The β-Secretase BACE1 Drives Fibroblast Activation in Systemic Sclerosis through the APP/β-Catenin/Notch Signaling Axis

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BACE1 is well-known for its role in the development of Alzheimer's disease. Recent publications, including our own, have demonstrated a role for this enzyme in other chronic diseases. The aim of this study was to investigate the role of BACE1 in the autoimmune disease systemic sclerosis (SSc). BACE1 protein levels were elevated in the skin of patients with SSc. Inhibition of BACE1 with small-molecule inhibitors or small interfering RNA blocked SSc and fibrotic stimuli–mediated fibroblast activation. Furthermore, we show that BACE1 regulation of dermal fibroblast activation is dependent on β-catenin and Notch signaling. The neurotropic factor brain-derived neurotrophic factor negatively regulates BACE1 expression and activity in dermal fibroblasts. Finally, sera from patients with SSc show higher β-amyloid and lower brain-derived neurotrophic factor levels than healthy controls. The ability of BACE1 to regulate SSc fibroblast activation reveals a therapeutic target in SSc. Several BACE1 inhibitors have been shown to be safe in clinical trials for Alzheimer's disease and could be repurposed to ameliorate fibrosis progression.

Keywords: Autoimmunity, Fibrosis, Notch, Wnt, Bace1

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INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease that presents with fibrotic involvement of the skin and internal organs, including the lungs and heart. The fibrosis is driven by activated fibroblasts (myofibroblasts) characterized by increased expression of alpha-smooth muscle actin (α-SMA) and secretion of extracellular matrix proteins. Several pathways have been implicated in the activation of fibroblasts in SSc, including the TGF-β, Wnt/β-catenin, and Hedgehog pathways (Beyer et al, 2012; Gillespie et al, 2018; Wasson et al, 2022, 2020b).

BACE1 is well-known for its role in the development of Alzheimer's disease through the generation of β-amyloid (Hampel et al, 2021). However, our recent findings have demonstrated a role for this enzyme in other chronic diseases associated with vascular damage, including type 2 diabetes and cardiovascular diseases (Taylor et al, 2022; Meakin et al, 2018).

Several links exist implicating a potential role for BACE1 in SSc. Vasculopathy is a common feature in SSc pathogenesis (Del Galdo et al, 2020), developing through the disease. We have shown that BACE1-knockout (KO) mice are protected from vascular damage in the diet-induced obesity mouse model (Meakin et al, 2020). Further studies showed that inhibition of BACE1 attenuates palmitate-induced endoplasmic reticulum stress and inflammation in skeletal muscles (Botteri et al, 2018), and endoplasmic reticulum stress has previously been shown to enhance fibrosis (Heindryckx et al, 2016). BACE1 can also target IL-1R2 for cleavage, modulating systemic IL-1 activity through soluble IL-1R2 release (Kuhn et al,

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Abbreviations: a-SMA, alpha-smooth muscle actin; APP, amyloid precursor protein; BACE1, β-amyloid precursor protein cleaving enzyme 1; BDNF, brain-derived neurotrophic factor; FBS, fetal bovine serum; HC, healthy control; Hh, Hedgehog; hTERT, human telomerase reverse transcriptase; KO, knockout; NICD, Notch intracellular domain; SAG, smoothened agonist; siRNA, small interfering RNA; SSc, systemic sclerosis; WT, wild-type

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2007). This is interesting because several IL-1 family members, including IL-33, have been implicated in SSc (Iannazzo et al, 2023). Furthermore, BACE1 expression levels are upregulated by NF-κB through binding of the p65 subunit to the BACE1 promoter (Chen et al, 2012). Taken together, these data suggest that BACE1 levels could be elevated in SSc through inflammatory responses and participate in vasculopathy and fibrosis progression.

The aim of this study was to determine whether BACE1 plays a role in the pathogenesis of fibrosis in SSc. We show that BACE1 protein levels are increased in SSc skin, fibroblasts, and a mouse model of fibrosis. Accordingly, the BACE1 products Aβ40 and Aβ42 are higher in sera from patients with SSc, whereas the BACE1-negative regulator brain-derived neurotrophic factor (BDNF) is reduced compared with that in healthy control (HC). In in vitro studies, we show that inhibition of BACE1 activity was sufficient to block expression of profibrotic markers, including α-SMA and collagen type 1 and actin-depending contraction in SSc fibroblasts. Mechanistically, BACE1 promoted fibroblast activation through its ability to activate Notch1 and Wnt3a signaling.

RESULTS

BACE1 expression is increased in the skin and fibroblasts of patients with SSc

To inform the potential role for BACE1 in SSc pathogenesis, we first assessed BACE1 protein expression in healthy (n = 5) and SSc (n = 5) forearm skin biopsies. BACE1 expression level was much higher in the epidermis than in the dermis, both in healthy skin biopsies and skin biopsies of patients with SSc (Figure 1a). However, dermal BACE1 protein levels were significantly elevated in SSc skin compared with that in healthy skin. In particular, we observed high levels of BACE1 in fibroblasts and endothelial cells in the SSc dermis (Figure 1a, inset). The specificity of BACE1 staining in the skin samples was confirmed with an isotype control antibody. The high levels of BACE1 staining found in the SSc dermis and epidermis was lost when stained with an isotype control (Supplementary Figure S1).

BACE1 protein levels remained increased in isolated immortalized dermal fibroblasts from patients with early diffuse SSc (>2-fold) compared with those in healthy dermal fibroblasts (Figure 1b and c). Similar results were observed between primary healthy and SSc dermal fibroblasts (Supplementary Figure S2a). In addition, we observed high levels of phosphorylated SMAD3 in the SSc fibroblasts, which confirmed the activated TGF-β phenotype in SSc fibroblasts (Figure 1b). Interestingly, *BACE1* transcript levels did not correlate with protein expression (Figure 1d), suggesting that the increase in BACE1 protein levels is regulated at the post-translation level, similar to that seen in Alzheimer's disease (Wen et al, 2022). In support of this, we found that levels of phosphorylated BACE1 at Ser498 were reduced in SSc fibroblasts compared with those in HC, and previous studies have shown that the phosphorylation of BACE1 can reduce its stability (Zhao et al, 2023)

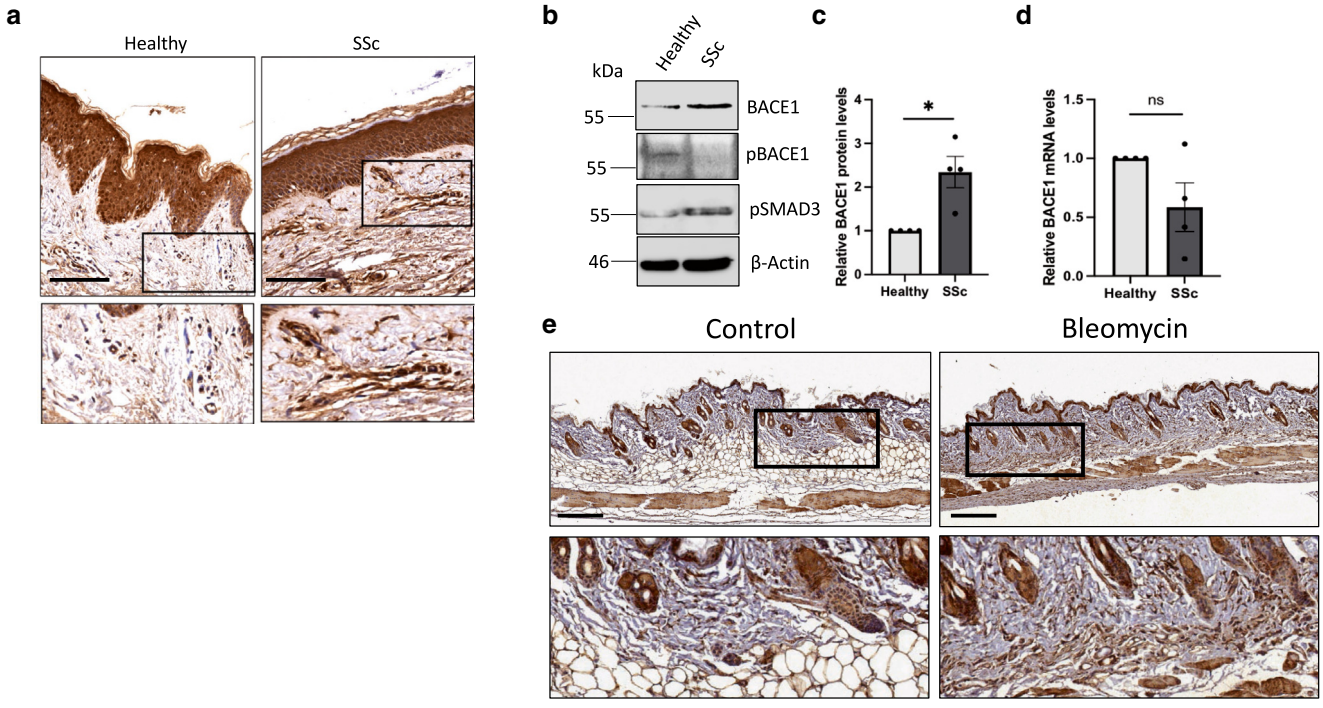

Figure 1. BACE1 levels are increased in the skin and fibroblasts of patients with SSc and in vivo skin fibrosis models. (**a**) Healthy skin biopsies and skin biopsies of patients with SSc were stained with an antibody specific to BACE1. Skin sections were counterstained with hematoxylin. Bars = 50 μM. Protein and RNA were extracted from healthy and early diffuse SSc dermal fibroblasts. (**b**) BACE1, pBACE1 (Ser498), and pSMAD3 protein levels were analyzed by western blot. β-Actin was used as a loading control. (**c**) Densitometry analysis of western blots. (**d**) *BACE1* transcript levels were assessed by qPCR. Graphs represent the mean and standard error. (**e**) Representative skin sections from vehicle- and bleomycin-treated mice (n = 4 each group) were stained with an antibody specific to BACE1 (brown). Bars = 200 μm. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. ns, not significant; pSMAD3, phosphorylated SMAD3; SSc, systemic sclerosis.

To assess the role of BACE1 in skin fibrosis establishment in vivo, we analyzed BACE1 levels in a previously described mouse model of SSc by bleomycin treatment of immunodeficient mice after xenotransplantation of human plasmacytoid dendritic cells (Ross et al, 2021). Subcutaneous injection of bleomycin induced skin fibrosis, loss of subcutaneous adipose tissue, and a thickening of the dermis. We observed higher levels of BACE1 in the keratinocytes and endothelial cells in both the control and bleomycin-treated skin (Figure 1e); however, BACE1 was specifically upregulated in the dermal fibroblasts in the bleomycin-treated fibrotic skin (Figure 1e), similar to that observed in the skin of patients with SSc (Figure 1a). This suggests that BACE1 expression is increased in response to induction of skin fibrosis in vivo.

BACE1 plays an important role in SSc and stimuli-induced myofibroblast activation

Next, we set out to determine the role of BACE1 in SSc fibroblast biology using structurally dissimilar small-molecule inhibitors against BACE1 (M3 and AZD3839). AZD3839 has completed phase 1 clinical trials for Alzheimer's disease with a good safety profile (clinical trial number: NCT01348737). We treated healthy and SSc fibroblasts with these inhibitors and assessed profibrotic gene expression. Both inhibitors led to a reduction in *COL1A2* (Figure 2a) and *α-SMA* (Figure 2b) transcript levels in SSc fibroblasts to a level indistinguishable from that of HC fibroblasts. The inhibitors had no effect on fibrotic marker expression in healthy fibroblasts. Similarly, α-SMA and CCN2 protein levels were reduced in SSc fibroblasts treated with M3 and AZD3839 (Figure 2c). AZD3839 reduced profibrotic gene expression (*COL1A2, CCN2*, and *α-SMA*) in a dose-dependent manner in SSc fibroblasts (Figure 2d–f). M3 had a similar effect in primary SSc and healthy fibroblasts (Supplementary Figure S2b). In the gel contraction assay, AZD3839 (1 μM) decreased the contracting ability of SSc fibroblasts (Figure 2g). The collagen gels containing SSc fibroblasts weighed less than HC gels (healthy = 157 ± 41 mg, SSc = 101 ± 14 mg). This phenotype was partially ameliorated when the SSc fibroblast gels were incubated with AZD3839 (SSc = 119 ± 13 mg; $P = .0356$), but the inhibitor had no significant effect on the weight of collagen gels containing healthy fibroblasts.

