cited2Δ−/− ESCs (Fig. 1E). In addition, the ATP content was reduced by ~30% in Cited2Δ−/− ESCs (Fig. 1F). These results show that WT and Cited2Δ−/− ESCs have a differential capacity to metabolize glucose during short incubation periods, suggesting a role of Cited2 in controlling glucose metabolism in mESCs.

Mouse ESCs possess bivalent metabolic traits (glycolysis and oxidative phosphorylation) to regulate pluripotency, proliferation, and differentiation (35). Because Cited2 deletion did not affect pluripotency in undifferentiated ESCs from our previous study (22), we tested whether enhanced glycolysis in Cited2Δ−/− ESCs supports ESC proliferation and survival. Cell proliferation was similar in WT and Cited2Δ−/− ESCs during 48 h of incubation, whereas Cited2Δ−/− ESCs had modestly increased cell proliferation at 72 h of incubation compared with WT control (Fig. 1G). In 48 h of sustained culture (starting cell density: 602 cells/cm²/ml), Cited2Δ−/− ESCs consumed significantly more glucose (35.57 ± 2.49 versus 27.80 ± 2.22 μmol, p < 0.05) and produced more lactate (68.33 ± 5.10 versus 48.09 ± 3.54 μmol, p < 0.05), when compared with WT cells. Because one molecule of glucose is converted to two molecules of lactate during glycolysis, glycolytic activity in WT and Cited2Δ−/− ESCs was
evaluated by the relative percentage of glucose converted to lactate (\(\mu\text{mol}/\mu\text{mol}\)). During 48 h of incubation, the majority (86.8 ± 1.3%) of glucose consumed was converted to lactate in WT ESCs, whereas \textit{Cited2}^{−/−} ESCs had a significantly greater glycolytic activity (96.1 ± 3.1% of glucose was converted to lactate). In agreement with undifferentiated ESCs tightly

FIGURE 1. Undifferentiated \textit{Cited2}^{−/−} ESCs have an increased rate of aerobic glycolysis and reduced glucose oxidation. 

A and B, glucose consumption and lactate production in undifferentiated WT and \textit{Cited2}^{−/−} ESCs. 4 \times 10^6 ESCs were resuspended in 2.0 ml of DMEM for 3 h and media were collected for determination of glucose consumption (A) and lactate production (B), \(n = 3\). C–E, oxidation of glucose, acetate, and glutamate was measured in undifferentiated WT and \textit{Cited2}^{−/−} ESCs. Undifferentiated ESCs were resuspended in 2.0 ml of DMEM containing [U-14C]glucose (C), [2-14C]acetate (D), and [U-14C]glutamate (E), respectively. The \(^{14}\text{CO}_2\) release during 4 h of incubation was determined. HEK293 cells were used as a positive control for the oxidation of acetate and glutamate, \(n = 3–4\). F, the ATP content of WT and \textit{Cited2}^{−/−} ESCs grown in complete ESC media was determined using a firefly luciferase-based assay and further normalized by the cell number, \(n = 3–4\). G, proliferation of WT and \textit{Cited2}^{−/−} ESCs. 7,500 cells were seeded on gelatin-coated 96-well plates in 5 replicates and 20 \(\mu\text{L}\) of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt)/phenazine methosulfate solution was added to each well at the indicated time points. Absorbance at 490 nm was recorded and data from two independent experiments are presented. H, effect of 2-DG treatment on survival of undifferentiated WT and \textit{Cited2}^{−/−} ESCs. ESCs were seeded on 0.1% gelatin-coated 6-well plates with or without 2-DG. The starting concentration of glucose and 2-DG was 10 and 1 mM, respectively. 72 h later, cells were fixed with 1% paraformaldehyde and subjected to alkaline phosphatase staining. Note that WT and KO ESCs formed colonies with positive AP staining (in arrows) in glucose media and no ESC colonies survived by 2-DG treatment. *, \(p < 0.05\) compared with WT controls.
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dependent on aerobic glycolysis for maintaining survival and pluripotency (15), WT and Cited2Δ/− ESCs treated with specific glycolysis inhibitor 2-deoxyglucose (2-DG) were not able to retain undifferentiated colonies with positive alkaline phosphatase (AP) staining (Fig. 1H). Collectively, these data demonstrate that Cited2Δ/− ESCs display enhanced aerobic glycolysis and reduced glucose oxidation, and that alternations in glucose metabolism in undifferentiated Cited2Δ/− ESCs did not affect ESC pluripotency and proliferation.

