SLIT3 deficiency attenuates pressure overload-induced cardiac fibrosis and remodeling

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In pulmonary hypertension and certain forms of congenital heart disease, ventricular pressure overload manifests at birth and is an obligate hemodynamic abnormality that stimulates myocardial fibrosis which leads to ventricular dysfunction and poor clinical outcomes. Thus, an attractive strategy is to attenuate the myocardial fibrosis to help preserve ventricular function. Here, by analyzing RNA-sequencing databases and comparing the transcript and protein levels of fibrillar collagen in wild-type and global knockout mice, we found that SLIT3 was predominantly present in fibrillar collagen-producing cells and that SLIT3 deficiency attenuated collagen production in the heart and other non-neuronal tissues. We then performed transverse aortic constriction or pulmonary artery banding in wild-type and knockout mice to induce left and right ventricular pressure overload, respectively. We discovered that SLIT3 deficiency abrogates fibrotic and hypertrophic changes and promotes long-term ventricular function and overall survival in both left and right ventricular pressure overload. Furthermore, we found that SLIT3 stimulated fibroblast activity and fibrillar collagen production, which coincided with the transcription and nuclear localization of the mechanotransducer YAP1. These results indicate that SLIT3 is important for regulating fibroblast activity and fibrillar collagen synthesis in an autocrine manner, making it a potential therapeutic target for fibrotic diseases, especially myocardial fibrosis and adverse remodeling induced by persistent afterload elevation.

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Title: SLIT3 Deficiency Attenuates Pressure Overload-induced Cardiac Fibrosis and Remodeling

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Abstract

In pulmonary hypertension and certain forms of congenital heart disease, ventricular pressure overload manifests at birth and is an obligate hemodynamic abnormality that stimulates myocardial fibrosis which leads to ventricular dysfunction and poor clinical outcomes. Thus, an attractive strategy is to attenuate the myocardial fibrosis to help preserve ventricular function. Here, by analyzing RNA-sequencing databases and comparing the transcript and protein levels of fibrillar collagen in wild-type and global knockout mice, we found that SLIT3 was predominantly present in fibrillar collagen-producing cells and that SLIT3 deficiency attenuated collagen production in the heart and other non-neuronal tissues. We then performed transverse aortic constriction or pulmonary artery banding in wild-type and knockout mice to induce left and right ventricular pressure overload, respectively. We discovered that SLIT3 deficiency abrogates fibrotic and hypertrophic changes and promotes long-term ventricular function and overall survival in both left and right ventricular pressure overload. Furthermore, we found that SLIT3 stimulated fibroblast activity and fibrillar collagen production, which coincided with the transcription and nuclear localization of the mechanotransducer YAP1. These results indicate that SLIT3 is important for regulating fibroblast activity and fibrillar collagen synthesis in an autocrine manner, making it a potential therapeutic target for fibrotic diseases, especially myocardial fibrosis and adverse remodeling induced by persistent afterload elevation.
Introduction

Neonatal pulmonary hypertension and certain forms of complex congenital heart disease subject the cardiac ventricle to pressure overload early in life (1-3). While this hemodynamic stress is well tolerated in the short term, chronic elevation in afterload can result in adverse ventricular remodeling characterized by fibrosis and hypertrophy which then contributes to ventricular dysfunction (4, 5). This is especially important in systemic right ventricle congenital heart disease and pediatric primary pulmonary hypertension where patient survival is determined by ventricular function (1, 6, 7). Therefore, in these clinical conditions where chronic pressure overload is obligate and unavoidable, preventing the adverse remodeling response would have an important clinical benefit (8).

Mammalian SLITs (SLIT1-3) are highly-conserved, secreted glycoproteins that were originally described to mediate repulsive axonal guidance during central nervous system development by binding to Roundabout (ROBO) receptors (9-12). Interestingly, SLIT3 is primarily expressed in non-neuronal tissues (13-16). The most striking manifestation of SLIT3 deficiency in mice is central congenital diaphragmatic hernia (CDH) (13, 17) and osteopenia (14, 18), while in humans, genetic variants and low SLIT3 serum levels have been associated with height (19) and osteoporosis (18), respectively.

Fibrillar collagen is the major component of extracellular matrix (ECM) in a variety of load-bearing tissues including the central diaphragm tendon, bone, and heart (20, 21). In the developing heart, SLIT3 is intensively expressed in mesenchymal cushions, which ultimately transforms into dense connective tissue, including the membranous ventricular septum as well as the atrioventricular and semilunar valves (22). Correspondingly, membranous ventricular septum defects, as well as atrioventricular and aortic valves abnormalities, are exhibited in SLIT3 mutant mice (22, 23). Congenital heart disease involving Tetralogy of Fallot, septal and outflow tract defects is associated with SLIT3 variants in humans (24).

Considering the above-mentioned association between SLIT3 deficiency and these collagen-rich tissue defects, we sought to further understand the function of SLIT3 in postnatal non-neuronal tissues, especially in the heart, where excessive fibrillar collagen accumulation leads to myocardial fibrosis and...
maladaptive remodeling. We found that SLIT3 is present at high levels in fibrillar collagen-producing cells, and SLIT3 deficiency reduces $Col1a1$ and $Col3a1$ transcript levels as well as the total collagen content in multiple non-neuronal tissues. More importantly, $Slit3$-targeted mice are markedly protected from pressure overload-induced left and right ventricular fibrosis and remodeling as well as associated mortality. These results identify the axon-guidance molecule SLIT3 as a fibroblast-secreted collagen regulating factor to promote type I collagen expression and as a potential therapeutic target for attenuating fibrillar collagen accumulation and adverse cardiac remodeling induced by pressure overload.
Results

SLIT3 is present at high levels in fibrillar collagen-producing cells. Fibrillar collagen is a sub-group of the collagen family, participates in the formation of striated fibrils, and provides robust mechanical strength (25). Collagen types I and III are the most common fibrillar collagens and are the major components of the myocardial collagen matrix (26). As the biosynthesis of fibrillar collagen is highly regulated at the transcript level (27), and SLIT3 deficiency impacts collagen-rich tissues (13, 14, 17, 18), we first interrogated the cellular transcript levels of SLITs, ROBOs, COL1A1, and COL3A1 in the FANTOM5 (human) (28) and Tabula Muris (Mus musculus) (29) projects. We found that SLIT3 transcripts were present at high levels in human fibroblasts, synoviocytes, tenocytes, adipocytes, mesenchymal cells, and smooth muscle cells (SMCs) (Supplemental Table 1), all of which belong to the family of connective-tissue cells distinguished by their ability to synthesize and deposit type I/III collagen (30). Linear regression analysis between the transcript levels of SLIT3 and COL1A1 exhibited a positive correlation across 512 human cell lines (R² = 0.1764, P < 10^-15) (Figure 1A and Supplemental Figure 1A). Also, in the single-cell RNA sequencing (RNA-seq) study of mouse tissues, Slit3 transcripts were present at high levels in mesenchymal stem cells (MSCs), stromal cells, and fibroblasts, and Slit3, Col1a1, and Col3a1 appeared to be coexpressed in the same cell populations (Figure 1B and Supplemental Figure 1B-C).

