Supplementary Experimental Procedures

Isolation, culture, and transfection of primary mammary epithelial organoids

Primary mammary epithelial organoids were prepared from 8-week-old CD1 mice (Charles River) as previously described (Nelson *et al.*, 2006). Inguinal glands were removed aseptically, minced with razor blades, and incubated with agitation (350 rpm) at 37°C in DMEM/F12 supplemented with 0.2% collagenase (Roche), 0.2% trypsin (Sigma), 5% FBS, 5 μg/ml insulin, and 50 μg/ml gentamicin. The digested tissue was centrifuged at 1800 rpm for 10 min. The supernatant was removed, centrifuged three times, and the resulting cell pellet was resuspended in DMEM/F12 supplemented with 20 U/ml DNase I (Sigma). The organoids were separated from single cells (mainly fibroblasts) using differential centrifugation. Organoids were resuspended in DMEM/F12 supplemented with 10% FBS, 5 ng/ml EGF, insulin/transferrin/sodium selenite (ITS; Sigma), penicillin/streptomycin (Sigma), and gentamicin, and immediately embedded in collagen gels or plated on collagen-coated plastic for transfection.

Transfection complex was prepared by diluting Fugene HD (Roche):DNA at a 4:1 ratio in reduced serum media and added to the primary mammary epithelial cells immediately after plating on collagen-coated plates. After 24h, fresh transfection complex was added to the primary cells and incubated for 24h.

Immunohistochemistry

Murine mammary glands obtained from 4-week-old CD-1 mice were fixed in 10% neutralized formalin, paraffin-embedded and sectioned into 5-μm slices, mounted on positively charged slides, hydrated through xylene and graded ethanol, and washed with PBS. Endogenous

peroxidase activity was quenched using 3% H_2O_2 in methanol and non-specific binding was blocked by incubation with blocking solution. Snail1 (Abcam), Snail2 (Cell signaling) or E47 (Abcam) antibodies were used to stain sections, which were counterstained with hematoxylin, dehydrated through graded ethanol into xylene, and mounted.

EdU incorporation

To determine cell proliferation during growth factor-induced branching morphogenesis of organotypic culture of mammary epithelial cells, EdU incorporation was performed using the Click-iT EdU Alexa Fluor 594 imaging Kit (Invitrogen) according to the manufacturer's protocol.

Supplementary Tables

Table S1. Primers used for Quantitative Real-Time Reverse-Transcription PCR

Gene	Sequences
β-actin	Fwd: GCCACAGGATTCCATACC Rev: GTGCGTGACATCAAAGAG
Snail2	Fwd: TGTGCTTACCAACCAAATAGTATG Rev: CTGTCTGTCTGTCTGTCTGTC
Snail1	Fwd: CCTGGTTCCTGCTTGGCTCTC Rev: GGCTCTGGGCGGGTACAA AG
E47	Fwd: TTCCCTCCCTGACCTCTC Rev: GGCTACTGATGCGATTTCC
Vimentin	Fwd: CCCTGAACCTGAGAGAAACTA AC Rev: GGTCATCGTGATGCTGAGAAG
MMP3	Fwd: CTCAAGATGATGTAGATGGTATTC Rev: TGGTGATGTCTCAGGTTCC
MMP9	Fwd: GCATCCGAGCAAGAAGAC Rev: GCATGTGAACATAACCTCATA C
FSP1	Fwd: AGAAGTGAAGACTCCTCAGATG Rev : ATGTGCGAAGAAGCCAGAG
E-cadherin	Fwd: CAACGAATCCCTCAAAGACC Rev: GATAGAGAAGCCATTGAAAA
N-cadherin	Fwd: AGAAGGTGGAGGAGAAGAAGAC Rev: TGTGGCTCAGCATGGATAGG
Mucin-1	Fwd: CAA TGG CAC CTC AGT CAC Rev: TTG TGG TCT GGA ATG ATA GC
Syndecan-1	Fwd: GCC CTC CCG CAA ATT GTG Rev: CCA GAG AAG TTG TCA GAG TCA TC
p53	Fwd: GCTACCTGAAGACCAAGAAG Rev: CATAAGACAGCAAGGAGAGG
BID	Fwd: GAGTGTATCTGAAGAGTTTACG Rev: GAACAGTCACTTGGCATTAC

Table S2. Sequence for shRNA (sense)

shRNA	The RNAi consortium number	Mature sense sequences
shSnail1-a	TRCN0000096619	GCCACCTTCTTTGAGGTACAA
shSnail1-e	TRCN0000096623	GCCCAACTATAGCGAGCTGCA
shSnail2-c	TRCN0000096226	CCCATATCTCTATGAAAGTTA
shSnail2-d	TRCN0000096227	GCAGACCCACTCTGATGTAAA
shSnail2-e	TRCN0000096228	ACCCTATACCTGTCATACCAA
shE47-a	TRCN0000086613	CCTTAACTATGTAAGACGGAA
shE47-c	TRCN0000086617	CCGGATCACTCCAGCAATAAT
shE47-d	TRCN0000086614	CCAGCAATAATTTCTCACCTA
shTwist1-a	TRCN0000095075	GCAGTCGCTGAACGAGGCGTT
shTwist1-b	TRCN0000095076	GCCCTCGGACAAGCTGAGCAA
shTwist1-e	TRCN0000095074	CCATACTTCCTCTGAAGGAAA

