### Highlights
- \( \text{ITGB3} \) (integrin \( \beta 3 \)) is regulated by the Polycomb protein \( \text{CBX7} \)

- \( \beta 3 \) regulates senescence by activating TGF-\( \beta \) in a paracrine and autocrine fashion

- \( \beta 3 \) is highly expressed in OIS and induces senescence via ligand-independent pathway

- There is a positive correlation between \( \beta 3 \) levels and aging in different tissues

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### In Brief
Rapisarda et al. show that integrin \( \beta 3 \) subunit expression induces senescence by activating TGF-\( \beta \), while \( \beta 3 \) knockdown overcomes senescence. \( \beta 3 \) is dynamically upregulated in OIS and has ligand-independent activity. They also find a positive correlation between \( \beta 3 \) levels and aging in a subset of tissues.

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Integrin Beta 3 Regulates Cellular Senescence by Activating the TGF-β Pathway

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SUMMARY

Cellular senescence is an important in vivo mechanism that prevents the propagation of damaged cells. However, the precise mechanisms regulating senescence are not well characterized. Here, we find that ITGB3 (integrin beta 3 or β3) is regulated by the Polycomb protein CBX7. β3 expression accelerates the onset of senescence in human primary fibroblasts by activating the transforming growth factor β (TGF-β) pathway in a cell-autonomous and non-cell-autonomous manner. β3 levels are dynamically increased during oncogene-induced senescence (OIS) through CBX7 Polycomb regulation, and downregulation of β3 levels overrides OIS and therapy-induced senescence (TIS), independently of its ligand-binding activity. Moreover, cilenitide, an αvβ3 antagonist, has the ability to block the senescence-associated secretory phenotype (SASP) without affecting proliferation. Finally, we show an increase in β3 levels in a subset of tissues during aging. Altogether, our data show that integrin β3 subunit is a marker and regulator of senescence.

INTRODUCTION

Cellular senescence is characterized by a proliferative arrest induced to prevent the propagation of damaged cells in a tissue. This arrest is mainly driven by the activation of two important pathways, p53/p21CIP1 and RB/p16INK4A. The senescence program can be triggered by a number of stressors, like the activation of oncogenes (termed oncogene-induced senescence; OIS), drug treatment (therapy-induced senescence; TIS) or deregulation of Polycomb Repressive Complex 1 (PRC1) proteins, including the polycomb protein chromobox 7 (CBX7) (Gil and O’Loghlen, 2014; Salama et al., 2014). Although arrested, senescent cells are metabolically and transcriptionally functional, and they actively communicate with their surroundings (Pérez-Mancera et al., 2014). In fact, senescent cells secrete an array of inflammatory proteins, growth factors, and metalloproteases that collectively constitute the SASP (senescence-associated secretory phenotype). The SASP recruits the immune system in order to eliminate senescent cells and induces changes in the extracellular matrix (ECM), thus facilitating tissue homeostasis and regeneration. The presence of senescent cells has been found in vivo in preneoplastic lesions (Muñoz-Espin and Serrano, 2014), in wound healing (Jun and Lau, 2010), during embryonic development (Muñoz-Espin et al., 2013; Storer et al., 2013), and in different tissues throughout aging (Baker et al., 2016; Krishnamurthy et al., 2004; Ressler et al., 2006). Interestingly, a recent study has demonstrated that p16INK4A-positive cells accumulate during aging and contribute to age-related dysfunctions in different tissues. Thus, the elimination of senescent cells reverses the aging phenotype and stimulates tissue regeneration, demonstrating that the activation of senescence is a direct cause of aging and opening avenues for targeting senescent cells as a therapy to extend healthy lifespan (Baker et al., 2016).

Senescence activation is governed by intracellular and extracellular signals and highly depends on the interaction of the cells with ligands in the ECM (Gutiérrez-Fernández et al., 2015; Jun and Lau, 2010; Parrinello et al., 2005). The most common ECM-receptor interaction proteins are integrins, which are heterodimeric cell-surface transmembrane receptors that provide cellular adhesion (Hynes, 2002). Upon ligand binding, intracellular proteins are recruited to clusters of integrin heterodimers in the plasma membrane, forming focal adhesion (FA) complexes, which mediate downstream signals to the cell. Integrin signaling affects numerous cellular processes, including cell adhesion, migration, proliferation, survival, and differentiation (Seguin et al., 2015) and, thus, has key roles during development, immune responses, and different pathologies such as cancer (Legate et al., 2009). Interestingly, integrins can also mediate signaling cascades independent of ligand binding. In fact, integrin αvβ3 can induce apoptosis (Stupack et al., 2001), tumor progression (Desgroisellier et al., 2009), and tumor stemness (Seguin et al., 2014) in an anchorage-independent manner.

