The Beta secretase BACE1 drives Systemic Sclerosis fibroblasts activation through β-catenin and Notch signalling

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Abstract

Background

The beta-amyloid precursor protein cleaving enzyme 1 (BACE1) is well known for its role in the development of Alzheimer's disease via the generation of β-amyloid. Recent publications, including our own, have demonstrated a role for this enzyme in other chronic inflammatory diseases, including type 2 diabetes and cardiovascular disease. However, to date there has been no studies looking into the role of BACE1 in the autoimmune condition Systemic Sclerosis (SSc). The aim of this study was to investigate the role of BACE1 in SSc tissue fibrosis.

Methods

Patient fibroblasts were obtained from full thickness forearm skin biopsies from healthy and early diffuse SSc patients. BACE1 was inhibited with the small molecule inhibitors M-3 and AZD3839 and siRNA specific to BACE1. Morphogen signalling was activated with recombinant TGF-β, Wnt-3a or the smoothened agonist SAG. Xenotransplant mouse model using patient pDC was used to interrogate in vivo expression of BACE1 in fibrosis.

Results

Here we show that BACE1 protein levels are elevated in SSc patient skin biopsies, as well as dermal fibroblasts and in mouse skin during fibrosis. Inhibition of BACE1 with small molecule inhibitors or siRNA blocked SSc and morphogen mediated fibroblast pro-fibrotic activation. Furthermore, we show that BACE1 regulation of dermal fibroblast activation is dependent on the β-catenin and Notch signalling pathway activation.

Conclusions

The ability of BACE1 to regulate SSc fibroblast activation reveals an exciting new therapeutic target in SSc. Several BACE1 inhibitors, including AZD3839, have been shown to be safe in clinical trials for Alzheimer's disease.

Keyword: Systemic Sclerosis, Fibrosis, BACE1, Notch

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Introduction

Systemic sclerosis (SSc) is an autoimmune disease that initially presents in the skin of the hands and feet and it variably progresses to affect proximal skin as well as internal organs including the lungs and heart, leading to pulmonary hypertension and heart failure in the most severe cases. An autoimmune related injury is believed to induce fibrosis in the skin. The fibrosis is driven by activated fibroblasts (myofibroblasts) characterised by increased expression of 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 sonic hedgehog (SHh) pathways (1, 2, 3, 4).

The beta-amyloid precursor protein cleaving enzyme 1 (BACE1) is well known for its role in the development of Alzheimer's disease via the generation of β-amyloid (5). However, our recent findings have demonstrated a role for this enzyme in other chronic inflammatory diseases associated with vascular damage, including type 2 diabetes and cardiovascular disease (6, 7). Several links exist implicating a role for BACE1 in SSc. Vasculopathy is a common feature in SSc pathogenesis (8), developing through the Scleroderma disease. We have shown that BACE1 KO mice are protected from vascular damage in the obesity induced diet mouse model (9). Further studies showed that inhibition of BACE1 attenuates Palmitate induced ER stress and inflammation in skeletal muscles (10). ER stress has previously been shown to enhance fibrosis (11). BACE1 can also target the IL-1 receptor 2 (IL-1R2) for cleavage, modulating systemic IL-1 activity through soluble IL1R2, (12). This is interesting as a number of IL-1 family members including IL-33 have been implicated in SSc (13). BACE1 expression levels are modulated by NFκ-B. The p65 subunit directly binds to the BACE1 promoter (14). Taken together this data suggests BACE1 levels could be elevated in SSc through inflammatory responses.

The aim of this study was to determine if BACE1 played a role in the pathogenesis of fibrosis in SSc. We show BACE1 protein levels are increased in SSc fibroblasts and a mouse model of fibrosis. Inhibition of BACE1 activity blocked expression of pro-fibrotic markers including

α-SMA and Collagen type 1. We also found that BACE1 regulates fibroblast activation through its ability to activate the Notch 1 and Wnt3a signalling pathways.

Results

BACE1 expression is increased in SSc patient skin and fibroblasts

To inform the potential role for BACE1 in SSc pathogenesis, we assessed BACE1 protein expression in healthy and SSc skin. BACE1 IHC on skin biopsies obtained from patient forearms revealed high levels of BACE1 protein expression in the epidermis of both healthy and SSc patient skin (Figure 1A). Conversely, dermal BACE1 protein levels were elevated only in SSc skin, with little or no expression in healthy controls. In particular, we observed high levels of BACE1 protein expression in fibroblasts and endothelial cells in the SSc dermis (Figure 1A inset). To further investigate BACE1 expression profile in dermal fibroblasts, we collected protein and RNA lysates from explanted healthy and early diffuse SSc dermal fibroblasts and analysed BACE1 levels by western blot and qPCR. We found BACE1 protein levels were increased in dermal fibroblasts from early diffuse patients (2-fold) compared to healthy dermal fibroblasts (Figure 1B-C). In addition, we observed high levels of phosphorylated SMAD3 (pSMAD3) in the SSc fibroblasts which confirms the SSc fibroblast TGF-β activated phenotype (Figure 1B). Interestingly BACE1 transcript levels did not correlate with protein levels and found no difference in mRNA levels between healthy and SSc fibroblasts (Figure 1D). This suggests the increase in BACE1 protein levels is mediated at the post-translation level, similar to that seen in Alzheimer's disease (15). To assess the role of BACE1 in skin fibrosis *in vivo* we analysed BACE1 levels in the bleomycin skin model using Xenotransplant mice injected with human pDCs (previously described in 16). Subcutaneous injection of bleomycin to the skin of mice induced skin fibrosis including a loss of the adipose and a thickening of the dermal layer of the skin. We found high levels of BACE1 in the keratinocytes and endothelial cells in both the control and bleomycin skin (Figure 1E). Interestingly we found an increase in BACE1 levels in the dermal fibroblasts in

the bleomycin skin (Figure 1E insert), similar to that observed in SSc patient skin (Figure 1A) This suggests the induction of skin fibrosis drives BACE1 levels *in vivo.*

BACE1 plays an important role in SSc and morphogen induced myofibroblast activation

Next, we set out to determine a role for BACE1 in SSc fibroblast biology using structurally diverse small molecule inhibitors against BACE1 (M-3 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 pro-fibrotic gene expression. Both inhibitors led to a reduction in Col1a2 (Figure 2A) and α-SMA (Figure 2B) transcript levels in SSc fibroblasts. The inhibitors had no effect on fibrotic marker expression in healthy fibroblasts. AZD3839 was shown to block pro-fibrotic gene expression in a dose-dependent manner in SSc fibroblasts (Supplementary Figure 1A-C). Importantly we observed similar results when we knocked down BACE1 using siRNA. BACE1 knockdown reduced Col1a2 (Figure 2C), α-SMA (Figure 2D) and CTGF (Figure 2E) transcript levels in SSc fibroblasts to similar levels seen in healthy fibroblasts. In a complementary approach, we overexpressed BACE1 in healthy dermal fibroblasts to determine if elevated BACE1 protein expression is sufficient to drive fibroblast activation. Healthy dermal fibroblasts were transfected with mammalian expression vectors containing wildtype BACE1 (WT) and an inactive form ($D_{92}A$ and $D_{289}A$; Mut) (7) (Figure 2F). While both BACE1 proteins expressed at similar levels (WT=3.6, Mut=3.7-fold increase in expression compared to empty vector), only the wildtype BACE1 protein induced α-SMA expression in the healthy fibroblasts. This confirms BACE1 can trigger fibroblast activation and its secretase activity is essential for this function.

Next, we assessed the effects of BACE1 KO on skin fibrosis *in vivo.* We assessed pro-fibrotic marker expression in dermal fibroblasts isolated from wildtype and BACE1 KO mice. We observed no differences in α-SMA protein levels between the wildtype and BACE1 KO

dermal fibroblasts (Figure 2G). This suggests BACE1 requires fibrotic stimuli in order to regulate pro-fibrotic gene expression.

