**INTRODUCTION**

Cellular senescence is a principle causative factor in organismal aging (1). It is induced by accumulated DNA damage, telomere erosion, and cellular stress, resulting in permanent withdrawal from the cell cycle. Senescent cells are characterized by elevated p16^Ink4a levels, with increased (2) and mitochondrial dysfunction. It is often accompanied by the senescence-associated secretory phenotype (SASP) (2) in which production of multiple inflammatory and remodeling factors exerts domi-

---

**RESULTS**

**βPIX expression during aging**

We assessed the levels of multiple FA proteins, including the Rac/Cdc42 GEF βPIX, as a function of age in mouse tissues. Western blotting showed declining βPIX protein levels with age, which reached approximately 10 to 20% of starting levels by 24 months in the lung, kidney, spleen, heart, and skin (Fig. 1A). GIT1/2 levels similarly decreased, with minor variations among organs. No significant changes were detected in the levels of Rac1, Cdc42, or Pak1. Our
initial investigation focused on βPIX. Immunohistochemical examination of bronchioles in mouse lung confirmed age-dependent decline in βPIX levels (Fig. 1A and C). Human lung tissue showed a similar decline (Fig. 1D; quantified in Fig. 1E) as did human diploid fibroblasts (HDFs) with passage in culture (Fig. 1F). Thus, βPIX declines with age in multiple in vivo and in vitro systems.

**Control of cellular senescence by βPIX expression**

The reduction in βPIX with age coincided with higher levels of p16, a hallmark of replicative senescence (18), in both mouse and human lungs (Fig. 1, A and C) and in HDFs (Fig. 1F). We also examined the senescence markers SA-β-Gal (19) and nuclear phosphorylated γH2AX, which marks DNA damage–induced heterochromatin foci associated with cellular senescence (20). All of these senescence markers were substantially up-regulated in old compared to young mice (fig. S1). Old HDFs also showed higher levels of SA-β-Gal than young HDFs (Fig. 1G; quantified in Fig. 1H).

These results led us to investigate whether the reduction of βPIX played a causal role in cellular senescence. Silencing βPIX in HDFs using two different small interfering RNA (siRNA) sequences was sufficient to induce expression of SA-β-Gal (Fig. 2, A to C) and γH2AX (Fig. 2A, inset). Reexpression of wild-type (WT) siRNA-resistant βPIX (si-rPIX) (fig. S2, A and B) rescued the increase in senescence markers after βPIX siRNA (fig. S2, A to E), excluding off-target effects. When βPIX was silenced by two different short hairpin RNAs (shRNAs), SA-β-Gal activity and p16 levels were similarly up-regulated [Fig. 2, D and E]. We next asked whether the GEF activity of βPIX is required. To this end, we expressed GEF-inactive βPIX [Db homology domain (DH) mutant; L238R/L239S] (fig. S2, A and B), which rescued βPIX depletion–induced senescence and WT si-rPIX, as assessed by SA-β-Gal activity and p16 (fig. S2, C to E).

Cellular senescence is associated with cell cycle arrest, which can be due to either increased expression of the cyclin-dependent kinase inhibitors (CKIs) or up-regulation of p53 (21). We observed increased p16 levels after βPIX knockdown in both mouse and human lungs (Fig. 1, B to E) and in HDFs (fig. S3A). In the HDFs, this coincided with reduced expression of the related CKI p21, decreased cyclin E levels, and hypophosphorylated Rb protein (fig. S3, A and B). By contrast, p19, cyclin D, and CDK4 were unchanged, as were p53 protein levels and S15 phosphorylation (fig. S3B). Consistent with this result, βPIX-depleted cells showed a cell cycle arrest in G1 (fig. S3C) and stopped proliferating in culture (fig. S3D). To test the roles of p16 and p53 in senescence, we depleted these proteins in HDFs via lentiviral shRNA expression (confirmed in fig. S3E). Depletion of p16, but not p53, blocked the increase in SA-β-Gal expression following βPIX knockdown (fig. S3F). Together, these results demonstrate that reduced βPIX levels can induce cellular senescence through the p16 pathway. Another characteristic feature of senescent cells is SASP (2). Analysis of the cytokine array revealed up-regulation of interleukin-6, monocyte chemoattractant protein–1, and tissue inhibitor of matrix metalloproteinase–2 after βPIX depletion (fig. S4).

To examine the effects of βPIX depletion in vivo, we used a system targeting mouse bronchial epithelium via intratracheal delivery of viral or lipid particles (22). Delivery of βPIX siRNA into lungs of young
mice (scheme in Fig. 2F) resulted in elevated SA-β-Gal staining in bronchial epithelial cells 4 weeks after treatment (Fig. 2G; quantified in Fig. 2H). Staining for both p16 and γH2AX was also markedly increased (Fig. 2G; quantified in Fig. 2H). Conversely, overexpression in the lungs of 12-month-old mice by delivery of lenti–green fluorescent protein (GFP)–βPIX (scheme in Fig. 2I), which were analyzed 4 weeks later, increased βPIX staining ~3-fold but reduced staining for SA-β-Gal, p16, and γH2AX (Fig. 2J; quantified in Fig. 2K). This decrease in staining is modest, as expected, presumably by preventing the increase that would have occurred over this 4-week time period.

