Adipocyte-Specific Deletion of Sine Oculis Homeobox Homolog 1 Inhibits Lipolysis and Reduces Skin Fibrosis

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Abstract

Dermal fibrosis is a cardinal feature of systemic sclerosis (SSc) for which there are limited Systemic sclerosis (SSc) is characterized by dermal fibrosis accompanied by loss of dermal white adipose tissue (DWAT), yet the mechanisms linking adipocyte depletion to fibroblast activation remain unclear. Here we identify the transcription factor SIX1 as a central regulator coupling adipogenic repression with profibrotic signaling. SIX1 expression was increased in skin biopsies from two independent SSc cohorts and localized to fibroblast and perivascular stromal cells. In mice, ubiquitous or adipocyte-specific deletion of *Six1* preserved DWAT, reduced collagen accumulation, and selectively decreased pro-fibrotic mediators. In cultured fibroblasts, CRISPR/Cas9-mediated *Six1* loss enhanced adipogenic markers while reducing profibrotic mediators and directly suppressed PAI-1 (*SERPINE1*) promoter activity. Together, these data position SIX1 as a transcriptional switch that promotes adipocyte reprogramming and fibrotic progression, and highlight SIX1 inhibition as a potential therapeutic strategy to preserve adipocyte identity and limit dermal fibrosis.

Keywords: systemic sclerosis, scleroderma, fibrosis, adipose

Short Summary:
SIX1 is elevated in systemic sclerosis skin and linked to adipose-associated biology. Deleting SIX1 in adipocytes reduces fibrosis, making it a potential therapeutic target.

Introduction

Systemic sclerosis (SSc; or scleroderma) is a rare and heterogeneous autoimmune connective tissue disorder.(1, 2) The multiorgan dysfunction has been characterized as a triad of immune dysregulation, vasculopathy, and excessive extracellular matrix (ECM) deposition by myofibroblasts, leading to skin and internal organ fibrosis.(2-4) Skin thickening and tightening is responsible for considerable morbidity in this debilitating disease.(5, 6) The extent of skin fibrosis defines the two subclasses of SSc: limited cutaneous (lcSSc) and diffuse cutaneous SSc (dcSSc).(3) There are currently no FDA-approved treatments for skin involvement in SSc. More extensive skin involvement at the time of diagnosis is associated with higher levels of disability, severity of pain, and decreased survival.(7, 8) This underscores the urgent need to identify novel targeted therapies for the treatment of SSc.(9)

Thus, understanding the early pathogenesis of disease represents a critical step towards novel therapeutic approaches.(9) The mechanisms that lead to organ fibrosis in SSc are not fully understood; however, similar mediators have been identified to play a role in the lung and skin. For example, increased adenosine, hyaluronan, and IL-6 have been implicated in the pathophysiology of both lung and skin fibrosis(10-14). However, these mechanisms have largely focused on fibroblast and epithelial biology, with limited studies in adipocytes. This is important as an early hallmark of SSc is skin-associated adipose tissue atrophy and replacement by extracellular matrix, leading to dermal thickening.(15, 16) Clinically, this may directly contribute to rigidity and tethering observed in early lesional SSc skin (15, 16). Dermal white adipocyte tissue (DWAT) adipocytes display

highly distinct features compared to other white adipocytes, including significant plasticity(17-19). They can cycle through de-differentiation and re-differentiation as part of a physiologic response to hair cycling, aging, and energy demands.(17, 18, 20) Under certain conditions, de-differentiated pre-adipocytes escape the normal cycle of redifferentiation and *trans*-differentiate into ECM-producing myofibroblasts. This process is referred to as the adipocyte-to-myofibroblast transition (AMT).(21, 22) AMT has been well-studied in wound healing (21, 23) and recent evidence suggests it may also contribute to pathological skin fibrosis.(22, 24) Mature adipocytes in the skin produce PDGF ligands and BMPs, both of which are implicated in wound healing and fibrosis. (25) In addition, the adipose secretome has also been identified to play a role in the pathophysiology of SSc. (26) Lipid-filled adipocytes cross-talk with other cell types in the stroma-vascular fraction of adipose tissue. The interaction between the dermal fibroblasts and adipocytes has also been appreciated as a contributor to irregular inflammation and aberrant wound healing through pro-inflammatory signaling between cell types (27) and adipocyte-driven regulation of fibroblast ECM production. (28) A robust physiologic axis also exists between adipocytes and endothelial cells, by which cell signaling can be bidirectionally regulated. (29) However, mechanisms by which adipocytes contribute to the pathogenesis of skin fibrosis in SSc remain poorly understood.

Our group and others identified sine oculis homeobox homolog 1 (SIX1) as a novel mediator in lung fibrosis, promoting the release of pro-fibrotic mediators by alveolar epithelial cells.(30-32) Further, SIX1 has also been implicated in asthmatic lung fibrosis (31, 32) and in liver fibrosis defined by excessive myofibroblast activation and ECM

deposition (33). Lung fibrosis is an important complication of SSc that is typically observed following onset of skin fibrosis, and while epithelial and fibroblast-based mechanisms are most highly studied, the contribution of the adipocyte to the fibrotic process is not fully known.(34) However, whether SIX1 plays a role in skin fibrosis in SSc is not known; in particular, how adipocyte SIX1 regulates dermal fibrosis remains under investigated. SIX1 is a member of an evolutionarily conserved family of developmental transcription factors.(35) SIX1 plays a critical role in regulating the expression of genes that control precursor cell survival and proliferation during embryogenesis. In healthy adults, SIX1 is negligibly expressed in most tissues.(36) Perhaps the most well-studied role of SIX1 in adulthood is in the context of cancer, where it is a critical regulator of trans-differentiation of pre-cancerous cells into mesenchymal cells with metastatic features.(37-41) Although *SIX1* transcript expression has been identified in healthy subcutaneous adipose tissue(42), to date its exact role in adipose tissue biology remains poorly investigated. Brunmeir et al.(43) were the first to identify the direct transcription regulation by SIX1 and the interaction between SIX1 and major regulators of adipogenesis, in mature fat cells. Recently, it was demonstrated that *in vivo* SIX1 overexpression in mouse hepatocytes exacerbates diet-induced liver inflammation, metabolic disruption, and hepatic steatosis, and activates liver-specific receptors to induce de novo lipogenesis.(44) This work is founded upon the fundamental and newly developing understanding of the roles of SIX1, particularly in the realm of lipolysis and the release of pro-fibrotic factors that regulate dermal fibrosis. We hypothesize that SIX1 contributes to dermal lipoatrophy and skin fibrosis in SSc. Surprisingly, our data suggests that SIX1 is not involved in

modulating adipocyte phenotype, but instead it regulates the release of the pro-fibrotic mediator, PAI-1 that promotes dermal fibrosis.

Results

Increased *SIX1* levels correlate to skin fibrosis in SSc patients.

Previously, our group identified increased SIX1 in lung fibrosis(30) and *SIX1* expression has been identified to be present in subcutaneous adipocytes, particularly in those exhibiting aberrant function (42). As such we aimed to determine whether *SIX1* was elevated in skin samples from SSc patients. To do this, we first determined expression of *SIX1* from two distinct cohorts: The GENISOS (Genetics versus ENvironment In Scleroderma Outcome Study), cohort, which includes patients with limited cutaneous (lc) (lcSSc) and diffuse cutaneous (dcSSc) at different stages of disease, and the Prospective Registry for Early Systemic Sclerosis (PRESS) cohort, enriched for patients with early-stage dc-SSc. *SIX1* transcript levels were elevated in SSc skin in limited SSc, diffuse SSc, and in early diffuse SSc compared to control skin in both independent cohorts (**Figure 1**). In the PRESS cohort, which is enriched for patients with early dcSSc, RNA-sequencing revealed increased *SIX1* signals (**Figure 1a**). In the GENISOS cohort, which encompasses dcSSc and lcSSc at different stages of disease, *SIX1* signal intensity, denoting expression levels, was higher in patients with dcSSc compared to lcSSc (**Figure 1b**). To determine whether *SIX1* is associated with the genomic landscape of a particular cell type, we correlated *SIX1* expression levels with cell type-specific signature scores previously utilized by our group.(45, 46) The bioinformatic analysis identified genes that are expressed at comparatively higher levels in a specific cell type and created a signature score for each cell type being evaluated. The subcutaneous adipose signature was the most highly correlated with *SIX1* expression in both the GENISOS (r = 0.76) and PRESS cohorts (r = 0.79). This points to *SIX1* is an important mediator that is elevated in

adipose tissue in SSc (**Figure 1c**). Higher expression of genes specific to fibroblasts, vascular and lymphatic endothelial cells, significantly correlated with higher expression of *SIX1,* suggesting that *SIX1* may regulate mesodermal derived cells during fibrosis but not the ectodermally derived epithelium. Individual gene correlation analysis revealed that genes associated with adipocyte biology were enriched amongst those genes most highly correlated with *SIX1,* specifically in early dcSSc skin (**Figure 1d; Supplementary Table 3**). These included genes encoding proteins required for adipocyte differentiation and triglyceride metabolism, including *ADIPOQ* and *PPARG*.(47-49) Functional annotation of all differentially expressed genes (DEGs) in the skin of dcSSc patients in the PRESS cohort compared to healthy controls showed significant enrichment for “regulation of lipolysis in adipocytes” in addition to “ribosome” and “AMPK signaling pathway” (**Figure 1e**).

Increased adipocyte *SIX1* levels correlate with loss of dermal white adipose tissue (DWAT) in SSc

Several studies have shown that the lipoatrophy and loss of DWAT is present in skin fibrosis in SSc(15) and even precedes the fibrotic matrix deposition(50). In addition, AMT (22, 24) and the adipose secretome (26) have been implicated in the pathophysiology of SSc. Thus, we next aimed to determine whether *SIX1* levels were increased in the diminishing DWAT layer in the skin. Herein, we assessed tissue samples from the GENISOS cohort that include SSc-affected skin samples with varying disease durations were compared to an age, sex, and ethnicity-matched control sample which demonstrating a progressive loss of DWAT areas as disease progresses (**Figure 2a**).

This is important as DWAT levels are known to reduce as dermal fibrosis develops, thus the GENISOS cohort allows us to temporally assess loss of adipose tissue and SIX1 levels during progression of disease. Demographic and clinical features of these individuals are listed in **Supplementary Table 4**. Compared to the control biopsy, there was notably less DWAT in the skin of all dcSSc-affected individuals, with the patient with the most established form of disease presenting with no discernible DWAT areas (**Figure 2a**). Thus, having established a correlation between changes in adipocyte biology, stromal cells, and *SIX1* gene expression in SSc skin; we sought out to identify those cell types that produce *SIX1* in SSc skin with abnormal dermal fat.

We selected 8 SSc patients who retained dermal fat, most of whom were within three years of developing diffuse disease. Additionally, skin biopsies from 4 healthy controls were included. To localize the *SIX1* gene in these samples, we employed single-molecule in situ hybridization. DWAT in human skin is localized around adnexal glands and hair follicles, and within and below the deep dermis(51). *SIX1* was detected in both peri-adnexal and deep dermal adipocytes. Mature adipocytes are identifiable by a single large lipid droplet surrounded by a thin ring of cytoplasm and a peripheral nucleus, which expresses Fatty Acid Binding Protein 4 (*FABP4)* (**Figure 2b**). Our morphometric quantification revealed increased expression of *SIX1* in SSc tissue (**Figure 2c**). We acknowledge that some fibroblasts also express *FABP4*, however, the unique morphology of adipocytes as described above makes them easily distinguishable from the small, spindle-shaped fibroblast with a dominant, central nucleus. Although $FABP4^{+}$ cells are consistent with preadipocytes, it is plausible for subset of cells to include

macrophage- or vascular-associated populations. In addition based on landmark scRNA-seq studies in SSc skin(52), increased SIX1 expression levels in fibroblast and pericyte subtypes, with limited expression in keratinocytes, macrophages and reduced expression in endothelial cells (**Supplementary Table 5**). It is important to note that no datasets were present for mature adipocytes. The clinical relevance of these findings was supported by positive correlations between the expression of *SIX1* in SSc skin, and the extent and severity of SSc skin fibrosis. Spearman's rank-order correlation analysis showed a positive correlation between whole skin *SIX1* expression and modified Rodnan skin score (mRSS) ($r=0.40$, $p<0.001$), and local skin score ($r=0.38$, $p<0.001$) near the site of the biopsy. To our knowledge, dermal fat *SIX1* has not previously been identified *in situ*, the significance of which is supported by clinical data linking *SIX1* to more severe and extensive SSc skin involvement. Together, these data from two SSc study cohorts provided a strong premise for investigating *SIX1* in SSc disease mechanisms.

