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Polymerase delta-interacting protein 2 regulates collagen accumulation via activation of the Akt/mTOR pathway in vascular smooth muscle cells

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Abstract

Objectives—Polymerase delta interacting protein 2 (Poldip2) has previously been implicated in migration, proliferation and extracellular matrix (ECM) production in vascular smooth muscle cells. To better understand the role of Poldip2 in ECM regulation, we investigated the mechanism responsible for collagen I accumulation in Poldip2$^{+/-}$ mouse aortic smooth muscle cells (MASMs).

Approach and results—Protein degradation and protein synthesis pathways were investigated. Depletion of Poldip2 had no effect on proteasome activity, but caused a partial reduction in autophagic flux. However, the rate of collagen I degradation was increased in Poldip2$^{+/-}$ vs. Poldip2$^{+/+}$ MASMs. Conversely, activation of the PI3K/Akt/mTOR signaling pathway, involved in regulation of protein synthesis, was significantly elevated in Poldip2$^{+/-}$ MASMs as was β1-integrin expression. Suppressing mTOR signaling using Akt inhibitor or rapamycin and reducing β1-integrin expression using siRNA prevented the increase in collagen I production. While collagen I and fibronectin were increased in Poldip2$^{+/-}$ MASMs, overall protein synthesis was not different from that in Poldip2$^{+/+}$MASMs, suggesting selectivity of Poldip2 for ECM proteins.

Conclusions—Poldip2$^{+/-}$ MASMs exhibit higher β1-integrin expression and activity of the PI3K/Akt/mTOR signaling pathway, leading to increased ECM protein synthesis. These findings have important implications for vascular diseases in which ECM accumulation plays a role.

Keywords

Poldip2; Vascular smooth muscle; Extracellular matrix; mTOR

1. Introduction

Atherosclerosis is the major cause of occlusive arterial disease leading to myocardial infarction and ischemic stroke [1,2]. Histological observations suggest that ruptured plaques with superimposed thrombi tend to exhibit thinning of the fibrous cap. Collagen I, produced by vascular smooth muscle cells (VSMCs), is the main extracellular matrix (ECM)

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component of the fibrous cap in atheroma. Because ruptured caps contain less collagen I than intact caps, collagen I is thought to play a role in plaque stability and vulnerability to rupture [2,3].

We recently reported that mice deficient in polymerase delta interacting protein 2 (Poldip2) have fractured elastic lamellae and increased medial collagen in their aortas [4]. This excess collagen accumulation is likely dependent on the ability of Poldip2 to regulate Nox4, as it is reversed by incubation with glucose oxidase. Moreover, Poldip2$^{+/-}$ mice are protected against vascular dilatation in experimental aneurysm. We also showed that Poldip2$^{+/-}$ mice produce less H_2O_2 during hindlimb ischemia, which is associated with reduced MMP2 and MMP9 activity and negatively influences collateral vessel formation for functional recovery [5]. Thus, it is clear that Poldip2 has major effects on ECM; however, the mechanism by which it controls collagen accumulation is unknown and likely to be multifactorial.

Excess collagen can result from impaired degradation or enhanced synthesis. Our previous analysis of aortic collagen I α1 and fibronectin mRNA levels showed no differences between wild-type and Poldip2$^{+/-}$ mice [4], leading us to conclude that accumulation of ECM proteins results from post-transcriptional mechanisms. While some proteins are degraded by the proteasome, autophagy has been implicated in degradation of long-lived proteins and in particular of collagen [6]. Autophagy is widely implicated in pathophysiological processes such as cancer, neurodegenerative disorders and cardiovascular diseases [7]. The mechanistic target of rapamycin (mTOR) kinase negatively regulates early steps in autophagosome formation under nutrient-rich conditions by preventing autophagy-related gene (Atg) 1 homolog and unc-51 like autophagy activating kinase (ULK)1/2 kinase complex activity [8,9]. On the other hand, AMP-activated protein kinase, a cellular energy sensor, activates ULK1 to promote its release from mTOR and subsequent localization in the region of autophagosome formation under starved conditions [10,11].

The mTOR signaling pathway also positively controls protein synthesis. Growth factors and integrins activate the mTOR complex 1 through stimulation of phosphoinositide-3-kinase (PI3K) [12,13], which results in the activation of protein kinase B (Akt) via phosphoinositide-dependent kinase-1 [14]. As a downstream target of Akt, mTOR regulates cell growth, proliferation, differentiation, and survival by phosphorylating at least two translational modulators, 70-kDa ribosomal S6 kinase (p70S6k) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) [15–17]. The phosphorylation of 4E-BP1 results in its dissociation from eIF4E, promoting the recruitment of other translation initiation factors to form the eIF4E complex and initiate cap-dependent translation [18,19].

Because mTOR regulates both autophagy and protein synthesis, we hypothesized that one mechanism by which Poldip2 regulates the accumulation of ECM proteins such as collagen I is via activation of the mTOR pathway. We show that in mouse aortic smooth muscle cells (MASMs) from Poldip2$^{+/-}$ mice, β1-integrin expression and the activity of the PI3K/Akt/mTOR signaling pathway are upregulated, leading to a selective increase in ECM protein synthesis. These findings lend new insight into the multifunctional role of Poldip2 in the vasculature.

2. Materials and methods

2.1. Isolation of MASMs

MASMs were isolated from thoracic aortas of Poldip2$^{+/+}$ and Poldip2$^{+/-}$ mice as described previously [4]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 4.5 g/l glucose, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ ml streptomycin and used between passages 6 to 10. Key results were verified in two independent cell isolates.

2.2. Collagen I production and secretion from cultured MASMs

MASMs from Poldip2$^{+/+}$ and Poldip2$^{+/-}$ mice were grown in 100-mm dishes to 90% confluence and were then incubated for 3 days with medium containing 10% fetal calf serum, 50 μg/ml ascorbic acid, and 50 μg/ml β-aminopropionitrile. Collagen I released in cell culture medium was measured by western blot analysis using primary anti-collagen I rabbit polyclonal Ab (Abcam Inc., Cambridge, MA). Protein loading was normalized using a band from Ponceau S staining of the membrane. Cell lysates were then collected to measure intracellular collagen I by western blotting as described previously [4].