A role for integrin signaling

As βPIX is known to regulate FA dynamics (13), we next addressed a possible role for altered integrin signaling. βPIX depletion in HDFs increased phosphorylation of FAK at Y397 and Y576 and paxillin at Y118 (Fig. 3A), consistent with more numerous FAs and denser actin stress fibers (Fig. 3B, left; quantified in Fig. 3, C and D). To determine whether the enhanced FAs contribute to the senescent phenotype, we treated βPIX-depleted HDFs with arginylglycylaspartic acid (RGD) peptides that antagonize integrins and destabilize FAs. This treatment reduced FA size and the robust actin stress fiber bundles typical of senescent cells (compare control versus RGD peptide in Fig. 3, B to D) and completely prevented the increase in SA-β-Gal staining (Fig. 3E; quantified in Fig. 3F). Because of its role as a principle mediator of integrin signaling, we asked whether the increased FAK activity was functionally important. The addition of a chemical inhibitor of FAK activity strongly reduced senescence after βPIX depletion in HDFs, whereas control (vehicle) had no effect (Fig. 3G). To test whether similar effects could be observed in vivo, we delivered FAK siRNA to mouse lungs 2 weeks after βPIX siRNA administration (scheme in Fig. 3H). This treatment also prevented the increase in senescence markers in mouse lungs at 5 weeks (Fig. 3I; quantified in Fig. 3J), confirming the pharmacological result. Thus, βPIX depletion induces senescence in vivo and in vitro through altered integrin signaling.

Rac1 and its effector PAK act downstream of integrins (23). Rac1 can directly activate NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidases (NOXs) to produce ROS (24), which are implicated in senescence (25). Paradoxically, Rac1 and PAK1 activity increased after βPIX knockdown even in the absence of serum (fig. S5A). Their activation was inhibited by RGD peptides, indicating signaling from integrin to Rac/ROS (fig. S5B). Silencing NOX1, an effector of Rac1 (7), strongly inhibited βPIX depletion–induced up-regulation of SA-β-Gal (fig. S5C). ROS generation assessed with the fluorescent sensor H2DCFDA was also strongly elevated by βPIX siRNA (fig. SSD). As expected, the ROS scavenger N-acetyl cysteine blocked induction of SA-β-Gal staining in this...
system (fig. S5E; quantified in fig. S5F). Thus, ROS generated through an integrin-Rac-NOX pathway mediates βPIX-dependent cellular senescence.

**FA dynamics**

We next investigated how βPIX controls integrin signaling. βPIX controls FA disassembly (13), which has been reported to involve both integrin endocytosis, which is induced following microtubule targeting of FAs, and calpain cleavage of FA proteins such as talin (15). We measured internalization of β1 integrin in HDFs using the nocodazole washout protocol that induces rapid and synchronous endocytosis (17). βPIX depletion strongly suppressed β1 integrin endocytosis in this assay (Fig. 4A; quantified in Fig. 4B). As a control, we also examined endocytosis of Alexa Fluor 594–conjugated transferrin as a marker of general CME. To our surprise, βPIX siRNA also inhibited transferrin uptake (Fig. 4, C and D), indicating a general block of CME. These effects were rescued by si-rPIX (Fig. 4, E and F; βPIX expression in fig. S2D). To investigate these effects in vivo, we used the mouse lung system. In vivo siRNA-mediated βPIX depletion (confirmed by βPIX staining; Fig. 4G) strongly reduced transferrin uptake in mouse bronchial epithelial cells (Fig. 4G; quantified in Fig. 4H). Thus, βPIX is required for CME of multiple receptors, including but not limited to, β1 integrin. These results prompted us to consider effects on conserved mediators of CME.

**Calpain cleavage of AMPH1**

Calpain also promotes adhesion turnover (14, 15), although the relationship to the microtubule/endocytosis pathway is unknown. To test the effects of reducing FA turnover via this pathway, we treated HDFs with the calpain inhibitor, N-Acetyl-Leu-Leu-Methional (ALLM). This treatment reversed the blockade in β1 integrin endocytosis after βPIX knockdown (fig. S6A). Furthermore, inhibiting calpain with ALLM (fig. S6B) or knockdown with specific siRNAs (fig. S6, C and D) in HDFs prevented rather than increased SA-β-Gal activity following βPIX depletion. These unexpected results prompted us to examine calpain’s function more closely. AMPH1 is a major endocytic adapter in CME and a known calpain substrate (26). βPIX knockdown in HDFs induced cleavage of ~63% of AMPH1 to a ~50-kDa N-terminal fragment, which was reduced by calpain inhibition (fig. S6E). To test relevance in vivo, mice received siRNA to deplete βPIX in the lung. We observed reduced AMPH1 staining in bronchiolar epithelial cells 4 weeks after treatment (fig. S6F; quantified in fig. S6G). In old mice, AMPH1 in the lung was almost completely degraded; there was also a slight reduction of dynamin II (fig. S6H). Consistent with these
results, transferrin uptake was markedly reduced in the lung from old mice compared to young mice (fig. S6I; quantified in fig. S6J). These results therefore led us to consider the role of calpain cleavage of AMPH1 in the reduced endocytosis and increased senescence after βPIX knockdown.