Cited2Δ/− ESCs Have Altered Morphology and an Increase in the Number of Mitochondria—A reduced rate of glucose oxidation by Cited2Δ/− ESCs suggested that mitochondrial functions might be affected in these cells. We next compared the ultrastructure of WT and Cited2Δ/− ESCs by EM. WT ESCs had a low number of mitochondria with typical morphology, which is consistent with previous reports (36) (Fig. 2, A and B). Interestingly, swollen mitochondria and an increased number of mitochondria were found in Cited2Δ/− ESCs (Fig. 2, A–C). The ratio of total mitochondrial DNA (mtDNA) copy to nuclear DNA copy reflects mitochondrial content. The increased mitochondrial number parallels the higher ratio of the mtDNA copy (ND1 and cytochrome b) to nuclear DNA (18S rDNA) copy in Cited2Δ/− ESCs (Fig. 2D). Swollen mitochondria are associated with reduced mitochondrial membrane potential, increased apoptosis, accumulated ROS in the mitochondria, and are defective autophagy in multiple cell types (37–40). To test whether the mitochondrial membrane potential is affected by abnormal morphology, we performed tetramethylrhodamine methyl ester (TMRM) staining and found that mitochondrial membrane potential was increased by ~20% in Cited2Δ/− ESCs (Fig. 3A). The increased TMRM staining of Cited2Δ/− ESCs and comparable basal apoptosis in WT and Cited2Δ/− ESCs (22) suggests that the swollen mitochondria in Cited2Δ/− ESCs are independent of apoptosis. Furthermore, WT and Cited2Δ/− ESCs had comparable levels of the reduced form of ROS in their mitochondria (Fig. 3B), indicating that ROS is not the main contributor for the swollen mitochondria in these cells. Swollen mitochondria are associated with accumulated ubiquitin and mitophagy markers, such as LC3 and p62/SQSTM1 (39, 40). In complete ESC media, Cited2Δ/− ESCs had increased conversion of LC3-I into LC3-II, the lipidated form of LC3, whereas p62 protein expression was comparable in WT and Cited2Δ/− ESCs (Fig. 3C). Upon nutrient deprivation, Cited2Δ/− ESCs not only had an increased rate of conversion of LC3-I into LC3-II but also an accumulation of p62 protein (Fig. 3C). Taken together, Cited2Δ/− ESCs display altered mitochondrial morphology and number, which is associated with a reduced rate of glucose oxidation, increased mitochondrial membrane potential, and impaired autophagy.