Importantly, we observed similar results when we knocked down BACE1 expression using small interfering RNA (siRNA). BACE1 knockdown reduced *COL1A2* (Figure 2h), *α-SMA* (Figure 2i), and *CCN2* (Figure 2j) transcript levels in SSc fibroblasts to levels similar to those seen in healthy fibroblasts. BACE1 knockdown in primary SSc fibroblasts resulted in a reduction in CCN2 protein expression (Supplementary Figure S2c)

In a complementary approach, we overexpressed BACE1 in healthy dermal fibroblasts to determine whether elevated BACE1 protein expression is sufficient to drive fibroblast activation. Healthy dermal fibroblasts were transfected with mammalian expression vectors encoding wild-type (WT) BACE1 or an inactive version with 2 genetic alterations (D92A/D289A) (Meakin et al, 2018) (Figure 2k). Although both BACE1 proteins expressed at similar levels (WT = 3.6, mutant = 3.7-fold increase in expression over endogenous levels in empty vector–transfected cells), only the WT BACE1 protein increased α-SMA and CCN2 expressions at protein level in healthy fibroblasts. This confirms that increased levels of BACE1 can trigger fibroblast activation and that its proteolytic activity is essential for this function.

Next, we isolated skin fibroblasts from WT and BACE1-KO mice to explore the role of BACE1 in basal profibrotic marker expression. We observed no differences in α-SMA protein levels between the WT and BACE1-KO dermal fibroblasts (Figure 2l). This agrees with data showing no effect of BACE1 inhibition or BACE1 siRNA-mediated knockdown on α-SMA in healthy fibroblasts transfected with the BACE1 siRNA. This suggests that BACE1 requires fibrotic stimuli to regulate profibrotic gene expression.

Because BACE1 inhibition had no effect on basal profibrotic marker expression in healthy fibroblasts, we tested whether BACE1 is necessary for fibroblasts activation in response to defined profibrotic stimuli. TGF-β, Wnt, and hedgehog (Hh) signaling is dysregulated in SSc fibroblasts and plays an important role in SSc disease progression (Gillespie et al, 2018; Wasson et al, 2022, 2020). The profibrotic stimuli enhance the expression of several profibrotic genes in fibroblasts, including *α-SMA* (Wasson et al, 2022). Stimulation with either TGF-β, Wnt3a, or smoothened agonist (SAG) (agonist of the Hh pathway) led to healthy fibroblasts activation (increased α-SMA levels) but had no effect on BACE1 levels (Figure 3a). Next, we investigated the potential role of BACE1 as a downstream mediator of TGF-β–, Wnt3a-, and SAG-induced fibroblast activation. To test this, we stimulated healthy fibroblasts with TGF-β in combination with each BACE1 inhibitor. Both BACE1 inhibitors blocked the TGF-β–mediated increase in α-SMA protein (Figure 3b). Similar results were observed using BACE1 siRNA (Figure 3c). Silencing of BACE1 attenuated TGF-β ability to increase α-SMA levels in dermal fibroblasts. This was also the case for Wnt3a-mediated fibroblast activation (Figure 3d and e). Finally, SAG-dependent fibroblast activation was also sensitive to BACE1 inhibition and BACE1 siRNA (Figure 3f and g). Together, these data suggest that BACE1 is an important downstream regulator of fibroblast activation when fibroblasts are challenged with profibrotic stimuli.

BACE1 activity is required for β-catenin stabilization by Wnt3a

Next, we sought to determine the mechanism through which BACE1 regulates the profibrotic effect of Wnt3a, TGF-β, and Hh signaling. The well-established BACE1 substrate amyloid precursor protein (APP) has been recently shown to act as a Wnt decoy receptor, binding Wnt3a and Wnt5a through its cysteine-rich domain (Liu et al, 2021). We hypothesized that increased processing of APP by BACE1 in SSc as a consequence of higher expression levels could increase the availability of Wnt ligands for Frizzled-dependent activation of canonical Wnt/β-catenin signaling. In support, inhibition of BACE1 with AZD3839 or M3 reduced β-catenin protein levels in SSc fibroblasts—30 and 15%, respectively—but had no effect on protein levels in HC fibroblasts (Figure 4a). These results were replicated in primary SSc dermal fibroblasts treated with M3 (Supplementary Figure S2b). In addition, we observed a dose-dependent decrease in β-catenin expression

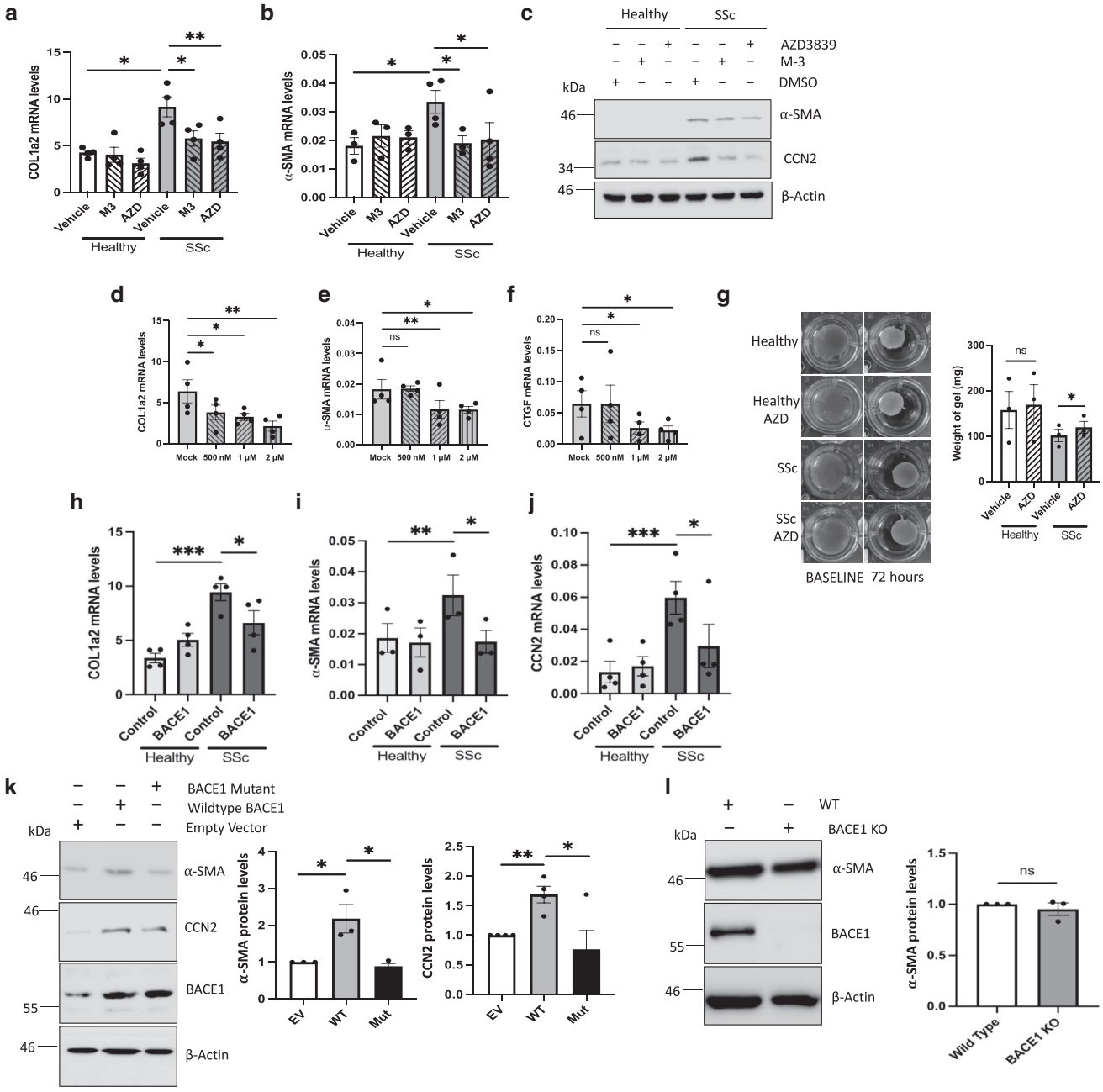

Figure 2. Modulation of BACE1 affects SSc fibroblast activation. RNA was extracted from healthy and SSc fibroblasts. In addition, fibroblasts were treated with the BACE1 inhibitors (**a**–**c**) M3 (1 μM) and AZD3839 (1 μM) or (**d**–**f**) AZD3839 (500 nM to 2 μM) or (**h**–**j**) BACE1 siRNA for 48 hours. COL1A2, α-SMA, and CCN2 protein and transcript levels were assessed. (**g**) Healthy and SSc fibroblasts were suspended in collagen gels in the presence or absence of AZD3839 (1 μM). Images were obtained for each condition, and the weight of the gels was measured. (**k**) Healthy fibroblasts were transfected with mammalian vectors containing WT or *BACE1* mutant (mut). (**l**) Fibroblasts were isolated from skin biopsies of WT and BACE1-KO mice (n = 3 per group). Graphs represent the mean and standard error. **P* < .05, ***P* < .01, and ****P* < .001. α-SMA, alpha-smooth muscle actin; KO, knockout; ns, not significant; siRNA, small interfering RNA; SSc, systemic sclerosis; WT, wild-type.

levels with AZD3839 in SSc fibroblasts (Supplementary Figure S3a). BACE1 siRNA reduced β-catenin in immortalized and primary SSc fibroblasts (Figure 4b and Supplementary Figure S2c). We also investigated whether BACE1 could also modulate β-catenin stability in response to exogenous Wnt3a. Healthy fibroblasts stimulated with Wnt3a showed increased levels of β-catenin (Figure 4c and d), which was reversed in the presence of the BACE1 inhibitors AZD3839 and M3. Overexpression of BACE1 in healthy dermal fibroblasts increased basal β-catenin expression levels (Figure 4e) in an activity-dependent manner because the BACE1 inactive mutant was unable to increase β-catenin expression levels.