AMPH1 has three potential calpain cleavage sites (fig. S6K) that are consistent with the size of the observed N-terminal fragment (fig. S6E). We therefore constructed AMPH1 mutants (S333G, S377G, and V392G) that should confer resistance to calpain at these sites (26). When expressed in 293T cells and ionomycin added to maximally activate calpain, only AMPH1-V392G was proteolysis-resistant under these conditions (fig. S6L). This mutant similarly behaved after βPIX knockdown in HDF cells (fig. S7A) and restored endocytosis of both transferrin (fig. S7B; quantified in fig. S7C) and β1 integrin (fig. S7D) in βPIX knockdown cells to nearly the control level, whereas WT AMPH1 rescued only weakly (fig. S7, B to D). Consistent with these results, AMPH1-V392G inhibited βPIX knockdown–induced SA-β-Gal staining (fig. S7E). To test in vivo, we delivered AMPH1-V392G or WT AMPH1 to mouse lungs, which were subsequently treated with βPIX siRNA (scheme in Fig. 5A). AMPH1-V392G prevented the decrease in transferrin uptake (Fig. 5B; quantified in Fig. 5C) and reduced the expression of senescence markers after βPIX depletion (Fig. 5D; quantified in Fig. 5E). These results show that decreased βPIX induces calpain-mediated cleavage of AMPH1, which reduces CME and results in cellular senescence.

**Molecular competition model**

How might βPIX control AMPH1 cleavage by calpain? The βPIX-GIT1 complex binds paxillin, which is a known calpain substrate (14). βPIX knockdown slightly increased paxillin cleavage, which was
amplified by ionomycin and abrogated by ALLM (fig. S8A); however, only a few percentage of the paxillin was affected, arguing against a functional role for paxillin degradation. This result led us to ask whether paxillin might serve as an adapter for calpain rather than a substrate. We observed coimmunoprecipitation of paxillin with calpain-2 (fig. S8B) and AMPH1 (fig. S8C) but not dynamin II (fig. S8B). Depletion of βPIX strongly increased the association with calpain-2 and AMPH1. Immunoprecipitates of calpain-2 and AMPH1 contained paxillin and the other two constituents (fig. S8D), suggesting a ternary complex containing paxillin, calpain-2, and AMPH1. Purified recombinant calpain-2 and the N-terminal AMPH1 fragment pulled down paxillin from cell lysates, whereas the AMPH1 C terminus was inactive (fig. S8, E and F). Thus, βPIX depletion induces association of calpain-2 and AMPH1, leading to calpain-2–mediated proteolysis of AMPH1 (diagram in fig. S8G).

The tight βPIX-binding partners GIT1 and GIT2 (10) associate with paxillin via specific conserved binding sites that mediate recruitment of the βPIX-GIT1/2 complex to FAs (11). We found that depletion of GIT1 and GIT2 also induced formation of the paxillin-calpain-AMPH1 complex (fig. S8C) and degradation of AMPH1 (fig. S8H). GIT1/2 depletion also inhibited endocytosis of both transferrin (fig. S8I) and β1 integrin (fig. S8J). Unexpectedly, βPIX depletion blocked the coimmunoprecipitation of GIT1 or GIT2 with paxillin (fig. S9A) and their colocalization with paxillin in peripheral FAs (fig. S9B; quantified in fig. S9C). To accurately evaluate the effect of siRNAs, we examined Cy-5–positive cells that took up siRNA (fig. S9B). Together, these results show that βPIX is required for GIT association with paxillin and localization of GIT to FA; thus, the loss of either βPIX or GIT results in disappearance of this complex from FA, which correlates with increased localization of calpain-2 to FAs (fig. S9D; quantified in fig. S9E), consistent with increased association of calpain-2 and paxillin (fig. S8, B and C).

These data suggest a simple model in which GIT1/2 and calpain-2 compete for binding to paxillin. To test this idea, we performed a competition assay in vitro with the GIT1 C terminus (GIT1-CT; amino acids 376 to 770) that contains the paxillin-binding site. Calpain-2 bound to paxillin, which was inhibited by GIT1-CT in a dose-dependent manner (Fig. 6A, left). In a control experiment, glutathione S-transferase (GST) did not affect calpain-2 binding to paxillin (Fig. 6A, right). According to this competition model, overexpression of GIT-CT would prevent βPIX depletion–induced decrease in CME and senescence. When overexpressed in HDFs, GIT-CT coprecipitated and colocalized with paxillin in FAs (fig. S10, A and B; quantified in fig. S10C) and prevented βPIX depletion–induced up-regulation of SA-β-Gal activity (fig. S10D). Consistent with these in vitro results, lentivirus-mediated delivery of GIT-CT into

---

*Fig. 5. Calpain-resistant amphiphysin (V392G) prevents βPIX knockdown–induced events in vivo. (A) Experimental scheme to analyze calpain-resistant amphiphysin I. (B) Uptake of Alexa Fluor 594–transferrin was visualized in bronchioles of siRNA-treated mouse lung expressing GFP (control) and amphiphysin (AMPH-WT or AMPH-V392G). Scale bar, 20 μm. siPIXm1, mouse specific siRNA for silencing βPIX. (C) Quantification of transferrin uptake. n = 3 mice per group (five bronchioles per mouse). *P < 0.001, t test. (D) SA-β-Gal and immunohistochemical staining for senescence markers were performed in the bronchioles of siRNA-treated mice expressing GFP (control) and amphiphysin (AMPH-WT or AMPH-V392G). Red arrows indicate positive staining. Scale bars, 10 μm. (E) Quantification of senescence markers. n = 3 mice per group (five bronchioles per mouse). *P < 0.001, t test. For all panels, quantified values are means ± SEM.*
βPIX-depleted mouse lungs maintained transferrin uptake (scheme in Fig. 6, B and C; quantified in Fig. 6D) and suppressed SA-β-Gal staining (Fig. 6E; quantified in Fig. 6F). These results show that GIT binding to paxillin displaces calpain-2, thereby protecting AMPH1 from calpain-mediated cleavage (Fig. 6G, left). βPIX appears to promote GIT1/2 binding to paxillin, which is likely to occur through the effects on GIT1/2 conformation. Thus, the βPIX-GIT complex protects cells from senescence via a competition mechanism in which AMPH1 is protected from proteolysis to support normal CME (Fig. 6G).