Cited2Δ/− ESCs Have an Enhanced Oxygen Consumption Capacity—Because we observed reduced glucose oxidation, swollen mitochondria, and an increased number of mitochondria in Cited2Δ/− ESCs, we tested whether these alternations are coupled with oxygen consumption. The rate of basal oxygen consumption (OCR) was comparable for adherent WT and Cited2Δ/− ESCs in the media containing 25 mM glucose, 2.5 mM pyruvate, and 2 mM l-alanyl-glutamine (Glutamax) (Fig. 3D). OCR was blocked by the ATP synthase inhibitor oligomycin to
Mitochondrial membrane potential, ROS production, autophagy induction and oxygen consumption in WT and Cited2 Δ/Δ ESCs. A, the mitochondrial membrane potential was measured by TMRM staining through flow cytometry and representative data are presented. Dashed line, WT ESCs; solid line, Cited2 Δ/Δ ESCs. B, the levels of the reduced forms of mitochondrial ROS were determined by MitoTracker Orange CM-H2TMRos staining and representative data are presented. Dashed line, WT ESCs; solid line, Cited2 Δ/Δ ESCs. C, conversion of LC3-I into LC3-II and p62 protein expression in WT and Cited2 Δ/Δ ESCs. Cells were grown in complete ESC media and upon nutrient deprivation (in Hanks’ balanced salt solution for 6 h) proteins were isolated and analyzed by Western blot assay. D, respiration of intact ESC mitochondria was determined using a Seahorse XF-24-based platform. WT and Cited2 Δ/Δ ESCs (1.2 × 10^5/well) were attached to a XF-24 plate in non-buffered assay media (containing 25 mM glucose, 2 mM L-alanyl-glutamine, and 2.5 mM sodium pyruvate). The OCR at basal, and upon sequential injection of oligomycin, FCCP, or rotenone was recorded and normalized by total protein mass. The experiment was repeated 4 times independently and one set of representative data are presented. E, the respiration of mitochondria was monitored in permeabilized ESCs by Oxigraph-2K-based workshop. ESCs were re-suspended in MiR05 buffer. Compared with WT control, basal oxygen consumption of Cited2 Δ/Δ ESCs in the MiR05 mitochondria buffer was reduced (Fig. 3E), whereas maximal mitochondrial respiration capacity upon FCCP treatment was also enhanced in intact Cited2 Δ/Δ ESCs, as compared with the WT control cells (data not shown). Digitonin-mediated cellular permeabilization resulted in ~50% reduction in OCR in WT and Cited2 Δ/Δ ESCs, reflecting the typical response to endogenous ADP depletion by digitonin. The addition of ADP and glutamate did not stimulate oxygen consumption in WT and Cited2 Δ/Δ ESCs, whereas FCCP treatment of permeabilized ESCs caused only a ~20% increase in mitochondrial respiration in both groups (Fig. 3E). Interestingly, WT and Cited2 Δ/Δ ESCs had a ~50% reduction of OCR in the presence of rotenone, an
inhibitor of Complex I of the respiratory chain, indicating that both ESCs maintain mitochondrial respiration. Collectively, intact Cited2\(^{−/−}\) ESCs have modestly increased oxygen consumption compared with the WT control, whereas permeabilized WT and Cited2\(^{−/−}\) ESCs do not respond to ADP stimulation, yet remain sensitive to rotenone inhibition of oxygen consumption.

Cited2 Regulates Expression of Metabolism-associated Genes in Undifferentiated ESCs—Because undifferentiated Cited2\(^{−/−}\) ESCs exhibited enhanced aerobic glycolysis, we investigated whether glycolysis associated genes are affected in these cells. The activity of LDH is known to be elevated during mouse embryonic development from the one-cell stage to blastocyst formation (embryonic day 0 to 3–4), whereas there is an increase in the activity of hexokinase (HK) (14). Glycogen synthesis activity is decreased during embryonic day 3–4, whereas phosphofructokinase 1 (PFK1) activity remains the same during embryonic development to blastocysts (14). We determined mRNA transcripts of HK (1 to 4 isoforms), PFK1 (liver (L) and muscle (M) isoforms), pyruvate kinase (PK), LDH (A to D isoforms) in Cited2\(^{−/−}\) and reconstituted Cited2\(^{−/−}\) ESCs by stably transfected with Cited2 expression plasmid. We have previously shown that Cited2 regulates Oct4 gene expression in ESCs (14). It is therefore not surprising that expression of the gene for HK1, an Oct4 target gene (41), was reduced to 1.6-fold in reconstituted Cited2\(^{−/−}\) ESCs (Fig. 4A). PFK1 is a rate-limiting enzyme in glycolysis; we noted that mRNA for PFK1-L, but not PFK1-M was increased to 2-fold in reconstituted Cited2\(^{−/−}\) ESCs (Fig. 4A). In addition, the mRNA for PK, another key regulatory enzyme in glycolysis was not affected in reconstituted Cited2\(^{−/−}\) ESCs (Fig. 4A). LDHB is a major regulator of glycolysis in normal and cancer cells (42–44) and LDH mRNA was not changed in reconstituted Cited2\(^{−/−}\) ESCs (Fig. 4A). Furthermore, reintroduction of Cited2 significantly enhanced the reduced level of glucose oxidation in Cited2\(^{−/−}\) ESCs (Fig. 4B). The direct role of Cited2 in the regulation of HK1 gene transcription was further evidenced by ~5-fold enrichment by Cited2 antibody against IgG control on the R3 region of the HK1 promoter (Fig. 4C), with modest enrichment in the R7 region and no enrichment in the R2 and R6 regions of the HK1 gene promoter (Fig. 4C). Collectively, Cited2 is recruited to the HK1 gene promoter to directly control HK1 gene expression and regulate glucose metabolism.