As BACE1 KO has no effect on α-SMA expression on healthy fibroblasts, we therefore assessed the effects of BACE1 inhibition and KO on healthy fibroblasts stimulated with pro-fibrotic stimuli such as the morphogens. Morphogen signalling (TGF-β, Wnt-3a and Hedgehog) is deregulated in SSc fibroblasts and play an important role in SSc disease progression (2, 3, 4). The morphogens enhance the expression of several pro-fibrotic genes in fibroblasts, including α-SMA (3). To determine if the morphogens play a role in regulating BACE1 expression in dermal fibroblasts, we stimulated healthy dermal fibroblasts with TGF-β, Wnt-3a or SAG (smoothened agonist that activates the hedgehog pathway) and assessed BACE1 protein levels. Stimulation with either TGF-β, Wnt-3a or SAG led to 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. The BACE1 inhibitors blocked the TGF-β-mediated increase in α-SMA protein (Figure 3B). Similar results were observed with BACE1 siRNA (Figure 3C). Depletion of BACE1 protein attenuated TGF-β ability to increase α-SMA levels in dermal fibroblasts. This was also the case for Wnt3a-mediated fibroblast activation. Inhibition (Figure 3D) and depletion (Figure 3E) of BACE1 prevented Wnt3a mediated induction of α-SMA expression. Finally SAG dependent fibroblast activation was also blocked by BACE1 inhibition. The ability of SAG to induce α-SMA protein levels was blocked with the BACE1 inhibitors and BACE1 siRNA (Figure 3F-G). Together, this data suggests BACE1 is an important downstream regulator of morphogen-mediated fibroblast activation.

Next, we sought to determine the mechanism in which BACE1 regulates morphogen signalling. The well-established BACE1 substrate amyloid precursor protein (APP) is known to be a conserved Wnt receptor (17). The processing of APP by BACE1 could increase the levels of Wnt ligands in the supernatant to activate the canonical signalling pathways

(through the frizzled receptor) in SSc. We hypothesised that BACE1 could regulate morphogen-mediated fibroblast activation through regulating β-catenin expression levels. Accordingly, we observed a dose-dependent decrease in β-catenin expression levels with AZD3839 in SSc fibroblasts (Figure 4A). BACE1 siRNA suppressed β-catenin expression levels in SSc fibroblasts (Figure 4B). BACE1 could also modulate β-catenin levels in the presence of Wnt ligands. Healthy fibroblasts stimulated with Wnt-3a resulted in increased levels of β-catenin (Figure 4C-D) which was reversed in the presence of the BACE1 inhibitors AZD3839 and M-3. Overexpression of BACE1 in healthy dermal fibroblasts led to increased β-catenin expression levels (Figure 4E) and this was dependent on its secretase activity, as the secretase dead BACE1 protein was unable to increase β-catenin expression levels. Interestingly BACE1 did not regulate the activity of the TGF-β canonical transcription factor SMAD3. Inhibition of BACE1 (Figure 4F) or BACE1 knockdown (Figure 4G) had no effect on phosphorylation of SMAD3 in TGF-β stimulated healthy fibroblasts. Previous studies have shown the three morphogen signalling pathways work in tandem in dermal fibroblasts (3). β-catenin was shown to be an important intermediator for TGF-β mediated α-SMA expression. Inhibition of β-catenin with the small molecule inhibitor FH535 blocked TGF-β ability to induce α-SMA expression (3). Here we show TGF-β stimulation induces β-catenin expression in dermal fibroblasts (Figure 4G) and this is partially blocked with the BACE1 siRNA (Figure 4G).

BACE1 regulates myofibroblast activation through the activation of Notch1 signalling

The Notch signalling pathway is important for cell-to-cell communication, including fibroblast biology. The Notch receptor resides at the cell surface and upon ligand binding (Jagged-1 (JAG1) or Delta like ligand-1 (DLL1)) is cleaved into the active Notch intracellular domain (NID) which induces transcription of downstream target genes (Hes1/Hey1). In SSc fibroblasts, Notch signalling is enhanced and characterised by increased NID and Hes1 expression. Previous work has shown the lncRNA HOTAIR plays an important role in

enhancing Notch1 transcription, leading to accumulation of NID in the SSc fibroblasts (4, 18). Generation of NID is orchestrated by the gamma secretase enzyme complex and inhibition of this secretase blocks SSc fibroblast activation (18). We explored the hypothesis that BACE1 regulates SSc fibroblast activation through the activation of Notch1. Therefore, we assessed Notch signalling in SSc fibroblasts treated with the BACE1 inhibitors. Notch 1 transcript levels were upregulated in SSc fibroblasts but the BACE1 inhibitors had no impact (Figure 5A). Interestingly, Hes1 transcript levels (downstream transcriptional target of Notch) were reduced in SSc fibroblasts treated with the BACE1 inhibitors in a dose dependent manner (Figure 5B, Supplementary Figure 1E). Hes1 levels were unaffected in healthy fibroblasts treated with the inhibitors. This suggests BACE1 regulates the activation of Notch signalling in SSc fibroblasts. Further analysis of the Notch signalling pathway revealed the BACE1 inhibitors reduced the levels of the NID in SSc fibroblasts in a dose-dependent manner (Figure 5C-D, Supplementary Figure 1), suggesting BACE1 regulates Notch signalling through the activation of the Notch1 receptor. BACE1 does not cleave the Notch1 protein, however BACE1 has been shown to selectively regulate the cleavage of membrane bound JAG1 in Schwann cells (19). Regulation of JAG1 by extracellular domain shedding may play a role in the activation of Notch 1 in SSc fibroblasts.

The BACE1 inhibitors reduced the expression of the Hedgehog transcription factor GLI2 in SSc fibroblasts (Figure 5C). GLI2 has previously been shown to be a downstream target of the Notch pathway in SSc fibroblasts (4). Accordingly, the inhibition of BACE1 led to suppression of downstream targets of Notch. Interestingly we also observed inhibition of the Notch signalling pathway in BACE1 depleted SSc fibroblasts. BACE1 siRNA did not alter Notch1 (Figure 5E) transcript levels, similar to the inhibitor data, but did reduce Hes1 (Figure 5F) and GLI2 (Figure 5G) transcript levels, as well as NID and GLI2 protein levels (Figure 5H). Overexpression of BACE1 in healthy dermal fibroblasts resulted in increased accumulation of NID (Figure 5I), while overexpression of the secretase mutant was unable to induce NID accumulation. This confirms the importance of BACE1 and its activity in the regulation of the Notch signalling pathway in SSc fibroblasts.

Further investigations found inhibition and knockdown of BACE1 reduced the ability of TGF-β to modulate NID in healthy fibroblasts (Figure 6A-B). We observed increased NID protein levels in TGF-β stimulated fibroblasts, which was reversed when the fibroblasts were treated with M-3, AZD3839 (Figure 6A) or BACE1 siRNA (Figure 6B). Cooperation between TGF-β and Notch signalling pathways is well characterised with Notch signalling required for the regulation of a number of TGF-β responsive genes (20). Similar results were observed in Wnt-3A (Figure 6C-D) and SAG (Figure 6E and F) stimulated fibroblasts. Inhibition of BACE1 with siRNA and the small molecule inhibitors blocked Wnt3a and SAG mediated activation of Notch signalling. This is consistent with data in the literature where the Notch ligand Jag1 is a target of the canonical Wnt/β-catenin signalling pathway in hair Follicles (21) and Hedgehog stimulation induces Notch target gene expression in vascular smooth muscle cells (22). Together our data shows BACE1 regulates Notch signalling in SSc fibroblasts and this is important for BACE1 ability to regulate morphogen dependent and independent myofibroblast activation.

Discussion

This is the first study describing a role of BACE1 in SSc fibroblast activation. BACE1 levels are increased in SSc patient skin and dermal fibroblasts. The elevated expression levels are important for SSc disease pathogenesis, as blocking BACE1 activity (with small molecule inhibitors) or expression levels (siRNA) led to a suppression of fibroblast activation in SSc.

The link between BACE1 and Alzheimer's disease progression is interesting in the context of SSc. SSc patients with Alzheimer's disease as a co-morbidity have a higher mortality rate (23). This could be a result of the higher levels of BACE1 in SSc patients resulting 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 novel functions

for BACE1 in another disease setting and highlights the need to study BACE1 in other cell types and disease settings.

The data presented here shows BACE1 regulates Notch1 signalling in dermal fibroblasts. The precise mechanism behind this activation is unknown. To date there is no experimental evidence to show Notch 1 is a direct substrate of BACE1. Another more likely hypothesis is BACE1 cleaves the Notch ligand Jagged 1 from the cell surface as previously described in neuronal cells (19). This in turn would lead to increased levels of soluble Jagged 1 which could induce paracrine Notch signalling in the fibroblasts (Figure 7). Future work is needed to identify the precise mechanism of Notch activation.

