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ORIGINAL ARTICLE

Glutamine deficiency drives transforming growth factor-β signaling activation that gives rise to myofibroblastic carcinoma-associated fibroblasts

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Abstract

Tumor-promoting carcinoma-associated fibroblasts (CAFs), abundant in the mammary tumor microenvironment (TME), maintain transforming growth factor-β (TGF-β)-Smad2/3 signaling activation and the myofibroblastic state, the hallmark of activated fibroblasts. How myofibroblastic CAFs (myCAFs) arise in the TME and which epigenetic and metabolic alterations underlie activated fibroblastic phenotypes remain, however, poorly understood. We herein show global histone deacetylation in myCAFs present in tumors to be significantly associated with poorer outcomes in breast cancer patients. As the TME is subject to glutamine (Gln) deficiency, human mammary fibroblasts (HMFs) were cultured in Gln-starved medium. Global histone deacetylation and TGF-β-Smad2/3 signaling activation are induced in these cells, largely mediated by class I histone deacetylase (HDAC) activity. Additionally, mechanistic/mammalian target of rapamycin complex 1 (mTORC1) signaling is attenuated in Gln-starved HMFs, and mTORC1 inhibition in Gln-supplemented HMFs with rapamycin treatment boosts TGF-β-Smad2/3 signaling activation. These data indicate that mTORC1 suppression mediates TGF-β-Smad2/3 signaling activation in Gln-starved HMFs. Global histone

Abbreviations: acetyl-CoA, acetyl coenzyme-A; acH3/4, acetyl-histone H3/4; BAMBI, BMP and activin membrane-bound inhibitor homolog; CAF, carcinoma-associated fibroblast; DCIS, ductal carcinoma in situ; Gln, glutamine; GSEA, Gene Set Enrichment Analysis; HDAC, histone deacetylase; HMF, human mammary fibroblast; mTORC1, mechanistic/mammalian target of rapamycin complex 1; myCAF, myofibroblastic CAF; NEDD4L, neural precursor cell expressed developmentally downregulated gene 4-like; PEG10, paternally expressed gene 10; pS6, phosphorylation of S6 at serine 235 and 236; pS6K, phosphorylation of p70 S6 kinase at threonine 389; S6K, S6 kinase; SAHA, suberoylanilide hydroxamic acid; Smad2, SMAD family member 2; TGF-β, transforming growth factor-β; TME, tumor microenvironment; TSA, trichostatin A; TβR-I, TGF-β receptor I; α-SMA, α-smooth muscle actin.

deacetylation, class I HDAC activation, and mTORC1 suppression are also observed in cultured human breast CAFs. Class I HDAC inhibition or mTORC1 activation by high-dose Gln supplementation significantly attenuates TGF-β-Smad2/3 signaling and the myofibroblastic state in these cells. These data indicate class I HDAC activation and mTORC1 suppression to be required for maintenance of myCAF traits. Taken together, these findings indicate that Gln starvation triggers TGF-β signaling activation in HMFs through class I HDAC activity and mTORC1 suppression, presumably inducing myCAF conversion.

KEYWORDS
class I HDAC, global histone deacetylation, glutamine starvation, mTORC1, myofibroblastic carcinoma-associated fibroblast, TGF-β/SMAD

1 | INTRODUCTION

Carcinoma cells interact with various types of stromal cells to generate the tumor microenvironment (TME), which in turn influences tumor hallmarks, such as tumor growth and progression.[1–4] Carcinoma-associated fibroblasts (CAFs), abundant in the TME, are common in several human malignancies. Various cells of origin and different stimuli from the TME result in the generation of distinct fibroblast subpopulations, mainly α-smooth muscle actin (α-SMA)-positive myofibroblastic CAF (myCAFs), inflammatory CAFs, and antigen-presenting CAFs,[5–8] although precise cause of the CAF subpopulation induction remains poorly understood.

We previously obtained evidence that resident human mammary fibroblast (HMFs) convert to myCAFs through interaction with intratumoral breast carcinoma cells. During tumor progression the establishment of self-stimulating autocrine TGF-β-Smad2/3 signaling mediates the myofibroblastic state and tumor-promoting ability of these fibroblasts.[9] Activated tumor-promoting traits in CAFs occur in part through epigenetic dysregulation of particular gene expressions attributable to alterations in DNA methylation, histone methylation, and/or chromatin remodeling.[10–14] Treatments with class I and II histone deacetylase (HDAC) inhibitors exert antifibrotic effects by attenuating TGF-β signaling and ECM production in cultured myofibroblasts and human breast CAFs.[15–18]

Acetylation of lysine on histones H3 and H4 is associated with transcriptional activation.[19] Histone acetylation turnover is dynamically balanced through reciprocal reactions catalyzed by histone acetyl transferases and HDACs. Histone acetylation is also influenced by intracellular glutamine (Gln) and glucose that fuel citrate of the tricarboxylic acid cycle to generate acetyl-CoA, an acetyl group donor for histone acetylation.[20–22]

Uptake of Gln is essential for tumor cell growth[23–25] but tumors often show inefficient diffusion of nutrients and oxygen from the circulation due to leaky and collapsed tumor vasculatures,[26] resulting in Gln shortage in the TME.[27,28] Glutamine deficiency also suppresses activity of mechanistic/mammalian target of rapamycin complex 1 (mTORC1), the evolutionarily conserved pathway for sensing growth factors and nutrients,[29,30] modulating metabolism in various cancer cells for their growth and proliferation.[31]

However, it is yet to be elucidated whether Gln deprivation in the TME influences mTORC1 and HDAC activities in HMFs, and its involvement in TGF-β signaling and the myCAF state during tumor progression remains to be determined. We sought to clarify roles of Gln deprivation regulating HDAC and mTORC1 activities in HMFs relevant to their phenotypic conversion to myCAFs.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Cell lines including HMFs, control fibroblasts, and CAFs used in this study were already established in our previous research.[9] In brief, primary HMFs were extracted from a healthy human breast tissue sample, prior to introduction of the retroviral pMIG (MSCV-IRES-GFP) vector expressing both human telomerase reverse transcriptase and GFP to facilitate their immortalization, and a pBabe-puro vector encoding a puromycin resistance gene.[9]

Materials and methods including cell culture, animal experiments, immunohistochemistry, tissue microarray, immunoblotting, and statistical analysis are described in Data S1.