Table S3. Primers used for cloning

Gene	Sequences
YFP	**Fwd:** GCCACAGGATTCCATACC **Rev :** GTGCGTGACATCAAAGAG
Snail1	**Fwd:** TGTGCTTACCAACCAAATAGTATG **Rev :** CTGTCTGTCTGTCTGTCTGTC
Snail2	**Fwd:** CCTGGTTCCTGCTTGGCTCTC **Rev :** GGCTCTGGGCGGGTACAA AG
E47	**Fwd:** CAACGAATCCCTCAAAGACC **Rev :** GATAGAGAAGCCATTGAAAA

Supplementary Figure Legends

Supplementary Figure 1. EMT markers are expressed during branching morphogenesis of mammary epithelial organoids and mammary glands *in vivo* (A) Clusters of mammary epithelial cells were embedded in collagen gel and treated with EGF, HGF, or no growth factor (No GF). Total RNA was isolated at indicated times and used to determine the mRNA levels of Twist1 using qRT-PCR. The mRNA levels were normalized to the levels of β-actin in each sample and each value was expressed relative to the levels in No GF; shown are mean ± SEM (n = 3). *, $P < 0.05$; **, $P < 0.01$ *vs* 3h No GF (2-way ANOVA with Bonferroni comparison). **(B)** Primary mammary epithelial organoids were cultured in collagen gels and treated with EGF for the indicated times. The expression of Snail1, Snail2, and E47 were analyzed by immunofluorescence staining. Scale bars, 100 μm. **(C)** Mammary epithelial clusters were embedded in collagen gel and treated with No GF or EGF for 24h, and then fixed and stained for E-cadherin. Scale bars, 100 μm. **(D)** Total RNA was isolated from mammary epithelial clusters cultured in collagen gels and treated with No GF, EGF, or HGF for 24h and used to determine the mRNA levels of syndecan-1 (SDC1), mucin-1 (Muc1), vimentin, fibroblast-specific protein-1 (FSP1), MMP3, and MMP9 by qRT-PCR. The mRNA levels were normalized to the levels of β-actin in each sample and each value was expressed relative to the levels in No GF; shown are mean ± SEM (n = 3). *, $P < 0.05$; **, $P < 0.01$ *vs* No GF (1-way ANOVA with Bonferroni comparison). **(E)** Inguinal mammary glands were isolated from 4-week-old CD1 mice and analyzed by immunohistochemistry for expression of Snail1, Snail2, and E47. Snail1 is expressed in the nuclei of luminal epithelial, myoepithelial, and stromal cells; Snail2 is expressed in the nuclei of myoepithelial cells; E47 is expressed in luminal epithelial and myoepithelial cells. Scale bars, 50 μm.

Supplementary Figure 2. Loss of Snail2 has modest effects on protein levels of E-cadherin. **(A)** Mammary epithelial cells transfected with shSnail2 or scrambled shRNA (Sc) were treated with or without growth factor for indicated times. Total protein was analyzed using immunoblot to determine the levels of E-cadherin and β-actin. **(B)** Quantification of the relative levels of E-cadherin from panel (A). **(C)** Mammary epithelial cells transfected with shSnail2 or scrambled RNA control (Sc) were treated with or without EGF, fixed, and stained for E-cadherin (red) or nuclei (blue). Scale bars, 20 μm.

Supplementary Figure 3. Modulation of Snail1, Snail2, and E47 regulates branching morphogenesis of primary mammary organoids cultured in collagen gels. (A) Primary mammary epithelial organoids were transfected with Flag-YFP to monitor transfection efficiency. Scale bars, 50 μm. **(B)** Primary mammary epithelial organoids were transfected with shSnail1, shSnail2, shE47, or scrambled shRNA (Sc), embedded in collagen gels, and treated with or without EGF for 24h. Shown are nuclei (blue) and actin (green). Scale bars, 100 μm. **(C)** Quantification of the percentage of branching shown in panel (B). Shown are mean ± SEM (n=35). **, $P < 0.01$ *vs* Sc (No GF); ##, $P < 0.01$ *vs* Sc (EGF) (2-way ANOVA with Bonferroni comparison). **(D)** Primary mammary organoids were transfected with Flag-tagged Snail1, Snail2, E47, or YFP and embedded in collagen gels. Shown are nuclei (blue) and actin (green). Scale bars, 100 μm. **(E)** Quantification of the percentage of branching shown in panel (D) Shown are mean ± SEM (n=35). **, $P < 0.01$ *vs* Flag-YFP (2-way ANOVA with Bonferroni comparison).

Supplementary Figure 4. Loss of Twist1 does not affect growth factor-induced mammary epithelial branching morphogenesis. (A-B) Mammary epithelial cells were transfected with three independent shTwist1, scrambled shRNA (Sc), or no shRNA cassette (NT). After 48h,

transfected cells were treated with no growth factor (No GF), EGF, or HGF for 9h. Total RNA was isolated for determination of **(A)** Twist1 and **(B)** E-cadherin mRNA levels by qRT-PCR. The mRNA levels were normalized to the levels of β-actin in each sample and each value was expressed relative to the levels in No GF of scrambled shRNA transfected cells (Sc); shown are mean ± SEM (n = 3). **, $P < 0.01$ *vs* Sc (No GF) (2-way ANOVA with Bonferroni comparison). **(C)** Mammary epithelial cells were transfected with shTwist1 and used to generate microfabricated mammary epithelial tubules. Tubules were treated with no growth factor (No GF), EGF, or HGF for 24h. Shown are frequency maps of branching from 50 tubules. Scale bars, 50 μm. Color bar indicates frequency. **(D)** Branching was quantified as described above.