Previous studies have shown that β-catenin is an important mediator of TGF-β–mediated α-SMA expression because inhibition of β-catenin with the small-molecule inhibitor FH535 blocked TGF-β ability to induce α-SMA expression (Wasson et al, 2022). To determine whether the

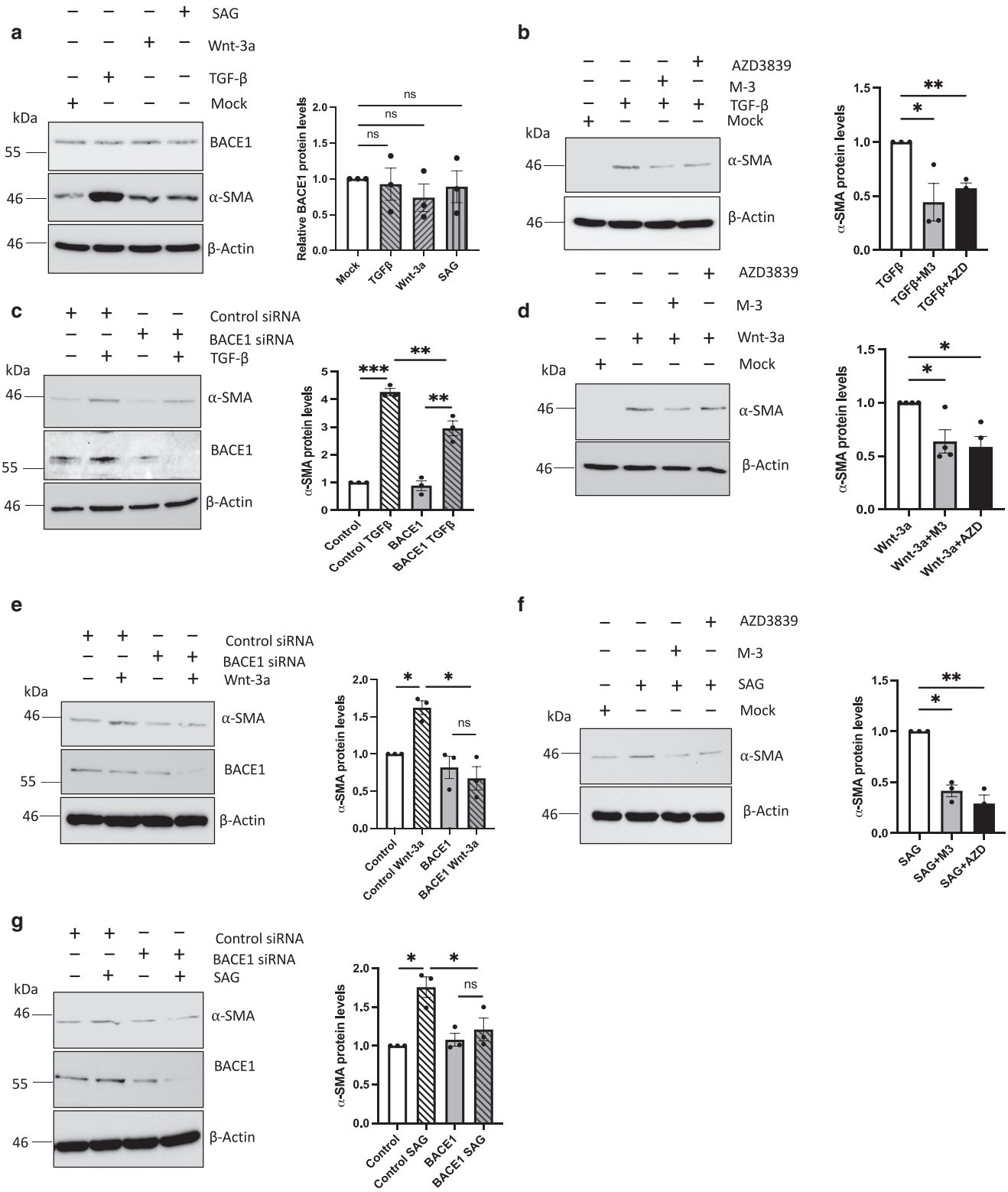

Figure 3. BACE1 is important for morphogen-mediated fibroblast activation. Healthy dermal fibroblasts were grown in serum-depleted media and stimulated with (**a**–**c**) TGF-β, (**a, d, e**) Wnt3a, or (**a, f, g**) SAG for 48 hours. BACE1 and α-SMA protein levels were assessed by western blot. β-Actin was used as a loading control. In addition, healthy dermal fibroblasts (**b, d, f**) were treated with the BACE1 inhibitors M3 or AZD3839 for 48 hours or (**c, e, g**) transfected with scramble or BACE1-specific siRNA for 48 hours. Graph represents densitometry analysis of the α-SMA western blots. $*P < .05$, $**P < .01$, and $***P < .001$. α-SMA, alpha-smooth muscle actin; ns, not significant; SAG, smoothened agonist; siRNA, small interfering RNA.

role of BACE1 in fibroblast activation by TGF-β is solely dependent on β-catenin or whether it also regulates the TGF-β/SMAD3 axis, we studied SMAD3 phosphorylation. Interestingly, inhibition of BACE1 (Figure 4f) or BACE1 knockdown (Figure 4g) had no effect on phosphorylation of SMAD3 in response to TGF-β–stimulated healthy

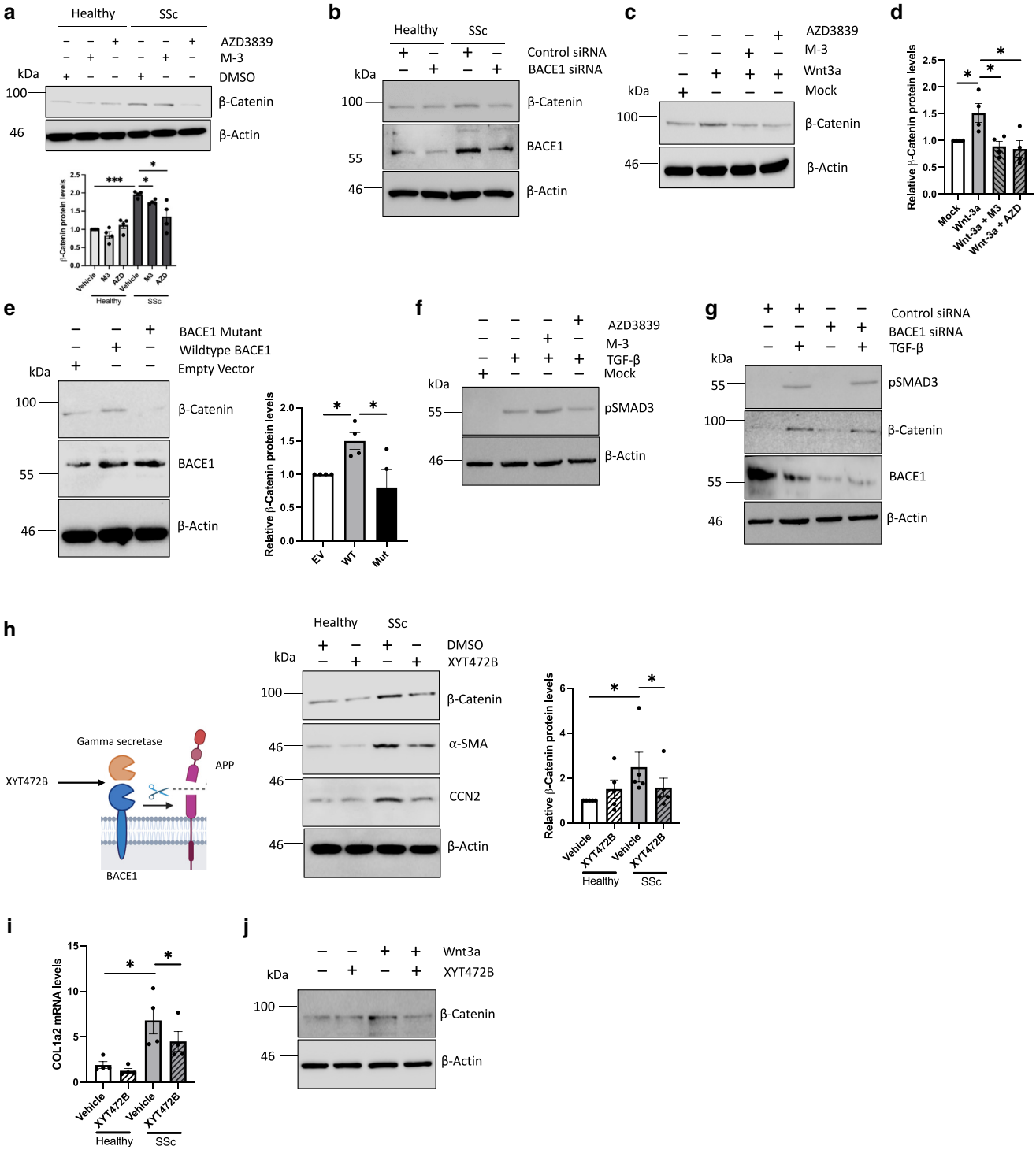

Figure 4. BACE1 regulates β-catenin expression in the presence and absence of Wnt ligands. Protein was extracted from healthy and SSc fibroblasts. In addition, the fibroblasts were treated with the (**a**) BACE1 inhibitors AZD3839 (denoted as AZD) and M3 and (**b**) BACE1 siRNA. Healthy fibroblasts were stimulated with (**c, d, j**) Wnt3a or (**f**) TGF-β for 48 hours in the presence and absence of BACE1 inhibitors. (**e**) Healthy fibroblasts were transfected with mammalian vectors containing WT BACE1 or *BACE1* mutant. (**g**) Healthy fibroblasts were transfected with scramble or BACE1-specific siRNA and stimulated with TGF-β for 48 hours. (**h**–**i**) RNA and protein were extracted from healthy and SSc fibroblasts. In addition, healthy and SSc fibroblasts were treated with the BACE1 inhibitors XYT472B (10 μM)) for 48 hours. *$P < .05$, **$P < .01$, and ***$P < .001$. ns, not significant; siRNA, small interfering RNA; SSc, systemic sclerosis; WT, wild-type.

fibroblasts. In this study, we show that TGF-β stimulation increases β-catenin levels in dermal fibroblasts, and this was partially blocked with the BACE1 siRNA (Figure 4g). This suggests that BACE1 regulates the TGF-β signaling pathway downstream of SMAD3 through the modulation of β-catenin.

Having demonstrated that BACE1 activity is necessary for β-catenin increase in response to Wnt3a, we tested our

hypothesis that BACE1 promotes Wnt signaling by proteolysis of full-length APP. To test this hypothesis, we used XYT472B, an inhibitor that specifically prevents BACE1 from processing APP but importantly does not affect the ability of BACE1 to cleave other substrates (Cui et al, 2015). After treatment of healthy and SSc fibroblasts with the compound (Figure 4h–j). We observed a reduction in β-catenin protein levels in SSc fibroblasts treated with XYT472B to levels similar to those in HC fibroblasts (Figure 4h). As expected, the reduction in β-catenin levels resulted in reduced α-SMA, CCN2 protein levels (Figure 4i), and *COL1A2* transcript levels (Figure 4j) in SSc fibroblasts treated with XYT472B to levels similar to those of the other inhibitors (Figure 2). In addition, XYT472B blocks Wnt3a-mediated induction of β-catenin expression. Healthy fibroblasts stimulated with Wnt3a had increased β-catenin expression, and this was attenuated with addition of XYT472B (Figure 4j). These data suggest that BACE1 modulates canonical Wnt signaling through processing of APP, a Wnt decoy receptor.

BACE1 regulates myofibroblast activation through the activation of Notch1 signaling

The Notch signaling pathway has been implicated in SSc dermal fibrosis (Wasson et al, 2020a), and there is evidence that the pathway is regulated by BACE1 (Hu et al, 2017). The Notch receptor resides at the cell surface, and upon ligand binding (JAG1 or DLL1), it is cleaved to release a soluble, the active Notch intracellular domain (NICD), which induces transcription of downstream target genes (*HES1*/*HEY1*). In SSc fibroblasts, Notch signaling is enhanced as assessed by increased NICD and *HES1* expressions. Generation of NICD is orchestrated by consecutive action of an extracellular protease and the γ-secretase enzyme complex, and inhibition of γ-secretase blocks SSc fibroblast activation (Wasson et al, 2020a). To explore the possibility that BACE1 contributes to Notch1 processing in SSc, we assessed Notch signaling in SSc fibroblasts treated with the BACE1 inhibitors. Notch1 expression was upregulated in SSc fibroblasts, but the BACE1 inhibitors had no impact on *NOTCH1* transcript levels (Figure 5a). Nevertheless, *HES1* transcript levels (downstream transcriptional target of Notch) were significantly reduced in SSc fibroblasts treated with the BACE1 inhibitors in a dose-dependent manner (Figure 5b and Supplementary Figure S3b). In the same experimental setting, HES1 levels in healthy fibroblasts were not affected by the inhibitors. The BACE1 inhibitors reduced the levels of NICD in SSc fibroblasts in a dose-dependent manner (Figure 5c and d and Supplementary Figure S3a and c). This suggests that BACE1 regulates the activation of Notch signaling in SSc fibroblasts through the activation of the receptor. The Hh transcription factor GLI2 has previously been shown to be a downstream target of the Notch pathway in SSc fibroblasts (Wasson et al, 2020b). The BACE1 inhibitors reduced the expression of the Hh transcription factor GLI2 in SSc fibroblasts (Figure 5c). Consistent with these data, BACE1 knockdown by siRNA did not alter *NOTCH1* (Figure 5e) transcript levels but did reduce *HES1* (Figure 5f) and *GLI2* (Figure 5g) transcript levels as well as NICD and GLI2 protein levels (Figure 5h). In a complementary, gain-of-function set of experiments, overexpression of BACE1 resulted in increased accumulation of NICD (Figure 5i), whereas overexpression of the secretase mutant was unable to induce NICD accumulation.