An intriguing element of the study is the uncovering of a role for BACE1 downstream of Morphogen signalling. Inhibition of BACE1 via siRNA or small molecule inhibitors blocked Morphogen mediated fibroblast activation (Figure 3). Interestingly APP is a conserved Wnt receptor (17). APP binds to Wnt3a and Wnt5a resulting in the recycling and stabilisation of APP. Thus the ability of BACE1 to process APP may enable more Wnt ligands to induce canonical Wnt signalling and fibroblast activation (Figure 7). In addition previous studies have shown an interplay between BACE1 and GSK3β which is a member of the β-catenin destruction complex (24). We observed reduced β-catenin levels in Wnt3a stimulated fibroblasts when BACE1 was inhibited (Figure 4C-D) supporting this hypothesis. Further analysis revealed BACE1 does not regulate the activity of the TGF-β transcription factor SMAD3 (Figure 4E-F). Therefore BACE1 could regulate TGF-β signalling through β-catenin. We have previously shown co-operation between the Wnt3a and TGF-β signalling pathways in dermal fibroblasts. β-catenin inhibitors blocked the ability of TGF-β to induce signalling (3). The ability of BACE1 to regulate Hedgehog mediated fibroblast activation (Figure 3F-G) could be due to its ability to regulate β-catenin. Previous studies have shown Wnt-3a/β-catenin can induce the expression of the hedgehog transcription factor GLI2 (3).

In addition, BACE1 can regulate Notch signalling through the modulation of Morphogen signalling. Morphogen signalling induced Notch receptor cleavage and activation and this

activation by morphogens is blocked by the BACE1 inhibitors (Figure 6). 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 (21). Therefore β-catenin could drive Notch signalling by increasing ligand expression in SSc fibroblasts

Systemic sclerosis fibroblasts have previously been shown to share several similarities with cancer associated fibroblasts (25). Interestingly cancer associated fibroblasts secrete amyloid-beta and this drives the formation of neutrophil extracellular traps (NETs) in the associated tissue. NETs are decondensed chromatin that the neutrophils extrude into the extracellular space and this has a microbicidal effect. BACE1 inhibition reduces amyloid-beta secretion and in turn reduces NETs from neutrophils in the associated tissue (26). This has implications for SSc, as NETosis in patient plasma in the early stages of the disease correlates with vascular complications (27). This further highlights the potential role of BACE1 in multiple aspects of SSc disease progression.

One exciting element to this study is a number of small molecule inhibitors targeting BACE1, including AZD3839, have progressed through clinical trials for the treatment of Alzheimer's disease indicating good tolerance by the patients. This opens up the possibility that these compounds could be re-purposed for the treatment of SSc and provide a novel therapy which is desperately needed.

Materials and Methods

Patient cell lines

Full thickness skin biopsies were surgically obtained from the forearms of four adult healthy controls and four adult patients with recent onset SSc, defined as a disease duration of less than 18 months from the appearance of clinically detectable skin induration. All patients satisfied the 2013 ACR/EULAR criteria for the classification of SSc and had diffuse cutaneous clinical subset as defined by LeRoy et al (28). 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 (2). Primary cells were immortalized using human telomerase reverse transcriptase (hTERT) to produce healthy control hTERT and SSc hTERT.

Cell culture

hTERT patient fibroblasts were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% FBS (Sigma) and penicillin-streptomycin (Sigma). fibroblasts were treated with BACE1 inhibitors (M-3 (500nM-4μM, Sigma-Aldrich) or AZD3839 (500nM-2μM, AstraZeneca)) for 48 hours in a humidified incubator at 37C and 5% CO_2.

Morphogen stimulation

Healthy dermal fibroblasts were serum starved for 24 h in DMEM containing 0.5% FBS and stimulated with 10 ng/ml TGF-β (R&D systems), 100 ng/ml Wnt-3a (R&D systems) or 100 nM SMO agonist (SAG) for 48 hr in combination with the BACE1 inhibitors.

siRNA transfections

A pool of four siRNAs specific for different regions of BACE1 or a negative control scrambled siRNA (Qiagen) were transfected into fibroblasts using Lipofectamine 2000 (Thermo Fisher). Fibroblasts were incubated for 48 h prior to harvesting.

BACE1 overexpression

Healthy dermal fibroblasts were transfected with 0.5μg of WT and Mutant BACE1 (Contained within pcDNA3.1 mammalian expression vector) (7) using lipofectamine 2000. Fibroblasts were incubated for 48 h prior to harvesting.

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) and probed with antibodies specific for alpha smooth muscle actin (Abcam), BACE1 (Sigma), Notch 1 (Cell signalling), GLI2 (R&D systems), β-catenin (cell signalling), phosho-SMAD3 (S423/S425) (Abcam) and β-Actin (Sigma). Immunoblots were visualized with species-specific HRP conjugated secondary antibodies (Sigma) and ECL (Thermo/Pierce) on a Biorad chemiDoc imaging system.

Quantitative Real time PCR

RNA was extracted from cells using commercial RNA extraction kits (Zymo Research). RNA (1ug) was reverse transcribed using cDNA synthesis kits (Thermo). QRT-PCRs were performed using SyBr Green PCR kits on a Thermocycler with primers specific for BACE1 (Forward: GCAGGGCTACTACGTGGAGA, Reverse: GTATCCACCAGGATGTTGAGC), alpha SMA (Forward; TGTATGTGGCTATCCAGGCG Reverse; AGAGTCCAGCACGATGCCAG), Collagen type 1A2 (Forward; GATGTTGAACTTGTTGCTGAGC Reverse; TCTTTCCCCATTCATTTGTCTT), CTGF (Forward; GTGTGCACTGCCAAAGATGGT Reverse; TTGGAAGGACTCACCGCT), Notch 1 (Forward; CCAGAACTGTGAGGAAAATATCG Reverse; TCTTGCAGTTGTTTCCTGGAC), Hes1 (Forward; TACCCAGCCAGTGTCAAC Reverse; CAGATGCTGTCTTTGGTTTATCC), GLI2 (Forward; TTTATGGGCATCCTCTCTGG Reverse; TTTTGCATTCCTTCCTGTCC) and GAPDH (Forward; ACCCACTCCTCCACCTTTGA Reverse; CTGTTGCTGTAGCCAAATTCGT). Data were analysed using the ΔΔ Ct method. GAPDH served as a housekeeping gene.

Immunohistochemistry

Immunohistochemistry was performed as previously described (26). Sections were stained with a BACE1 antibody (1/200) (Sigma), visualised using an HRP conjugated mouse secondary and counterstained with haematoxylin.

Bleomycin Mouse Model

Mice used in the bleomycin study were severe combined immunodeficient (CB17/Icr-Prkdcscid/IcrIcoCrl, Charles River). 100µl of bleomycin (200µg/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 euthanized and the skin was harvested using a punch biopsy (16).

BACE1 KO mouse study

Mice were given free access to food and water and maintained on a 12 h 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 wild type control mice were generated using a BACE1 heterozygote breeding strategy (9). Punch biopsies (3mm) were obtained from the skin of these mice and fibroblasts were isolated from the biopsies, as previously described (16).

Statistical analysis

Data are presented as the mean ± standard error. Statistical analysis was performed using a two-tailed, paired Student's t-test**.**

Ethics Approval and consent to participate

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

Author Contribution

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Figure Legends

Figure 1: BACE1 levels are increased in SSc patient skin, fibroblasts and in vivo skin fibrosis models. (A) *Healthy and SSc patient skin biopsies were stained with an antibody specific to BACE1 (Brown). Skin sections were counterstained with Haematoxylin (Blue). Scale Bars represent 50μM. Protein and RNA were extracted from healthy and early diffuse SSc dermal fibroblasts. (B) BACE1 and pSMAD3 protein levels were analysed by western blot. β-actin was used as a loading control. (C) Densitometry analysis of BACE1 western blots. (D) BACE1 transcript levels were assessed by qPCR. Graphs represent the mean and standard error for 4 healthy and 4 SSc patient fibroblast lines. (E) Representative skin sections from vehicle and bleomycin treated mice (N=4 each group) were stained with an antibody specific to BACE1 (Brown). Skin sections were counterstained with Haematoxylin (Blue). Scale bars represent 200μM*

Figure 2: Modulation of BACE1 affects SSc fibroblast activation. *RNA was extracted from healthy and SSc fibroblasts. In addition healthy and SSc fibroblasts were treated with the BACE1 inhibitors M-3 (1µM) and AZD3839 (1µM) for 48 hours. Col1a2 (A) and α-SMA (B) transcript levels were assessed by qPCR. Healthy and SSc fibroblasts were transfected with siRNA specific for BACE1 for 48 hours. Col1a2 (C), α-SMA (D) and CTGF (E) transcript levels were assessed by qPCR. Graphs represent the mean and standard error for healthy and SSc patient fibroblast lines. (F) Healthy fibroblasts were transfected with mammalian vectors containing WT BACE1 or BACE1 mutant that disrupts the secretase activity. BACE1 and α-SMA protein levels were assessed by western blot. β-actin was used as a loading control. Graph represents densitometry analysis of α-SMA western blots. (G) Fibroblasts were isolated from skin biopsies of WT and BACE1 KO mice (N=3 per group). Protein was isolated from the fibroblasts. α-SMA and BACE1 protein levels were analysed by western blot. β-actin was used as a loading control. *p<0.05, **p<0.01, ***p<0.001*

