markedly and significantly increased in BSMs of the diseased mice (Figure 1F). The real-time qRT-PCR analyses revealed that, in this animal model of asthma, the miR-140-3p expression was significantly reduced in BSMs ($P < 0.05$ by unpaired Student's *t* test; Figure 1G). Although the mechanism of reduction of miR-140-3p is unclear now, our preliminary study also revealed a reduction of its 5p strand, miR-140-5p, which suggests an idea that the transcription of *mir-140* gene might be reduced.

In conclusion, the current findings suggest that RhoA protein expression is negatively regulated by miR-140-3p in BSMs. In BSMs of the repeatedly antigen-challenged mice, a downregulation of miR-140-3p might cause an upregulation of RhoA, presumably resulting in an augmentation of the contraction. The crucial roles of RhoA and its downstream Rho-kinases were also demonstrated in contraction of human BSM (14). The RhoA/Rho-kinase pathway has now been proposed as a new target for the treatment of AHR in asthma (15). Thus, the current study might provide us a new insight into the treatment of the AHR; however, further studies, such as those using different animal models and human specimens, are required to fully validate the role of miR-140-3p in allergic asthma. ■

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TBX4 Transcription Factor Is a Positive Feedback Regulator of Itself and Phospho-SMAD1/5

To the Editor:

TBX4 (T-box transcription factor 4) encodes an early embryonic transcription factor that plays an essential role in pulmonary vascular and airway branching (1, 2). *TBX4* mutations are associated with pulmonary arterial hypertension (PAH); however, the cellular and molecular mechanisms behind this remain unknown (3–6). Decreased BMP (bone morphogenetic protein)–Smad1/5/8 phosphorylation and increased TGFβ (transforming growth factor β)–SMAD2/3 phosphorylation is linked to PAH in humans and animal models, highlighting the

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This letter has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

importance of this pathway (7). Interestingly, BMP–Smad signaling is closely related to TBX function, and several *TBX* family members are direct Smad targets (8–10). On the basis of the close relationship between the TBX family members and the BMP pathway and the critical role of the BMP pathway in PAH pathogenesis, we hypothesized that *TBX4* mutations might affect intracellular BMP signaling.

TBX4 is expressed in pulmonary fibroblast, endothelial, and arterial smooth muscle cells (11). We first determined whether the *TBX4* expression would affect phospho -Smad1/5 (p-Smad1/5) concentrations in cells. We found that overexpression of *TBX4* in fetal lung fibroblast (FLF) and pulmonary artery smooth muscle cells (PASMCs) resulted in increased p-Smad 1/5 concentrations (Figures 1A, 1B and E1A in the data supplement) as determined by Western blot analysis, whereas TBX4 knockout (CRISPR-Cas9–mediated) decreased p-Smad1/5 (Figure 1C). Furthermore, *TBX4* knockdown resulted in partial attenuation of the *ID1*, *ID2*, and *ID3* mRNA, which are direct targets of p-Smad1/5, compared with controls (data not shown).

To determine the functional TBX4 domain for this effect, we focused on the highly conserved DNA binding T-box domain. Seventy-five percent of missense mutations seen in TBX4 occur within the T-box domain (Figure E1B) (1, 12). We found that two-thirds of these mutations were in the conserved amino acids

