BOLA (BolA Family Member 3) Deficiency Controls Endothelial Metabolism and Glycine Homeostasis in Pulmonary Hypertension

BACKGROUND: Deficiencies of iron-sulfur (Fe-S) clusters, metal complexes that control redox state and mitochondrial metabolism, have been linked to pulmonary hypertension (PH), a deadly vascular disease with poorly defined molecular origins. BOLA3 (BolA Family Member 3) regulates Fe-S biogenesis, and mutations in BOLA3 result in multiple mitochondrial dysfunction syndrome, a fatal disorder associated with PH. The mechanistic role of BOLA3 in PH remains undefined.

METHODS: In vitro assessment of BOLA3 regulation and gain- and loss-of-function assays were performed in human pulmonary artery endothelial cells using siRNA and lentiviral vectors expressing the mitochondrial isoform of BOLA3. Polymeric nanoparticle 7C1 was used for lung endothelium-specific delivery of BOLA3 siRNA oligonucleotides in mice. Overexpression of pulmonary vascular BOLA3 was performed by orotracheal transgene delivery of adeno-associated virus in mouse models of PH.

RESULTS: In cultured hypoxic pulmonary artery endothelial cells, lung from human patients with Group 1 and 3 PH, and multiple rodent models of PH, endothelial BOLA3 expression was downregulated, which involved hypoxia inducible factor-2α-dependent transcriptional repression via histone deacetylase 1–mediated histone deacetylation. In vitro gain- and loss-of-function studies demonstrated that BOLA3 regulated Fe-S integrity, thus modulating lipoate-containing 2-oxoacid dehydrogenases with consequent control over glycolysis and mitochondrial respiration. In contexts of siRNA knockdown and naturally occurring human genetic mutation, cellular BOLA3 deficiency downregulated the glycine cleavage system protein H, thus bolstering intracellular glycine content. In the setting of these alterations of oxidative metabolism and glycine levels, BOLA3 deficiency increased endothelial proliferation, survival, and vasoconstriction while decreasing angiogenic potential. In vivo, pharmacological knockdown of endothelial BOLA3 and targeted overexpression of BOLA3 in mice demonstrated that BOLA3 deficiency promotes histological and hemodynamic manifestations of PH. Notably, the therapeutic effects of BOLA3 expression were reversed by exogenous glycine supplementation.

CONCLUSIONS: BOLA3 acts as a crucial lynchpin connecting Fe-S–dependent oxidative respiration and glycine homeostasis with endothelial metabolic reprogramming critical to PH pathogenesis. These results provide a molecular explanation for the clinical associations linking PH with hyperglycinemic syndromes and mitochondrial disorders. These findings also identify novel metabolic targets, including those involved in epigenetics, Fe-S biogenesis, and glycine biology, for diagnostic and therapeutic development.
Clinical Perspective

What Is New?

- We demonstrate that epigenetic and hypoxic repression of the iron-sulfur biogenesis protein BOLA3 (BolA Family Member 3) promotes pulmonary artery endothelial metabolic reprogramming and dysfunction.
- To do so, BOLA3 deficiency induces alterations of mitochondrial electron transport, glycolysis, and fatty acid oxidation.
- BOLA3 deficiency also represses lipoate biosynthesis, thus inhibiting the glycine cleavage system, increasing glycine accumulation, and promoting endothelial proliferation.
- In vivo, we find that BOLA3 deficiency is both necessary and sufficient to regulate endothelial glycine metabolism and to promote hemodynamic and histological manifestations of pulmonary hypertension.

What Are the Clinical Implications?

- These findings define BOLA3 as a crucial lynchpin connecting oxidative metabolism and glycine homeostasis with endothelial dysfunction in pulmonary hypertension.
- These results provide a molecular explanation for the enigmatic clinical associations linking pulmonary hypertension with hyperglycinemic syndromes and mitochondrial disorders, such as those driven by endogenous BOLA3 mutations.
- These findings also identify novel metabolic targets, including those involved in epigenetics, iron-sulfur biogenesis, and glycine homeostasis, for diagnostic and therapeutic development in this devastating disease.

Pulmonary hypertension (PH), its severe subtype pulmonary arterial hypertension (or World Health Organization Group 1 PH), and its subtype characterized by hypoxic lung diseases (World Health Organization Group 3 PH) are enigmatic vascular diseases characterized by profound metabolic reprogramming in multiple vascular cell types, often driven by the master transcription factors of hypoxia, hypoxia inducible factor (HIF)-1α and HIF-2α. We and others have explored the pathogenic impact of mitochondrial dysfunction in the pulmonary artery endothelial cells (PAECs), but the full relevance of this principle to PH has been incompletely defined.

We previously reported that hypoxia decreased ISCU1/2 (iron-sulfur [Fe-S] cluster assembly proteins 1/2), which are essential for Fe-S cluster biogenesis. Fe-S clusters ([4Fe-4S] and [2Fe-2S]) are bioinorganic prosthetic groups that mediate electron transport and cellular redox processes. We found that downregulation of ISCU1/2 decreased Fe-S–dependent mitochondrial respiration and promoted glycolysis (Warburg-like effect seen in cancer cells). Long-term repression of ISCU1/2, particularly in PAECs, drove pulmonary vascular remodeling and PH.

Beyond ISCU1/2, Fe-S biogenesis in human cells is controlled by a conserved set of >30 assembly proteins. Rare mutations in the Fe-S scaffold protein BOLA3 (BolA Family Member 3) are an underlying cause of a fatal autosomal recessive disorder, multiple mitochondrial dysfunctions syndrome subtype 2 (MMD52). A manifestation of MMD5 includes PH, accompanied by emerging links (reported by the Uniprot Consortium) with BOLA3 mutations. BOLA3 is crucial in the mitochondria for Fe-S maturation downstream of ISCU1/2. BOLA3 also controls Fe-S–dependent synthesis of lipoic acid, via facilitating Fe-S clusters to act as sulfur donors for lipoate. Lipoate is transferred as a covalent moiety to lysine residues of multiple mitochondrial enzymes, including the E2 subunits of pyruvate dehydrogenase (PDH), thus affecting oxidative metabolism. The mitochondrial H protein of the glycine cleavage system (GCSH) also depends on lipoate modification for modulation of glycine production and cellular homeostasis of this amino acid. Glycine levels are critical mediators of cellular proliferative capacity and are an overarching regulator of growth in cancer cells. However, glycine and its links to Fe-S biology have not been explored in pulmonary vascular disease. Thus, we endeavored to determine whether BOLA3 and its effects on both Fe-S–specific oxidative respiration and the glycine cleavage system in pulmonary arterial endothelium are key pathogenic drivers in multiple forms of PH.