3 | RESULTS

3.1 | Global histone deacetylation in myCAFs present in tumor stroma of breast cancer patients

The TME often lacks Gln, essential for generating acetyl-CoA: an acetyl group donor for histone acetylation.[20,23,27,28] We thus investigated the global histone acetylation state on tumor sections prepared from a breast cancer patient by immunohistochemistry using antibodies for acetyl-histone H3 (acH3) and acH4, global histone

acetylation markers. Carcinoma cells showed weaker acH3 and acH4 staining than normal epithelial cells in noncancerous regions. Previous reports consistently reported global histone deacetylation in cancer cells of human breast carcinomas.[32,33]

We also observed very slight or absent acH3 and acH4 staining in stromal fibroblasts in tumor regions (Figure 1A), in contrast to the strong staining in those in noncancerous regions of breast tissue from the same individual. The decreased $acH3^+$ and $acH4^+$ fibroblast proportions were also observed in tumor-associated stroma from a total of 10 breast cancer patients (Figure 1B). Immunofluorescence using anti-acH4 and anti-α-SMA antibodies indicated an abundance of $acH4^-\alpha\text{-}SMA^+$ myCAFs in human breast cancerous regions, in sharp contrast to the $acH4^+\alpha\text{-}SMA^-$ stromal fibroblasts in noncancerous regions (Figure 1C).

We also observed lower proportions of $acH3^+$ and $acH4^+$ fibroblasts in the tumor-associated stroma in two different murine breast cancer models: tumor xenografts from human breast ductal carcinoma MCF10DCIS.com (DCIS) cells[34] injected orthotopically into immunodeficient mice (Figure 1D,E) and spontaneous tumors generated in mouse mammary tumor virus-polyoma-middle tumor antigen (MMTV-PyMT) transgenic mice (Figure 1F,G). Collectively, these findings indicate global histone deacetylation in CAFs of both human and murine breast tumors.

To further examine the potential clinical relevance of this observation, we used anti-acH4 antibody for immunohistochemistry in another cohort including 231 breast cancer patients. The acH4 staining was positive in tumor-associated stroma in 138 of the 231 tumors (59.7%), while being negative in 93 (40.3%). The negative stromal acH4 staining was associated with poorer outcomes by Kaplan–Meier analysis and univariate Cox regression analysis (Figure 1H and Table 1). Multivariate Cox regression analysis also revealed negative stromal acH4 staining to be an independent prognostic factor, but there were no correlations with any clinical parameters in breast cancer patients (Tables 1 and 2).

These observations suggest global histone deacetylation in CAFs in the TME to be a poor prognostic marker in human breast carcinoma patients.

3.2 | Global histone deacetylation and TGF-β-Smad2/3 signaling activation are induced in Gln-starved HMFs in a class I HDAC-dependent fashion

As global histone deacetylation was seen in myCAFs present in breast tumors susceptible to Gln deficiency (Figure 1C), we investigated whether global histone deacetylation is also triggered in HMFs cultured in Gln-starved medium. Expression levels of acetylated H3 and H4 relative to their total proteins (acH3/H3 and acH4/H4 ratios, indicative of global histone acetylation) were indeed attenuated in Gln-starved HMFs by 65.7% and 53.8%, respectively, as compared to those in Gln-supplemented fibroblasts (Figure 2A).

Given that global histone deacetylation is shared between Gln-starved fibroblasts in culture and myCAFs present in breast carcinomas, we reasoned that Gln starvation could trigger TGF-β signaling activation, a hallmark of myCAFs. As anticipated, the ratio of phosphorylated Smad2 relative to Smad2/3 protein (pSmad2/Smad2/3, indicative of canonical TGF-β signaling), was upregulated in Gln-starved HMFs by 3.6-fold as compared to that in Gln-supplemented fibroblasts (Figure 2B). *TGFB1* and *TGFB2*, and TGF-β-target genes such as *SERPIN1* and *SMAD7* but not *ACTA2*, the gene encoding α-SMA protein, were also significantly upregulated at the mRNA level (Figures 2C and S1A).

Next, to address whether TGF-β signaling relies on HDAC activity in Gln-starved HMFs, these fibroblasts were treated with different concentrations of a class I and class II HDAC inhibitor, trichostatin A (TSA). The acH3/H3 and acH4/H4 ratios were dramatically upregulated while the pSmad2/Smad2/3 ratio and TGF-β-target gene expressions were dose-dependently downregulated, as compared to control DMSO treatment (Figures 2D and S1B). Another class I and class II HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), and a selective inhibitor for class I HDACs, entinostat,[35] also strongly inhibited elevations of the pSmad2/Smad2/3 ratio in Gln-starved HMFs (Figure 2E). These observations indicate that Gln deficiency induces global histone deacetylation and TGF-β signaling activation in HMFs, mediated by class I HDAC activity.

3.3 | Roles of mTORC1 suppression on TGF-β signaling in Gln-starved HMFs

We anticipated that TGF-β signaling activation in Gln-starved HMFs is due to their increased TGF-β production that can act in an autocrine fashion, although active TGF-β production was undetectable in conditioned medium harvested from these cells (Figure S1C). We then checked mTORC1 signaling, an evolutionarily conserved sensing pathway for amino acids including Gln,[29,30] employing immunoblotting using an antibody for phosphorylation of p70 S6 kinase at the threonine 389 (pS6K), a target site of mTORC1. The pS6K/S6K ratio, indicative of mTORC1 signaling activity was indeed strongly attenuated in Gln-starved HMFs (Figure 3A). Inhibition of mTORC1 signaling in Gln-supplemented HMFs by rapamycin also increased the pSmad2/Smad2/3 ratio by 2.6-fold, but not α-SMA expression, as compared to DMSO treatment (Figure 3A,B). These data indicate that mTORC1 suppression mediates TGF-β-Smad2/3 signaling activation in Gln-starved HMFs.

To further investigate the link among Gln, mTORC1, and TGF-β signaling, Gln-starved HMFs were supplemented with 4 mM Gln leading to mTORC1 activation, as indicated by the increased pS6K/S6K ratio (Figure 3C,D). As anticipated, TGF-β-Smad2/3 signaling was strongly attenuated in these fibroblasts (Figure 3C,E). Importantly, mTORC1 suppression by rapamycin restored the decreased TGF-β-Smad2/3 signaling by 42.3% (Figure 3C–E), further indicating