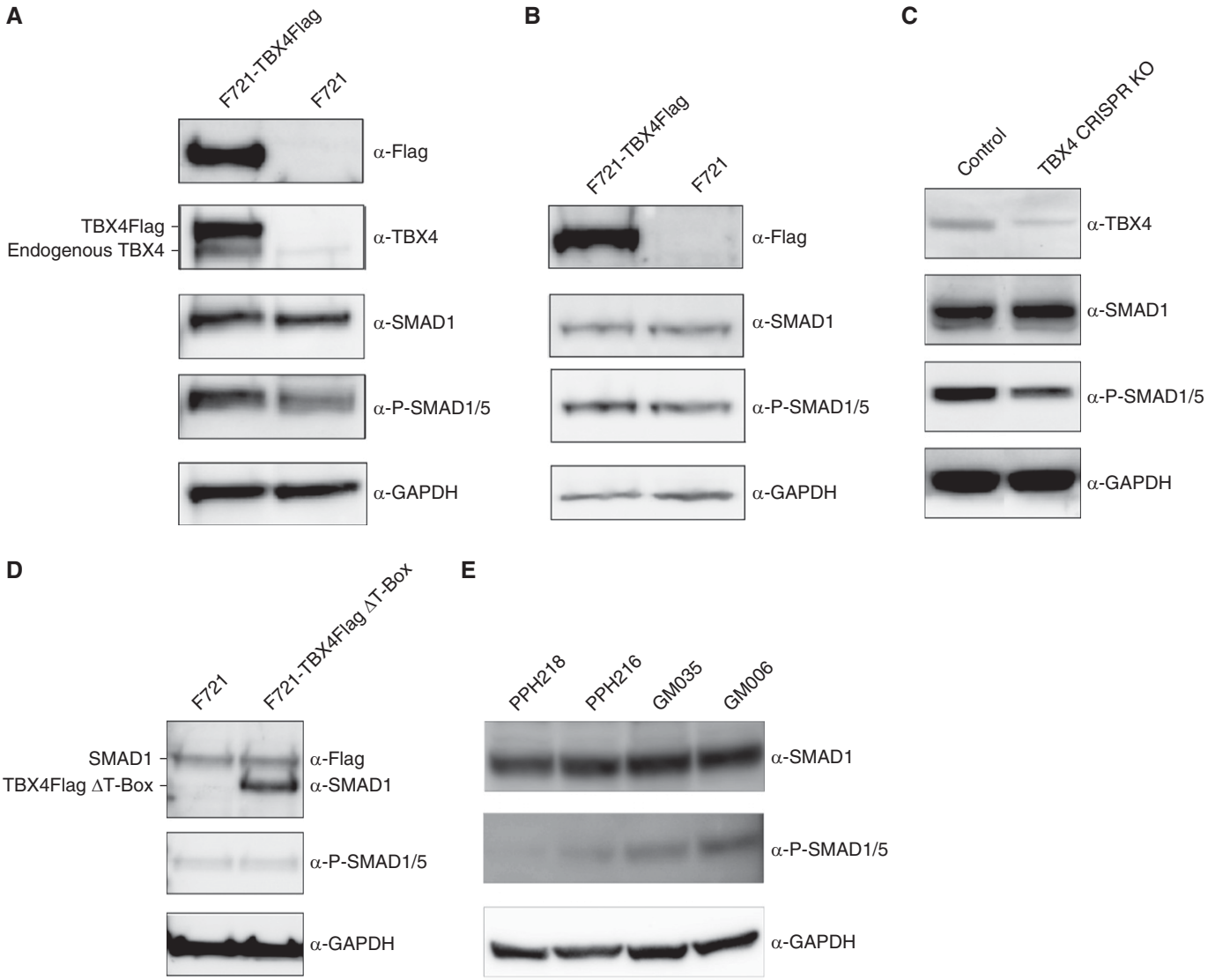

Figure 1. TBX4 (T-Box 4) enhances intracellular phospho-Smad 1/5 concentrations. (*A–D*) Western blot of cell lysate from *TBX4* overexpression in fetal lung fibroblast (FLF) (*A*), pulmonary artery smooth muscle cells (*B*), CRISPR-Cas9–mediated *TBX4*-knockout FLF (*C*), and FLF cells transiently transfected with F721 or F721-*TBX4*Flag ΔT-Box (*D*). (*E*) Lymphoblasts derived from patients with primary pulmonary hypertension (PPH218 and PPH216) and healthy control subjects (GM035 and GM006). PPH216 is a c.538_547 (CCCTTTGGCC) deletion mutation that leads to frameshift; PPH218 is a *TBX4* deletion mutation (chr17:57,972,342–60,472,864). The corresponding antibodies used, anti-Smad1, anti–phospho-Smad1/5, anti-Tbx4, and anti-GAPDH, are shown in the figure. KO = knockout; P = phospho.

as identified by Ruvinsky and colleagues (Figure E1C) (13), and one-third were either adjacent to or just one amino acid away from the conserved amino acids (Figure E1C). We deleted the T-Bbox domain (71aa to 251aa of TBX4) in the expression vector F721-TBX4Flag ΔT-Box. The expression of this vector in FLF showed that TBX4 without T-box failed to increase p-Smad1/5 compared with control (compare Figure 1A to Figure 1D). These results showed that *TBX4* affected intracellular p-Smad1/5 concentrations through its T-box domain.

