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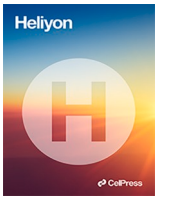

Research article

Protein interactome analysis of ATP1B1 in alveolar epithelial cells using Co-Immunoprecipitation mass spectrometry and parallel reaction monitoring assay

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ABSTRACT

Aims: Alveolar epithelial barrier integrity is essential for lung homeostasis. Na, K-ATPase β1 subunit (ATP1B1) involves alveolar edema fluid clearance and alveolar epithelial barrier stability. However, the underlying molecular mechanism of ATP1B1 in alveolar epithelial cells still needs to be understood.

Main methods: We utilized Co-Immunoprecipitation mass spectrometry proteomic analysis, protein-protein interaction (PPI) analysis, enrichment analysis, and parallel reaction monitoring (PRM) analysis to investigate proteins interacting with ATP1B1 in A549 cells.

Key findings: A total of 159 proteins were identified as significant proteins interacting with ATP1B1 in A549 cells. Ribosomal and heat shock proteins were major constituents of the two main functional modules based on the PPI network. Enrichment analysis showed that significant proteins were involved in protein translation, posttranslational processing, and function regulation. Moreover, 10 proteins of interest were verified by PRM, and fold changes in 6 proteins were consistent with proteomics results. Finally, HSP90AB1, EIF4A1, TUBB4B, HSPA8, STAT1, and PLEC were considered candidates for binding to ATP1B1 to function in alveolar epithelial cells.

Significance: Our study provides new insights into the role of ATP1B1 in alveolar epithelial cells and indicates that six proteins, in particular HSP90AB1, may be key proteins interacting with and regulating ATP1B1, which might be potential targets for the treatment of acute respiratory distress syndrome.

1. Introduction

Alveolar epithelial barrier integrity plays a crucial role in maintaining homeostasis in the lung. Disruption of the alveolar epithelial barrier could cause increased epithelial permeability and impaired gas exchange and ultimately develop into acute respiratory distress syndrome (ARDS). ARDS is an acute respiratory illness characterized by hypoxemia and bilateral non-cardiogenic pulmonary edema, with hospital mortality greater than 30 % [1,2]. In the setting of ARDS, the capacity to remove alveolar edema fluid is reduced, which is associated with poor prognosis [3]. As a key structure of the lung epithelium, alveolar type II cells are pivotal in surfactant production and secretion, alveolar surface liquid regulation, innate immune response, and alveolar epithelial regeneration. Damage to

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alveolar type II cells could not only exacerbate the progression of ARDS but also interfere with alveolar epithelial repair and regeneration and eventually lead to lung fibrosis [4–6]. Thus, exploring the molecular mechanisms in alveolar epithelial cells, especially alveolar type II cells, is of great importance to improving the outcomes of ARDS patients.

Na, K-ATPase, also known as sodium pump, is localized to the basolateral plasma membrane of alveolar epithelial cells and transports sodium and potassium ions in an ATP-dependent manner to maintain lung fluid balance [7]. Na, K-ATPase consists of α subunit, β subunit, and FXYD subunit [8]. As part of the functional core of Na, K-ATPase, β subunit is required for the structure, function, and localization of Na, K-ATPase, and is involved in cell adhesion [8–10]. There are three isoforms of the β subunit. Among them, the β1 subunit is highly expressed in alveolar epithelial cells, and the combination of the α1 subunit and β1 subunit is thought to be the predominant isozyme [8,9]. Several studies have demonstrated the vital role of the Na, K-ATPase β1 subunit (ATP1B1) in alveolar fluid clearance (AFC) of non-cardiogenic [9,11–13] or cardiogenic [14] pulmonary edema by enhancing alveolar ion and fluid transport. Moreover, a current study provides evidence that ATP1B1 could increase alveolar epithelial barrier integrity by an ion transport-independent mechanism [15]. The underlying molecular mechanism of ATP1B1 in alveolar epithelial cells deserves further investigation.

Based on Immunoprecipitation mass spectrometry (IP-MS) and Co-IP assays, myotonic dystrophy-related Cdc42-binding kinase alpha (MRCKα) is proven to specifically bind to ATP1B1 in alveolar type I cells and is required for the regulation of alveolar barrier function [15]. However, a comprehensive interactome analysis of ATP1B1 in alveolar epithelial cells, especially alveolar type II cells, is still limited. Co-IP-MS technology is considered a powerful screening tool for the composition of protein complexes and has been widely used in studies of targeted protein interactions [16,17]. In our previous study, Co-IP-MS was conducted to investigate significant proteins interacting with Na, K-ATPase α1 in LPS-A549 cells and control-A549 cells [18]. Parallel reaction monitoring (PRM) is a novel targeted quantification method with high resolution and mass accuracy [19]. Recently, the PRM-based targeted method has been successfully applied to validate quantification results from proteomics studies [20], which provides a feasible strategy for us to validate the interactomics results of ATP1B1.

In the present study, the human type II alveolar epithelial A549 cell line was used to explore the molecular mechanism of ATP1B1 in alveolar epithelial cells. Our study identified proteins that might interact with ATP1B1 in alveolar epithelial cells using diverse ways, including Co-IP-MS proteomics, protein-protein interaction (PPI) analysis, enrichment analysis, and PRM validation. Finally, we demonstrated six proteins as potential candidates interacting with ATP1B1, providing a clue to the molecular mechanism of ATP1B1 in regulating lung fluid balance and maintaining epithelial cell stability.