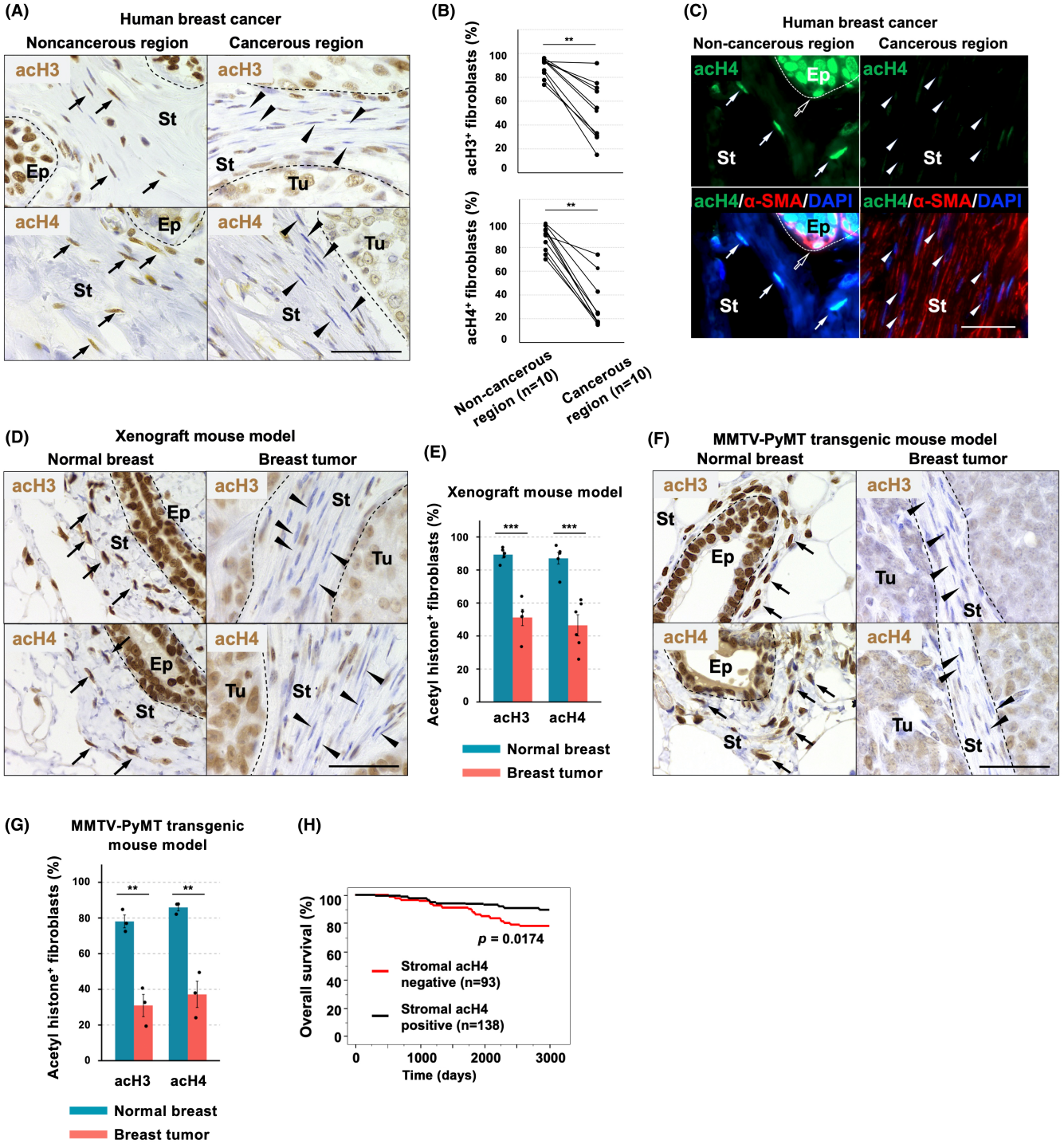

FIGURE 1 Global histone deacetylation in myofibroblastic carcinoma-associated fibroblasts (myCAFs) of mammary tumors associated with poorer outcomes in breast cancer patients. (A) Immunohistochemistry of sections prepared from human breast cancer tissues using anti-acetyl-histone H3 (acH3) and -acH4 antibodies. acH3$^+$ and acH4$^+$ fibroblasts (arrows) as well as acH3$^-$ and acH4$^-$ CAFs (arrowheads) are present in noncancerous and cancerous regions, respectively. (B) Quantification of acH3$^+$ and acH4$^+$ fibroblasts in noncancerous and cancerous regions in tumor-associated stroma in specimens from 10 human breast cancer patients. $^{**}p < 0.01$ by paired *t*-test. (C) Double immunofluorescence for sections prepared from human breast cancer tissues using anti-acH4 and -α-smooth muscle actin (α-SMA) antibodies. acH4$^+$α-SMA$^-$ fibroblasts (closed arrows) and acH4$^-$α-SMA$^+$ CAFs (arrowheads) are present in the stroma. α-SMA$^+$ myoepithelial cells (open arrow) can be seen in the epithelium. (D–G) Immunohistochemistry of sections prepared from tumor xenografts developed by human breast cancer DCIS cells injected orthotopically into NOG mice (D) or autochthonous tumors developed in mammary glands of MMTV-PyMT mice (F). acH3$^+$ and acH4$^+$ fibroblast-like cell proportions (%) are quantified in breast tumors and normal breast tissues (E, G). $^{**}p < 0.01$, $^{***}p < 0.001$ by Student's *t*-test ($n = 5$–6, E; $n = 3$, G). Error bars indicate SE. Dots indicate tumors or mammary fat pads (E, G). (H) Kaplan–Meier plot indicating overall survival of breast cancer patients. Patients are grouped according to the indicated stromal acH4 staining in breast carcinoma specimens. *p* value was determined by applying log-rank test. Scale bars, 40 μm (A, D, F) and 30 μm (C). Ep, epithelium; St, stroma; Tu, tumor (A, C, D, F).

mTORC1 suppression to be required for induction of TGF-β-Smad2/3 signaling in Gln-starved HMFs.

We next explored cross-talk between class I HDAC activity and mTORC1 signaling following TGF-β signaling activation in Gln-starved HMFs. Rapamycin treatment failed to influence the global histone acetylation state in Gln-supplemented HMFs (Figure 3F,G). Treatment with TSA did not raise mTORC1 suppression in Gln-starved HMFs to mediate the TGF-β-Smad2/3 signaling suppression (Figure 3H). These observations indicate little cross-talk between HDAC activity and mTORC1 signaling in these cells.

Collectively, these data suggest that class I HDAC activity and mTORC1 suppression independently contribute to TGF-β-Smad2/3 signaling activation in Gln-starved HMFs.

3.4 | Histone deacetylase activity is required for TGF-β signaling activation and myofibroblastic state in human breast CAFs

We next investigated whether global histone acetylation is also attenuated in cultured human breast CAFs by using several human breast CAF lines (designated exp-CAFs), as exemplified by TGF-β signaling activation and the myofibroblastic state.[9] As anticipated, lower acH3/H3 and acH4/H4 ratios were observed in all of the different exp-CAFs (544, 542M, and 546M) as compared to those in the control fibroblast line (522) and the parental HMF line (218TGpp) (Figures 4A and S2A,B). The exception was another control fibroblast line (533) whose ability to affect tumor growth had been poorly examined (Figure S2A,B). For the following experiments, we thus used well-characterized fibroblast lines from our previous in vitro and in vivo studies[9,36]: tumor-promoting exp-CAF, 544 (designated exp-CAF2 cells) and a scarcely tumor-promoting control fibroblast line, 522 (designated control fibroblasts).