Interestingly, overexpression of BACE1 reduced full-length JAG1 protein levels in dermal fibroblasts (Figure 5i). BACE1 has been shown to selectively regulate the cleavage of membrane-bound JAG1 in Schwann cells (Hu et al, 2017). Regulation of JAG1 by extracellular domain shedding may play a role in the activation of Notch1 in SSc fibroblasts. Our data suggest that BACE1 cleaves JAG1 from the cell surface, resulting in a soluble JAG1, which is enough to induce Notch signaling in a paracrine signaling cascade. The reduction in JAG1 levels was not observed in fibroblasts overexpressing the secretase dead mutant (Figure 5i). Further analysis of JAG1 levels in healthy and SSc fibroblasts treated with the BACE1 inhibitors revealed low levels of JAG1 in SSc fibroblasts, and this was partially reversed with both AZD3839 and M3 (Figure 5c). This suggests that BACE1 might cleave JAG1 from the surface of SSc fibroblasts, which in turn could lead to increased soluble JAG1 that is able to induce Notch signaling.

Next, we assessed the effects of the APP-specific BACE1 inhibitor XYT472B on Notch signaling. XYT472B was unable to inhibit or *HES1* transcription (Figure 5j) or NICD expression (Figure 5k) in SSc fibroblasts. This suggests that BACE1 ability to induce the Notch signaling cascade is independent of its ability to target APP.

Further investigations found that inhibition of or silencing BACE1 reduced the ability of TGF-β, Wnt, and Hh signaling to modulate NICD in healthy fibroblasts (Supplementary Figure S4a and b). We observed increased NICD protein levels in TGF-β–stimulated fibroblasts, which was reversed when the fibroblasts were treated with M3, AZD3839 (Supplementary Figure S4a), or BACE1 siRNA (Supplementary Figure S4b). Cooperation between TGF-β Notch signaling pathways is well-characterized with Notch signaling required for the regulation of a number of TGF-β–responsive genes (Niimi et al, 2007). Similar results were observed in Wnt3a- (Supplementary Figure S4c and d) and SAG- (Supplementary Figure S4e and f) stimulated fibroblasts. Together our data show that BACE1 regulates Notch signaling in SSc fibroblasts, which in turn contributes to fibroblast activation.

In addition to assessing the effects of BACE1 modulation on the Wnt3a/β-catenin and Notch signaling pathways in the context of SSc dermal fibroblasts, we investigated the effects on a number of other signaling pathways previously implicated in SSc (namely, the focal adhesion kinase [Shi-Wen et al, 2012; Mimura et al, 2005] and Hippo [Ma et al, 2024] pathways). Overexpression of BACE1 (WT or mut) in healthy dermal fibroblasts did not modulate the activation of focal adhesion kinase (phosphorylation site Tyr397) or the expression of the Hippo-associated transcription factor Taz (Supplementary Figure S5a). Further analysis showed that the elevated levels of Taz found in SSc fibroblasts were not disrupted when BACE1 was disrupted with small-molecule inhibitors (Supplementary Figure S5b) or siRNA (Supplementary Figure S5c). Finally, inhibition of BACE1 with the small-molecule inhibitors did not affect TGF-β–mediated activation of focal adhesion kinase (Supplementary Figure S5d). These data suggest that BACE1 modulates myofibroblast activation independent of these pathways.

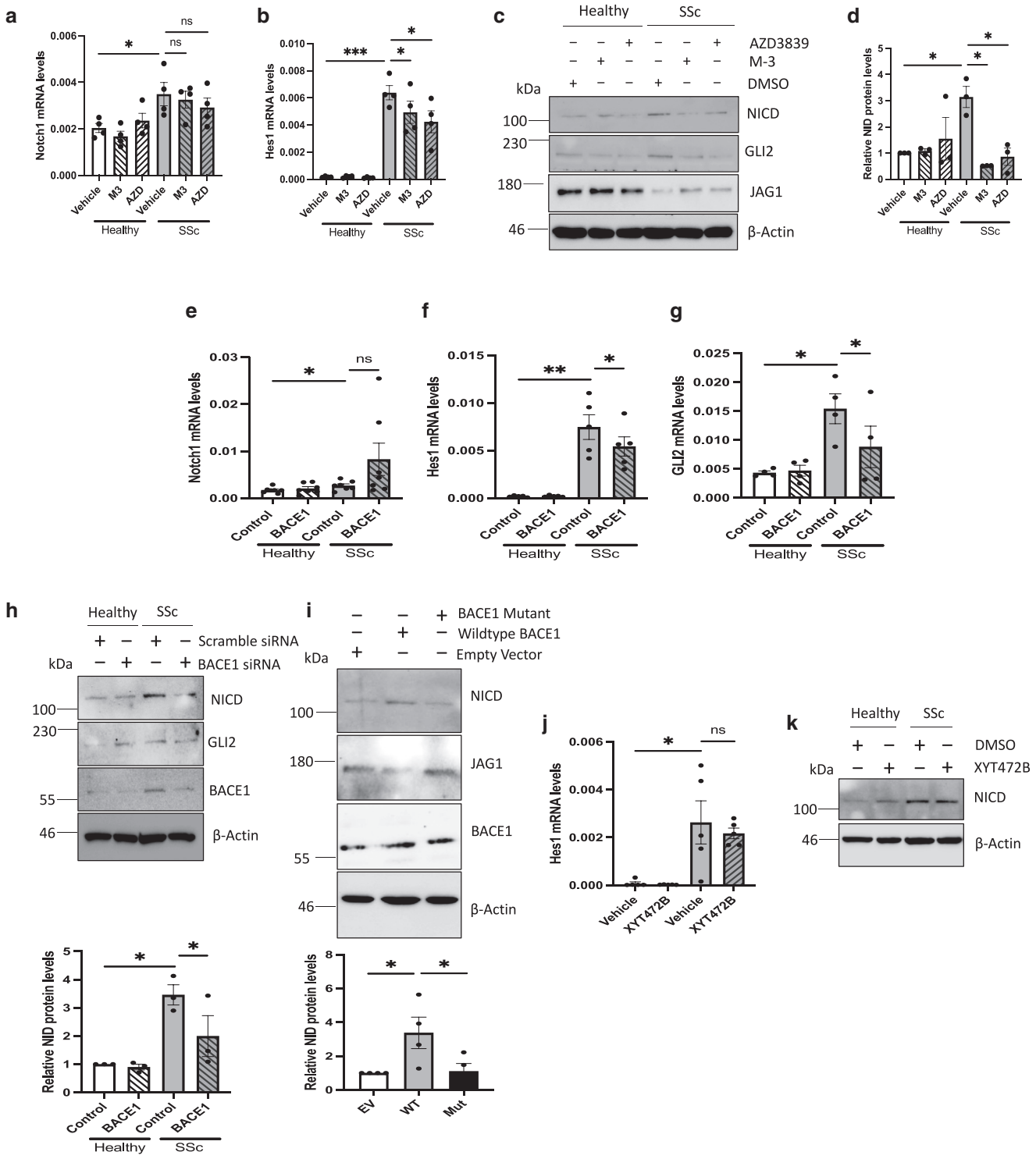

Figure 5. Inhibition of BACE1 leads to reduced Notch signaling in SSc fibroblasts. Healthy and SSc fibroblasts were treated with the (**a**–**d**) BACE1 inhibitors M3 and AZD3839 and (**e**–**h**) BACE1 siRNA. *Notch1* and *Hes1* transcript levels were assessed by qPCR, and NICD, GLI2, and JAG1 protein levels were assessed by western blot. (**i**) Healthy fibroblasts were transfected with mammalian vectors containing WT BACE1 or *Bace1* mutant. BACE1, NICD, and JAG1 protein levels were assessed by western blot. β-Actin was used as a loading control. RNA was extracted from healthy and SSc fibroblasts. In addition, healthy and SSc fibroblasts were treated with the BACE1 inhibitors XYT472B (10 μM)) for 48 hours. (**j**) *Hes1* transcript levels were assessed by qPCR, and (**k**) NICD protein levels. **P* < .05, ***P* < .01, and ****P* < .001. NICD, Notch intracellular domain; ns, not significant; siRNA, small interfering RNA; SSc, systemic sclerosis; WT, wild-type.

BACE1 expression and profibrotic function are attenuated by BDNF

We have identified that BACE1 is upregulated in SSc dermal fibroblasts. To date, the mechanism behind the elevated BACE1 expression levels is unresolved. A number of pathways known to regulate BACE1, in other diseases and tissues, are dysregulated in SSc fibroblasts. Therefore, we aimed to determine the pathway(s)

involved in regulating BACE1 expression in SSc fibroblasts.

First, a number of potential mediators were excluded. Overexpression of epigenetic factors such as the long non-coding RNA HOTAIR (Wasson et al, 2020a) in dermal fibroblasts did not affect BACE1 levels (Supplementary Figure S6a). Oxidative stress is dysregulated in SSc (Antinozzi et al, 2021; Di Luigi et al, 2020) and has been shown to regulate BACE1 in neuronal cells (Mouton-liger et al, 2012). Induction of oxidative stress in dermal fibroblasts increased *BACE1* transcription but not protein levels (Supplementary Figure S6b and c). BACE1 is known to interact with the lipid raft protein caveolin 1 (Hattori et al, 2006), and we have previously shown that caveolin 1 is downregulated in SSc fibroblasts (Liakouli et al, 2018; Del Galdo et al, 2008). Knockdown of caveolin 1 in dermal fibroblasts reduced BACE1 levels (Supplementary Figure S6d). These data suggest that other factors are at play in the overexpression of BACE1 in SSc fibroblasts.

Previous studies have shown that BDNF plays an important role in regulating the activity of BACE1 (Baranowski et al, 2021). BDNF was shown to suppress BACE1 activity in mouse neuronal cells (Baranowski et al, 2021). To determine whether BDNF regulates BACE1 expression and activity levels in dermal fibroblasts, we stimulated healthy and SSc dermal fibroblasts with recombinant BDNF. Interestingly, BDNF stimulation reduced BACE1 expression and β-catenin levels in both healthy and SSc dermal fibroblasts (Figure 6a). BDNF is known to signal through TRKB in a range of cell types. Interestingly, there is evidence that shows that activation of TRKB attenuates liver fibrosis by inhibiting TGF-β signaling (Song et al, 2023). Therefore, we wanted to determine whether BDNF suppressed SSc fibroblast activation through its canonical receptor. Healthy dermal fibroblasts were stimulated with BDNF in combination with the TRKB inhibitor ANA-12. BDNF stimulation suppressed BACE1, β-catenin, α-SMA, and CCN2 levels and increased JAG1 levels (Figure 6b), and these effects were prevented by TRKB inhibition. This suggests that BDNF alters BACE1 expression through its well-characterized receptor TRKB.