We analyzed available RNA-sequencing expression data (GSE27661) (14) and found that TBX4 expression is upregulated when pulmonary artery smooth muscle cells PASMCs and human umbilical vein endothelial cells are exposed to BMPs (Figure E2A). To further confirm these data, we treated pulmonary artery smooth muscle cells with BMP4 and found that congruent with the RNA-sequencing data, *TBX4* mRNA concentrations were higher in the cells treated with BMP4 (Figure E2B). These data suggested that *TBX4* may be a target of Smad pathway. We scanned *TBX4* proximal promoter region (−558 to −1) and found a TBX binding motif, a Smad binding element, as well as a cluster of BMP response elements/5GC Smad binding element sites (Figure E3A) (14, 15). To determine whether TBX4 and p-Smad1/5 bind to this region, we performed chromatin IP (ChIP)-qPCR assay. We found that p-Smad1/5 and TBX4 specifically bound the *TBX4* upstream region compared with controls (Figure E3B). These data showed that the *TBX4* upstream region is a direct target of p-Smad1/5 and TBX4, suggesting that *TBX4* is autoregulated by TBX4 and regulated by p-Smad1/5.

We then used a luciferase reporter assay to confirm the ChIP data. We designed an expression vector in which the region −717 bp to −8 bp of *TBX4* proximal promoter (contains all the TBX and Smad1/5 binding motifs) was inserted in front of a luciferase reporter herein referred to as pGL4.23-*TBX4* reporter (Figure E3A). We then coexpressed this vector together with an increasing dose of vector F721-*TBX4* (expressing TBX4) or F721-*TBX4*ΔT-Box in FLF and PASMCs. We found that in both PASMCs and FLF, pGL4.23-*TBX4* reporter luciferase activity increased in a dose-dependent manner with an increasing dose of F721-*TBX4* but not with the control reporter pGL4.23. Coexpression of F721-TBX4ΔT-Box did not increase pGL4.23-*TBX4* luciferase expression (Figures E4A and E4B). These data show that TBX4 can transactivate its transcription, which is consistent with the ChIP data showing that TBX4 bound to *TBX4* promoter (Figure E3B).

We then compared two PAH patient-derived cultured lymphocyte cell lines with two control lines. One cell line, PPH216, carries a frameshift deletion, whereas PPH218 has a complete deletion of *TBX4*. We found that the p-Smad1/5 concentrations were significantly lower in the *TBX4*-mutant cell lines compared with the control cell lines (Figures 1E and E5A).

The data presented in this letter show that overexpression of TBX4 increased intracellular concentrations of p-Smad1/5, and its downregulation decreased p-Smad1/5. Furthermore, BMP stimulation upregulated *TBX4* transcription and increased p-Smad1/5 concentrations. And, TBX4 bound to the *TBX4* upstream proximal promoter region, suggesting a positive feedback loop between TBX4 and p-Smad1/5. Importantly p-Smad1/5 concentrations were significantly lower in the *TBX4*-mutant patient with PAH–derived cell lines (*see* model in Figure E5B).

Given these data, we hypothesize that *TBX4* mutations may lead to a lower baseline intracellular p-SMAD1/5 concentration, resulting in decreased activity of downstream BMP signaling cascades. Diminished BMP signaling may subsequently increase the risk for abnormal lung development, response to injury, or both, which could increase the risk of developing PAH. This hypothesis could provide an initial framework to explore how *TBX4* mutations may cause PAH in future studies. ■

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Mycoplasma pneumoniae–Specific IFN-γ–Producing CD4$^+$ Effector-Memory T Cells Correlate with Pulmonary Disease

To the Editor:

Mycoplasma pneumoniae (*Mp*) is a major cause of community-acquired pneumonia (CAP) in children (1). However, the pathogenesis of *Mp* CAP is not well understood. Lymphocyte responses against *Mp* have been reported to promote either protection or immunopathology in mice (1, 2). In humans, intradermal injection of *Mp* antigen elicited a delayed-type hypersensitivity skin reaction in patients with *Mp* infection (3). The size of the delayed-type hypersensitivity skin induration, which depends mainly on infiltrating CD4$^+$ T helper 1 (Th1) cells, correlated with the severity of pulmonary infiltrates in those patients (3). These observations suggest that the *Mp*-specific T-cell response contributes to *Mp* pulmonary disease.