Given global histone deacetylation in the different exp-CAFs (Figures 4A and S2A,B), we speculated that HDAC activity mediates TGF-β signaling activation in these cells. The TSA and SAHA treatment indeed upregulated global histone acetylation (Figures 4B and S3A) and progressively attenuated the pSmad2/Smad2/3 ratio in exp-CAF2 cells by 58.0% (at 12h) and 61.7% (at 18h), respectively, as compared to DMSO treatment (Figures 4C and S3B). *ACTA2* mRNA and α-SMA protein expressions were also substantially and dose-dependently decreased in exp-CAF2 cells treated with different concentrations of TSA (Figure 4D,E). The GSEA revealed greater enrichment of TGF-β-target genes in exp-CAF2 cells relative to control fibroblasts (Figures 4F and S3C). Treatment of exp-CAF2 cells with TSA or SB431542, a TGF-β receptor I (TβR-I) inhibitor, attenuated the markedly increased TGF-β target gene enrichment, indicating HDAC activity and TβR-I to mediate TGF-β signaling in these fibroblasts (Figures 4F and S3C). These experiments, taken together, indicate that HDAC activity is required for maintenance of TGF-β-Smad2/3 signaling activation and the myofibroblastic state in myCAFs.

We next addressed the molecular mechanisms underlying attenuated TGF-β signaling in TSA-treated CAFs. We found upregulated mRNA expressions and greater acetylated histone H3 lysine 27 (H3K27ac) enrichment at promoters in three TGF-β negative regulators, that is, *PEG10*,[37] *NEDD4L*,[38] and *BAMBI*,[39] in TSA-treated exp-CAF2 cells (Figure S4A–D). However, siRNAs that strongly inhibited PEG10 or NEDD4L protein expressions in these cells failed to significantly restore TGF-β signaling, as compared to the control siRNA (Figure S4E). Collectively, our observations indicate that these TGF-β negative regulators contribute minimally, if at all, to the attenuated TGF-β signaling in TSA-treated exp-CAF2 cells.

TABLE 1 Cox proportional hazards model analysis of stromal acetyl-histone H4 (acH4) staining and other prognostic factors in breast cancers

Variable	Hazard ratio	95% CI	Unfavorable/favorable	*p* value
Univariate analysis				
Stromal acH4 expression	2.24	1.131–4.435	Negative/positive	0.0207*
Age (years)	1.293	0.618–2.704	≥65/<65	0.4946
Grading	1.59	0.796–3.176	G2–3/G0–1	0.1887
Luminal type status	1.366	0.696–2.678	Others/luminal type	0.3647
HER2 status	2.109	0.955–4.66	HER2 type/others	0.0650
pT factor	3.656	1.415–9.449	T2–3/T1	0.0074*
pN factor	5.769	2.512–13.252	N1–2/N0	<0.0001*
Multivariate analysis				
Stromal acH4 expression	2.134	1.076–4.23	Negative/positive	0.0300*
pT factor	2.45	0.927–6.476	T2–3/T1	0.0709
pN factor	4.589	1.957–10.761	N1-2/N0	0.0005*

Note: Cox proportional hazards model analysis was carried out by using tissue microarray data from 231 breast cancer specimens.

Abbreviations: CI, confidence interval; HER2, human epidermal growth factor receptor type 2.

*$p < 0.05$ by Wald test.

Parameter		Total	Stromal acH4 expression		
			Positive	Absent	
		231	138	93	*p* value
Age (years)	<65	173	100	73	0.3541
	≥65	58	38	20	
Grading	G0–1	113	68	45	>0.9999
	G2–3	118	70	48	
pT factor	T1	86	50	36	0.7816
	T2–3	145	88	57	
pN factor	N0	133	82	51	0.5006
	N1–2	98	56	42	
Luminal type status	Luminal type	137	84	53	0.5866
	Others	94	54	40	
HER2 status	HER2 type	32	17	15	0.4412
	Others	199	121	78	

Note: Associations of stromal acH4 staining with clinical parameters were statistically evaluated by using tissue microarray data from 231 breast cancer specimens. *p* values were determined by Fisher's exact test.

TABLE 2 Association of stromal acetyl-histone H4 (acH4) staining with clinical parameters of 231 breast cancer patients

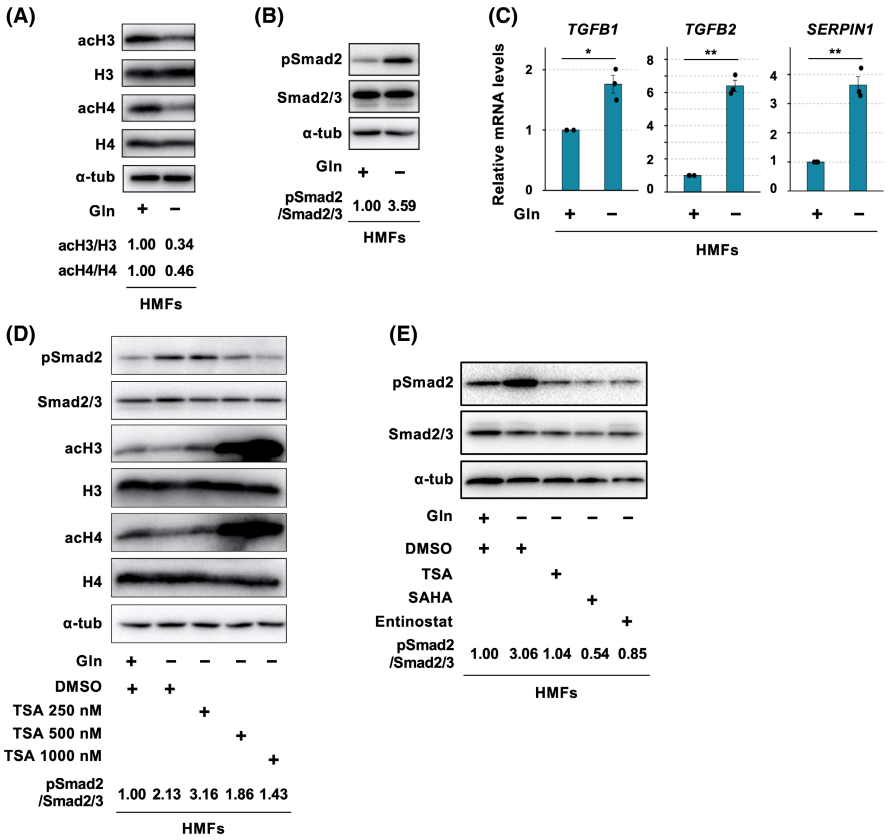

FIGURE 2 Glutamine (Gln) starvation induces global histone deacetylation and transforming growth factor-β (TGF-β)-Smad2/3 signaling activation in human mammary fibroblasts (HMFs) in a class I histone deacetylase-dependent fashion. (A–C) Immunoblotting (A, B) and real-time PCR (C) of HMFs cultured in medium with or without Gln for 24 h. $^{*}p<0.05$, $^{**}p<0.01$ by Welch's *t*-test ($n=3$, C). Error bars, SE. Dots indicate biological replicates (C). (D, E) Immunoblotting of HMFs cultured in medium with or without Gln for 24 h. Fibroblasts were simultaneously incubated with DMSO, trichostatin A (TSA; 1 μM), suberoylanilide hydroxamic acid (SAHA; 10 μM), or entinostat (10 μM) for 24 h. α-tub, α-tubulin.