METHODS

The materials and methods that support the study findings are available from the corresponding author on reasonable request. Several approaches were used in this study: in situ staining of human and rodent PH lungs, in vitro studies of cultured primary cells, and in vivo studies of PH mice in which the consequences of manipulating BOLA3 and glycine levels were investigated. The corresponding author had access to all data and takes responsibility for the integrity and data analysis. Detailed description of materials and methods is provided in the online-only Data Supplement.

Human and Animal Subjects and Ethical Considerations

Tables I through XII in the online-only Data Supplement describe human PH specimens; non-PH human lung specimens were described previously. Procedures were approved by institutional review boards at Partners Health Care; the University of California, Los Angeles; Boston Children’s Hospital; the University of Pittsburgh; and the New England Organ Bank. Ethics approval and informed consent were obtained through the Declaration of Helsinki. All animal experiments were approved by the University of Pittsburgh.
**Statistical Analysis**

Data are represented as mean±SEM or mean±SD. For cell culture data, 3 independent experiments were performed in triplicate. Animal numbers were calculated to measure ≥20% difference between means of experimental and control groups with a power of 80% and an SD of 10%. Normality of data was confirmed by Shapiro-Wilk testing. For comparisons between 2 groups, a 2-tailed Student t test was used for normally distributed data. For comparisons among groups, 1-way or 2-way ANOVA and post hoc Tukey testing was performed. A value of P<0.05 was considered significant.

**RESULTS**

**BOLA3 Expression Is Hypoxia Dependent and Downregulated in Pulmonary Vascular Endothelial Cells in PH**

In human and rodent models of PH in which HIF-1α and HIF-2α are known to be active, BOLA3 expression was reduced within endothelial and smooth muscle cells of small diseased pulmonary arterioles in human pulmonary arterial hypertension (Table I in the online-only Data Supplement) and Figure 1A) and Group 3 PH with idiopathic pulmonary fibrosis (Table I in the online-only Data Supplement and Figure 1B). Similarly, in inflammatory and hypoxic PH mice relevant to Group 1 and Group 3 PH, pulmonary BOLA3 was decreased in wild-type mice with hypoxic PH (Figure 1C and 1D and Figure IA in the online-only Data Supplement) and in mice harboring a pulmonary-specific transgene expressing the inflammatory cytokine interleukin-6 (IL-6),15 with or without hypoxia (Figure 1C). Pulmonary vascular BOLA3 (Figure IA and IE in the online-only Data Supplement) was decreased in mice with a variant model16 of severe fibrotic lung disease induced by exposure to bleomycin and hypoxia (Figure 1E in the online-only Data Supplement). Female PH mice displayed similar decreases of BOLA3 via exposure to hypoxia alone or hypoxia plus bleomycin (Figure IE in the online-only Data Supplement). Decreases of BOLA3 were observed in inflammatory models of PH such as chronic Schistosoma mansoni infection in mice (Figure 1E), monocrotaline exposure (Figure 1F and Figure IIA in the online-only Data Supplement), and SUS416+hypoxic exposure in rats (Figure IIB in the online-only Data Supplement). BOLA3 downregulation was observed in CD31-positive endothelial cells from diseased versus control lungs of hypoxic PH mice (Figure 1G). Correspondingly, we found that hypoxia downregulated BOLA3 in cultured PAECs (Figure 1H and 1I). BOLA3 transcript in cultured PAECs was not affected by all inflammatory cytokines or deficiency of factors genetically associated with PH (Figure IIC and IID in the online-only Data Supplement). Although BOLA3 was decreased in diseased pulmonary artery smooth muscle cells (PASMCs) in situ (Figure 1), BOLA3 expression in cultured PASMCs was not altered by hypoxia (Figure IIE in the online-only Data Supplement), suggesting at least partial predilection of this biology for endothelial cells. Also considering the importance of ISCU1/2 in endothelial cells to drive PH,2,4 we focused on studying BOLA3 in PAECs.

**Hypoxia Represses BOLA3 Transcription via a HIF-2α–Histone Deacetylase 1–Dependent Epigenetic Axis**

We concentrated on the roles of HIF-1α and HIF-2α in driving endothelial BOLA3 downregulation in hypoxia. Knockdown of HIF-2α, but not HIF-1α, partially rescued BOLA3 in hypoxic PAECs (Figure 2A), and overexpression of constitutively active HIF-2α in normoxic PAECs reduced BOLA3 (Figure 2B). Although no HIF-2α binding site was predicted in the BOLA3 promoter, 2 sites (A/B) were predicted as histone binding sites and thus modulated by lysine 9 acetylation of histone 3 (H3K9; Figure 2C). Chromatin immunoprecipitation was performed in PAECs to pull down acetylated H3K9, coupled with polymerase chain reaction (PCR) of chromatin immunoprecipitation contents (Table III in the online-only Data Supplement) to detect these BOLA3 promoter sites. Binding of sites A and B with complexes containing acetylated H3K9 proteins was decreased in hypoxia or with forced expression of constitutive HIF-2α (Figure 2C), indicating a more closed chromatin state at these sites less conducive to active transcription compared with normoxia. In hypoxic PAECs, BOLA3 expression was rescued with enhanced histone acetylation via inhibition by histone deacetylase 1 (HDAC1) siRNA or the HDAC inhibitor valproic acid (Figure 2D–2G). Although HDAC1 expression was unchanged under hypoxia (Figure 2H), HDAC1 knockdown enhanced enrichment of acetylated H3K9 at the BOLA3 promoter both in hypoxia (Figure 2I) and during HIF-2α expression (Figure 2J). By chromatin immunoprecipitation–PCR pulldown of HDAC1, HDAC1 binding was enriched at the BOLA3 promoter sites (Figure 2K). However, HIF-2α did not affect that binding, indicating that the role of HDAC1 on H3K9 acetylation at these sites is more complex and does not rely on altered HDAC1 expression or binding. Thus, the downregulation of BOLA3 expression in hypoxia depends in large part on HIF-2α activity coupled with epigenetic control of H3K9 promoter acetylation.