In order to confirm the above sequencing results by real-time PCR, we isolated and cultured primary aortic adventitial, cardiac, and pulmonary fibroblasts, as well as aortic vascular smooth muscle cells from adult wild-type (WT) CD-1 mice. Similar to the expression pattern of Col1a1, Slit3 transcript levels were low in LV tissue and negligible in freshly isolated cardiomyocytes, fibroblasts from the heart and other tissues, yet were significant in aortic vascular smooth muscle cells (Figure 1C). Interestingly, the expression pattern of Robo1, which is considered as the receptor for SLIT3-mediated effects on fibrillar collagen formation in bone (14, 18), is different from that of Slit3 by sequencing or real-time PCR. Compared to the high intensity concentrated and overlapping expressions of Slit3 and Col1a1, the expression of Robo1 expands beyond these cell populations (Figure 1A-C and Supplemental Figure 1A-C). In addition, we also confirmed SLIT3 and ROBO1 protein expression in adult primary murine fibroblasts by
immunocytochemistry (Figure 1D and Supplemental Figure 8). These results indicate that fibrillar collagen-producing fibroblasts express SLIT3 and also possess its primary receptor ROBO1 (14, 17, 18, 22, 23, 31), indicating a possible autocrine axis.

*Phenotypic manifestations of Slit3−/− CD1 mice.* Due to the widespread distribution of fibrillar collagen-producing cells and the critical importance of fibrillar collagen in providing structural support for high-pressure structures such as the cardiac ventricles, we sought to investigate the influence of SLIT3 deficiency on the postnatal cardiovascular system. Since previous studies in inbred Slit3−/− (knockout) mice precluded analyses of the postnatal cardiovascular system because of the confounding phenotype of diaphragmatic hernia (13, 14, 17, 18), we utilized knockout mice on a CD-1 (outbred) background (F:M=1:1), which have a lower penetrance of central CDH (approximately 5%, Supplemental Figure 2F) and normal left ventricular (LV) systolic function in the first year of life (Supplemental Table 2). Compared to age-matched WT mice, the body weight, tibia length, and hair follicle density of Slit3−/− mice were all significantly reduced (Supplemental Figure 2A-E). Further, the amounts of fibrillar collagen fibers found in the arterial adventitia and perivascular/interstitial regions of multiple non-neuronal organs (Figure 2A), including that of the heart (Figure 3A, E), were reduced in Slit3−/− mice while myocardial capillary density was preserved (Figure 3A). Interestingly, previous studies in inbred Slit3−/− mice described that the liver was always found in the hernia sac and adhered to its wall without the formation of the falciform ligament (13, 17). However, we observed in a CD-1 knockout mouse that the stomach, rather than the liver, was the only hernia sac content (Supplemental Figure 2F), which suggests that the formation of central CHD is not necessarily related to liver or falciform ligament development.

**SLIT3 deficiency reduces fibrillar collagen production in vivo and impacts LV biomechanical toughness.** Given the histological changes observed in Slit3−/− mice, we next evaluated Col1a1 and Col3a1 transcript levels as well as total collagen protein levels in the aortic adventitia, lung, spleen, kidney, bone, skin, and heart. Col1a1 and Col3a1 transcripts (Figure 2B and Figure 3B), as well as hydroxyproline levels (Figure 2D and Figure 3F) were all significantly reduced in Slit3−/− compared with WT mice. The transcript levels of Slit3 and Col1a1 among individual WT mice exhibited a strong positive and linear correlation in all
analyzed tissues (Figure 2C and Figure 3C). Interestingly, the indexed femur and heart weight (normalized to tibia length), as well as cardiomyocyte size, were also decreased in Slit3−/− mice (Figure 3I and Supplemental Figure 6A).

Because cardiac fibroblasts are responsible for most of the fibrillar collagen production in the heart and are widely distributed in the left and right ventricles that have different embryological origins and physiological characteristics (32, 33). We next determined if there was a ventricular-specific expression pattern for Slit3. Although the ventricles possessed widely varying levels of Slit3 transcripts between animals, we found that Slit3 transcript levels were strongly correlated in the RV and LV in the same individual (R² = 0.98, Figure 3D), indicating a tightly-regulated balance of Slit3 ventricular expression which is also consistent with the common embryological origin of fibroblasts in both ventricles (34-36). As SLIT3 deficiency reduces the myocardial fibrillar collagen content, which plays crucial mechanical roles in the ventricle, we then investigated its effect on LV toughness i.e., the amount of energy absorbed prior to irreversibly fracturing/tearing. Using gradual inflation of the cardioplegia-relaxed LV, the energy density required to rupture the LVs from Slit3−/− mice was significantly reduced compared with WT mice (Figure 3G). Consistently, in 1-year-old mice, SLIT3 deficiency increased mitral E/A ratio, which is a sensitive indicator of LV compliance (Figure 3E-H) (37). To further confirm these findings, we evaluated WT and knockout mice in a left anterior descending coronary artery ligation myocardial infarction (MI) model since myocardial collagen is needed to maintain the integrity of the infarcted myocardium, thereby preventing ventricular rupture (38, 39). We found that the 1-week survival rate was significantly reduced in Slit3−/− compared with WT mice. Among twenty-five WT and twenty-four Slit3−/− mice after surgery, a total of seven Slit3−/− mice died in the first week, six of which suffered LV rupture, characterized by a large amount of blood in the thoracic cavity around the heart, and an area of transmural rupture in the ischemic area of the LV (Figure 3H).

SLIT3 deficiency attenuates LV fibrosis and adverse remodeling. Chronic pressure overload of the LV and RV occurs in acquired and congenital cardiovascular disease and leads to adverse myocardial remodeling characterized by fibrillar collagen accumulation and myocyte hypertrophy. To investigate the
role of SLIT3 in the LV and RV fibrotic response to pressure overload, we subjected WT and Slit3⁻/⁻ body
weight matched mice to transverse aortic constriction (TAC) or pulmonary artery banding (PAB) with
similar initial peak pressure gradients (Figure 4C and 5E, Supplemental Figure 3A, C, 4A and 5A).