Adipose tissue loss is evident in a bleomycin model of dermal fibrosis

We selected the murine subcutaneous (SQ) bleo model of skin fibrosis(53) as the pre-clinical model to study the effects of *SIX1* on fibrosis and lipoatrophy.(11, 54) Unlike human skin, rodent skin DWAT is separated from the SWAT by a thin layer of skeletal muscle (the panniculus carnosus), allowing us to distinguish dermal adipocytes from subcutaneous adipocytes without the use of additional markers.(55, 56) Further, the fibrotic changes that occur over the 28 days of SQ bleo treatment allow us to study the role and expression of SIX1 during the pathogenesis of dermal fibrosis.(22, 50, 57)

We demonstrated that serial injections of SQ bleo recapitulated lipodystrophy and dermal sclerosis mirroring SSc manifestations (**Figure 3a)**.(22, 49, 56) Using Masson's trichrome staining, we showed progressive bleo-induced atrophy of the DWAT, increased collagen deposition, and dermal thickening observable at weekly time points up to 28 days (**Figure 3b**). Attrition of DWAT was appreciable on histology as early as day 7 of bleo treatment, when dermal thickening was less pronounced. Quantification of dermal thickness and DWAT area confirmed these observations. When compared to day 7, dermal thickening was significant after 28 days of bleo (**Figure 3c**). This change lagged behind the significant decline in DWAT area (**Figure 3d**). Transcriptomic analysis revealed increased signals for *Six1* (**Figure 3e**) and despite minimal histological changes in the dermis after 7 days of bleo, prominent extracellular matrix genes collagen 1a1 (*Col1a1*), collagen 1a2 (*Col1a2*), and collagen 6a1 (*Col6a1*), transforming growth factor beta (*Tgfb1*) and of serine proteinase inhibitor E 1 (*Serpine1*) were upregulated (**Figure 3f-j**). Intriguingly, we did not detect increased expression for peroxisome proliferator-activated receptor gamma (*Pparg) on day 7 of SQ bleo (***Figure 3k***).* These results support that a reduction in DWAT and upregulation of fibrotic genes precedes significant dermal expansion following SQ bleo treatment. Next, we investigated whether adipocyte *Six1* expression, as observed in SSc skin, was recapitulated in the SQ bleo model. Dual in situ hybridization probed for skin *Six1* and *Adipoq* transcript expression after 7 days of SQ vehicle or bleo injections. *Adipoq* is highly and specifically expressed in lipid-laden adipocytes.(48) Mirroring findings in human SSc skin whereby *SIX1* was elevated in early disease, *Six1* transcripts levels were upregulated in the dermal adipocytes after 7 days of SQ bleo treatment (**Figure 4 a -c**). We also assessed *Six1* expression levels in fibroblasts using smooth

muscle actin (SMA) as a readout, these studies did not reveal increased *Six1* in SMA+ cells. (**Figure 4 d, e**). Collectively, this data demonstrates that adipocyte loss is evident prior to fibrotic deposition and that SIX1 levels are increased in adipocytes by day 7 of bleo treatment.

Transgenic *Six1* deletion attenuates bleomycin-induced skin fibrosis

We next investigated whether genetic inhibition of *Six1* could prevent skin fibrosis. After a 28-day course of SQ bleo to induce an end-stage fibrosis phenotype, the affected skin of tamoxifen-treated mice with (iUbc^{Cre}) and without the *Six1* allele (iUbc-Six1$^{-/-}$) was analyzed by gene expression profiling and histology (**Figure 5 a**). *Six1* depletion in iUbc-Six1$^{-/-}$ skin following tamoxifen was confirmed morphometrically using dual FABP4 IHC and Rnascope for *Six1* (**Figure 5 b, c**) and by RT-qPCR (**Figure 5 d**). Expression of profibrotic agents *Col1a1, Col1a2, Fn1, elastin, Acta2, Tgfb1 and Serpine1 but not Mif* was decreased in the skin of iUbc-Six1$^{-/-}$ compared to iUbc^{Cre} mice (**Figure 5 e-l**). To investigate whether *Six1* modulates latent TGF-β complex components, we measured Latent Transforming Growth Factor Beta Binding Protein (*Ltbp*)1–4 transcripts. Only *Ltbp4* but not *Ltbp1-3* was significantly decreased in iUbc-Six1$^{-/-}$ mice, (**Figure 5, m-p**). These data suggest that *Six1* may selectively affect latent TGF-β sequestration/availability through *Ltbp4.* We did not detect changes in expression of adipocyte markers*: Adiponectin, Cebpa, or Pparg* (**Figure 5 q-s**). Masson's trichrome staining of bleo-affected skin showed maintenance of the DWAT layer in iUbc-Six1$^{-/-}$ mice compared to iUbc^{Cre} mice (**Figure 6a**). *Six1*-deficient mice had more prominent DWAT with lipid-laden adipocytes (**Figure 6b).** Perilipin 1 immunostaining was used to

specifically detect adipocyte lipid droplets (**Figure 6c).** Adipocyte droplets in iUbc-Six1$^{-/-}$ were significantly larger compared to iUbc^{Cre} mice (**Figure 6d).** The deposition of collagen 6, which is enriched in adipose tissue, was analyzed using dual immunofluorescent staining with perilipin 1 to identify the DWAT (**Figure 6c**).(58, 59) iUbc-Six1$^{-/-}$ had lower collagen 6 density in the DWAT compared to iUbc^{Cre} mice (**Figure 6e**). There was no significant difference in collagen 6 deposition in the dermis, or in dermal thickening (data not shown). In summary, whole-body depletion of *Six1* followed by 28 days of SQ bleo revealed that *Six1* deletion may halt pro-fibrotic gene expression and maintain DWAT in skin fibrosis.

To determine whether adipocyte-derived SIX1 contributes to dermal fibrosis, we examined the effect of adipocyte-specific deletion of Six1 in the bleomycin-induced skin injury model

To build on our data demonstrating elevated *SIX1* in biopsies from individuals with early SSc and adipose *Six1* expression in a mouse model of dermal fibrosis, when DWAT has begun to atrophy. We next investigate the potential role of adipocyte-specific Six1 in early disease. We developed a transgenic mouse model to knock-out *Six1* in cells with an active *Adipoq* promoter after tamoxifen treatment, conferring adipocyte-specific *Six1* depletion in adult mice(48). We challenged mice with SQ vehicle or bleo for 14 days, as we found this duration of bleo induced DWAT atrophy before late fibrosis is established. Gene expression and histological analyses were performed to determine the contribution of adipocyte *Six1* in early events in skin fibrosis. Masson's trichrome staining and dermal thickness measurements revealed a thinner dermis in iAdipo-Six1$^{-/-}$ mice after SQ bleo compared to iAdipo^{Cre} mice (**Figure 7a-b**), demonstrating how adipocyte-specific *Six1* deletion prevented dermal thickening compared to *Six1*-competent mice. *Six1* deletion

was assessed morphometrically in dual RNAscope for *Six1* and adiponectin revealed reduced *Six1* puncta in Bleo-treated iAdipo-Six1$^{-/-}$ mice. Gene expression analysis revealed reduced *Serpine1* but elevated *Adiponectin* expression on day 14 of bleo exposure in in iAdipo-Six1$^{-/-}$ vs control iAdipo^{Cre} mice (**Figure 7e, f**). Consistent with improved dermal thickness at day 14 of Bleo, we report reduced *Col1a1* and *Col1a2* following SQ bleo in iAdipo-Six1$^{-/-}$ vs control iAdipo^{Cre} mice (**Figure 7 g, h**).

Next, we performed perilipin 1 immunostaining to identify adipocytes in the DWAT and measured intracellular lipid droplet size (**Figure 8a**). No difference in droplet size was observed in vehicle-treated mice regardless of *Six1* expression. However, adipocyte *Six1* deletion inhibited the reduction in droplet size induced by bleo that precedes loss of adipose tissue (**Figure 8b**). These findings suggest that *Six1* plays a role in dermal lipid droplet size during the early development of skin fibrosis. To determine whether adipocyte preservation was accompanied by reduced DWAT collagen deposition, we performed immunofluorescent staining to detect collagen 6. While collagens 1, 4, and 6 are abundantly expressed in adipocytes, collagen 6 is the most predominant collagen in fat depots (59), thus the measurement of such would be expected to have the highest sensitivity for significant changes in the ECM. Deletion of *Six1* in adipocytes significantly reduced DWAT collagen 6 deposition after 14 days of bleo (**Figure 9 a, b**). This is evident in dual IF for perilipin and collagen 6 demonstrating maintenance of perilipin structures and reduced COL6 in SQ Bleo treated iAdiponectinCre-Six1$^{-/-}$ vs iAdiponectin^{Cre} controls (**Figure 9c**). Adipocyte lipid droplet shrinkage and increased ECM deposition cause dramatic changes to DWAT architecture and function in skin fibrosis. We have here

established adipocyte *Six1* as a driver of both lipid droplet size and ECM deposition, and a candidate target for therapeutic intervention.

Collectively, our data demonstrates that *Six1* deletion helps maintain lipid droplet size and ECM deposition in bleomycin-treated mice. To identify the SIX1 downstream mechanisms, we treated 3T3L1 fibroblasts with a *siSIX1* (or control siRNA) and an adipocyte differentiation cocktail to promote differentiation to adipose cells. Cells were collected on days 0, 2, 4, 6 and 9. These experiments demonstrated a reduction *SIX1* knockdown on day 4 and 6 by western blots (**Supplementary Figure 1 a, b**).

Next, we performed a gene analysis using the nCounter platform targeting fibrotic gene expression on Day 6 of 3T3 treated fibroblasts with and without *siSIX1*. These unbiased experiments and subsequent heat maps for ECM synthesis and TGF-β signaling revealed that KD of *SIX1* reduced levels of serine proteinase inhibitor E 1 (SERPINE1) the gene that encodes plasminogen activator inhibitor 1 (PAI-1), (**Supplementary Figure 2 a, b)**. These results also demonstrated reduced *Col4a1*, *Col4a2*, *Col5a1* and *Col5a3* and *Col6a3* in addition to *Tgfb1*. Analysis of the TGF-β signaling pathway revealed reduced downstream mediators following siSix1 such as *Tgfbr1*, *Crebbp*, or *Furin* (**Supplementary Figure 2 a, b**). *Serpine1* was selected for further validation based on a volcano plot demonstrating that it was one of the most significantly downregulated genes following siSix1. (**Supplementary Figure 3**).

To further investigate the role of *Six1* as a regulator of *Serpine1,* we treated mouse 3T3L1 fibroblasts with a pLenti-CRISPR/Cas9 *Six1* gRNA vector and an adipocyte differentiation

cocktail to promote differentiation to adipose cells. Clone 2 was selected for its efficacy at depleting SIX1 levels, consistent with reduced PAI-1 levels (**Figure 10 a**). Cells were collected on days (D) 0, 2, 4, 6, 9 and 12, herein *Six1* deletion resulted in reduced Serpine1 levels on D, 2, 4, 6 and 9 (**Figure 10 b**). The reduction in *Serpine1* expression levels was consistent with reduced PAI-1 signals in DWAT areas in Bleo-treated iAdiponectinCreSix1$^{-/-}$ mice vs control Adiponectin^{Cre} mice, assessed morphometrically (**Figure 10 c, d**). We confirmed *SERPINE1* promoter binding by SIX1 using a full-length h*SERPINE1* promoter Gaussia Luciferase–expressing plasmid. Luciferase activity was significantly increased in *Six1*OE within 12 hours indicating a direct activation of the promoter (**Figure 10 e**). Expression of SERPINE1 was also upregulated 11.5-fold and 3.8-fold in the PRESS and GENISOS cohorts respectively (**Table 1**). Although the increased expression of SERPINE1 could reflect secondary processes in fibrosis, our findings support the conclusion that SIX1 upregulation directly promotes SERPINE1/PAI-1 expression as a downstream driver of dermal fibrosis.