**DISCUSSION**

Together, these results define a previously unknown molecular connection between cell-matrix adhesion and senescence. In this model, the decline in βPIX-GIT levels is a significant rate-limiting step in cellular aging. Loss of βPIX-GIT leads to the formation of a paxillin-calpain-AMPH1 complex and AMPH1 cleavage. The resulting inhibition of integrin endocytosis leads to abnormal FAs that activate Rac and thus ROS production, which induces cellular senescence through the well-studied p16Ink4a-pRb pathway. Similar effects were seen in cultured human fibroblasts and mouse bronchial epithelium, demonstrating both species conservation and consistent effects in vitro and in vivo. These results provide a functional and mechanistic explanation for the long-standing observation that cellular senescence is associated with a hyperadhesive phenotype with enhanced FAs and actin stress fibers, as well as poor cell motility.

The finding that CME is generally reduced with age is consistent with previous reports (27, 28), although the phenomenon has not been explored in much detail. Reduced CME was attributed to low AMPH1 levels in senescent fibroblasts (27), consistent with the present study. Reduced endocytic capacity of aged rat liver sinusoidal endothelial cells was observed, but the mechanism was not identified (28). The current study defined calpain-dependent proteolysis as the key...
mechanism underlying AMPH1 down-regulation in senescent fibroblasts and lung epithelium. Decreased CME in aged cells may also explain decreased functions such as hyposponsiveness to external stimuli (27) and defective clearance of waste products (28). More recently, Rajarajacholan et al. (29) reported that the Inhibitor of Growth 1a tumor suppressor induces senescence through altered endocytosis in fibroblasts. Our study now reveals a molecular mechanism and an in vivo role for decreased CME in senescence using the lung as our model. Cellular senescence in the lung has recently been recognized as causally associated with ARDs including idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease (30, 31). It will therefore be interesting to investigate whether decreasing βPIX or GIT levels in old age contribute to pathogenesis of these lung diseases.

The finding that βPIX knockdown increased Rac activity was unexpected in light of the βPIX GEF activity toward Rac. However, integrins have been reported to trigger Rac activation through a variety of GEFs, including ROCK1, Vav1 and Vav2, and TIAM1 (32). Furthermore, βPIX has been found to regulate cell behavior via GEF-independent functions (33). In this setting, the predominant effect of βPIX appears to be mediated through its regulation of FA dynamics. βPIX promotes FA turnover (13), a function linked to both Rac1 activation and Rac-independent protein interactions such as binding to Myosin 18 (34). Abnormal FAs with elevated Rac then signal to induce excessive ROS, a well-known inducer of cellular senescence (35). How abnormal FA signaling induces sustained ROS production remains unknown. Integrins can signal to ROS via both the NOX (36, 37) and mitochondrial metabolism (38), with ROS from both sources implicated in cellular senescence (35). Constitutive integrin activation blocks cell migration and proliferation in some settings (39, 40), consistent with abnormal signaling under conditions of reduced turnover.

βPIX depletion abolished GIT1/2 targeting to FAs. How then does βPIX control GIT targeting? The finding that GIT-CT alone binds paxillin and competes with calpain-2 for binding to paxillin suggests that βPIX affects GIT conformation, which determines binding to paxillin. However, this model remains to be experimentally tested, and alternative mechanisms cannot be excluded.

One model for the role of βPIX in FA disassembly is based on its ability to bind myosin II that is activated and assembled into bipolar filaments with high adenosine triphosphatase activity (41). Myosin II inactivation triggers FA disassembly, suggesting that βPIX released from disassembling myosin II filaments might translocate to nearby FAs to promote their turnover (13). An alternative model is that transport of the βPIX-GIT complex might occur via the microtubule motor kinesin, as suggested by direct recruitment of βPIX from a perinuclear location to FAs (42).

Our data point toward several new directions for future research. Dissecting the differential signaling under conditions of reduced integrin turnover may reveal specific targets for interrupting the abnormal ROS production without affecting normal functions. While β integrin would not appear as an attractive therapeutic target, FAK inhibitors are being tested in human clinical trials, and antibodies to specific integrins are used clinically (43). Understanding the basis for age-dependent suppression of βPIX and the protein interactions required for amphiphysin cleavage could reveal previously unidentified targets. Future studies to identify novel therapeutic targets for aging-related diseases within the integrin-βPIX-AMPH1-ROS pathway therefore seem warranted.