2. Materials and methods

2.1. Cell culture

A549 human lung adenocarcinoma cells (CL-0016; Pricella, China) were cultured in DMEM (Gibco, USA) with 10 % fetal bovine serum (Gibco, USA) and 1 % penicillin/streptomycin (Cytiva, USA) and grown at 37 °C with 5 % CO2.

2.2. Co-IP

Two biological replicate samples were performed Co-IP. 50 μL anti-IgG Dynabeads (Thermo Scientific, USA) were used for each sample. Wash beads with 500 μL PBSN (PBS supplemented with 1 % Nonidet P 40 (Sangon Biotech, China)) and shake gently three times. Dynabeads were resuspended at 4 °C for 1 h in a mixture of PBSN and antibodies against ATP1B1 or IgG. Then, Dynabeads were washed three times with PBSN, and the supernatant was discarded. The sample lysate and Dynabeads mixture was shaken at 4 °C for 2 h. Subsequently, Dynabeads were washed three times with PBSN/PBS mixed with a Protease inhibitor cocktail (Kangchen Bio-tech, China) successively, and the supernatant was discarded. 50 μL of 1 % Trifluoroacetic Acid (Sigma-Aldrich, USA) was added to Dynabeads to elute binding proteins, and the mixture was incubated with high-speed shaking at 37 °C for 10 min. The supernatant was transferred to a new tube. Then, the elution step was repeated, and the elutions were pooled and adjusted to a neutral pH with 10 % ammonium hydroxide (Sigma-Aldrich, USA). Finally, 100 μL of Ammonium bicarbonate buffer (Sigma-Aldrich, USA) was added for trypsin digestion.

2.3. Tryptic digestion and peptide desalting

5 mM tris (2-carboxyethyl) phosphine (Sigma-Aldrich, USA) was added to each sample and incubated and mixed at 55 °C for 10 min. After the samples were cooled to room temperature, 10 mM iodoacetamide (Sigma-Aldrich, USA) was added and incubated in the dark for 15 min. Then, 1 μL 0.5 μg/μL trypsin (Promega, USA) solution was added to each sample, mixed and incubated at 37 °C by Thermo mixer overnight, and finally carried out quench reaction with Trifluoroacetic Acid. The peptide solution was desalted using the C18 tip column (Sigma-Aldrich, USA) and resuspended in 10 L 0.1 % Formic acid (FA; Sigma-Aldrich, USA).

2.4. LC-MS analysis

LC-MS analysis was performed using a nano-UPLC (EASY-nLC1200) coupled to Q-Exactive mass spectrometry (Thermo Finnigan). Samples were separated by a reversed-phase column (100 μm, ID × 15 cm, Reprosil-Pur 120C18-AQ, 1.9 μm, Dr. Math). The flow rate was set to 300 nL/min, and the gradient was 120 min (8-30 % for 92 min, 30-40 % for 20 min, 40-100 % for 2 min, 100 % for 2 min,

100-2 % for 2 min and 2 % for 2 min; mobile phase A = 0.1 % FA, 2 % ACN in water and phase B = 80 % ACN, 0.1 % FA in water).

The data-dependent acquisition was performed in profile and positive mode with Orbitrap analyzer at a resolution of 70,000 at 200 m/z (fwhm) with *m/z* range of 350–1600 for MS1 and a resolution of 17,500 at 200 m/z (fwhm) for MS2. The automatic gain control (AGC) target was set to 1.0 E+6 with a max IT 100 ms for MS1 and 5.0 E+4 with a max IT 200 ms for MS2. The top 10 most intense ions were fragmented by higher energy collisional dissociation (HCD) with normalized collision energy (NCE) of 27 % with an isolation window of 2 m/z. The dynamic exclusion time window was 20 s.

2.5. MaxQuant database

MaxQuant is a proteomic software widely used for analyzing large-scale tandem MS data [21]. Raw MS files were processed with MaxQuant (Version 1.5.6.0). The protein sequence database (Uniprot_organism_2016_09) was downloaded from UNIPROT [22]. Subsequently, UNIPROT and its reverse decoy were searched against by MaxQuant software. Both peptide and protein FDR should be less than 0.01. Unique and razor peptides were used for quantification. All the other parameters were reserved as default.

2.6. Bioinformatics analysis

The STRING database was used to predict interactions of proteins interacting with ATP1B1 [23]. The PPI network was downloaded and processed with the MCODE plug-in in Cytoscape software to analyze functional modules [24]. GO and KEGG databases were used to analyze the functions and signaling pathways of proteins interacting with ATP1B1. The Hypergeometric test was used to identify enriched GO terms and KEGG pathways with $P < 0.05$ as statistically significant.

2.7. PRM validation

The LC-MS/MS setup was as described in the previous subsection. The scheduled PRM was run at a resolution of 17,500 at 200 m/z (fwhm), with an AGC target of 5.0 E+4, a max IT of 200 ms, an NCE of 27 %, and an isolation window of 2.0 m/z.