Supplementary Figure 5. Ectopic expression of Snail1, Snail2 and E47 induces the expression of mesenchymal markers in mammary epithelial cells. (A-D) Mammary epithelial cells were transfected with Flag-tagged Snail1, Snail2, E47, YFP or nothing (NT). Total RNA was isolated for determination of **(A)** MMP3, **(B)** MMP9, **(C)** FSP1, and **(D)** vimentin mRNA levels by qRT-PCR. The mRNA levels were normalized to the levels of β-actin in each sample and each value was expressed relative to the levels in YFP-transfected cells; shown are mean ± SEM (n = 4). *, $P < 0.05$; **, $P < 0.01$ *vs* Flag-tagged YFP (1-way ANOVA with Bonferroni comparison).

Supplementary Figure 6. Ectopic Snail2 increases the expression of Snail1 to induce mammary epithelial branching. (A-D) Mammary epithelial cells were transfected with Flag-tagged Snail2 or YFP, together with two independent shSnail1, scrambled shRNA (Sc) or nothing (NT). **(A)** Total protein was assayed by immunoblot to determine the expression levels of Snail1, Snail2, and E-cadherin. Total RNA was isolated for determination of **(B)** E-cadherin,

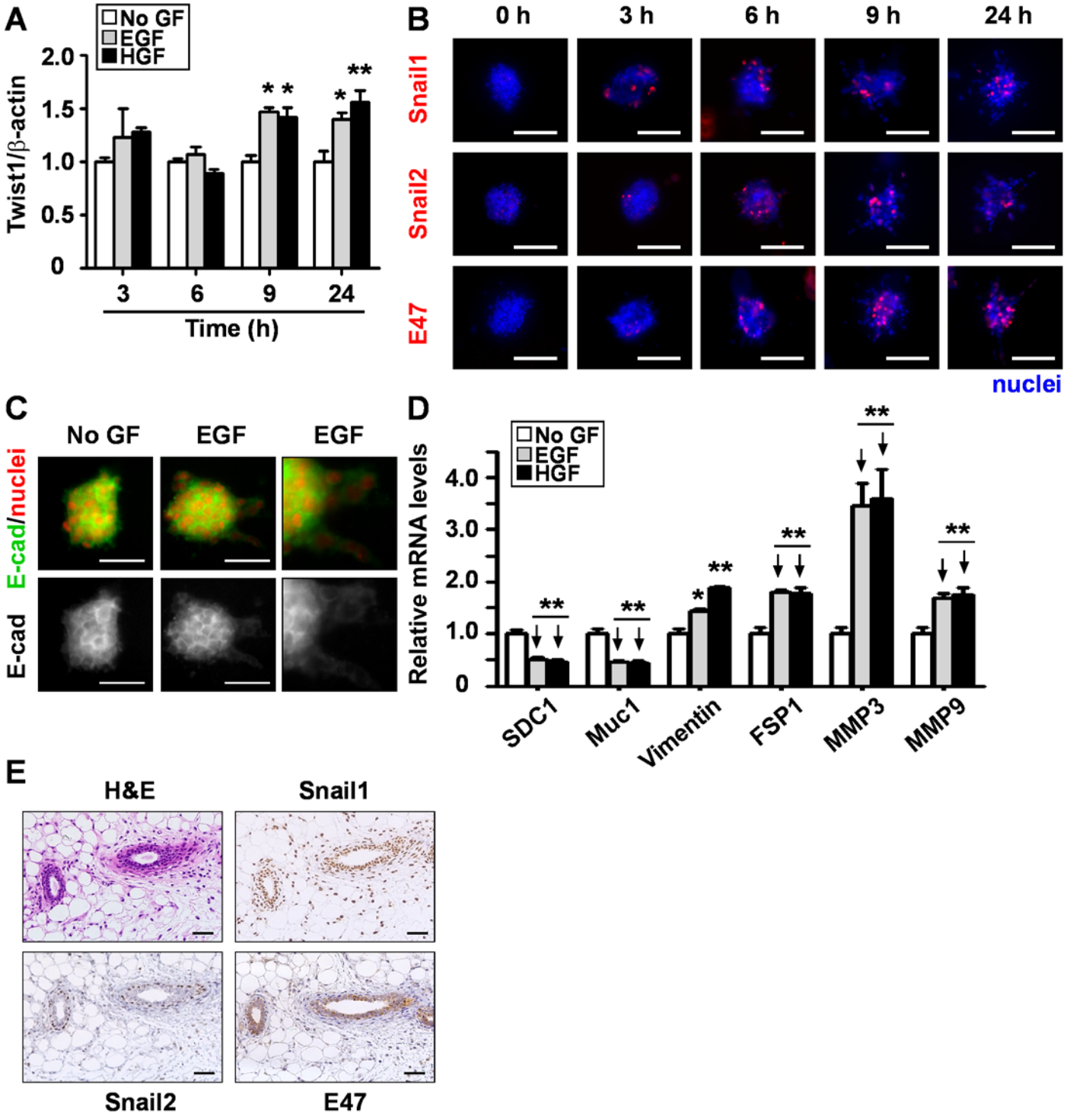
(C) N-cadherin, and **(D)** vimentin mRNA levels by qRT-PCR. The mRNA levels were normalized to the levels of β-actin in each sample and each value was expressed relative to the levels in Flag-tagged YFP transfected cells; shown are mean ± SEM (n = 4). *, $P < 0.05$; **, $P < 0.01$ *vs* Flag-tagged YFP (NT); #, $P<0.05$ *vs* Flag-tagged Snail2 with Sc (2-way ANOVA with Bonferroni comparison). **(E)** Mammary epithelial cells were co-transfected with Flag-tagged Snail2 or YFP, and two independent shSnail1, Sc or NT and used to generate microfabricated mammary epithelial tubules. Shown are frequency maps of branching from 50 tubules. Scale bars, 50 μm. Color bar indicates frequency. **(F)** Branching was quantified as described above; shown are mean ± SEM (n = 5). **, $P < 0.01$ *vs* Flag-tagged YFP (No GF); ##, $P < 0.01$ *vs* Flag-Snail2 with Sc (2-way ANOVA with Bonferroni comparison).