Here, we establish integrin beta 3 (β3) subunit as a marker and regulator of senescence. Our findings highlight the importance of the β3 subunit signaling in regulating senescence by activating the transforming growth factor β (TGF-β) pathway in an autocrine and paracrine fashion. We found that β3 levels are dynamically upregulated during OIS, by CBX7 transcriptional regulation.
and that αvβ3 regulates OIS independently of its ligand-binding activity. Importantly, an αvβ3 antagonist exerts dual activity by regulating interleukin (IL)-6/IL-8 secretion but not the growth arrest during OIS. Additionally, we found an increase in the levels of β3 during aging in a subset of mouse tissue and human samples, where the manipulation of β3 levels in fibroblasts derived from old human donors overcomes the accumulation of different markers of senescence and aging. Our results demonstrate the importance of cellular adhesion during senescence and identify integrins as potential therapeutic targets during early carcinogenesis and aging.

RESULTS
SILAC Screen Identifies Proteins Grouped in the ECM-Receptor Interaction Pathway as Putative Regulators of Senescence
We have previously shown that CBX7 loss of function by short hairpin RNA (shRNA) in primary cells induces cellular senescence (Gil and O’Loghlen, 2014; O’Loghlen et al., 2015a, 2015b) (Figures 1A and 1B). This has been primarily attributed to the derepression of the CDKN2A locus, which encodes the cell-cycle inhibitor p16INK4A. As PRC1 targets multiple genes, and we see additional markers of senescence present upon CBX7 knockdown (Figures 1B and S1A), we hypothesized that other unknown regulators could be inducing senescence in this model. To this end, we performed a quantitative proteomic analysis to determine changes occurring upon CBX7 knockdown in human primary fibroblasts, taking advantage of the SILAC (stable isotope labeling by amino acids in culture) technology. Human primary breast fibroblasts (BFs) transduced with an shRNA targeting CBX7 (shCBX7) were grown in media supplemented with “heavy” (forward experiment)- or “light” (reverse experiment)-labeled amino acids and compared with BFs expressing an empty vector (Figure S1B). Combination of the results from both forward and reverse experiments show 82 proteins with a 2-fold expression difference in both experiments, including CDKN2A (Figure 1C). Annotation of differentially expressed proteins into functional pathways (Kyoto Encyclopedia of Genes and Genomes; KEGG) shows the ECM-receptor-interacting and focal adhesion pathways upregulated upon CBX7 knockdown (Figure 1D). As the SILAC screen was performed in a CBX7-depleted background, we hypothesized that the genes encoding the proteins found in the SILAC screen could be regulated by CBX7. Thus, we compared the SILAC data with a published
genome-wide binding profile for CBX proteins (chromatin immunoprecipitation sequencing; ChIP-seq) in human fibroblasts (Pemberton et al., 2014). We found 20 proteins whose genes are potential targets for CBX proteins, including CDKN2A (Figures 1E and 1F). In fact, knockdown of CBX7 led to more than 2-fold upregulation of the mRNA levels of the majority of the 20 genes, as shown by qPCR (Figures 2A and S1C), while overexpression of murine Cbx7 resulted in gene silencing and transcriptional repression (Figures 2B and S1D). This was repeated using a different strain of human fibroblast, IMR-90 (Figure S1E). To confirm that these genes are regulated by CBX7, we performed ChIP for endogenous CBX7 in BFs and analyzed the enrichment of CBX7 at the transcription start site (TSS) of the 20 genes. Our data show enrichment for CBX7 at the TSS of the analyzed genes, including INK4A (encoding p16\textsuperscript{INK4A}), but not the negative controls ARF (encoding p14\textsuperscript{ARF}) or ACTB (encoding β-actin) (Figure 2C).