Next, to identify whether SIX1 can directly regulate stromal cell fate in dermal fibrosis we assessed lipid expression levels in mouse 3T3L1 fibroblasts exposed to adipocyte differentiation cocktail. These studies revealed increased *adiponectin* and *Cebpa* expression levels on D6, D9 (*Cebpa* only) and D12 of CRISPRCas9 deletion of *Six1* (**Supplementary Figure 4 a, b**). *Fabp4* and *Pparg* followed the same trend with reduced expression in SIX1 KD cells of both of these genes on D2 and D9 (*Pparg* only) and increased expression on D4, D6 and D12 (**Supplementary Figure 4 c, d**). Fibro-genic gene expression revealed increased *Fn1* expression levels on D0 and D2 but reduced expression on D6 and D9 (**Supplementary Figure 4 e**) in SIX1 KD cell. *Mif* expression

levels reduced on D4, D6 but increased on D12 (**Supplementary Figure 4 f**) following loss of SIX1. *Tgfb1* expression levels were reduced on D2, D9 and D12 but elevated on D6, in SIX1 deficient cells (**Supplementary Figure 4 g)**. Intriguingly, the downstream mediator of TGFβ, *Smad3*, did not follow the same trend as *Tgfb* except for D12 where it was also reduced; instead, it was reduced on D0, and elevated on D2 and D9 (**Supplementary Figure 4 h**). Despite the variation in gene expression, these data point to deletion of SIX1 resulting in increased lipid mediators and reduced pro-fibrotic gene expression by D12. This temporal pattern parallels the *in vivo* sequence, where adipocyte depletion precedes collagen deposition, suggesting that SIX1 orchestrates early transcriptional events determining stromal fate after injury. This is consistent with IF denoting a more adipocyte-like cell following SIX1 KD compared to control cells stimulated with a differentiation cocktail (**Supplementary Figure 4 i**). These *in vitro* studies suggest that SIX1 acts to repress adipocyte differentiation and favor a more fibroblast-like, profibrotic transcriptional state when treated with a adipocyte differentiation cocktail *in vitro.*

Discussion

This study demonstrates the previously undescribed clinical and translational relevance of the developmental transcription factor *SIX1* in adipocyte-mediated skin fibrosis. We identified increased expression of the developmental transcription factor *SIX1* in skin-associated adipose tissue in SSc skin samples from two well-described cohorts encompassing 161 patients. The GENISOS cohort contains both lcSSc and dcSSc, allowing us to identify a further increase in *SIX1* in diffuse disease in a large cohort. *SIX1* was also elevated in the skin of individuals with dcSSc diagnosed within three years of disease onset, obtained from the PRESS cohort. In agreement with expression data showing enrichment of *SIX1* in dcSSc, *SIX1* skin expression positively correlated with clinical measurements consistent with worse skin disease extent and severity. Previous work established a subcutaneous adipose signature in human skin.(45, 46, 60) We found this signature to be strongly correlated with *SIX1* expression in both SSc cohorts. It is important to note that a specific dermal adipose signature has not yet been established.(56) This observation suggests that in SSc skin, *SIX1* expression may be associated with genomic changes in adipocyte function. Expression data was supported by histology findings in SSc skin samples from the GENISOS cohort. We reproduced previously reported observations(15, 50) that dermal fat mass declines early and remains atrophic in SSc skin. Using two novel transgenic mouse models, we found evidence for a role for *Six1* in lipodystrophy and fibrotic features observed in the SQ bleo model. Intriguingly, however, only the deletion of *Six1* from adipocytes but not in UBC^{Cre} expressing mice resulted in a reduction of dermal thickening. Possible explanations for this include a more efficient and selective inhibition of *Six1* in adipocytes through the

adiponectin Cre system compared to UBC^{Cre} expression or a potential protective response of *Six1* in other cells that could include mesenchymal or inflammatory cells. We propose a working model in which SIX1 helps to drive skin fibrosis by interacting with lipolysis-associated molecular pathways to promote intracellular lipid loss, a critical first step towards transforming a healthy adipocyte to a disease-driving cell type. However, it is also possible for the adipocyte layer to function as a protective element against the development of fibrosis that is lost as tissue atrophies.

We found that *SIX1* expression in SSc skin correlated with genes related to lipid metabolism. We and others have shown that lipid droplets in DWAT are smaller in the SQ bleo-treated mice.(22, 61) Mice with *Six1*-deficient adipocytes (*Six1*$^{-}$ WAs) had larger intracellular lipid droplets than *Six1*$^{+}$ WAs after SQ bleo. Changes in size of unilocular lipid droplets in WAs is one surrogate measurement used to profile an adipose depot as being lipogenic or lipoatrophic.(51, 62) A critical step in AMT is the release of free fatty acids into the local tissue environment, a process which permits the transition of a lipid droplet-containing adipocyte into a precursor cell(20). AMT is a fluid process involving dynamic and complex changes in cellular phenotypes and gene expression(24, 63). Dynamic control of lipid storage is required for differentiation and transdifferentiation (47, 64, 65). We found *Six1* to be expressed in mouse DWAT after just 7 days of bleo. While the majority of *Six1*-positive (Six1^{+}) cells were also positive for *Adipoq* (*Adipoq*$^{+}$), we observed a minority of *Six1*$^{+}$ *Adipoq*$^{-}$ cells, that is, cells other than lipid-laden adipocytes. *Adipoq*$^{-}$ cells in the stroma vascular fraction (SVF) include diverse cell types.(66) Further, *ADIPOQ* was highly correlated with *SIX1* in SSc skin. Given the exclusivity of *Six1* expression to the DWAT, we propose that these cells most likely represent those of

adipocyte lineage. These studies and the known capacity of SIX1 to promote trans-differentiation (38, 67) point at a role for SIX1 in mediating AMT. In line with this, our *in vitro* findings provide mechanistic insight into our *in vivo* observations showing that SIX1 deletion maintains DWAT and adipocyte barrier following fibrotic injury. In cultured 3T3-L1 fibroblasts, loss of SIX1 enhanced adipogenic differentiation, as evidenced by increased expression of *Adiponectin*, *Cebpa*, *Pparg*, and *Fabp4*, and reduced expression of profibrotic mediators including *Fn1*, *Mif*, *Tgfb1*, and *Smad3*. This pattern mirrors the phenotype observed in SIX1-deficient mice, where adipocytes were preserved and fibrotic remodeling was attenuated after bleomycin treatment. Together, these data support a model in which SIX1 promotes AMT by repressing adipogenic transcriptional programs and activating fibrogenic signaling. Deletion of SIX1 prevents this reprogramming, thereby stabilizing adipocyte identity and preserving the DWAT barrier. Given the observed modulation of *Mif* and PAI-1 expressions, SIX1 may also regulate paracrine signaling between adipocytes and fibroblasts, reinforcing profibrotic activation within the dermal microenvironment. To further uncover the mechanism that leads to SIX1-mediated paracrine dermal fibrosis, we turned to a non-biased approach using the nCounter platform. Here siRNA deletion of *Six1* in 3T3L1 cells inhibited ECM components such as *Col6a3*, consistent with our IHC for COL6A and other collagens such as *Col4a1*, *Col4a2*, *Col5a1* and *Col5a3* in addition to *Tgfb1*. In line with previous studies linking *Mif*(30) , *Tgfb1*(67) and *Pparg*(43) as targets of SIX1 our studies demonstrated that these mediators we altered following SIX1 deletion using CRISPRCas9. To further explore upstream pathways that may link SIX1 to fibrotic remodeling, we examined expression of *Ltbp* family, which regulates extracellular sequestration and activation of latent TGF-β

complexes(68). Among these, only *Ltbp4* was significantly reduced in Bleo-iUbc-Six1$^{-/-}$ mice. Because LTBP4 participates in matrix tethering and bioavailability of latent TGF-β, its selective downregulation may attenuate TGF-β activation in the fibrotic niche. These findings raise the possibility that SIX1 modulates dermal fibrosis in part through regulation of latent TGF-β signaling. Since LTBP4 regulates extracellular sequestration and activation of latent TGF-β complexes(69), its reduction in SIX1-deficient mice may contribute to attenuated TGF-β activation and diminished fibrotic remodeling

A common mediator that was altered in both heat maps was *Serpine1*, the gene encoding for PAI-1. These results revealed reduced *Serpine1* following *Six1* deletion by CRISPRCas9, a result that was validated by RT-qPCR and western blots and by IHC in skin sections from bleo-treated mice, revealing reduced PAI-1 signals in mice lacking *Six1* in adipocytes. This was further confirmed with a luciferase assay demonstrating that SIX1 is able to bind to the Serpine1 promotor. These results are significant since elevated PAI-1 has been shown to be elevated in skin lesions from SSc patients(70, 71) and its inhibition improved dermal inflammation and fibrosis in bleo treated mice.(71) These findings suggest SIX1 as upstream from PAI-1 and as a mediator that predisposes adipocytes to a pro-fibrotic phenotype.

Although SIX1 expression was most prominent in dermal fibroblasts, increased SIX1 expression was also observed in macrophage-like and endothelial-like cells in both the GENISOS and PRESS cohorts. The functional significance of SIX1 in these non-fibroblast populations remains to be determined. It is conceivable that SIX1 contributes to profibrotic signaling through modulation of cytokine or angiogenic pathways, consistent with its described roles in other systems (72, 73). Although SIX1 expression was strongest in

fibroblast and perivascular clusters, its induction across stromal compartments suggests a broader contribution to the activated dermal niche in SSc.

Targeted therapeutics in SSc are limited as a result of our fragmented understanding of disease mechanisms.(74) Two medications, nintedanib and tocilizumab, have been approved by the Food and Drug Administration (FDA) for SSc-related interstitial lung disease.(75) However, neither these drugs or others are FDA-approved for SSc skin involvement. SIX1 has been shown to be a potential therapeutic target in pulmonary fibrosis.(30) We proposed that SIX1 might also play a role in skin fibrosis. We have shown that genetic deletion of *Six1* is able to reduce dermal adipose tissue atrophy and fibrotic changes in a rodent model of skin fibrosis. These studies demonstrate that adipose tissue homeostasis not only has anti-fibrotic effects, but that its preservation is a potential therapeutic approach for skin manifestations in SSc. Although our data support a primary role for SIX1 in adipocyte fate determination, its regulation of inflammatory mediators such as MIF suggests that SIX1 may also influence immune-stromal interactions contributing to sustained fibrosis. Together, these findings suggest that pharmacological inhibition of SIX1, or its downstream mediator PAI-1, may represent a novel strategy for preventing or treating dermal fibrosis in SSc.

Methods

Sex as a biological variant

In our human and mouse studies female and male sexes were examined no significant differences were observed between sexes.

Study populations

GENISOS cohort

The prospective cohort study, GENISOS , is a collaboration between The University of Texas Health Science Center, Houston, The University of Texas Medical Branch at Galveston, and the University of Texas Health Science Center at San Antonio. All participants met the diagnosis of SSc according to the American College of Rheumatology preliminary classification.(76) Details of recruitment and selection criteria have been previously published.(77) As described by (78), full-thickness skin biopsies (forearm or back) were collected under local anesthesia. Total RNA was extracted using TRIzol reagent (Invitrogen) followed by RNeasy column purification (Qiagen). RNA integrity was confirmed (RIN > 7) before hybridization to the Illumina HumanHT-12 v4 Expression BeadChip.

Individuals with dcSSc and lcSSc are enrolled within five years of disease onset, defined as the first non-Raynaud's symptom. The study was approved by the institutional review board of all participating sites, and written informed consent was obtained from all study subjects.

The mRSS was calculated by a board-certified rheumatologist with extensive experience in the assessment of scleroderma skin (79). The mRSS is determined by assessment of the skin thickness of 17 body areas by physical examination. The mRSS serves as a surrogate for disease activity, severity and mortality in patients with SSc (80). Healthy

control individuals are enrolled to serve as controls. SSc-affected individuals and controls are matched at a ratio of 3:1 based on age, sex, and ethnicity. Gene expression analysis from SSc-affected skin and skin from controls has been previously described.(78) Raw probe-level intensities were imported into BRB-ArrayTools v4.7.1, $\log_2$-transformed, and quantile-normalized. Probes with > 20 % missing values or mean signal below background were excluded prior to differential expression analysis. Briefly, global gene expression is assessed using the Illumina HumanHT-12 bead array. Raw data were analyzed with BRB ArrayTools. 113 SSc-affected individuals and 44 unaffected controls had available *SIX1* expression levels.