Supplementary Figure 7. Snail2 regulates branching and apoptosis through Snail1. (A) Mammary epithelial cells were transfected with shSnail2 together with Flag-tagged YFP or Snail1 and used to generate microfabricated mammary epithelial tubules. Shown are frequency maps of branching from 50 tubules. Scale bars, 50 μm. Color bar indicates frequency. **(B-C)** Mammary epithelial cells were transfected with Flag-tagged Snail2 or YFP, together with two independent shSnail, scrambled shRNA (Sc) or nothing (NT). Total RNA was isolated for determination of **(B)** p53 and **(C)** BID mRNA levels using qRT-PCR. The mRNA levels were normalized to the levels of β-actin in each sample and each value was expressed relative to the levels in Flag-tagged YFP; shown are mean ± SEM (n = 4). *, $P < 0.05$; **, $P < 0.01$ *vs* Flag-YFP; # , $P < 0.05$ (2-way ANOVA with Bonferroni comparison). **(D)** Mammary epithelial cells were transfected with Flag-tagged Snail2 or YFP, together with shSnail1 or scrambled shRNA (Sc) and used to generate microfabricated mammary epithelial tubules. Tubules were cultured for 20h and stained for cleaved-caspase3 and nuclei. Scale bars, 50 μm. **(E)** Percent area of cleaved-

caspase3 was quantified as described above; shown are mean ± SEM (n=30). **, $P < 0.01$ (1-way ANOVA with Bonferroni comparison).

Supplementary Figure 8. Cell proliferation is increased at branch sites. (A-B)