2.3. Real-time PCR

Quantitative PCR was carried out on a LightCycler instrument (Roche Applied Science, Indianapolis, IN) in glass capillaries, using PlatinumTaq DNA polymerase (Invitrogen) and SYBR green (Invitrogen) dye. Primer sequences were: Collagen I α1, 5'-CCTAAGGGTCCCCAATGGTGAGACG-3' (sense) and 5'-GGGGGTTGGGACAGTCCAGTTCTTC-3' (antisense); fibronectin, 5'-CTCAACCTCCCTGAAACGGCCAACT-3' (sense) and 5'-TCTTGGGGTGCCAGTGGTCTCTTGT-3' (antisense). Data analysis was performed using the mak3 module of the qpcR software library in the R environment [4].

2.4. Measurement of CTX-1

Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs were cultured in DMEM for 10 days. CTX-1 in the medium was measured with the mouse CTX-1 ELISA kit (NeoScientific, Cambridge, MA) following the manufacturer's instructions.

2.5. Measurement of autophagy

Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs were incubated with DMEM supplemented with amino acids with or without 1 μM E64 (EMD Chemicals, Inc., San Diego, CA) and 1 μM Pepstatin A (Sigma-Aldrich, Inc., Saint Louis, MO) and without serum and glucose for 18 h. Lysates were collected and western blotting was used to measure LC3B and p62 expression with anti-LC3B (D11) XP rabbit mAb (Cell Signaling Technology, Inc., Danvers, MA) and anti-p62 mouse mAb (Abcam Inc., Cambridge, MA). Autophagic flux was analyzed by assessing LC3B or p62 turnover [20].

2.6. Collagen I degradation assay

Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs were incubated with medium containing 10% serum, 50 μg/ml ascorbic acid, and 50 μg/ml β-aminopropionitrile for 3 days. Medium was then changed to DMEM supplemented with 50 μM cycloheximide, serum and glucose. MASMs were harvested 0, 6, 12 and 24 h later and collagen I expression was evaluated by western blotting.

2.7. Western blotting

Samples were lysed in lysis buffer (50 mM HEPES, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM Na pyrophosphate, 1 mM Na orthovanadate, 1% Triton X-100 and protease inhibitors, pH 7.5). After sonication for 10 s, samples were centrifuged at 12,500 ×*g* for 5 min. After calculating the concentration of protein with the Bradford Assay (Bio-Rad Laboratories, Inc., Hercules, CA), protein lysates were boiled and separated on SDS-PAGE gels and transferred to PVDF (0.2 μm) membranes. Following blocking, blots were incubated with primary antibodies (diluted 1:1000 in 5% BSA/PBS): anti-collagen I rabbit polyclonal Ab (Abcam Inc., Cambridge, MA), anti-ubiquitin (P4D1) rabbit polyclonal Ab, anti-K48-linkage specific polyubiquitin (D9D5) rabbit mAb, anti-Hsc70 (1B5) rat mAb (Abcam Inc., Cambridge, MA), anti-LC3B (D11) XP rabbit mAb, anti-p62 mouse mAb, anti-PI3K p85 (19H8) rabbit mAb, anti-PTEN (D4.3) XP rabbit mAb, anti-Akt rabbit polyclonal Ab, anti-phospho-Akt (Ser473) rabbit polyclonal Ab, anti-phospho-Akt (Thr308) rabbit polyclonal Ab, anti-mTOR (7C10) rabbit mAb, anti-phospho-mTOR (Ser2448) (D9C2) XP rabbit mAb, anti-4E-BP1 (53H11) rabbit mAb, anti-phospho-4E-BP1 (Thr37/46) (236B4) rabbit mAb, anti-p70 S6 Kinase (49D7) rabbit mAb, anti-phospho-p70 S6 Kinase (Thr389) rabbit polyclonal Ab, (Cell Signaling Technology, Inc., Danvers, MA), anti-integrin β1 rabbit polyclonal Ab (Santa Cruz Biotechnology, Inc., Dallas, TX), and Poldip2 rabbit mAb (Abcam Inc., Cambridge, MA) as described previously [4]. After overnight incubation with primary antibody, membranes were incubated with secondary anti-rabbit (Cell Signaling Technology, Inc., Danvers, MA) or anti-mouse peroxidase-coupled antibodies (Amersham plc, Amersham, UK), diluted 1:2000 in 5% BSA/PBS. Bands were visualized by enhanced chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and quantified by densitometry using ImageJ 1.38 software.

2.8. Immunoprecipitation

MASMs were lysed in standard lysis buffer as described above. Whole cell lysates were utilized for immunoprecipitation, as described previously [21,22]. For all immunoprecipitation experiments, lysis buffer alone was incubated with primary antibody and beads as a negative control. Proteins were separated using SDS-PAGE and transferred to PVDF membranes, blocked with 5% non-fat milk and incubated with appropriate primary antibodies. Chemiluminescent signal was detected using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Band intensity was quantified by densitometry using ImageJ 1.38 software.

2.9. PTEN and PI3K activity assay

Samples were immunoprecipitated using primary antibodies against PI3K p85 (19H8 rabbit mAb) and PTEN (D4.3 XP rabbit mAb) (Cell Signaling Technology, Inc., Danvers, MA). After collecting PTEN or PI3K protein, immunoprecipitates were incubated with PI(3,4,5)P_3 for the PTEN assay or PI(4,5)P_2 for the PI3K assay. Products were measured using a competitive ELISA method (Echelon Biosciences Inc., Salt Lake City, UT) according to the manufacturer's instructions.

2.10. Small interfering RNA transfection

MASMs were grown to 40–50% confluence, washed with OPTI-MEM, incubated with small interfering (si)RNA plus Lipofectamine RNAiMAX complexes for 5 h, and harvested 72 h after transfection. Double-stranded siRNA against mouse β1-integrin: sense 5'-GGUCCAUGUCUAGCGUCAAd(TT)-3', antisense 5'-UUGACGCUAGACAUGGACCd(AG)-3' and AllStars negative control were used at 6.25 nM. These predesigned siRNAs were purchased from Qiagen (Valencia, CA).

2.11. Protein synthesis

Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs were serum starved for 24 h and then incubated for 24 h with [^{3}H]leucine (0.5 μCi/ml) in the presence or absence of 10% FBS. After incubation, plates were washed twice with phosphate buffered saline, followed by incubation with 5% trichloroacetic acid for 5 min for protein precipitation. NaOH (0.4 N, 1 ml) was added and aliquots of 0.4 ml were acidified with 0.2 ml of 1 N HCl. Samples were added to 10 ml of Liquiscint (National Diagnostics Solutions, Lake Mary FL), mixed and incubated for 30 min at room temperature. [^{3}H]leucine incorporation was measured in a liquid scintillation spectrometer.