Skyline is a freely available, open-source application for analyzing the data collected for chromatography-based quantitative MS experiments [25]. The acquired LC-MS/MS data were imported into Skyline for transition extraction. The precursor charges were set as 2 and 3, and the daughter ion charges were set as 1 and 2. The b and y ions of precursor-last were selected for chromatographic peak extraction and quantification. The final quantified transition was then manually screened based on the peak pattern, intensity, and signal-to-noise ratio of the extracted ion chromatogram. The method match tolerance was 0.005 m/z. We applied GraphPad Prism 8 to compare the fold change of ten proteins in proteomics and PRM analysis.

2.8. Western blot

The protein samples were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, followed by blocking using TBST (a mixture of Tris-buffered saline (TBS) and 0.05 % Tween-20) containing 5 % fat-free dry milk. Then, these membranes were incubated in rabbit primary antibodies (HSP90AB1, 1:1000, Abcam) or mouse primary antibodies (ATP1B1, 1:1000, Santa) overnight at 4 °C. After washing three times in TBST, they were incubated in anti-rabbit IgG-HRP (1:1000, Beyotime) or anti-mouse IgG-HRP (1:1000, Beyotime) for 2 h at 37 °C. The protein bands were detected using NcmECL Ultra (NCM Biotech, China) and captured by Tanon-5200 Chemiluminescent Imaging System (Tanon, China).

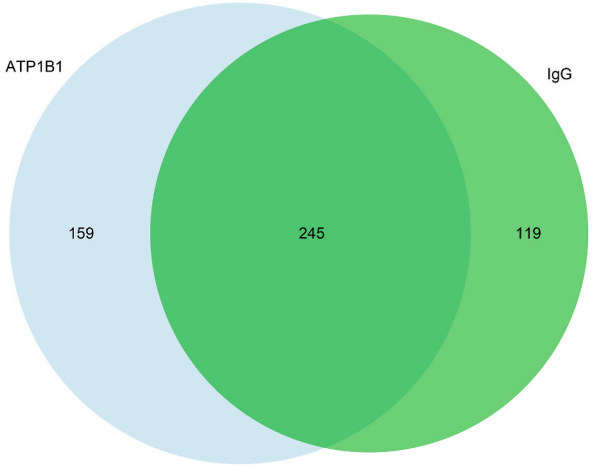

Fig. 1. Venn diagram of the different interacting proteins between ATP1B1 and IgG protein group. ATP1B1, Na, K-ATPase β1 subunit.