**ITGB3 Is Regulated by PRC1**

Among the potential inducers of senescence regulated by CBX7, we focused on ITGB3 (which encodes for the integrin β3 subunit) because: (1) it is a component of the two most representative pathways upon CBX7 knockdown in the SiLAC; (2) we found CBX7 at the TSS of ITGB3 in the ChIP-seq dataset; and (3) it is the gene that is most upregulated upon CBX7 knockdown. Interestingly, two additional PRC1 proteins, CBX8 and RING1B, were also found at the TSS of the ITGB3 locus (Figure 2D), further supporting the regulation of ITGB3 by PRC1. To confirm whether the latter changes at the mRNA level correlate with protein levels, we checked the levels of the integrin heterodimer αvβ3 by immunofluorescence (IF) (Figure 2E) and the β3 subunit by immunoblot upon CBX7 knockdown (Figure S1F) or Cbx7 ectopic expression (Figure 2F). Importantly, we observed that shCBX7 increases the number of cells presenting αvβ3-stained FA complexes by IF (Figure 2E). The regulation of β3 protein levels by CBX7 was also confirmed in IMR-90 fibroblasts (Figure S1G). Altogether, these data show that the ITGB3 locus is regulated by the Polycomb protein CBX7 in human primary fibroblasts.

**ITGB3 Induction of Senescence Is Dependent on the p53/p21\textsuperscript{CIP} Pathway**

The aforementioned findings suggest that ITGB3 could be a regulator of cellular senescence. Indeed, expression of a retroviral vector encoding ITGB3 in BFs reduces their proliferation rate, quantified by measuring the percentage of cells incorporating bromodeoxyuridine (BrdU) (Figure 3A). A retroviral construct expressing the oncogene H-Ras\textsuperscript{G12V} (RAS) was used as a positive control (Serrano et al., 1997). Concomitant with the growth arrest, we observed an increase in the protein levels of the cell-cycle inhibitors p21\textsuperscript{CIP} and p53 by IF (Figure 3B) and CDKN2B mRNA levels by qPCR, with no changes observed in CDKN2A or CDKN1B (Figure 3C). Consistent with the activation of senescence, ITGB3 expression led to an increase in the number of cells staining positive for senescence-associated β-galactosidase (SA-β-Gal) activity (Figure 3D), an accumulation of reactive oxygen species (ROS) (Figure 3E), and a mild increase in the mRNA levels of different SASPs (Figure 3F). However, we failed to observe a DNA-damage response upon ITGB3 expression (Figure S2A). Importantly, ITGB3 expression also induced senescence in IMR-90 fibroblasts, indicating that this response is not strain specific (Figures S2B–S2D). We confirmed that the activation of senescence by ITGB3 expression in BFs is dependent on the p53 pathway. Using a previously characterized shRNA targeting TP53 (shp53) (Acosta et al., 2008), we impaired not only the proliferation arrest induced by ITGB3 (Figure 3G, left panel) but also the increase in SA-β-Gal activity (Figure 3G, right panel). The use of a short interfering RNA (siRNA) targeting TP53 (sip53) also impaired the growth arrest induced by ITGB3 expression (Figure S2E). Thus, ITGB3 ectopic expression induces senescence in human primary fibroblasts, which is dependent on the p53/p21\textsuperscript{CIP} pathway.

**ITGB3 mRNA Levels Are Dynamically Regulated during Senescence**

We next decided to determine whether ITGB3 was endogenously regulated during senescence. As OIS is a potent tumor suppressor mechanism both in vitro and in vivo (Muñoz-Espir and Serrano, 2014), we used BFs stably expressing the oncogene RAS. Staining for αvβ3 by IF shows a substantial increase in its expression levels upon RAS induction. In addition, αvβ3 co-localizes with F-actin, indicating that it is part of FA complexes during RAS activation (Figure 4A). We confirmed that β3 protein and transcript levels are endogenously upregulated upon RAS expression compared to the vector control in an additional strain of human and also in mouse fibroblasts (Figure S3A). We also observed the upregulation of β3 in BFs during DNA-damage-induced senescence (DDIS) induced by etoposide treatment (Figures 4B and S3B). Moreover, treatment of two different cancer cell lines, MCF7 (breast) and SK-HEP-1 (liver) (Bollard et al., 2016), with a CDK4/6 inhibitor (Palbociclib or Palb), mimicking TIS, also triggered endogenous upregulation of β3 (Figures 4C, S3C, and S3D). Intriguingly, the upregulation of ITGB3 mRNA levels in SK-HEP-1 cells could only be observed after 10 days of treatment with Palbo, concomitant with the establishment of senescence. To determine the temporal changes of ITGB3 during senescence, we took advantage of IMR-90 fibroblasts expressing an endoplasmic reticulum (ER):RAS fusion protein (ER:RAS). Upon treatment with 4-hydroxytamoxifen (4OHT), senescence is progressively established, displaying an initial mitotic arrest followed by full senescence induction after 4–6 days treatment. Though the mRNA levels of ITGB3 at early timepoints of the induction of senescence were downregulated, we did observe a consistent upregulation of ITGB3 during the establishment of senescence (Figure S3E). This pattern highly resembles the recently described temporal changes induced by NOTCH1 during OIS (Hoare et al., 2016).