2.12. Statistical analysis

Differences in western blot exposures between experiments were corrected using percent control or normalization. In the latter case, correction factors were calculated as the average signal of all blots, divided by the average signal of each blot. Results are expressed as mean ± SE from at least 3 independent experiments. Statistical significance was assessed using ANOVA and Bonferroni's multiple comparisons test. In some cases, a Student's t-test was used to assess significance between groups when no other variables were considered. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Collagen I expression and crosslinking in Poldip2 heterozygous MASMs

The characteristics of Poldip2$^{+/-}$ MASMs are shown in Fig. 1. The protein levels of Poldip2 in Poldip2$^{+/-}$ MASMs are significantly lower (43 ± 13%) compared to Poldip2$^{+/+}$ MASMs ($P < 0.05$ vs. Poldip2$^{+/+}$) (Fig. 1A). Both collagen I expression in Poldip2$^{+/-}$ MASMs and collagen I secretion into the culture medium are significantly increased (Fig. 1C, D).

Previously we reported that mRNA levels of collagen I α1 expression were not changed in Poldip2$^{+/-}$ aortas [4]. However, in cell culture, Poldip2$^{+/-}$ MASMs showed a small but

significant increase in collagen I α1 mRNA compared to Poldip2$^{+/+}$ MASMs, while mRNA for a second matrix protein, fibronectin, that is also upregulated by Poldip2 depletion [4], was unchanged (data not shown). These data suggest that Poldip2 has additional effects beyond mRNA regulation.

To test the hypothesis that enhanced crosslinking, and therefore increased stability, of collagen is responsible for the remarkable increase in collagen in Poldip2$^{+/-}$ MASMs, we measured the C-Telopeptide of Type I Collagen (CTX-1), which is released when collagen undergoes crosslinking [23,24]. There was no difference in CTX-1 levels between Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs (Fig.1B), suggesting that the collagen I accumulation is unlikely to be due to stabilization.

3.2. Dysfunction of the degradation system in Poldip2$^{+/-}$ MASMs is not the critical cause of collagen I accumulation

Because neither mRNA upregulation nor increased crosslinking is sufficient to explain the increase in collagen protein, we next focused on pathways regulating protein synthesis and degradation. We initially hypothesized that loss of Poldip2 impairs collagen I degradation. One of the major routes for intracellular protein degradation, the ubiquitin-proteasome system, was evaluated by blotting for Lys-48 specific ubiquitin, which is most commonly associated with proteins targeted for proteasomal degradation. To compare proteasome activity in Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs, we measured accumulation of ubiquitinated proteins. Total and Lys-48 specific ubiquitination were similar in Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs (Suppl. Fig. IA and B in the online-only data supplement). Moreover, inhibiting the proteasome with MG132 (Suppl. Fig. IC in the online-only data supplement) had no effect on collagen I expression in Poldip2$^{+/-}$ MASMs (Suppl. Fig. ID in the online-only data supplement), suggesting that differences in proteasomal degradation are unlikely to be responsible for the observed collagen accumulation.

The other major degradation system is autophagy, which degrades long-lived proteins and dysfunctional organelles. Although we did not see a difference in expression of Heat shock cognate protein 70 (Hsc70), which regulates chaperone-mediated autophagy, between Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs (Suppl. Fig. IIA in the online-only data Supplement), macroautophagy, the most extensively investigated mechanism, showed a significant difference between Poldip2$^{+/-}$ and Poldip2$^{+/+}$ MASMs. We induced autophagy with 18 h of starvation, and found that the markers of autophagy p62 and microtubule-associated protein 1A/1B-light chain 3B II (LC3B II), were increased by lysosomal inhibitors in Poldip2$^{+/+}$ MASMs, as expected. While the induction of p62 was nearly abolished in Poldip2$^{+/-}$ cells, basal levels of LC3B II were higher, but showed only a non-significant increase after lysosomal inhibition (Fig. 2A and B). When we analyzed autophagic flux, we found no difference in processing of LC3B II, but p62 accumulation was significantly less in Poldip2$^{+/-}$ compared to Poldip2$^{+/+}$ MASMs (Fig. 2C). This result suggests that a partial defect in autophagy might underlie collagen accumulation in Poldip2$^{+/-}$ MASMs, following starvation. In contrast, little autophagy was detected when cells were not starved (Suppl. Fig. IIB and C in the online-only data supplement). To further test this hypothesis, we investigated the degree of collagen I degradation in both MASM genotypes. Basally,

Poldip2$^{+/-}$ MASMs have approximately 2.5-fold more collagen I compared to Poldip2$^{+/+}$ MASMs; however, contrary to our expectation, collagen I degradation was faster in Poldip2$^{+/-}$ than Poldip2$^{+/+}$ MASMs after 24 hour starvation (data not shown) and in the presence of serum (Fig. 2D). This result indicates that the increase in accumulation of collagen I in Poldip2$^{+/-}$ MASMs is unlikely to be due to a partial dysfunction in autophagy in the absence of starvation, prompting us to examine collagen synthesis.

3.3. mTOR signaling pathway is activated in Poldip2$^{+/-}$ MASMs

To assess pathways responsible for protein synthesis, we first checked the phosphorylation of p70S6K and 4EBP-1, known to be activated downstream of mTOR and to be central regulators of protein synthesis. Western blot analysis showed that the phosphorylation of both p70S6K and 4EBP-1 were significantly higher in Poldip2$^{+/-}$ MASMs (Fig. 3A). These changes are expected to lead to phosphorylation of the 40S ribosomal S6 protein and to disrupt 4EBP-1 interaction with the eIF-4E initiation factor, allowing eIF-4E to participate in assembly of a translation initiation complex. Moreover, upstream phosphorylation of Akt/PRAS40/mTOR was also significantly increased in Poldip2$^{+/-}$ MASMs (Fig. 3B and Suppl. Fig. IIIA in the online-only data supplement). Taken together, these results suggest that Poldip2$^{+/-}$ MASMs exhibit inappropriate activation of mTOR signaling, indicating an increased ability to induce protein synthesis.

We have previously shown that the reduction in H_2O_2 that accompanies loss of Poldip2 contributes to collagen accumulation [4]. Accordingly, we tested the ability of the H_2O_2 generator, glucose oxidase, to reverse Akt activation. As shown in Suppl. Fig. IV in the online-only data supplement, glucose oxidase failed to alter the elevation of Akt phosphorylation in Poldip2$^{+/-}$ MASMs, indicating that the effects of Poldip2 on the Akt/mTOR pathway are likely independent of modifications in reactive oxygen species.