PRESS cohort

The PRESS cohort is a multi-site observational cohort of individuals with dcSSc enrolled within three years of the onset of the first non-Raynaud's symptom.(9) All participants fulfill the 2013 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for SSc.(81) RNA sequencing data from the skin of PRESS participants and controls, previously utilized by our group,(46) was queried for expression of *SIX1*. Forty-eight SSc-affected individuals and thirty-three controls had available *SIX1* expression levels. RNA-seq libraries were generated from total RNA using the Illumina TruSeq Stranded mRNA kit and sequenced (2 × 100 bp) on a HiSeq 2500. Reads were aligned to the human GRCh38 reference genome using STAR v2.7, and raw counts were normalized and tested for differential expression with edgeR (82). The R Bioconductor package edgeR6 analysis was utilized to identify differentially expressed

546 transcripts between SSc patients and healthy controls with a false discovery rate cutoff
547 of 0.05 and fold change cutoff of >1.5 or <0.67
548
549 **Cell type-specific expression signatures**
550
551 Cell type-specific expression signatures were originally developed as previously
552 described (60) and have been utilized by our group.(46) A “cell-type specific signature
553 score” denotes a set of genes for which expression in a given cell type is notably higher
554 than expression in the other evaluated cell types. Signature matrices were derived from
555 previously published SSc skin transcriptomic datasets (83, 84). For each sample, the
556 mean fold-change (SSc vs control) across 125 signature genes defined the cell-type
557 score. Spearman’s rank correlations were computed between each score and SIX1
558 expression to assess lineage association. The numerical value of each score was
559 calculated based on fold-change estimates (SSc versus control) for 125 “signature genes”
560 of a given cell type. The methodology used is described in detail in (60, 78, 85), for gene
561 expression analysis, data were imported into BRB-ArrayTools as processed signal
562 values. Values were excluded if the mean signal was not significantly greater than the
563 background. Values were then log2 transformed, followed by quantile normalization.
564 Genes with >20% missing values across arrays were filtered out. The remaining gene
565 values were used for analyses.
566
567 **Pathway Analysis**
568 Herein, we used the same protocol described previously(60, 78, 85), genes that were
569 differentially expressed on average in SSc compared to control at a false discovery rate

(FDR) of <0.05 were uploaded to Ingenuity Pathway Analysis (Qiagen). The reference set was 'Human Genome CGH 44 K'; only experimentally observed direct and indirect relationships were included. Canonical pathway and upstream-regulator analyses were performed at FDR-adjusted $P < 0.05$ and confirmed with FDR < 0.1.

Correlation analyses

The correlation between signature score and *SIX1* expression was evaluated for each sample, and then the mean Spearman's rank correlation coefficient was reported. Individual gene correlations were analyzed by Spearman's rank correlation. Correlation coefficients are reported as "r." All correlation analyses were performed in R v4.2 using the 'Hmisc' package; significance was defined as two-tailed $P < 0.05$. Plots were generated in ggplot2.

Functional annotation of all differentially expressed genes in PRESS

All DEGs in the skin biopsies of PRESS cohort participants with SSc were input into The **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (DAVID) for functional annotation (Available at https://david.ncifcrf.gov/summary.jsp). Analyses were conducted in DAVID v6.8(86) using the full human genome as background. Biological processes and molecular functions were considered enriched at Bonferroni-adjusted $P < 0.05$. A Bonferroni cut-off of 0.5 was used to determine significantly enriched biological pathways.

Animal studies

All studies were reviewed and approved by UTHealth Houston Animal Welfare Committee (AWC-19-0029, AWC-22-0028). Six to eight-week-old male and female C57/BL6J mice were used for experiments with wild-type mice. Mice with dorsal skin in the telogen phase of hair cycle were used. Regions of skin in the anagen phase were excluded. When possible, littermates were equally distributed between groups. Group sizes were determined by power analysis ($\alpha = 0.05$, power = 80%) and restricted to telogen-phase skin, as exclusion of anagen-phase regions reduces biological variability and provides reliable statistical power with fewer animals(87).

Detailed experimental procedures are available in the supplement, including a complete list of SYBR green primers is listed in Supplementary Table 1.

Statistical analysis

Prism software (v9.0; GraphPad or higher) was used for all statistical analyses. ROUT outlier test was performed on all datasets. Outliers were excluded if FDR was greater than 1%. Two-tailed t-test with Welch's correction was used for 2-group comparisons. Two-way ANOVA with multiple comparisons and correction using the Sidak method was used for 3 or more groups. Detailed statistical analysis for each experiment is shown in the figure legends.

Declarations:

Author Contributions: NW, SA, and HKQ conducted experiments, acquired data, analyzed data, and wrote the manuscript. TM, SC, MW, LR, RG, ML, BS, and WB

conducted experiments, acquired data, and analyzed data. WS analyzed data and provided research material. MA, YY and WJZ performed bioinformatic analyses and AF and HK perform ran the nCounter experiments. All authors read and approved the manuscript for submission.

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Figure Legends

Figure 1. *SIX1* is elevated in SSc skin and correlates with adipose-related genes and pathways.

a) *SIX1* expression in PRESS SSc skin samples and controls. FPKM = fragments per kilobase million based on a false discovery rate cutoff of 0.05 and fold change cutoff of >1.5 or <0.6 **b**) *SIX1* expression in baseline SSc skin samples and controls in the GENISOS cohort based on Student's t-test **c**) Heatmap showing correlation ("r") between skin *SIX1* expression and cell type signature scores on Spearman's Rank order correlation. Bolded pathways indicate correlations with p-values <0.05 in both cohorts. **d**) Volcano plot showing individual gene-*SIX1* correlations in PRESS cohort SSc skin samples. PLIN1=perilipin 1; G0S2=G0/G2 switch gene 2; ADIPOQ=adiponectin; HSL=hormone sensitive lipase; FABP4=fatty acid binding protein 4; LEP=leptin; ATGL=adipose triglyceride lipase; PPARG= peroxisome proliferator activated receptor gamma based on a Spearman's rank coefficient analysis. **e)** KEGG pathway annotation of all DEGs in SSc skin of PRESS cohort participants using GoStats. Significance levels *** P ≤ 0.001 refer to a Bonferroni cut-off of 0.5 analysis (panel a) or a the R Bioconductor package edgeR6 analysis for to identify differentially expressed transcripts between SSc patients and healthy controls with a false discovery rate cutoff of 0.05 and fold change cutoff of >1.5 or <0.67 for panel b.

Figure 2. Dermal White Adipose Tissue (DWAT) atrophy and increased adipocyte SIX1 expression in SSc a) Hematoxylin and eosin staining of human skin biopsies from GENISOS cohort participants. **top** Control skin. **middle** early SSc-representative

image **bottom** established representative SSc-skin sample. Clinical and demographic features of biopsies individuals are provided in Supplementary Table 4. Boxes contain dermal white adipose tissue. Dotted boxes denote DWAT areas. **b)** Representative images of dual *in situ* hybridization for *SIX1* (teal) and *FABP4* (pink) in 3 SSc and 1 demographically matched control biopsies. Hematoxylin (purple) co-stain labels nuclei. “A” marks a dermal adnexal structure. Black arrows point to *SIX1* transcript signal. Dash dot outlines denote the interior periphery of adipocytes. **c)** Representative morphometric quantification for dual positive FABP4/SIX1 cells in healthy vs SSc tissue. Significant levels * P ≤0.05 refer to Mann-Whitney comparisons between healthy (N=4) vs SSc (N=8).

Figure 3. Dermal white adipose loss precedes fibrosis in the murine model of bleomycin induced skin fibrosis. a) Schematic representation of SQ bleo model of skin fibrosis in mice (Created with BioRender) **b)** Representative images of Masson’s trichrome staining of dorsal mouse skin after 7, 14, 21, and 28 days of SQ vehicle or bleo treatment. White arrows indicate dermal thickness. Scale bar = 200µm. **c)** Dermal thickness and **d)** area of DWAT at 7, 14, 21, and 28 days reported in a dot plot ±SD of bleo-injected mice normalized to the average of all vehicle-injected mice at that time point N=4-5 for each time point. Transcript expression levels for sine oculis homeobox homolog 1 (*Six1,* **e**),collagen 1a1 (*Col1a1,* **f**), collagen 1a2 (*Col1a2,* **g**), collagen 6a1 (*Col6a1,* **h**), transforming growth factor beta 1 (*Tgfb1,* **i**), serpin family E member 1 (*Serpine1*, **j**) and peroxisome proliferator activated receptor gamma (*Pparg,* **k**) in 7-day

skin samples by RT-qPCR. Expression was normalized to 18s rRNA. DWAT = dermal white adipose tissue. Significance levels * P ≤0.05, ** P ≤0.01, *** P ≤0.001, and **** P ≤0.0001 refer to a simple linear regression for panels c-d and an unpaired t-test for panels e-k. Each individual plot represents a biological N number. N=3-7.

Figure 4. Adipocyte *Six1* expression precedes white adipose loss in SQ bleomycin-treated mice.

Representative images of mouse dorsal skin injected with SQ bleo for 7 days (n=6). **a):** Masson's trichrome staining. **b)**: Dual *in situ* hybridization for *Adiponectin* (pink) and *Six1* (teal). Arrows point to *Six1* signal. DWAT=dermal white adipose tissue. **c)** Quantification of *Six1* puncta in Adiponectin expressing cells from vehicle (PBS, N=4) or Bleo treated mice (N=7). **d)** Quantification of *Six1* puncta in smooth muscle actin (SMA) expressing cells from vehicle (PBS, N=5) or from Bleo (N=4) treated mice. **e)** Dual immunohistochemistry for SMA (blue signals) and RNAScope for *Six1*, magenta puncta fibroblasts *f* denote fibroblasts and *v* refer to vessels. Scale bar represents 50μm panel a, 10μm panel b and 20μm panel e. Signifiance level * P ≤0.05 refers to an un unpaired t-test for panels c and d.

Figure 5. Inducible global deletion of Six1 inhibits fibrotic gene expression in SQ bleomycin treated mice.

a) Schematic representation of experimental design (Created with BioRender). Following IP tamoxifen administration, iUbc^{Cre} and iUbc-Six1$^{-/-}$ were given 28 days of SQ vehicle (PBS) or bleo. **b**) Representative dual *Six1 RNAscope (*teal) and *adiponectin*

(magenta) from bleomycin iUbc^{Cre} and iUbcSIX1$^{-/-}$ treated mice and **c**) corresponding *Six1* puncta quantification. Arrows point at *Six1* signals and scale bar represent 20μm. Transcript expression levels for sine oculis homeobox homolog 1 (*Six1,* **d**),collagen 1a1 (*Col1a1,* **e**), collagen 1a2 (*Col1a2,* **f**), fibronectin (*Fn1,* **g**), elastin (**h**), actin alpha 2 (*Acta2*, **i**), serpin family E member 1 (*Serpine1*, **j**), transforming growth factor beta 1 (*Tgfb1,* **k**), macrophage migration inhibitory factor (*Mif,* **l**), Latent Transforming growth factor beta binding protein (*Ltbp1,* **m**), (*Ltbp2,* **n**), (*Ltbp3,* **o**), (*Ltbp4,* **p**), *adiponectin* (**q**), CCAAT enhancer binding protein alpha (*Cebpa,* **r**), peroxisome proliferator activated receptor gamma (*Pparg,* **s**) at day 28 of bleomycin treatment. Expression was normalized to 18s rRNA.. Significance levels * P ≤0.05, ** P ≤0.01, *** P ≤0.001, and **** P ≤0.0001 refer to an unpaired t-test for panels c-s Each individual plot represents a biological n number. N=3-8.

Figure 6. Global *Six1*-deletion prevents lipolysis and collagen 6 accumulation induced by SQ bleomycin a) Masson's trichrome staining of skin biopsies from iUbcCre and iUbc-Six1$^{-/-}$ mice treated with 28 days of SQ bleo. **b)** High-magnification Masson's trichrome images of DWAT area marked by square bracket. **c)** Representative brightfield (left panels) and dual immunofluorescent staining for collagen 6 (yellow signals) and perilipin 1 (red signals) in skin from 28-day bleo-injected iUbc^{Cre} and iUbc-Six1$^{-/-}$ mice (right panels). **d)** Quantification of droplet size of perilipin 1-positive adipocytes in the DWAT. Left: High magnification (100X, zoomed) image of lipid droplet within DWAT stained with perilipin 1. **e)** Quantification of collagen 6 as percent positive area in the DWAT. Significance levels * P ≤0.05 refer to an unpaired t-test.

Each individual plot represents a biological N number. N=4-6.V denotes vessels; SM denotes skeletal muscle. Scale bar represents 200 μm (panel a), 50 μm (panel b), and 20 μm (panel c).