Increased BACE1 activity markers and reduced BDNF levels in sera from patients with SSc

As described earlier, BDNF is an important negative regulator of BACE1 in fibroblasts of patients with SSc. To investigate whether the upregulated BACE1 activity in SSc could be linked to reduced BDNF, we assessed BDNF levels in sera samples from 121 patients with SSc, 34 HCs (aged 51.1 ± 14.4 years, 23.5% males), and 24 subjects with secondary Raynaud's phenomenon (age 48.6 ± 14.4 years, 16.9% males) through Luminex multiplex assays (Myriad Rules-Based Medicine). Demographic and clinical characteristics of the involved patients with SSc are reported in Supplementary Table S1. BDNF levels were lower in patients with SSc (19.20 ± 6.14 ng/ml) than in both HCs (22.00 ± 4.61 ng/ml, adjusted $P = .022$), and those with secondary Raynaud's phenomenon (23.40 ± 5.99 ng/ml, adjusted $P = .005$) (Figure 6c).

BACE1 cleaves APP to release soluble Aβ40 and Aβ42. Consistent with BDNF serum concentration data, serum Aβ40 levels were higher in patients with SSc (0.40 ± 0.13 ng/ml) than in both HCs (0.35 ± 0.08 ng/ml, adjusted $P = .043$) and those with secondary Raynaud's phenomenon (0.35 ± 0.07 ng/ml, $P = .043$) (Figure 6d). Similarly, Aβ42 was detectable in 56.4% of patients with SSc compared with 20.6% of HCs (adjusted $P = .002$) (Figure 6e). When the undetectable Aβ42 was approximated to the lower limit of detection, median Aβ42 values were higher in patients with SSc (0.18, interquartile range = 0.14–0.59) than in HCs (0.14, interquartile range = 0.14–0.14; adjusted $P = .001$) (Figure 6e). Altogether, these data suggest that the low levels of circulating BDNF in the sera from patients with SSc could underlie the increased BACE1 expression and activity in SSc.

Serum Aβ40 and BDNF levels predict prognosis in patients with SSc

In the subgroup of 45 patients with high-resolution computed tomography–proven interstitial lung disease, 21 (46.7%) experienced a progression in the 24 months after serum collection according to ERICE criteria (George et al, 2020). These progressor patients had higher baseline levels of Aβ40 (0.47 ± 0.15 ng/ml) than patients who were clinically stable over time (0.37 ± 0.12 ng/ml, $P = .028$) (Figure 6f). In the subgroup of 40 patients affected by diffuse cutaneous SSc variant, 6 (15.0%) experienced a clinically meaningful modified Rodnan skin score progression (Khanna et al, 2019) in a 12-month period after serum collection. Also in this case, patients who experienced skin progression showed higher Aβ40 levels (0.49 ± 0.12 ng/ml) than the rest of the cohort (0.39 ± 0.16 ng/ml); however, statistical significance was not reached given the low number of progressors ($P = .099$) (Figure 6g).

Finally, 44 patients (36.4%) died during a 10-year follow-up, and 19 (15.7%) of them died as a direct consequence of cardiopulmonary SSc involvement. For survival analysis, patients with SSc were assigned to different risk categories according to their BDNF and Aβ40 levels compared with the HCs. High-Aβ40 patients (14.0%) were defined as those with serum levels 2 SDs above the mean in the HC group, and low-BDNF patients (19.8%) were those with serum levels 2 SDs below the mean in the HC group. The survival distributions of the defined risk categories were statistically different, with a poorer SSc-specific survival for high-Aβ40 patients than for low-Aβ40 patients ($\chi 2[2] = 11.0$, $P < .001$) (Figure 6h) and for low-BDNF patients than for high-BDNF patients ($\chi 2[2] = 3.9$, $P = .05$) (Figure 6i).

DISCUSSION

This study demonstrates, to our knowledge, a previously unreported role for BACE1 in SSc fibroblast activation. BACE1 levels are increased in the skin of patients with SSc and isolated dermal fibroblasts. The elevated expression levels are important for SSc disease pathogenesis because blocking BACE1 activity (with small-molecule inhibitors) or expression levels (siRNA) led to a suppression of fibroblast activation in SSc. Moreover, the BACE1 products Aβ40 and Aβ42 were higher, and its negative regulator BDNF was lower in sera from patients with SSc than in those from HCs. From a clinical perspective, a status systemic fibroblast hyperactivation suggested by these markers identifies the patients with a

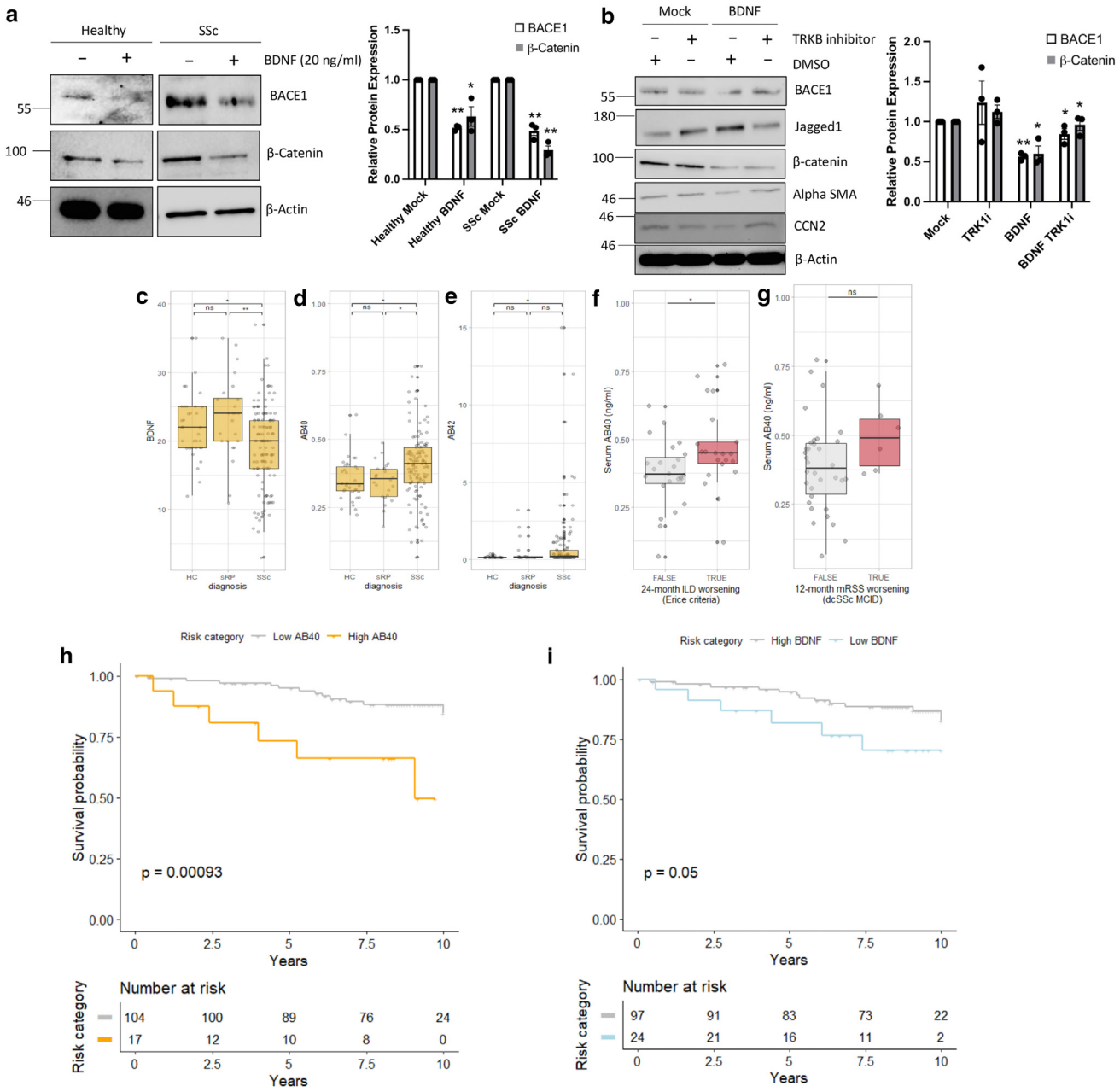

Figure 6. BACE1 expression levels are regulated by BDNF in SSc. Healthy and SSc fibroblasts were stimulated with (**a**) BDNF or (**b**) in the presence of TRKB inhibitor. BACE1, JAG1, α-SMA, and β-catenin protein levels were assessed. Graphs represents densitometry analysis for both BACE1 and β-catenin western blots. Graph represents (**c**) BDNF, (**d**) AB40, and (**e**) AB42 protein expression levels in healthy sera (34), sera of patients with sRP, (24) and sera of patients with SSc (121). (**f**) Baseline serum AB40 levels in patients with SSc-ILD according to the occurrence of ERICE progression at 24-month follow-up. (**g**) Baseline serum AB40 levels in patients with dcSSc according to occurrence of MCID mRSS worsening at 12-month follow-up. The 10-year survival probability charts of patients with SSc categorized with high and low (**h**) AB40 and (**i**) BDNF. α-SMA, alpha-smooth muscle actin; BDNF, brain-derived neurotrophic factor; dcSSc, diffuse cutaneous systemic sclerosis; HC, healthy control; ILD, interstitial lung disease; MCID, minimal clinically important difference; mRSS, modified Rodnan skin score; ns, not significant; sRP, secondary Raynaud's phenomenon; SSc, systemic sclerosis.

poorer prognosis in terms of pulmonary and possibly skin fibrosis as well as overall mortality. The data presented in this study are preliminary, and longitudinal outcome data may be confounded by other baseline factors. Therefore, further studies in validation cohorts will need to be performed to fully assess the potential of BDNF, Aβ40, and Aβ42 as predictive biomarkers in SSc.

The link between BACE1 and Alzheimer's disease progression is interesting in the context of SSc. Patients with SSc with Alzheimer's disease as a comorbidity have a higher mortality rate (Watad et al, 2018). This could be a result of the higher levels of BACE1 (in patient skin) and β-amyloid (in sera from patients with SSc), which results in exacerbated Alzheimer's disease progression.

To date, most studies describing BACE1 functions have been performed in neuronal cell types in the context of Alzheimer's disease. Therefore, this study uncovers functions for BACE1 in another disease setting and highlights the

need to study BACE1 in other cell types and disease settings.

An intriguing element of the study is the uncovering of a role for BACE1 downstream of profibrotic signaling (TGF-β, Wnt3a, and Hh). Inhibition of BACE1 through siRNA or small-molecule inhibitors blocked TGF-β, Wnt3a, and smoothened-mediated fibroblast activation (Figure 3). Interestingly, APP is a conserved Wnt receptor (Liu et al, 2021). APP binds to Wnt3a and Wnt5a, resulting in the recycling and stabilization of APP. Thus, the ability of BACE1 to process APP may enable more Wnt ligands to induce canonical Wnt signaling and fibroblast activation (Supplementary Figure S7). Cleavage of APP may result in its degradation, which in turn would release the bound Wnt ligands. In addition, previous studies have shown an interplay between BACE1 and GSK3β, which is a member of the β-catenin destruction complex (Ly et al, 2013). We observed reduced β-catenin levels in Wnt3a-stimulated fibroblasts when BACE1 was inhibited (Figure 4c and d), supporting this hypothesis. Furthermore, a BACE1 inhibitor (XYT472B) (Cui et al, 2015), which prevents the ability of BACE1 to process APP but does not affect any other BACE1 substrate, blocked profibrotic gene expression in SSc dermal fibroblasts (Figure 4h) and attenuated β-catenin accumulation in response to Wnt3a in healthy fibroblasts (Figure 4j).