Increased Glycolytic Activity and Impaired Hypoxic Differentiation in Cited2\(^{−/−}\) ESCs—Because glycolase metabolism was altered by Cited2 deletion but did not affect mESC pluripotency and proliferation in undifferentiated ESCs, we further explored whether enhanced glycolysis and reduced glucose oxidation caused by Cited2 deletion impact ESC differentiation. During ESCs differentiating to cardiomyocytes, the rate of lactate production, normalized by time and total protein mass was reported to be reduced to 2-fold, supporting the notion that metabolic reprogramming is essential for proper ESC differentiation (19). We previously demonstrated that Cited2\(^{−/−}\) ESCs exhibit defective cardiomyocyte and hematopoietic differentiation during normoxia (22). In WT and Cited2\(^{−/−}\) EBs favoring cardiomyocyte differentiation, glucose oxidation was reduced by 30% in Cited2\(^{−/−}\) EBs compared with the WT control (Fig. 5A), indicating Cited2\(^{−/−}\) EBs retain defects in glucose oxida-
ment with a previous study (46). In contrast, the majority of glucose (~95%) was still converted to lactate in Cited2Δ/− EBs (Fig. 5D) as did in undifferentiated Cited2Δ/− ESCs. Furthermore, ~2.5-fold increase in glycolytic activity resulted in ~4.5-fold higher cell proliferation rate in Cited2Δ/− EBs (Fig. 5E). These results suggest that deletion of Cited2 results in reduced glucose oxidation and enhanced glycolytic activity in both undifferentiated and differentiating ESCs.

We next tested whether metabolic defects of Cited2Δ/− ESCs affects proper ESC differentiation under hypoxia by examining the expression of the pluripotency gene (Oct4) and differentiation genes (Fgf5, T, Gata-6, and Cdx2) (47). Oct4 gene expression was gradually reduced in differentiating WT ESCs during hypoxia, indicative of proper ESC differentiation (Fig. 6A). In contrast, the expression of Oct4 remained high in differentiating Cited2Δ/− ESCs (Fig. 6A), which is consistent with our previous report that Cited2 regulates expression of the Oct4 gene to control ESC differentiation during normoxia (22). Fgf5, a primitive epiblast marker for ESC differentiation, was induced in WT EBs at day 3.5 and the expression level was gradually reduced at EB days 4.5 and 5.5. Compared with WT controls, Fgf5 mRNA was induced to similar levels at EB day 3.5, whereas there was increased induction of Fgf5 at EB days 4.5 and 5.5 in Cited2Δ/− EBs. Induction of T and Gata-6, a mesoderm marker and an endoderm marker, respectively, was significantly reduced in Cited2Δ/− EBs at days 3.5 and 4.5 as compared with WT controls (Fig. 6A). Cited2Δ/− EBs had a lower level of expression of Cdx2, a trophoderm marker, at EB days 3.5 and 4.5, whereas there was a higher induction at EB day 5.5 compared with WT controls (Fig. 6A). Because Cited2Δ/− EBs had a defective induction of the mesoderm marker T gene, we performed a colony formation unit (CFU) assay to examine whether differentiation to hematopoietic cells, a bona fide mesoderm lineage, is affected. Indeed, cfu-erythroid (CFU-E), CFU-granulocyte-macrophage (CFU-GM), and CFU-mixed colonies were significantly reduced in Cited2Δ/− ESCs under hypoxic conditions (Fig. 6B). Collectively, we show that Cited2Δ/− ESCs have impaired differentiation during hypoxia, as evidenced by their inability to down-regulate pluripotency genes, an improper induction of lineage marker genes, and defective hematopoietic cell differentiation. Taken together, increased glycolytic activity, reduced glucose oxidation, and the inability to switch off glycolysis in differentiating Cited2Δ/− ESCs may contribute to defective cell lineage specification.