Figure 7. Adipocyte SIX1 deletion prevented bleomycin-induced dermal white adipose atrophy and dermal thickness. a) Representative images of Masson's trichrome staining from full-thickness skin biopsies. **b)** Quantification of dermal thickness from Masson's trichrome staining on day 14 of vehicle (PBS) or Bleo SQ administration from iAdipo^{Cre} and iAdipo-Six1$^{-/-}$ mice **c**) Representative dual *Six1 RNAscope (*teal) and *adiponectin* (magenta) from bleomycin iAdipo^{Cre} and iAdipo-SIX1$^{-/-}$ treated mice and **d**) corresponding *Six1* puncta quantification. Arrows point at *Six1* signals and scale bar represent 20μm. Transcript expression levels for *Serpine 1* (**e**), adiponectin (**f**), collagen 1a1 (*Col1a1,* **g**), or collagen 1a2 (*Col1a2,* **h**), from SQ Bleo treated from iAdipo^{Cre} and iAdipo-Six1$^{-/-}$ mice on day 14 (panels e, f) or day 28 (panels g, h). Significance levels * P ≤0.05 and ***P≤0.001 refer to a One-way ANOVA with a Bonferroni correction for panel b or un-paired t-test for panels d-h. Each individual plot represents a biological N number. N=5-13

Figure 8. Lipid droplets are preserved in bleomycin-treated adipocyte-Six1 deficient mice treated with bleomycin. a) Representative images of immunofluorescent staining for perilipin 1 in skin from iAdipoc^{Cre} and iAdipo-Six1$^{-/-}$ mice treated with 14 days of SQ vehicle (PBS) or bleo. Left: brightfield image, Right: Iimmunofluorescence for perilipin (red signals) images. **b)** Quantification of droplet size

of perilipin-positive adipocytes in the DWAT. Significance levels ** P ≤0.01 and ***P≤0.001 refer to a Two-way ANOVA with multiple comparison employing a Sidak correction for panel b. Each individual plot represents a biological N number V denotes vessels; SM denotes skeletal muscle. Scale bar represents 20μm.

Figure 9. iAdipo^{Cre} -*Six1* KO mice have decreased collagen 6 deposition in the DWAT after 14 days of SQ bleomycin. a) Representative images of immunofluorescent staining for collagen 6 (yellow) in skin samples from iAdipo^{Cre} or iAdipo-Six1$^{-/-}$ mice treated with 14 days of SQ bleo. *Left*: Brightfield image and .*Right*: Immunofluorescence for collagen 6 (yellow signals). Area within dotted lines represents DWAT. **b)**. Quantification of collagen 6 as brightness intensity within the DWAT. Significance levels ***P≤0.001 refer to a Two-way ANOVA with multiple comparison employing a Sidak correction for panel b. Each individual plot represents a biological N number N=5. **c**) representative dual immunofluorescence for collagen 6 (red signals) or perilipin (green signals) focusing on the DWAT area of either Bleomycin treated iAdipo^{Cre} (upper panel) iAdipoCreSIx1$^{-/-}$ mice (lower panel). V denotes vessels; SM denotes skeletal muscle. Scale bar represents 20μm (panel a) and 50μm (panel b).

Figure 10. PAI-1 levels are upregulated in bleomycin induced skin fibrosis and track with SIX1 expression levels (**a**) protein levels for β-actin, SIX1 and PAI-1, and from 3T3L1 cells treated with a differentiation cocktail for adipocytes and transfected with either control plasmic (blue bars, panel b) or CRISPR/Cas9 *Six1* plasmid (green

bars, panel a). **b)** *Serpine1* transcript levels and **c**) quantification of PAI-1 signals in DWAT from (**d**) IHC for PAI-1 from bleomycin exposed mouse skin for from 2 independent iAdipoCre or iAdipoSix1$^{-/-}$ mice. PAI-1 signals are shown in magenta. Significance levels ***P≤0.001 refer to a Two-way ANOVA with multiple comparison employing a Sidak correction for panel b. Significance levels *P≤0.05 refer to an unpaired t-test for panel c. **(e)** Ratios of Gaussian luciferase/secreted embryonic alkaline phosphatase (G-Luc/SEAP) showing levels of *SERPINE1*-promoter activity from *Six1*OE cells compared with GFP controls at 12 hours. N=3 (panels b and e) and N=8 (panel c). Scale bars represent 20μm.

Table 1. SERPINE1 expression levels in GENISOS and PRESS cohorts

Cohort	**GENISOS**	**PRESS**
Ensembl_ID	ENSG00000106366.8	
Gene name	SERPINE1	
Gene type	Protein coding	
logFC	1.928211	3.524306
logCPM	2.589225	4.593314
PValue	**2.64E-12**	**4.17E-33**
FDR	2.05E-09	9.2E-30
FC	**3.805831**	**11.50593**
CPM	6.017755	24.13933
Regulation in SSc group	Up	up

FC: Fold Change, CPM: Counts per million

Figure 1

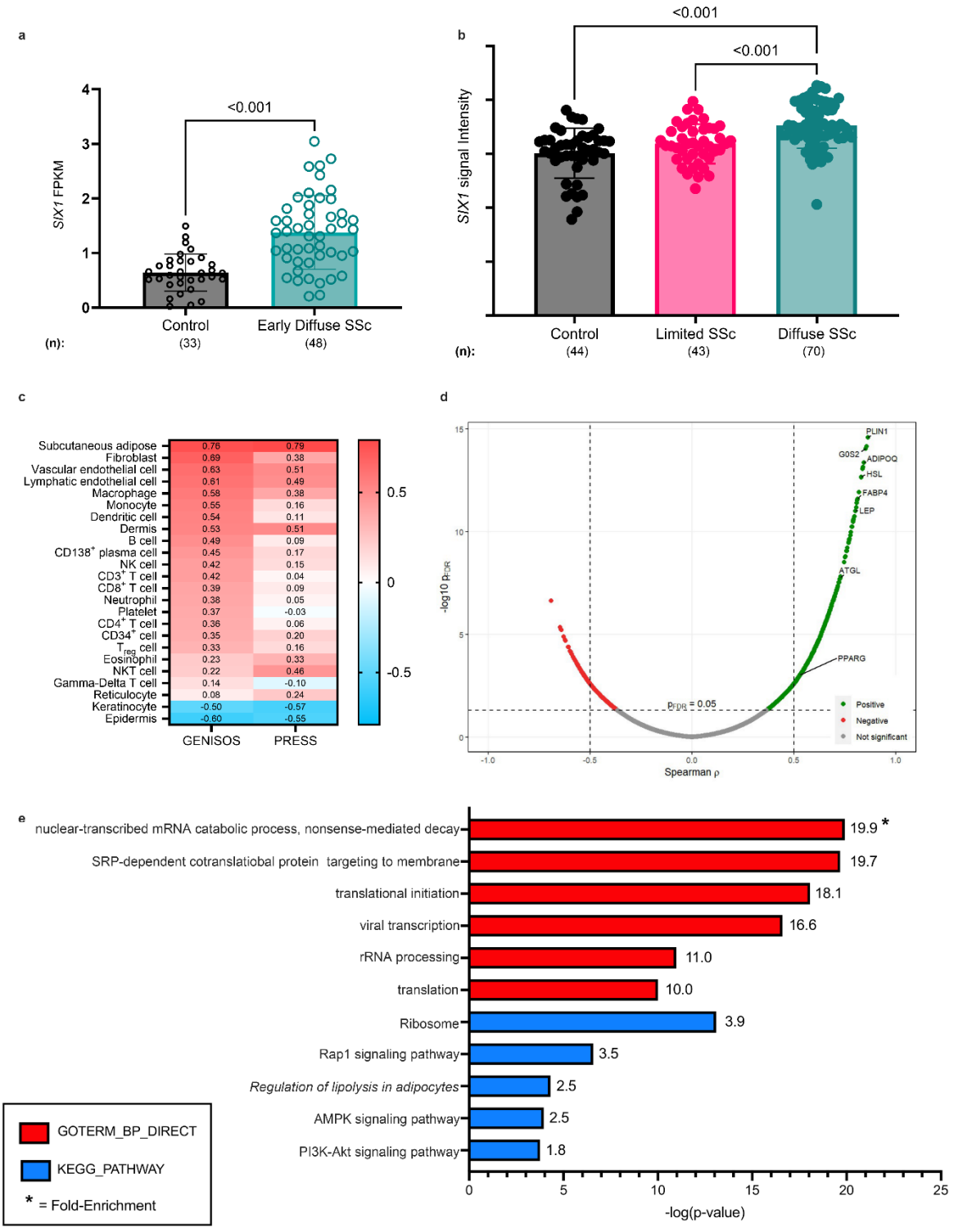

a
SIX1 FPKM
<0.001
Control
Early Diffuse SSc
(n):
(33)
(48)
b
SIX1 signal Intensity
<0.001
<0.001
Control
Limited SSc
Diffuse SSc
(n):
(44)
(43)
(70)
c
Subcutaneous adipose
Fibroblast
Vascular endothelial cell
Lymphatic endothelial cell
Macrophage
Monocyte
Dendritic cell
Dermis
B cell
CD138+ plasma cell
NK cell
CD3+ T cell
CD8+ T cell
Neutrophil
Platelet
CD4+ T cell
CD34+ cell
Treg cell
Eosinophil
NKT cell
Gamma-Delta T cell
Reticulocyte
Keratinocyte
Epidermis
0.76 0.79
0.69 0.38
0.63 0.51
0.61 0.49
0.58 0.38
0.55 0.16
0.54 0.11
0.53 0.51
0.49 0.09
0.45 0.17
0.42 0.15
0.42 0.04
0.39 0.09
0.38 0.05
0.37 -0.03
0.36 0.06
0.35 0.20
0.33 0.16
0.23 0.33
0.22 0.46
0.14 -0.10
0.08 0.24
-0.50 -0.57
-0.60 -0.55
GENISOS
PRESS
0.5
0
-0.5
d
-log10 pDR
PLIN1
G0S2
ADIPOQ
HSL
FABP4
LEP
ATGL
PPARG
pDR = 0.05
Positive
Negative
Not significant
Spearman ρ
e
nuclear-transcribed mRNA catabolic process, nonsense-mediated decay
SRP-dependent cotranslatiobal protein targeting to membrane
translational initiation
viral transcription
rRNA processing
translation
Ribosome
Rap1 signaling pathway
Regulation of lipolysis in adipocytes
AMPK signaling pathway
PI3K-Akt signaling pathway
19.9 *
19.7
18.1
16.6
11.0
10.0
3.9
3.5
2.5
2.5
1.8
GOTERM_BP_DIRECT
KEGG_PATHWAY
* = Fold-Enrichment
-log(p-value)

Figure 2

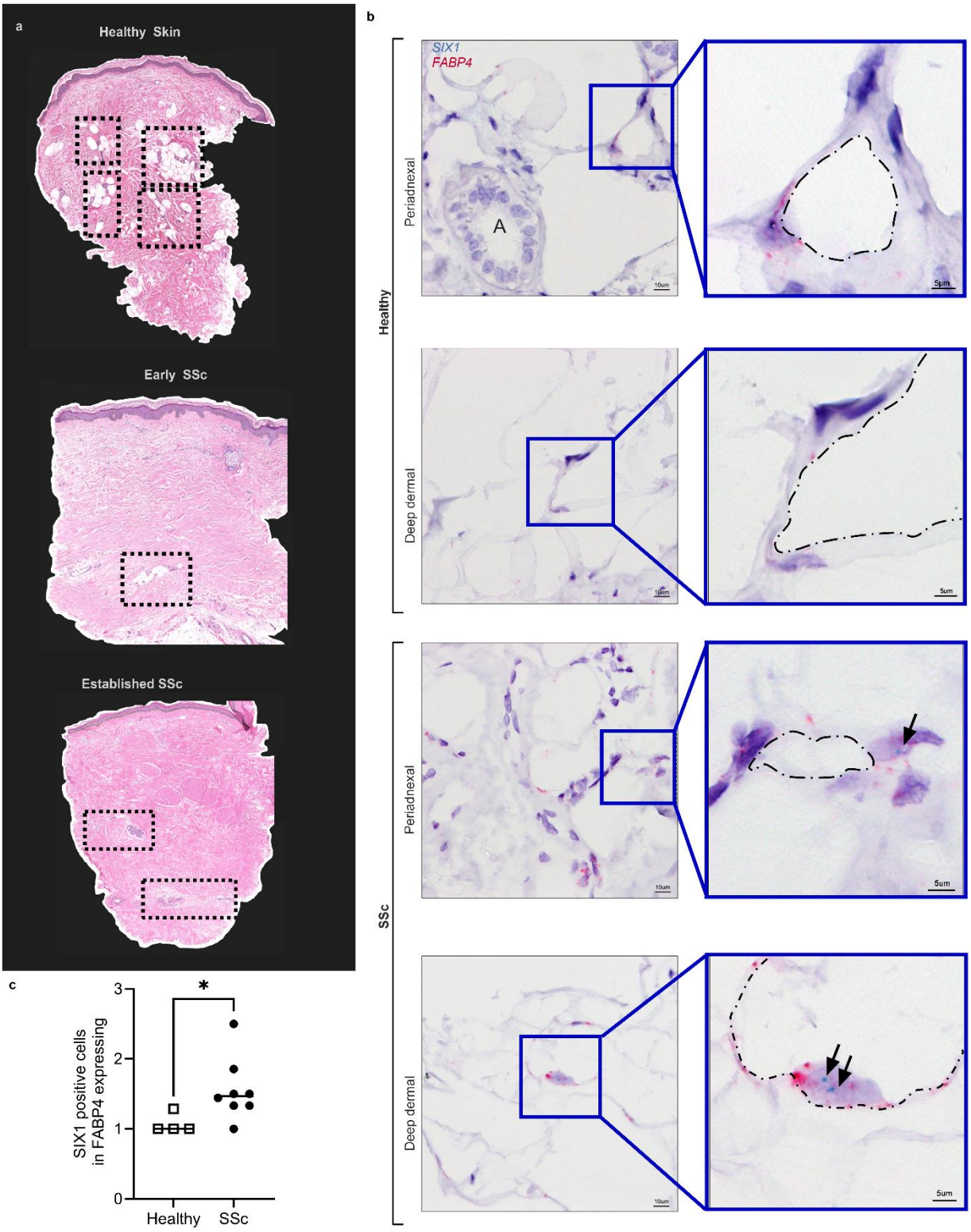

a
Healthy Skin
Early SSc
Established SSc
b
SIX1
FABP4
Periadnexal
Deep dermal
Healthy
SSc
A
10um
5um
c
SIX1 positive cells in FABP4 expressing
3
2
1
0
*
Healthy
SSc