The ability of BACE1 to reduce fibroblast activation by the other signaling pathways (TGF-β and Hh) could be linked to its ability to modulate Wnt3a/β-catenin. We have previously shown co-operation between the Wnt3a and TGF-β signaling pathways in dermal fibroblasts because β-catenin inhibitors blocked the profibrotic ability of TGF-β (Wasson et al, 2022). In this study, we show that BACE1 does not regulate the activity of the TGF-β transcription factor SMAD3 (Figure 4f and g). The ability of BACE1 to regulate Hh-mediated fibroblast activation (Figure 3f and g) could also be due to its ability to regulate β-catenin because we previously showed that Wnt3a/β-catenin induces expression of the Hh transcription factor GLI2 (Wasson et al, 2022).

In addition, BACE1 promotes Notch signaling in response to TGF-β, Wnt3a, and SAG (Supplementary Figure S3). This suggests an integrated pathway involving BACE1, Notch, and β-catenin involved in SSc fibroblast activation. β-Catenin has previously been shown to induce the expression of the Notch ligand JAG1 (Estrach et al, 2006). Therefore, β-catenin could drive Notch signaling by increasing ligand expression in SSc fibroblasts. One caveat to this is that the APP-specific BACE1 inhibitor XYT472B does not affect *HES1* mRNA levels in SSc fibroblasts, suggesting a different substrate, such as JAG1. BACE1 has been reported to cleave the Notch ligand JAG1 from the cell surface in neurons (Hu et al, 2017), and recombinant soluble JAG1 can trigger Notch signaling and fibroblast activation (Dees et al, 2011). In this study, we show that BACE1 reduces the level of full-length JAG1 in dermal fibroblasts (Figure 5).

SSc fibroblasts have previously been shown to share several similarities with cancer-associated fibroblasts (Wasson et al, 2021). Interestingly, cancer-associated fibroblasts secrete β-amyloid, and this drives the formation of neutrophil extracellular traps in the associated tissue. Neutrophil extracellular traps are decondensed chromatin that the neutrophils extrude into the extracellular space, and this has a microbicidal effect. BACE1 inhibition reduces β-amyloid secretion and in turn reduces neutrophil extracellular traps from neutrophils in the associated tissue (Munir et al, 2021). This has implications for SSc because NETosis in patient plasma in the early stages of the disease correlates with vascular complications (Didier et al, 2020). This further highlights the potential role of BACE1 in multiple aspects of SSc disease progression.

We have shown that BDNF reduces BACE1 expression levels in both healthy and SSc dermal fibroblasts in a TRKB-specific manner (Figure 6). The role of BDNF in fibrosis is a conflicting story. Our data suggest that BDNF is inversely correlated with SSc progression, and mechanistic studies in dermal fibroblasts point toward an antifibrotic function. However, BDNF was previously shown to have a profibrotic role in idiopathic pulmonary fibrosis, suggesting a disease-specific role (Cherubini et al, 2017). Analysis of a published proteomic antibody microarray from healthy versus SSc sera revealed a similar downward trend in BDNF sera levels between healthy control and patients with SSc with interstitial lung disease and pulmonary hypertension (Huang et al, 2024). This strengthens our hypothesis that BDNF is antifibrotic in the context of SSc.

The translational potential of this study is underscored by number of small-molecule inhibitors targeting BACE1, including AZD3839, which have progressed through clinical trials for the treatment of Alzheimer's disease, indicating safety and good tolerance by the patients. This opens up the possibility that these compounds could be repurposed for the treatment of SSc and provide a therapy, which is desperately needed.

MATERIALS AND METHODS

Patient cell lines

Full-thickness skin biopsies were surgically obtained from the forearms of 4 adult healthy controls and 4 adult patients with recent-onset SSc, defined as a disease duration of <18 months from the appearance of clinically detectable skin induration. All patients satisfied the 2013 American College of Rheumatology/European League Against Rheumatism criteria for the classification of SSc and had diffuse cutaneous clinical subset as defined by LeRoy and Medsger (2001). All participants provided written informed consent to participate in the study. Informed consent procedures were approved by NRES-011NE to FDG. Fibroblasts were isolated and established as previously described (Gillespie et al, 2018). Primary cells were immortalized using human telomerase reverse transcriptase (hTERT) to produce healthy control hTERT and SSc hTERT (Gillespie et al, 2018).

Cell culture

hTERT patient fibroblasts were maintained in DMEM (Gibco, 21885-025) supplemented with 10% fetal bovine serum (FBS) (Gibco, A5256801) and penicillin–streptomycin (Sigma-Aldrich, P433). Fibroblasts were treated with BACE1 inhibitors (M3 [500 nM–4 μM, Merck, 565788], AZD3839 [500 nM–2 μM, AstraZeneca, obtain through discovery scheme], or XYT472B [10 μM, synthesized in house]) for 48 hours in a humidified incubator at 37 °C and 5% carbon dioxide. For hydrogen peroxide, the fibroblasts were treated with 100 μM hydrogen peroxide for 24 hours. For BDNF stimulation,

healthy and SSc dermal fibroblasts were grown in sera-depleted media and stimulated with 20 ng/ml BDNF (R&D Systems, 11166-BD) for 48 hours.

Morphogen stimulation

Healthy dermal fibroblasts were serum starved for 24 hours in DMEM containing 0.5% FBS and stimulated with 10 ng/ml TGF-β (Sigma-Aldrich, T7039), 100 ng/ml Wnt3a (R&D Systems, 5036-WN), or 100 nM SAG for 48 hours in combination with the BACE1 inhibitors.

siRNA transfections

A pool of 4 siRNAs (70 nM total concentration) specific for different regions of BACE1 (created using Qiagen's flexitube gene solution, 3567213) or a negative control scrambled siRNA (Qiagen) were transfected into fibroblasts using Lipofectamine 2000 (1 μg/ml) (Thermo Fisher Scientific,11668-030). Fibroblasts were incubated for 48 hours prior to harvesting.

BACE1 overexpression

Healthy dermal fibroblasts were transfected with 0.5 μg of WT and mutant BACE1 (contained within pcDNA3.1 mammalian expression vector) (Meakin et al, 2018) using lipofectamine 2000. Fibroblasts were incubated for 48 hours prior to harvesting.

Caveolin-1 short hairpin RNA

Caveolin-1 was silenced by shRNAmir GIPZ lentiviruses transduction (Open Biosystems, Surrey, United Kingdom). Briefly, healthy dermal fibroblasts were transduced with lentiviruses containing scramble control shRNA and caveolin-1 short hairpin RNA and incubated for 48 hours. Transduced fibroblasts were selected with 1.0 μg/ml puromycin (Life Technologies, P9620) for 10 days (Del Galdo et al, 2008).

Gel contraction assay

A total of 200,000 fibroblasts were suspended in Collagen Gel Working Solution (Cambridge Biosciences, catalog CBA-201). After collagen polymerization, 1 ml of 1% FBS culture medium was added on top of each collagen gel and incubated overnight at 37 °C with 5% carbon dioxide. Next day, the sides of the cell–collagen gels were detached using sterile spatulas, ensuring that the gels were circular. Images and weight of the gels were collected after 72 hours.

Western blotting

Total proteins were extracted from fibroblasts in RIPA buffer and resolved by SDS-PAGE (10–15% Tris-Glycine). Proteins were transferred onto Hybond nitrocellulose membranes (Amersham biosciences, 10600033) and probed with antibodies specific for α-SMA (Abcam, ab7817, dilution 1:2000), BACE1 (Sigma-Aldrich, B0681, dilution 1:1000), Notch1 (Cell Signaling Technology, 3608, dilution 1:1000), GLI2 (R&D Systems, AF3635, dilution 1:500), β-catenin (Cell Signaling Technology, 8480, dilution 1:1000), phosphorylated SMAD3 (S423/S425) (Abcam, ab52903, dilution 1:2000), caveolin 1 (Santa-Cruz Biotechnology, sc-894, dilution 1:1000), JAG1 (Cell Signaling Technology, 2620, dilution 1:500), and β-actin (Sigma-Aldrich, A6441, dilution 1:5000). Immunoblots were visualized with species-specific horse radish–conjugated secondary antibodies (Cell Signaling Technology, dilution 1:5000) and ECL (Thermo Fisher Scientific/Pierce, 34578) on a Bio-Rad chemiDoc imaging system.

qRT-PCR

RNA was extracted from cells using commercial RNA extraction kits (Zymo Research, R1055). RNA (1 μg) was reverse transcribed using cDNA synthesis kits (Thermo Fisher Scientific, 4368814). qPCR were performed using SyBr Green PCR kits on a Thermocycler with primers specific for *BACE1* (forward: GCAGGGCTACTACGTGGAGA, reverse: GTATCCACCAGGATGTTGAGC), *α-SMA* (forward: TGTATGTGGCTATCCAGGCG, reverse: AGAGTCCAGCACGATGCCAG), *COL1A2* (forward: GATGTTGAACTTGTTGCTGAGC, reverse: TCTTTCCCCATTCATTTGTCTT), CTGF (forward: GTGTGC ACTGCCAAAGATGGT, reverse: TTGGAAGGACTCACCGCT), *NOTCH1* (forward: CCAGAACTGTGAGGAAAATATCG, reverse: TCTTGCAGTTGTTTCCTGGAC), *HES1* (forward: TACCCAGCCAG TGTCAAC, reverse: CAGATGCTGTCTTTGGTTTATCC), *GLI2* (forward: TTTATGGGCATCCTCTCTGG, reverse: TTTTGCATTCCTT CCTGTCC), and *GAPDH* (forward: ACCCACTCCTCCACCTTTGA, reverse: CTGTTGCTGTAGCCAAATTCGT). Data were analyzed using the ΔΔ Ct method. *GAPDH* served as a housekeeping gene.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Ross et al, 2021). Sections were stained with a BACE1 antibody (1/200) (Sigma-Aldrich, B0681), visualized using a horse radish–conjugated rabbit secondary, and counterstained with hematoxylin. An anti-Rabbit Isotype control antibody (Invitrogen, 08-6199) was used as a negative control, and this staining was performed alongside the BACE1 staining.

Bleomycin mouse model

Mice used in the bleomycin study were severe combined immunodeficient (CB17/Icr-Prkdcscid/IcrIcoCrl, Charles River Laboratories). A total of 100 μl of bleomycin (200 μg/ml in PBS) was injected into a single location on the shaved back of the mice. Bleomycin was administered once every other day for 3 weeks. The mice were killed, and the skin was harvested using a punch biopsy (Ross et al, 2021).

BACE1-KO mouse study

Mice were given free access to food and water and maintained on a 12-hour light/dark cycle (PPL PP2103311). BACE1-KO mice, C57BL/6J background, were obtained from the Jackson Laboratory (B6.129-Bace1tm1Pcw/J), and BACE1-KO and WT control mice were generated using a BACE1 heterozygote breeding strategy (Meakin et al, 2020). Punch biopsies (3 mm) were obtained from the skin of these mice, and fibroblasts were isolated from the biopsies, as previously described (Ross et al, 2021).

Lentiviral transduction

Fibroblasts were grown from healthy control forearm biopsies and immortalized using retrovirus expressing human telomerase (hTERT) as previously outlined (Gillespie et al, 2018). HOTAIR expression was then induced by transduction with GIPZ lentiviruses carrying *HOTAIR* gene sequence or scrambled RNA sequence as control in frame with puromycin resistance gene and GFP fluorochrome gene (Open Biosystems). For this purpose, fibroblasts were seeded at 50% confluence and infected with lentiviral particles in serum-free DMEM and incubated for 6 hours, after which an additional 1 ml of DMEM containing 10% fetal calf serum was added, and the cells were incubated for further 72 hours. Stably transduced fibroblasts were positively sorted for GFP fluorescence employing FACS in sterile conditions (BD INFLUX). Positively sorted cells were further selected in media containing 1.0 μg/ml puromycin (Life Technologies) for 10 days.