**HIF-1 Regulates Expression of Differentiation Genes in Cited2Δ/− ESCs**—Because Cited2Δ/− ESCs during hypoxic differentiation had increased glycolytic activity and defective gene expression, we next investigated whether HIF-1α plays a major role.
role in the initiation of differentiation program in Cited2Δ−/− ESCs. During hypoxic differentiation, a knockdown of HIF-1α (Fig. 7A) had no effect on the aberrant activation of Oct4 mRNA in Cited2Δ−/− EBs (Fig. 7B). The expression of Fgf5 was not affected at EB days 3.5 and 4.5 but was further up-regulated at EB day 5.5 in Cited2Δ−/− EBs with HIF-1α knockdown (Fig. 7B). Interestingly, defective induction of T and Gata-6 mRNA in Cited2Δ−/− EBs was significantly resumed (Fig. 7B), whereas impaired trophoderm marker Cdx2 mRNA induction in Cited2Δ−/− EBs was not affected by HIF-1α knockdown (Fig. 7B). Thus, during hypoxic differentiation, a knockdown of HIF-1α modestly corrects the defective induction of the lineage differentiation genes T and Gata-6 in Cited2Δ−/− EBs, indicating that HIF-1 is in part responsible for the defective mesoderm/endo- derm differentiation in Cited2Δ−/− ESCs.

DISCUSSION

Cited2 is a well established transcription co-activator that competes with HIF-1α for binding to the CH1 domain of CBP/p300 (45). Accordingly, a knock-out of p300 or Cited2 in ESCs results in several similar phenotypes, such as delayed silencing of ESC pluripotency genes and defective hematopoietic differentiation (22, 48, 49). The role of p300 in ESC metabolism remains elusive. By monitoring glucose consumption and differentiation (22, 48, 49), the role of p300 in ESC metabolism results in several similar phenotypes, such as delayed silencing of ATP by the conversion of extracellular glucose to intracellular lactate was abrogated in response to hypoxia and hypoglycemic stresses (55, 56). Stabilization of HIF-1α in ESCs reduces mitochondrial respiration, whereas the rate of extracellular acidification is not significantly affected (35). Importantly, enhanced HIF-1 activity in ESCs is associated with the up-regulated expression of genes encoding glycolytic enzymes and enzymes involved in energy metabolism, which primes transition of mESC to epiblast stem cell (35). Targeted deletion of a histone deacetylase, Sirtun-6 (SIRT6), in ESCs also results in increased glucose uptake and lactate production, whereas mitochondrial respiration is attenuated in SIRT6−/− ESCs (57). Mechanistically, the switch from glucose oxidation to CO2 in the TCA cycle to glycolysis in SIRT6−/− ESCs is mediated by increased protein stability of HIF-1α, and the corresponding up-regulation of HIF-1 target genes, such as LDHB, PDK4, and PDK1 (57). As a key regulator of metabolism, HIF-1 is an important transcription factor controlling multiple cell fate decisions, including mESC differentiation. During hypoxic differentiation, a knockdown of HIF-1α in Cited2Δ−/− EBs mod-
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**FIGURE 7. Regulation of differentiation genes in Cited2−/− ESCs by HIF-1.** A, knockdown of HIF-1α in Cited2−/− ESCs. HIF-1α mRNA and protein expression in Cited2−/− ESCs stably infected with lentivirus that expresses shGFP or shHIF-1α during hypoxia. B, effect of HIF-1α knockdown on the expression of pluripotency and differentiation genes in Cited2−/− EBs during hypoxia. Cited2−/− ESCs stably infected with lentivirus that expresses shGFP or shHIF-1α were induced to differentiate under hypoxia (2%) as described in the legend to Fig. 6A and respective gene expression was examined. *, mean ± S.E.; p < 0.05 compared with Cited2−/− ESCs with shGFP controls.