**BOLA3 Controls Fe-S Integrity and Lipoate-Dependent Activity of the Mitochondrial Enzyme PDH**

To determine whether BOLA3 controls Fe-S levels in PAECs, we used a fluorescent quantitative detection
system of intracellular [2Fe-2S] clusters dependent on glutaredoxin 2 protein homodimerization as described. Although fluorescent signal was unchanged with non–Fe-S–dependent sensors (GCN4), BOLA3 knockdown reduced glutaredoxin 2 fluorescence (Figure 3A), reflecting a downregulation of Fe-S levels. Moreover, BOLA3 knockdown modestly decreased expression of Il-6 transgenic mice (without or with hypoxia), and hypoxic wild-type (WT) mice (C). BOLA3 transcript expression was decreased in lung from hypoxic PH mice (10% O2; D), PH mice infected with Schistosoma mansoni (E), and monocrotaline (MCT)-exposed PH rats (F). G, Similarly, BOLA3 transcript expression was decreased in CD31-positive pulmonary endothelial cells (ECs) isolated from chronically hypoxic PH mice. H and I, By RT-qPCR and immunoblotting, BOLA3 was decreased at the transcript (H) and protein (I) levels, respectively, in cultured hypoxic human pulmonary artery endothelial cells (PAECs)...

Figure 1. Downregulation of BOLA3 (BolA family member 3) across multiple in vitro and in vivo models of pulmonary hypertension (PH). A through C, Fluorescence microscopy of human lung from Group 1 pulmonary arterial hypertension (PAH; A) and Group 3 PH (B) vs patients without PH, interleukin-6 (IL-6) transgenic mice (without or with hypoxia), and hypoxic wild-type (WT) mice (C) revealed reduced BOLA3 expression in endothelium (CD31 label) and smooth muscle (α-smooth muscle actin [α-SMA] label). Scale bar, 50 μm. D through G, By reverse transcription–quantitative polymerase chain reaction (RT-qPCR), BOLA3 transcript expression was decreased in lung from hypoxic PH mice (10% O2; D), PH mice infected with Schistosoma mansoni (E), and monocrotaline (MCT)-exposed PH rats (F). G, Similarly, BOLA3 transcript expression was decreased in CD31-positive pulmonary endothelial cells (ECs) isolated from chronically hypoxic PH mice. H and I, By RT-qPCR and immunoblotting, BOLA3 was decreased at the transcript (H) and protein (I) levels, respectively, in cultured hypoxic human pulmonary artery endothelial cells (PAECs). In I, a representative immunoblot is shown with densitometry calculated across 3 separate replicates. In all panels, mean expression in controls (no PAH, no PH, WT, vehicle, control, normoxia) was normalized to a fold change of 1, to which relevant samples were compared (n=3–8 per group). Data represent the mean±SEM. A.U. indicates arbitrary units. *P<0.05. **P<0.001.
components of Fe-S–dependent mitochondrial complex I (NDUFV2) and II (SDHB), leading to complex I activity repression in normoxia and hypoxia (Figure 3B and 3D). Conversely, forced BOLA3 expression in hypoxia via lentiviral transgene delivery rescued NDUFV2, SDHB, and complex I activity (Figure 3C and 3E). Thus, BOLA3 directly controls Fe-S integrity and mitochondrial complex protein levels to regulate respiratory complex activity in PAECs.

Beyond regulating Fe-S integrity, BOLA3 has been reported to control the Fe-S–dependent generation of lipoate, a sulfur-containing moiety and posttranslational modification crucial to some metabolic enzymes such as PDH. In PAECs, we also found that the lipoate-modified form of PDH was downregulated by BOLA3 inhibition, accompanied by concomitant decrease in PDH enzymatic activity (Figure 3F and 3G). Conversely, lipoylated PDH and PDH activity were rescued in hypoxia by forced BOLA3 expression (Figure 3I and 3J). As expected from the known consequences of inhibiting PDH, BOLA3 knockdown increased glycolytic enzymes (including lactate dehydrogenate and PDH kinase 1; Figure 3H), whereas forced BOLA3 expression reversed changes in these gene networks in hypoxia (Figure 3K).

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**Figure 2.** Hypoxia mediates transcriptional repression of BOLA3 (BolA family member 3) via hypoxia-inducible factor-2α (HIF-2α)/histone deacetylase 1 (HDAC)/histone acetylation pathway.