Microfabricated mammary epithelial tubules were cultured with or without EGF in the presence of EdU. Shown are EdU incorporation for **(A)** 8h or **(B)** 24h. Scale bars, 50 μm. **(C)** Clusters of mammary epithelial cells were cultured in collagen gels with or without EGF in the presence of EdU. Scale bars, 100 μm.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HGF
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1.0
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24
Time (h)
B
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3 h
6 h
9 h
24 h
Snail1
Snail2
E47
nuclei
C
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EGF
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E-cad
D
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2.0
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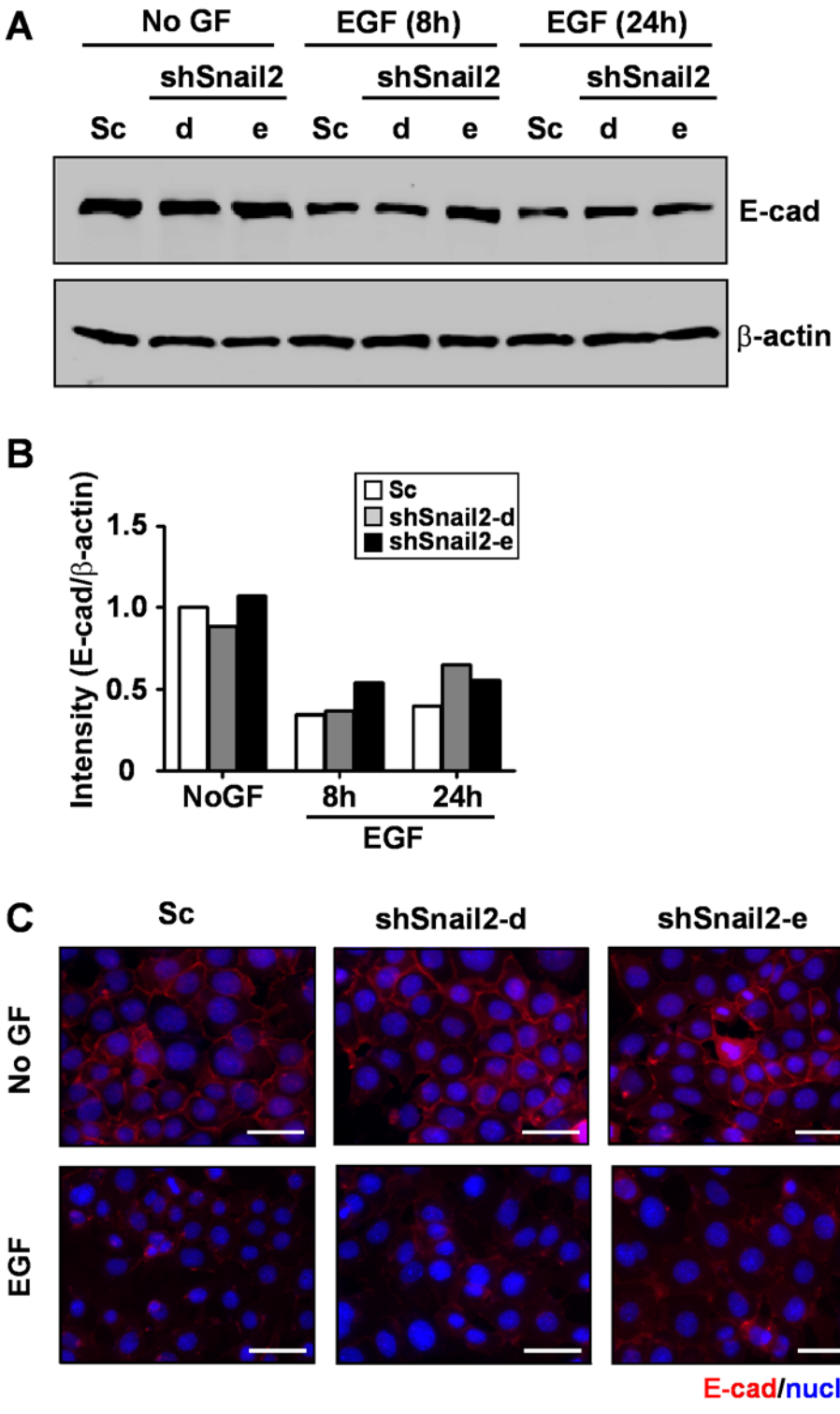
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Intensity (E-cad/β-actin)