Successful TAC in mice induced LV fibrillar collagen accumulation and cardiomyocyte hypertrophy, but
this pressure overload-induced cardiac remodeling was attenuated in Slit3⁻/⁻ mice as compared to WT
mice. Perivascular fibrillar collagen area was decreased in Slit3⁻/⁻ mice at 1, 3 and 8 weeks after TAC
(Figure 4A-B), which corresponded to decreased transcript levels of fibrosis-related genes (Col1a1, Col3a1, Ctgf, Fn1, and Acta2) at 1 and 3 weeks after TAC in these animals (Figure 4D and Supplemental
Figure 4D). Unexpectedly, we also observed that myocardial hypertrophy was blunted in Slit3⁻/⁻ mice after
TAC (Figure 4A and Supplemental Figure 4C), consistent with a reduced heart weight/tibia length ratio,
LV cardiomyocyte cross-sectional area at 1, 3 and 8 weeks after TAC (Figure 4A-B), LV end-diastolic
posterior wall thickness, mass, end-diastolic volume, and end-systolic volume at 3, 7 and 16 weeks
(Supplemental Figure 4E). In agreement with these observations, expression of hypertrophic-related
genes (Nppb, Nppa, and Myh7) was also reduced in Slit3⁻/⁻ mice (Figure 4D and Supplemental Figure 4D).

Further, the LV function was preserved at 7 and 16 weeks in Slit3⁻/⁻ mice after TAC (Figure 4F and
Supplemental Figure 4B). Most importantly, the long-term survival was significantly enhanced in Slit3⁻/⁻
mice where null mice remained viable over a 1-year time course compared with more than 60% mortality
in the control group (Figure 4G).

SLIT3 deficiency attenuates RV fibrosis and adverse remodeling. Next, we subjected animals to PA
banding to determine the importance of SLIT3 in RV pressure overload. The effects of SLIT3 deficiency
were even more apparent in blunting the adverse remodeling response in the context of RV pressure
overload, a hemodynamic condition found in congenital heart disease that can lead to RV failure and has
no effective medical treatment besides heart transplantation (40). The RV free wall fibrosis area,
cardiomyocyte cross-sectional area, end-diastolic diameter and area, RV weight-to-LV plus septum
weight ratio (RV/LV+S), RV/LV end-systolic diameter ratio (Figure 5A-D and Supplemental Figure 5B, D),
and transcript levels of Col1a1, Nppb, Col3a1, Nppa, and Myh7 (Figure 5F and Supplemental Figure 5F)
were all reduced in \textit{Slit3}−/− mice at 2 and 4 weeks, altogether indicating that knockout animals had a decreased adverse fibrotic and hypertrophic response. In addition, after PAB, \textit{Slit3}−/− mice had preserved RV fractional shortening (FS) at 2 weeks, reduced liver congestion at 4 weeks, and markedly increased survival (Figure 5 H-I and Supplemental Figure 5 C, E).

Interestingly, the transcript levels of \textit{Slit3}, as well as that of \textit{Col1a1}, and \textit{Col3a1}, were all increased significantly at 1-2 weeks, and then returned to baseline levels in WT mice after TAC and PAB (Supplemental Figure 3B, D) (41). Similarly, the peak aortic gradients in post-TAC WT mice increased significantly from day 3 to day 21, as described previously and attributed to the development of compensatory LV hypertrophy and the recovery of peak systolic wall stress index (42), and then returned to initial levels after day 42 with the transition from compensated to decompensated heart failure as indicated by the deterioration of LV function (Supplemental Figure 3A). By contrast, in \textit{Slit3}−/− mice, peak-to-baseline ratios of fibrosis-related gene transcript levels were all decreased after TAC and PAB, and changes in peak gradients and LV function were both relatively mild throughout the study period without a time-dependent linear trend after TAC (Supplemental Figure 3C). Although, \textit{Col1a1} and \textit{Col3a1} transcript levels were significantly different between baseline WT and \textit{Slit3}−/− mice, there was a genotypic effect on the transcription of these fibrillar collagen genes in response to pressure overload surgery, and \textit{Slit3}−/− mice had a weaker fibrotic response to TAC and PAB than WT mice (two-way ANOVA, \(P<0.05\), Supplemental Figure 3E-F).

\textit{SLIT3} deficiency inhibits fibroblast activity. Growth factors such as transforming growth factor beta 1 (TGFβ1) and platelet-derived growth factor BB (PDGF-BB) are overexpressed and act as important mediators of fibroblast activation in the pathogenesis of cardiac fibrotic and hypertrophic remodeling (8, 43-45). Therefore, TGFβ1 and PDGF-BB were used to activate primary adult fibroblasts in vitro. Compared with passage-matched WT cells, \textit{Slit3}−/− fibroblasts treated with or without either PDGF-BB or TGFβ1 displayed significantly inhibited proliferation, migration and contraction activities (Figure 6A-C). Two-way ANOVA demonstrated significant interaction between SLIT3 and PDGF-BB in fibroblast proliferation assays, although it accounted for only 4.5\% of all variation. No other significant interactions
were observed in the contraction and migration assays. In the proliferation and contraction assays, PDGF-
BB and TGFβ1 each contributed more than 82% of the total variation, whereas the genotypic effect of
Slit3 presence or absence in the fibroblasts only accounted for 5.9% or 12.3%, respectively (Figure 6A-
B). Overall, these functional results indicated that SLIT3’s stimulation of fibroblast activity appears to be
relatively independent of rTGFβ1 for the concentrations of the factors that we used in our experiments. To
further confirm the effect of SLIT3 on fibroblast biological activity in vivo outside of the heart, a splinted
skin excisional wound model was performed (46), and consistently, we found that dermal wound healing,
which is critically dependent upon fibroblast function and fibrillar collagen production (47), was also
delayed in Slit3−/− mice (Figure 6D).