MATERIALS AND METHODS

Materials

Invivofectamine, Lipofectamine 2000, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Opti-MEM, Alexa Fluor 594–conjugated transferrin, and Alexa Fluor–conjugated secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA). shRNA lentiviruses for p16 and p53 were purchased from Santa Cruz Biotechnology (Dallas, TX). Lentiviruses for amphiophsins, si-rPIXS, and GIT-CT were provided by Sung from Korea Research Institute of Bioscience and Biotechnology (Cheongju, Korea). Lentiviruses for GFP (LVP690) and GFP-βPIX (LVP718951) were purchased from Abm Inc. (Richmond, Canada). Calpain inhibitors II (ALLM), SA–β–Gal staining solution, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant paxillin–His protein was purchased from RayBiotech (Peachtree Corners, GA). Human inflammation antibody array was purchased from Abcam (Cambridge, UK). siRNAs for GIT1 (SI02224467) and GIT2 (HS114794) were obtained from Qiagen (Germantown, MD). siRNAs for βPIX, calpain-2, and siFAK were obtained from Thermo Fisher Scientific and Bioneer (Daejeon, Korea). siRNA or shRNA sequences are summarized as follows: siPIX1, 5′-UCAACUGGUAGAAGCAAAAGUUU; siPIX2, 5′-UUGACCGCAGAUCGAGCAGGAGC; shPIXa, 5′-AACCTCTACCTACGTATTGTG; siCtrl1, 5′-CCUACGCGCA-CAUUUCGU; hCalpain-2, 5′-GGCAUUAAGAAAGACGGAGU; siPIXm1, 5′-CAACUGGUAGAAGCAGCAAGGUU (UGAUGUCAUCAUGCACA); siPIXm2, 5′-GAGGACCUAGGAUCAUGGAA; siFAK, AACCACUGGGCCAGUUUAAUA. Animal experiments were performed in accordance with the approved animal protocols and the guidelines established by the Ethics Review Committee of the Chungbuk National University for Animal Experiments (CBNUA-901-15-01). Mice were obtained from DahanBioLink (Seoul, Korea). Human lung samples were obtained from patients with pneumothorax who received surgery at the Chungbuk National University Hospital (Cheongju, Korea). These patients did not show any other pathology in the lung. The study was reviewed and approved by the Institutional Review Board of the Chungbuk National University Hospital (2014-02-009-009).

Antibodies

Anti-pFAK (Y576) (#3281; 1:500), FAK (#3258; 1:1000 for immunoblotting/1:200 for immunohistochemistry), pp53 (#2524; 1:1000), p53 (S15) (#9284; 1:500), pPAK1 (T423) (#2610; 1:500), paxillin (Y118) (#2541; 1:500), and pYH2AX (S139) (#9713; 1:200) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-pFAK (Y397) (611806; 1:500), paxillin (610051; 1:1000), GIT2 (P94020; 1:1000), Cdk2 (610145; 1:500), Cdk4 (610147; 1:500), Cyclin D (610279; 1:500), Cyclin E (551159; 1:500), pRB (610884; 1:500), ppRB (610490; 1:500), and p19 (610530; 1:500) antibodies were purchased from BD Biosciences (San Jose, CA). Antibodies against calpain-2 (sc-373966; 1:1000) and calpain-4 (sc-30065; 1:1000), p16 (sc-28260; 1:500 for immunoblotting/1:200 for immunohistochemistry), p21 (sc-6246; 1:500), GIT1 (sc-9657; 1:500), and amphiphysin I (sc-376402 and sc-39028; 1:1000) were purchased from Santa Cruz Biotechnology (Dallas, TX). GFP (NB600-308; 1:1000) for immunoblotting/1:200 for immunohistochemistry) antibody was purchased from Novus Biologicals (Centennial, CO). The active integrin β1 (MAB2079Z; 1:50) and GST (A00895; 1:1000) antibodies were purchased from Merck Millipore (Burlington, MA) and
GenScript (Piscataway, NJ), respectively. Anti–β₃ integrin antibody (ab30394; 1:50), and 6×His-tag (ab18184; 1:1000) antibodies were from Abcam (Cambridge, UK). Anti–βPIX antibody (1:1000 for immunoblotting/1:200 for immunohistochemistry) was raised against the C-terminal region (amino acids 439 to 648) of βPIX.

**Plasmids and DNA constructs**

βPIX constructs, si-rPIX (WT), and si-rPIX (DHmt) were cloned into pHR-CMV SV40 for lentiviral expression. Mutagenesis for si-rPIX (WT) and si-rPIX constructs was performed by using a QuickChange II site-directed mutagenesis kit (Agilent). N-terminal (amino acids 1 to 351) and C-terminal (amino acids 346 to 695) amphiphysin I constructs and calpain-2 were cloned into pGEX4T-1. WT and MT (V392G) constructs of amphiphysin I were cloned into pHR-CMV SV40 for lentiviral expression. shPIXs were cloned to PLKO.1 for lentiviral expression. Calpain-2 was cloned into pGEX4T-1. Calpain-2 complementary DNA was purchased from OriGene. GIT1-CT (amino acids 376 to 770) was cloned into pGEX4T-1 for bacterial expression and pHR-CMV SV40 for lentiviral expression, respectively.