As integrins play a predominant role in cellular signaling and adhesion, we next investigated whether other integrin beta subunits were deregulated during OIS. To avoid the confounding effects of integrin changes during the initial phases of the establishment of senescence, we decided to measure the mRNA expression levels of ITGB1-8 using BFs stably expressing RAS. Our data show a noticeable deregulation of integrin beta subunits during OIS, with upregulation of ITGB1, 3, 4, and 6 during...
RAS expression, ITGB3 being the subunit most upregulated (Figure 4D). Therefore, we show that ITGB3 mRNA levels increase concomitantly with the establishment of senescence.

CBX7 Regulates ITGB3 Locus during OIS
Since ITGB3 locus is regulated by CBX7 (Figure 2), we reasoned that the endogenous upregulation of ITGB3 mRNA upon RAS
Figure 3. ITGB3 Ectopic Expression Induces Senescence via p21CIP/p53 Pathway

(A–F) Overexpression of a retroviral construct encoding ITGB3 in BFs induces senescence. H-RasG12V (RAS) is used as a positive control for inducing senescence. (A) We show a reduction in proliferation in BFs expressing ITGB3 by measuring the percentage of cells incorporating BrdU (left panel: quantification levels; right panel: representative pictures). Proliferation was assessed 4–5 days after plating. BFs expressing ITGB3 show (B) an increase in p21CIP and p53 protein (legend continued on next page).
expression could be due to epigenetic regulation by CBX7. To test this hypothesis, we performed ChIP for endogenous CBX7 on the ITGB3 locus in BF5s transduced with vector or RAS. Our data show a reduced binding of CBX7 to the ITGB3 TSS during OIS (Figure 4E), suggesting that the endogenous upregulation of ITGB3 during OIS is due to the transcriptional deregulation of the locus by the loss of CBX7 binding. As expected, we did not observe changes in CBX7 binding in vector or RAS BF5s in a control-coding region (Figure 4E).

**β3 Regulates Senescence Independently of Its Binding Activity**

To test whether β3 has a functional role during senescence, we manipulated ITGB3 mRNA levels during OIS and TIS, once senescence was fully established. The transduction of BF5s expressing RAS with an shRNA targeting ITGB3 (shITGB3) or transfection with two different siRNAs (siITGB3) impaired the proliferation arrest induced by RAS (Figures 4F, S3F, left panel, and S3G), and partially reverted the increase in the cell-cycle inhibitor p21Waf1/CIP1 induced upon OIS, as shown by IF (Figure S3F, right panel). Furthermore, ablation of ITGB3 mRNA by siRNA also overcame the proliferation arrest induced by Palb treatment in MCF7 cells (Figure 4G). We next treated BF5s stably expressing RAS with the βvβ3/βvβ5 antagonist cilenitide, as we reasoned that inhibiting βvβ3 could be a therapeutic treatment to overcome senescence. Surprisingly, treatment of BF5s expressing RAS with cilenitide could not reverse the proliferation arrest (Figure 4H) or the upregulation of p21Waf1/CIP1 or p16INK4A protein levels (Figure S4A). This suggested that β3 induces senescence independently of its ligand-binding activity, which was further confirmed by the ectopic expression of a mutant β3, defective for the ligand-binding domain (ITGB3ΔT119A) (Loftus et al., 1990) that also induced senescence (Figures S4B–S4E). Excitingly, although cilenitide treatment during OIS could not reverse the proliferation arrest induced by RAS or the upregulation of p16INK4A or p21Waf1/CIP1 protein levels, we did observe a significant reduction of the SASP, as shown by measuring the levels of IL-8 and IL-6 secreted to the supernatant by immunoblot (Figure 4I). Therefore, βvβ3 inhibition is able to uncouple the SASP release from the proliferation arrest in OIS.

**β3 Regulates Cellular Senescence in a Cell-Autonomous Fashion by Activating the TGF-β Pathway**

To determine the pathway by which β3 induces senescence in BF5s, we next used a panel of small molecule inhibitors. We assessed proliferation levels by quantifying BrdU incorporation from the proliferation arrest in OIS. Therefore, β3 inhibition is able to uncouple the SASP release from the proliferation arrest in OIS.