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1.0
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Sc
shSnail2-d
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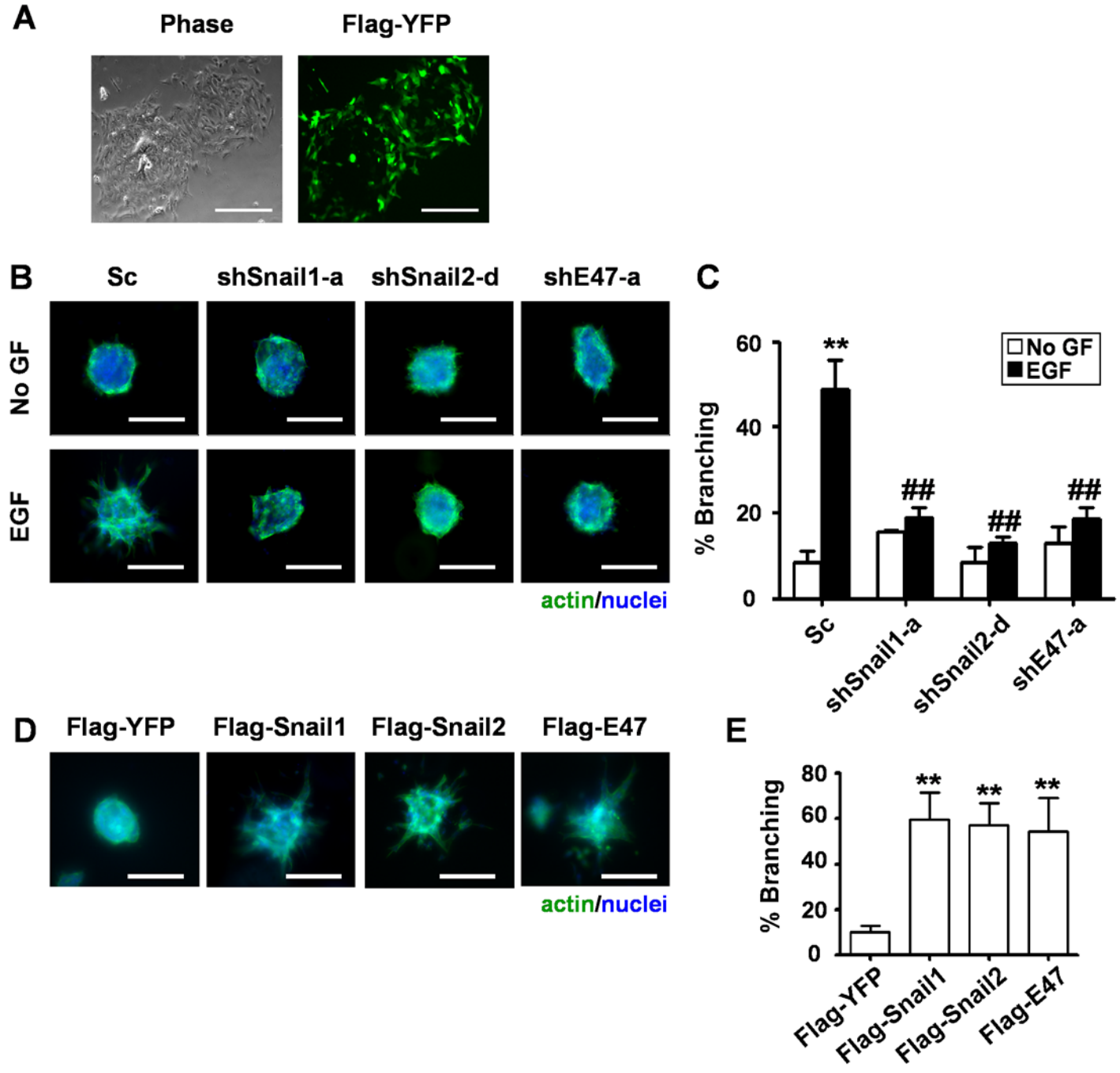
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actin/nuclei
C
% Branching
No GF
EGF
**
##
Sc
shSnail1-a
shSnail2-d
shE47-a
D
Flag-YFP
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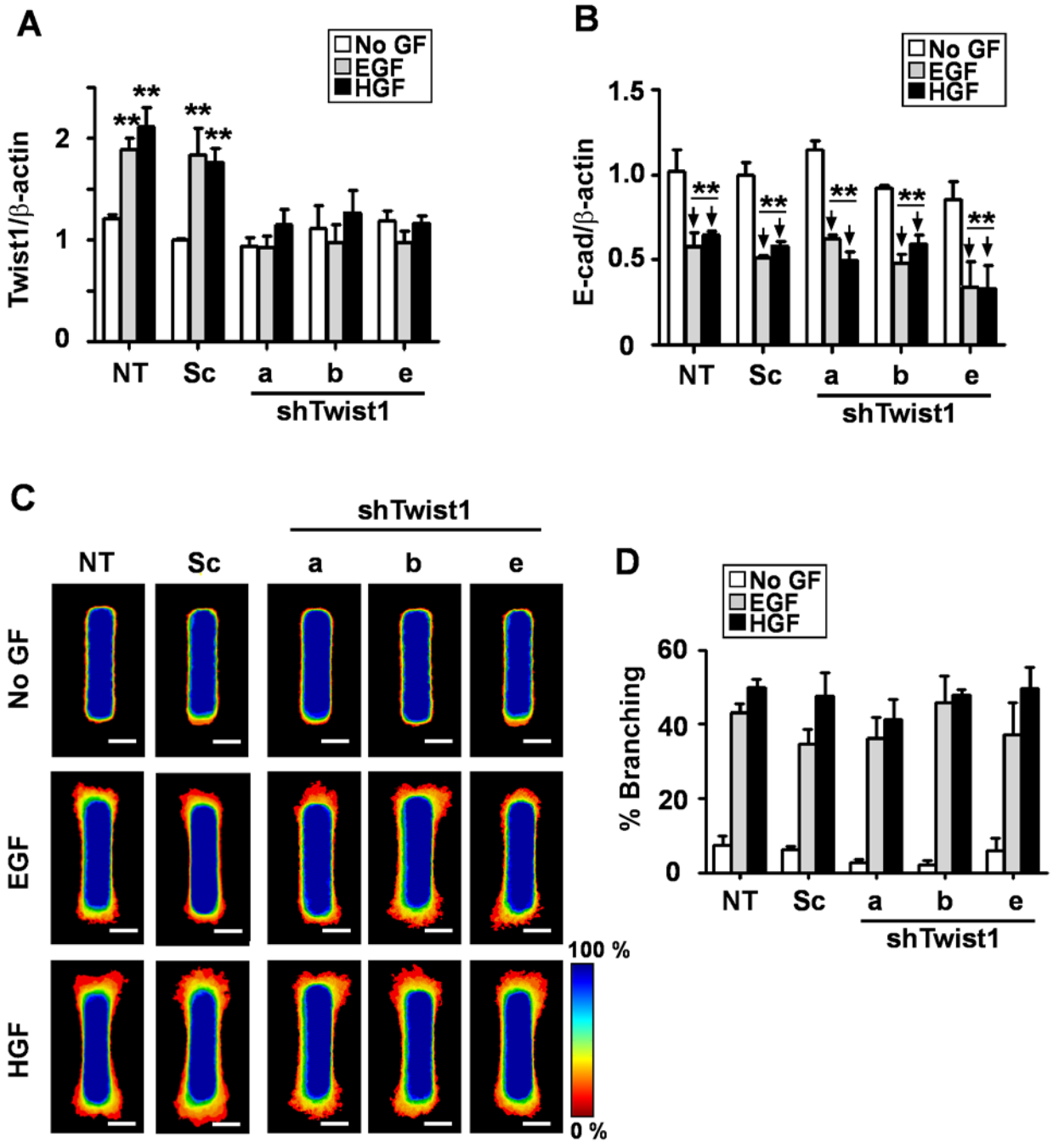