Figure 3

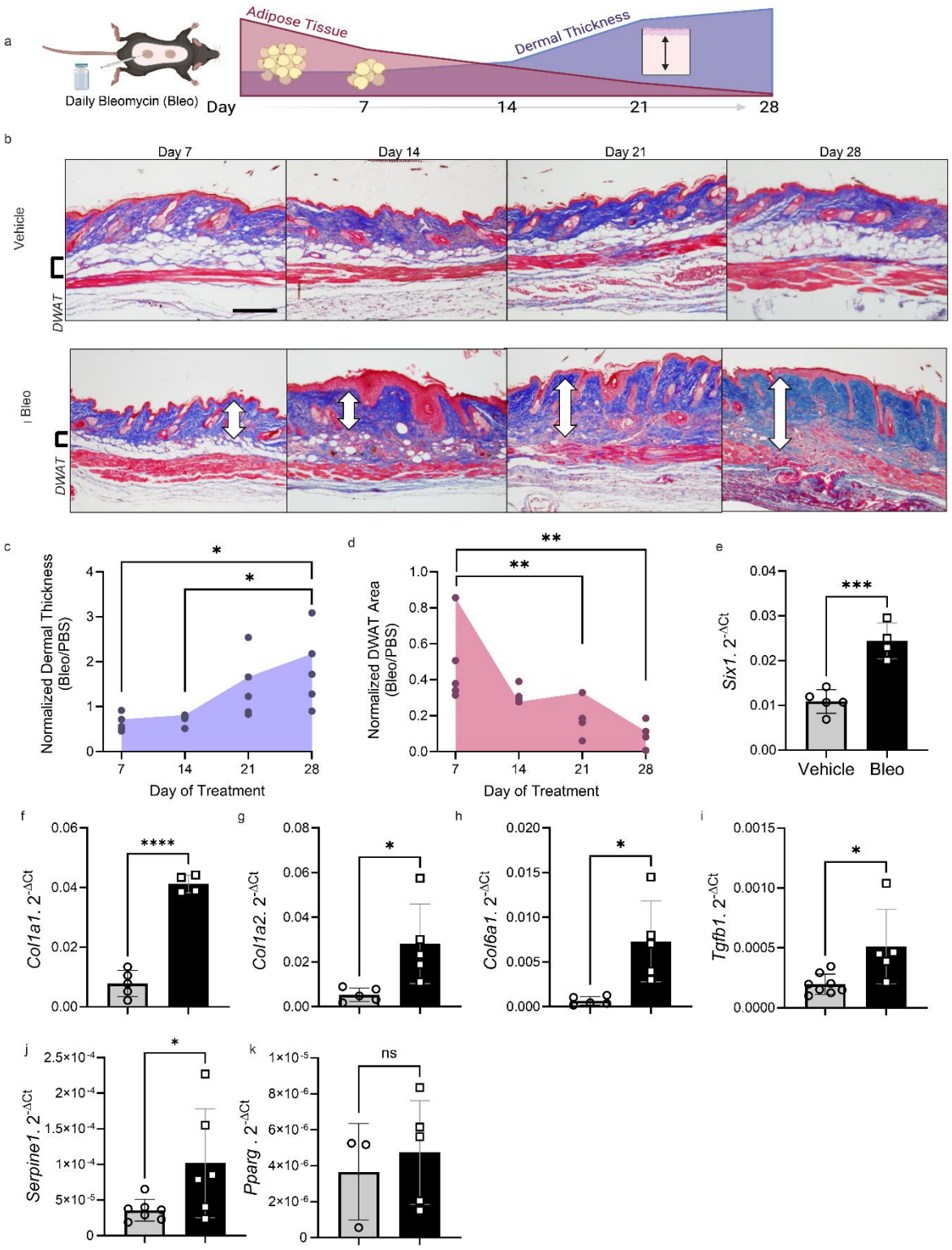

a
Adipose Tissue
Dermal Thickness
Daily Bleomycin (Bleo)
Day
7
14
21
28
b
Day 7
Day 14
Day 21
Day 28
Vehicle
Bleo
DWAT
c
Normalized Dermal Thickness (Bleo/PBS)
Day of Treatment
d
Normalized DWAT Area (Bleo/PBS)
Day of Treatment
e
Six1. 2-ΔCt
Vehicle
Bleo
f
Col1a1. 2-ΔCt
g
Col1a2. 2-ΔCt
h
Col6a1. 2-ΔCt
i
Tgfb1. 2-ΔCt
j
Serpine1. 2-ΔCt
k
Pparg . 2-ΔCt
ns

Figure 4

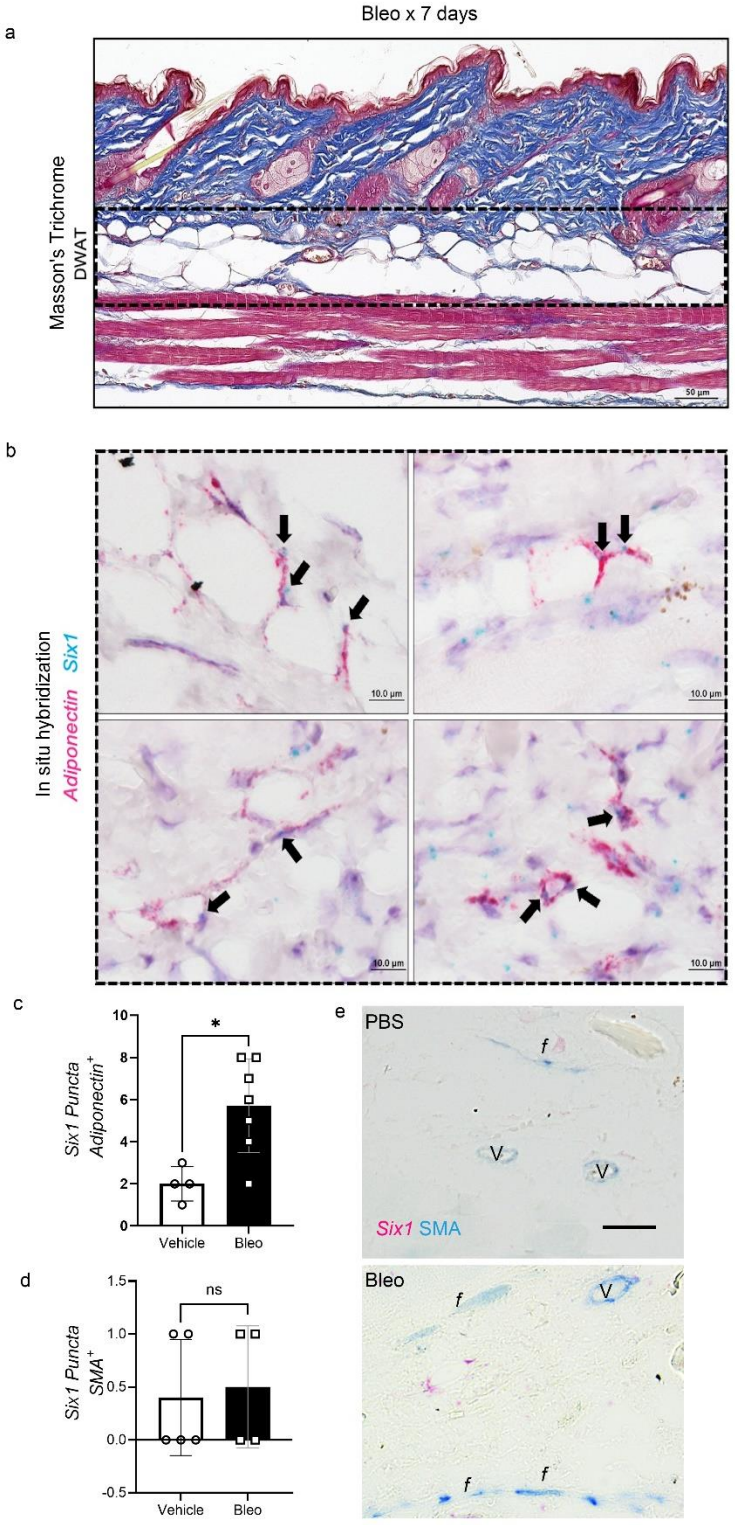

Bleo x 7 days
a
Masson's Trichrome
DWAT
50 µm
b
In situ hybridization
Adiponectin Six1
10.0 µm
c
Six1 Puncta
Adiponectin+
*
Vehicle
Bleo
d
Six1 Puncta
SMA+
ns
Vehicle
Bleo
e
PBS
f
V
Six1 SMA
Bleo