**In vivo delivery of siRNA, lentivirus, or transferrin**

The intratracheal delivery technique was modified from a previous procedure (22). Briefly, mice were anesthetized with avertin (2, 2, 2-tribromoethanol, 0.45 mg/g of body weight) by intraperitoneal injection and placed on a platform that holds the mouse’s top front teeth on the bar. A 2.54-cm, 22-gauge Safelet IV catheter with blunted needle was located into the trachea by peering into the mouth and looking for white light emission from the trachea. After ensuring that the catheter was in the trachea, the blunted needle was removed from the catheter. The Invivofectamine–RNA interference complex (75 μl) was instilled in the trachea, the blunted needle was removed from the catheter, and the catheter was in the trachea, the blunted needle was removed from the catheter. The Invivofectamine–RNA interference complex (75 μl of liposomes) was prepared according to the manufacturer’s protocol, and lentivirus particles or Alexa Fluor 594–conjugated transferrin was pipetted directly into the opening of the catheter.

**Cell culture**

HDF (human diploid fibroblast) and 293T cells were cultured in DMEM supplemented with 10% FBS and antibiotics in a 5% CO₂ incubator at 37°C.

**SA-β-Gal assay**

SA-β-Gal activity was measured at pH 6.0 as described (19) with slight modifications. Briefly, cells were washed with phosphate-buffered saline (PBS), fixed with 3% formaldehyde for 5 min, and washed with PBS. Cells were then incubated in SA-β-Gal staining solution (Sigma-Aldrich) for 13 to 14 hours at 37°C and then stained with Hoescht 33258 for 30 min to count the cell number. Cellular senescence was scored as a percentage of SA-β-Gal–positive cells (blue staining) relative to the total cell number. For tissues, animals were anesthetized and perfused with saline. Tissues were flash-frozen in liquid nitrogen and embedded in optimal cutting temperature compound. Tissues were immediately cut into 10-μm sections, fixed with 1% formaldehyde in PBS, washed with PBS, and incubated in SA-β-Gal staining solution for 13 to 14 hours at 37°C. After incubation, the nuclei were stained with Safranin-O and mounted with VECTASHIELD mounting medium (Vector Laboratories Inc., Burlingame, CA).

**In vitro binding assay**

GST–tagged or 6×His–tagged proteins were purified with glutathione or Ni–nitrilotriacetic acid affinity chromatography, respectively. Purified proteins were incubated on binding buffer [20 mM Hepes (pH 7.4), 0.15 M NaCl, 1 mM dithiothreitol, 0.2% Triton X-100, 5 μM MgSO₄, and protease inhibitor] for 30 min at room temperature. Beads were washed five times with binding buffer, then subjected to SDS–polyacrylamide gel electrophoresis (PAGE), and immunoblotted with specific antibody.

**FA analysis**

HDF cells were plated on fibronectin-coated coverslips. After 1 day, cells were transfected with siRNAs, then treated with control or RGD peptides for 3 days, and stained with antibody for paxillin and Alexa Fluor 568–conjugated phalloidin. FAs were observed and photographed by Olympus FluoView FV1000, or 1X81-ZDC inverted microscope (Olympus, Japan) equipped with a cool charge-coupled device camera, Cascade 512B (Photometrics). FA number and actin intensity were determined using ImageJ software.

**Immunohistochemistry**

Tissues were fixed with 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Sections (4 μm thick) were cut from formalin-fixed, paraffin-embedded tissue blocks. After deparaffinization, slides were subjected to an antigen retrieval procedure in 10 mM sodium citrate buffer (pH 6.0) for 10 min using a pressure cooker (Decloaking Chamber, Biocare Medical), followed by incubation with blocking solution [0.3% Triton X-100, 1% bovine serum albumin (BSA), 0.05% Tween 20, 0.1% cold-water fish gelatin, and 0.05% sodium azide in PBS] for 1 hour at room temperature. Primary antibodies were incubated with the sections overnight at 4°C. After five washes with 0.1% Tween 20, 0.1% BSA in PBS, slides were incubated with an Alexa Fluor–conjugated secondary antibody (1:200) for 1 hour in a dark chamber at room temperature. After several washes, slides were counterstained with Hoescht 33258 and mounted with VECTASHIELD mounting medium (Vector Laboratories Inc.). For diaminobenzidine–HCl (DAB) staining, slides were incubated in methanol containing 0.3% hydrogen peroxide for 20 min at room temperature to block endogenous peroxidase activity before applying blocking solution. Slides were then incubated with a biotin-conjugated secondary antibody for 30 min at room temperature and lastly with peroxidase-conjugated streptavidin for 30 min at room temperature. Peroxidase activity was detected using the substrate DAB. For negative controls, sections were treated with tris-buffered saline (TBS) without a primary antibody. For histologic evaluation, sections were stained with hematoxylin and eosin.

**Immunocytochemistry**

Cells were fixed for 15 min with 3.7% paraformaldehyde, permeabilized for 5 min with 0.2% Triton X-100, and blocked for 30 min at 25°C with 2% BSA in PBS. For antigen staining, cells were incubated with a primary antibody for 1 hour at 25°C, followed by incubation with a secondary Alexa Fluor–conjugated antibody for 1 hour. To visualize F-actin, cells were stained with Alexa Fluor 568–conjugated...
Immunoblotting and immunoprecipitation

Cells were lysed with cold lysis buffer [50 mM Heps (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 500 μM EDTA, 200 μM sodium pyruvate, and 50 mM β-glycerophosphate], and supernatants immunoprecipitated with the indicated primary antibody for 18 hours at 4°C. Immunoprecipitates were fractionated by 8 to 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane in a tris-glycine-methanol buffer (25 mM tris base, 200 mM glycine, and 20% methanol). Membranes were blocked with 3% BSA in TBS (TBS-T; 50 mM tris, 150 mM NaCl, and 0.1% Tween 20) for 30 min, incubated with a primary antibody for 1 hour at room temperature, and washed three times with TBS-T. Membranes were then incubated with a secondary horseradish peroxidase-conjugated antibody for 1 hour at room temperature and washed three times with TBS-T. Signals were detected using an enhanced chemiluminescence reagent.