3.5 | Histone deacetylase 1 is required for increased TGF-β-Smad2/3 signaling and myofibroblastic state in CAFs

As HDAC1 and HDAC2 are major isoforms for class I HDACs, their expressions were investigated in several exp-CAFs as well as control fibroblast lines. HDAC1 and HDAC2 expressions were similar among different fibroblasts (Figure S5A,B). However, GSEA analysis indicated HDAC1 and HDAC2 target genes to be enriched not only in exp-CAF2 cells, but also in tumor-associated stroma from laser microdissected human breast carcinoma specimens, available as public data (GSE14548)[40] (Figure 4G,H), indicating that HDAC1

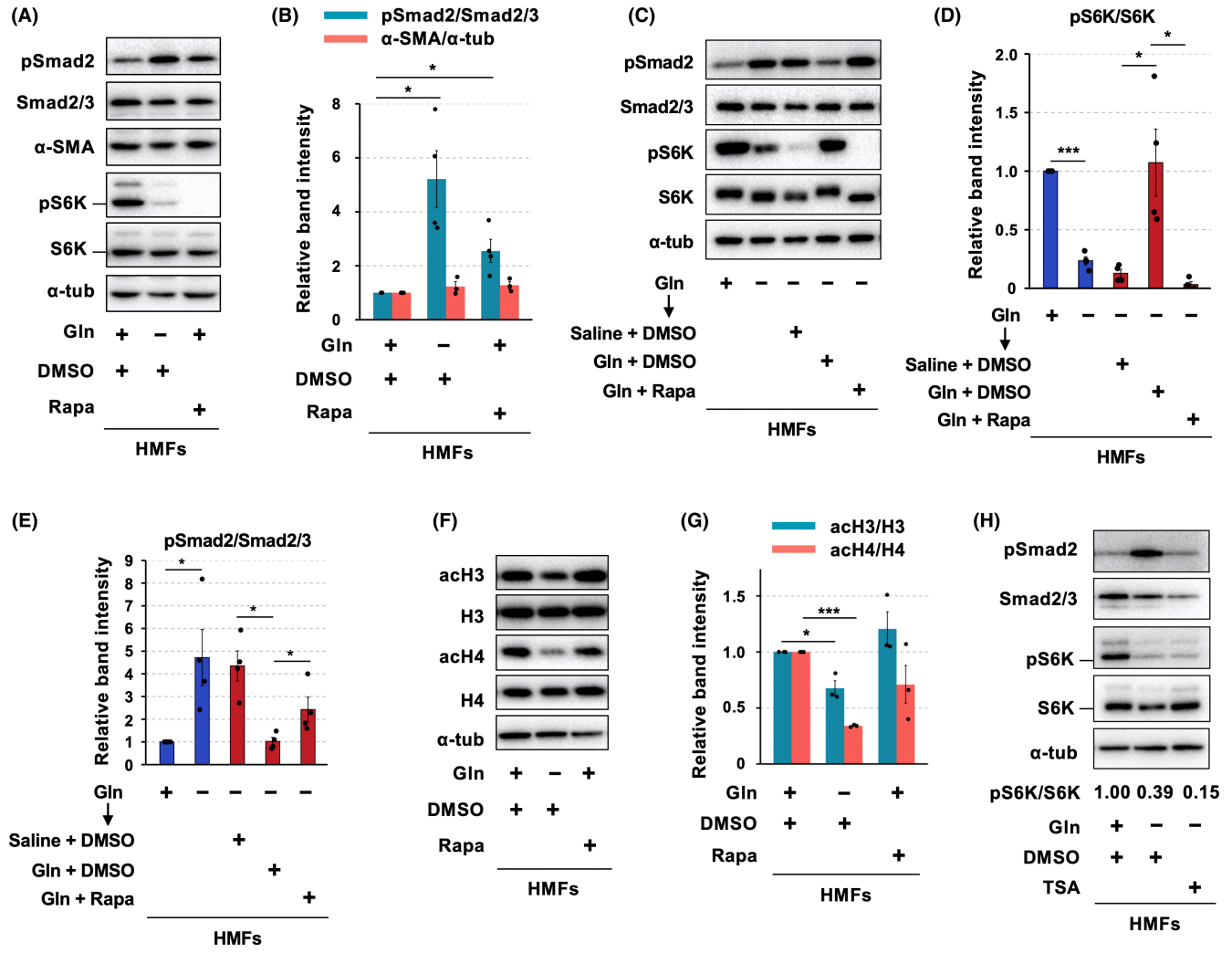

FIGURE 3 Glutamine (Gln) starvation induces TGF-β-Smad2/3 signaling activation in HMFs through mTORC1 suppression. (A, B) Immunoblotting of HMFs cultured in medium with or without Gln for 24 h. Fibroblasts were simultaneously incubated with DMSO or rapamycin (Rapa; 20 nM) for 24 h. *$p < 0.05$ by Welch's *t*-test (pSmad2, $n = 4$; α-SMA, $n = 3$; B). (C–E) HMFs cultured in medium with or without 4 mM Gln for 12 h (lanes 1 and 2). Gln-starved cells were subsequently treated with saline (lane 3) or 4 mM Gln with DMSO or rapamycin (25 nM) (lanes 4 and 5) for 12 h. Signal intensity ratios are indicated in (D) and (E). *$p < 0.05$, ***$p < 0.001$ by Student's *t*-test ($n = 4$). (F, G) Immunoblotting for the same samples as in (A, B). *$p < 0.05$, ***$p < 0.001$ by Welch's *t*-test (G, $n = 3$). (H) Immunoblotting of HMFs cultured in medium with or without Gln for 24 h. DMSO or TSA (1 μM) was simultaneously administered for 24 h. Horizontal lines indicate specific bands detecting phospho- or pan-p70 S6 kinase (S6K) (A, H). Error bars, SE (B, D, E, G). Dots indicate biological replicates (B, D, E, G).

and HDAC2 activities are elevated in CAFs present in human breast carcinomas.