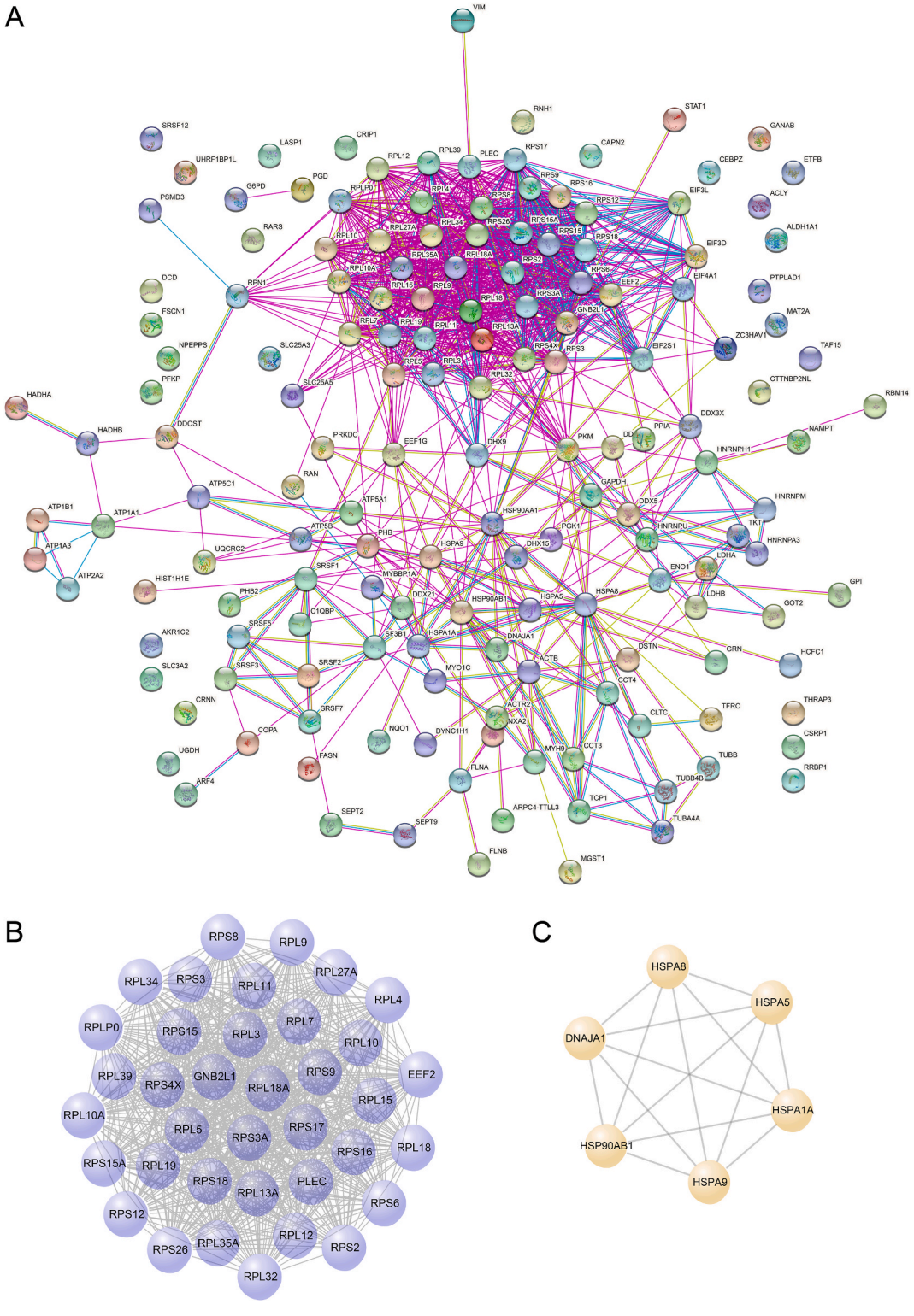

Fig. 2. Protein-protein interaction networks of significant proteins interacting with ATP1B1. (A) Protein-protein interaction network of 159 significant proteins. (B)(C) Two functional modules of 159 significant proteins. ATP1B1, Na, K-ATPase β1 subunit.

3. Results

3.1. Proteomics of significant proteins interacting with ATP1B1 in A549 cells

A Co-IP-MS proteomic analysis was applied to analyze the interacting proteins of ATP1B1 in alveolar epithelial cells. Total proteins were extracted from A549 cells, and proteomics was carried out using an antibody against ATP1B1 or IgG. We identified 2876 peptides and 593 proteins, and 523 proteins were retained after filtering. A Venn diagram was drawn to show the differences between the

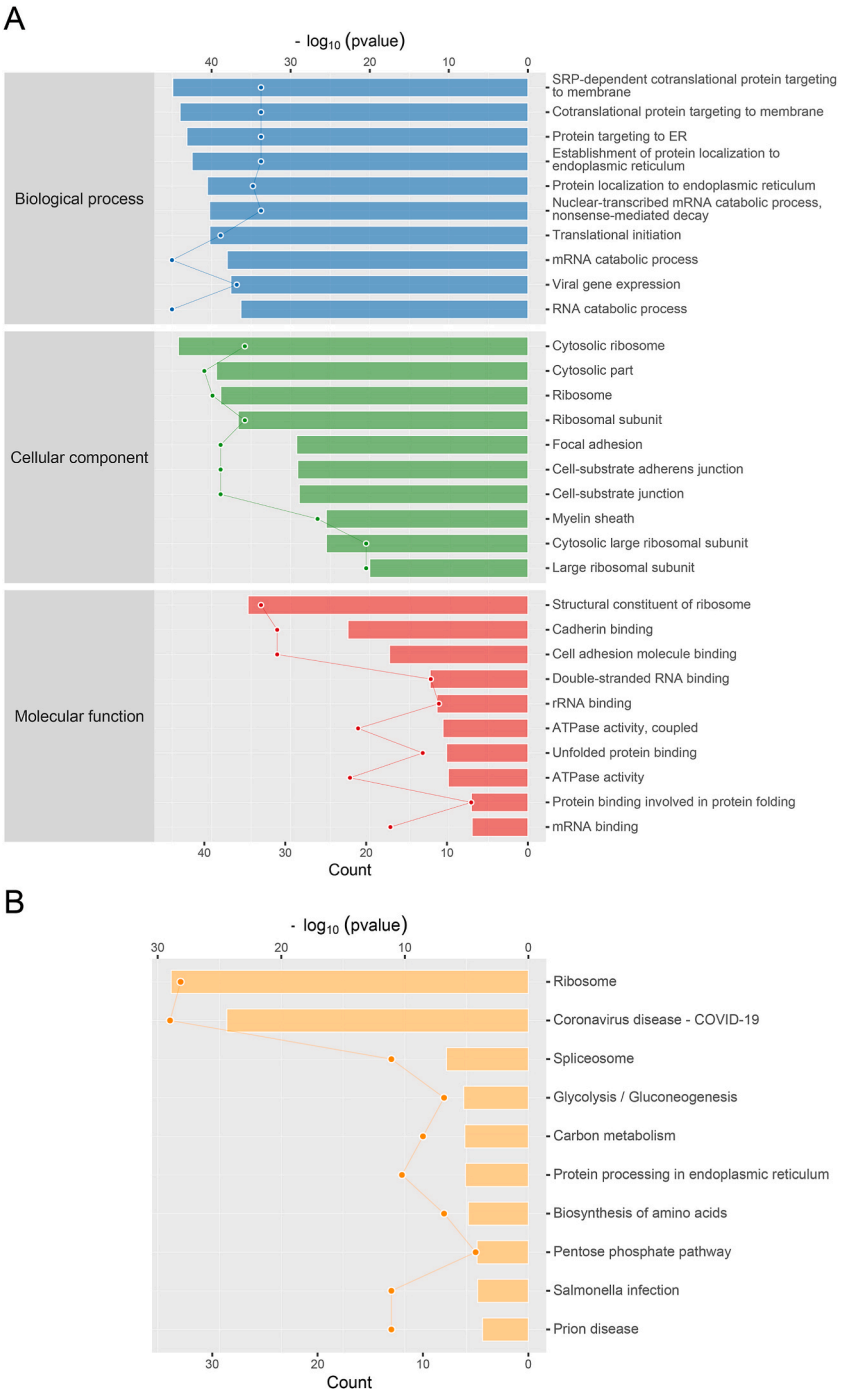

Fig. 3. GO analysis (A) and KEGG pathway enrichment analysis (B) of significant proteins interacting with ATP1B1. ATP1B1, Na, K-ATPase β1 subunit; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

ATP1B1 group and the IgG group (Fig. 1). A total of 159 proteins were considered significant proteins binding to ATP1B1 (fold change >1.5 and unique peptide ≥2).