Figure 3: BACE1 is important for morphogen mediated fibroblast activation. *Healthy dermal fibroblasts were grown in serum depleted media and stimulated with (A,B,C) TGF-β, (A,D,E) Wnt-3a 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 were treated with the BACE1 inhibitors M-3 or AZD3839 for 48 hours (B,D,F) or transfected with scramble or BACE1 specific siRNA for 48 hours (C,E,G). Graph represents densitometry analysis of the α-SMA western blots. *p<0.05, **p<0.01, ***p<0.001*

Figure 4: BACE1 regulates β-catenin expression in the presence and absence of Wnt ligands. *(A) SSc fibroblasts treated with the BACE1 inhibitor AZD3839 (AZD) for 48 hours. β-catenin protein levels were assessed by western blot. β-actin was used as a loading control. (B) Healthy and SSc fibroblasts were transfected with BACE1 siRNA with BACE1*

*and β-catenin protein levels were assessed by western blot. Healthy fibroblasts were grown in serum depleted media and stimulated with Wnt-3a (C) or TGF-β (F) for 48 hours in the presence and absence of BACE1 inhibitors (M-3 or AZD3839). Graph represents densitometry analysis of the β-catenin western blots. (E) Healthy fibroblasts were transfected with mammalian vectors containing WT BACE1 or BACE1 mutant that disrupts the secretase activity. BACE1 and β-catenin protein levels were assessed by western blot. β-actin was used as a loading control. Graph represents densitometry analysis of the β-catenin western blots (G) Healthy dermal fibroblasts were transfected with scramble or BACE1 specific siRNA and stimulated with TGF-β for 48 hours. β-catenin, pSMAD3 and BACE1 protein levels were analysed by western blot. β-actin was used as a loading control. *p<0.05, **p<0.01, ***p<0.001*

Figure 5: Inhibition of BACE1 leads to reduced Notch signalling in SSc fibroblasts. *Healthy and SSc fibroblasts were treated with the BACE1 inhibitors M-3 and AZD3839 for 48 hours. Notch 1 (A) and Hes1 (B) transcript levels were assessed by qPCR. Graphs represent the mean and standard error for healthy and SSc patient fibroblast lines. (C) NID and GLI2 protein levels were assessed by western blot. β-actin was used as a loading control. (D) Densitometry analysis of NID western blots. Healthy and SSc fibroblasts were transfected with siRNA specific to BACE1. Notch 1 (E), Hes1 (F) and GLI2 (G) transcript levels were assessed by qPCR. (H) NID, GLI2 and BACE1 protein levels were assessed by western blot. β-actin was used as a loading control. Graph represents densitometry analysis of NID western blots. (I) Healthy fibroblasts were transfected with mammalian vectors containing WT BACE1 or BACE1 mutant that disrupts the secretase activity. BACE1 and NID protein levels were assessed by western blot. β-actin was used as a loading control. *p<0.05, **p<0.01, ***p<0.001*

Figure 6: BACE1 regulates morphogen mediated Notch signalling. *Healthy fibroblasts were grown in serum depleted media and stimulated with TGF-β (A), Wnt-3a (C) or SAG (E)*

*for 48 hours in the presence or absence of BACE1 inhibitors, M-3 or AZD3839. NID protein levels were analysed 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 TGF-β (B), Wnt-3a (D) or SAG (F) for 48 hours. NID protein levels were analysed by western blot. β-actin was used as a loading control. Graph represents densitometry analysis of NID western blots. *p<0.05, **p<0.01, ***p<0.001*

Figure 7: BACE1 regulates SSc fibroblast activation through a β-catenin/Notch axis. *BACE1 can regulate β-catenin expression and this in turn can drive Notch signalling potentially through regulating the expression of the Notch ligand Jag1*

Supplementary Figure 1: 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 (Range 500nM to 2uM) for 48 hours. Col1a2 (A), α-SMA (B), CTGF (C), Hes1 (E) transcript levels were assessed by qPCR. Graphs represent the mean and standard error for three 3 independent experiments. (D) NID protein levels were assessed by western blot. β-actin was used as a loading control. (F) Protein was extracted from SSc fibroblasts treated with the BACE1 inhibitors M3 (Range 500nM to 4uM) for 48 hours. NID protein levels were assessed by western blot. β-actin was used as a loading control *p<0.05, **p<0.01, ***p<0.001*

Figure 1: BACE1 levels are increased in SSc patient skin, fibroblasts and in vivo skin fibrosis models