SLIT3 regulates YAP1 and fibrillar collagen production. Fibrillar collagen contributes to the biomechanical
properties of connective tissue (21), where resident fibroblasts are commonly quiescent (48) and
protected from persistent external loads by dynamically regulated ECM in vivo (49). Resident cardiac
fibroblasts are also quiescent cells with a low proliferation and ECM production under normal
hemodynamic conditions (50), and are the principal source of activated fibroblasts in injured mammalian
hearts (51, 52). We observed that acutely increasing ventricular wall stress with TAC or PAB resulted in
the activation of resident fibroblasts and increase of myocardial production of fibrous collagen and SLIT3
(Figure 4E and Figure 5G) (53). The mechanical microenvironment is known to be an important
determinant of fibroblast activation in vitro and in vivo (54). To further investigate the effect of the
mechanical microenvironment on the role of SLIT3-induced fibroblast activation in vitro, aortic adventitial
fibroblasts (AAFs), cardiac fibroblasts (CFs), and lung fibroblasts (LFs) were cultured on stiff plastic tissue
surfaces or in soft collagen type I gel, as fibroblasts adaptate their internal stiffness to match that of their
substrate (55) and local stiffness acts as an important mechanical effector (56). Importantly, we found
that transcript levels of Acta2, Col1a1, Slit3 and Yap1 were all significantly increased by culturing
fibroblasts on a stiff surface (Figure 7A-B), indicating that SLIT3 transcription may be regulated by the
surrounding mechanical microenvironment.
As the transcription factors YAP1/TAZ have been identified as an important mediator of mechanotransduction signals in fibroblasts and widely regulates fibroblast biological activities, including proliferation, migration and fibrillar collagen production (57-60), the potential association of SLIT3 and YAP1/TAZ were then examined. We found that the transcript levels of Yap1, rather than Taz, and its regulated gene Ctgf in the cardiac ventricles after TAC were reduced significantly in Slit3⁻/⁻ compared with WT mice (Figure 7C and Supplemental Figure 6B) (61). In vitro, both the transcript levels of Yap1 and Ctgf, as well as the nuclear translocation of YAP1, were reduced significantly in Slit3⁻/⁻ as compared to WT fibroblasts (Figure 7D-F). Further, there was a strong linear association between the transcript levels of Slit3 and Yap1 of fibroblasts cultured on both soft and stiff surfaces ($R^2=0.91$, Figure 7G), and recombinant SLIT3 (rSLIT3, aa 34-1116) treatment significantly increased the transcript levels of Yap1 as well as its downstream gene targets Ctgf and Col1a1 in fibroblasts (62) (Figure 7H), suggesting that SLIT3 has a positive regulatory effect on Yap1 transcription. In addition, although TGFβ1 treatment of fibroblasts significantly reduced the transcript levels of Slit3, there was a genotypic effect on Ctgf expression response to TGFβ1; i.e., Slit3⁻/⁻ fibroblasts had a blunted response to TGFβ1 as compared to WT fibroblasts (two-way ANOVA, $P<0.001$, Figure 7J).
Discussion

Since its original description as an axon guidance molecule (63), there has been mounting evidence that SLIT3, a large, secreted glycoprotein (64), has other important extra-neuronal functions (14, 16, 17, 65-68). Here we provide evidence that SLIT3 plays an important role in the function of fibroblasts, which express ROBO1 (Figure 1D), also the principal Roundabout receptor for SLIT3-mediated osteogenesis (14, 18), and, consequently, the content of fibrillar collagen in the heart during conditions of homeostasis as well as stress. Furthermore, our results demonstrate that SLIT3 also regulates fibroblasts and the collagen content in extracardiac tissues, underscoring SLIT3’s importance in collagen-rich tissues and, in turn, their biomechanical properties.

Similar to previous reports, outbred SLIT3 deficiency mice have reduced bone mass (Figure 3I). Interestingly, for this phenotype, Xu et al (14) and Kim et al (18) reported contradictory mechanisms, and mainly focused on the effective sources of SLIT3. However, using multiple lines of evidence, Li et al (69) recently demonstrated that skeletal SLIT3 is mainly secreted by osteoblasts rather than osteoclasts. Osteoblasts are differentiated from mesenchymal stromal cells and has also been regarded as a sophisticated fibroblast (70). It is possible that osteoporosis (14, 18), congenital central diaphragmatic hernia (13, 17), and membranous ventricular septal defects (22, 23) all observed in SLIT3 deficient animals are the organ-specific manifestation of the systemic collagen reduction caused by SLIT3 deficiency, as fibrillar collagen types I is the main and functional component of all of these tissues.

By inhibiting the biosynthesis of fibrillar collagen, targeting SLIT3 may be counterproductive in the setting of osteoporosis (14, 18), bone fracture (14), wound healing (Figure 6D), and post myocardial infarction (Figure 3H); however, it may have beneficial effects in other contexts such as pressure-overload adverse remodeling of the ventricle. After TAC or PAB, ventricular pressure overload increases peak wall stress and induces reactive tissue remodeling that is thought to occur as a compensatory response to normalize wall stress (42, 71). Within three weeks after TAC, the aortic peak gradient was positively correlated with myocardial hypertrophy (Supplemental Figure 3G), and correspondingly, the early-stage remodeling was compensatory in both WT and Slit3-/- mice (Figure 4F) (42). Notably, different degrees of reactive
remodeling induced by similar initial hemodynamic load were observed in WT and Slit3−/− post-TAC mice (71), as blunted fibrillar collagen synthesis together with a stable aortic gradients may have permitted the recovery of LV wall stress in the absence of excessive hypertrophy in Slit3−/− mice. Furthermore, suppressing reactive remodeling by SLIT3 deficiency yielded a long-term cardioprotective effect in the setting of persistent afterload stress in LV, as the response in WT mice was compensatory, but also excessive and ultimately maladaptive (Figure 4F-G) (72, 73).

Unlike the more robust and pressure-resistant LV (74), pressure overload of the RV induced by PAB resulted in stronger reactive remolding, including significant free wall fibrosis which is an important pathophysiological factor correlating with disease severity (75-77) and promotes diastolic dysfunction by reducing myocardial compliance (7, 78, 79). Perhaps preserving RV compliance and diastolic function by reducing collagen accumulation induced by PAB, SLIT3 deficiency attenuated its dilation (Figure 5C-D), prevented the transition from adaptive concentric remodeling to maladaptive hypertrophy and fibrosis associated with decompensated failure (Supplemental Figure 5B-C), and, importantly, exhibited an excellent long-term cardioprotective effect in the setting of persistent afterload (Figure 5H-I) (7, 80, 81). Interestingly, compared with other forms of pulmonary arterial hypertension (PAH), the RV of patients with Eisenmenger’s syndrome exhibit significantly less fibrosis and are better adapted to increased pressure overload (82).

Although several preclinical studies have demonstrated that inhibiting load-induced myocardial hypertrophy might be beneficial in the short term despite persistence of the initiating stimulus (53, 83-87). It is also evident that excessive inhibition of the required compensatory response to normalize increased wall stress is associated with cardiomyocyte damage, depressed systolic function, and increased mortality (42, 71, 88, 89). This did not seem to be the case in SLIT3 deficient animals undergoing TAC as only 3 of the total 13 (23%) animals demonstrated localized fibrosis possibly occurring after cardiomyocyte apoptosis or necrosis (90) at the junctional area of the posterior ventricular septum and free wall and 1 animal (0.08%) suffered from transient cardiac dysfunction that recovered within 15 days while, in general, the cardiac function of Slit3−/− mice was well preserved after TAC (Supplemental Figure 7A-F). These
findings indicate that the inhibitory effect of SLIT3 deficiency on the hypertrophy response induced by pressure overload is moderate and may be secondary to defective fibroblast: cardiomyocyte crosstalk, although a direct effect of SLIT3 on cardiomyocytes is also possible (86, 91-93).