Although oxidative phosphorylation is one of the typical features of ESC metabolism, the rate of oxygen consumption is extremely low in ESCs (15, 36). Our results demonstrated that 14C-labeled acetate and glutamate oxidation in ESCs are barely detectable; permeabilized ESCs are not responsive to ADP addition, suggesting that mitochondria in ESCs are not as active as when they are at the homeostatic state. However, oxygen consumption in the ESCs is sensitive to rotenone treatment and this may reflect the potential ability of ESCs in responding to various differentiation cues and stress conditions; for example, treatment of ESCs with CCCP affects their differentiation (64). Furthermore, the knockdown of mitochondrial uncoupling protein 2 (UCP2) in human ESCs inhibits glycolysis, whereas the expression of ESC pluripotency markers, such as Oct4 and SSEA1, is not affected (32). Ectopic expression of UCP2 in human ESCs reduces glucose oxidation, impairs EB formation, and hampers early differentiation (32). Importantly, the mitochondrial metabolic stress-activated checkpoint in the control of ESC differentiation has been attributed to Ptpmt1 (protein-tyrosine phosphatase, mitochondrial 1), a mitochondrial PTEN-like phosphatidylinositol phosphate phosphatase (29); enhanced aerobic glycolysis, decreased oxygen consumption, compromised mitochondrial fusion/dynamics, and blocked differentiation was observed in Ptpmt1 knock-out ESCs (29). This implicates mitochondria as the key player in ESC differentiation and metabolic reprogramming, which may represent a unique way to modulate the fate of ESCs. To our knowledge, Cited2 is the first pro-differentiation gene whose deletion affects glucose oxidation and in turn ESC differentiation. Thus, our findings may provide a molecular basis for screening small molecules targeting Cited2 to modulate mitochondrial functions and control ESC pluripotency and differentiation.

The current investigation of the metabolic role of Cited2 also has significant implications for regenerative and clinical medicine. Metabolic reprogramming of glycolysis can greatly increase the efficiency of generating induced pluripotent stem...
cells from somatic fibroblasts (21). It is thus imperative to investigate whether MEFs with a deletion in Cited2 have an enhanced rate of glycolysis and reduced glucose oxidation via the TCA cycle, as noted with differentiating EBs with Cited2 deletion. Small molecule inhibitors that target Cited2 and thus favor glycolysis may represent a novel method to increase induced pluripotent stem efficiency. On the other hand, embryonic stem cells and cancer cells are metabolically similar in that they utilize glycolysis to supply energy for proliferation. The metabolic changes in Cited2−/− ESCs may also apply to cancer cells in which the gene for Cited2 has been silenced. In human hepatoma cells, Cited2, a direct effector of peroxisome proliferator-activated receptor γ, is down-regulated (65). Because many cancer initiating/stem cells reside in hypoxic regions of tissues and are resistant to routine therapy, it is plausible that alterations in the activity of Cited2 could be a potential therapeutic alternative to modulate metabolic processes, as suggested by the current study.

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