**Non-Cell-Autonomous Effect of β3 on Human Primary Fibroblasts**

Integrins can activate TGF-β embedded in the ECM by increasing the expression of matrix-degrading enzymes (matrix metalloproteinases; MMPs) and the proteolytic release of TGF-β to the media. Therefore, we determined the expression levels by IF 4–5 days after plating (left panel: percentage of cells stained positive for p21Waf1/CIP1 and p53; right panel: representative pictures for p21Waf1/CIP1 staining) and (C) an increase in CDKN2A (encoding p16INK4A) mRNA levels by qPCR. No changes were observed in CDKN1B (encoding p21Waf1/CIP1) or CDKN2A (p16INK4A). (D) Expression of ITGB3 also induced an increase in senescence-associated β-galactosidase activity (SA-β-Gal). Data represent the percentage of cells staining positive for SA-β-Gal ± SD. Staining was performed 7–10 days after plating; (E) an increase in the levels of ROS, measured by 8-oxoG staining, and (F) a mild increase of the mRNA levels of different SASP by qPCR. Cells were subjected to analysis (either by IF or qPCR) 4–5 days after plating.

(G) An shRNA against p53 (shp53) prevents the activation of senescence induced by ITGB3 ectopic expression, as shown by the reversion in the percentage of BF5s incorporating BrdU induced by ITGB3 (left graph) and the decrease in SA-β-Gal activity (right graph). BrdU was added 24 hr prior to fixing the cells for IF. Data represent the mean ± SD of more than two independent experiments. Scale bars, 100 μm. *p < 0.05; **p < 0.01; ***p < 0.001.
A. Oncogene-induced senescence
IF: αvβ3/F-Actin staining

BF fibroblasts

2.2% ± 0.4
36% ± 5*

B. DNA-damage senescence

ITGB3

mRNA levels

DMSO Etop

C. Therapy-induced senescence

MCF-7 cells

Palbo β3 subunit p53 β-Actin

D. Integrin subunit β expression

mRNA levels

ITGB1 ITGB2 ITGB3 ITGB4 ITGB5 ITGB6 ITGB7 ITGB8

Vector RAS

DMSO Etop

E. ChIP at ITGB3 locus

Coding TSS

Vector RAS Vector RAS

IgG CBX7

F. Knockdown of ITGB3 overcomes OIS

Infection Infection Experiment

Plate Readout

Days 0 7 14-21 +4-5

Proliferation

Relative cell number

Vector RAS

β3 subunit β-Actin

G. BrdU

% positive cells

DMSO Palbo

Scr silITGB3, 3 silITGB3, 4

H. ITGB3 bypass of OIS is ligand-independent

Proliferation

Relative cell number

DMSO αvβ3 inh. TGFBR1

I. Conditioned Media (CM)

RAS αvβ3 inh.

IL-8 IL-6 Coomassie

(legend on next page)
levels of different MMPs in BFs expressing ITGB3 and found up-regulation of the mRNA levels of MMP1 and MMP9, indicating that ITGB3 expression can directly activate the TGF-β pathway (Figure 5G). We next tried to determine whether TGF-β was being released to the supernatant in BFs expressing ITGB3 and had a non-cell-autonomous role on surrounding cells. While we could not detect TGF-β in the supernatant (data not shown), we did find that the conditioned media (CM) from cells expressing ITGB3 had an effect on normal BFs by inducing the stabilization of p53 protein (Figure 5H), the nuclear translocation of SMAD2/3 (Figure 5I), and a reduced proliferation rate (Figure S5C) in normal BFs. Furthermore, treatment with a pan-specific neutralizing anti-TGF-β1-3 antibody (Figure 5H) or an inhibitor for TGFBR1 (Figure 5I) abrogated the effect of the CM from ITGB3 cells, suggesting that the non-cell-autonomous effect of ITGB3 cells is dependent on TGF-β.