On the premise of normalizing wall stress and preventing myocyte death, our results indicate that minimizing, but not completely abrogating, the reactive remodeling, especially fibrosis induced by pressure overload, is a promising strategy to pursue therapeutic long-term cardioprotective effects in both the LV and RV. Our results indicate that global SLIT3 deficiency appears to yield such a beneficial effect, achieving the “Goldilocks Zone” of tempering hypertrophy and fibrosis but not completely abolishing them. While SLIT3 is mainly expressed in fibroblast and other connective tissue cell lineages, additional work with lineage-specific and inducible knockout animals is needed to confirm that fibroblast mediated SLIT3 is responsible for the phenotype that we observed in our global knockout animals. Nonetheless, from a therapeutics and translational perspective, targeting SLIT3 and its downstream signals via, for example small molecule antagonists, will likely target most cells indiscriminately and thus may reflect the situation in our global knockouts with the favorable phenotype in the setting of ventricular pressure overload.

It has been previously shown that pressure overload by TAC induces an acute increase in ventricular wall stress that is later normalized within 10 days due to remodeling (42), which is nearly contemporaneous with the peak expression of Slit3 (Figure 4E and Figure 5G) and Col1a1 (Supplemental Figure 3B, D) that we observed in our model (41). Interestingly, the signaling activity of TGFβ1, known to play an important role in fibrosis (94, 95), has been demonstrated to start and progressively increase up to at least 9 weeks post TAC (96). These results, taken together with our in vitro findings that the mechanical microenvironment can stimulate Slit3 transcription, suggest that pressure overload may provide a mechanical stimulus for Slit3 transcription. Further supporting a role for SLIT3 in mechanotransduction is our finding that fibroblasts from SLIT3 deficient animals have decreased nuclear and total YAP1 content, as well as the decreased transcription of TEAD target gene Ctgf (Figure 7D-F). In addition, exposing SLIT3-deficient fibroblasts to a ROBO1-binding rSLIT3 N-terminal fragment can acutely stimulate Yap1 and Ctgf transcription (Figure 7H), indicating that SLIT3 deficiency attenuated the effect of mechanical
stimulation on fibroblasts by downregulating the transcript levels of Yap1, an important sensor and mediator of mechanical cues (Graphical Abstract) (59).

The findings of this study are especially relevant to children with congenital heart disease or pulmonary hypertension who are chronically exposed to either left or right ventricular pressure overload and whose outcomes are dependent upon the maintenance of ventricular function. Importantly, children with pressure overload of the RV have no effective medical treatment beyond heart transplantation (1, 6), and thus targeting SLIT3 or fibroblast-specific YAP1 activation (58, 97, 98), rather than cardiomyocyte-specific YAP1 activation (99), may a promising strategy to inhibit adverse remodeling and preserve ventricular function by reducing fibrosis (100). Furthermore, myocardial infarction is rare in children, and hence myocardial rupture after infarction as a potential side effect of SLIT3 inhibition would be unusual in children with ventricular pressure overload. In sum, the results of this study identify SLIT3 as a potential therapeutic target to prevent adverse remodeling and to preserve ventricular function in the setting of chronic ventricular pressure overload.
Methods

FANTOM 5 data processing. Expression levels of the genes of interest in primary cell types with replicates were downloaded from the FANTOM5 database (119 cell types). Analysis was performed as described by Schafer et al (101). Briefly, the gene expression level was obtained by first adding all counts that were assigned to a specific gene and then normalized by the library size to derive the transcripts per million (TPM) for each gene. We determine the TPM for our genes of interest in 512 different primary cell samples that included cell types from all lineages.

Slit3−/− (knockout) mice. Slit3−/− mice on a CD1 background were obtained from Dr. Sean McLean, University of North Carolina. Wild-type CD1 mice (WT) were purchased from Charles River Laboratories (CRL). Similar to the inbred mice (13), for the genotyping analysis of CD1 WT and Slit3−/− mice, three primers were used. The wild-type allele amplified a 250-bp fragment (primers a and b), whereas the null allele amplified a 410-bp fragment (primers a and c). PCR conditions were 35 cycles at 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s. The oligonucleotide sequences used for genotyping were primer a (5′ GCG CCT CCT CGG GCT CCT CGT GTC 3′, sense), primer b (5′ TGC GGG GGA TGC CCC GAG GAA 3′, antisense), and primer c (5′ CGG ATT CTC CGT GGG AAC AAA CGG 3′, antisense).

Mouse models. Mouse cardiac procedures, including transverse aortic constriction (TAC), pulmonary artery banding (PAB), and left anterior descending (LAD) coronary artery ligation, were performed as previously described with the aid of an operating microscope (102). Details of these procedures are provided in the Supplemental Methods.

The mouse excisional wound splinting model was performed as previously described (46). After hair removal, the dorsal skin of the chest was folded and punched with a 5 mm diameter biopsy punch to create two full-thickness excisional wounds besides the midline. Donut-like splints with a 6 mm diameter hole were glued and sutured to the skin around the wound. Digital photographs of individual wounds were taken on days 0 and 15 after surgery.
**Mouse left ventricle toughness.** After anesthesia with inhalational isoflurane, a sternotomy was performed, and the ascending aorta was clamped. The heart was removed, and the LV was perfused with 4°C histidine-tryptophan-ketoglutarate solution to obtain arrest in diastole. A balloon-tipped catheter was inserted into the LV through the mitral valve, fixed by a purse-string suture, and gradually inflated until LV rupture. The heart was then removed and weighed. Passive inflation was accomplished with a Gilson MINIPULS 3 pump using a constant rate. During passive inflation of the LV, pressure was monitored with a pressure transducer (ADInstruments MLT0670), and data was acquired with a PowerLab DAQ device and recorded with LabChart software. Pressure-volume (PV) curves were then generated, and the area under the PV curves after subtracting the PV curve obtained with the balloon only was calculated as the energy of LV rupture, which was then normalized to the heart mass to obtain the energy density.

**Echocardiography.** Trans-thoracic echocardiography was performed by the University of Michigan Cardiovascular Phenotyping Core, as previously described (103) and in the Supplemental Methods.

**Primary murine cell isolation and culture.** Cells were isolated from 6-8 week old CD1 WT and Slit3−/− mice. Cardiac fibroblasts and cardiomyocytes (105), lung fibroblasts (106), as well as aortic adventitial fibroblasts and vascular smooth muscle cells (107) were isolated and cultured as described in the Supplemental Methods.