3.4. PI3K and PTEN activity/expression in Poldip2$^{+/-}$ MASMs

Next, we investigated the activity and expression of the upstream signaling proteins in this pathway, PI3K and phosphatase and tensin homolog deleted from chromosome 10 (PTEN). To measure PI3K activity, we collected PI3K protein by immunoprecipitation (Fig. 4A), and then measured phosphorylation of the PI(4,5)P_2 substrate. PI3K activity was significantly higher in Poldp2$^{+/-}$ MASMs (Fig. 4C), while PI3K expression showed no difference between Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs (Fig. 4E). To evaluate PTEN activity, we also collected PTEN protein by immunoprecipitation (Fig. 4B), and then measured the conversion of PI(3,4,5)P_3 substrate to PI(4,5)P_2. The activity of PTEN was not different between Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs (Fig. 4D). Similarly, the protein expression of PTEN showed no difference between the two groups (Fig. 4F).

3.5. Akt inhibitor and rapamycin suppress the increased collagen I accumulation in Polidp2$^{+/-}$ MASMs

We next investigated if inhibition of mTOR signaling by Akt inhibitor (iAkt) or rapamycin decreases the excess collagen I production in Poldip2$^{+/-}$ MASMs. The iAkt (5 nM) significantly inhibited phosphorylation of Akt at both Thr308 and Ser473 (Suppl. Fig. IIIB and Suppl. Fig. VA, respectively, in the online-only data supplement) and decreased the

production of collagen I in Poldip2$^{+/-}$ MASMs without cytotoxicity (Fig. 5A). Rapamycin (100 nM) inhibited phosphorylation of mTOR significantly (Suppl. Fig. VB in the online-only data supplement) and decreased collagen I expression significantly in Poldip2$^{+/-}$ cells (Fig. 5B). It should be noted that to measure collagen accumulation in the media, we incubated cells with β-aminopropionitrile and ascorbic acid for 3 days to prevent collagen crosslinking (Fig. 5A and B). Under these conditions, the increase in Akt and mTOR phosphorylation is not evident in Poldip2$^{+/-}$ cells (Suppl. Fig IIIB, V and VI in the online-only data supplement). However, we do see increased Akt and mTOR phosphorylation 1–2 days after addition of the inhibitors before we begin to detect soluble collagen (Suppl. Fig. VI in the online-only data supplement), suggesting that either collagen I accumulation or increased confluency of the cells provides negative feedback by day 3.

3.6. Polidp2$^{+/-}$ MASMs express higher β1-integrin, which influences collagen I expression

Because the effect of Poldip2 is upstream of PI3K, we considered whether Poldip2 might affect receptor expression. Of note, collagen I binds to β1-integrin receptors [25], raising the possibility that a positive feedback loop is in play. Accordingly, we investigated the effect of heterozygous deletion of Poldip2 on β1-integrin expression. As shown in Fig. 5C, Poldip2$^{+/-}$ MASMs express significantly higher β1-integrin levels compared to Poldip2$^{+/+}$ MASMs. To determine the role of this increase in β1-integrin on collagen I expression, we knocked down β1-integrins using siRNA. Collagen I protein expression was unaffected by transfection of control siRNA, but was significantly decreased in cells treated with siβ1-integrin (Fig. 5D), suggesting that the increased collagen I produced by Poldip2$^{+/-}$ MASMs binds to β1-integrins, creating a positive feedback loop in which activation of β1-integrin leads to Akt/mTOR activation and collagen I expression that then further activates β1-integrin. This suggests that Poldip2 normally acts as a brake in the integrin-collagen signaling pathway.

3.7. Protein synthesis

While the increase in collagen accumulation in Poldip2$^{+/-}$ MASMs is clearly due, at least in part, to increased activation of protein synthesis via the Akt/mTOR pathway, we questioned whether overall protein synthesis is also increased, or if it is specific for collagen and other ECM proteins. As shown in Fig. 6A overall protein synthesis is similar between genotypes, suggesting that activation of the Akt/mTOR pathway mediated by loss of Poldip2 may selectively target ECM proteins. We therefore examined expression of fibronectin, an ECM protein, and laminin, a basement membrane protein (Fig. 6B), and found that fibronectin, but not laminin, is elevated in Poldp2$^{+/-}$ MASMs, in agreement with our previous results using siRNA to knock down Poldip2 [4]. These results suggest that Poldip2-mediated activation of the PI3K/Akt/mTOR pathway specifically upregulates ECM proteins (Fig. 6C).

4. Discussion

Under basal cellular conditions, the fine-tuned balance between protein degradation and synthesis contributes to the maintenance of cellular homeostasis and tissue structure [3,26]. In this study, we investigated the mechanism of excess collagen I accumulation in Poldip2$^{+/-}$ MASMs. We found that cross-linking is unaffected and that ubiquitin-proteasome activity appears normal after loss of Poldip2, while autophagic flux is partially inhibited. However,

the decrease in autophagy did not appear to be responsible for collagen accumulation; rather, increased activity of the PI3K/Akt/mTOR pathway led to increased collagen synthesis. Enhanced protein synthesis was not a general effect of loss of Poldip2, but seemed to be restricted to ECM proteins because other ECM proteins such as fibronectin were similarly affected.

We first focused on the relationship between Poldip2 and protein degradation as a potential mechanism for alterations in collagen accumulation because Nox4, a known effector of Poldip2, has been implicated in both proteasomal degradation and autophagy [27–29]. It has been reported that the ubiquitin proteasome system (UPS) and autophagy can serve in a complementary manner to each other to maintain cellular homeostasis [30]. Neither total ubiquitin, K48-linkage specific polyubiquitin levels, nor Hsc70, a marker of chaperone-mediated autophagy [26], was altered in Poldip2$^{+/-}$ MASMs (Suppl. Figs. I and II in the online-only data supplement). In contrast, autophagic mechanisms to degrade long lived proteins such as collagen, proteoglycan and elastin [31] were partially impaired. It has been reported that reactive oxygen species such as H_2O_2 are necessary for autophagosome formation by inducing downregulation of Atg4, which enables the conversion of LC3-I into LC3-II, lipidation and insertion into the autophagosome [32]. Since then, accumulating evidence has demonstrated that Nox4 plays a role in upregulating autophagy and protecting cells from external stressors [27,29,33,34]. Administration of 7-ketocholesterol, a major component of oxidized lipoprotein, to VSMCs promoted increased H_2O_2 through activation of Nox4, inhibited Atg4B activity, and induced autophagy [27]. In the case of cardiomyocytes, H_2O_2 produced by Nox4 in the ER induced autophagy through activation of PERK/eIF-2α/ATF4 pathway, protecting against energy deprivation [29]. Because the activity of Nox4 is positively regulated by Poldip2 in VSMCs [35], H_2O_2 production is actually lower in Poldip2$^{+/-}$ than Poldip2$^{+/+}$ MASMs [4]. Consistent with these previous findings, Poldip2$^{+/-}$ MASMs exhibit a partial defect in autophagic flux, compared to Poldip2$^{+/+}$ MASMs (Fig. 2A–C) [31]. However, contrary to our expectation, collagen I degradation was faster in Poldip2$^{+/-}$ than in Poldip2$^{+/+}$ MASM (Fig. 2D). This result indicates that the accumulation of collagen I in Poldip2$^{+/-}$ MASMs is likely independent of the partial dysfunction in autophagy, especially in the absence of starvation, although this possibility cannot be fully excluded.