β3 Subunit Expression Increases during Replicative Senescence

Activation of senescence has been described in a variety of physiological and pathological conditions, including aging. In fact, the activation of cellular senescence is considered one of the hallmarks of aging (López-Otín et al., 2013). In order to determine whether the β3 subunit is upregulated during aging, we used a retroviral construct encoding the dominant-negative allele of the telomeric repeat binding factor 2 (TRF2ΔM). Expression of TRF2ΔM in primary fibroblasts rapidly mimics the process of replicative senescence and aging (Karseder et al., 1999). Similar to our previous results, where β3 is upregulated in senescence, TRF2ΔM-expressing BFs presented an increase in β3 subunit (Figure 6A). This was further confirmed in murine hepatic stellate cells (mHSCs) extracted from an adult mouse harboring a doxycycline (Dox)-inducible construct to express shp53 (Lujambio et al., 2013). Upon Dox withdrawal, senescence is induced by re-expression of p53 and mHSCs showed an increase in a number of markers of senescence (Figure S6A), concomitant with the accumulation of Itgb3 (Figure 6B).

ITGB3 mRNA Levels Are Dynamically Upregulated during Aging in Mice

To determine whether Itgb3 expression is changing during aging in vivo, we extracted RNA from liver tissue of C57BL/6J female mice aged 4, 19, and 25 months. Livers from mice aged 19 months presented a dynamic increase at the mRNA levels of Cdkn2a and Itgb3, but the highest increase was observed in 25-month-old mice, where additional markers of senescence were observed (Figures 6C and S6B). This is in agreement with our in vitro data that show a concomitant upregulation of ITGB3 with CDKN2A mRNA levels (Figure S3E). Upregulation of Itgb3 mRNA and other markers of senescence were also observed in kidney (Figures 6D and S6B) and, to a lesser extent, in the intestine in 25-month-old mice (Figures S6B and S6C).

β3 Subunit and TGF-β Components Are Highly Expressed in Fibroblasts Derived from Old Human Donors

Next, we took advantage of primary skin fibroblasts derived from young (<10 years old) and old (>80 years old) human donors and tested whether a correlation between senescence and β3 subunit existed during aging (Figure S6D). In order to confirm that fibroblasts derived from old donors behaved as senescent cells, we analyzed different senescence markers. We could, indeed, observe that fibroblasts derived from old donors presented a reduced proliferative capacity measured by relative cell number (Figure S6E) and had a significant increase in p16^INK4A and p21^CIP protein levels, as measured by IF, compared to young donor cells (Figure S6F). We then analyzed the expression levels of β3 subunit between fibroblasts from young and old donors and observed an increase in β3 by immunoblotting (Figure S6E), an increase in the percentage of αvβ3 staining in FA complexes by IF (Figures 6F and S6G), and an increase in ITGB3 at the RNA level.
level (Figure S6H) in fibroblasts from old compared to young donors. We have previously shown that activation of senescence by β3 is dependent on the TGF-β pathway. To determine whether the same activation pathway applies to fibroblasts derived from human donors, we analyzed the expression levels of different regulators of the TGF-β pathway. Interestingly, we could also observe an increase in the mRNA levels of different regulators of the TGF-β pathway, including TGF-β receptors 1 and 2 and SMAD3 and 4 (Figure 6G). Altogether, these data show the existence of a positive correlation between senescence and the expression levels of β3 subunit and different regulators of the TGF-β pathway in aging.

**ITGB3 Plays a Role in Aging in Fibroblasts Derived from Old Human Donors**

We next decided to manipulate the expression levels of ITGB3 in fibroblasts derived from young and old donors to identify whether ITGB3 plays a role in this model. As expected, ectopic expression of either ITGB3 or RAS in fibroblasts derived from two different young donors induced senescence-like growth arrest, as observed by a reduction in the percentage of cells incorporating BrdU (Figure 7A) and an upregulation of cells staining positive for p21CIP (Figure 7B). We next decided to determine whether reducing the endogenous levels of ITGB3 mRNA in cells derived from old donors could attenuate aging. To this end, we chose the two fibroblasts from old donors that expressed the highest levels of β3, and we reduced ITGB3 expression levels using RNAi. Transfection with two different siRNAs (siITGB3) and sip3, overcame the proliferation arrest characteristic of old fibroblasts (Figure 7C) and partially reverted the increase in the cell-cycle inhibitor p21CIP (Figure 7D). This was further confirmed using shITGB3 (Figure S7A). As our previous data show that ITGB3 induces senescence independently of its ligand-binding activity, we investigated whether this mechanism was conserved during aging. Treatment of old donor cells with ciliogtide could not attenuate senescence, neither the proliferation arrest nor p21CIP upregulation (Figures 7E and 7F), suggesting that the role for ITGB3 in aging in human primary fibroblasts is independent of its ligand-binding activity. Altogether, these data suggest that ITGB3 is a regulator of aging in the human primary fibroblasts derived from old donors in this study.