3.2. PPI analysis of the 159 proteins interacting with ATP1B1

To explore interaction relationships between proteins binding to ATP1B1, we used the STRING database to construct a PPI network of 159 proteins (Fig. 2A). The PPI network consisted of 130 nodes and 1047 edges. We then used the MCODE plug-in in Cytoscape to analyze functional modules. The result showed two modules with an established score greater than 5, with scores of 37 and 6, respectively (Fig. 2B and C). Of these two modules, the highest scoring module, consisting of 37 nodes and 666 edges, was mainly composed of ribosomal proteins, including both small (RPS) and large (RPL) subunits. The other module comprised 6 nodes and 15 edges, including heat shock protein family A (HSP70) member 1 A (HSPA1A), member 5 (HSPA5), member 8 (HSPA8), member 9 (HSPA9), heat shock protein 90 alpha family class B member 1 (HSP90AB1), and DnaJ heat shock protein family (HSP40) member A1 (DNAJA1).

3.3. Enrichment analysis of the proteins interacting with ATP1B1

Functional and signaling pathway enrichment analysis was performed based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to better understand the roles of these proteins in alveolar epithelial cells.

3.3.1. GO analysis

GO analysis revealed that 798, 137, or 232 GO terms were statistically enriched (pvalue <0.05) in biological process (BP), cellular component (CC), or molecular function (MF), respectively. In the BP domain, the enriched terms were related to cotranslational protein targeting to membrane, protein targeting to endoplasmic reticulum, and protein localization to endoplasmic reticulum. In the CC domain, the enriched terms were concerned with ribosome, cell-substrate junction, and myelin sheath. In the MF domain, the enriched terms were involved in structural constituent of ribosome, cadherin (cell adhesion molecule) binding, double-stranded RNA binding, rRNA binding, ATPase activity, unfolded protein binding, protein binding involved in protein folding, and mRNA binding. The top 10 GO terms in these three domains are presented in Fig. 3A.

3.3.2. KEGG pathway enrichment analysis

KEGG pathway enrichment analysis showed 10 pathways in which these proteins were likely involved. According to Fig. 3B, the most significantly enriched pathway was ribosome, in which RPS and RPL proteins were enriched. The other nine pathways included coronavirus disease-Corona Virus Disease 2019 (COVID-19), spliceosome, Glycolysis/Gluconeogenesis, carbon metabolism, protein processing in endoplasmic reticulum, biosynthesis of amino acids, pentose phosphate pathway, salmonella infection, and prion disease.

Our results showed that proteins binding to ATP1B1 were mainly involved in protein translation and posttranslational processing and had regulatory effects on protein functions, such as cadherin-mediated cell adhesion and enzyme activity.

3.4. PRM validation for ten proteins of interest

Since we were more concerned with the mechanism of ATP1B1 in alveolar edema fluid clearance and alveolar epithelium barrier stabilization, three enriched terms, including cadherin binding, ATPase activity, and protein folding, were focused. Finally, ten proteins of interest were selected from the relevant protein clusters for further PRM validation, including eukaryotic translation initiation factor 4A1 (EIF4A1), tubulin beta 4 B class IVb (TUBB4B), signal transducer and activator of transcription 1 (STAT1), solute carrier family 3 member 2 (SLC3A2), heat shock protein 90 alpha family class A member 1 (HSP90AA1), HSP90AB1, HSPA8, eukaryotic translation elongation factor 2 (EEF2), myosin heavy chain 9 (MYH9), and plectin (PLEC). The target peptide sequences, retention time

Table 1
The PRM parameters of ten target proteins. PRM, parallel reaction monitoring.

Protein	Peptide sequence	*m/z*	Retention time window [min]	NCE
EIF4A1	ATQALVLAPTR	570.8404	49.56–69.56	27
TUBB4B	ALTVPELTQQMFDAK	846.4373	100.00–120.00	27
STAT1	KLEELEQK	508.7847	9.80–29.80	27
SLC3A2	VAEDEAEAAAAAK	623.3015	25.67–45.67	27
HSP90AA1	DQVANSAFVER	618.3044	37.86–57.86	27
HSP90AB1	EQVANSAFVER	625.3122	35.40–55.40	27
HSPA8	DAGTIAGLNVLR	600.3408	90.64–110.64	27
EEF2	YEWDVAEAR	569.7618	62.53–82.53	27
MYH9	LQQELDDLLVDLDHQR	650.6691	100.00–120.00	27
PLEC	LAAIGEATR	451.2587	23.41–43.41	27

windows, and *m/z* are depicted in Table 1. The qualitative and quantitative results of polypeptides of these ten proteins are shown in Fig. 4A and B.

