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Jingru Zhao April 8, 2018

Differential Ubiquitination of Profilin-1 in Hypoxia-induced Pulmonary Hypertension

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Abstract

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Pulmonary hypertension (PH) is a chronic illness characterized by increased pulmonary arterial pressure due to constriction and thickening of the arterial walls. In part, this is due to smooth muscle cell proliferation. Protein ubiquitination is an important regulator of cellular proliferation. Ubiquitin ligases tag target proteins with ubiquitin moieties at lysine (K) residues to alter protein stability and expression. We used mass spectrometry (MS) to screen lung tissue from chronic hypoxia mice exposed to 10% O_2 for 3 weeks for changes in ubiquitination. Profilin-1, a mediator of actin-polymerization, had decreased ubiquitination on K54 (fold change -1.86) and K126 (fold change -1.40). Overexpression of profilin-1 is associated with increased wall thickness in aortas of spontaneously hypertensive rats, but its role in PH is not well characterized. Profilin-1 promotes actin polymerization, a process that is known to play a key role in cellular proliferation, cell motility, and muscle contraction and is up-regulated in PH. We hypothesized that hypoxia-induced changes in ubiquitination of profilin-1 changes its regulation of actin polymerization, leading to proliferation of smooth muscle cells. In our hypoxia exposed human pulmonary artery smooth muscle cell (hPASMC) and chronic hypoxia mouse lung models, we found no difference in profilin-1 protein level when compared to normoxia controls. This suggests that ubiquitination of profilin-1 could be a novel mechanism of regulating its activity rather than stability. Knockdown of profilin-1 with siRNA or disrupting actin polymerization using 200nM latrunculin B or 0.25 μM cytochalasin D leads to significant decrease in hPASMC proliferation. Preliminary fractionation results also show an increased association of profilin-1 and G-actin in hypoxia. Together, our results indicate that reduced ubiquitination of profilin-1 may increase actin polymerization and contribute to PH pathogenesis.

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CHAPTER 1

Introduction

Pulmonary Hypertension

Pulmonary hypertension (PH) is a chronic illness characterized by increased pressure in the pulmonary arteries and pulmonary vascular resistance. Clinically, PH is defined as having a sustained mean pulmonary artery pressure (mPAP) of greater than 25 mm Hg at rest in the supine position [1] and 30 mm Hg during exercise [2]. In healthy adults, the mPAP ranges from 10.7 mm Hg to 17.3 mm Hg at rest [3].

There are five classes of PH defined by the World Health Organization [4]. Group I PH is defined as pulmonary arterial hypertension (PAH) due to idiopathic or heritable disorders, certain drugs, heart abnormalities in newborns, and other conditions including connective tissue disorders and HIV infections. PAH is characterized by the formation of constrictive lesions such as concentric laminar intimal and acellular intimal proliferation in the small pulmonary arteries and arterioles. Complex lesions are also formed, including plexiform lesion, dilation, and arteritis [5]. Group II PH results from left-sided heart disease associated with interstitial fibrosis and adventitial thickening. According to Foshat and Boroumand [5], group II PH is characterized by medial hypertrophy of arterioles and veins, dilation of lymphatics, interstitial fibrosis, interstitial edema, and hemosiderosis [5]. Group III is PH secondary to chronic lung disease or because of hypoxemia. It is associated with medial hypertrophy of small pulmonary arteries and the extension of smooth muscle peripherally [5]. Group IV is due to chronic thrombotic or embolic disease. Histologically, it is characterized by intravascular fibrous septa or webs in the arteries and eccentric intimal fibrosis. Unlike other groups, medial hypertrophy is mild or absent [5]. Group V is PH associated with other conditions such as blood disorders,

systemic disorders, metabolic disorders, or others, such as tumors that press on the pulmonary arteries [6]. This group is characterized by pulmonary hemosiderosis and capillary proliferation that are localized to the lung that the capillaries invade, including the pulmonary interstitium, the pulmonary vasculature, and the airways [5].

Because of the diversity of conditions that may cause PH and the complications in identifying the pathogenesis of PH, this new classification of PH was redefined in 2003 to allow investigators to better group patients in clinical trials [7]. In a study conducted from 1993 to 2012, the frequency of each group within the adult PH population was 68.5% in group 2, 47.0% in group 3, and 9% in group 4 [8].

Pulmonary hypertension can be difficult to diagnose as its symptoms are non-specific and it can be masked by other diseases. Symptoms of PH include fatigue, chest pain, racing heartbeat, shortness of breath, and pain in upper right side of abdomen. From 1993 to 2012, the annual prevalence of PH increased from 99.8 to 127.3 cases per 100,000 population [8]. In 2002, PH was responsible for 15,668 deaths and 260,000 hospital visits [9]. High hospitalization rates, morbidity, and mortality all indicate a critical need for research examining the causes of PH.

Hypoxia-Induced Pulmonary Hypertension

Patients with group 3 PH, or PH resulting from hypoxemia or lung diseases, comprises a quarter of all patients with increased pulmonary arterial pressure [10]. One of the main causes of

hypoxemia in a person without lung disease is high altitude exposure. Upon histological examination of pulmonary vessels from high-altitude residents after they died from a cause other than pulmonary disease, Arias-Stella et al. [11] have found typical patterns of pulmonary hypertension, including bulging of the main pulmonary artery and right ventricular hypertrophy. Furthermore, pulmonary arterial pressure (PAP) is also higher in children and young adults living in high-altitude areas [12, 13].

Many common lung diseases also lead to Group 3 PH, including chronic obstructive pulmonary disease (COPD), interstitial lung disease (ILD), and obstructive sleep apnea (OSA). However, of all the lung diseases, COPD is most commonly associated with group III PH. In fact, COPD is the second most frequent cause of PH of any group, with the first being left heart diseases [1].

In patients with lung disease, the presence of PH is strongly associated with decreased survival and functional capacity and increased complications [14]. COPD patients with PH had double the hospitalization rate of those without [15]. Even moderate PH in the presence of lung disease is associated with greater morbidity and mortality. Pi et al. [16] found that patients with group III PH had a four-fold greater risk of death compared to disease controls without PH. Oswarld-Mammosser et al. [17] have shown that, in COPD patients without PH, the mean 5-year survival rate was 62.2% while in those with PH, it was 36.3%.

Furthermore, the severity of PH also affects survival rate. COPD patients with severe PH, arbitrarily defined as having a mPAP $\geqslant$40 mmHg, had a 1-year survival rate that was 13% less than those with mild to moderate PH and a 5-year survival rate that was 22% lower than the

mild to moderate group [18]. Studies suggest that the development of severe group III PH can be due to greater susceptibility to hypoxia in the alveoli [19], the coexistence of group I PH [20], remodeling of the pulmonary arteries initiated by inflammatory factors [21-23] or damage of the capillary vascular bed [24].

The incidence of PH secondary to lung disease varies depending on the initial disease and across studies. In COPD, the incidence of PH found in most studies ranges from 30% to 70% [14]. Five to twenty percent of COPD patients develop severe PH, with the disease being the only major contributing factor [20, 25, 26]. The incidence varies from study to study due to the differences in the severity of COPD in the population under study, some minor differences in the definition used to define PH, and the differences in the techniques used to measure PAP. In most studies, the most widely accepted mPAP used to define PH is above 25 mm Hg but some studies have used 20 mm Hg [3]. For interstitial lung disease, one study of 2525 patients listed for lung transplant with the United Network for Organ Sharing and the Organ Procurement and Transplant Network registry found the incidence rate of PH to be 46% [27]. For other diseases such as obstructive sleep apnea, which also occurs in patients with PAH, most studies suggest that the incidence rate of PH is about 20-30% of patients [14]. Obesity contributes significantly to the incidence rate of PH secondary to sleep disordered breathing. In fact, most sleep disordered breathing occurs more frequently in patients with obesity. Obesity and the associated metabolic irregularities including insulin resistance, endothelial dysfunction, and increased oxidative stress are all risk factors for the development of PH [28].

Currently, there are no approved medical therapies nor recommended treatments for PH secondary to chronic lung disease. The main approach to treating group III PH is through treating the underlying lung disease. However, advanced lung diseases are usually not reversible. When the lung disease cannot be cured, therapies approved for group I PAH may be used, however, they do not improve the patient outcomes [14]. As PH develops, it can lead to right ventricular hypertrophy and eventually right ventricular failure, leading to death [29, 30]. Therefore, it's critical to understand the underlying mechanisms that lead to group III PH so that treatment options can be developed in the future.

Group III PH Pathophysiology

The pulmonary circulation is a low-pressure system, with most healthy adults having a mPAP of 14.0 ± 3.3 mm Hg at rest [3]. Normally, the circulation maintains a small pressure gradient between the pulmonary arterial and pulmonary venous circulations. During exercise, the increase in blood flow and carbon monoxide (CO) is accommodated by the dilation of perfused blood vessels and the recruitment, or perfusion, of under-perfused vessels [31]. Therefore, in a healthy adult, there would be little increase in the PAP during exercise. Chronic lung disease patients have fewer peripheral pulmonary blood vessels [31], suggesting that patients with chronic lung diseases have a reduced number of vessels that could be recruited during exercise. This could explain the higher prevalence of PH in COPD induced by exercise. In one study, 66% of COPD patients had a mPAP greater than or equal to 30mm Hg induced by exercise [32].

Most of the group III PH patients have mild to moderate PH, with most studies reporting a mPAP ranging from 25 to 35 mm Hg and only about 3-4% of patients have a mPAP greater than 40mmHg [14]. In Group 1 PH, on the other hand, most studies have found that the average mPAP is 50-55 mmHg [33], indicating that the pathophysiology of PAH differs from group III PH in key ways.

The pathophysiology of group III PH involves vasoconstriction, neomuscularization, and the thickening of the pulmonary arterioles [34]. During hypoxia, the pulmonary vascular tone increases. This is unlike the microcirculation of the systemic vascular bed, which dilates in response to decreased oxygen tension. This pattern of hypoxic pulmonary vasoconstriction (HPVC), is seen in response to both alveolar hypoxia and pulmonary arterial hypoxemia [35]. HPVC diverts pulmonary blood flow away from the areas of alveolar hypoxia, maintaining normal arterial O_2 tension. However, when large areas of the lung experience hypoxia, HPVC greatly increases pulmonary vascular tone to maintain the normal O_2 saturation [14]. While this can be fixed with supplemental oxygen in patients without lung diseases, in patients with diseases leading to chronic hypoxia exposure, the damaging effects can only be partially reversed. Over time, this produces increased muscularization of the pulmonary arteries and remodeling of the distal pulmonary circulation [14].

Aberrant expression of certain inflammatory mediators and growth factors contribute to the vasoconstriction seen in PH. Endothelin 1 (ET-1), an endothelium-derived vasoconstrictor, was found to increase in expression in patients with PAH [36] and plasma levels of ET-1 is increased in patients with emphysema and interstitial lung disease [37]. Circulating levels of ET-

1 are also correlated with the severity of PH [36]. In rats exposed to chronic hypoxia, a model for group III PH, ET-1 was found to enhance actin polymerization in the pulmonary arteries, leading to increased constriction of the pulmonary arteries and greater agonist-induced vasoconstrictor activity [38]. Nitric oxide levels also play an important role in the progression of PH. Prior to vasoconstriction in the pulmonary arteries following hypoxia, reductions in the lung NO levels were found [39]. Another study found that the decrease in NO synthesis caused by tobacco smoke leads to apoptosis of healthy pulmonary vascular endothelial cells, furthering the development of emphysema and PH [40].