Transferrin endocytosis

Cells were incubated on ice for 10 min in cold live cell image solution [LCIS; 140 mM NaCl, 20 mM Heps, 2.5 mM KCl, 1.8 mM CaCl2, and 1.0 mM MgCl2 (pH 7.4)] containing 20 mM glucose and 1% BSA. Cells were incubated with Alexa Fluor 594–transferrin (20 μg/ml) in LCIS containing 20 mM glucose and 1% BSA for 15 min at 37°C, washed with PBS, and fixed with 4% paraformaldehyde for 10 min. To analyze transferrin endocytosis in mouse lung, 0.1% BSA. Cells were incubated with Alexa Fluor 594–transferrin in mouse lung, 0.1 μM Alexa Fluor 594–transferrin was inserted directly into the opening of the catheter for intratracheal delivery. After 1 hour, animals were perfused with PBS through the heart. Tissues were dissected and flash-frozen in liquid nitrogen and then immediately sliced into a thickness of 10 μm with the cryomicrotome and fixed with cold acetone for 10 min. Sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) and transferred to a polyvinylidene fluoride membrane in a tris-base washing buffer. Membranes were blocked with 3% BSA in TBS and incubated with a primary antibody for 1 hour, washed three times with TBS-T, and incubated with a secondary horseradish peroxidase-conjugated antibody for 1 hour at room temperature and washed three times with TBS-T. Signals were detected using an enhanced chemiluminescence reagent.

Active β1 integrin endocytosis

Starved cells were pretreated with 10 μM nocodazole for 20 min and incubated with anti–active β1 integrin antibody (1:200) for 40 min at 37°C. Unbound antibodies and nocodazole were washed away with PBS, and a 60-min chase was performed. Cells were then washed with warm PBS, followed by acid rinse [0.5% acetic acid and 0.5 M NaCl (pH 3.0)] to remove surface antibodies. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Tween 20. Internalized active β1 integrin was detected by incubation with Alexa Fluor–conjugated secondary antibody (1:200) for 1 hour.

Statistical analysis

Data are expressed as means ± SEM; representative data from at least three independent experiments were analyzed. Statistical significance was assessed by an unpaired Student’s t test (t test), or Wilcoxon-Mann-Whitney test using SigmaPlot (version 12) for Windows. P < 0.05 was considered statistically significant. To incorporate source data, bar graphs were modified by GraphPad Prism software (version 8).
SCIENCE ADVANCES | RESEARCH ARTICLE

25. Y. D. C. Schilder, E. H. Heiss, D. Schachner, J. Ziegler, G. Reznicek, D. Sorescu, V. M. Dirsch, NADPH oxidases 1 and 4 mediate cellular senescence induced by resveratrol in human endothelial cells. *Free Radic. Biol. Med.* 46, 1598–1606 (2009).

26. Y. Wu, S. Liang, Y. Oda, I. Ohmori, T.-I. Nishi, K. Takei, H. Matsui, K. Tomizawa, Truncations of amphiphysin I by calpain inhibit vesicle endocytosis during neural hyperexcitation. *EMBO J.* 26, 2981–2990 (2007).

27. J. S. Park, W. Y. Park, K. A. Cho, D. I. Kim, B. H. Jhun, S. R. Kim, S. C. Park, Down-regulation of amphiphysin-1 is responsible for reduced receptor-mediated endocytosis in the senescent cells. *FASEB J.* 15, 1625–1627 (2001).

28. J. Simon-Santamaria, I. Malovic, A. Warren, A. Oteiza, D. Le Couteur, B. Smidsrød, P. McCourt, K. K. Sorensen, Age-related changes in scavenger receptor-mediated endocytosis in rat liver sinusoidal endothelial cells. *J. Gerontol. A Biol. Sci. Med. Sci.* 65, 951–960 (2010).

29. U. K. Rajarajacholan, S. Thalappilly, K. Riabowol, The ING1a tumor suppressor regulates the on-off relationship of Rho and Rac during integrin-endothelial cells. *FASEB J.* 21, 833–843 (2010).

30. M. J. Schafer, T. A. White, K. Iijima, A. J. Haak, G. Ligresti, E. J. Atkinson, A. L. Oberg, J. Birch, H. Salmonowicz, Y. Zhu, D. L. Mazula, R. W. Brooks, H. Fuhrmann-Stroissnigg, T. Pirtskhalava, Y. S. Prakash, T. Tschonka, P. D. Robbins, M. C. Aubry, J. F. Passos, J. L. Kirkland, D. J. Tschumperlin, H. Kita, N. K. LeBrausser, Cellular senescence mediates fibrotic pulmonary disease. *Nat. Commun.* 8, 14532 (2017).