**DISCUSSION**

Intercellular communication is an important feature to maintain tissue homeostasis, where the activation of cellular senescence plays a crucial role. In fact, previous reports have found ECM remodeling to regulate fibrosis by activating the senescence program (Jun and Lau, 2010; Krizhanovsky et al., 2008; Lujambio et al., 2013). Apart from inflammation and ECM remodeling, cells can communicate via the secretion of extracellular vesicles (Tkach and Thery, 2016), cell–cell contact (Hoare et al., 2016), or intercellular protein transfer (Biran et al., 2015). Here, we provide evidence that the integrin β3 subunit plays a role in senescence through activation of the TGF-β pathway.

A great deal of information exists regarding the biological function of integrins and their regulation of the microenvironment, but relatively little is known about the transcriptional regulation of integrins themselves. A recent report has found that MYC overexpression leads to a direct downregulation of ITGB3, inducing decreased motility and invasiveness (Liu et al., 2012). Our results further add PRC1 complex as a regulator of the ITGB3 locus in normal fibroblasts and for CBX7 during OIS, where CBX7 binding to the ITGB3 TSS is reduced. In fact, ITGB3 has been previously identified as a Polycomb target by ChIP sequencing in other biological contexts, such as mouse embryonic stem cells (ESCs) (Morey et al., 2013) and human primary fibroblasts (Pemberton et al., 2014), suggesting that the ITGB3 locus is epigenetically regulated in several biological contexts.

Integrin signaling regulates diverse functions in cancer, angiogenesis, stemness, and drug resistance (Desgrozeller and Cheresh, 2010). In addition, integrins also regulate fibrosis and wound healing (Margadant and Sonnenberg, 2010). Our findings establish the β3 subunit as a regulator for cellular senescence. We show that β3 subunit expression accelerates the onset of senescence in human primary fibroblasts, which is dependent on the...
activation of the p21\textsuperscript{CIP}/p53 pathways. This is complementary with a previous study, which shows activation of fibroblast senescence by the ECM protein CCN1 that binds to α6β1, activating ROS production (Jun and Lau, 2010). Our results also show a robust expression of β3 upon senescence activation induced by a variety of stimuli, while interference with its expression levels disrupts the senescence phenotype. In contrast, it was found that αvβ3 expression escapes OIS in glioblastoma by activating PAK4 (Franovic et al., 2015) and that mice expressing β1-deficient tumors show reduced tumor burden and activation of senescence (Kren et al., 2007). Furthermore, mice lacking β3 accelerate wound-healing closure, which could be by restricting the induction of senescence. However, in contrast with our findings, the authors observed an increase in TGF-β signaling in β3 null mice (Reynolds et al., 2005). All these seemingly paradoxical behaviors of integrin-signaling activity could be due to differences in the cellular and environmental contexts during senescence activation, as it has been previously described for H-Ras\textsuperscript{G12V} (Serrano et al., 1997) and the chemokine receptor CXCR2 (Acosta et al., 2008).

Cellular adhesion is a key feature of senescence. In agreement with our results, several reports have found differential expression of integrins during cellular senescence activation. Analysis of published datasets show that the “cellular adhesion” pathway and integrins are differentially expressed during senescence activation (Fridman and Tainsky, 2008; Storer et al., 2013). Likewise, a number of studies have found that TGF-β ligands are part of the SASP and play an important role in senescence through p21\textsuperscript{CIP} regulation, in agreement with our data (Acosta et al., 2013; Hoare et al., 2016; Muñoz-Espín et al., 2013; Storer et al., 2013). The TGF-β superfamily controls numerous cellular and biological processes, such as development, regeneration, fibrosis, and cancer (Macias et al., 2015). Accumulating evidence indicates that a cross-talk between integrins and TGF-β exists, in particular to regulate fibrosis, wound healing, and cancer (Asano et al., 2005; Margadant and Sonnenberg, 2010). However, even if senescence is known to regulate all these biological processes, none of these studies have reported the existence of a cross-talk between integrins and TGF-β in senescence or aging. Our data show that β3 regulates senescence by activating TGF-β via cell-autonomous and
non-cell-autonomous mechanisms. The use of small molecule inhibitors, RNAi technology, and the analysis of the expression levels of various members of the TGF-β pathway authenticate a role for TGF-β during senescence induced by ITGB3 expression.