As treatment with scriptaid, an HDAC1/3/8 inhibitor, reportedly inhibited α-SMA expression in human breast CAFs,[16] we prioritized determining the specific roles of HDAC1 in exp-CAF2 cells. Inhibition of HDAC1 expression by each of two shRNAs (shHDAC1, 2) produced substantial decreases in the pSmad2/Smad2/3 ratio, α-SMA protein, and *ACTA2* mRNA expressions (Figure 4I,J), while the acH3/H3 and acH4/H4 ratios tended to increase (Figure S5C,D), in exp-CAF2 cells. These data therefore indicate that HDAC1 expression is required for maintenance of TGF-β-Smad2/3 signaling activation in these fibroblasts.

We also noted a tendency for decreases in TGF-β1, 2, and 3 mRNA expression and active TGF-β protein in exp-CAF2 cells expressing shHDAC1-1 and -2 (Figure S5E,F), suggesting that HDAC1 expression mediates TGF-β signaling activation in exp-CAF2 cells, possibly through active TGF-β production.

To investigate the role of HDAC1 expression in the tumor-promoting ability of CAFs, we comixed DCIS cells with exp-CAF2 cells expressing shHDAC1-1, prior to subcutaneous injection into immunodeficient mice. Inhibition of stromal HDAC1 expression by shRNA reduced the weights of tumors (by 11.8%) as compared to the effect of control shGFP, but without statistical significance (Figure S5G). These data indicate that HDAC1 activation mediates activated TGF-β-Smad2/3 signaling and the myofibroblastic state while only minimally impacting tumor-promoting abilities in CAFs.

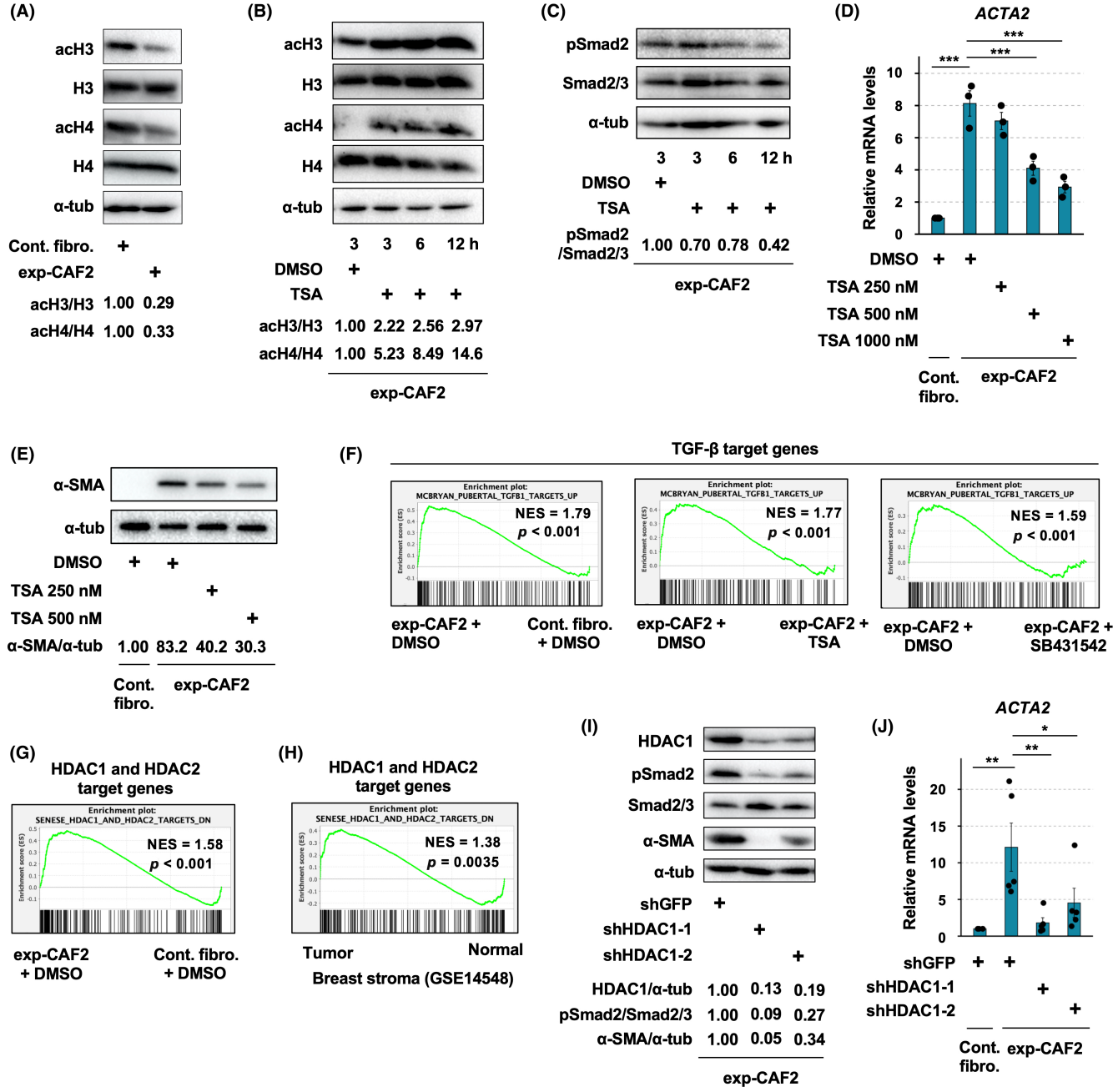

FIGURE 4 Histone deacetylase 1 (HDAC1) expression is required for increased TGF-β-Smad2/3 signaling and myofibroblastic characteristics in exp-CAF2 cells. (A) Immunoblotting of control fibroblasts (522; Cont. fibro.) and exp-CAF2 cells (544). (B, C) Immunoblotting of exp-CAF2 cells treated with DMSO and TSA (1 μM) for indicated times. (D) Real-time PCR of the indicated cells treated with DMSO or TSA for 24 h. ***$p < 0.001$ by Dunnett's test ($n = 3$). (E) Immunoblotting of control fibroblasts and exp-CAF2 cells treated with DMSO or TSA (250 and 500 nM) for 48 h. (F) Enrichment plots of Gene Set Enrichment Analysis (GSEA) showing significant enrichment of TGF-β downstream targets in DMSO-treated exp-CAF2 cells relative to DMSO-treated control fibroblasts (left), exp-CAF2 cells treated with TSA (1 μM, middle), or SB431542 (10 μM, right) for 24 h. (G, H) GSEA enrichment plots of gene sets regulated by HDAC1 and HDAC2 between the indicated cells (G) and between human mammary tumor-associated stroma and normal stroma (GSE14548) (H). *p* values were determined by applying permutation test (F–H). (I, J) Immunoblotting (I) and real-time PCR (J) of the described cells expressing GFP- and HDAC1-shRNAs. *$p < 0.05$, **$p < 0.01$ by Dunnett's test ($n = 5$). Error bars, SE (D, J). Dots indicate biological replicates (D, J). NES, normalized enrichment score.