A, Transfection of siRNA targeting hypoxia-induced factor (HIF)-2α, but not HIF-1α, partially rescued BOLA3 expression in hypoxic human pulmonary artery endothelial cells (PAECs). B, Lentivirus (LV)-mediated forced expression of a constitutively active HIF-2α in PAECs inhibited BOLA3. C, Chromatin immunoprecipitation (ChIP)–quantitative polymerase chain reaction (qPCR) via immunoprecipitation of acetylated histone 3 lysine 9 (H3K9) and PCR detection of BOLA3 promoter sites indicated acetylated H3K9 enrichment of the BOLA3 promoter (sites A and B), which was decreased by hypoxia (left) or constitutive HIF-2α expression (right). D through G, siRNA knockdown of histone deacetylase 1 (HDAC1) and valproic acid (VPA; 3 mmol/L), a HDAC inhibitor, both rescued BOLA3 transcript and protein expression in hypoxic PAECs. H, Expression of HDAC1 remained unchanged in hypoxic PAECs. I and J, siRNA knockdown of HDAC1 enhanced enrichment of H3K9 acetylation at the BOLA3 promoter in hypoxia (I) or with constitutive HIF-2α expression (J). K, ChIP-qPCR via immunoprecipitation of HDAC1 indicated HDAC1 enrichment at BOLA3 promoter sites that was not altered with constitutive HIF-2α expression. In all panels measuring fold change, mean expression in control groups (si-NC, lentivirus [LV]–green fluorescent protein [GFP], normoxia, vehicle) was normalized to fold change of 1, to which relevant samples were compared (n=3 per group). Data represent the mean±SEM. AU indicates arbitrary units. *P<0.05. **P<0.01.
Notably, phosphorylation of PDH kinase 1 was also upregulated by BOLA3 knockdown (Figure IVA in the online-only Data Supplement), which would specifically reinforce the inhibition of activity of nonlipoylated PDH. Correspondingly, exposure to dichloroacetate, a PDK phosphorylation inhibitor tested therapeutically...
Figure 4. BOLA3 (BolA family member 3) deficiency modulates glycine metabolism by inhibiting glycine cleavage system H protein (GCSH) in pulmonary artery endothelial cells (PAECs).

A, Via immunoblot and densitometry, in PAECs, BOLA3 knockdown inhibited GCSH in normoxia, thus phenocopying hypoxia alone. B, BOLA3 knockdown in normoxia promoted intracellular glycine accumulation, again similar to hypoxia. C and D, Conversely, in hypoxia, forced BOLA3 expression reversed the reduction of GCSH and blunted consequent glycine accumulation. E and F, By mass spectrometry, BOLA3 knockdown in normoxia (Nx) phenocopied hypoxia (Hx) by increasing accumulation of glycine and its relevant metabolites, serine and sarcosine, as predicted by known metabolic schema. G and H, GCSH knockdown augmented glycine accumulation particularly in hypoxia, whereas forced expression of GCSH reversed the accumulation of glycine in hypoxia. I through L, GCSH knockdown further enhanced lactate dehydrogenase (LDHA; I) and PDH kinase1 (PDK1; J) expression in hypoxia, which was reversed by GCSH overexpression (K and L). M and N, GCSH knockdown increased PAEC proliferation, as measured by cell number count (M) and BrdU incorporation (N). In all panels, mean expression of control groups (Nx si-NC, Nx lentivirus–green fluorescent protein [LV-GFP]) was normalized to fold change of 1, to which relevant samples were compared (n=3 per group). Data represent the mean±SEM. AU indicates arbitrary units. *P<0.05. **P<0.01.
BOLA3 Deficiency Activates Glycine Production by Upregulating the GCSH

We hypothesized that the control of lipoate synthesis by BOLA3 would influence other lipoate-dependent enzymes such as the GCSH, a protein that catabolizes glycine. Consistent with the downregulation of lipoate synthesis (Figure 3F), BOLA3 knockdown in PAECs downregulated GCSH (Figure 4A) and increased intracellular glycine content in normoxia (Figure 4B), thus phenocopying the effects of hypoxia on glycine in PAECs (Figure 4B) and in other cancer cells.19 Conversely, forced BOLA3 expression reduced glycine accumulation in hypoxia by upregulating GCSH (Figure 4C and 4D). High-resolution liquid chromatography mass spectrometry of metabolite levels resulting after BOLA3 knockdown confirmed increased glycine and the relevant downstream metabolites serine and sarcosine, which would be expected to increase consequent to high glycine (Figure 4E and 4F).

Neither glycine dehydrogenase, a subunit in the glycine cleavage enzyme, nor serine hydroxymethyltransferase, which catalyzes reversible conversion of serine to glycine, was altered with BOLA3 knockdown (Figure VA in the online-only Data Supplement). Furthermore, GCSH knockdown (Figure VB in the online-only Data Supplement) upregulated glycine, most prominently during hypoxia but less substantially in normoxia (Figure 4G), suggesting that overall hypoxic reprogramming provided a permissive environment that allows BOLA3 deficiency and its downstream downregulation of GCSH to carry their most robust effects on glycine. With either BOLA3 knockdown (Figure VH in the online-only Data Supplement) or endogenous BOLA3 deficiency in hypoxia (Figure 4H), forced GCSH expression (Figure VC in the online-only Data Supplement) reversed the elevation of glycine, thus establishing the essential role of GCSH in mediating the effects of BOLA3 deficiency on glycine metabolism. Finally, this decreased lipoylation activity, decreased GCSH, and increased glycine production as driven by BOLA3 deficiency in PAECs were phenocopied in primary fibroblasts derived from a patient with MMDS2 carrying homozygous missense mutations in BOLA3 (Figure VI in the online-only Data Supplement), thus revealing the relevance of these findings to the inherited predisposition to PH in these patients.

Elevation of glycine has been linked to cancer cell proliferation and increased glycolysis in ischemia and hypoxia.13,20 Correspondingly, in hypoxia, exogenous glycine augmented increases of glycolytic enzymes lactate dehydrogenate and PDH kinase1 (Figure VD and VE in the online-only Data Supplement) and promoted PAEC proliferation (Figure VF and VG in the online-only Data Supplement), phenocopying the effects of GCSH knockdown (Figure 4I, 4J, 4M, and 4N). GCSH reversed increases of lactate dehydrogenate and PDH kinase1 during hypoxia (Figure 4K and 4L) and siRNA-induced BOLA3 knockdown (Figure VI–VJ in the online-only Data Supplement). Consequently, GCSH reversed, at least partially, the changes in multiple functional indices of oxidative metabolism, including the decreases of mitochondrial complex I and PDH activity induced by BOLA3 deficiency (Figure VK and VL in the online-only Data Supplement). Together, these data demonstrate that BOLA3 deficiency increases glycine via downregulation of GCSH, influencing additive pathobiology beyond the effects of Fe-S deficiency on glucose metabolism.