We next focused on the possibility that excessive activation of PI3K/ Akt/mTOR signaling pathway causes increased collagen I accumulation in Poldip2$^{+/-}$ MASMs. This pathway is activated in response to upstream cellular signals, such as growth factors, stress and nutrients, and is a critical regulator of cell growth, protein synthesis, proliferation, and differentiation. We speculated that excess activation of this pathway, as reflected in phosphorylation of key transregulators such as p70S6k and 4E-BP1 [15–17,36], causes alterations in matrix accumulation. In Poldip2$^{+/-}$ MASMs, not only was PI3K/Akt/mTOR signaling significantly increased, but so too was downstream phosphorylation of p70s6k and 4E-BP1 (Figs. 3 and 4). These results account for higher accumulation of collagen I in Poldip2$^{+/-}$ MASMs and mouse aortic media compared to Poldip2$^{+/+}$ cells and tissue, as confirmed by inhibition of collagen I accumulation by the Akt inhibitor and rapamycin (Fig. 5A and B). Significant inhibition of collagen I production by both agents, in conjunction with the observed increase in PI3K activity (Fig. 4A and C), suggests that Poldip2 acts

upstream from PI3K. One possibility is that the increased extracellular collagen may create a positive feedback loop by activating integrin receptors [25,37] which play important roles in matrix remodeling via survival signals from PI3K/Akt [25,38]. This was in fact what we found: Poldip2$^{+/-}$ MASMs express more β1-integrin (Fig. 5C) compared to Poldip2$^{+/+}$ MASMs, and accumulation of collagen I was partially inhibited by downregulation of β1-integrin (Fig. 5D). These results suggest that Poldip2 may normally function to limit integrin activation by collagen by regulating its expression, an important potential future avenue of research.

The finding that synthesis of extracellular matrix proteins is preferentially upregulated in Poldip2$^{+/-}$ MASM is intriguing. There are several possible explanations for this putative specificity. Poldip2 may stimulate transcription of miRNAs, such as miR-29 [39,40], or specifically interfere with translation of collagen I and other ECM components, as has been shown for miRNAs in other cell types [41,42]. Alternatively, Poldip2 may target LARP6, a protein that has been shown to bind collagen I and III mRNA 5'-UTR system loop (5'-SL), promoting collagen I and III translation in VSMCs [43]. Finally, Poldip2 may simultaneously activate a pathway inhibitory to general protein synthesis, permitting synthesis of only selected proteins. Further work will be necessary to distinguish among these possibilities.

The results reported here underline the complexity of mechanisms regulating collagen accumulation. While the bulk of our data suggest that the major role of Poldip2 is via regulation of protein synthesis, it is clear that other mechanisms contribute as well, including potential transcription, impaired autophagy (Fig. 2A–C), and inhibition of MMP activity. Because glucose oxidase reversed collagen accumulation in Poldip2$^{+/-}$ MASMs [4], but had no effect on the elevation in Akt activity here (Suppl. Fig. IV), these data suggest that reactive oxygen species control another aspect of collagen regulation by Poldip2. The most likely target is MMPs, which we have previously shown to be inhibited in Poldip2$^{+/-}$ mice [5].

A role for Poldip2/β1-integrin/PI3K/Akt/mTOR in collagen accumulation has obvious potential consequences for vascular development and disease. We have previously shown that Poldip2$^{+/-}$ mice have disordered structure of large arteries due to excess collagen deposition and breaks in the elastic fibers [4]. We found that the vessels of Poldip2$^{+/-}$ mice were protected against vascular dilation in experimental aneurysm due to the excess ECM [4]. Others have shown that rupture of atherosclerotic plaques is often associated with a thinned-out, collagen-depleted fibrous cap [1,2], suggesting that Poldip2 inhibition might be beneficial in this situation. The phenotype of increased collagen I expression in the aortic media of Poldip2$^{+/-}$ mice may confer resistance to plaque rupture by converting the plaque composition from the lipid-rich, collagen-depleted fibrous cap to the lipid-poor, collagen-rich stable plaque, a possibility that remains to be explored. Alternatively, excess matrix production could actually increase plaque size. Further analysis of the role of Poldip2 in atherosclerosis in vivo is warranted.

5. Conclusions

In conclusion, Poldip2 appears to play a role in limiting integrin-collagen signaling. Poldip2$^{+/-}$ MASMs exhibit less autophagy, higher β1-integrin expression and basal activation of the PI3/Akt/mTOR signaling pathway. One of the consequences of activation of this pathway is an accumulation of extracellular collagen, which may in some cases be protective, as with aneurysm formation, but in others deleterious (e.g., increased neointimal formation). The clear link between Poldip2 and matrix thus has important implications for understanding the molecular basis of vascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Akt	protein kinase B
Atg	autophagy-related gene
ECM	extracellular matrix
4E-BP1	eukaryotic initiation factor 4E binding protein 1
eIF4E	eukaryotic translation initiation factor 4E
Hsc70	heat shock cognate protein 70
LC3B	microtubule-associated protein 1A/1B-light chain 3
MASMs	mouse aortic smooth muscle cells
MMP	matrix metalloproteinase
mTOR	mechanistic target of rapamycin
PI3K	phosphoinositide-3-kinase
Poldip2	polymerase delta interacting protein 2
PRAS40	proline-rich Akt substrate of 40 kDa
PTEN	phosphatase and tensin homolog deleted from chromosome 10
p70S6k	70-kDa ribosomal S6 kinase
VSMCs	vascular smooth muscle cells
UPS	ubiquitin proteasome system

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2016.01.016.