**Recombinant proteins.** Human TGFβ1 (240-B, R&D Systems), Rat PDGF-BB (520-BB/CF, R&D Systems), Human FGF-basic (03-0002, STEMGENT), Mouse Slit3, (aa 34-1116, 9296-SL-050, R&D Systems) were used at the indicated concentrations.

**Cellular proliferation and functional assays.** The cell proliferation, scratch wound and migration, and floating collagen gel contraction were detailed in the Supplemental Methods.

**Tissue samples preparation and hydroxyproline assay.** Mice were euthanized by cervical dislocation under anesthesia with 2% isoflurane. Next, the chest, abdomen, and right atrium were opened, and the LV was perfused with 20 ml 4°C PBS to flush the blood from the vessels. The heart, left lung, spleen, left kidney, aorta, dorsal skin, and right femur were sequentially harvested and stored at 4°C in HBSS. Then
any attached adjacent tissues were removed from the organs with the aid of an operating microscope. For the hydroxyproline assay, tissues were weighed (80-90 mg were utilized) and placed into pressure-tight 2.0 ml screw-top polypropylene tubes. For real-time qPCR, 5-10 mg tissues were placed into 1.5 ml tubes. All samples were then immediately stored at -80°C. The hydroxyproline assay kit (Sigma, catalog no. MAK008) was used to quantify the total tissue collagen content according to the manufacturer's protocol. Briefly, 6 M HCl was added to the 2.0 ml screw-top polypropylene tubes as 20 μl per 1 mg tissue and these samples were hydrolyzed for 24 hours under a tight lid at 110°C. Next, the tubes were mixed and centrifuged at 10,000 x g for 3 minutes. 10 μl supernatant from the tubes of bone and skin was diluted with 190 μl water in new 1.5 ml tubes. Then 20 μl (heart, kidney, and spleen), 10 μl (lung), and 20 μl diluted (bone and skin) supernatants were transferred to 96-well plates, respectively. The 96-well plates were placed in a 60°C oven to dry samples. Then 100 μl Chloramine T/Oxidation buffer mixture was added to each reaction well and standard well. The plates were incubated at 25°C for 10 minutes. 100 μl Diluted DMAB Reagent was added to each reaction well and standard well and then the plates were incubated in a 60°C oven for 90 minutes. The absorbance of each well was measured at 560 nm with a spectrophotometer.

Real-time qPCR (RT qPCR) gene transcript level analysis. Total RNA was extracted from frozen tissues stored at -80°C or cells cultured in plates using RNeasy Mini Kit (Cat No./ID: 74106, QIAGEN). About 0.5-1 μg of total RNA was used to synthesize the cDNA with the cDNA Synthesis kit (Lot 028755, Quanta bio) and according to the manufacturer’s protocol. Quantitative RT qPCR gene transcript level analysis was performed on duplicate samples with SYBR green (Lot 028416, Quanta bio) technology using a StepOnePlus™ (Applied Biosystem). The amplification protocol consisted of 40 cycles, including denaturation at 95°C for 15 s, annealing, and extension at 60°C for 60 s. The gene expression levels were normalized to that of the housekeeping gene GAPDH. The \(2^{-\Delta\Delta Ct}\) method was used to calculate the fold change. Sequences of the RT qPCR primer pairs are provided in the Supplemental Methods.

Western blot. Total protein was extracted from cultured cells in RIPA buffer supplemented with EDTA-free Halt protease inhibitor cocktail (Thermo Fisher Scientific). Cytoplasmic and nuclear proteins were
extracted using a NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer’s instruction. Equal amounts of protein were loaded onto a precast NuPAGE 4% 12% Bis-Tris mini protein gel (Invitrogen) and transferred to a nitrocellulose membrane by Trans-Blot Turbo transfer system (Bio-Rad). Transferred membranes were blocked for 1 hour in TBS containing 5% non-fat dried milk followed by overnight incubation at 4°C in the corresponding primary antibodies. The following antibodies purchased from Cell Signaling were used at 1:1000 dilution: β-tubulin, Histone H3, YAP, phospho-YAP (Ser127). After three washes for 5 min in TBST, following incubation with Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 680 and Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 800 (1:5,000; Thermo Fisher Scientific) and then visualized by using an Odyssey CLx imaging system (LI-COR Biosciences).

Histology. Tissues from mouse models were fixed in 10% formalin, dehydrated and embedded in paraffin, and sectioned at 5 µm. Masson’s trichrome staining was used to detect collagen fibers, while hematoxylin and eosin (H&E) staining was used to determine cardiomyocyte cross-sectional area, skin hair follicle density and wound healing process. The images of sections were captured, scanned and then analyzed with Aperio Image Scope (version 12.1) and Image-Pro Plus (version 7).

Immunofluorescence/Immunohistochemistry. Cells were fixed and permeabilized in 4% paraformaldehyde (PFA) and 0.1% Triton in PBS, respectively, then blocked with 3% BSA in PBS for 30 minutes. Subsequently, cells were stained with primary antibodies targeting SLIT3 (SAB2104337: Sigma-Aldrich) or ROBO1 (ab7279: abcam) at 1:200 in blocking solution at 4°C overnight. Then, cells were washed and incubated with the appropriate secondary antibody donkey anti-rabbit IgG (H+L) highly cross-adsorbed, Alexa Fluor Plus 488 (Invitrogen) in dark for 60 minutes at room temperature. Nuclei were stained with 1 µg/mL DAPI. Images were obtained using a Nikon A1 confocal microscope.

Heart paraffin sections were deparaffinized with xylene and rehydrated with serial dilutions of ethanol. After antigen retrieval, the sections were blocked with 5% donkey serum/3%BSA/0.1% Triton X-100/PBS for 1 hour at room temperature. The following primary antibodies were used at 1:200 dilution overnight at 4°C: CD31 (AF3628: R&D Systems) and collagen type I (Abcam ab21286). After washing three times for
5 min each, secondary antibody donkey anti-goat Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 564 (Invitrogen) were incubated with a 1:500 dilution for 1 hour at room temperature. Sections were then stained with DAPI. Images were acquired on an inverted Nikon A1 confocal microscope (Nikon, Japan).

**Statistical analysis.** All statistical analyses, including unpaired two-tailed student’s t-test, one-way and two-way ANOVA with following multiple comparisons test, linear regression and trend, as well as survival curve were performed using GraphPad Prism software (version 8). Outliers were identified by GraphPad outlier calculator (Alpha = 0.05) and removed before analysis. P values of less than 0.05 were regarded as statistically significant.