BOLA3 Deficiency Activates PAEC Glycolysis and Fatty Acid Oxidation and Drives the Production of Reactive Oxygen Species

On the basis of these alterations of metabolic gene expression and enzyme activity, we determined whether BOLA3 deficiency modulates endothelial respiration. Via extracellular flux analysis, oxygen consumption rate (OCR) and extracellular acidification rate (a marker of glycolysis) in cultured PAECs were assessed, with or without expression of a constitutively active form of HIF-2α (Figure VIIA, VIIC, and VIIE in the online-only Data Supplement). BOLA3 knockdown in normoxia alone (Figure VIIA, VIIC, and VIIE in the online-only Data Supplement). BOLA3 knockdown in the presence of HIF-2α further promoted basal glycolysis. As expected, HIF-2α decreased basal OCR (Figure VIIB and VIID in the online-only Data Supplement). Surprisingly, BOLA3 knockdown, either in normoxia or with constitutive HIF-2α expression, increased basal and maximal OCR. To reconcile this observation with the Pasteur effect that shunts glucose away from oxidative phosphorylation, we hypothesized that BOLA3 deficiency mobilizes fatty acid (FA) flux and FA oxidation to increase oxygen consumption, despite the decreased, but not entirely absent, reservoir of Fe-S–dependent mitochondrial activity. Correspondingly, when FA uptake was inhibited by etomoxir, a carnitine palmitoyl transferase inhibitor, BOLA3 knockdown still drove increased basal glycolysis (Figure VIIIF and VIWH in the online-only Data Supplement).
BOLA3 Deficiency Reprograms PAECs to Control Proliferation, Apoptosis, Angiogenesis, and Generation of Vasoconstrictive Factors

Downstream of Fe-S–dependent and glycine-dependent metabolic reprogramming events, we found that BOLA3 deficiency drives key phenotypic changes to promote endothelial activation and dysfunction. BOLA3 knockdown activated PAECs in normoxia and hypoxia, increasing proliferative potential (Figure 5A and Figure IXA in the online-only Data Supplement) and decreasing apoptotic signaling (Figure 5B). Conversely, forced expression of BOLA3 in hypoxia decreased proliferation (Figure 5C) and increased apoptotic caspase activity (Figure 5D), thus revealing that BOLA3 deficiency blunts the proapoptotic hypoxic state and acts to maintain proliferation close to normoxic levels. BOLA3 deficiency also decreased angiogenic tube formation potential in vitro (Figure 5E and 5F), as well as in vivo angiogenesis, by Matrigl plug assay (Figure IXB and IXC in the online-only Data Supplement). This was accompanied by repressing the expression of the angiogenic factor vascular endothelial growth factor (Figure 5G). Furthermore, BOLA3 overexpression stimulated angiogenic tube formation in hypoxia (Figure 5H). Finally, human PASM Cs cultured in gel were overlaid with conditioned media from normoxic or hypoxic PAECs previously exposed to BOLA3 siRNA or control. As assessed by gel contraction, PASM C contraction was increased by media from BOLA3-deficient PAECs in normoxia and, more so, in hypoxia (Figure 5I), consistent with increased vasoconstrictor endothelin-1 (Figure 5J) and decreased endothelial nitric oxide synthase (Figure 5K) in PAECs. Forced BOLA3 expression in PAECs reduced PASM C contraction (Figure 5L), decreased endothelin-1, and increased nitric oxide synthase (Figure 5M and 5N). Consistent with proliferative effects of glycine in cancer cells,13 forced expression of GCSH blunted proliferation and increased production of endothelin-1 driven by BOLA3 deficiency. It did not reverse the decrease of apoptotic caspase activity, a pathophenotype possibly more dependent on Fe-S–dependent mitochondrial function than glycine production (Figure IXD–IXF in the online-only Data Supplement). To ensure specificity of BOLA3 and GCSH knockdown, a second set of siRNAs were used to confirm key findings (Figure X in the online-only Data Supplement). Taken together, these data demonstrate that BOLA3 deficiency, dependent in part on its effects on GCSH, is both necessary and sufficient to promote a proproliferative and antiapoptotic endothelial state, characterized by decreased angiogenesis and increased production of vasoconstrictive effectors.

Endothelial Knockdown of BOLA3 Dysregulates Glycine Metabolism and Promotes Hemodynamic and Histological Manifestations of PH

Revealing the relevance of this BOLA3-dependent pathway in human PH, we found that pulmonary vascular lipids and GCSH levels were downregulated in patients with group 1 and group 3 PH (Figure XI in the online-only Data Supplement), consistent with the downregulation of BOLA3 (Figure 1A and 1B). To demonstrate the
importance of BOLA3 and its control of Fe-S–dependent and glycine-dependent mechanisms in PH, we assessed the in vivo consequences of repressing BOLA3 in the endothelium. To do so, we used the 7C1 nanoparticle intravenous system to serially deliver siRNAs across 4 weeks directly to the vascular endothelium of mice.

Figure 5. BOLA3 (BolA family member 3) deficiency increases proliferation, inhibits apoptosis and angiogenesis, and promotes vasoconstriction in glycine cleavage system H protein (GCSH) in pulmonary artery endothelial cells (PAECs).