Structural changes also occur in the pulmonary arteries during the progression of the disease. Pulmonary remodeling associated with PH is present even in mild COPD [41]. Structural changes include arterial thickening, neomuscularization, reduction in the total number of pulmonary vessels, and pulmonary thrombosis.

Arterial thickening is a characteristic development of PH. Colombat et al. [42] examined diseased lungs of idiopathic pulmonary fibrosis patients removed during transplant procedures and found that 65% of patients had thickening of the pulmonary arteries and occlusion of small pulmonary veins and venules. Even smokers with normal lung function may develop intimal thickening in the muscular pulmonary arteries [37]. A key cause of arterial thickening is the proliferation of vascular smooth muscle cells. Both *in vitro* and *in vivo* studies demonstrate that hypoxia promotes proliferation of pulmonary artery smooth muscle cells (PASMC) [43-46]. Further, hypoxia-inducible factor 1 alpha (HIF-1α) plays an important role in hypoxia-induced pulmonary vascular remodeling. In hypoxia, HIF-1α expression is upregulated (54 kwon),

leading to its accumulation and dimerization with HIF-1β to induce hypoxia-specific gene expression [47]. Further, HIF-1α knock down in PASMC reduces proliferation [45, 48].

In addition, hypoxia exposure leads to muscularization the alveolar walls, which are normally non-muscular [49, 50]. Hypoxia leads to an increase in cells that express α-smooth muscle actin, which could be caused by the migration of smooth muscle cells and cellular proliferation, including the trans-differentiation of endothelial cells into the mesenchymal-type cells, and differentiation of fibroblasts [49, 50].

A reduction in the peripheral blood vessels contributes to a reduced cross-sectional area of the pulmonary vascular bed [50].

Models for Hypoxia-Induced PH

For *in vivo* models of hypoxia-induced PH, animals are exposed to suboptimal oxygen concentrations rather than normoxic atmospheric levels at 21% O_2. The most widely used model for chronic hypoxia in rodents, also the technique that's used in our lab [51], involve exposing the animals to 10% O_2, about the level of oxygen at an altitude of 18,000 feet, for 3-5 weeks. Hypoxia induces the proliferation of smooth muscle cells in the blood vessels and the muscularization of small pulmonary arterioles leading to increased right ventricular systolic pressure, vascular occlusion, and hypertrophy without significant proliferation of endothelial cells in rodent models. Therefore, this study employs this chronic hypoxia model for group III PH.

Physiologically, at the cellular level, hypoxia is defined as the O_2 level that cells and tissues must actively respond to main homeostasis, which is typically 3-7% O_2 [52, 53]. This study also uses an *in vitro* model of hypoxia-induced PH, in which human pulmonary artery smooth muscles cells (HPASMCs) are exposed to 1% O_2 for 72 hours [51]. The cells exhibit increased cell proliferation and signaling changes similar to what is seen in group III PH.

Ubiquitin and Protein Ubiquitination

Ubiquitin is a small 76-residue protein that can be tagged on to target proteins by ligases. The addition of ubiquitin consists of three steps [54-56]. First, ubiquitin is activated in the presence of ATP by ubiquitin-activating enzyme (E1). Next, the now activated ubiquitin is transferred to the catalytic site of ubiquitin-conjugating enzymes (E2) through formation of a thioester bond. The ubiquitin ligases (E3) then interact with E2s to attach the activated ubiquitin to a specific target. This process creates an isopeptide linkage between the carboxy terminus of ubiquitin and the ε-amino group of lysine on the target protein [57]. Additional ubiquitin moieties can then be bound to previously attached ubiquitin on one of its 7 internal lysine residues (K6, K11, K27, K29, K33, K48, and K63), also through an isopeptide linkage [56, 57]. In proteins lacking lysine, the poly-ubiquitination might occur at the amino terminal residue instead [58]. The specificity of ubiquitination is determined by both E2s and E3s [59, 60].

There are many outcomes of protein-ubiquitination, including proteasomal degradation through the ubiquitin proteasome system (UPS), endocytosis, regulation of protein activity, and others. The outcome of ubiquitination is determined by the linkage type. Although all seven lysine residues are used for chain formation [61], the most commonly studied are K48 and K63. Polyubiquitination using K48 to create ubiquitin chains often results in proteasomal degradation by the 26S proteasome, where proteins are broken down and recycled through the ubiquitin proteasome system (UPS) [56]. On the other hand, polyubiquitination at K63 results in a variety of functions, including endocytosis, degradation of lysosomes, DNA repair, regulation of transcription, and activation of protein kinases [62-64].

Both mono-ubiquitination and poly-ubiquitination can play a role in endocytosis, endosome regulation, gene expression, DNA repair, virus budding, and nuclear export [65-68]. One example is proliferating cell nuclear antigen protein (PCNA) where mono-ubiquitination of PCNA changes the function of the protein from high-fidelity DNA-replication to a low-fidelity translesion DNA synthesis bypass following UV irradiation [69]. However, polyubiquitination of PCNA plays a critical role in the error-free repair of damaged DNA and its protective pathway [64]. Another role of mono-ubiquitination is the regulation of membrane protein trafficking signals [70]. A protein can be modified through mono-ubiquitination of several different lysine residues. This can lead to ligand internalization and endocytosis [71, 72]. Mosesson et al. [72] found that multi-mono-ubiquitination of epidermal growth factor receptors can lead to endocytosis and degradation by cells. Another mechanism, branched polyubiquitination, may regulate protein activity, though the exact function remains unclear [73-76].

Ubiquitination has been shown to be an important regulator of a variety of cellular processes [57], including metabolism [77], cell proliferation [78], inflammation [77, 79], antigen presentation [80], and autophagy [79, 81]. Besides its role in regulating protein expression through the ubiquitin proteasome system (UPS), ubiquitination can also regulate protein stability, interactions with other proteins, localization, and other biological functions [82]. The function that ubiquitination serves depends on the specificity of ubiquitinated substrates, such as its subcellular localization, or the number and location of the ubiquitin moieties, which can change the proteins three-dimensional structure [57, 83]. The three-dimensional signal can be interpreted by cells through interaction with ubiquitin-associated protein domains (UBA) [65].

Further, ubiquitin-interacting motifs (UIM) can be found in not only the proteasome but also other proteins that are involved in ubiquitination and ubiquitin metabolism or can interact with ubiquitin-like modifiers [84].

Ubiquitination is a vital post-translational modification, which can be reversibly regulated by both E2s and de-ubiquitinase (DUB) [85]. Deubiquitination by is a rapid process that switches off the ubiquitin signal or changes the modification on the same lysine residue [86]. Deubiquitination has been implicated in many diseases. For example, ubiquitin-specific peptidase 4 (USP4) suppresses stress-induced cell apoptosis and facilitates tumor metastasis in melanoma [83].

Disruption of ubiquitin-dependent processes can often lead to disease. For example, many cancers and several inherited diseases are associated with the dysregulation of ubiquitin-dependent proteolysis [57]. CDC34, a ubiquitin-conjugating enzyme, controls expression of p27Kip1, a protein associated with proliferation that is highly expressed in many cancers [87]. Cancer-associated mutations of Parkin, an E3 ubiquitin ligase, disrupts its downregulation of HIF-1α through ubiquitination and abolish its function in inhibiting cancer metastasis [78]. Rac1, a small signaling G-protein that regulates actin dynamics and cell-cell adhesion, is down regulated by the UPS in the beginning of epithelial cell scattering and is associated with tumor cell invasion and some stages of embryonic development [88]. Changes in ubiquitination can also lead to disease if the site of ubiquitination is blocked by mutation, other posttranslational modifications, or by abnormal binding of other proteins at the site. For example, the

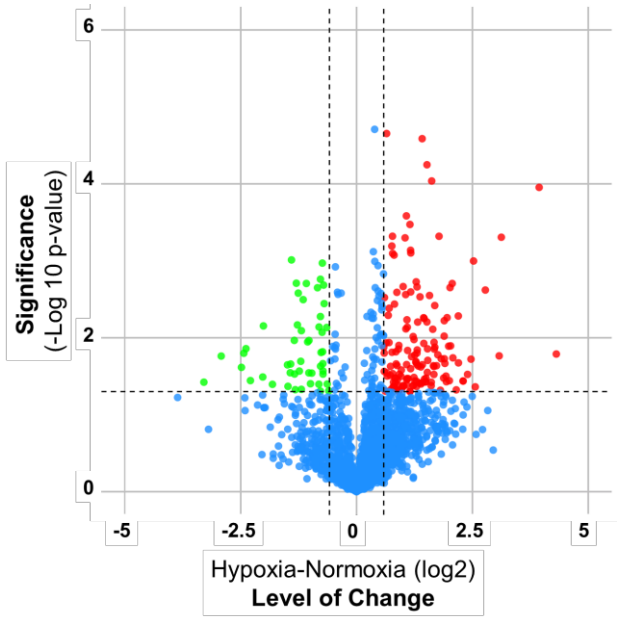
transcription factor C-Jun associates with HIF-1α masking its ubiquitination sites, thereby stabilizing HIF-1α and leading to angiogenesis-related events [88, 89].

Protein Ubiquitination in PH

Dysregulation of ubiquitination has also been implicated in PH. Smurf1, a ubiquitin ligase, downregulates bone morphogenetic protein receptor (BMPR) and its signaling molecules. In hypoxia induced PH models, Smurf1 expression is increased, inducing changes in BMPR signaling that are associated with the abnormal smooth muscle cellular proliferation in PH [90-93]. Further, caveolin 1 (cav-1), an important regulator of endothelial cell function and vascular homeostasis, is downregulated through ubiquitination in the plexiform lesions in lung dissections from idiopathic PH patients [94]. The increase in ubiquitination and degradation of cav-1 could contribute to the dysfunction of endothelial cells in PH [95].

To further investigate the effects of hypoxia-induced PH on ubiquitination, Wade et al. screened lung tissue from mice exposed to chronic hypoxia for changes in protein ubiquitination and found a greater than 30% decrease in protein ubiquitination and a significant change in ubiquitination in 198 peptides (Figure 1.1) in 131 proteins. Many are known to play important roles in cell growth and other cellular processes that are disrupted in PH; however, some of these proteins have not been implicated in group III pulmonary hypertension, including profilin-1 [96].