We observed that HSP90AB1, EIF4A1, TUBB4B, HSPA8, STAT1, and PLEC showed a significant fold change (fold change >1.5),

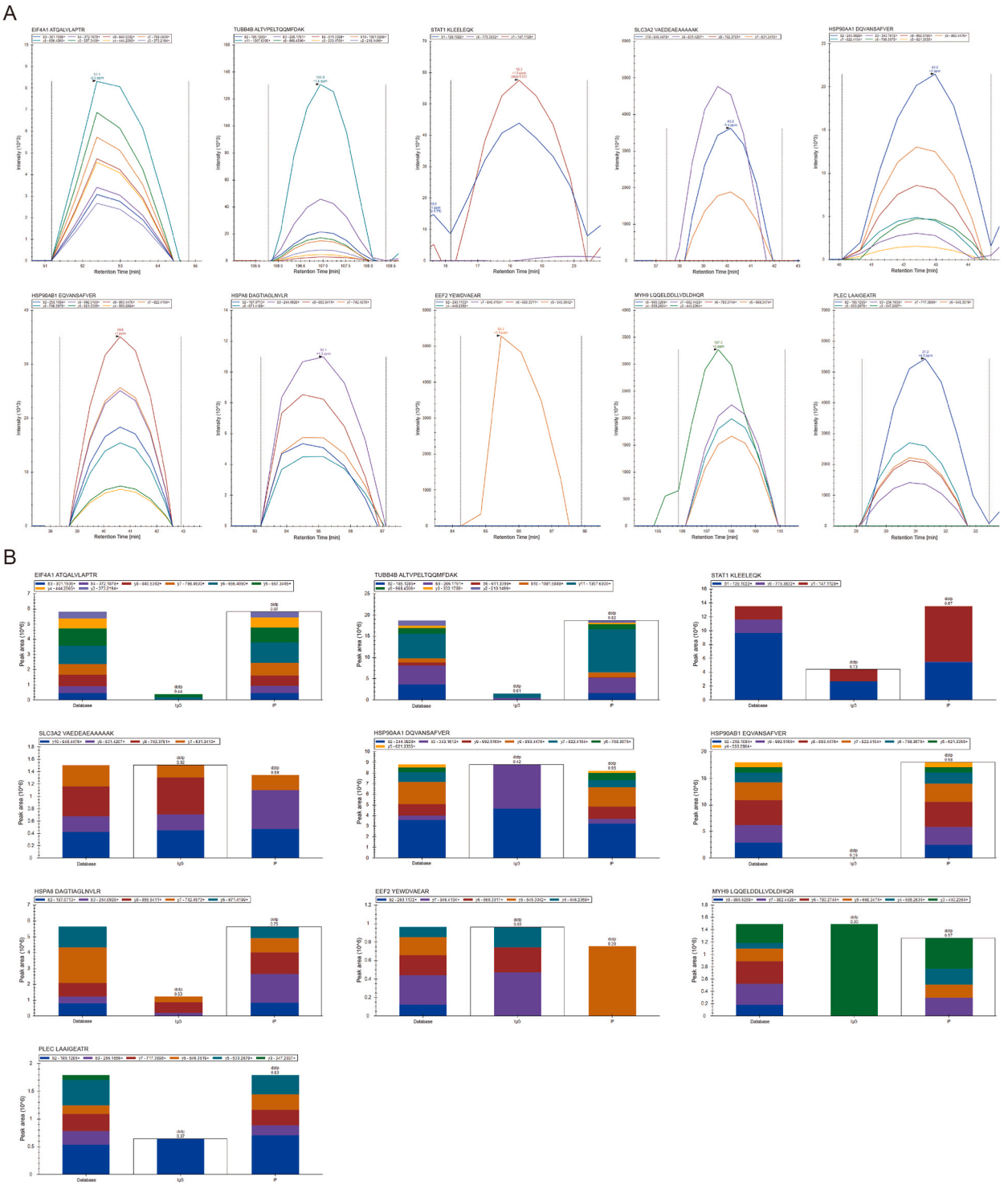

Fig. 4. The qualitative and quantitative results of ten target proteins. (A) The qualitative results of ten proteins. Different colors represent different fragment ions of the same peptide. If these fragment ions have the same co-elution, this polypeptide was identified; if they do not have the same co-elution, this polypeptide was not identified. (B) The quantitative results of ten proteins.

consistent with the proteomic analysis (Fig. 5A). Intriguingly, HSP90AB1 showed the most significant fold change (560.8964-fold) in the PRM analysis, suggesting a highly probable interaction between HSP90AB1 and ATP1B1. To further confirm the interaction between HSP90AB1 and ATP1B1, we performed a Co-IP experiment in A549 cells. As shown in Fig. 5B, the endogenous ATP1B1 protein was co-precipitated by an HSP90AB1-specific antibody, and endogenous HSP90AB1 was co-precipitated by an ATP1B1-specific antibody. Our results demonstrated that six proteins, especially HSP90AB1, were potential candidates to interact with ATP1B1 in alveolar epithelial cells.

4. Discussion

Under physiological conditions, the alveolar-capillary barrier is highly impermeable, primarily due to the alveolar epithelial barrier [26]. Alveolar epithelial barrier injury is the key to alveolar edema and severe gas exchange disorder and may eventually result in ARDS [1]. Several studies have demonstrated the vital role of ATP1B1 in alveolar edema fluid clearance and alveolar epithelial barrier integrity [9,11–15], while studies on the molecular mechanisms targeting ATP1B1 in alveolar epithelial cells, especially alveolar type II cells, are still lacking. In response to this gap, our study was designed to identify interacting proteins of ATP1B1 in A549 cells and explore the molecular mechanisms by which they regulate ATP1B1.