A and B, As reflected by BrdU incorporation and apoptotic caspase 3/7 activation, BOLA3 knockdown increased PAEC proliferation and reduced apoptosis in both normoxia and hypoxia. C and D, Conversely, forced BOLA3 expression inhibited proliferation and promoted apoptotic signaling in hypoxia. E and F, BOLA3 knockdown inhibited in vitro angiogenic potential, as measured by tube formation in matrix gel in normoxia and hypoxia (E). White arrows indicate representative full tubes and branch point quantification (F). Scale bar, 200 μm. G, BOLA3 knockdown downregulated vascular endothelial growth factor (VEGF) as assessed by immunoblot. H, Conversely, forced BOLA3 expression increased in vitro tube formation in hypoxic PAECs. I, As quantified by a gel matrix contraction assay encompassing the exposure of pulmonary artery smooth muscle cells (PASMCs) in gel matrix to conditioned serum-free medium from PAECs, knockdown of BOLA3 in PAECs produced conditioned media that increased PASMC contraction in normoxia and hypoxia. J, As quantified by ELISA, BOLA3 knockdown increased secreted endothelin-1 (ET-1) production in normoxia and hypoxia. K, As assessed by immunoblot and densitometry, BOLA3 knockdown in normoxia inhibited nitric oxide synthase 3 (NOS3) expression, at least partially phenocopying the more robust downregulation in hypoxia. L, Conditioned serum-free medium from PAECs constitutively expressing BOLA3 transgene blunted the hypoxic induction of PASMC contraction in matrix gel. M, Forced BOLA3 expression inhibited ET-1 release in normoxia and hypoxia. N, More evident in hypoxia, constitutive BOLA3 transgene expression rescued NOS3 expression. In G, K, and N, mean expression of control group (Nx si-NC or Nx lentivirus–green fluorescent protein [LV-GFP]) was normalized to fold change of 1, to which relevant samples were compared. For all panels, n=3 to 6 replicates per group. Data represent the mean±SEM. AU indicates arbitrary units. *P<0.05. **P<0.01.
in vivo\textsuperscript{21} (Figure 6A). A specific siRNA targeting murine BOLA3 was screened for optimal efficiency of BOLA3 knockdown in cultured murine PAECs in Figure XIIA in the online-only Data Supplement. Detection of BOLA3 siRNA in pulmonary vascular CD31-positive endothelial cells after 4 weeks of treatment confirmed the successful delivery of the siRNA in 7C1 nanoparticle formulation (Figure XIIB in the online-only Data Supplement). Although 7C1 offered efficient pulmonary endothelial delivery, dosing notably resulted in modest delivery in right ventricular (RV) tissue (Figure XIIB and X XIIIB in the online-only Data Supplement). Yet, such treatment did not significantly alter systemic vascular or left ventricular parameters (Figure XIID–XIIIH in the online-only Data Supplement). Compared with control siRNA-treated mice, mice administered BOLA3 siRNA demonstrated a selective reduction of BOLA3 in pulmonary vascular CD31-positive endothelial cells (Figure 6B and Figure X XIIIC in the online-only Data Supplement). Furthermore, consistent with our in vitro findings, BOLA3 knockdown resulted in a concurrent downregulation of lipoate and GCSH expression in CD31\(^+\) (endothelial) cells (Figure 6C and 6D), thus increasing glycine content in mouse lung tissue (Figure 6E). In endothelial and vascular smooth muscle cells, BOLA3 knockdown increased the proliferation marker proliferating cell nuclear antigen (PCNA) and decreased the apoptosis marker cleaved caspase 3 (Figure 6F–6H). Consequently, pulmonary vascular remodeling (Figure 6I–6K) and RV systolic pressure (RVSP; Figure 6L) were increased. RV remodeling (Fulton index) was not significantly altered (Figure 6M). Nonetheless, when considering the vascular-specific effects of BOLA3, we find that these data demonstrate that BOLA3 deficiency drives a combination of Fe-S–dependent and lipoate-dependent metabolic reprogramming in PAECs to promote pulmonary vascular proliferation and PH.

**PH Is Prevented by Overexpression of Pulmonary Vascular BOLA3 and Is Reversed by Glycine Supplementation**

To investigate whether forced BOLA3 expression could rescue these PH pathophenotypes, mice received orotracheal administration of a recombinant adeno-associated virus (rAAV) carrying the BOLA3 transgene 4 weeks before a 3-week exposure to hypoxia (Figure 7A). rAAV was chosen for long-term transgene delivery, as described,\textsuperscript{22} and serotype 6 was empirically selected for optimal endothelial delivery (Figure XIV in the online-only Data Supplement). We confirmed that vector DNA (Figure XIVB in the online-only Data Supplement) and green fluorescent protein reporter gene (Figure XIVC and XIXD in the online-only Data Supplement) were present in mouse pulmonary vascular CD31-positive endothelial cells after 4 weeks after AAV6 delivery. Although modest rAAV6-BOLA3 expression was observed in RV tissue (Figure XVIIIc and XIXId in the online-only Data Supplement), delivery did not affect systemic vascular or left ventricular parameters (Figure XIXh–XIXIv in the online-only Data Supplement). Rather, rAAV6-BOLA3 delivery increased BOLA3 in pulmonary vascular CD31-positive endothelial cells (Figure 7B and Figure XIXC and XIXD in the online-only Data Supplement). In these male, chronically hypoxic mice, rAAV6-BOLA3 increased lipoate and GCSH levels in the pulmonary vasculature (Figure 7C–7E) and reduced glycine accumulation in mouse lungs (Figure 7F). Correspondingly, rAAV6-BOLA3 decreased the proliferation marker PCNA and increased the apoptosis marker cleaved caspase 3 in CD31\(^+\) (endothelial) and \(\alpha\)-SMA\(^+\) (smooth muscle) pulmonary arteriolar cells compared with rAAV–green fluorescent protein mice (Figure 7G and 7H). As a result, rAAV6-BOLA3 significantly decreased pulmonary arteriolar remodeling and muscularization (Figure 7I and 7J), as well as RVSP (Figure 7K), but not Fulton index (Figure 7L).