31. C. D. Lawson, K. Burridge, The on-off relationship of Rho and Rac during integrin-mediated adhesion and cell migration. *Small GTPases* 5, e27958 (2014).

32. T. S. Pavlov, A. Chahdi, D. V. Ilatovskaya, V. Levchenko, A. Vandewalle, O. Pochynyuk, A. Sorokin, A. Staruschenko, Endothelin-1 inhibits the epithelial Na+ channel through IP/14-3-3/Nedd4-2. *J. Am. Soc. Nephrol.* 21, 833–843 (2010).

33. R.-M. Hsu, M.-H. Tsai, Y.-J. Hsieh, P.-C. Lyu, J.-S. Yu, Identification of MYO18A as a novel and inhibits Dbll family guanine nucleotide exchange factors: A possible link to Rho family GTPases. *J. Biol. Chem.* 2981–2990 (2010).

34. A. C. E. Shibata, L. H. Chen, R. Nagai, F. Ishidate, R. Chadda, Y. Miwa, K. Naruse, Y. M. Shirai, T. K. Fujiwara, A. Kusumi, Rac1 recruitment to the archipelago structure of the focal adhesion through the fluid membrane as revealed by single-molecule analysis. *Cytoskeleton (Hoboken)* 70, 161–177 (2013).

35. K. Kuwano, J. Araya, H. Harah, S. Minagawa, N. Takasaka, S. Ito, K. Kobayashi, K. Nakayama, Cellular senescence and autophagy in the pathogenesis of chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF). *Respir. Investig.* 54, 397–406 (2016).

36. M. J. Schafer, T. A. White, K. Iijima, A. J. Haak, G. Ligresti, E. J. Atkinson, A. L. Oberg, J. Birch, H. Salmonowicz, Y. Zhu, D. L. Mazula, R. W. Brooks, H. Fuhrmann-Stroissnigg, T. Pirtskhalava, Y. S. Prakash, T. Tschonka, P. D. Robbins, M. C. Aubry, J. F. Passos, J. L. Kirkland, D. J. Tschumperlin, H. Kita, N. K. LeBrausser, Cellular senescence mediates fibrotic pulmonary disease. *Nat. Commun.* 8, 14532 (2017).

37. R. W. Hurley, J. B. McCarthy, C. M. Verfaillie, Direct adhesion to bone marrow stroma via fibronectin receptors inhibits hematopoietic progenitor proliferation. *J. Clin. Invest.* 96, 511–519 (1995).

38. B. I. Lundell, J. B. McCarthy, N. L. Kovach, C. M. Verfaillie, Activation of μ1 integrins on CML progenitors reveals cooperation between μ1 integrins and CD44 in the regulation of adhesion and proliferation. *Leukemia* 11, 822–829 (1997).

39. B. I. Lundell, J. B. McCarthy, C. M. Verfaillie, Direct adhesion to bone marrow stroma via fibronectin receptors inhibits hematopoietic progenitor proliferation. *J. Clin. Invest.* 96, 511–519 (1995).

40. C.-S. Lee, C.-K. Choi, E.-Y. Shin, M. A. Schwartz, E.-G. Kim, Myosin II directly binds and inhibits Dbll family guanine nucleotide exchange factors: A possible link to Rho family GTPases. *J. Biol. Chem.* 2981–2990 (2010).

41. A. C. E. Shibata, L. H. Chen, R. Nagai, F. Ishidate, R. Chadda, Y. Miwa, K. Naruse, Y. M. Shirai, T. K. Fujiwara, A. Kusumi, Rac1 recruitment to the archipelago structure of the focal adhesion through the fluid membrane as revealed by single-molecule analysis. *Cytoskeleton (Hoboken)* 70, 161–177 (2013).

42. K. Ley, J. Rivera-Nieves, W. J. Sandborn, S. Shattil, Integrin-based therapeutics: Biological basis, clinical use and new drugs. *Nat. Rev. Drug Discov.* 15, 173–183 (2016).

Acknowledgments

Funding: This work was supported, in part, by grants from the National Research Foundation of Korea (2017R1A2B4002661 and 2017R1A2B3005714) and the Bio and Medical Technology Development Program of the NRF funded by the Korean government, MSIP (2017M3A9D8063627), and the USPHS grant RO1 GM47214 to M.A.S. Author contributions: E.-Y.S. designed and conducted most of the biochemical and cell biology experiments and analyzed data. S.-Y.T., J.-H.P., and O.-J.L. conducted IP/1 knockdown experiments in the lung of mice and humans. C.-S.L., S.-Y.W., J.-J.P., and J.S. performed a part of biochemical and cell biology experiments. N.-K.S. prepared lentiviruses for expression of amphiphysin I, si-PIXs, and GIT-CT and performed experiments. M.A.S. and E.-G.K. supervised the entire project and wrote the paper. All authors discussed the results and commented on the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 14 June 2019
Accepted 21 February 2020
Published 6 May 2020
10.1126/sciadv.aay3909

Citation: E.-Y. Shin, J.-H. Park, S.-T. You, C.-S. Lee, S.-Y. Won, J.-J. Park, H.-B. Kim, J. Shim, N.-K. Soyang, O.-J. Lee, M. A. Schwartz, E.-G. Kim, Integrin-mediated adhesions in regulation of cellular senescence. *Sci. Adv.* 6, eaay3909 (2020).