Patient clinical characterization and serum sample collection

Consecutive patients with SSc who were enrolled at the University of Leeds between January 2012 and December 2014 were included in this study and longitudinally evaluated every 6 months up to 10 years. Two control groups included respectively HCs and patients with secondary Raynaud's phenomenon defined for anticentromere or anti-Scl70 positivity without any other manifestations of connective tissue disease.

The medical history collection, a comprehensive clinical examination, the annual pulmonary function tests, and echocardiogram were used to define clinical characteristics, including disease cutaneous variant and duration from the first non-Raynaud symptom, anticentromere and anti-Scl70 antibody positivity, severity of skin sclerosis according to modified Rodnan skin score, and visceral organ involvement. High-resolution computed tomography and right-heart catheterization were performed when interstitial lung disease or pulmonary hypertension presence or progression were clinically suspected. Twenty-four-month interstitial lung disease progression according to ERICE working group criteria, 12-month modified Rodnan skin score progression according to minimal clinically important difference definition in patients with diffuse cutaneous variant, and 10-year SSc-related mortality were selected as outcome measures.

Luminex analysis of patient sera

BDNF, AB40, and AB42 serum protein analysis was performed by the Clinical Laboratory Improvement Amendments–certified Myriad Rules-Based Medicine (Austin, TX) using a Multi-Analyte Profiling–multiplexed immune Luminex assay.

Statistical analysis

Categorical variables were reported as numbers and percentages, whereas continuous variables were reported as mean ± SD, mean ± standard error, or median with interquartile range. Comparisons of continuous variables were performed with Student's *t*-test or Wilcoxon rank-sum test, as appropriate, with Benjamini–Hochberg adjustment in case of multiple comparisons. The Kaplan–Meier method with log-rank test was conducted to determine whether there were differences in the survival distributions according to preset clinical risk categories. Statistical significance was defined as a $P < .05$ for all the statistical analyses. All the tests were 2 tailed. Data were analyzed using RStudio (version 2022.02.3+492).

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by National Research Ethics Service committee North East-Newcastle & North Tyneside (Research Ethics Committee reference: 15/NE/0211 to FDG). All participants provided written informed consent to participate in this study. Informed consent procedure was approved by NRES-011NE to FDG by the University of Leeds. Project licence number for the mouse work is PP2103311, issued by the University of Leeds. Patients or the public were involved in the design, conduct, reporting, or dissemination plans of our research.

DATA AVAILABILITY STATEMENT

Data are available in the article itself and its supplementary materials.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: CWW, FDG, PJM; Data Curation: CWW, EDL, EMC, KAW, BC-R, CA; Formal Analysis: CWW, EDL, NAR-DG, LDL, CSM, FDG, PJM; Investigation: CWW, EMC, RLR, KAW, BC-R, CA, RW, GM, JMB; Methodology: CWW, EDL, RLR, GM, JMB, SJT, KJS, NAR-DG, LDL, CSM, FDG, PJM; Project Administration: FDG, PJM; Resources: SJT, KJS; Supervision: KJS, CSM, FDG, PJM; Writing - Original Draft Preparation: CWW, EDL, FDG, PJM; Writing - Review and Editing: CWW, EDL, EMC, RLR, KAW, BC-R, CA, RW, GM, JMB, SJT, KJS, NAR-DG, LDL, CSM, FDG, PJM

Disclaimer

The views expressed are those of the author and not necessarily those of the National Institute for Health Research or the Department of Health and Social Care.

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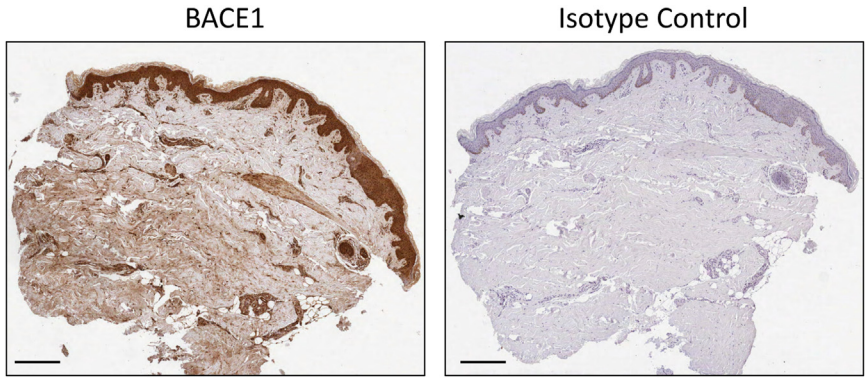
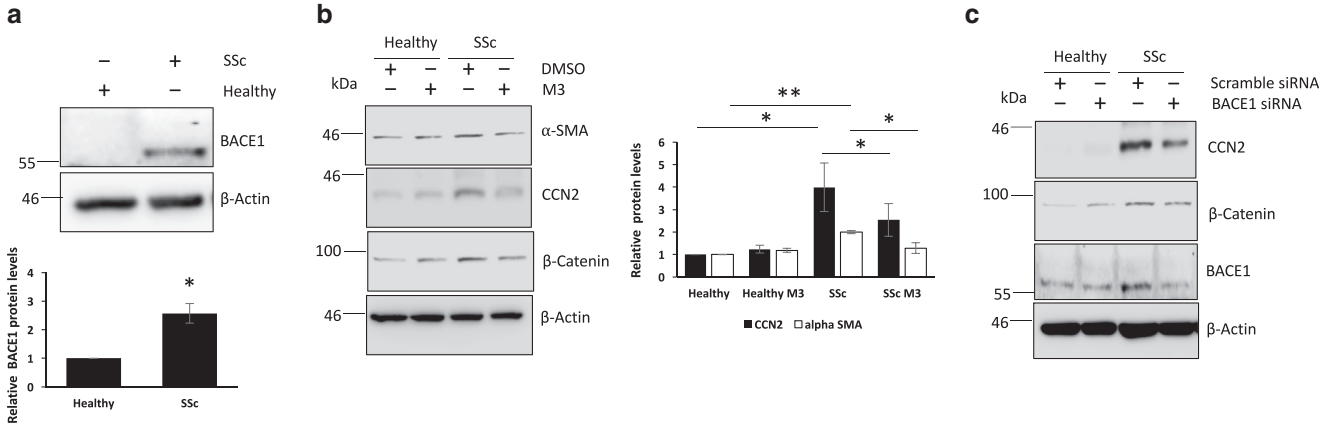
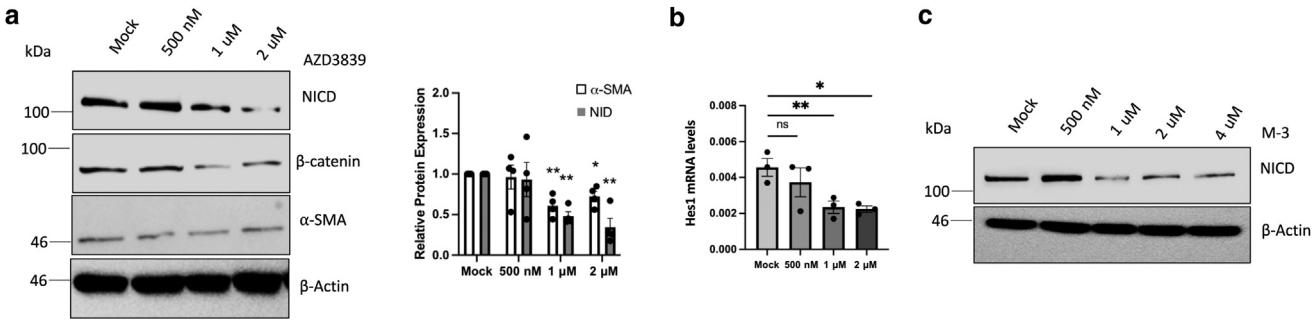


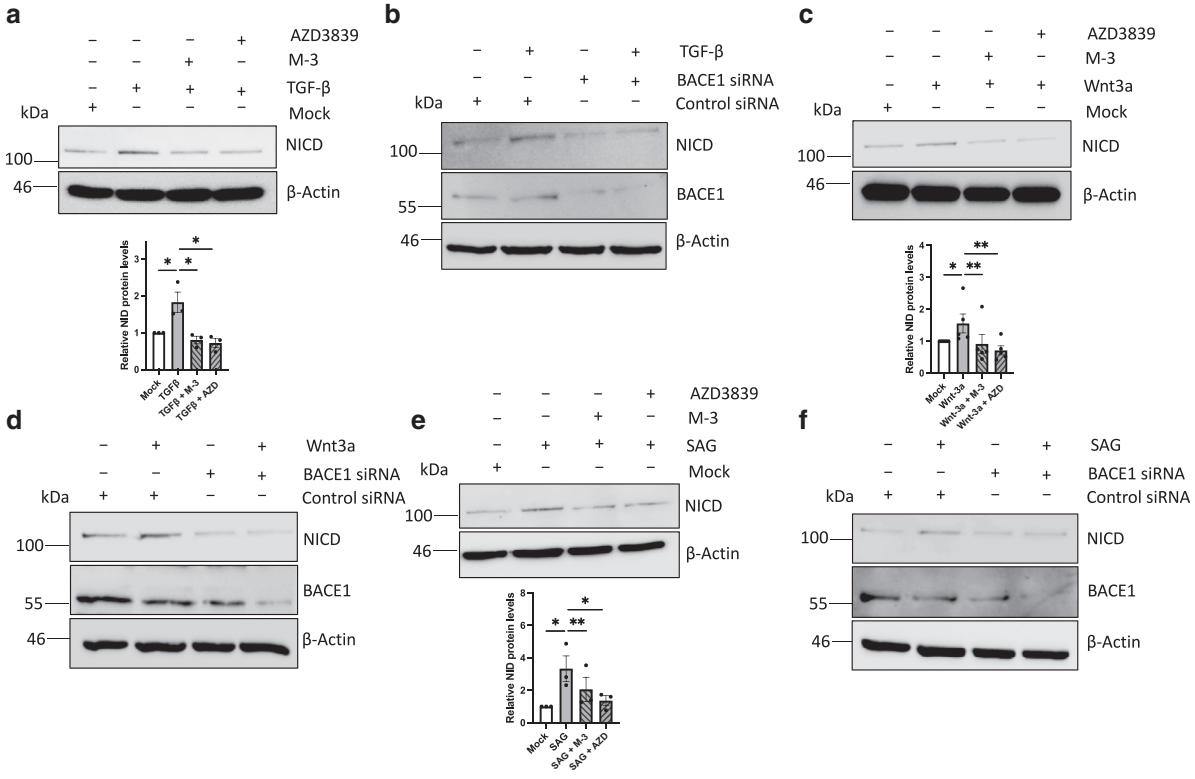
Supplementary Figure S1. BACE1 levels are increased in the skin of patients with SSc. Skin biopsies from patients with SSc were stained with an antibody specific to BACE1 alongside an isotype control antibody. Skin sections were counterstained with hematoxylin. Bars = 200 μm. SSc, systemic sclerosis.