We aimed to determine whether forced BOLA3 expression could protect against PH across several mouse models with varying levels of hemodynamic severity (Figure 1): female chronically hypoxic mice (Figure XVA in the online-only Data Supplement), male and female mice exposed to a combination of bleomycin and hypoxia (Figure X Vi and X VII in the online-only Data Supplement), and chronically hypoxic male transgenic IL-6 mice\textsuperscript{15} (Figure 8 and Figure XVIIIa in the online-only Data Supplement). As in male hypoxic mice, systemic hemodynamics were not affected by rAAV6-BOLA3 delivery (Figures XV and XVI, XVII–XVIII, and XVIIIId in the online-only Data Supplement). In all cases, rAAV6-BOLA3 increased endothelial lipoate and GCSH (Figures XVb, XVC, XVb, XVC, XVb, XVIC, XVIIIb and XVIIIc in the online-only Data Supplement) and downregulated PCNA (Figures XVIIa, XVD, XVId, and XVIE in the online-only Data Supplement), thus decreasing pulmonary arteriolar remodeling (Figures XVIIb, XVIIc, XVF, XVIIa, XVb, XVIIb in the online-only Data Supplement) and RVSP (Figures XVIIId, XVg, and XVIIc, XVIE in the online-only Data Supplement). This was accompanied by a decreased Fulton index in hypoxic IL-6 transgenic mice (Figure 8E) and in female (Figure XVIIIF in the online-only Data Supplement), but not male (Figure XVIIId in the online-only Data Supplement), mice exposed to hypoxia plus bleomycin. A trend toward a lower Fulton index was noted in hypoxic female mice (Figure XVh in the online-only Data Supplement) treated with rAAV6-BOLA3.

To determine the role of glycine in the actions of BOLA3 in PH, male mice were supplemented long term with glycine via osmotic pump and in drinking water. Doses were determined empirically to ensure a no more than 1.5-fold long-term increase of glycine in...
Figure 6. Endothelium-specific delivery of BOLA3 (BoLA family member 3) siRNA dysregulates lipoate synthesis and glycine metabolism, thus promoting pulmonary hypertension.

A, Wild-type C57Bl6 mice received intravenous injections of either a specific siRNA targeting murine BOLA3 (si-BOLA3):7C1 or si-NC:7C1 nanoparticles every 4 days for 28 days to target vascular endothelium of mice. B, Pulmonary vascular delivery of si-BOLA3:7C1 nanoparticles reduced BOLA3 RNA expression in isolated CD31+ pulmonary endothelial cells but not in CD31- pulmonary cells (n=4 per group). C through E, 7C1:si-BOLA3 reduced lipoate (white, left 3 columns) and glycine cleavage system H protein (GCSH; white, right 3 columns) expression by in situ fluorescence microscopy and increased glycine content by fluorometric assay in mouse lung tissues. F through H, 7C1:si-BOLA3 increased the proliferation marker proliferating cell nuclear antigen but inhibited the apoptotic marker cleaved caspase 3 (CC-3) in both CD31+ endothelial and α-smooth muscle actin (α-SMA)-positive cells in mouse vasculature. I through K, si-BOLA3:7C1 increased pulmonary vessel thickness (J) and muscularization (K) as assessed by hematoxylin and eosin staining (left, column, I), as well as α-SMA staining (green, right column, I). L and M, 7C1:si-BOLA3 elevated right ventricular systolic pressure measured by right heart catheterization but did not affect right ventricular hypertrophy (Fulton index, right ventricle/left ventricle+septum [RV/LV+S]); n=10 mice per group. In B, mean expression of control group (si-NC) was normalized to fold change of 1, to which relevant samples were compared. Data represent the mean±SEM. Scale bars, 50 μm. *P<0.05. **P<0.01. ***P<0.001.
lung tissue in normoxic wild-type mice (Figure XIXA in the online-only Data Supplement). Such supplementation also facilitated persistently elevated glycine levels even when rAAV6-BOLA3 was delivered in hypoxic mice (Figure 8F). Delivery did not affect heart rate, systemic mean arterial pressure, or left ventricular function (Figure XIXB–XIXK in the online-only Data Supplement). Consistent with the predicted pathogenic effects of glycine on the pulmonary vasculature, in normoxic mice, such glycine supplementation, independently of any BOLA3 deficiency (Figure XXA in the online-only Data Supplement), promoted a modest increase...
of PCNA (Figure XXB in the online-only Data Supplement) and vascular remodeling (Figure XXC and XXD in the online-only Data Supplement), accompanied by a slight rise in RVSP (Figure XXE in the online-only Data Supplement) but without alteration of Fulton index (Figure XXF in the online-only Data Supplement). More
importantly, in hypoxic PH mice, glycine supplementation abrogated the protective effect of rAAV6-BOLA3, allowing persistently elevated PCNA (Figure 8H), pulmonary vascular remodeling, RVSP, and Fulton index (Figure 8I–8L), despite forced expression of BOLA3. These results taken together, as defined via epistatic gain- and loss-of-function analyses both in vitro and in vivo, BOLA3 deficiency drives critical endothelial alterations, with a specific dependence on glycine metabolism, to promote pulmonary vascular proliferation, remodeling, and hemodynamic manifestations of PH.

**DISCUSSION**

These findings demonstrate that BOLA3 downregulation, from epigenetic, hypoxic, or genetic means, promotes pulmonary artery endothelial metabolic reprogramming via control of mitochondrial glucose metabolism and glycine homeostasis. In vivo, BOLA3 deficiency is both necessary and sufficient to regulate endothelial glycine metabolism and to promote PH, relevant to both Group 1 and Group 3 subtypes (Figure 8M). These results provide a molecular explanation for the enigmatic clinical associations linking PH with hyperglycinemic syndromes and mitochondrial disorders such as those driven by endogenous BOLA3 mutations. These findings also identify novel metabolic targets, including those involved in epigenetics, Fe-S biogenesis, and glycine homeostasis, for diagnostic and therapeutic development.

Our results provide crucial support for the notion of central dysregulation of Fe-S integrity in pulmonary vascular disease, whereby deficiency of BOLA3, a second Fe-S biogenesis protein beyond ISCU1/2, has now been proven to promote PH. Our data implicate the relevance of BOLA3 to multiple subtypes of PH, including Group 3 PH, a category displaying increasing prevalence and morbidity worldwide without effective treatment options. Our data report substantial consistencies of this mechanism await interrogation. Prior studies have suggested that Fe-S biogenesis genes may contribute to other clinical syndromes of altered mitochondrial respiration associated with PH,25–28 as well as iron deficiencies known to be important in rodents29,30 and humans31,32 with PH.