In this study, we analyzed the interacting proteins of ATP1B1 in A549 cells, and 159 proteins were considered significant proteins binding to ATP1B1. A previous study analyzed ATP1B1 interactome in 16HBE14o-cells and identified 138 proteins having potential interactions with ATP1B1 [15]. However, the top 10 interacting proteins of ATP1B1 reported in that study are not included among the significant proteins interacting with ATP1B1 in our study. We hypothesize that the selection of cell lines may be the reason for the different results in the two studies. Given that the cell line of choice in the present study was human type II alveolar epithelial A549 cells, our finding may better reflect the interactions of ATP1B1 in alveolar epithelial cells.

We performed PPI network construction for 159 proteins with significant interactions with ATP1B1. We found that ribosomal proteins and heat shock proteins were components of two major functional modules, respectively. Consistent with prior studies, ATP1B1 is involved in facilitating the translation of the Na, K-ATPase α1 subunit mRNA in the endoplasmic reticulum [27]. Heat shock proteins assist protein folding, assembly, and stability, thereby sustaining protein homeostasis [28]. Disorders of protein folding play a critical role in the development of ARDS and other lung diseases [29]. Previous research has shown that Na, K-ATPase β subunit is indispensable for appropriate folding, functional property acquisition, and correct membrane localization of the α subunit [30]. Moreover, specific proteins participate in the maturation of Na, K-ATPase through interaction with ATP1B1 [31]. However, the exact molecular mechanisms and more proteins regulating the protein folding of Na, K-ATPase through ATP1B1 remain to be determined.

Then, we conducted GO and KEGG pathway enrichment analysis of these significant proteins. In keeping with the result of functional module analysis, the proteins interacting with ATP1B1 were mainly involved in protein translation and posttranslational processing. It was also found that these proteins had regulatory effects on protein functions, such as cadherin-mediated cell adhesion and enzyme activity. E-cadherin, a member of the classical family of cadherins, is integral in cell adhesion and maintaining epithelial barrier integrity [32]. Rajasekaran et al. [33] confirmed that ATP1B1 is required for E-cadherin-mediated cell adhesion. Overexpression of both ATP1B1 and E-cadherin, but not of E-cadherin alone, could assist in the localization of E-cadherin and facilitate the formation of tight junctions of MSV-MDCK cells compared with normal MDCK cells. In addition, Na, K-ATPase has been reported to be

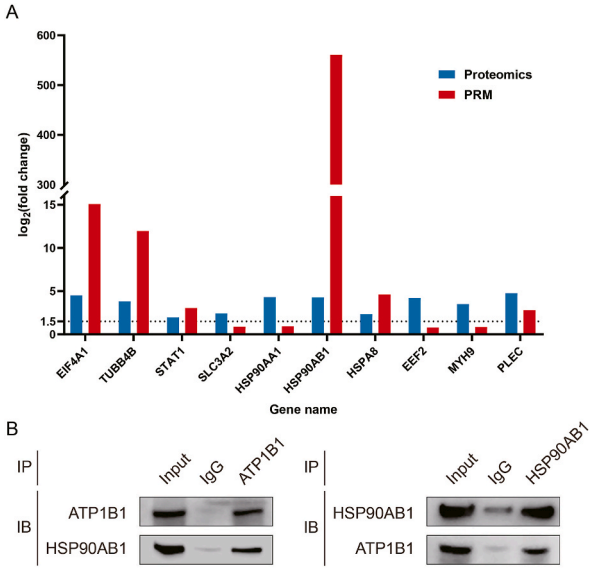

Fig. 5. PRM and Western bolt validation of ten target proteins. (A) The fold change comparison of ten proteins in proteomics and PRM analysis. (B) Endogenous Co-IP result of ATP1B1 and HSP90AB1. PRM, parallel reaction monitoring.

co-localized with E-cadherin [34]. Therefore, we further investigated whether there was a direct interaction between ATP1B1 and E-cadherin. However, proteomic analysis showed that ATP1B1 had no interaction with E-cadherin, which aligns with a previous study [35]. We speculate that specific proteins mediate the interaction of ATP1B1 and E-cadherin by forming a complex, which warrants further investigation.

In discovery-based proteomics, proteins are identified unbiasedly, which also means it is less effective for consistently detecting proteins of interest [20]. Recent studies have shown that PRM assay has emerged as an available method of targeted quantification with high resolution and mass accuracy [19]. Compared with the traditional Western blot validation, PRM has the advantages of being antibody-independent, having high throughput, high specificity, and a low false-positive rate. According to the proteomics results, our study selected ten proteins of interest from protein clusters of MF terms related to cadherin binding, ATPase activity, and protein folding for PRM analysis to verify the protein interactions with ATP1B1. Compared with proteomic and PRM analysis results, six proteins were identified as potential proteins interacting with ATP1B1, including HSP90AB1, EIF4A1, TUBB4B, HSPA8, STAT1, and PLEC. Among them, HSP90AB1 showed the most significant fold change (560.8964-fold) in PRM analysis, strongly pointing to an interaction between ATP1B1 and HSP90AB1. The Co-IP results further confirmed the interacting relationship of HSP90 and ATP1B1. To our knowledge, there are no reports of interactions between ATP1B1 and these six proteins. Thus, we extrapolate their possible roles binding with ATP1B1 based on the functions in which they are involved as well as the published literature.