All mice experiments were performed according to the animal experimental guidelines issued by the Institutional Animal Care & Use Committee (IACUC) of the University of Michigan.
Author Contributions

M.Si conceived, managed and arranged funding for the project. L.G., M.Si, S.We., S.E., J.S., A.W., S.Wa. designed the experiments. Wet lab experiments (cell culture, cell biology, molecular biology, histology, immunohistochemistry) were carried out by L.G., S.Wa., L.S, B.L., and X.L. In vivo mouse experiments were performed by L.G. and L.S. LV rupture testing and data analysis were performed by L.S., C.L., M.Sh., J.S., A.W., and M.Si. Data were analyzed by L.G., L.S., S.Wa., M.Si, S.We., S.E., A.E., Y.Y., and D.X. L.G., M.Si, S.We., and S.Wa. drafted the manuscript with input from the other coauthors.

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Competing interests

The authors declare no competing interests.

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Figure 1. SLIT3 is present at high levels in fibrillar collagen-producing cells. (A) Linear regression analysis between the transcript levels of SLIT3 and COL1A1, SLIT3 and COL3A1, ROBO1 and COL1A1 across 512 cell lines from the FANTOM 5 project (28). Transcripts per million, TPM. (B) Single cell transcriptome data from the Tabula Muris project (29), t-SNE plot of all cells collected by FACS, overlaid with the predominant cell type composing each cluster (n = 44,949 individual cells from 20 mouse organs). The clusters of cells expressing Slit3, Col1a1, and Robo1. (C) Transcript levels of Slit3, Col1a1, and Robo1 in cardiac fibroblasts, Left ventricle, freshly isolated cardiomyocytes, aortic adventitial fibroblasts, lung fibroblasts, aortic vascular smooth muscle cells (VSMC), aorta media, and aorta adventitia from WT mice. Samples taken directly from or isolated from living tissue were marked blue and samples of purified and cultured cells were marked red on panels. Each data point represents tissue/cells obtained from a single animal. (D) Confocal immunofluorescence images of mouse aortic adventitial fibroblasts stained with SLIT3 (green), ROBO1 (green) and, DAPI (blue) (n=2). Scale bars, 50 μm. *P < 0.05, **P < 0.01, ***P < 0.001 using the one-way ANOVA with Tamhane T2 multiple comparisons test (C).
Figure 2. SLIT3 deficiency reduces fibrillar collagen production in vivo. (A) Masson’s trichrome-stained histological sections of lung, spleen, and kidney in 8-week-old Slit3−/− and WT mice. Results are representative of samples obtained from 5 animals per genotype group. Scale bars, 100 μm, 60 μm, and 100 μm from top to bottom. (B) Transcript levels of Slit3, Col1a1, and Col3a1 in the aortic adventitia, lung, spleen, and kidney in 8-week-old Slit3−/− and WT mice (n=4-6 per group). (C) Linear regression analysis between the transcript levels of Slit3 and Col1a1 in the aortic adventitia, lung, spleen, and kidney from 8-week-old WT mice (n=5-7 per group). (D) Quantification of tissue collagen content by assessment of hydroxyproline concentrations in the femur, skin, lung, spleen, and kidney in 8-week-old Slit3−/− and WT mice (n=5-8 per group). Data are presented as mean±SD. Number (n), male (M), female (F). *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT mice using the unpaired two-tailed Student’s t-test (B, D).
Figure 3. SLIT3 deficiency reduces cardiac fibrillar collagen production and LV biomechanical toughness. (A) Representative Masson’s trichrome-stained histological sections of heart in 8-week-old and 1-year-old Slit3−/− and WT mice, representative confocal immunofluorescence images of heart stained with Collagen Types 1 (red), CD31 (green) and, DAPI (blue) in 8-week-old Slit3−/− and WT mice, and representative immunohistochemical stain for CD31 in sections of hearts from 8-week-old Slit3−/− and WT mice (n=3 per group). (B) Transcript levels of Slit3, Col1a1, and Col3a1 in the left ventricle (LV) and right ventricle (RV) in 8-week-old Slit3−/− and WT mice (n=4-6 per group). (C) Linear regression analysis between the transcript levels of Slit3 and Col1a1 in the LV and RV from 8-week-old WT mice (n=6 per group). (D) Transcript levels of Slit3 in LV and RV, and their linear regression analysis in 8-week-old wild-type mice (n=6 per group). (E) Quantification of perivascular collagen area of coronary arteries and mitral ratio of peak early to late diastolic filling velocity (mitral E/A ratio) in 8-week-old and 1-year-old Slit3−/− and WT mice (n=4-16 per group). (F) Quantification of cardiac collagen content by assessment of hydroxyproline concentrations in 8-week-old Slit3−/− and WT mice (female, n=8 per group). (G) Representative passive LV pressure-volume curve and quantification of energy density required to rupture the cardioplegia-relaxed LVs by balloon catheter inflation in 8-week-old Slit3−/− and WT mice (n=4 per group). (H) Representative image of myocardial rupture and overall survival curve of the first week after left anterior descending coronary artery ligation (MI) in 8-week-old Slit3−/− and WT mice. The blue arrowhead indicates the position of myocardial rupture hole and survival analysis was performed using the Kaplan-Meier method. Log-rank test, p=0.0039 (n=24-25 per group). (I) Representative image of femurs or hearts, as well as quantification of femur or heart weight/tibia length ratio in 8-week-old Slit3−/− and WT mice (n=6 per group). Scale bar, 1 mm. Data are presented as mean±SD. Number (n), male (M), female (F). *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT mice using the unpaired two-tailed Student’s t-test (B, E-G, I).