A)



B)

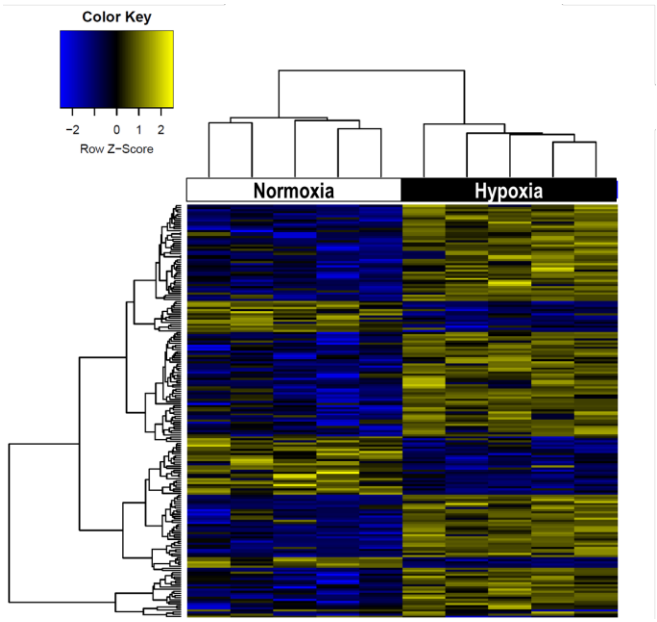


Figure 1.1 Hypoxia-induced changes in ubiquitination was found in 198 peptides and 131 proteins (n=5). A) A volcano plot plotting individual substrates showing a fold-change threshold of ≥ ±1.25. Significant decreases in ubiquitination are in green and significant increases in red ($p<0.01$). B) A heat map showing individual substrates in rows and individual samples in columns.

Profilin-1

Profilin-1 is a small (12-15 kD) actin-binding protein that is ubiquitously expressed [97]. There are four isoforms of profilin found in humans, though each isoform has different ligand affinities [98]. Haugwitz et al. [99] have shown that different profilin isoforms compensate for each other's functions in Dictyostelium but in humans, profilin isoforms do not compensate for each other [100], indicating key differences in function. Whereas profilin-1 is expressed in all cells, the other isoforms are tissue-specific. Profilin-2A and -2B are brain specific and are involved in neuronal development [100]. Profilin-2 forms complexes with membrane trafficking proteins such as synapsin and dynamin-I [98]. Studies have shown that while profilin-1 promotes protrusion, motility, and invasion, though these effects can be suppressed by profilin-2 [101]. Profilin-3, on the other hand, is expressed in the testis and kidney and in developing spermatids [102]. Profilin-4 plays a role in the formation of acrosome and morphogenesis of sperm [100]. Obermann et al. [103] have shown that profilin-3 and profilin-4 share only 30% amino acid homology with other profilins. When discussing regulation of actin dynamics, the discussion is often limited to just profilin-1 and profilin-2. Profilin-1 has approximately a fivefold greater affinity for actin than profilin-2 [104] and promotes protrusion, motility, and invasion while those processes are suppressed by profilin-2 [105].

Profilin-1 is a potent regulator of actin dynamics. The protein promotes the exchange of ADP to ATP bound to free globular actin (G-actin) monomer by forming a 1:1 profilin-actin complex which can then interact with the fast growing, free barbed, or plus, end of filamentous actin (F-actin) (Figure 1.2). The plus end is the barbed end while the minus end is the pointed

end. Because of profilin-1's low affinity for the barbed end, of the actin filament, profilin-1 will readily dissociate when the ATP-actin monomer is added to the growing filament [98, 106, 107]. Therefore, the elongating filament consists of ATP-actin. However, ATP-actin is slowly hydrolyzed to ADP-actin by the intrinsic ATPase activity of actin [98]. Depolymerization, with or without help of the accelerating actin-depolymerizing proteins, causes the ADP-actin to be slowly released from the pointed, or minus, end of the filament [108]. Under physiological conditions, G-actin is in equilibrium with actin-profilin and actin-actin complexes [109]. Profilin-1 and thymosin-β4 both contribute to sequestration of actin monomers, maintaining greater than half of cellular actin in the unpolymerized pool at a concentration that is 200- to 1000-fold higher than the critical concentration for barbed-end polymerization [106, 110, 111]. It has been shown that profilin-1 also captures actin monomers from thymosin-β4-actin complexes and catalyzes polymerization [112]. Although, compared to thymosin-β4 which binds actin monomers simultaneously, profilin-1 does not bind much actin, it can dramatically amplify changes in actin monomer concentration caused by other cellular events because of its interaction with various types of signaling pathways and its ability to lower the critical concentration for actin polymerization [106, 113, 114].

The importance of profilin-1 in normal cell growth and differentiation has been well characterized. Ligands for profilins include actin [115], actin-related protein (Arp2) [116], gephyrin [117], phosphatidyl-inositol-4,5-bisphosphate (PIP2) [118, 119], and other proline-rich domain-containing proteins. Arp2 modulate the assembly of actin and form large protein complexes that induce gene expression [120]. Mammalian enabled (Mena) and vasodilator

stimulated phosphoprotein (VASP), are both polyproline-rich proteins and are involved in the recruitment of profilin-actin complexes to membranes and the structural organization of actin filaments [121, 122]. Gephyrin mediates profilin-1's interaction with Mena/VASP proteins [117]. Through binding these ligands, profilin-1 can regulate major signaling pathways, affecting processes such as transcription factor expression [123], cell motility, and cytokinesis [99, 124].

Due to its ability to bind to many ligands, profilin-1 can regulate actin polymerization through signal transduction and transmembrane signaling [110, 125], leading to many cellular processes, such as proliferation and movement [126]. Genetic studies have shown that the disruption of the profilin gene leads to greatly impaired growth, motility, and cytokinesis in single cells [99, 124]. In human epithelial cells, a minimum level of profilin was found to be necessary for differentiation. In breast cancer cells, tumorigenicity is associated with low profilin-1 levels [127]. In normal human mammary epithelial cells, silencing expression of profilin-1 is associated with a decrease in cell-cell and cell-matrix adhesions and an increase in motility and scattering while induced overexpression of profilin-1 in breast cancer cells is associated with decreased invasiveness [101]. A similar effect of overexpression was seen in pancreatic cancer [128]. A contrasting effect was observed in other cancers. For example, in gastric cancer, increased expression of profilin-1 promoted gastric cancer progression [129]. In bladder cancer, suppressing profilin-1 levels is associated with decreased cell migration, adhesion, and tumor establishment [130]. Further, the levels of profilin-1 decreases with tumor progression in bladder and breast cancer [130, 131], suggesting that profilin-1 levels are

important in early tumorigenesis. Therefore, the role of profilin-1 in promoting cell migration and metastasis is specific to the cell or tissue type involved.

In vascular diseases, the role of profilin-1 is more consistent. Increased expression of profilin-1 is associated with vascular hypertrophy [132], inflammation [133], and remodeling (60 wang) [97]. For example, mechanical stress or hypertension in the vascular wall leads to higher expression of profilin-1 and actin polymerization, triggering the activation of hypertrophic signals such as Rho and Rho-associated protein kinase (ROCK), extracellular signal-regulated kinases (ERK1/2), and c-Jun N-terminal kinase (JNK) [132-134]. Selective overexpression of profilin-1 leads to greater actin polymerization in the aortic vascular smooth muscle cells of transgenic mice and is associated with greater stress fiber formation, activation of hypertrophic signaling cascades such as mitogen-activated protein kinase (MAPK) in the aorta, and growth of vascular smooth muscle cells (VSMC) [132]. These effects resulted in systemic vascular hypertrophy and increase in medial cross-sectional area and medial/lumen ratio in the micro vessels [132]. Importantly, Weise-Cross et al. [38] have found that actin polymerization is linked to increased basal pulmonary arterial constriction in rats exposed to chronic hypoxia in a ROS-, ROCK-dependent manner, although the role of profilin-1 was not examined.

Further, even small changes in the vascular lumen radius create stress in the vascular wall. Chronic mechanical stress, elevated blood pressure, and vascular inflammation lead to vascular remodeling to maintain the wall tension and stress constant [135-138]. One of the most prominent ways is by increasing wall thickness. Profilin-1 and the dynamic organization of

the actin cytoskeleton play an integral role in this process [133]. Song et al. [139] found that angiotensin-converting enzyme 2 (ACE2) activates the profilin-1/MAPK signaling pathway to lead to VSMC proliferation and downregulation of profilin-1 lessened this aberrant growth. Wang et al. [97] found that overexpression of profilin-1 is associated with significant increases in vascular wall thickness, vessel size, and collagen content along with severe infiltration under the intima, and an increase in interleukin-6 (IL-6) levels. Further, overexpression of profilin-1 also induces an increase in levels of inducible nitric oxide synthase iNOS and peroxynitrite [97, 133, 140]. iNOS expression is associated with the development of hypoxia-induced pulmonary vascular remodeling in the hypoxic lung [141] while peroxynitrite activates MAPK and is linked to vascular remodeling [142, 143]. In monocrotaline-treated rats that model inflammation induced PAH, the invasiveness and growth of VSMCs and the development of increased vascular resistance were positively correlated with profilin-1 expression and increased remodeling of subcortical actin filaments [144].

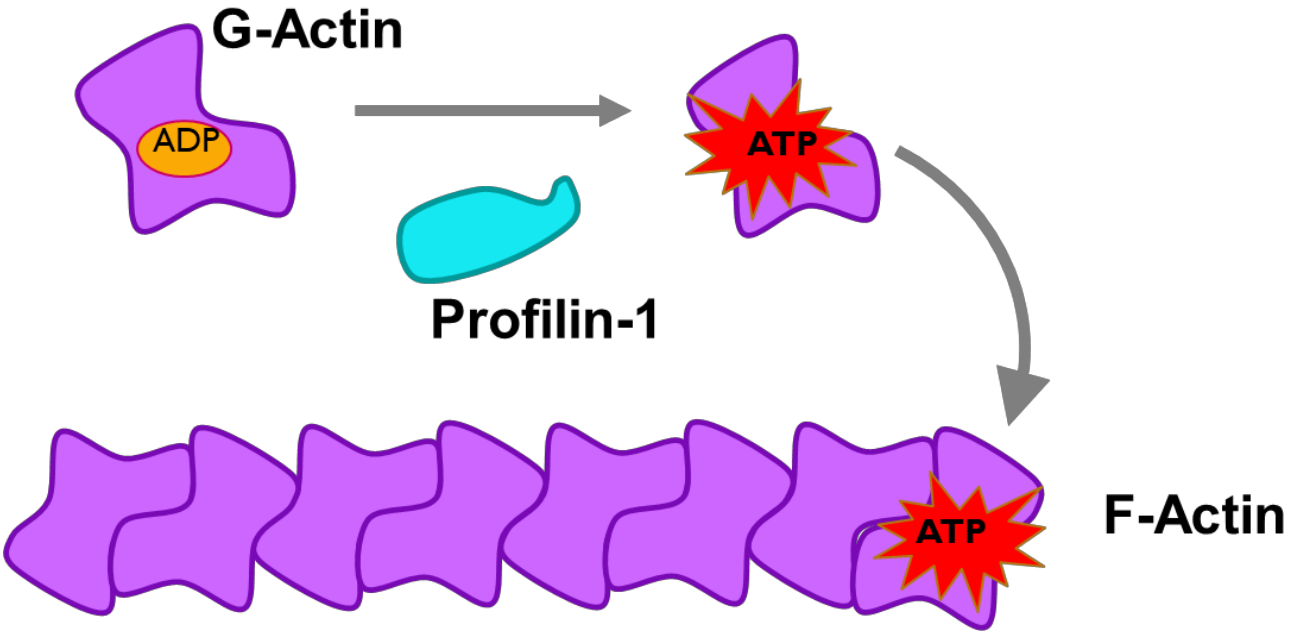


Figure 1.2 Profilin-1 binds ADP-actin monomer and catalyzes the nucleotide exchange to yield ATP-actin, which can be added to the barbed end of the growing actin filament.