Our findings also contribute to our evolving understanding of altered endothelial metabolism and activation, relevant to PH and other pathophysiological conditions. Downregulation of mitochondrial oxidative glucose consumption accompanied by increased glycolytic dependence has been observed in PH-diseased PAECs from humans and rodents.33–36 Our data demonstrate a causal role for BOLA3 deficiency in endothelial repression of respiratory complex function and augmentation of glycolytic flux. Yet, the metabolic shift controlled by BOLA3 increased glycolysis and oxygen consumption (Figure VIIA and VIIIB in the online-only Data Supplement), the latter driven by increased FA oxidation and ROS production. Endothelial upregulation of both glycolysis and oxygen consumption has been described in other contexts in which cellular quiescence transitions to an activated state.37 This observation may seem counter to the original Warburg-like effect in PH,1 but prior studies quantified oxygen consumption primarily in relation to glucose, but not FA, oxidation. Recent studies have demonstrated that FAs control endothelial proliferation via dependence on incorporating FA-derived carbons into nucleotide synthesis,38 particularly under stress.39 Therefore, our observations expand our understanding of the Warburg-like effect in PH and invoke new questions concerning the interconnected roles of these processes.

In addition, the control of glycine metabolism by BOLA3-dependent lipoate synthesis introduces an unexplored mechanism for promoting pulmonary vascular proliferation. Several genetic diseases, driven either by mutations in Fe-S biogenesis components (including the BOLA3-driven MMDS2) or by pathogeneses independent of Fe-S production, present with metabolic acidosis and hyperglycinemia and are associated with PH.7,40,41 Furthermore, a mutation in the lipoyltransferase gene LPT1 has been associated with lipoylation defects and PH,9 offering complementary support to our findings. Also lending credence to our observations, elevated glycine levels have been observed in a hypoxic PH mice,42 individuals nonacclimatized to high altitude and thus at risk for PH,43 and patients with scleroderma with PH.44

Whether other Fe-S biogenesis genes also regulate glycine levels in PH remains unclear, and complex features of this mechanism await interrogation. Prior studies have suggested that glycine may improve systemic hypertension by reducing oxidative stress and increasing nitric oxide45; other studies associated higher dietary glycine with more severe hypertension.46 Glycine may have a protective role against ischemia,47 an activity evident when hypoxia is known to promote efflux of endogenous glycine, to prevent its reuptake,48 and presumably to hamper cellular proliferation. In those cases, downregulation of BOLA3 would appear to act as a homeostatic brake to buffer against a substantial loss of net
levels of intracellular glycine under hypoxic stress, thus maintaining at least normoxic levels of this amino acid. Therefore, this may represent a crucial mechanism by which cellular proliferation in hypoxia can be maintained close to normoxic levels, an adaption needed to survive acute hypoxic injury but a detrimental pathophenotype in PAECs and PH during chronic HIF induction. Consequently, we expect that further characterization of the delicate control of glycine homeostasis by BOLA3 and perhaps other Fe-S biogenesis genes will offer substantial insights into the evolution of the proliferation kinetics of PAECs in PH and its subtypes. In addition to the proliferative response, BOLA3 deficiency led to a state of dysregulated endothelial activation that decreased apoptosis, increased elaboration of vasoconstrictive effectors, and decreased angiogenic potential (Figure 5 and Figure IX in the online-only Data Supplement). Although a spatiotemporal model of endothelial pathobiology was proposed for PH, our data predict an even greater degree of endothelial complexity than originally anticipated, in which, driven by BOLA3 deficiency, proproliferative yet dysfunctional angiogenic potential may coalesce in a single, rather than separate, cellular population. Our findings offer impetus for developing more effective diagnostic and therapeutic targets related to Fe-S biogenesis and glycine metabolism. Using glycine levels as a diagnostic or prognostic biomarker in PH could be possible, singly or in a panel. From a therapeutic perspective, high-throughput drug screening may be effective in identifying small molecules that can directly and effectively controls the levels of Fe-S clusters. Furthermore, altering glycine metabolism or exogenous depletion of glycine could be explored as a therapeutic maneuver. Yet, given the complexity of Fe-S–specific metabolism and glycine in various cell types, maximal efficacy of any targeted drug would necessitate strategy for timing and cell-specific delivery.

Study limitations exist. In vitro and in vivo, some responses to BOLA3/GCSH deficiency and to glycine were heightened in hypoxia, whereas normoxic deficiency to BOLA3 (eg, bleomycin exposure alone in mice) and normoxic glycine supplementation showed less robust phenotypes. Therefore, hypoxic reprogramming of the endothelial cells appears to provide a permissive state that allows BOLA3 and GCSH downregulation to carry their most robust effects. Although the explanation underlying these observations remains undefined, it is possible that there may be a compensatory molecular response to BOLA3 knockdown in normoxia to prevent as robust a response as in hypoxia. Furthermore, it is notable that some, but not all, in vivo models of PH studied displayed an alteration of RV remodeling that was dependent on siRNA or rAAV6-BOLA3 delivery. Although varying the hemodynamic severity across PH models may have contributed, confounding delivery of BOLA3 reagents to the RV cardiomyocytes and alteration of carnitine palmitoyl transferase and FA oxidation (Figure XIII in the online-only Data Supplement) may have partially compromised the effect of pulmonary vascular improvements on the RV. Such a notion more broadly sets the stage for studies to determine the exact cell-specific actions of BOLA3 in vascular and nonvascular (RV) cell types driving PH.

Conclusions
These findings define a paradigm whereby BOLA3 acts as a crucial linchpin connecting Fe-S–dependent oxidative respiration and glycine homeostasis with endothelial metabolic reprogramming critical to PH pathogenesis. These results alter our fundamental understanding of endothelial dysfunction in PH, offer a molecular explanation underlying the associations linking hyperglycinemia and mitochondrial disorders with PH, and present novel metabolic pathways for diagnostic and therapeutic development.

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