HSP90AB1, also known as HSP90beta, is a member of the HSP90 family and participates in protein folding, transport, and degradation [36]. HSP90AA1 and HSP90AB1 share 60 % overall homology [36]. However, according to the PRM results, there is limited interaction between HSP90AA1 and ATP1B1, which provides a clue to the interaction site between ATP1B1 and HSP90AB1. The result of GO analysis showed that HSP90AB1 is involved in cadherin binding and protein folding. The loss of E-cadherin is a key characteristic of the epithelial-mesenchymal transition (EMT), contributing to tumor progression and metastasis and involving ARDS development and regression [37–39]. Earlier studies have shown that HSP90AB1 could regulate EMT-related signaling pathways, such as the Wnt/β-catenin pathway [40] and the TGF-β/SMAD pathway [41], by maintaining the stability of interacting proteins. Interestingly, a recent study indicated that inhibition of the interaction of HSP90AB1 with B7-1 could significantly repress B7-1-induced expression of active β-catenin and restore the expression of Zona occludens 1 (ZO-1) and Podocalyxin in Mouse Podocyte Clone-5 cells [42]. Furthermore, they also found that HSP90AB1 mediated the interaction between integrin β1 and B7-1, suggesting that HSP90AB1 might directly participate in integrin activation and actin cytoskeleton reorganization. HSP90 inhibitors have been proven to prevent alveolar endothelial cell hyperpermeability induced by several agents, including LPS, TGFβ1, PMA, thrombin, and VEGF [43]. However, its role in the alveolar epithelial cells has not yet been demonstrated. Whether HSP90AB1 can regulate alveolar epithelial cell junctions directly or indirectly by interacting with ATP1B1 deserves further investigation.

EIF4A1, one of the mammalian isoforms of EIF4A, participates in forming the EIF4F heterotrimeric complex that facilitates translation initiation. Both EIF4A and EIF4F are indispensable for efficient ribosome binding [44,45]. TUBB4B belongs to the family of β-tubulin and is the main component of microtubules. A recent study has shown that TUBB4B and five other proteins are potential biomarkers in sepsis-related ARDS [46]. It was found that recruiting Na, K-ATPase to the plasma membrane could improve AFC in a rat chronic heart failure model [47], which is likely to be mediated by the cytoskeleton. The microtubule-stabilizing agents, such as taxol [48] and colchicine [49], have been reported to attenuate inflammation in acute lung injury. It would be interesting to investigate whether microtubule-stabilizing agents could affect ATP1B1 by modulating TUBB4B, thereby improving AFC and alveolar epithelial barrier integrity. HSPA8 is a molecular chaperone that participates in various cellular processes, such as endocytosis, protein folding, degradation, transport, and autophagy [50]. STAT1 belongs to the STAT family, which has essential effects on apoptosis, inflammation, and tumor inhibition [51,52]. Several studies have demonstrated that the STAT signaling pathway plays an important role in the pathogenesis of ARDS caused by COVID-19 [53]. A prior report indicated that the knockdown of STAT1 could lead to a decrease in the expression of E-cadherin [54], suggesting that STAT1 may regulate alveolar epithelial stability by affecting cell adhesion. PLEC, a member of the Plakin family, plays various roles as a cytoskeletal crosslinker and signaling scaffold [55]. Xu et al. [56] reported that downregulation of plectin inhibited hepatocellular carcinoma cell migration and EMT through ERK1/2 signaling.

Given the proteomics and PRM validation result, we believe HSP90AB1, EIF4A1, TUBB4B, HSPA8, STAT1, and PLEC are potential proteins interacting with ATP1B1 and may be involved in regulating the function of ATP1B1. It should be noted that we have only validated a small subset of the proteins of interest, and the potential interactions and value of other proteins identified by proteomics interacting with ATP1B1 should not be underestimated. Besides, although the A549 cell line is widely used as a model of alveolar epithelial type II cells, it still differs from human alveolar epithelial cells in the physiological state, implying that these proteins interacting with ATP1B1 need to be further validated in primary alveolar type II cells or in vivo. Despite these limitations, considering the critical role of ATP1B1 in the alveolar edema fluid clearance and alveolar epithelial barrier integrity in ARDS, it is significant to reveal interacting proteins and their potential regulatory roles in ATP1B1 in alveolar epithelial cells. We believe our study will help other researchers better understand the molecular mechanism of ATP1B1 in alveolar epithelial cells and provide a theoretical basis for precision medicine for ARDS.

5. Conclusion

Our study is the first to analyze the interactome of ATP1B1 in alveolar epithelial type II cells. We identified 159 proteins that significantly bind to ATP1B1 utilizing a Co-IP-MS approach. These proteins were highly enriched in protein translation, post-translational processing, and function regulation. Ten proteins of interest were selected for further PRM validation. Ultimately, six proteins, including HSP90AB1, EIF4A1, TUBB4B, HSPA8, STAT1, and PLEC, were first reported to be potential candidates involved in the function of ATP1B1 through protein interaction, providing new insights into the molecular mechanism of ATP1B1 in alveolar

epithelial cells and which could be targets for future ARDS drug design.

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Data availability statement

The datasets generated during and/or analyzed during the current study are available in the Mendeley Data repository, https://doi.org/10.17632/d6929dgh3c.1.

CRediT authorship contribution statement

Yu Zheng: Writing – original draft, Validation, Formal analysis. **Weiting Peng:** Writing – original draft, Visualization, Software, Funding acquisition, Formal analysis. **Xupeng Wen:** Writing – review & editing. **Qiquan Wan:** Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32579.

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