Summary and Hypothesis

Group III pulmonary hypertension (PH) is a chronic illness characterized by increased pressure in the pulmonary arteries and pulmonary vascular resistance secondary to lung diseases or chronic hypoxemia. There are currently no approved pharmaceutical therapies recommended to treat group III PH patients as many of the underlying mechanisms of its pathophysiology are still poorly understood. PH pathogenesis involves vasoconstriction, muscularization of non-muscular arteries, and the thickening of pulmonary [34].

Pulmonary remodeling due to hypoxia exposure is characterized by aberrant proliferation of pulmonary artery smooth muscle cells [43-46] . Ubiquitination plays an integral role in cellular proliferation due to its ability to regulate protein expression, stability, interactions, and localization among other biological functions [56, 64, 66, 68-70, 74]. Because of its role in important cellular processes and in cellular proliferation involved in PH [90-93], Wade et al. (in submission) [96] studied the effects of chronic hypoxia exposure on the ubiquitination of proteins from lung tissue of mice exposed to chronic hypoxia. Of the 131 proteins that had a significant change in ubiquitination, profilin-1 was one of them.

Profilin-1 plays an integral role in vascular hypertrophy, vascular remodeling, and vascular inflammation in many cancers and vascular diseases, including atherosclerosis [133] and hypertension due to inflammation. However, profilin-1, including expression levels after chronic hypoxia exposure and its role in chronic hypoxia induced PH has not been well characterized. Actin polymerization plays an important role in cellular proliferation [145] and is linked to promoting vasoconstriction in chronic hypoxia induced PH [38]. Therefore, we

hypothesize that hypoxia-induced changes in ubiquitination of profilin-1 changes its regulation of actin polymerization, leading to proliferation of smooth muscle cells.

CHAPTER 2

Methods

In vitro hypoxia model

Human pulmonary artery smooth muscle cells (HPASMC) were obtained from ScienCell Research Laboratories (Carlsbad, CA). As previously described by Adesina et al. [51] from our lab, the genetic backgrounds of HPASMC were characterized using STR profiling (Biosynthesis DNA Identity Testing Center, Lewisville, TX). The HPASMC were derived from five male patients. HPASMC were maintained in smooth muscle medium (ScienCell Research Laboratories, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) and provided growth factors at 37°C in a 75cm^2 cell culture flask (Corning, Corning, NY) in a humidified incubator with 21% O2 and 5% CO2. The HPASMC were used at passages lower than 8 and their elongated spindle morphology was confirmed with phase contrast microscopy before use. The cells were split at 70-80% confluency. The medium was first removed and before the HPASMCs were washed with sterile PBS 1X (Corning, Corning, NY). The PBS was then replaced with 2mL of Trypsin EDTA (0.05% Trypsin, 0.53mM EDTA). 6mL of fresh 2% FBS medium was added to the HPASMC after cells were incubated for 3 minutes in the humidified incubator. The mixture is then centrifuged. The resultant pellet is mixed with 3mL of 2% FBS medium before it was counted using the Countess II FL Automated Cell Counter (Life Technologies, Carlsbad, CA) with 0.4% Trypan Blue (Invitrogen, Carlsbad, CA). The cells were then either used for maintenance or plated for experiments.

Cell Toxicity

As described previously by Adesina et al. [51], HPASMC were plated in clear bottom black 96-well plates (Corning, Corning, NY), then grown in 0.5% FBS medium for 24 hours to synch cell growth. Medium was then replaced with 5% FBS medium. For hypoxia studies, the HPASMC were either exposed to normoxic (21% O_2) or hypoxic (1% O_2) conditions for 72 hours at 37°C. The MultiTox-Glo Multiplex Cytotoxicity Assay (Promega, Madison, WI) was used according to the manufacturer's instructions. Fifty microliters of diluted glycyl-phenylalanyl-aminofluoro-coumarin (GF-AFC) substrate was added to each well prior to incubation for 30 minutes at 37°C in a humidified incubator. The plates were then measured using a Wallac Victor 3 fluorimeter (Perkin Elmer, Waltham, MA) at 400 nm excitation/505 nm emission to detect live cells. The GF-AFC substrate causes the release of fluorogenic AFC through entering intact cells and being cleaved by live-cell proteases. The fluorescent signal detected is directly proportional to the number of live cells, allowing for comparisons between relative levels of HPASMC in each treatment group. Fifty microliters of luminogenic cell-impermeant peptide (AAF-aminoluciferin) substrate diluted in Assay Buffer was then added to each well without removing the diluted GF-AFC substrate. The plates were then incubated for 15-30 minutes at 37°C in a humidified incubator prior to measurement in a Wallac Victor 3 fluorimeter. The dead-cell luminescence is measured through photon emission. The AAF-aminoluciferin measures the activity of dead-cell proteases that were released from cells that lost membrane integrity.

Inhibition of Actin Polymerization in HPASMC

Actin polymerization was inhibited with either cytochalasin D (Cayman Chemical Company, Ann Arbor, MI) or latrunculin B (EMD Millipore, Burlington, MA). Varying concentrations: 1nM, 10nM, 50nM, 100nM, and 500nM latrunculin B or 0.05uM, 0.1uM, 0.25uM, 0.5uM, and 2uM cytochalasin D were added for 72 hours to HPASMC grown on black 96-well plates (Corning, Corning, NY) in normoxic and hypoxic conditions. The proliferation of HPASMC and the toxicity of the treatments given were assessed using the MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega, Madison, WI) as described previously. Significant decreases in proliferation were observed with 200nM of latrunculin B or 0.25uM of cytochalasin D.

Induction of Actin Polymerization in HPASMC

Jasplakinolide (Tocris Bioscience, Bristol, UK; Enzo Life Sciences, Farmingdale, NY) was used to induce actin polymerization in HPASMC. The cells were grown with 1μM jasplakinolide in media for 72 hours. The proliferation was then measured with MultiTox-Fluor Multiplex Cytotoxicity Assay.

Arrest of Actin Polymerization in HPASMC

A pharmacological cocktail containing Y27632, jasplakinolide, and latrunculin B was used to arrest the actin dynamics of HPASMCs [146]. After growing in hypoxia or normoxia conditions for 72 hours, the HPASMCs were then preincubated with 10μM Y27632 for 10 minutes. Next, 8μM jasplakinolide and 5μM latrunculin B were added for 30 seconds before the HPASMCs are washed with 1X PBS. The cells were then scraped with 1X PBS and used for fractionation. Cells grown under hypoxic conditions were extracted at 1% O_2 in a C-Shuttle Glove Box (BioSpherix, Lacona, NY).

Knockdown of Profilin-1 in HPASMC with siRNA

Transfection of HPASMC with siProfilin-1 (Santa Cruz Biotechnology, Dallas, TX) was achieved using the Lipofectamine® RNAiMAX Reagent (Invitrogen, Carlsbad, CA). The HPASMC were plated as described previously 24 hours prior to transfection and adherence was confirmed with phase contrast microscopy. The Lipofectamine® RNAiMAX Reagent was first mixed with serum free media and separately, 20nM, 30nM, and 40nM siProfilin-1 is mixed with serum free media. The diluted siRNA and reagent were then combined and incubated at 25°C for 5 minutes. The siRNA-lipid complex was then added to each well. The treated HPASMC were then incubated for 3 different time points, 24, 48, and 72 hours. Knockdown was confirmed with real-time PCR. 30nM was determined to be the optimal concentration of siProfilin-1.

qRT-PCR

Total RNA was extracted from HPASMCs with RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly, the HPASMC were lysed with RLT lysis buffer and 2% β-mercaptoethanol. Then, a volume of 70% ethanol equal to the RLT buffer was added and the samples were centrifuged for 15 seconds at 9000 rpm in the provided RNeasy Mini spin column. The flow-through was discarded and RW1 Buffer was added. The sample was spun for 15 seconds at 9000 rpm. The flow-through was discarded and RPE buffer was added to the column. After centrifugation, RPE buffer was added again and the samples were centrifuged for 4 minutes at 9000 rpm. The samples were then diluted and incubated in RNase free water for 5 minutes with a new collection tube before centrifugation for 3 minutes at 9000rpm. The concentration of RNA was determined using a Nanodrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

The samples were diluted to the same concentration using RNase free water and reverse transcribed to synthesize cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Target cDNA was then amplified using SYBR Green (Applied Biosystems, Hercules, CA). Real-time PCR was used to measure RNA levels of profilin-1 and GAPDH (Table 2.1). All data were normalized to GAPDH levels in the same sample.

Sample	Genotype	Sequence (5’ -> 3’)
GAPDH	Human	Forward: GCC CAA TAC GAC CAA ATC C Reverse: AGC CAC ATC GCT CAG ACA
Profilin-1	Human	Forward: CCT TCA ATG TCA CTG TCA CCA Reverse: ACC ACC GTG GAC ACC TTC T

Table 2.1 Sequences of all primers used for qRT-PCR

In Vivo Chronic Hypoxia model

Atlanta Veterans Affairs Medical Center Institutional Animal Care and Use Committee approved all animal studies. All studies were performed in accordance with National Institutes of Health guidelines from the *Guide for the Care and Use of Laboratory Animals*. As stated previously by Adesina et al. [51] from our lab, C57BL/6 mice, ages 6-9 weeks, were housed either in ambient air (normoxia, 21% O_2) or in hypoxic conditions (10% O_2) for 3 weeks using the ProOx 110 (BioSpherix, Lacona, NY) as a model for chronic hypoxia exposure in pulmonary hypertension. The right ventricle systolic pressure (RVSP) of the mice were determined after three weeks. Mice were first lightly anesthetized with isoflurane, and a 0.8 F micro-tip pressure transducer (Transonic, Ithaca, NY) was inserted through the right jugular vein and moved to the right ventricle. For 10 minutes, the RVSP was carefully monitored and recorded. The data was then analyzed using the Powerlab system (ADInstruments, Denver, CO). The mice were then euthanized by CO_2 exposure. The lungs were excised and perfused without blood with 1X sterile

PBS. Both lungs or the pulmonary artery were then flash frozen in liquid nitrogen prior to storage in a -80°C freezer.

Mass Spectrometry Screening of Mouse Lung Tissue

PTMScan® Ubiquitin Remnant Motif (K- ε-GG) Analysis (Cell Signaling Technology, Danvers, MA) was employed to compare the ubiquitination of peptides in lungs from mice exposed to normoxia or hypoxia conditions as previously described. Proteome Discoverer™ 2.0 (Thermo Fisher Scientific, Waltham, MA) was used for analysis.

Protein Quantification

Prior to western blot analysis, the protein concentration of sample (either homogenized mice lung tissue or HPASMC lysate) was quantified using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA). The proteins were then diluted in 50-parts of reagent A per 1-part reagent B for 30 minutes at 37°C. First, the biuret reaction chelates copper with protein in an alkaline environment, forming a light blue complex. Next, a purple reaction product is observed due to the chelation of two molecules of bicinchoninic acid (BCA) with one molecule of cuprous cation.

Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to compare relative levels of profilin-1, β-actin and GAPDH in vitro and in vivo. The cell and lung lysates were run through 10% Bis-Tris gels (Invitrogen) using 2-(N-morpholino) ethanesulfonic acid (MES) buffer and transferred onto nitrocellulose membranes (Millipore, Billerica, MA). The lysates were then probed with primary antibodies specific for profilin-1 1:500 (EPR6304 Abcam, Cambridge, UK), β-actin 1:1000 (sc-47778 Santa Cruz Biotechnology, Dallas, TX), or GAPDH 1:20000 (G8795 Sigma-Aldrich, St.Louis, MO) in either bovine serum albumin (BSA) (profilin-1), 5% milk (GAPDH), or Odyssey® Blocking Buffer (LI-COR Biosciences, Lincoln, NE)(β-actin). The membranes were incubated overnight on a rocker at 4°C. The membranes were then washed 3 times for 10 minutes at Room temp with PBS-Tris (what percent triton) and developed with the respective secondary antibody 1:20000. Immunodetection was done with a near-infrared fluorescent method (Li-Cor, Lincoln, NE).

Quantification of G- and F-actin

In vitro. The fractionation method was previously described by Parreno et al. in 2014 [147]. The HPASMCs obtained after the arrest of actin polymerization were centrifuged at 800 g for 3 minutes. The pellet was resuspended in PBS with 0.1% Triton, phosphatase inhibitor (Roche, Basel, Switzerland) and protease inhibitor (Sigma-Aldrich, St.Louis, MO) and incubated for 5 minutes on a rocker. The resultant supernatant containing mostly G-actin is separated into a

separate tube. The triton-insoluble pellet, containing mostly F-actin, is resuspended in RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors. Then, equal concentrations of both fractions were mixed with 5X Laemmli buffer (60mM Tris-Cl pH 6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and heated at 98°C for 10 minutes. The fractions were then quantified by densitometry analysis of western blot probed for β-actin and profilin-1.

CHAPTER 3

Results

Expression of Profilin-1

We conducted a mass spectrometry screen on lung tissue from mice exposed to normoxia or chronic hypoxia. In hypoxia we observed a decrease in ubiquitination of profilin-1 at two sites, K54 and K126, compared to normoxia control (Figure 3.1). K126 and K54 are both spatially near actin binding sites (Figure 3.1B).

We suspected a decrease in profilin-1 ubiquitination may leads to a decrease in rate of degradation, leading to a decrease in profilin-1 protein levels. Using western blot analysis, we measured profilin-1 protein in lung tissue from mice exposed to chronic hypoxia (Figure 3.2A) and human pulmonary artery smooth muscle cells (hPASMC) after hypoxia exposure (Figure 3.2B) then compared them with their respective normoxic controls. We found no difference in expression of profilin-1 in mouse lung nor in hPASMCs.

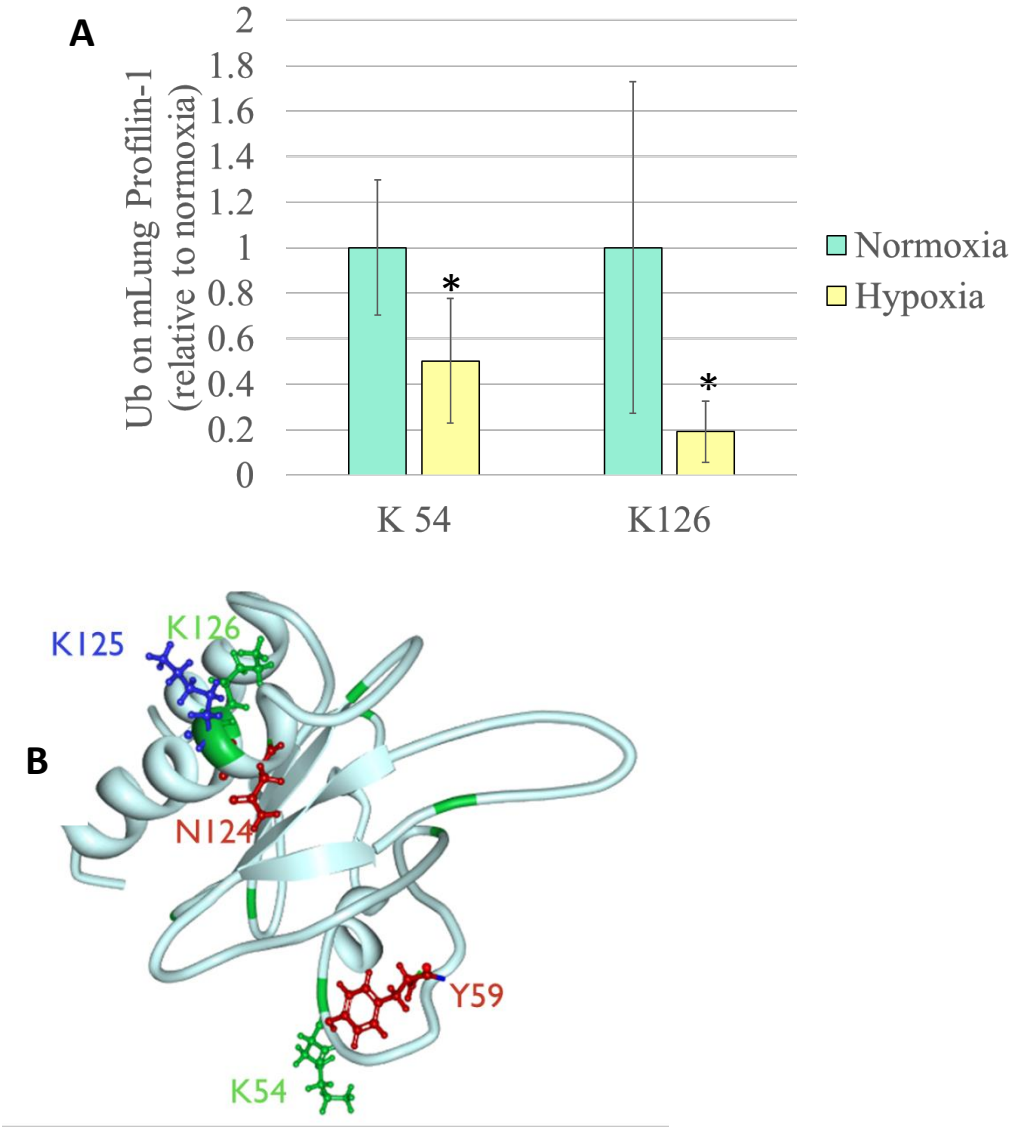


Figure 3.1. Profilin-1 ubiquitination. A) Profilin-1 ubiquitination of in mouse lung tissue after chronic hypoxia exposure (n=5) compared to normoxia control (n=5) at K54 and K126 **B)** Model of human Profilin-1. K126 and K54 are represented in green while nearby actin binding sites are represented in red and Phosphatidylinositol 4,5-bisphosphate in blue. *p<0.05. Error bars represent ± SEM.

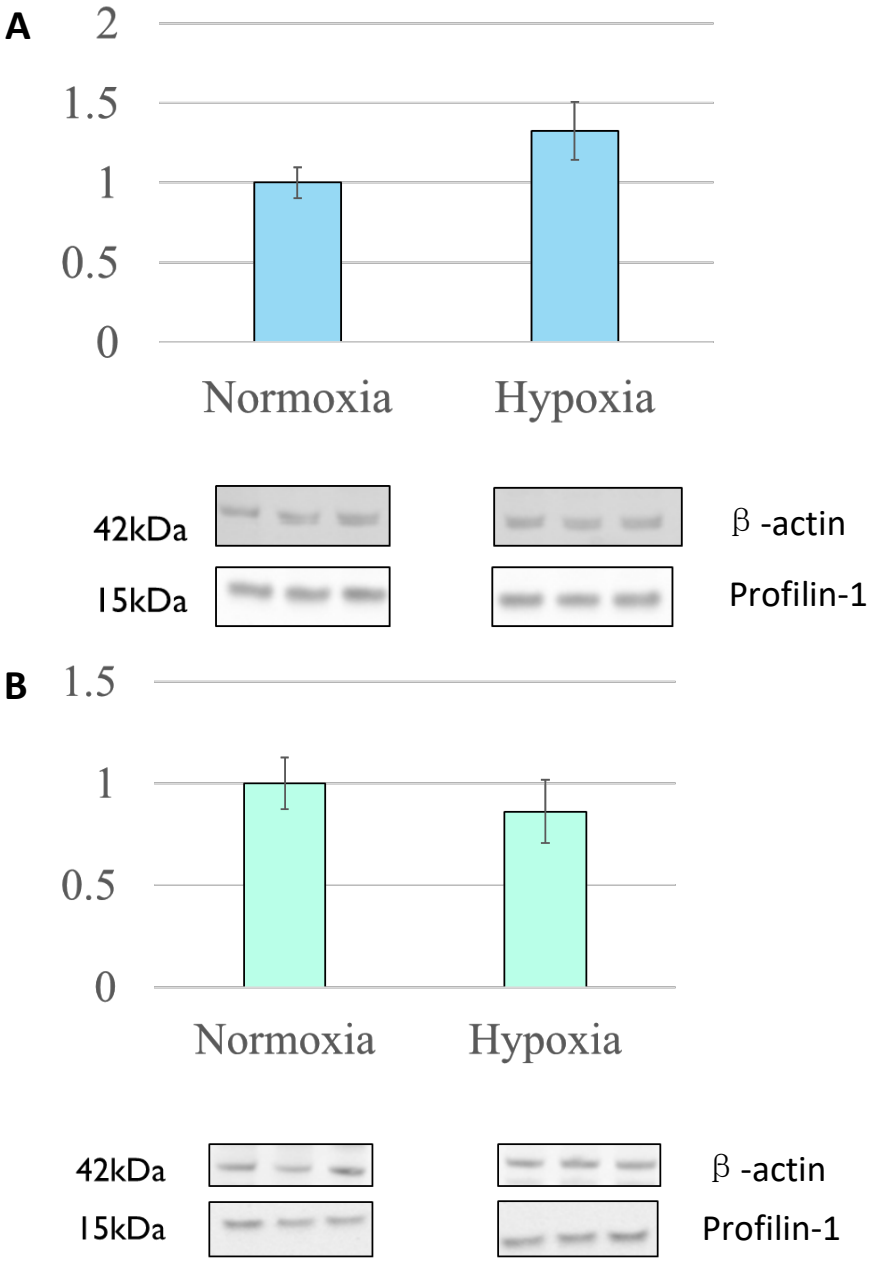


Figure 3.2 Profilin-1 protein levels in mouse lung and HPASMC. **A)** Profilin-1 protein measured in hPASMCs following 72 hours normoxia or hypoxia by western blotting and densitometry (n=9; p=0.08). **B)** Profilin-1 protein measured in normoxic or hypoxic mouse lung tissue (n=10; p=0.493). Error bars represent ± SEM.