Different reports have found that there is cross-talk between integrins and chemokine receptors (Desgrosellier and Cheresh, 2010). Although we could not detect any changes in the mRNA expression levels of CXCR2 in cells expressing ITGB3 (data not shown), it would be interesting to further investigate a potential connection in senescence.

Senescence regulates tissue-regenerative capacity and homeostasis. In fact, αvβ3 expression is increased in a number of cell types undergoing tissue remodeling (Asselin-Labat et al., 2007; Brooks et al., 1994). Furthermore, integrins can direct specific stemness-related reprogramming, providing an important role during development independent of their ligand-binding activity (Seguin et al., 2015). Interestingly, developmental senescence is activated to promote tissue remodeling and stem cell renewal (Muñoz-Espín and Serrano, 2014). Our data show that senescence induced by β3 presents a similar pattern to developmental senescence (activation of p21<sup>CIP</sup>, TGF-β/SMAD, and no DNA damage) and that it is independent of ligand binding. It would be interesting to investigate whether integrins also play a role in this context.

Our data show an increase in the expression levels of Itgb3 mRNA concomitant with an increase in different markers of senescence in tissue from old mice. Upregulation of β3 and senescence/aging markers, including TGF-β members, was further observed in fibroblasts from old human donors. This is in accordance with previous reports, which have found that p16<sup>INK4A</sup> levels correlate with chronological age in most tissues analyzed, both in mice (Baker et al., 2016; Krishnamurthy et al., 2004) and in humans (Reissler et al., 2006). Interestingly, knockdown of ITGB3 mRNA partially reversed the aging phenotype of fibroblasts derived from old human donors. However, the αvβ3 antagonist, cilengitide, could not reverse aging, suggesting that the role for β3 in this cellular system is independent of its ligand-binding activity.
activity. Our data show that cilengitide has a diverse effect on the SASP and on the senescence growth arrest. As senescent cells accumulate during aging, causing chronic inflammation (van Deursen, 2014), cilengitide could be a potential therapeutic route to block inflammation without affecting proliferation in aging.

In summary, here, we provide evidence for the β3 subunit being a marker and regulator of senescence. Our results demonstrate the importance of FA complex formation regulating the microenvironment during senescence activation and identify integrins as potential therapeutic targets to promote healthy aging.

**EXPERIMENTAL PROCEDURES**

The care and use of mice were in accordance with the UK Home Office regulations and the UK Animals (Scientific Procedures) Act of 1986.

**Cell Culture and Retroviral and Lentiviral Infections**

MCF7, SK-HEP-1, and IMR-90 were obtained from the American Type Culture Collection. BFs were described previously (Pemberton et al., 2011). Donor primary human fibroblasts were obtained from the Coriell Cell Repository. Cells were maintained in DMEM (Invitrogen) with 10% fetal bovine serum (FBS) (PAA Laboratories) and 1% antibiotic-antimycotic solution (Invitrogen). Mouse hepatic stellate cells were maintained in the same media supplemented with 1 μg/mL Dox. Methods used for retrovirus and lentivirus production and infection have been previously described (O’Loghlen et al., 2012).

**Treatment with Kinase Inhibitors**

BFs were seeded at the same density in 96-well or 24-well plates. Inhibitors for different signaling pathways were added at the concentrations detailed in Table S2. BFs were incubated with the inhibitors for 48 hr, renewing after 24 hr. Cells were fixed 24 hr later.

**Conditioned Media Experiments**

The indicated cells were cultured for 7 days in DMEM in 0.5% FBS. The conditioned media (CM) were collected and supplemented to generate 10% FBS. The conditioned media (CM) were stained with IL-8/IL-6 antibodies. was concentrated using Amicon Ultra Centrifugal Filters (Millipore) and stained CM, and normal cells were treated with or without pan-specific TGF-

**Statistics**

Results are expressed as the mean ± SD, and statistical analysis was performed using a Student’s t test. A p < 0.05 was considered significant. *p < 0.05; **p < 0.01; ***p < 0.001.

**ACCESSION NUMBERS**

The accession number for the proteomics dataset reported in this paper is PRIDE: PXD005717.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.02.012.

**AUTHOR CONTRIBUTIONS**

V.R. performed most of the experiments, except where specified. A.O., V.E., and A.P.S. performed and analyzed the SILAC data. CDK4/6 inhibitor experiments were performed by M.B., V.M., and A.L. A.O. conceived and designed the study and analyzed most data. A.O. wrote and edited the manuscript, with input from all the authors.

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