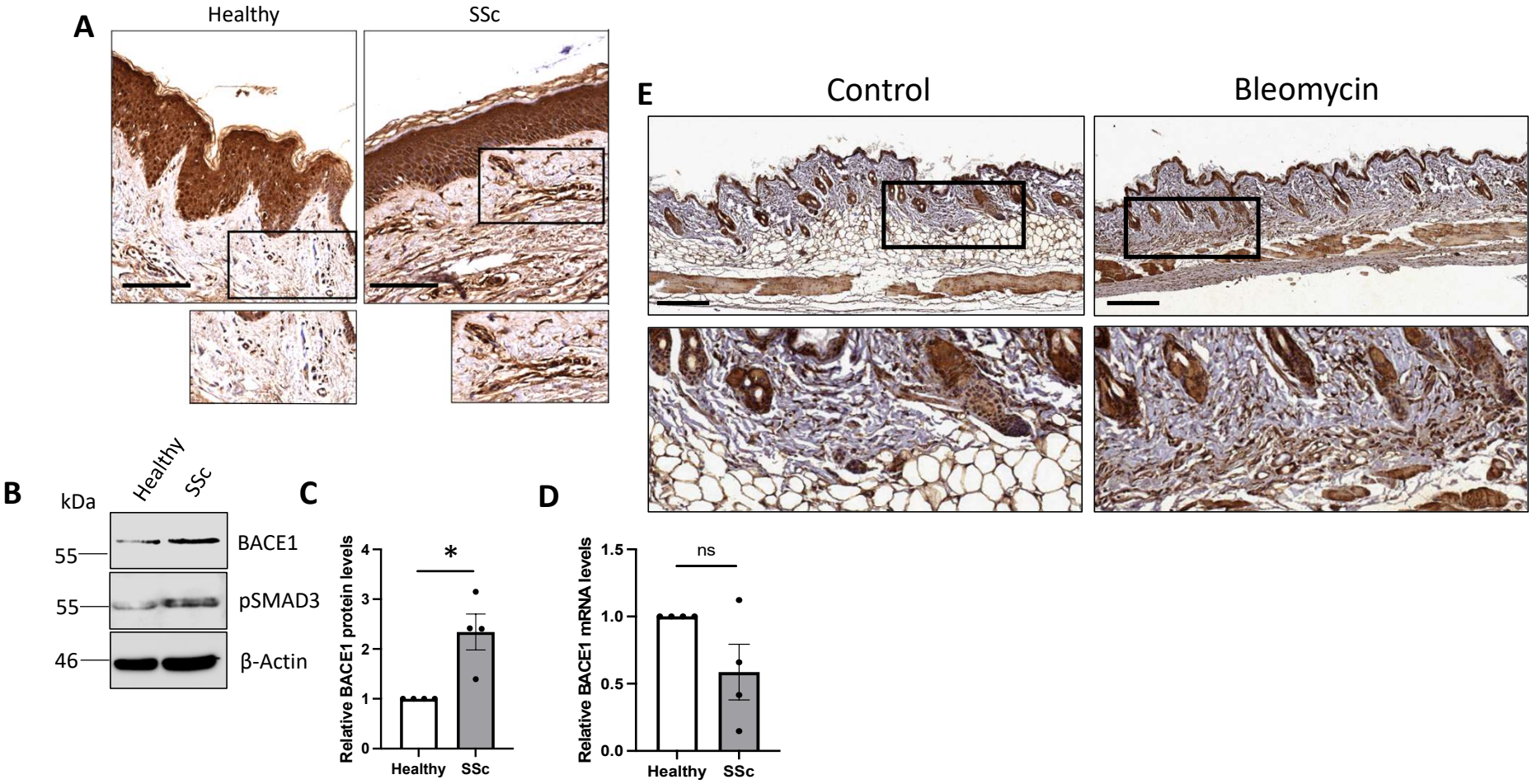


Figure 2: Modulation of BACE1 affects SSc fibroblast activation

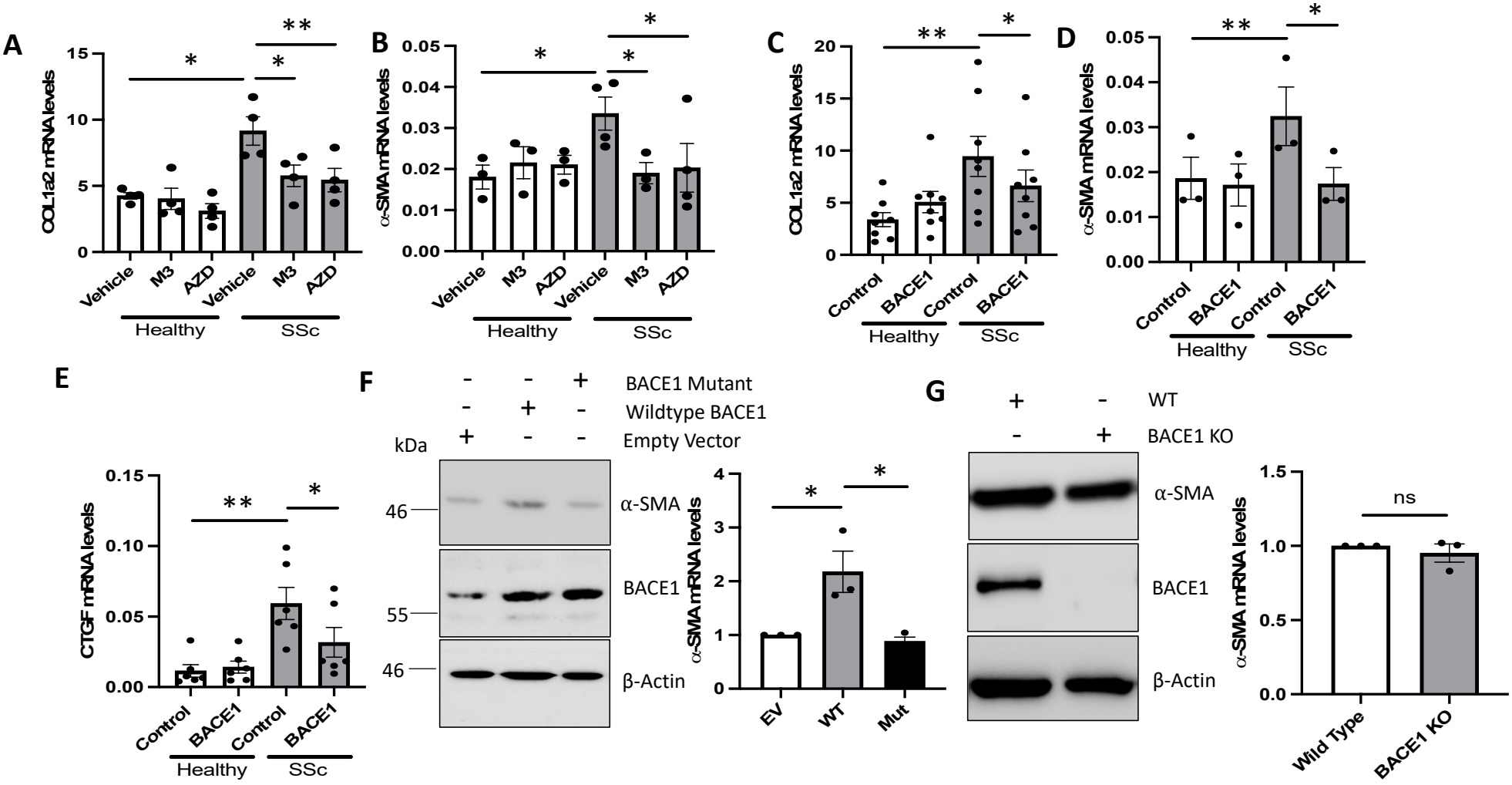


Figure 3: BACE1 plays an important role in Morphogen mediated fibroblast activation

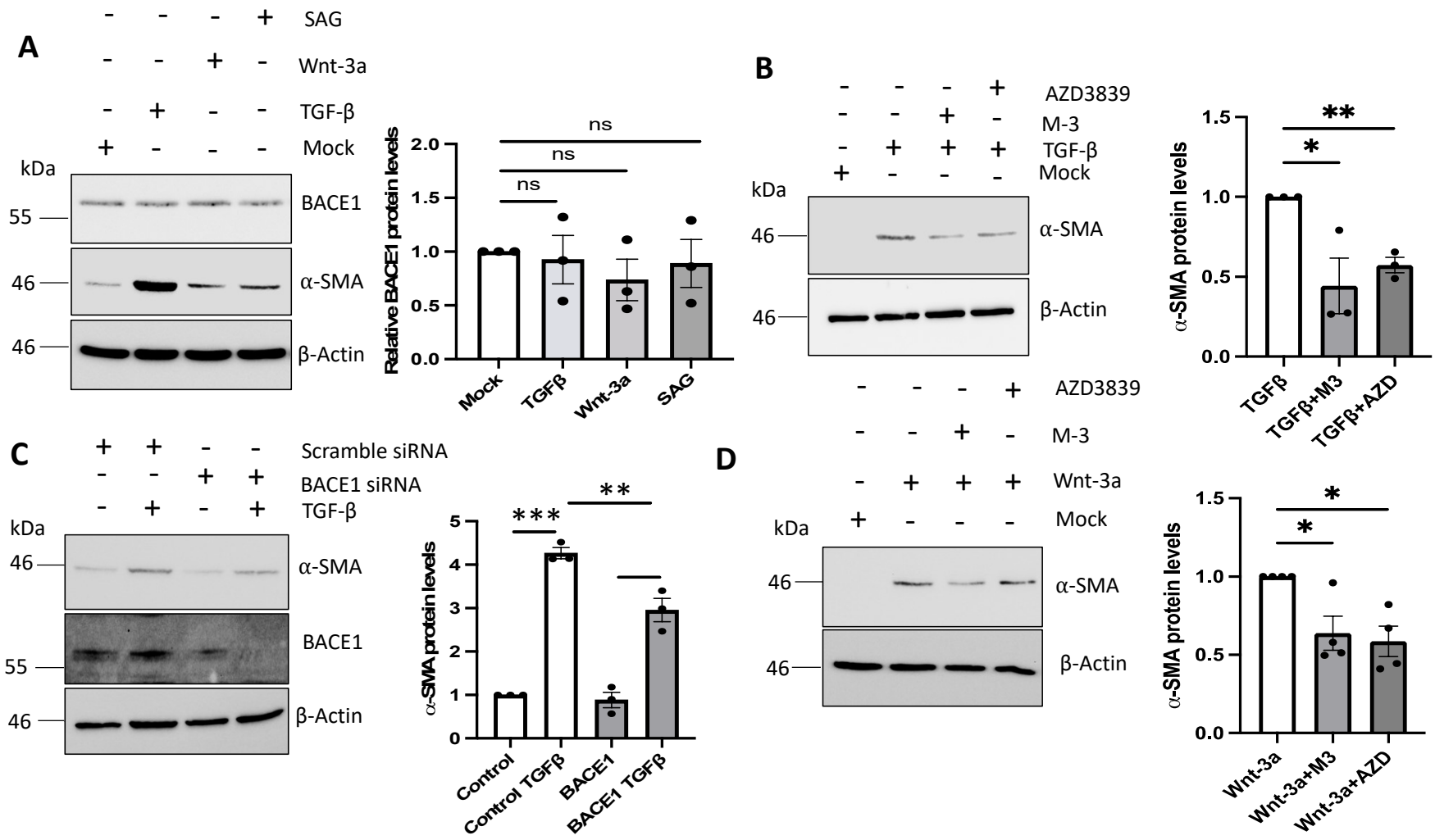


Figure 3 (Cont): BACE1 plays an important role in Morphogen mediated fibroblast activation