Actin Dynamics

A combination of Jasplakinolide, latrunculin B, and Y27632 (JYL) were used to arrest actin dynamics. Jasplakinolide binds actin filaments and prevents disassembly while also lowering the dissociation rate constant for barbed-end actin to essentially zero.At high doses or with long exposure, jasplakinolide results in the incorporation of almost all free actin (source). Latrunculin B, on the other hand, sequesters the free actin monomer pool and depolymerizes existing actin filaments (source). Y27632 a Rho kinase inhibitor, inhibits myosin II activation The efficacy of jasplakinolide to induce actin polymerization and latrunculin B to inhibit actin polymerization were independently examined in HPASMC. Each treatment was added to hPASMC samples immediately prior to G- and F-actin fractionation. and the combination of Y27632, jasplakinolide, and latrunculin B (JYL) to arrest actin dynamics, compared to no treatment, JYL had a lower proportion of G-actin, jasplakinolide had a lower proportion of G-actin, and latrunculin B had a higher proportion of G-actin (Figure 3.3).

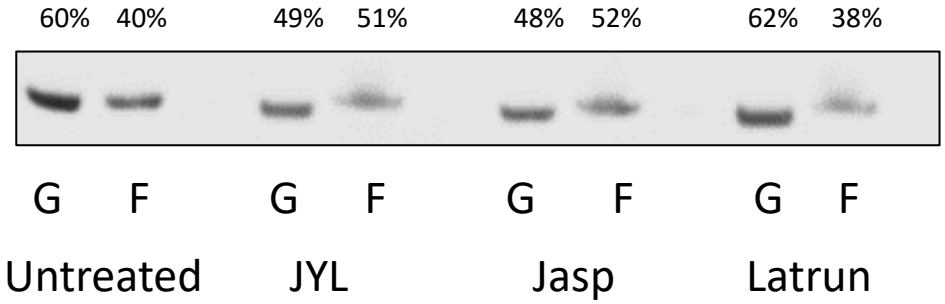


Figure 3.3 Western blot of hPASMCs treated with jasplakinolide-Y27632-latrunculin B cocktail, jasplakinolide, and latrunculin B.

Profilin-1 binding to G-actin in normoxic and hypoxic HPASMC ’s

Because the hypoxia-induced changes in ubiquitination were at sites that are close to actin and phosphatidylinositol 4,5-bisphosphate (PIP2) binding sites (Figure 3.1), we investigated whether the decrease in ubiquitination leads to a greater affinity of profilin-1 to bind to actin monomers, or G-actin.

After arresting actin polymerization with JYL treatment hPASMC lysates were subject to fractionation to separate the G- and F-actin fractions. The fractions were then subject to western blot analysis and probed with profilin-1 and actin. We found a lower proportion of G-actin in hypoxia compared to normoxic control (Figure 3.4A). Further, we also found a higher proportion of G-actin to be associated with profilin-1 in hypoxia (Figure 3.4B).

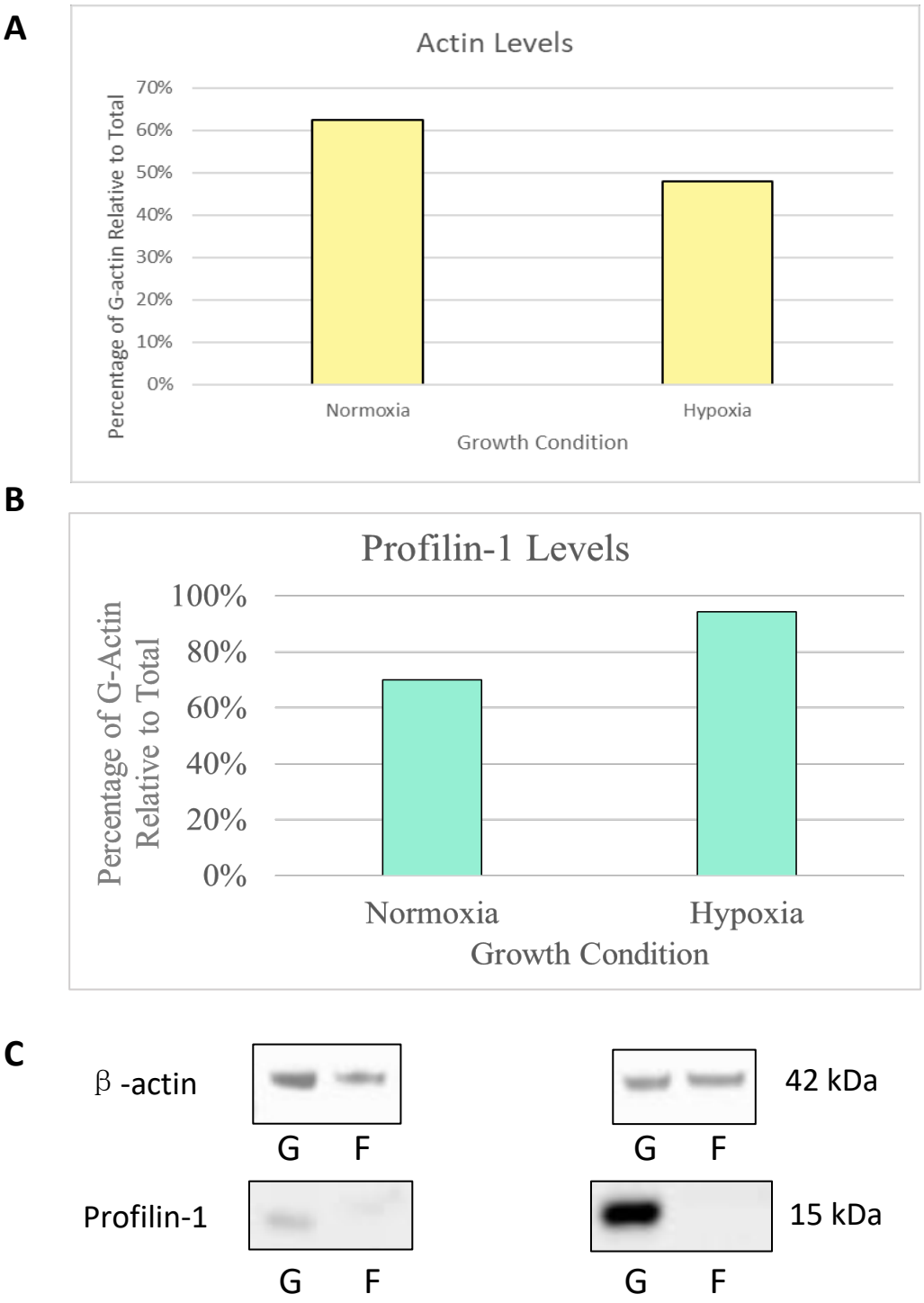


Figure 3.4 Profilin-1 association with G-actin. **A** G- and F-actin relative to total actin in HPASMC exposed to normoxia and hypoxia (n=1). **B** Profilin-1 associated with G- and F-actin in HPASMC

exposed to normoxia and hypoxia (n=1). **C** Western blot. A higher proportion of profilin-1 was associated with G-actin in chronic hypoxia hPASMC (n=1).

Inhibition of actin polymerization attenuated hPASMC proliferation

HPASMC were cultured with either latrunculin B or cytochalasin D for two hours, to investigate whether inhibiting actin polymerization would change hPASMC proliferation in hypoxia. As stated previously, latrunculin B inhibits actin polymerization by sequestering the free actin monomer pool and depolymerizes the existing actin filaments. In hypoxia we observe an increase in HPASMC proliferation (Fig. 3.5). A significant decrease in proliferation in hypoxia was observed with 200nM of Latrunculin B but not for normoxia ($n=5$, $p<0.05$; Figure 3.4). A concentration dependent decrease in cell proliferation was observed in both normoxic and hypoxic hPASMC. Cell death was not significantly different among groups (not shown).

In hPASMCs exposed to hypoxia, there is a significant decrease in live cells compared to normoxic negative control in the 200nM, 400nM, and 500nM latrunculin B treatment groups (Figure 3.4). There was a significantly greater number of cells in hypoxic negative control than normoxic negative control. In the 50nM treatment group, there was a significant difference between hypoxic and normoxic hPASMCs. Normoxic hPASMCs treated with 100nM, 300nM, 400nM, and 500nM latrunculin B had significantly lower live cell counts compared to normoxic negative control. Hypoxic hPASMCS treated with 200nM, 300nM, 400nM, 500nM latrunculin B had significantly lower live cell counts than the hypoxic DMSO group.

In cells treated with cytochalasin D, cell count in hypoxic negative control was significantly higher, by about 20%, than normoxic negative control (Figure 3.6). In hPASMCs exposed to hypoxia, there was a significant decrease in live cells compared to normoxic

negative control in the 0.25µM, 0.5µM treatment groups. In the 0.1µM, 0.25µM, 0.5µM, and 2µM treatment groups of hypoxic hPASMC, there was a significant decrease in cell count compared to hypoxic negative control. Normoxic hPASMCs treated with the 0.1µM, 0.25µM, 0.5µM, and 2µM cytochalasin D had a significantly lower cell count compared to normoxic negative control.

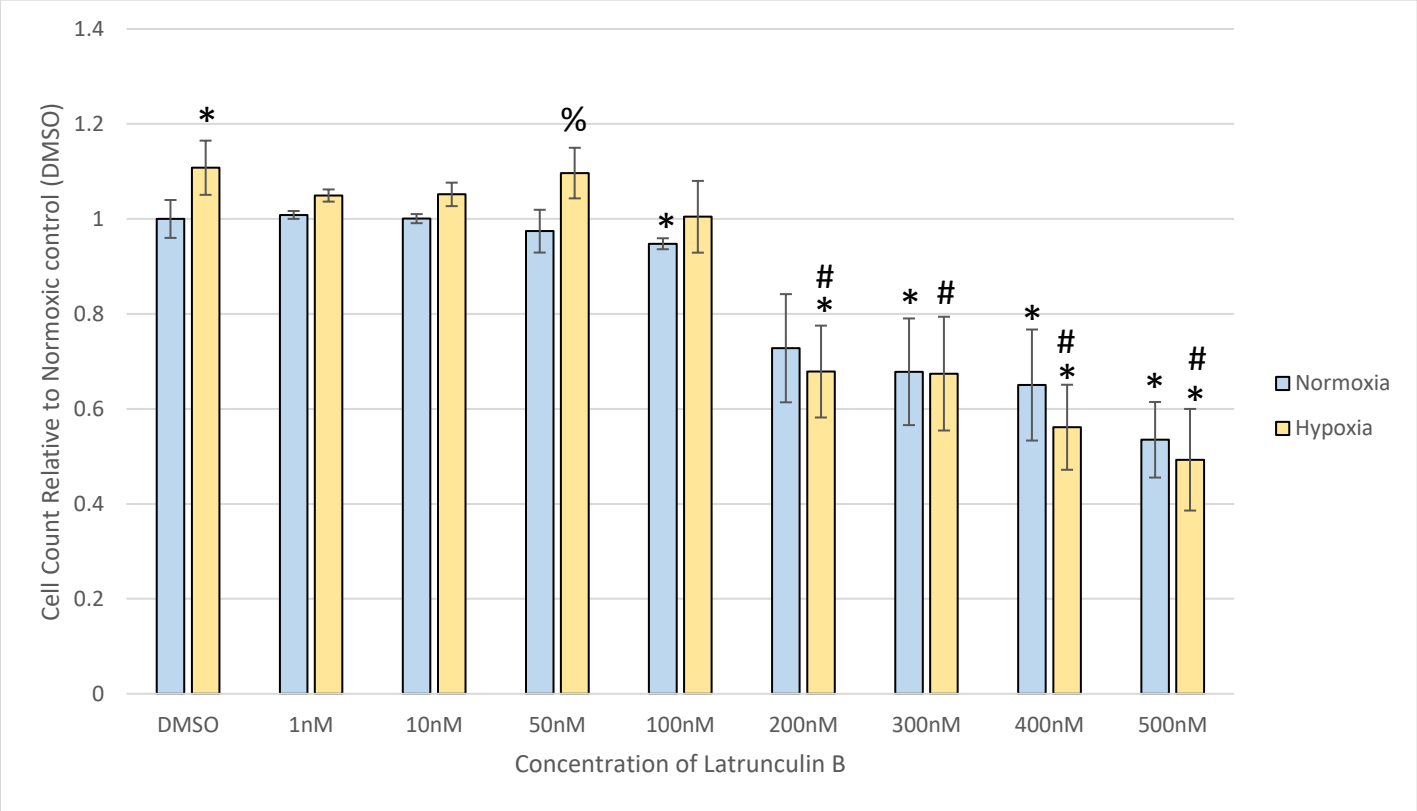


Figure 3.5 Number of live cells measured by a fluorimeter in normoxic and hypoxic hPASMCs after DMSO or differing concentrations of Latrunculin B were added for 72 hours (n=5). *p<0.05 compared to normoxic negative control. %p<0.05 compared to normoxic hPASMC in the same treatment group. #p<0.05 compared to hypoxic negative control. Error bars represent ± SEM.