We showed that the measurement of specific IgM antibody-secreting cells (ASCs) in blood discriminated patients with CAP with *Mp* infection from *Mp* carriers suffering from CAP caused by other pathogens (4). Using this well-diagnosed cohort, we here investigated the *Mp*-specific T-cell response and its contribution to pulmonary disease.

Children with CAP ($n = 35$) and healthy controls (HCs; $n = 16$) aged 3–18 years from a prospective longitudinal study (4, 5) from which peripheral blood mononuclear cells (PBMCs) were available were included in this study. Baseline characteristics of subjects are shown in Table E1 in the data supplement. The study was approved by the ethics committee of Zurich, Switzerland (no. 2016-00148). Detailed methods are shown in the data supplement. CAP disease severity was assessed based on chest radiograph (CXR) findings, hypoxemia (oxygen saturation as measured by pulse oximetry <93%) requiring oxygen supply, and inflammatory parameters (6). CXRs were graded with an adapted CXR severity scoring system (7), with grades 1, 2, and 3 representing increasing severity (Table E2 and Figure E1).

We first developed an *Mp*-specific IFN-γ enzyme-linked immunospot (ELISpot) assay (data supplement (5, 8)) and demonstrated its specificity by comparing patients with *Mp* PCR-positive (*Mp*$^+$) CAP and *Mp*$^+$ HCs (carriers), as well as patients with *Mp* PCR-negative (*Mp*$^-$) CAP and *Mp*$^-$ HCs (Figure 1A). The ELISpot assay detected IFN-γ released by PBMCs after stimulation with *Mp* antigen most frequently and pronounced in patients with *Mp*$^+$ CAP (Figures 1A and 1B). This is in line with IgM ASC ELISpot assay results, which confirmed *Mp* infection in those patients with *Mp*$^+$ CAP (Table E1). However, in contrast to IgM ASCs, which were short lived (5) and mainly present during the symptomatic stage (≤20 days after onset of symptoms), the *Mp*-specific IFN-γ response was significantly longer lasting and also detectable in the convalescent stage (>20 days) ($P = 0.0007$) (Figure 1C).

To identify *the* IFN-γ–producing cells, we depleted CD4$^+$ or CD8$^+$ T cells from PBMCs of a patient with *Mp*$^+$ CAP (Figure E2). Depletion of CD4$^+$ T cells reduced IFN-γ spot-forming units (SFUs) by 96% and 88% upon 24 hours and 48 hours preincubation with *Mp* antigen, respectively (Figure 1D). CD8 depletion did not markedly reduce IFN-γ SFUs. These findings were corroborated by flow cytometry: only IFN-γ–producing CD4$^+$ T cells, and almost no CD8$^+$ T cells, were detected (Figure 1E). Among these IFN-γ–producing CD4$^+$ T cells, a significant proportion coexpressed CD69 and CD40L, identifying antigen-responsive T cells (data not shown). Importantly, the majority of IFN-γ$^+$CD4$^+$ T cells were detected in the effector-memory T cell (T_{EM}) compartment (Figures 1F and E3).

Th1 cells have been reported to contribute to immune-mediated tissue damage in other infectious diseases (9–14). Therefore, we correlated the *Mp*-specific IFN-γ response with disease severity in *Mp*$^+$ CAP (Table E3). The extent of pulmonary disease reflected by increased CXR grading correlated positively with the degree of the specific IFN-γ response in symptomatic ($R = 0.49$, $P = 0.03$) and convalescent stage ($R = 0.62$, $P = 0.006$) (Figures 1G and 1H). Interestingly, in contrast to patients with CXR grade 1, those with CXR grades 2 and 3 showed even an increase in IFN-γ–producing cells over time (Figure 1I). The IFN-γ response was antigen dose dependent and most pronounced for patients with CXR grade 3 (Figures 1J and E4A). However, the IFN-γ response did not correlate with bacterial load in the upper respiratory tract. No relation was observed between CXR grading and bacterial load (Figure E4B) or the *Mp*-specific B-cell response (Figure E5). The acute IFN-γ response was also associated with C-reactive protein levels ($P = 0.009$; Figure 1K) and oxygen need

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