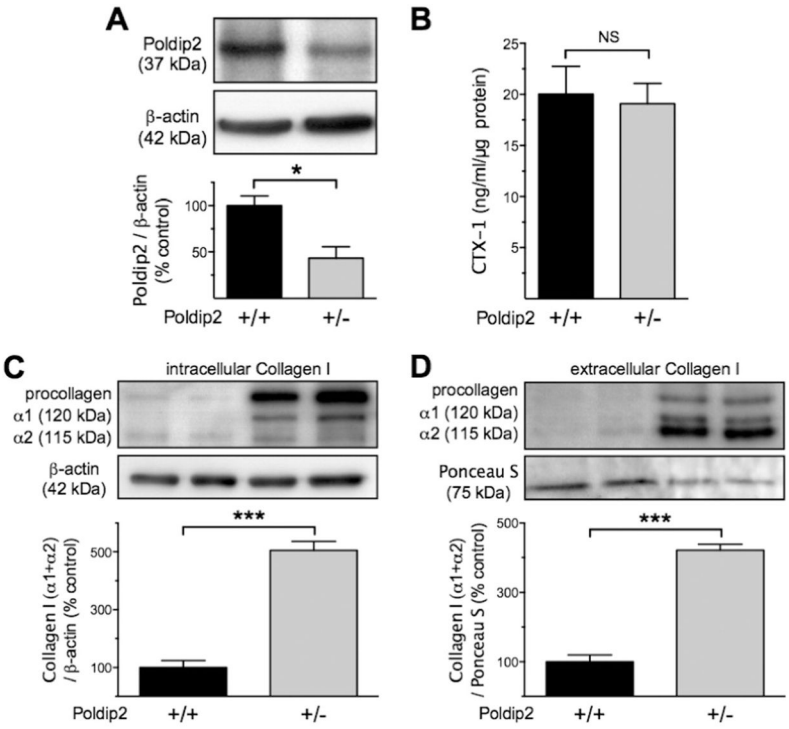

Fig. 1.

Collagen I accumulation is increased in Poldip2$^{+/-}$ MASMs. A, Protein levels of Poldip2 were evaluated by western blot analysis of Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASM lysates and normalized to β-actin. Results are expressed as percent of control ± SE from 4 independent experiments (*$P < 0.05$). B, Collagen crosslinking was evaluated by quantifying extracellular CTX-1 peptide, using an ELISA assay. Results, corrected for total intracellular protein, are expressed as average concentrations ± SE from 6 independent experiments (NS). C–D, Western blot analysis of collagen I in whole cell lysates (C) or medium (D) from cells treated with ascorbic acid (50 μg/ml) and β-aminopropionitrile (50 μg/ml) to prevent crosslinking of secreted collagen. Protein levels were normalized to β-actin (C) or Ponceau S staining (D), and the results are expressed as percent of control ± SE from 3 independent experiments (***$P < 0.001$).

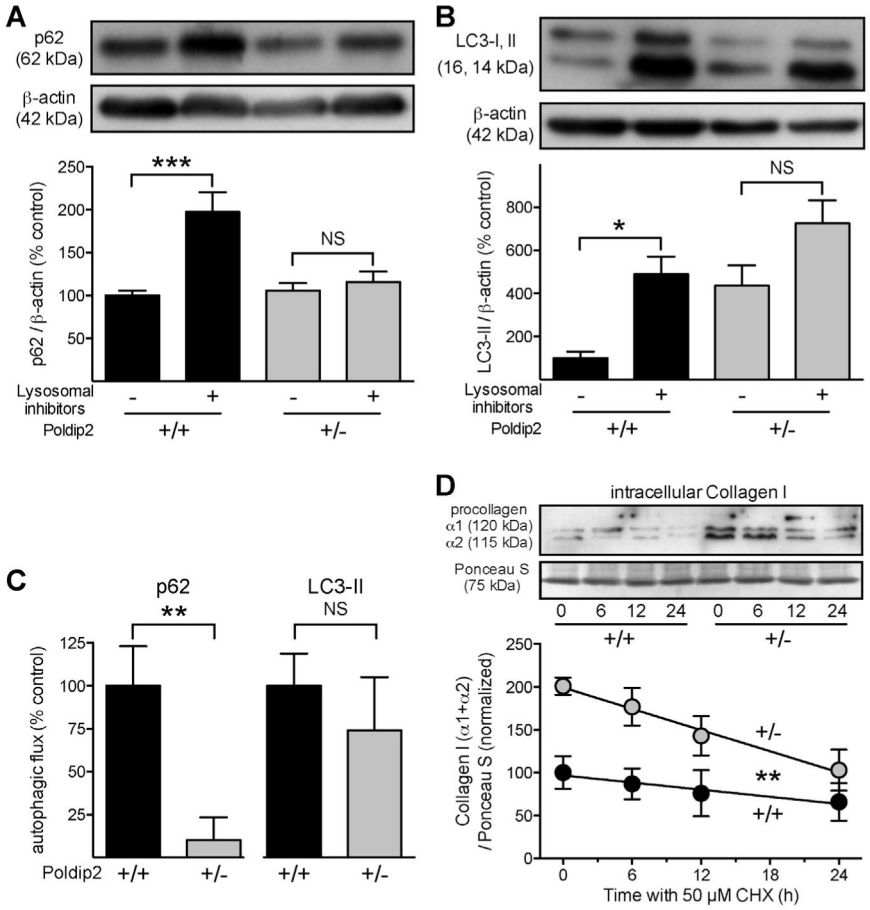

Fig. 2.

Autophagic flux is decreased in Poldip2$^{+/-}$ MASMs, but collagen I degradation is faster than in wild type cells. A–C, Cultured MASMs derived from Poldip2$^{+/+}$ and Poldip2$^{+/-}$ mice were incubated with DMEM without serum or glucose, with or without lysosomal inhibitors E64d (1 μM) and Pepstatin A (1 μM) for 18 h. Cell lysates were subjected to SDS-PAGE followed by western blotting with antibodies specific to p62 or LC3B. Protein levels were normalized to β-actin, and the results are expressed as mean percent of control ± SE from 4 to 6 independent experiments (***P < 0.001, *P < 0.05). C, Autophagic fluxes calculated from the data above as the difference in signals between presence and absence of lysosomal inhibitors. Results are expressed as mean percent of control ± SE (**P < 0.01). D, After a 3 day pretreatment with ascorbic acid (50 μg/ml) and β-aminopropionitrile (50 μg/ml) to prevent collagen crosslinking, MASMs derived from Poldip2$^{+/+}$ and Poldip2$^{+/-}$ mice, cultured in DMEM with serum and glucose were incubated with cycloheximide (50 μM) for the indicated times (0, 6, 12, 24 h). After lysis, samples were subjected to SDS-PAGE followed by western blotting with antibodies to collagen I. Protein levels were normalized to Ponceau S, and the results are expressed as average ± SE from 4 independent experiments (linear regression slopes **P < 0.01).