**Figure 4. SLIT3 deficiency attenuates LV fibrosis and adverse remodeling.** (A) Histological transverse sections of whole heart (first row, Masson's trichrome stain), coronary arteries (second row, Masson's trichrome stain), and LV myocytes (third row, haematoxylin and eosin stain) in *Slit3<sup>−/−</sup>* and WT mice before and at 3 and 8 weeks after transverse aortic constriction (TAC) (n=6) Scale bars, 2 mm, 60 or 200 μm, and 60 μm from top to bottom. (B) Quantification of heart weight/tibia length ratio, coronary perivascular fibrosis area, cardiomyocyte cross-sectional area in *Slit3<sup>−/−</sup>* and WT mice before and at 1, 3, and 8 weeks after TAC (n=4-9 per group). (C) TAC peak pressure gradient determined by echocardiography in *Slit3<sup>−/−</sup>* and WT mice after surgery (n=4-24 per group, initial gradients at day 3, 43±6.8 vs. 43±11 mmHg, p > 0.99). (D-E) Transcript levels of *Col1a1*, *Nppb*, and *Slit3* in the LV in *Slit3<sup>−/−</sup>* and WT mice before and at 1 and 3 weeks after TAC (n=6-8 per group). (F) LV ejection fraction (EF) determined by echocardiography in *Slit3<sup>−/−</sup>* and WT mice before and at 3, 7, and 16 weeks after TAC (n=5-19 per group). (G) Long-term overall survival curve of *Slit3<sup>−/−</sup>* and WT mice from day 1 after TAC. Survival analysis was performed using the Kaplan-Meier method. Log-rank test, p=0.0245 (n=25-30 per group). BSL: 7-9 weeks old baseline mice before surgery. Data are presented as mean±SD. Number (n). *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT mice using the unpaired two-tailed Student's t-test (B-D, F) and one-way ANOVA with Tamhane T2 multiple comparisons test (E).
Figure 5. SLIT3 deficiency attenuates RV fibrosis and adverse remodeling. (A) Histological transverse sections of whole heart or part of RV free wall (first row, Masson’s trichrome stain), RV free wall with coronary arteries (second row, Masson’s trichrome stain), RV free wall myocytes (third row, haematoxylin and eosin stain), and representative images of RV echocardiogram (fourth row, M-mode parasternal short-axis) in Slit3−/− and WT mice before and at 2 and 4 weeks after pulmonary artery banding (PAB) (n=6). Scale bars, 2 mm, 200 μm, 60 μm, and 2 mm from top to bottom. (B-D) Quantification of RV free wall fibrosis area, myocyte cross-sectional area, as well as end-diastolic diameter and area determined by echocardiogram in Slit3−/− and WT mice before and at 2 and 4 weeks after PAB (n=4-9 per group). (E) Quantification of PAB peak pressure gradient by echocardiography in Slit3−/− and WT mice at 3, 14 and 28 days after PAB (n=5-13 per group, initial gradients at day 3, 30±7.9 vs. 29±9.7 mmHg, p > 0.75). (F-G) Transcript levels of Col1a1, Nppb, and Slit3 in the RV in Slit3−/− and WT mice before and at 2 and 4 weeks after PAB (n=5-6 per group). (H) RV fractional shortening (FS) determined by echocardiography in Slit3−/− and WT mice at 2 weeks after PAB (n=5-11 per group). (I) Long-term overall survival curve of Slit3−/− and WT mice from day1 after PAB. Survival analysis was performed using the Kaplan-Meier method. Log-rank test, p=0.0494 (n= 18-19 per group). BSL: 7-9 weeks old baseline mice before surgery. Data are presented as mean±SD. Number (n). *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT mice using the unpaired two-tailed Student’s t-test (B, E, F), one-way ANOVA with Tamhane T2 multiple comparisons test (G), and two-way ANOVA with Tukey multiple comparisons test (C-D).
Figure 6. SLIT3 deficiency inhibits fibroblast biological activity. (A) MTT cell proliferation assay. Slit3−/− and WT aortic adventitial fibroblasts were seeded in 96-well plates (10% FBS media) and treated with PBS or PDGF-BB (100 ng/ml). Absorbance was measured at 560 nm at 0 and 48 hours after treatments (n=6-14 per group). (B) Floating collagen gel contraction assay. Representative images and area quantification of collagen gel. Slit3−/− and WT lung fibroblasts were seeded in PBS or TGFβ1 (5.0 ng/ml) added collagen gel (1mg/ml collagen type I, 0.5% FBS). The final to initial area ratio was determined at 24 hours after floating (n=3 per group). Scale bars, 5 mm. (C) Scratch wound healing assay. Representative images and migration distance quantification of scratch wound healing. Slit3−/− and WT lung fibroblasts were seeded in 6-well plates (1% FBS media) and treated with PBS or PDGF-BB (100 ng/ml). The migration distance was determined at 24 hours after scratching (n=4). Scale bars, 20 μm. (D) Excisional wound healing assay. Representative gross and histological images (hematoxylin and eosin staining) and quantification of wound area in 8-week-old Slit3−/− and WT mice at the day of surgery (Day 0) and 15 days (Day 15) after surgery (n=19-21 per group). Scale bars, 1 mm and 700 μm from top to bottom. Data are presented as mean±SD. In vitro experiments were performed at least 3 times independently. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT mice or cells using the unpaired two-tailed Student’s t-test (D) and two-way ANOVA with Two-stage step-up method of Benjamini, Krieger and Yekutieli multiple comparisons test (A-C).
Figure 7. SLIT3 regulates YAP1 and fibrillar collagen production. (A-B) Representative images and transcript levels of Acta2, Col1a1, Slit3, and Yap1 in adult primary fibroblasts cultured on stiff surfaces (6-well plastic tissue culture plate, 10% FBS) or in soft collagen gel (1mg/ml collagen type I, 1% FBS) for 24 hours. From left to right, aortic adventitial fibroblasts (AAFs), cardiac fibroblasts (CFs), and lung fibroblasts (LFs) (n=3 per group). Scale bars, 20 μm and 50 μm from top to bottom. (C) Transcript levels of Yap1/Taz ratio and Ctgf in the LV in Slit3−/− and WT mice before and at 1 week after TAC (n=6-8 per group). (D-E) Western blots of YAP1 and pYAP1 in Slit3−/− and WT CFs and LFs cultured on stiff surface (6-well plastic tissue culture plate) with quantification (n=2). (F) Transcript levels of Yap1 and Ctgf in Slit3−/− and WT AAFs and CFs in soft collagen gel (1mg/ml collagen type I, 1% FBS) for 24 hours (n=3 per group). (G) Linear regression analysis between the transcript levels of Slit3 and Yap1 in WT AAFs cultured on stiff surfaces (6-well plastic tissue culture plate, 10% FBS) or in soft collagen gel (1mg/ml collagen type I, 1% FBS) for 24 hours (R²=0.9114, n = 18). (H) Transcript levels of Yap1, Ctgf, and Col1a1 in Slit3−/− LFs cultured in PBS or recombinant SLIT3 (rSLIT3, aa 34-1116, 1 µg/ml) added collagen gel (1mg/ml collagen type I, 0.5% FBS) for 24 hours (n = 10 per group). (I) Transcript levels of Slit3 in WT AAFs and CFs cultured in PBS or TGFβ1 (5.0 ng/ml) added collagen gel (1mg/ml collagen type I, 0.5% FBS) for 24 hours (n = 3 per group). (J) Transcript levels of Yap1 and Ctgf n Slit3−/− and WT CFs cultured in PBS or TGFβ1 (5.0 ng/ml) added collagen gel (1mg/ml collagen type I, 0.5% FBS) for 24 hours (n = 3 per group). Data are presented as mean ± SD, Number (n). In vitro experiments were independently performed at least 3 times unless indicated otherwise. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT mice or cells using the unpaired two-tailed Student’s t-test (B, E-F, H-I) and two-way ANOVA with Tukey multiple comparisons test (C, J).