Figure 5

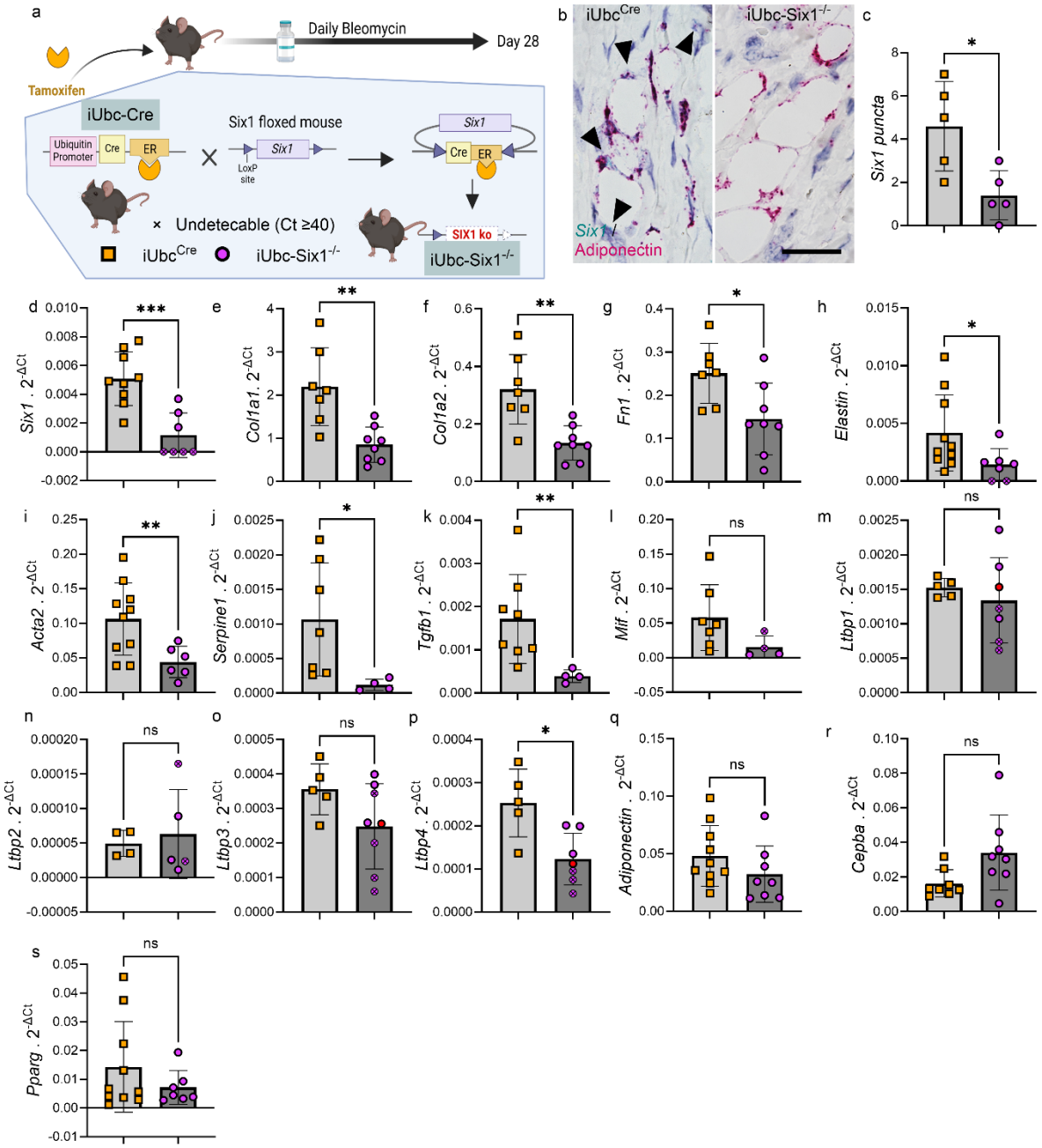

a
Daily Bleomycin
Day 28
Tamoxifen
iUbc-Cre
Ubiquitin Promoter
Cre
ER
Six1 floxed mouse
Six1
LoxP site
Undetecable (Ct ≥40)
iUbc^Cre
iUbc-Six1^-/-
SIX1 ko
b
iUbc^Cre
iUbc-Six1^-/-
Six1
Adiponectin
c
Six1 puncta
d
Six1 . 2^-ΔCt
e
Col1a1 . 2^-ΔCt
f
Col1a2 . 2^-ΔCt
g
Fn1 . 2^-ΔCt
h
Elastin . 2^-ΔCt
i
Acta2 . 2^-ΔCt
j
Serpine1 . 2^-ΔCt
k
Tgfb1 . 2^-ΔCt
l
Mif . 2^-ΔCt
m
Ltbp1 . 2^-ΔCt
n
Ltbp2 . 2^-ΔCt
o
Ltbp3 . 2^-ΔCt
p
Ltbp4 . 2^-ΔCt
q
Adiponectin . 2^-ΔCt
r
Cepba . 2^-ΔCt
s
Pparg . 2^-ΔCt
ns

Figure 6

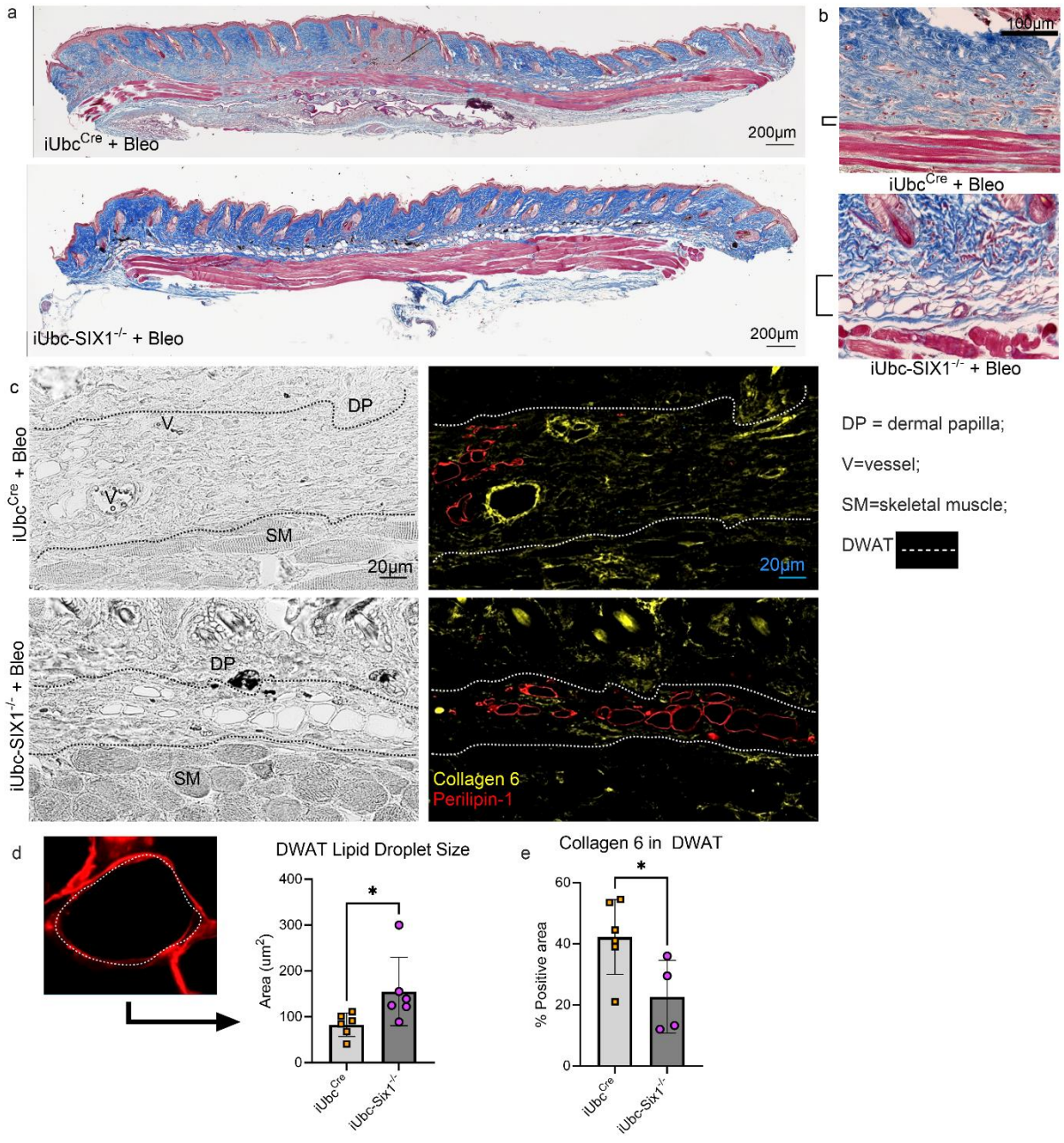

a
iUbc^Cre + Bleo
200μm
iUbc-SIX1^-/- + Bleo
200μm
b
100μm
iUbc^Cre + Bleo
iUbc-SIX1^-/- + Bleo
c
iUbc^Cre + Bleo
DP
V
V
SM
20μm
20μm
iUbc-SIX1^-/- + Bleo
DP
SM
Collagen 6
Perilipin-1
DP = dermal papilla;
V=vessel;
SM=skeletal muscle;
DWAT
d
DWAT Lipid Droplet Size
Area (um^2)
400
300
200
100
0
*
iUbc^Cre
iUbc-Six1^-/-
e
Collagen 6 in DWAT
% Positive area
60
40
20
0
*
iUbc^Cre
iUbc-Six1^-/-

Figure 7

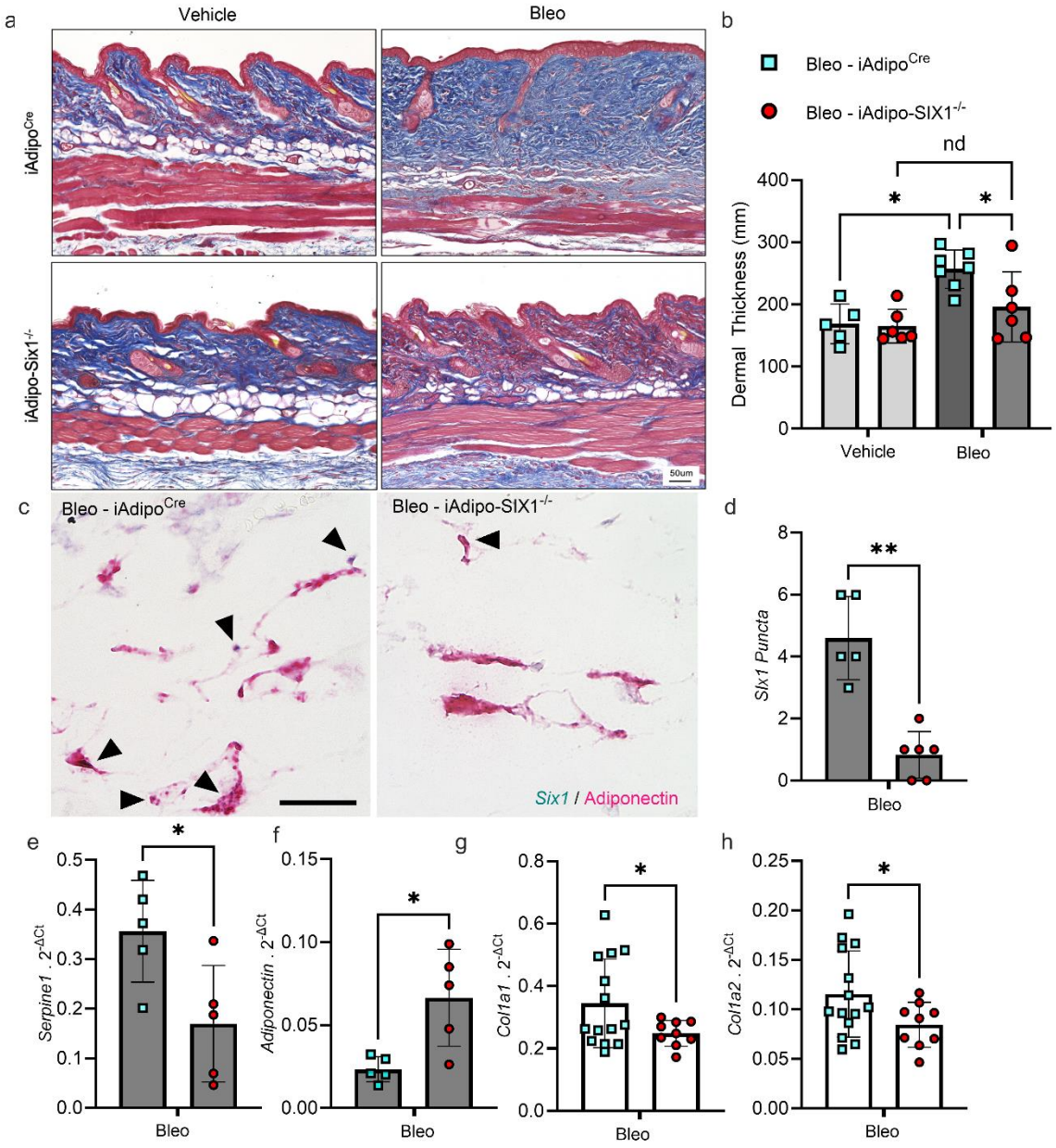

a
Vehicle
Bleo
iAdipoCre
iAdipo-Six1-/-
50um
b
Bleo - iAdipoCre
Bleo - iAdipo-SIX1-/-
nd
*
*
Dermal Thickness (mm)
400
300
200
100
0
Vehicle
Bleo
c
Bleo - iAdipoCre
Bleo - iAdipo-SIX1-/-
Six1 / Adiponectin
d
**
Six1 Puncta
8
6
4
2
0
Bleo
e
*
Serpine1 . 2-ΔCt
0.5
0.4
0.3
0.2
0.1
0.0
Bleo
f
*
Adiponectin . 2-ΔCt
0.15
0.10
0.05
0.00
Bleo
g
*
Col1a1 . 2-ΔCt
0.8
0.6
0.4
0.2
0.0
Bleo
h
*
Col1a2 . 2-ΔCt
0.25
0.20
0.15
0.10
0.05
0.00
Bleo