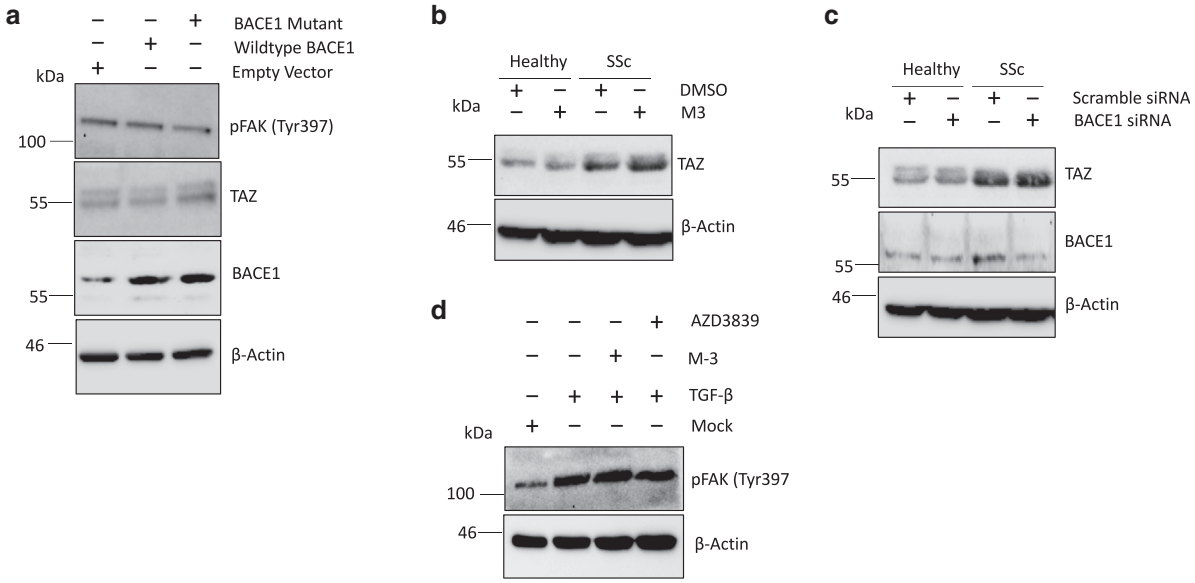
Supplementary Figure S2. Modulation of BACE1 affects SSc fibroblast activation. (**a**) Protein was extracted from primary healthy and SSc fibroblasts. BACE1 protein levels were assessed. Protein was extracted from primary healthy and SSc fibroblasts. In addition, fibroblasts were treated with the (**b**) BACE1 inhibitor M3 (1 μM) and (**c**) BACE1 siRNA for 48 hours. α-SMA, CCN2, and β-catenin protein levels were assessed. α-SMA, alpha-smooth muscle actin; siRNA, small interfering RNA; SSc, systemic sclerosis.



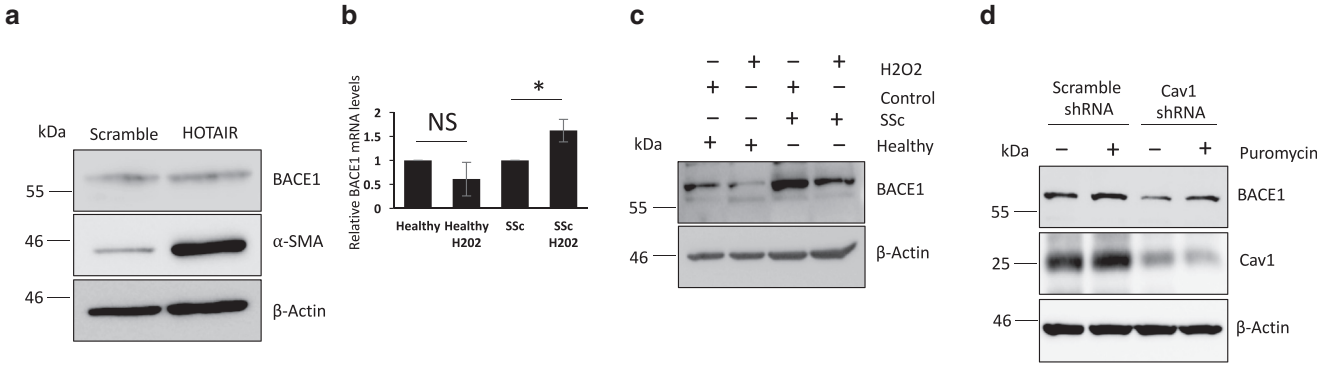
Supplementary Figure S3. The BACE1 inhibitors block SSc fibroblast activation in a dose-dependent manner. RNA and protein were extracted from SSc fibroblasts treated with the BACE1 inhibitors AZD (denoting AZD3839) (range = 500 nM to 2 μM) for 48 hours. (**a**) NICD, β-catenin, and α-SMA protein levels were assessed by western blot. β-Actin was used as a loading control. Graph represents densitometry analysis of western blots. (**b**) *Hes1* transcript levels were assessed by qPCR. (**c**) Protein was extracted from SSc fibroblasts treated with the BACE1 inhibitor M3 (range = 500 nM to 4 μM) for 48 hours. NICD protein levels were assessed by western blot. β-Actin was used as a loading control. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$. α-SMA, alpha-smooth muscle actin; NICD, Notch intracellular domain; ns, not significant; SSc, systemic sclerosis.



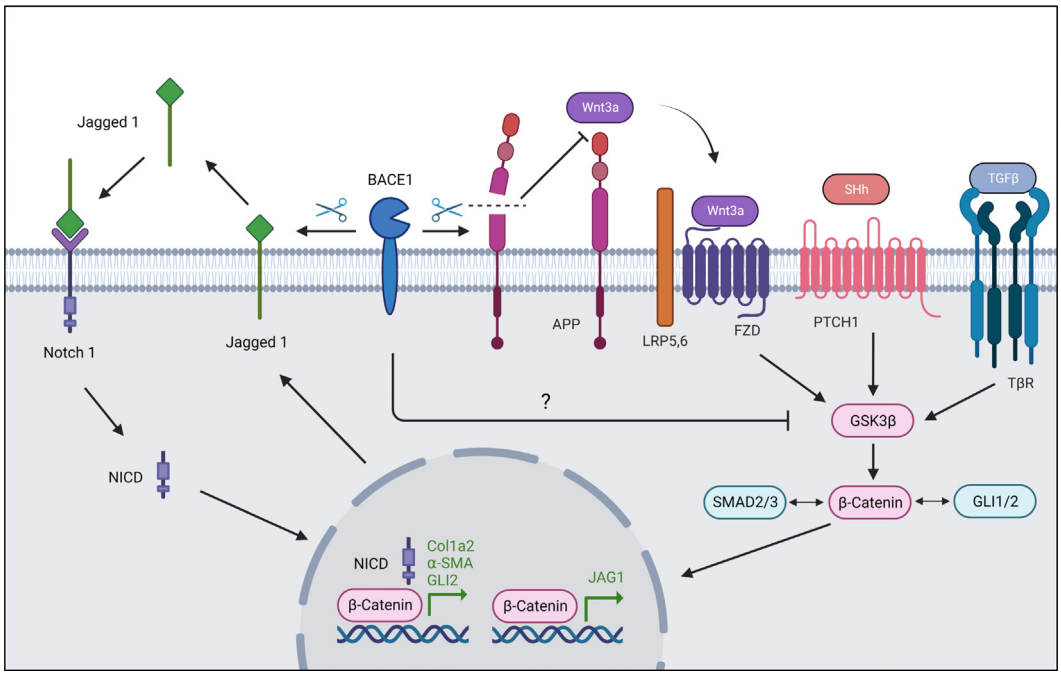
Supplementary Figure S4. BACE1 regulates morphogen-mediated Notch signaling. Healthy fibroblasts were grown in serum-depleted media and stimulated with (**a**) TGF-β, (**c**) Wnt3a, or (**e**) SAG for 48 hours in the presence or absence of BACE1 inhibitors, M3, or AZD3839. NICD protein levels were analyzed by western blot. β-Actin was used as a loading control. Healthy dermal fibroblasts were transfected with scramble or BACE1-specific siRNA and stimulated with (**b**) TGF-β, (**d**) Wnt3a, or (**f**) SAG for 48 hours. NICD protein levels were analyzed by western blot. β-Actin was used as a loading control. Graph represents densitometry analysis of NICD western blots. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$. NICD, Notch intracellular domain; SAG, smoothened agonist; siRNA, small interfering RNA.



Supplementary Figure S5. BACE1 does not regulate the Hippo or focal adhesion kinase signaling pathways in dermal fibroblasts. (**a**) Healthy fibroblasts were transfected with mammalian vectors containing WT BACE1 or *BACE1* mutant. Protein was extracted from primary healthy and SSc fibroblasts. In addition, fibroblasts were treated with the BACE1 inhibitors (**b**) M3 (1 μM) and (**c**) BACE1 siRNA for 48 hours. pFAK (Tyr397) and TAZ protein levels were assessed by western blot. (**d**) Healthy dermal fibroblasts were grown in serum-depleted media and stimulated with TGF-β for 48 hours. pFAK protein levels were assessed by western blot. β-Actin was used as a loading control. pFAK, phosphorylated focal adhesion kinase; siRNA, small interfering RNA; SSc, systemic sclerosis; WT, wild-type.



Supplementary Figure S6. Regulation of BACE1 expression in dermal fibroblasts. Protein was extracted from fibroblasts expressing the sequence for HOTAIR or a scramble control. (**a**) BACE1 and α-SMA protein levels were assessed by western blot. RNA and protein were extracted from healthy and SSc dermal fibroblasts. In addition, the fibroblasts were treated with H_2O_2 for 24 hours. (**b**) *BACE1* transcript levels were assessed by qPCR. Graphs represent the mean and standard error for 3 independent experiments. (**c**) BACE1 protein levels were assessed by western blot. Protein was extracted from healthy dermal fibroblasts transduced with lentiviral vectors containing Cav1 or scramble control shRNA. (**d**) BACE1 and Cav1 protein levels were assessed by western blot. α-SMA, alpha-smooth muscle actin; Cav1, caveolin 1; H_2O_2, hydrogen peroxide; NICD, Notch intracellular domain; SSc, systemic sclerosis.



Supplementary Figure S7. BACE1 regulates SSc fibroblast activation through a β-catenin/Notch axis. BACE1 can regulate β-catenin expression through the processing of APP. BACE1 can activate Notch signaling by regulating the expression of the Notch ligand JAG1. α-SMA, alpha-smooth muscle actin; APP, amyloid precursor protein; SSc, systemic sclerosis.

Supplementary Table S1. Demographic and Disease Characteristics of Patients with SSc Evaluated for Serum AB40, AB42, and BDNF

Characteristics	Patients with SSc n = 121
Age, y, mean ± SD	57.4 ± 12.5
Male gender, n (%)	21 (17.4)
Le Roy diffused cutaneous variant, n (%)	40 (33.1)
Disease duration, age, median (IQR)	9.0 (4.0–14.0)
ACA, n (%)	56 (46.3)
Anti-Scl70 antibody, n (%)	27 (22.3)
Active-late capillaroscopy pattern, n (%)	48 (64.9)
Late capillaroscopy pattern, n (%)	26 (34.7)
mRSS, median (IQR)	2.0 (1.0-5.0)
ILD on HRCT, n (%)	45 (37.2)
PH on RHC, n (%)	10 (8.3)
FVC, % of predicted, mean ± SD	100.5 ± 23.6
TLC, % of predicted, mean ± SD	91.8 ± 17.8
DLco, % of predicted, mean ± SD	62.8 ± 16.1
Digital ulcers, n (%)	65 (53.7%)
Skin calcinosis, n (%)	51 (42.1%)
Skin telangiectasias, n (%)	94 (77.7%)
Arthritis, n (%)	12 (9.9%)
Immunosuppressive treatment, n (%)	42 (34.7%)
Vasoactive treatment, n (%)	90 (74.4%)
AB40, ng/ml, mean ± SD	0.40 ± 0.13
AB42, ng/ml, median (IQR)	0.18 (0.14–0.59)
BDNF, ng/ml, mean ± SD	19.20 ± 6.14

Abbreviations: ACA, anticentromere antibody; BDNF, brain-derived neurotrophic factor; DLco, lung diffusion of carbon monoxide; FVC, forced vital capacity; HC, healthy control; HRCT, high-resolution computed tomography; IQR, interquartile range; ILD, interstitial lung disease; mRSS, modified Rodnan skin score; PH, pulmonary hypertension; RHC, right heart catheterization; sRP, secondary Raynaud's phenomenon; SSc, systemic sclerosis; TLC, total lung capacity.