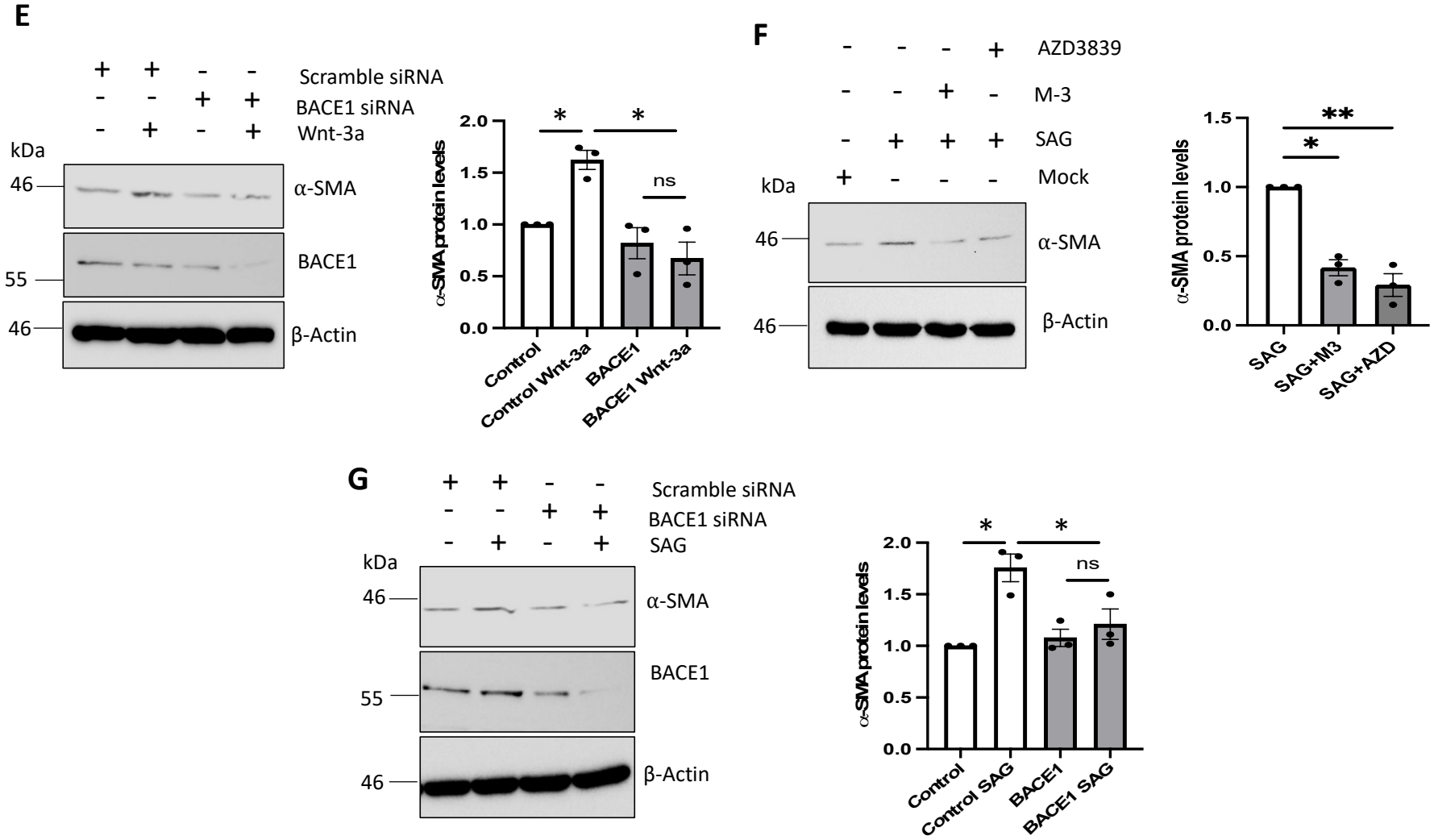


Figure 4: BACE1 regulates β-catenin expression in the presence and absence of Wnt ligands

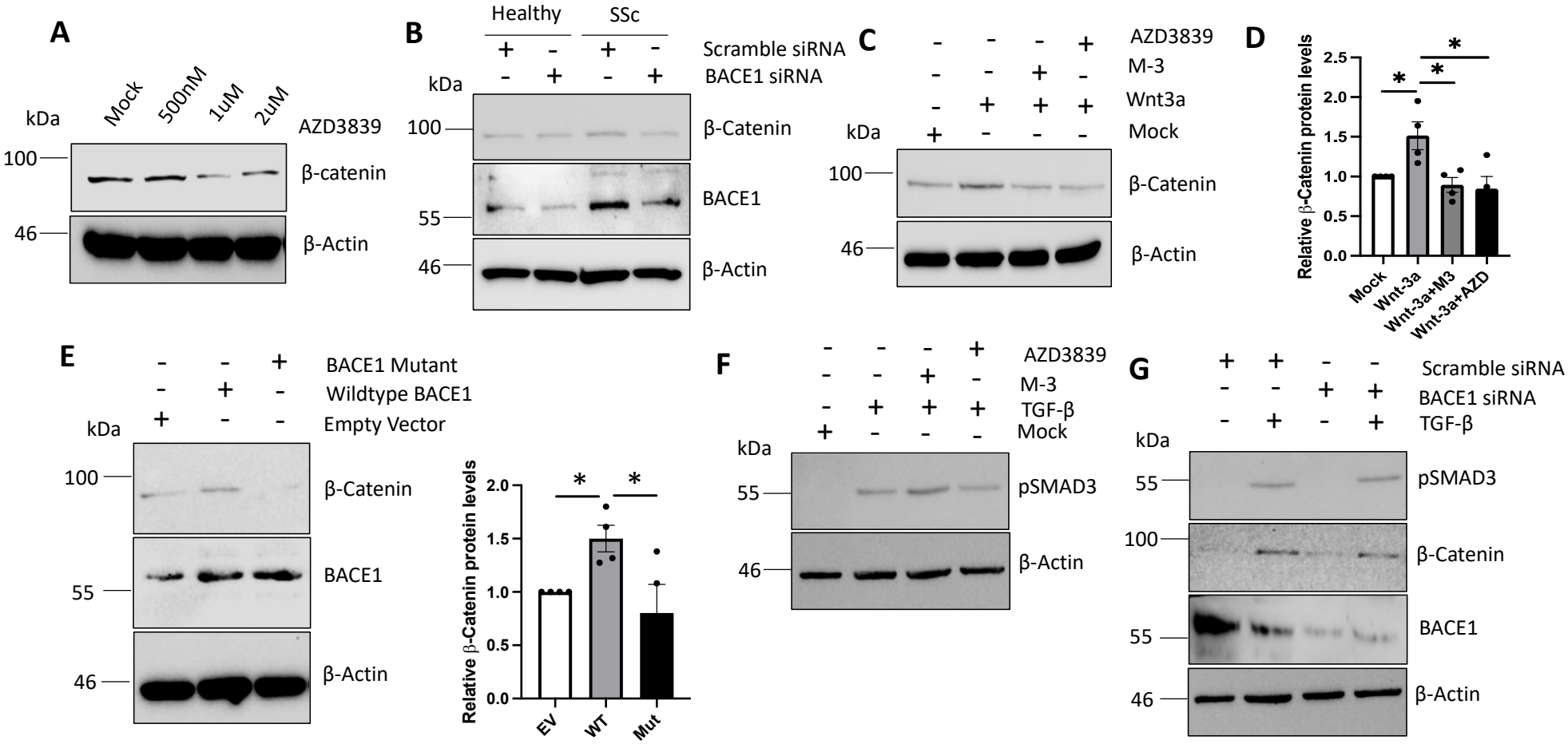


Figure 5: Inhibition of BACE1 leads to reduced Notch signaling in SSc fibroblasts