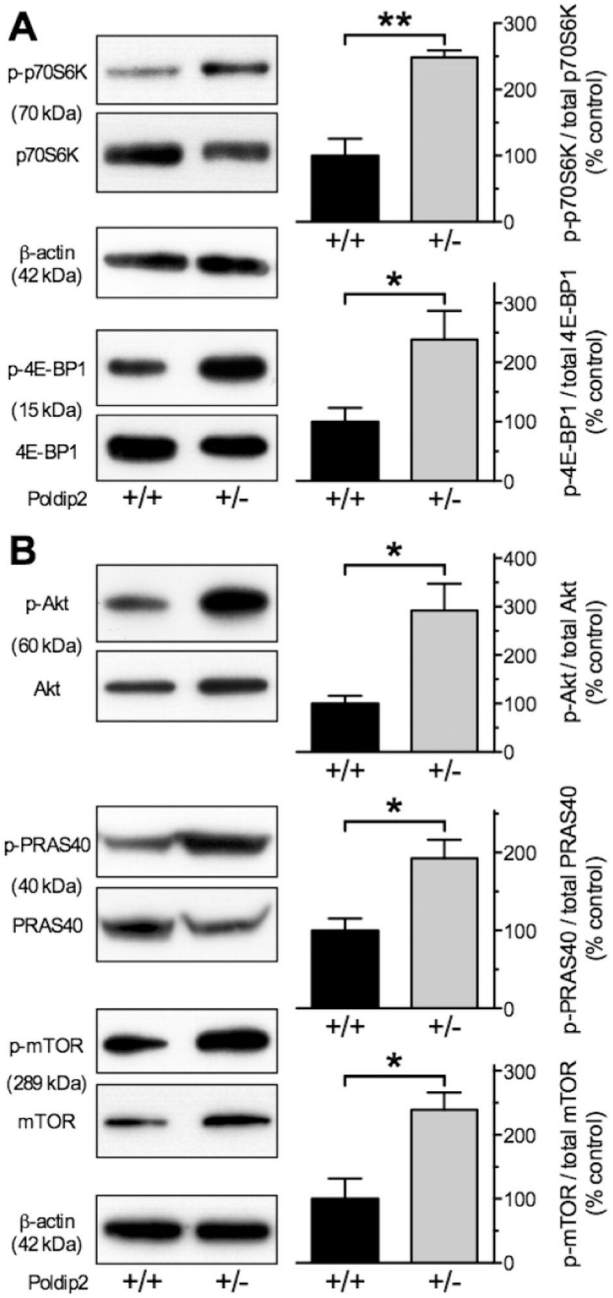

Fig. 3.

Activation of mTOR signaling pathway in Poldip2$^{+/-}$ MASMs. Phosphoproteins and total proteins were analyzed by western blotting and their ratios are expressed as percent of control ± SE from 4 to 5 independent experiments (**P < 0.01, *P < 0.05). A, p70s6k and 4E-BP1, both downstream of mTOR, were detected using antibodies specific to phospho-p70S6K at T389 and phospho-4E-BP1 at S37/46, respectively. B, Akt and PRAS40, both upstream of mTOR, as well as mTOR, were detected using antibodies specific to phospho-Akt at T308, phospho-PRAS40 at T246, and phospho-mTOR at S2448.

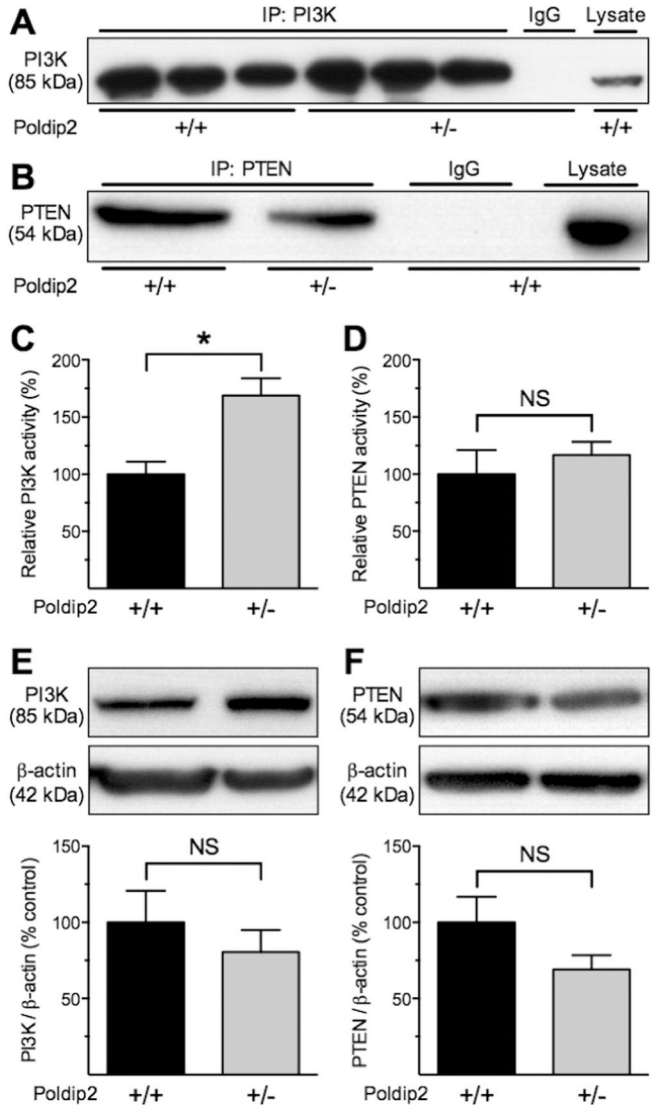

Fig. 4.

PI3K, but not PTEN, activity is increased in Poldip$^{+/-}$ MASMs. A–B, IP for PI3K or PTEN, using the 19H8 PI3K or D4.3 PTEN antibodies was followed by IB with same antibodies. IgG was used as a negative control. C, PI3K protein collected by IP was incubated with PI(4,5)P_2 substrate and its activity was quantified by measuring PI(3,4,5)P_3 by competitive ELISA (*$P < 0.05$, n = 5). D, PTEN protein collected by IP was incubated with PI(3,4,5)P_3 substrate and its phosphatase activity was quantified by measuring PI(4,5)P_2 by competitive ELISA (NS, n = 6). E–F, PI3K and PTEN proteins were evaluated by western blotting analysis of lysates from Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs. Protein levels were normalized to β-actin, and the results are expressed as the mean percent of control ± SE (NS, n = 4).