3.6 | mTORC1 suppression is necessary for TGF-β signaling activation and myofibroblastic state in CAFs

We next determined whether mTORC1 signaling is attenuated in human breast CAFs by immunoblotting using antibodies for pS6K and/or phosphorylation of S6 at serine 235 and 236 (pS6), another marker for mTORC1 signaling activation. As anticipated, we found lower pS6K/S6K and pS6/S6 ratios in exp-CAF2 cells by 36% and 64%, respectively, compared with those in control fibroblasts (Figure 5A). Additionally, mTORC1

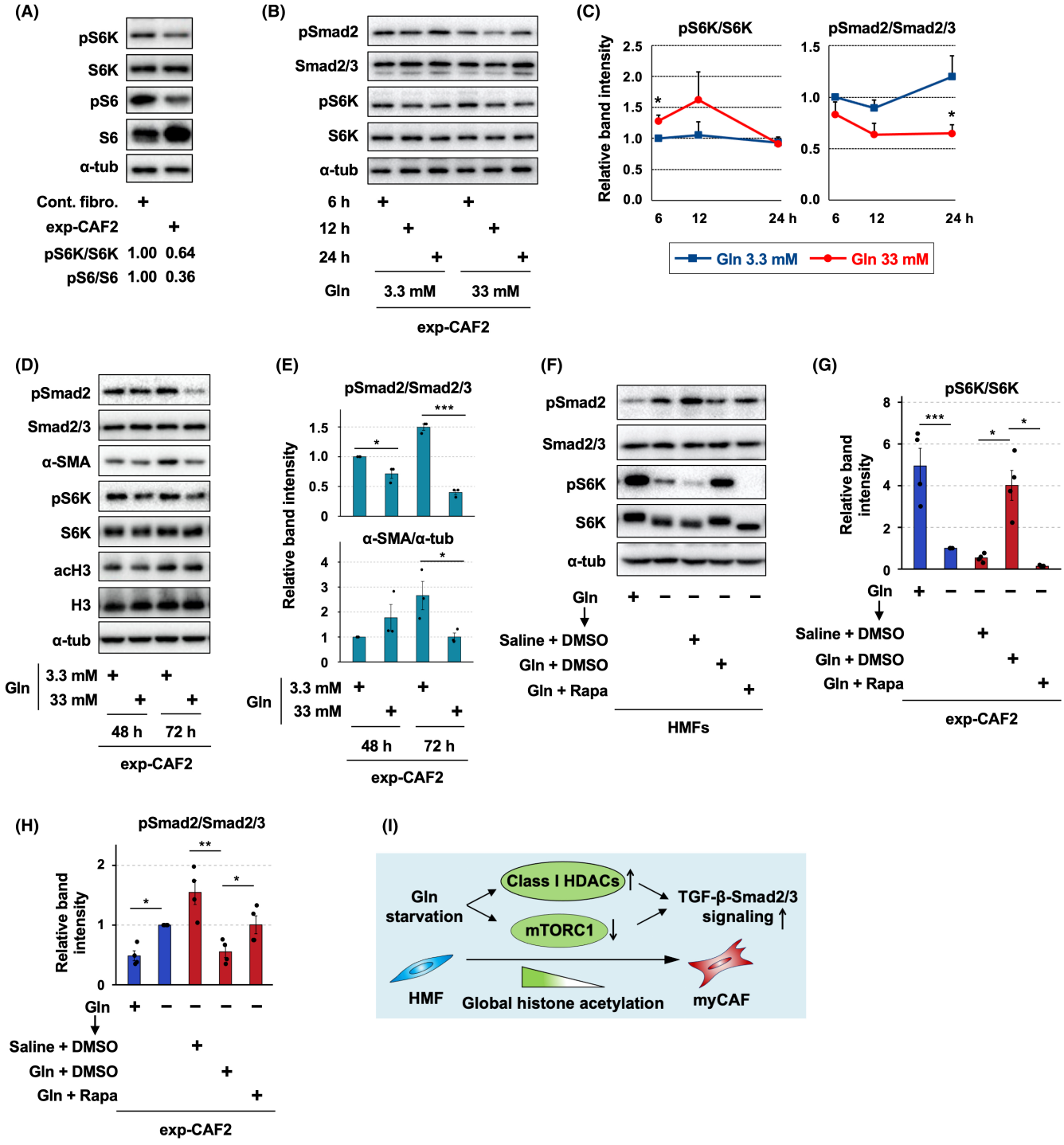

FIGURE 5 Suppression of mTORC1 is required for TGF-β-Smad2/3 signaling activation and myofibroblastic characteristics in exp-CAF2 cells. (A) Immunoblotting of control fibroblasts (522) and exp-CAF2 cells (544). (B–E) Immunoblotting of exp-CAF2 cells treated with medium supplemented with 3.3 or 33 mM Gln for 6, 12, and 24 h (B, C), and 48 and 72 h (D, E). Band intensity is indicated relative to 3.3 mM at 6 h (C) or at 48 h (E). $^{*}p < 0.05$, $^{***}p < 0.001$ by Student's *t*-test ($n = 5$, pS6K/S6K; $n = 4$, pSmad2/Smad2/3, C; $n = 3$, E). (F–H) exp-CAF2 cells cultured in medium with or without 4 mM Gln for 12 h (lanes 1 and 2). Gln-starved cells were treated subsequently with saline (lane 3) or 4 mM Gln with DMSO or rapamycin (20 nM) (lanes 4, 5) for 12 h. Signal intensity ratios are indicated in (G) and (H). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by Student's *t*-test ($n = 4$). (I) Gln starvation in the tumor microenvironment triggers class I HDAC activity and mTORC1 suppression to boost TGF-β-Smad2/3 signaling in HMFs, presumably inducing phenotypic conversion to myCAFs during tumor progression. Error bars, SE (C, E, G, H). Dots indicate biological replicates (E, G, H).

signaling was considerably suppressed in all other exp-CAF lines (Figure S6A,B). These observations indicate that mTORC1 suppression is sustained in CAFs, even when cultured with Gln-supplemented regular medium.

We thus speculated that mTORC1 suppression contributes to TGF-β-Smad2/3 signaling activation and the myofibroblastic state in CAFs. To address this possibility, exp-CAF2 cells were supplemented with high-dose Gln (33 mM) that resulted in an increased pS6K/S6K ratio for 6–12 h compared to those cultured in 3.3 mM Gln-supplemented medium (Figure 5B,C). Of note, pSmad2/Smad2/3 and α-SMA/α-tubulin ratios were decreased in these cells by 73.4% and 62.3%, respectively, at 72 h posttreatment (Figure 5D,E). These data therefore indicate that mTORC1 suppression is required for maintenance of activated TGF-β-Smad2/3 signaling and the myofibroblastic state in CAFs.