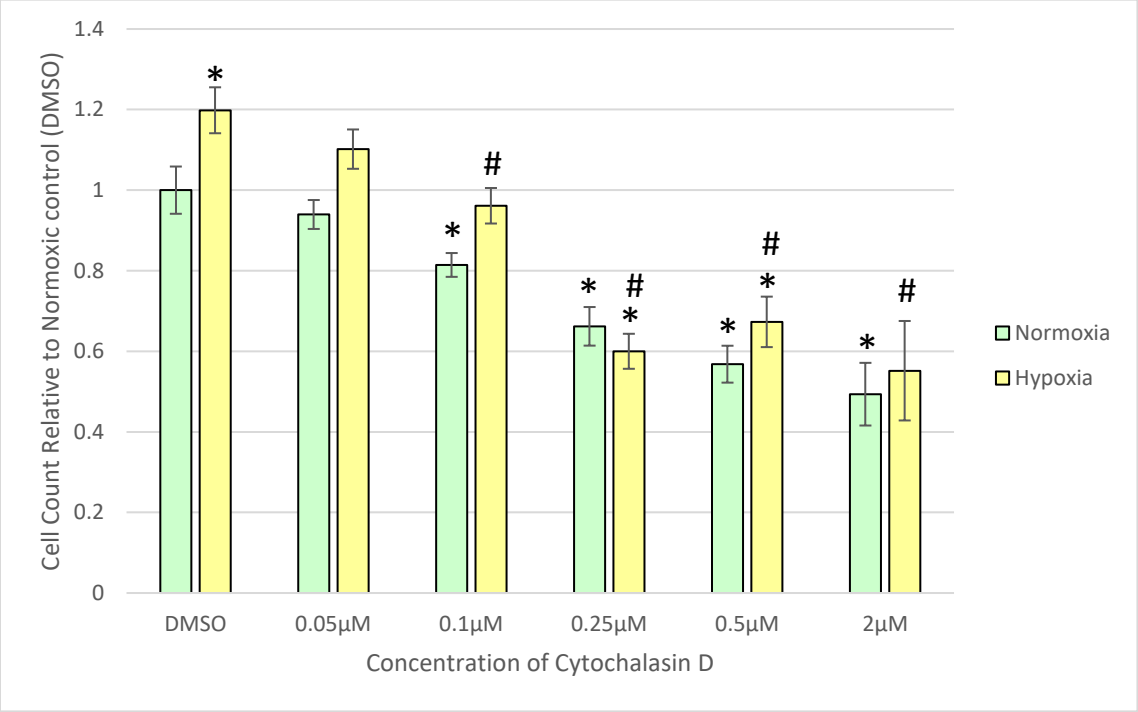


Figure 3.6 Number of live cells measured by a fluorimeter in normoxic and hypoxic hPASMCs treated with DMSO or differing concentrations of cytochalasin D for 72 hours. n=7 *p<0.05 hypoxic negative control compared to normoxic negative control. Hypoxic groups 0.25µM, 0.5µM treatment groups n=3 *p<0.05 compared to normoxic negative control. Hypoxic groups 0.1µM (n=7), 0.25µM (n=3), 0.5µM (n=3), and 2µM (n=3) #p<0.05 compared to hypoxic negative control. Normoxic groups 0.1µM (n=7), 0.25µM (n=3), 0.5µM (n=3), and 2µM (n=3) *p<0.05 compared to normoxic negative control. Error bars represent ± SEM.

Profilin-1 knockdown with siRNA

To study the effects of knocking down profilin-1RNA was extracted from hPASMC treated with 20nM, 30nM, or 40nM of profilin-1 siRNA for 24 and 48 hours (Figure 3.7). Treatment with 30nM and 40nM siProfilin-1 led to a consistent knockdown of Profilin-1.

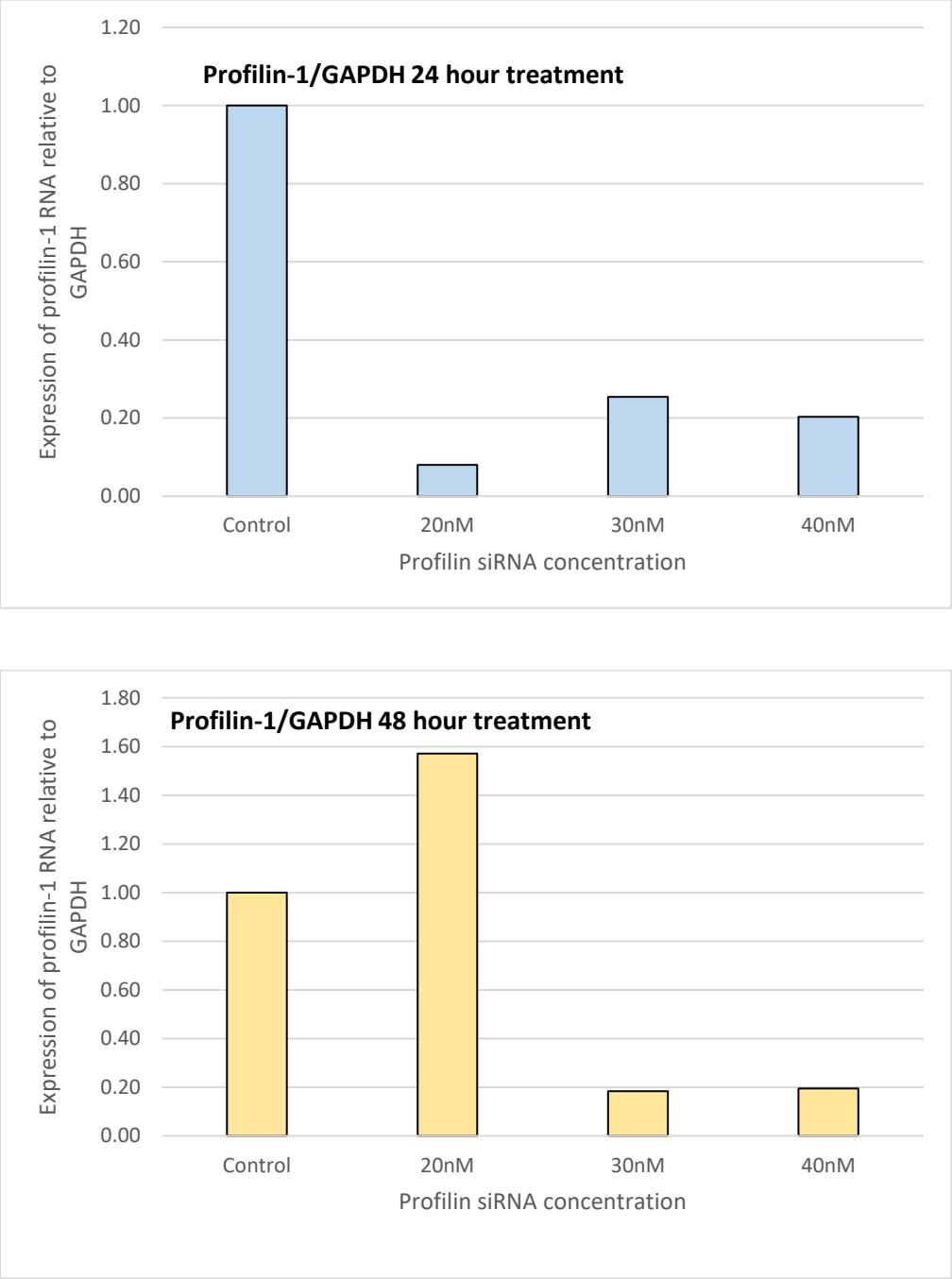


Figure 3.7 Expression of profilin-1 RNA after treatment with either control RNA or siProfilin-1 (n=1).

hPASMC proliferation with profilin-1 knockdown

To investigate the effects of profilin-1 expression on hPASMC proliferation, we inhibited proflin-1 expression with 30nM of siRNA. The cells were grown for 72 hours after siRNA knockdown of profilin-1. We found a significant decrease in live cells (Figure 3.8), about 80%, with no significant difference in cell death (not shown).

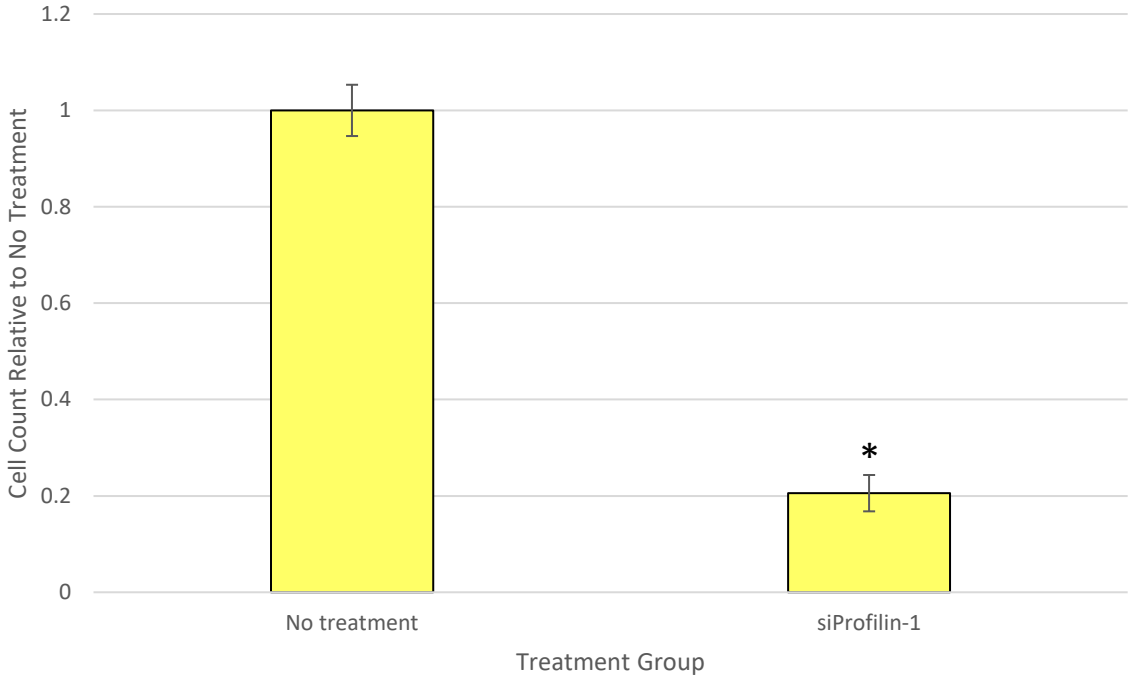


Figure 3.8 Relative cell count after 72-hour incubation with 30nM siProfilin-1 *p<0.05 (n=3). Error bars represent ± SEM.