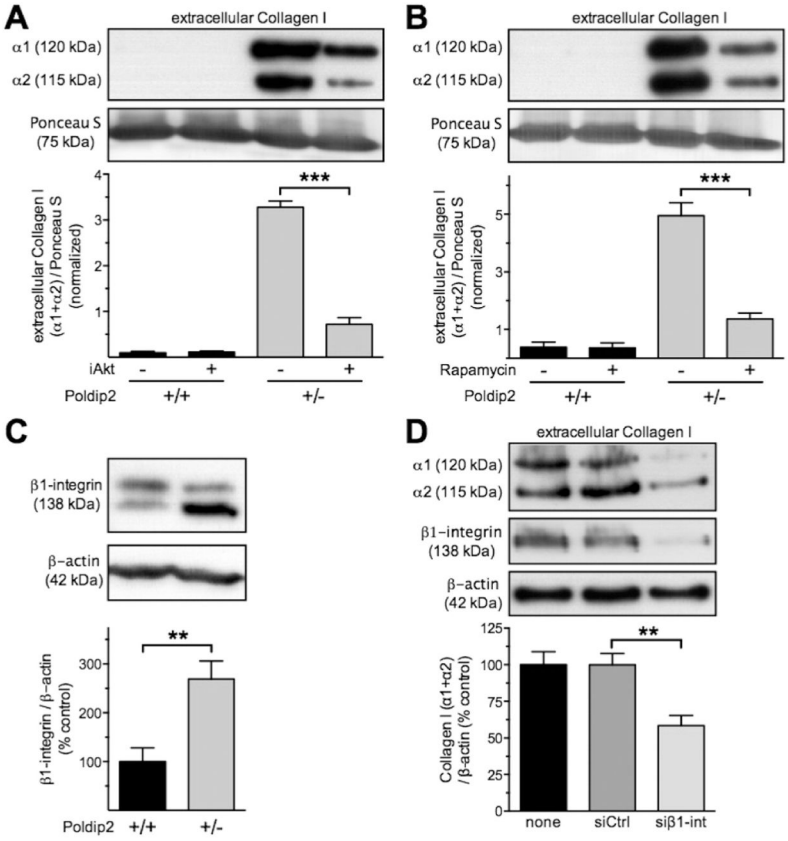

Fig. 5.

Collagen I accumulation in Poldip2$^{+/-}$ MASMs is mediated by β1-integrin upregulation and reversed by iAkt and rapamycin. A–B, Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs were cultured with ascorbic acid (50 μg/ml) and β-aminopropionitrile (50 μg/ml) with or without iAkt (5 nM) or rapamycin (100 nM) for 3 days. Media samples were subjected to SDS-PAGE followed by western blotting with antibodies to collagen I. Protein levels were normalized to Ponceau S, and results are expressed as the mean ± SE from 4 independent experiments (***P < 0.001). C, β1-integrin expression was measured in Poldip2$^{+/+}$ and Poldip2$^{+/-}$ MASMs by western blotting. Results were normalized to β-actin, expressed as percent of control average ± SE from 5 independent experiments (**P < 0.01). D, MASMs were cultured for 72 h with ascorbic acid (50 μg/ml) and β-aminopropionitrile (50 μg/ml), following no treatment (none) or transfection with control siRNA (siCtrl) or siRNA against β1-integrin (siβ1-int). Proteins were analyzed by western blotting of media (collagen I) or lysates (β1-integrin and β-actin). Results are expressed as percent of control average ± SE from 5 independent experiments (**P < 0.01).

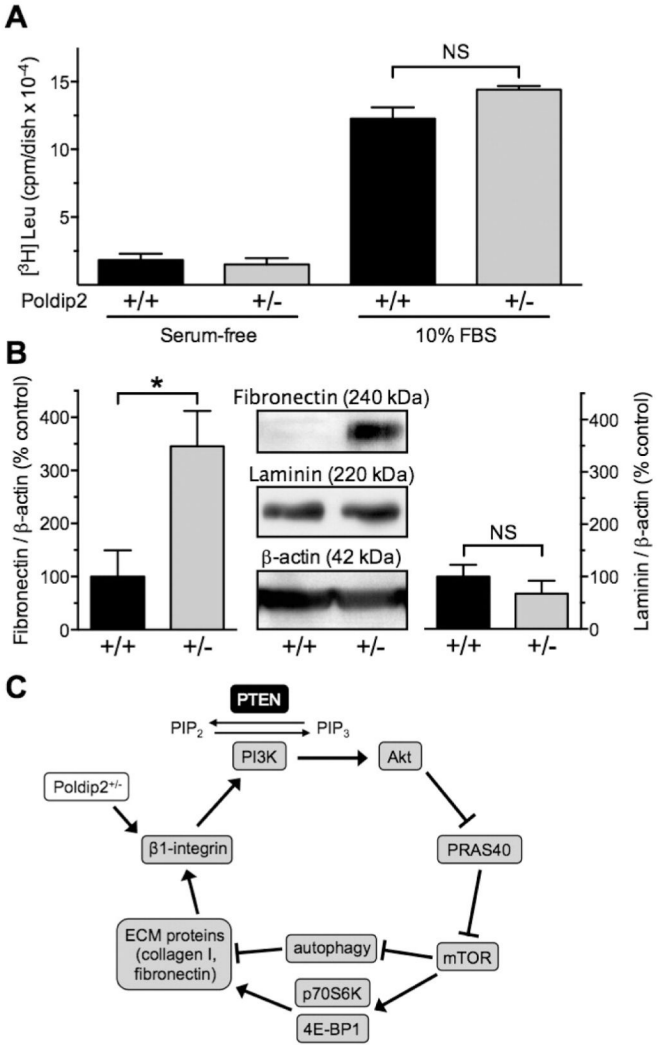

Fig. 6.

Increased protein synthesis is selective for ECM proteins. A, MASM were incubated with [^{3}H]Leucine for 24 h with or without 10% fetal bovine serum (FBS). Following washing and lysis, incorporated radioactivity was measured by scintillation spectrometry. Results are expressed as mean ± SE of 3 independent experiments. B, Western blot analysis of fibronectin and laminin was performed in samples from whole cell lysate. Protein levels were normalized to β-actin, and the results are expressed as the mean percent of control ± SE from 3 independent experiments (*$P < 0.05$). C, Partial loss of Poldip2 upregulates β1-integrin, thus triggering a vicious cycle of extracellular matrix protein accumulation, mediated by PI3K and the mTOR pathway.