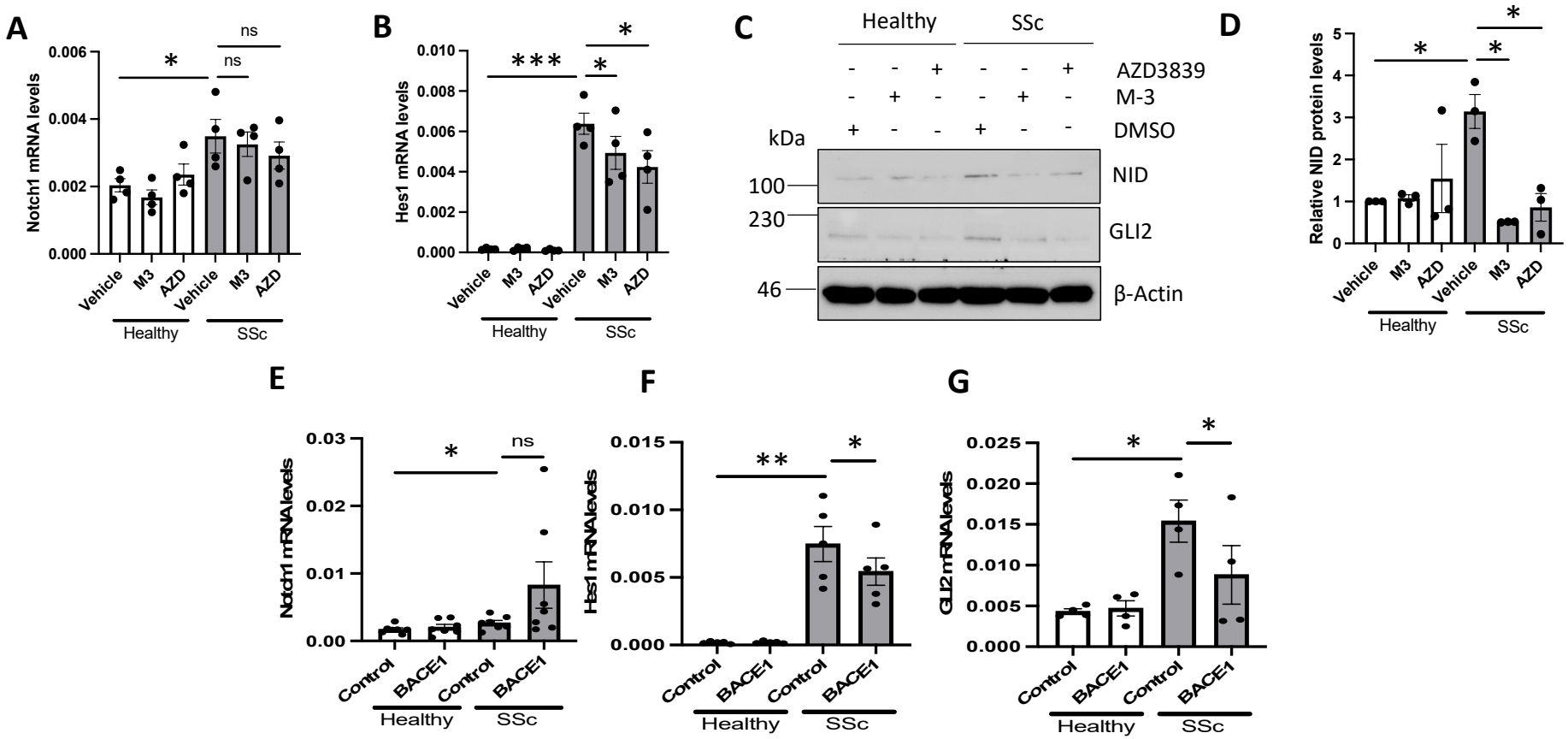


Figure 5 (Cont): Inhibition of BACE1 leads to reduced Notch signaling in SSc fibroblasts

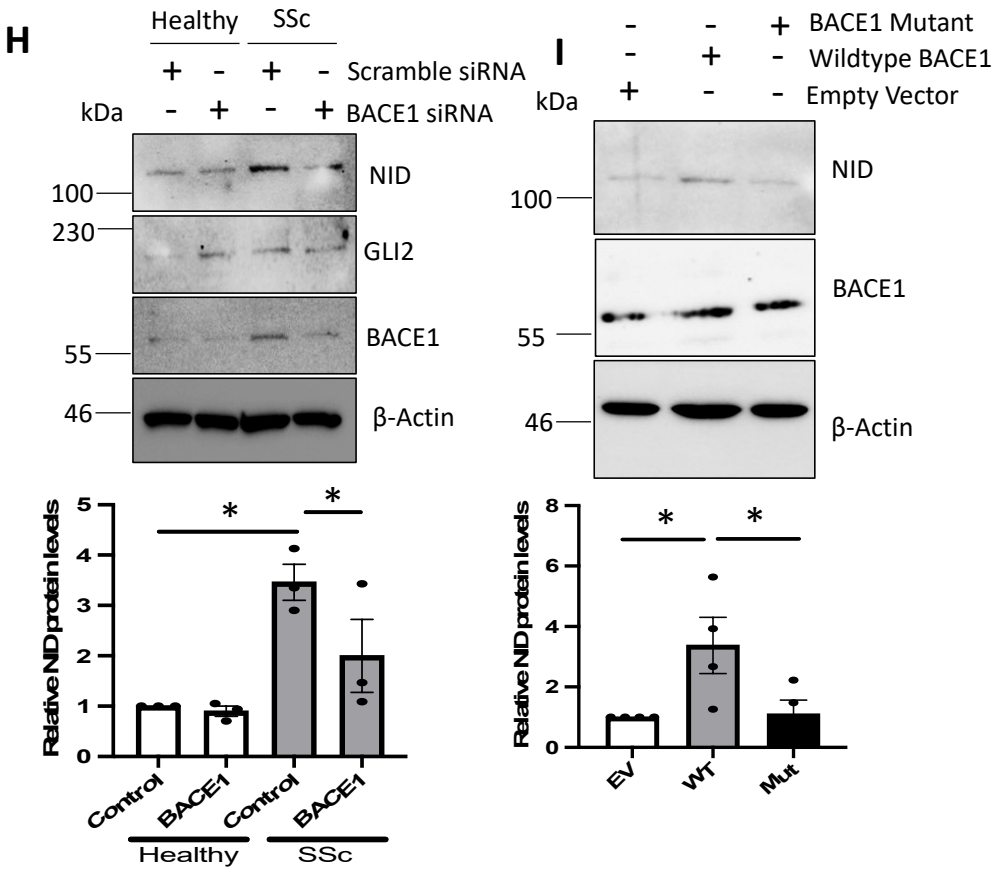


Figure 6: Inhibition of BACE1 prevents morphogen mediated Notch signaling in dermal fibroblasts