CHAPTER 4

Discussion

The current study provides supporting evidence for the role of hypoxia-induced changes in ubiquitination and its effect on the binding activity of profilin-1. Profilin-1 had a significant decrease in ubiquitination on two lysine residues, K54 and K126 in lungs from chronic hypoxia mice (Figure 3.1). These ubiquitination sites are conserved in humans. The most extensively researched actin binding sites in profilin-1 are residues 59-61, 69, 71-74, 82 90, 99, 118/119, 121, 122, 124, 125, 128, and 129 [110]. Two of which, F59 and K125, have been studied in the past and are close to the ubiquitination sites of interest. A F59A profilin-1 mutation in bovines leads to decreased affinity for actin by one-fold in magnitude while a K125A mutation led to a 50% reduction in profilin-1's binding to actin [148]. As K125 forms a salt bridge with E364 for actin binding [149], a mutation to a non-polar residue, alanine, could prevent this interaction. The study also provided evidence that these mutations lowered the motility and proliferation of amoebae [148]. A mutant profilin-1 K125N on the other hand, led to a tighter interaction with actin [149]. The ability of profilin-1 to bind to actin is crucial to profilin-1's regulation of many cellular processes. Wittenmayer et al. found that when the AA 60 of profilin-1 was blocked, it inhibited profilin-1's tumor suppressing functions on breast cancer cells [150]. Furthermore, Wu et al. have shown that profilin-1 mutants leading to the formation of ubiquitinated, insoluble aggregates display decreased actin binding activity and reduced F/G-actin ratio [151]. Together, these studies demonstrate a possible role for ubiquitination in profilin-1's ability to bind to actin.

We observed that profilin-1 protein levels do not change in mice or human pulmonary artery smooth muscle cells (hPASMC) subjected to chronic hypoxia exposure when compared to

normoxic controls. This finding suggests that hypoxia-induced changes in profilin-1 ubiquitination may result in a change in profilin-1 activity. Therefore, we investigated whether there is a greater association of profilin-1 with actin in hypoxic hPASMC. In preliminary studies, we found a lower G/F-actin ratio and a greater proportion of profilin-1 in the G-actin fraction in hypoxic hPASMC (Figure 3.2). Although this study needs to be repeated with more samples, this suggests that profilin-1 has greater actin binding activity in hypoxia. Further investigation at different time points is also needed, as actin polymerization is constantly changing.

Another molecule that binds to profilin-1 is phosphatidylinositol (4,5) ‑ bisphosphate (PIP2). PIP2 binds at a region that partially overlaps with the actin binding site [149]. PIP2 is important in regulation of actin polymerization as it connects the catalytic function of profilin-1 with signal transduction [152, 153]. PIP2 causes dissociation of ATP-actin from profilin-1 so that it can then be added to the growing filament. This provides a mechanism by which free ATP-actin could be concentrated and localized [154]. Further, a K125N mutation of profilin-1, although increasing binding to actin, decreases the ability of profilin-1 to bind to PIP2 [149]. As K125 is close to K126, where we observed a change in ubiquitination, this indicates that a lack of ubiquitination could also alter the binding of PIP2 to profilin-1, a pathway that could be investigated further in future studies. A filter assay which retains bound PIP2 and proflin-1 described by Skare and Karlsson [149] can be used to study the association of PIP2 with Profilin-1 and its changes in chronic hypoxia hPASMC. As PIP2's binding to profilin-1 promotes actin polymerization, we would expect a higher association of PIP2 with profilin-1 in chronic hypoxia hPASMC.

Furthermore, actin polymerization has been shown to be important in tension development and shortening or constriction in vascular smooth muscle [155-158]. Increased actin polymerization in mouse aorta triggers the activation of hypertrophic signaling cascades, leading to elevated blood pressure [152]. In addition, Weise-Cross et al. have shown that actin polymerization increases in rat pulmonary arteries after chronic hypoxia exposure and contributes to enhancing vasoconstrictor activity [38]. We show here that actin polymerization is also critical for hypoxia-induced smooth muscle cell proliferation. Blocking the plus or barbed-ends of actin filaments with cytochalasin D and promoting depolymerization or sequestering the free actin monomer pool with latrunculin B decreased cellular proliferation without increasing cell death (Figure 3.4 & 3.5). High doses of latrunculin B decreased hypoxic proliferation more than normoxic. This trend could be due to latrunculin B only sequestering free actin, which doesn't affect those that are already bound to proteins like profilin-1. Inhibiting actin polymerization using cytochalasin D reduced the effect of hypoxia on proliferation to a level similar to normoxic hPASMC. These data also suggest that actin polymerization plays an important role in the aberrant proliferation of PASMC we see in PH.

Knockdown of profilin-1 expression by approximately 80% with siRNA reduced HPASMC proliferation by 80%, demonstrating the importance of profilin-1 in normal cell growth (Figure 3.7). The role of profilin-1 in hypoxia-induced proliferation can be further explored using siRNA knockdown in HPASMC exposed to hypoxia. We expect that siRNA knockdown of Profilin-1 would reduce levels of hypoxic proliferation. The knockdown of profilin-1 in hypoxic hPASMC could also be used to investigate a change in G-/F-actin ratio. We expect that hypoxia-induced

increases in profilin-1's interaction with actin in hPASMC to be reversed by the profilin-1 knockdown. Therefore, we would expect the G-/F-actin to return to normoxic levels.

In conclusion, our study demonstrates a possible mechanism through which profilin-1 effects hPASMC proliferation (Figure 4.1) in hypoxia. A decrease in ubiquitination likely increases profilin-1's binding activity to actin, promotes actin polymerization, and increases hPASMC proliferation. To link ubiquitination with binding activity more directly, immunofluorescence staining would be used to observe the association of G-actin with profilin-1 in chronic hypoxia hPASMC with or without unubiquitinatable profilin-1 mutants. Considering we observed changes in profilin-1 ubiquitination at K54 and K126, unubiquitinatable K54R and K126R profilin-1 single and combined mutants could be used to study actin binding activity and resultant changes in cellular proliferation that occurs when blocking ubiquitination at these sites. As ubiquitination would be inhibited by replacing the lysine residue with an arginine residue in profilin-1, based on our current findings we would expect an increase in actin polymerization in normoxia. With both mutants combined, we would expect greater cellular proliferation as this would leave profilin-1 more open to actin binding. As PIP2 is known to interact with K125, a K126R mutant would possibly lead to more proliferation than K54R.

Other studies examining the effects of overexpressing profilin-1 in hPASMC, studying the effects of mutant proflin-1 *in vivo*, and studying a possible pathway that leads to the change in ubiquitination after chronic hypoxia exposure. One E3 ligase we can examine is C-terminus of Hsc70-interacting protein (CHIP). Depletion of CHIP leads to greater stability of profilin-1 [159] and oxygen-glucose deprivation is known to upregulate CHIP [160]. We can further study

hypoxia-induced changes on CHIP activity and the effects of CHIP downregulation using siRNA on the activity of profilin-1 in hPASMC.

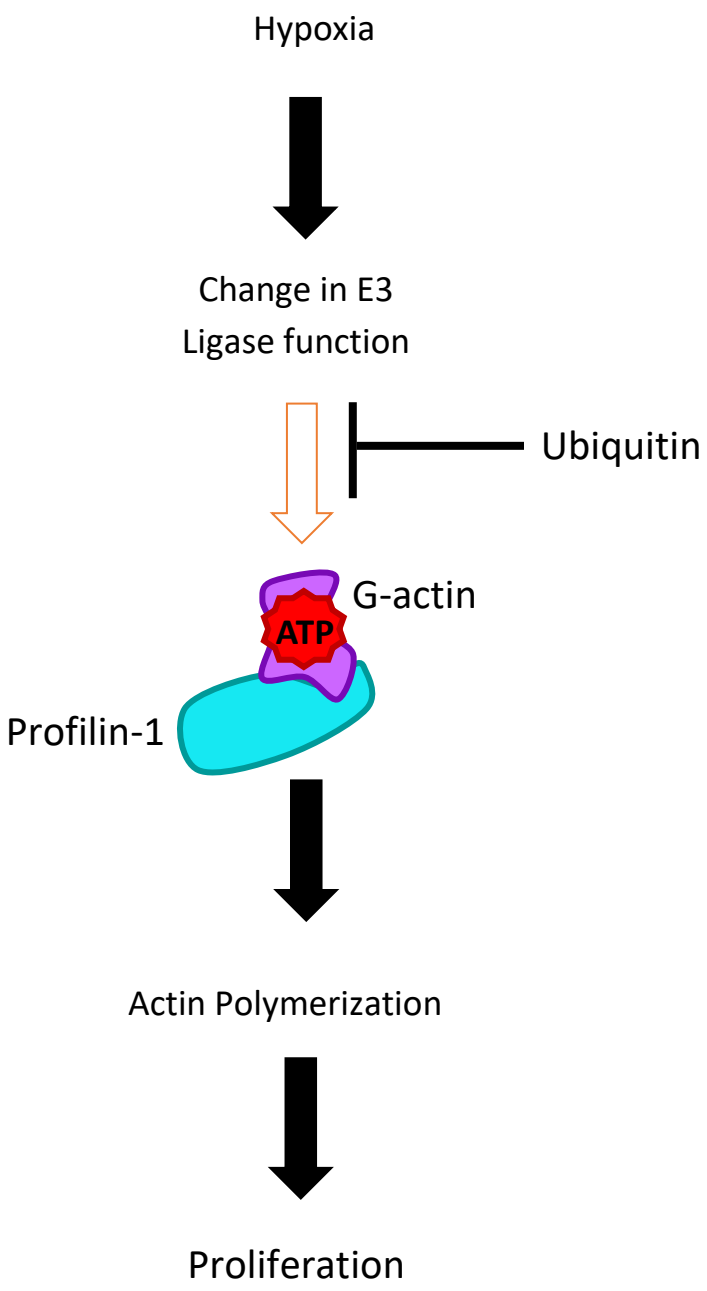


Figure 4.1 A schema of the proposed pathway. Black solid line represents what are known and orange line is what we suspect; t-shape indicates inhibition.

Tables and Figures

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