We also addressed whether CAFs with mTORC1 suppression are still sensitive to Gln starvation further attenuating mTORC1 signaling and increasing TGF-β signaling. exp-CAF2 cells were thus cultured with Gln-starved medium for 12 h, resulting in a markedly decreased pS6K/S6K ratio and an increased pSmad2/Smad2/3 ratio (Figure 5F–H). The increased pSmad2/Smad2/3 ratio was also attenuated by 4 mM Gln supplementation, and this effect was inhibited by rapamycin (Figure 5F–H), the response resembling that observed in HMFs (Figure 3C–E). These findings suggest that exp-CAF2 cells remain sensitive to the extracellular Gln level, further modulating mTORC1 and TGF-β-Smad2/3 signaling.

We next investigated cross-talk between HDAC activation and mTORC1 suppression in CAFs. High-dose Gln supplementation (33 mM) restored the lower acH3/H3 ratio in exp-CAF2 cells (Figure S6C,D). Glutamine supplementation (3.3 mM) also raised the markedly decreased acH3/H3 and acH4/H4 ratios in Gln-starved exp-CAF2 cells, but these elevations were not inhibited by rapamycin (Figure S6E), presumably indicating mTORC1-independent regulation of HDAC activity. Treatment with TSA also did not elevate mTORC1 signaling in exp-CAF2 cells to mediate TGF-β signaling suppression (Figure S6F). These data series indicate that mTORC1 suppression and HDAC1 activation independently mediate TGF-β-Smad2/3 signaling activation in CAFs, an observation mirroring that in Gln-starved HMFs (Figure 3F–H).

4 | DISCUSSION

Our previous work showed that resident HMFs acquire self-stimulating TGF-β-Smad2/3 autocrine signaling to be converted into myCAFs by interacting with mammary cancer cells in tumors.[9] However, epigenetic and metabolic alterations through which HMFs are converted into myCAFs during tumor progression remain uncertain.

Herein, we show global histone deacetylation in myCAFs within tumors to be significantly associated with poorer outcomes in breast cancer patients (Figure 1A–H). To mimic Gln deficiency in the TME, HMFs were cultured in Gln-starved medium. Global histone deacetylation and TGF-β-Smad2/3 signaling activation are notably induced in these cells through class I HDAC activity. Gln starvation also inactivates mTORC1 to mediate TGF-β-Smad2/3 signaling activation in HMFs. Importantly, global histone deacetylation, class I HDAC activation, and mTORC1 suppression are maintained in exp-CAF2 cells even when cultured in Gln-supplemented medium, during in vitro propagations (Figures 4A,G, 5A, S2A,B, and S6A,B), suggesting reprograming of these features in human breast CAFs. Inhibition of class I HDAC activation and restoration of mTORC1 suppression by high-dose Gln supplementation significantly inhibits TGF-β-Smad2/3 signaling activation and the myofibroblastic state in CAFs. These unprecedented findings reveal molecular mechanisms underlying myCAF conversion from HMFs: Gln starvation in the TME triggers class I HDAC activation and mTORC1 suppression to boost TGF-β-Smad2/3 signaling activation in HMFs, presumably mediating myCAF conversion (Figure 5I).

The myofibroblastic state is not induced in HMFs cultured in Gln-starved or rapamycin-treated medium (Figures 3A,B and S1A). This might be attributable to severe inhibition of protein synthesis including α-SMA and TGF-β under such culture conditions. Long-term treatment of HMFs with TGF-β under Gln-starved and hypoxic three-dimensional culture conditions, precisely mimicking the TME, might be required for complete and stable conversion into myCAFs.

Our results indicate that HDAC1 is required for maintenance of TGF-β-Smad2/3 signaling and the myofibroblastic state of CAFs (Figure 4I,J). Given that HDAC1 was similarly expressed in control fibroblasts and CAFs (Figure S5A,B), elevated class I HDAC activity in CAFs might be mediated by posttranslational modifications such as phosphorylation, acetylation, and ubiquitination of HDAC1.[41] A previous study showed that HDAC1 directly deacetylates Smad7, an inhibitory Smad, to facilitate ubiquitination leading to proteasomal degradation.[42] Molecular mechanisms underlying activation of class I HDACs including HDAC1 and the subsequent TGF-β-Smad2/3 signaling in CAFs awaits further study.

mTORC1 suppression mediates TGF-β-Smad2/3 signaling activation in Gln-starved HMFs and CAFs (Figures 3A–E and 5F–H). These findings are consistent with previous studies, demonstrating mTORC1 inhibition to upregulate TβR-I expression, thereby activating TGF-β-Smad2/3 signaling in affected murine skin keratinocytes deficient for Raptor, the mTORC1 component, and Rheb, the mTORC1 activator.[43] Valencia et al. also reported mTORC1 suppression and increased TGF-β1 and α-SMA expression in p62-deficient mouse prostatic fibroblasts,[44] raising the interesting possibility of p62 deficiency-induced mTORC1 suppression and TGF-β signaling activation in breast myCAFs. However, the molecular mechanisms underlying the mTORC1 suppression-induced TGF-β signaling activation have yet to be clarified.

This study suggests that Gln starvation-triggered class I HDAC activation and mTORC1 suppression serve as epigenetic and metabolic alterations that initiate TGF-β signaling activation and give rise to myCAF differentiation during tumor progression. These observations further our understanding of how myCAFs are induced,

and how their activated states are reprogramed to retain their tumor-promoting ability, key steps toward developing novel myCAF-targeted therapeutic approaches.

AUTHOR CONTRIBUTIONS

Y.M. and A.O. contributed to the concept, study design, and interpretation of the data. Y.M. and T.W. contributed to the acquisition and analysis of the data. Y.D., A.T., Y.M., T.Yo., and T.Ya. contributed to the tissue microarray studies. L.Y. and R.M. contributed to the ChIP-seq. H.S. contributed to the DNA microarray. Y.M. and A.O. drafted the manuscript. All authors read and approved the final manuscript for submission.

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CONFLICT OF INTEREST STATEMENT

Y.D. and H.S. are editorial board members of *Cancer Science*. The other authors have no conflicts of interest to disclose.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: Use of FFPE tissue specimens of human breast cancers in this study (Figure 1A–C) was approved by the Juntendo University ethics review board. For tissue microarray (Figure 1H and Tables 1 and 2), individual institutional ethics committees approved this study and the use of all clinical materials. Experiments met all guidelines and regulations indicated by these committees.

Informed consent: Informed consent was obtained.

Registry and the registration no. of the study/trial: N/A.

Animal studies: The animal experiments were approved by the Animal Research Ethics Committee of the Juntendo University Faculty of Medicine.

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SUPPORTING INFORMATION

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