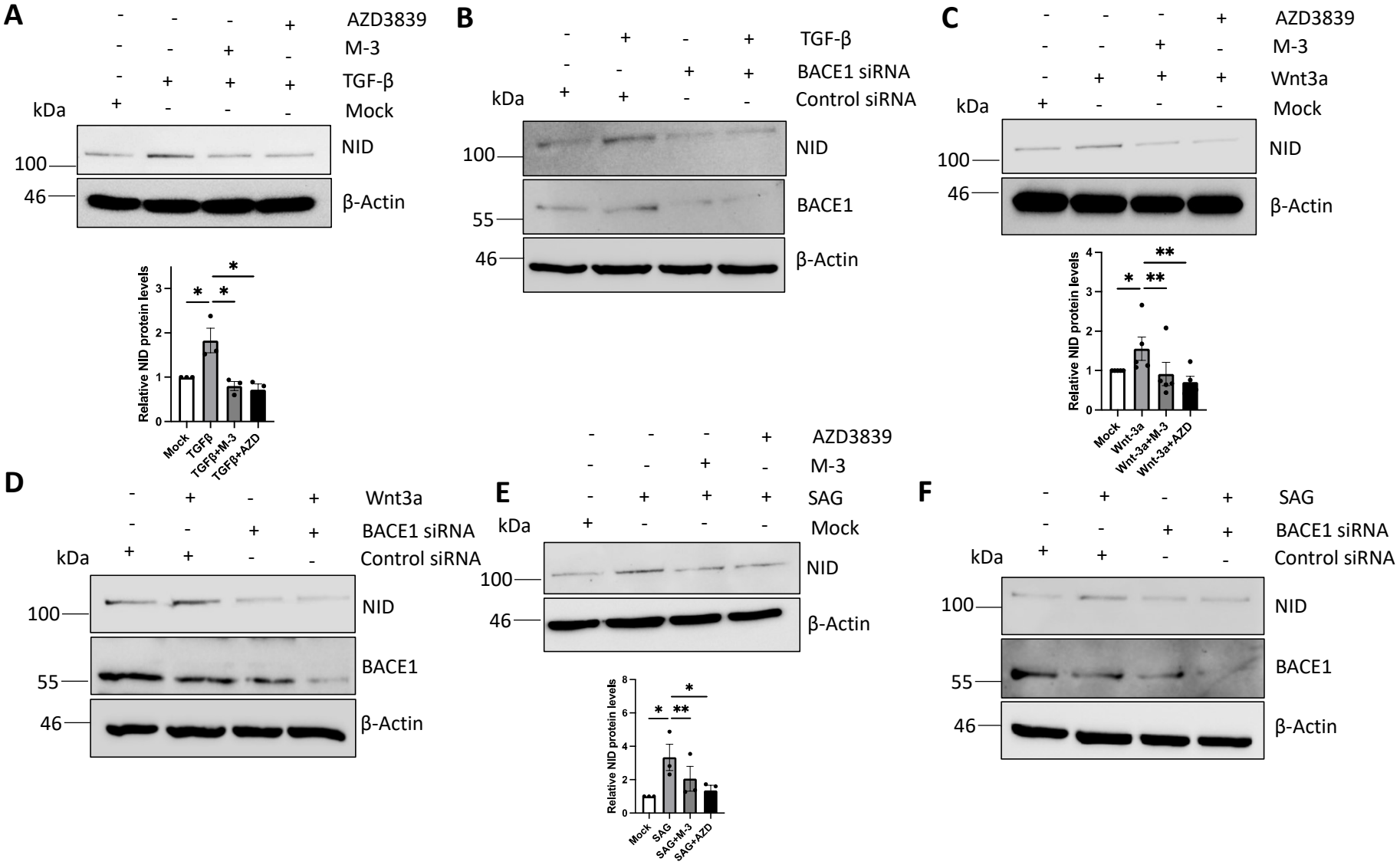
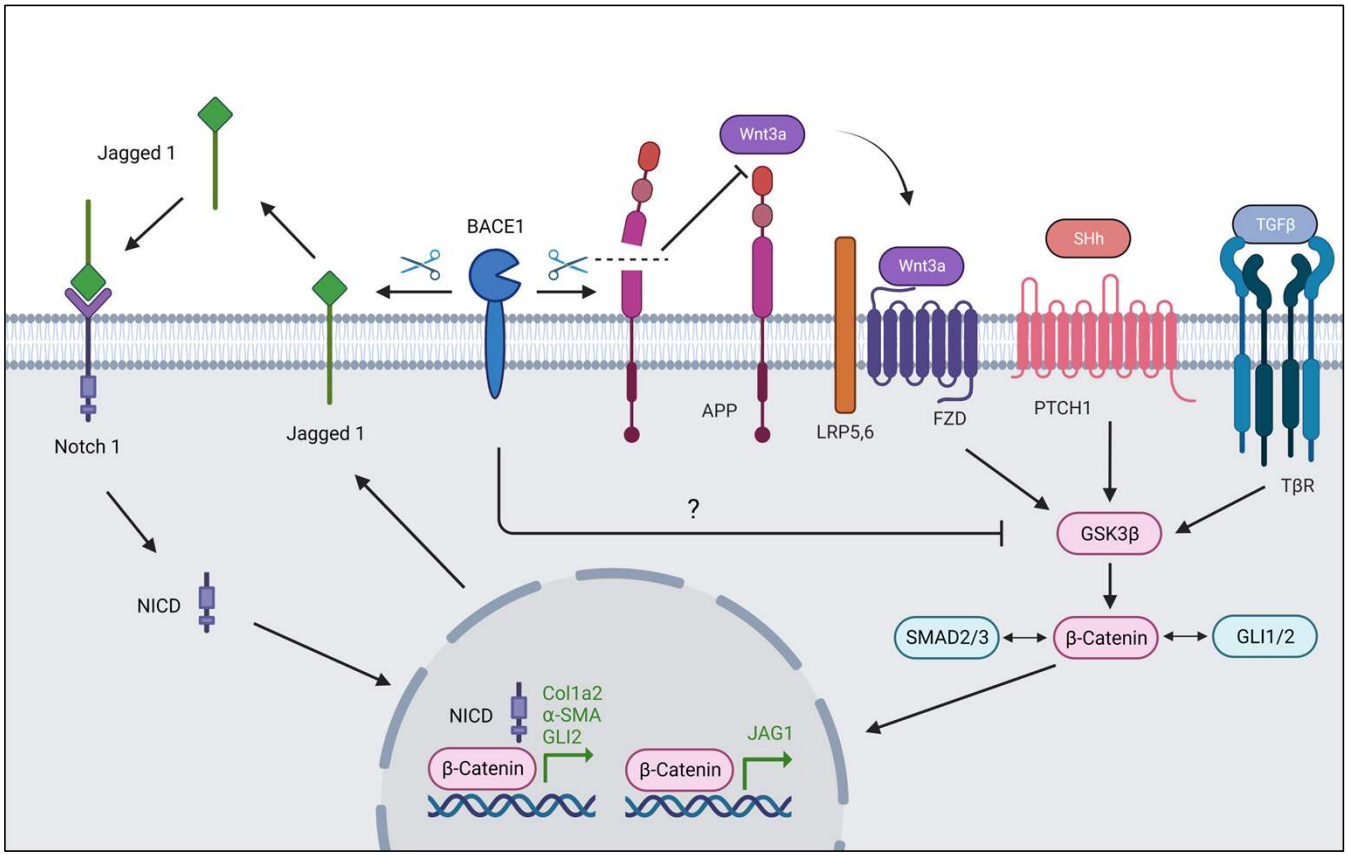
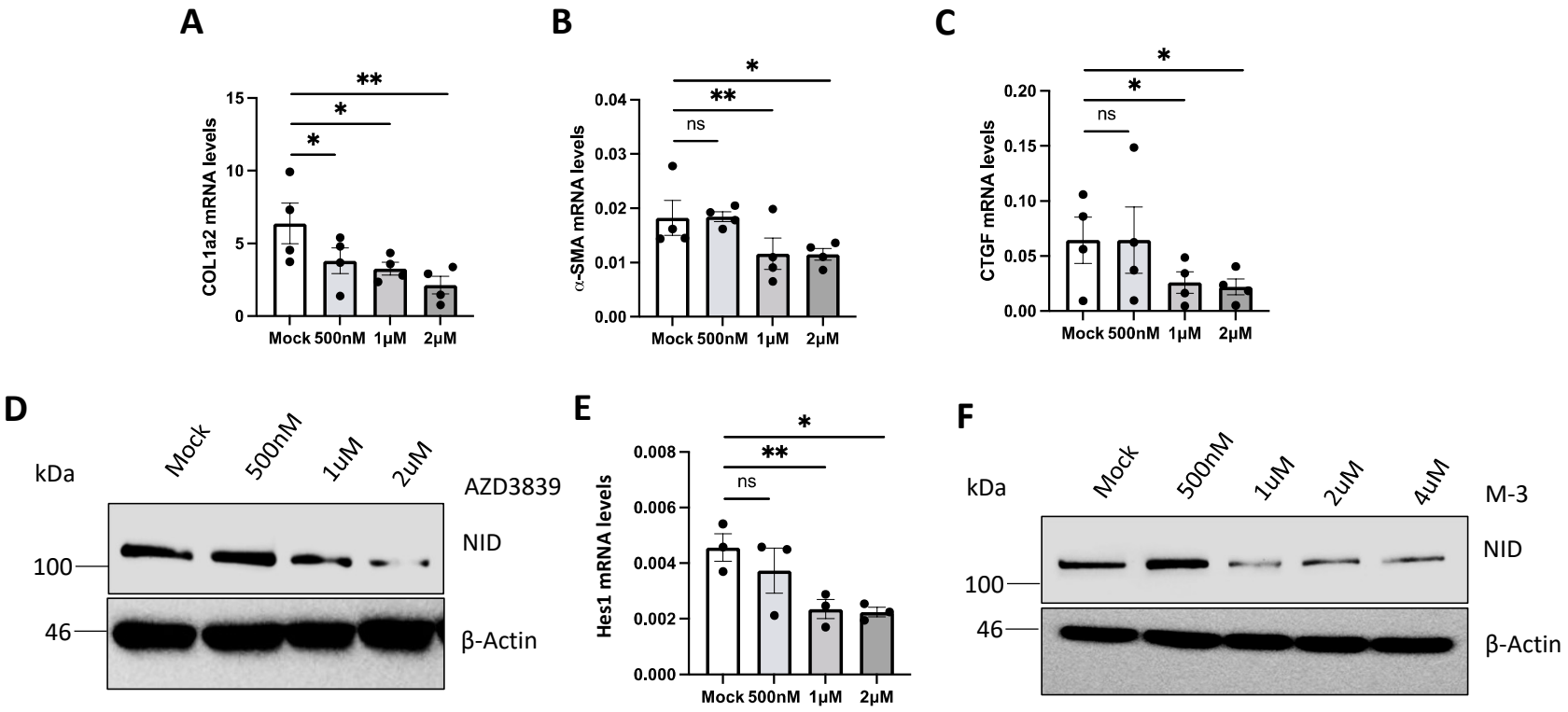


Figure 7: BACE1 regulates SSc fibroblast activation through a β-catenin/Notch axis



Supplementary Figure 1: The BACE1 inhibitors block SSc fibroblast activation in a dose dependent manner