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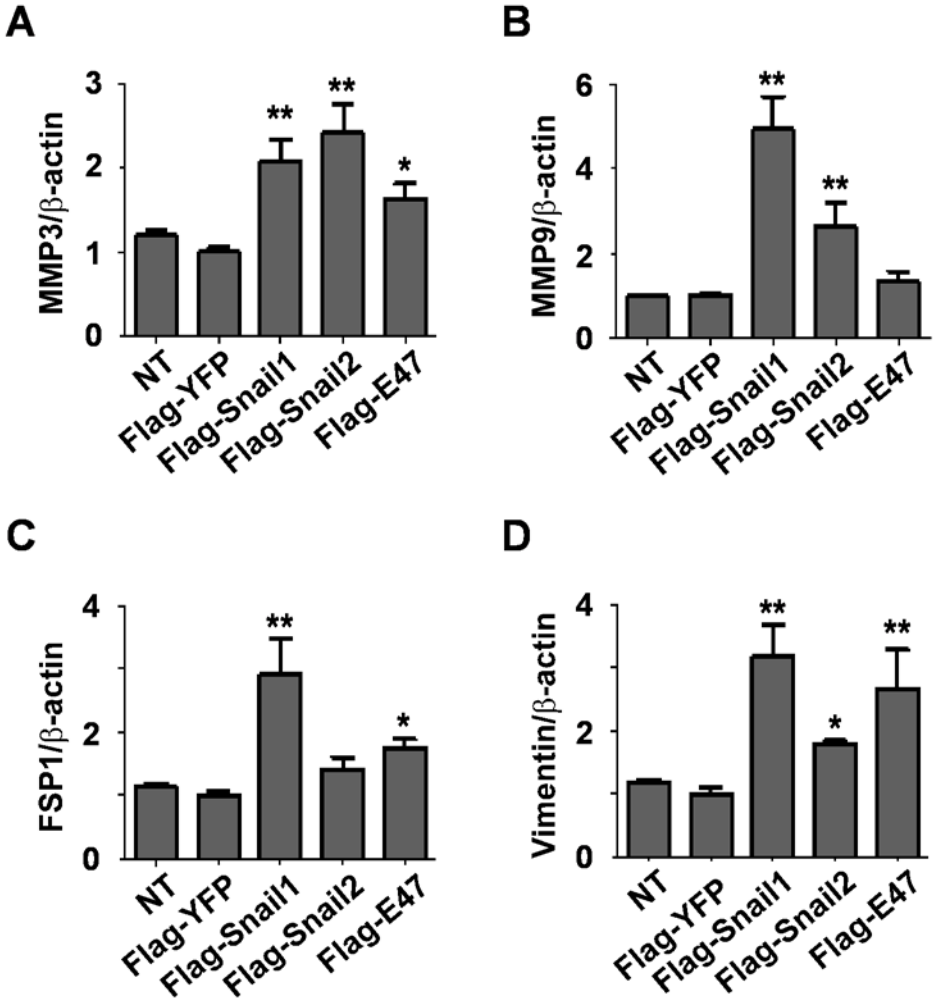
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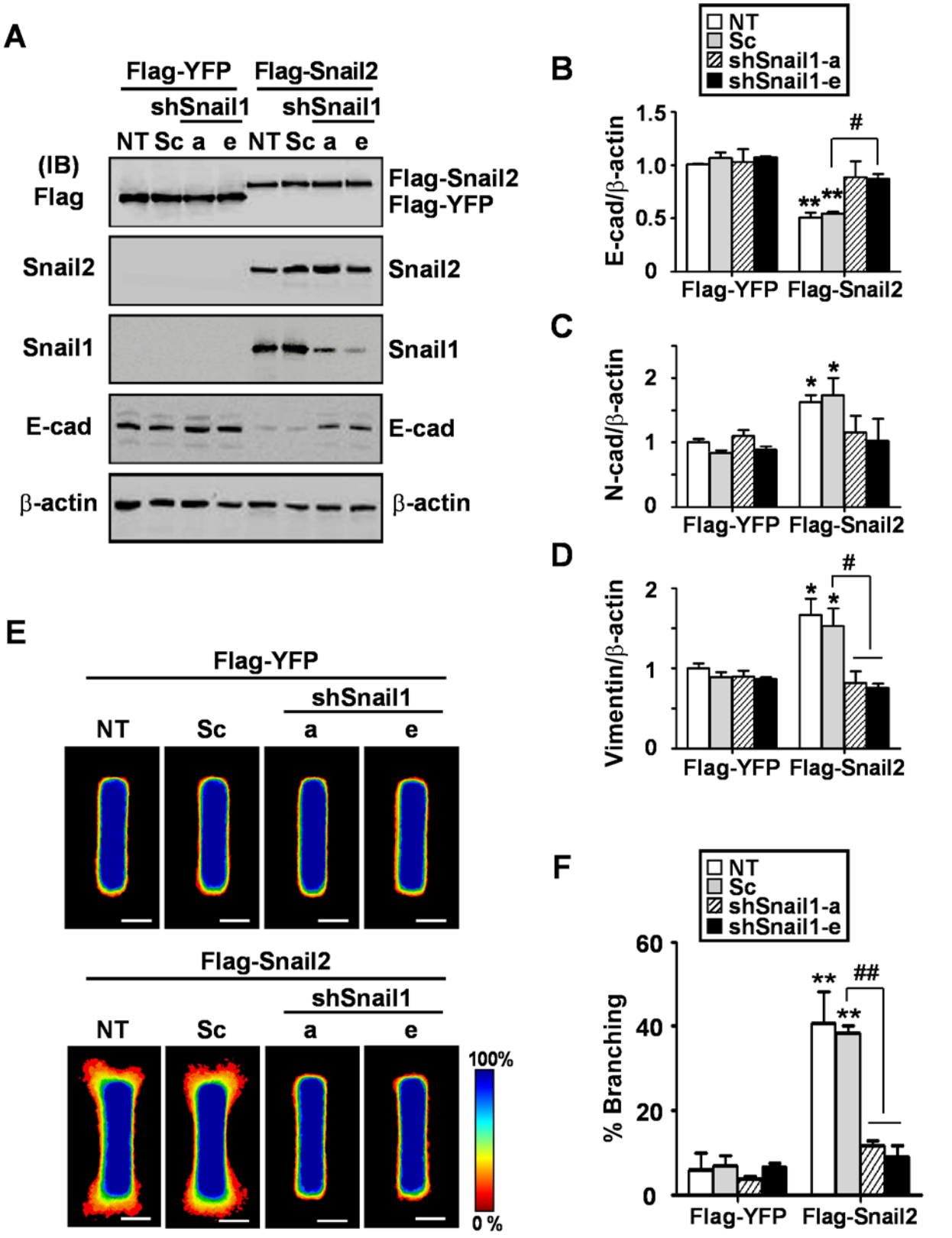
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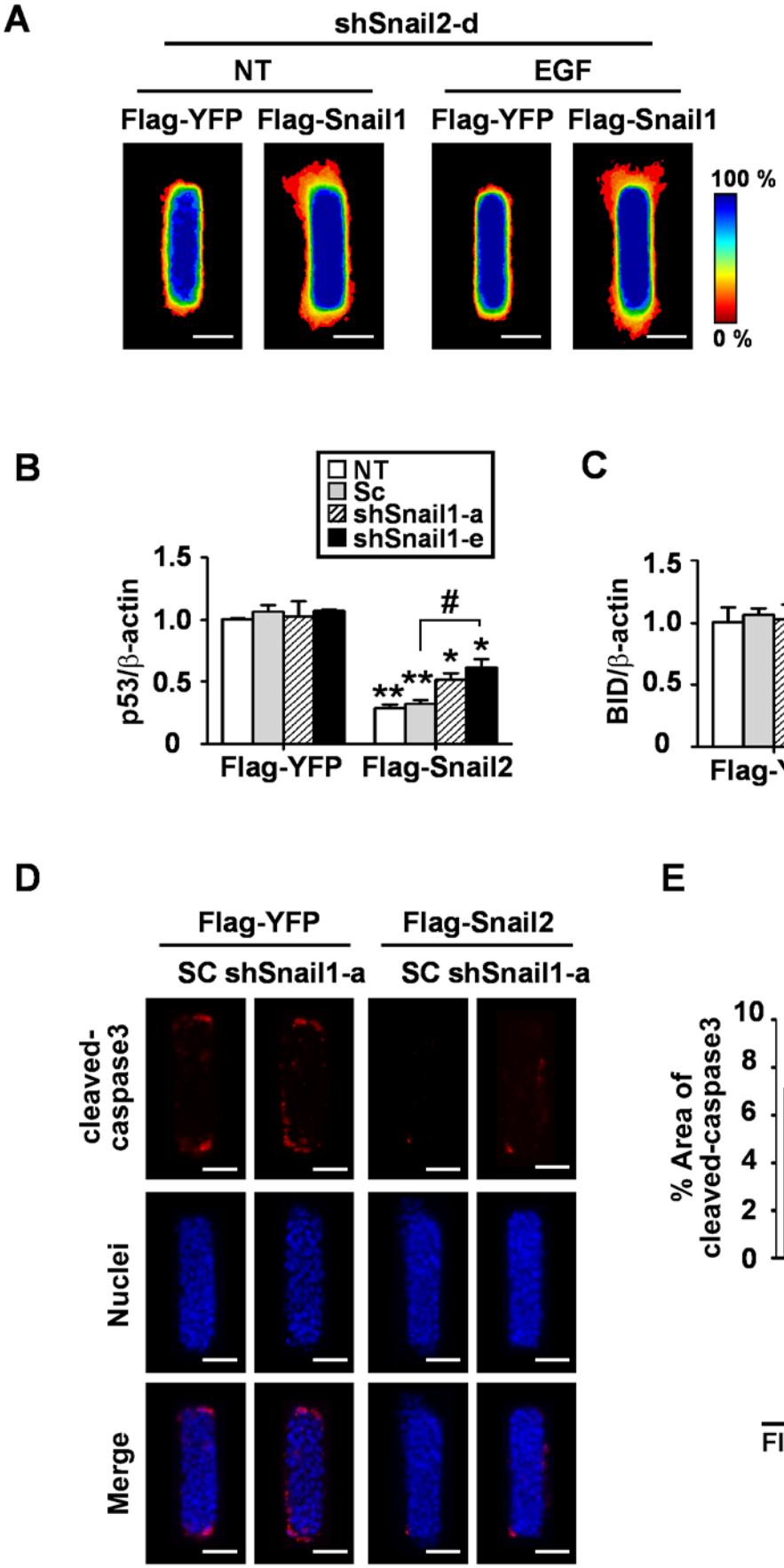
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Nuclei
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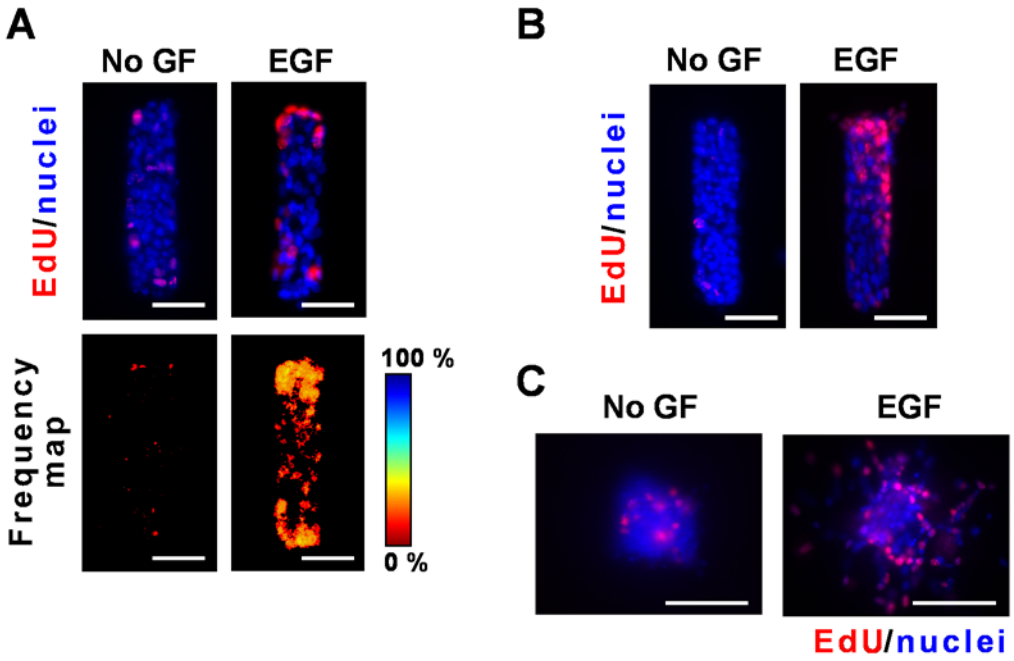
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