Figure 8

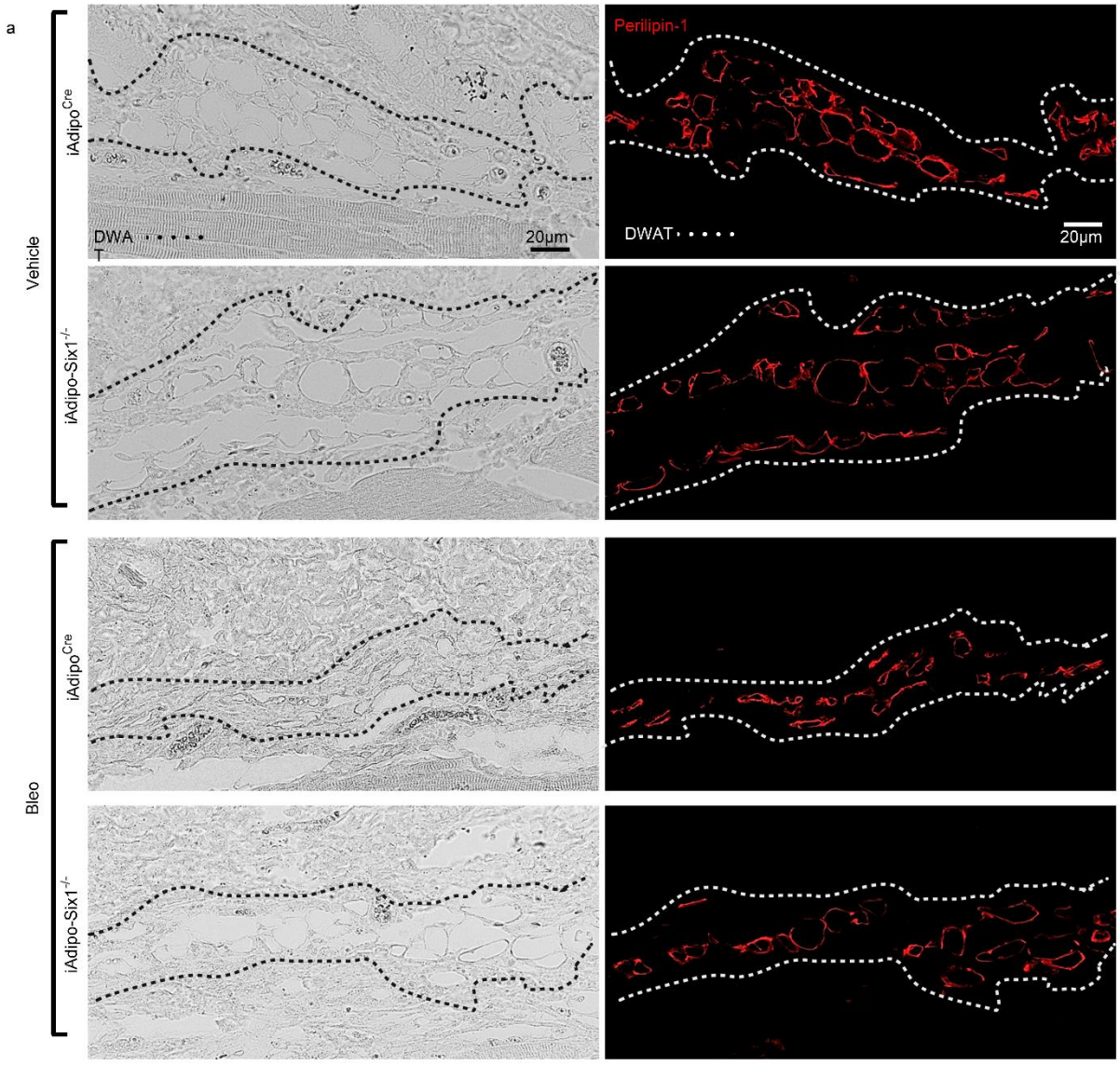

a
Vehicle
Bleo
iAdipoCre
iAdipo-Six1-/-
DWAT
Perilipin-1
20μm
b
DWAT Lipid Droplet Size at Day14
Area (μm)
250
200
150
100
50
0
Vehicle
Bleo
iAdipoCre
iAdipo-SIX1-/-
*

Figure 9

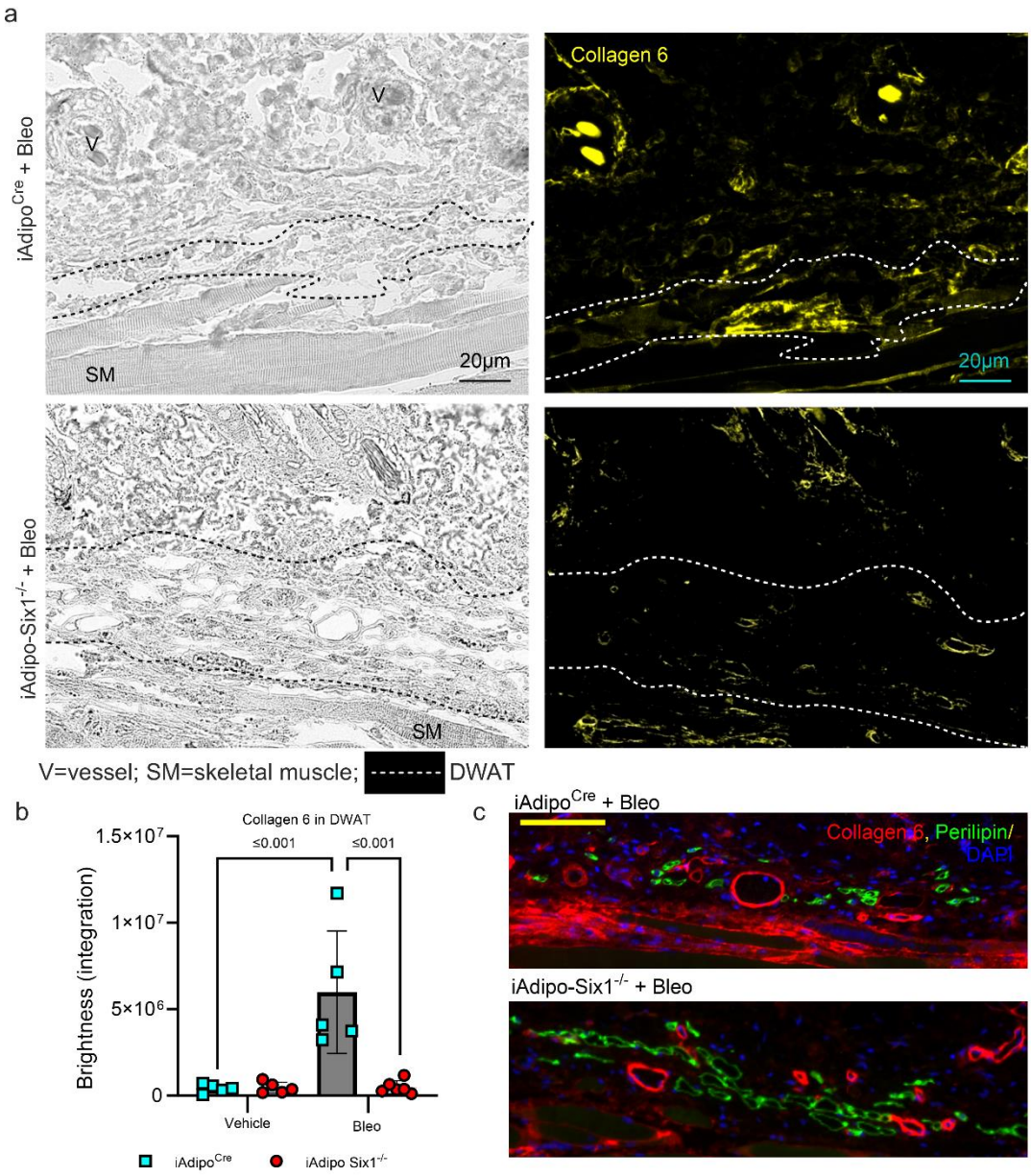
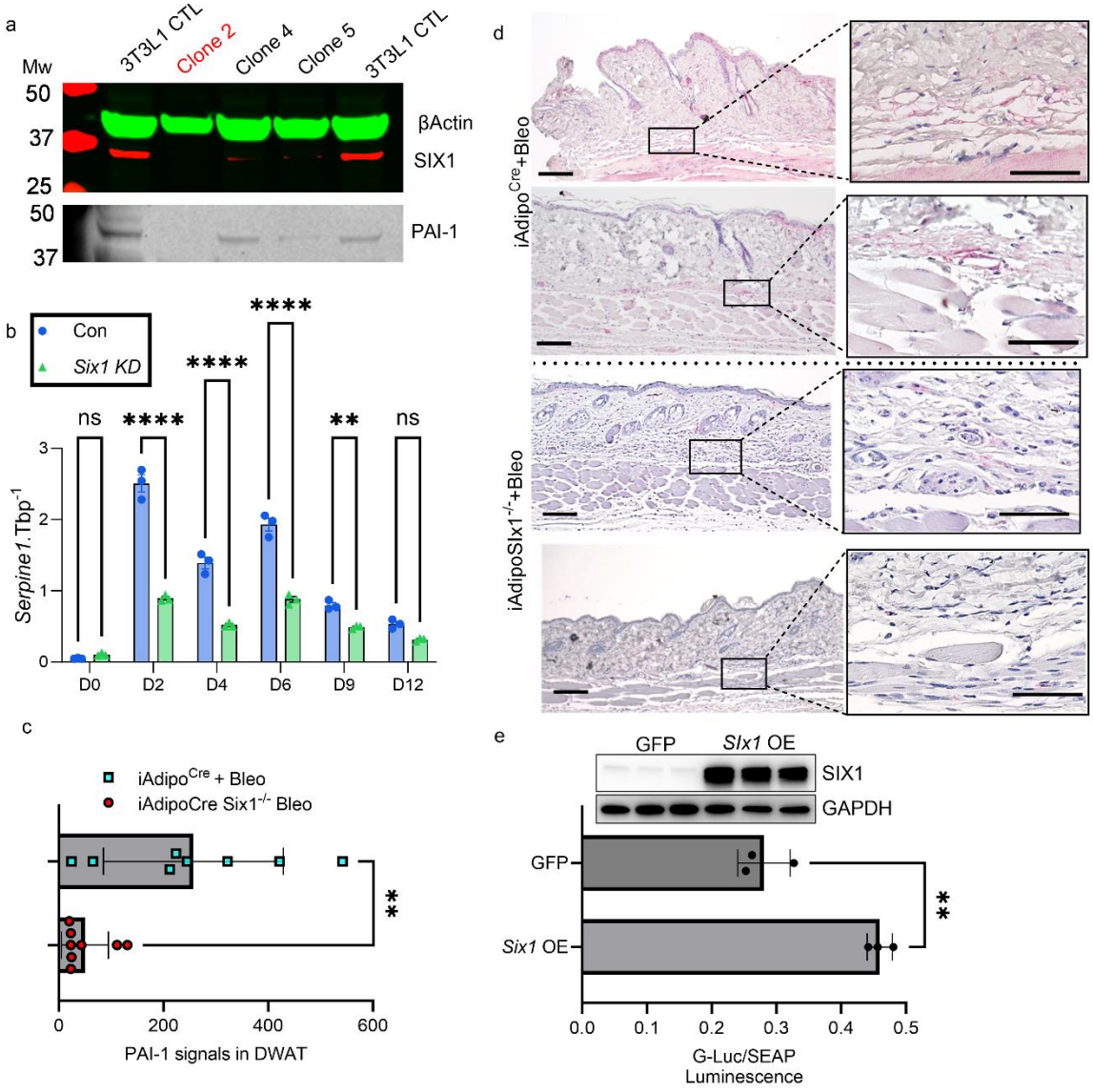

a
iAdipo^{Cre} + Bleo
iAdipo-Six1$^{-/-}$ + Bleo
V
V
SM
20μm
Collagen 6
20μm
SM
V=vessel; SM=skeletal muscle; DWAT
b
Collagen 6 in DWAT
≤0.001
≤0.001
1.5×10^7
1×10^7
5×10^6
0
Brightness (integration)
Vehicle
Bleo
iAdipo^{Cre}
iAdipo Six1$^{-/-}$
c
iAdipo^{Cre} + Bleo
Collagen 6, Perilipin/
DAPI
iAdipo-Six1$^{-/-}$ + Bleo

Figure 10


a
3T3L1 CTL
Clone 2
Clone 4
Clone 5
3T3L1 CTL
Mw
50
37
25
50
37
βActin
SIX1
PAI-1
b
Con
Six1 KD
ns
****
****
****
**
ns
Serpine1.Tbp-1
D0
D2
D4
D6
D9
D12
c
iAdipoCre + Bleo
iAdipoCre Six1-/- Bleo
**
0
200
400
600
PAI-1 signals in DWAT
d
iAdipoCre+Bleo
iAdipoSix1-/-+Bleo
e
GFP
Six1 OE
SIX1
GAPDH
GFP
Six1 OE
**
0.0
0.1
0.2
0.3
0.4
0.5
G-Luc/SEAP
Luminescence