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A Soft Microenvironment Protects from Failure of Midbody Abscission and Multinucleation Downstream of the EMT-Promoting Transcription Factor Snail

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Abstract

Multinucleation is found in more than one third of tumors and is linked to increased tolerance for mutation, resistance to chemotherapy, and invasive potential. The integrity of the genome depends on proper execution of the cell cycle, which can be altered through mechanotransduction pathways as the tumor microenvironment stiffens during tumorigenesis. Here, we show that signaling downstream of matrix metalloproteinase-3 (MMP3) or TGFβ, known inducers of epithelial–mesenchymal transition (EMT), also promotes multinucleation in stiff microenvironments through Snail-dependent expression of the filament-forming protein septin-6, resulting in midbody persistence, abscission failure, and multinucleation. Consistently, we observed elevated expression of Snail and septin-6 as well as multinucleation in a human patient sample of metaplastic carcinoma of the breast, a rare classification characterized by deposition of collagen fibers and active EMT. In contrast, a soft microenvironment protected mammary epithelial cells from becoming multinucleated by preventing Snail-induced upregulation of septin-6. Our data suggest that tissue stiffening during tumorigenesis synergizes with oncogenic signaling to promote genomic abnormalities that drive cancer progression.

Significance: These findings reveal tissue stiffening during tumorigenesis synergizes with oncogenic signaling to promote genomic abnormalities that drive cancer progression. *Cancer Res; 78(9); 2277–89. ©2018 AACR.*

Introduction

All cancers are genomically unstable and the most common type of genomic instability is chromosomal instability, or an increased rate of chromosome missegregation. Chromosomal instability is characterized by aneuploidy, which is defined as an abnormal number of chromosomes and is found in 85% of solid cancers, including 85% of breast tumors (1). Even in the absence of other defects, induction of aneuploidy is sufficient to promote tumorigenesis *in vivo* (2). Failure of mitosis that results in multinucleation can lead to aneuploidy—multinucleated cells that continue to divide will commonly produce aneuploid progeny of varying chromosomal arrangements (3), and there is mounting evidence that multinucleation is both common in cancer and indicative of tumor prognosis: An analysis of eleven types of cancer from The Cancer Genome Atlas found that 37% percent of tumors exhibit whole-genome doubling (WGD) and that WGD typically precedes other somatic copy-number alterations (4). A comparison of *in situ* breast carcinomas revealed that multinucleation is more common in pleomorphic lobular carcinoma than in the less invasive classical lobular or ductal carcinomas, suggesting that multinucleation may be associated with invasive potential (5). Furthermore, induction of tetraploidy in human cells promotes increased tolerance for mutation, resistance to chemotherapeutic drugs, and transformation in culture (6).

In addition to being genomically unstable, tumors are generally stiffer than normal tissue. Enhanced deposition and crosslinking increases the density, and consequently the stiffness, of the extracellular matrix (ECM) in tumors, and stiffness is a common diagnostic parameter. Mechanosensors mediate the balance between extracellular forces and intracellular cytoskeletal tension and in doing so translate physical changes in the microenvironment to chemical signals inside the cell. Signaling triggered by stiffening of the ECM can induce changes in phenotype and gene expression that result in deleterious consequences, including cancer progression. For example, the ability of matrix metalloproteinase-3 (MMP3; stromelysin-1) to induce epithelial–mesenchymal transition (EMT) in mammary epithelial cells depends on the subcellular localization of the Rac1 splice variant Rac1b, which is modulated by the stiffness of the surrounding microenvironment. MMP3 is a secreted protease commonly upregulated in cancer that induces expression of Rac1b. Stiff substrata, with compliances characteristic of breast tumors, cause activation and clustering of integrins, which promotes localization of Rac1b to the plasma membrane, activation of NADPH oxidase, production

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of reactive oxygen species (ROS), and elevated expression of the key EMT effector and transcription factor, Snail. Soft substrata, with compliances characteristic of normal mammary tissue, prevent Rac1b membrane localization and protect against EMT (7).

Through their primary role in remodeling the ECM, MMPs induce many changes in the surrounding cells, EMT serving as just one example. Notably, MMPs were previously linked to genomic instability in culture and *in vivo*. MMP3 in particular was found to increase the resistance of mouse mammary epithelial cells to *N*-(phosphonacetyl)-L-aspartate (PALA) through amplification of the *CAD* locus downstream of ROS, in addition to causing other genomic amplifications and deletions similar to those observed in transgenic mice that ectopically express MMP3 in the mammary gland (8, 9). Similarly, TGFβ is a potent inducer of EMT that has also been implicated in genomic instability, including promotion of multinucleation in MCF10A mammary epithelial cells (10). The ability of TGFβ to induce EMT in mammary epithelial cells is likewise regulated by substratum stiffness (11).

Although the mechanical properties of the microenvironment have previously been shown to regulate the cell cycle (12, 13), and proper regulation of the cell cycle is intrinsically connected to the stability of the genome, a link between abnormal mechanical properties in the extracellular microenvironment and induction of genomic instability has not been established. We show here that matrix stiffness regulates multinucleation in mammary epithelial cells. By evaluating how cells respond to EMT inducers when cultured on engineered two-dimensional (2D) polyacrylamide substrata of varying stiffness, we found that multinucleation is increased on stiff substrata through failure of midbody abscission as a consequence of the expression of septin-6, a novel target of Snail. A soft microenvironment appears to protect the stability of the genome in epithelial cells by preventing septin-6 overexpression. Taken together, our data provide evidence that tissue stiffening during tumorigenesis synergizes with EMT-associated pathways to promote genomic abnormalities that drive cancer progression.

Materials and Methods

Cell culture and reagents

p53-mutant SCp2 mouse mammary epithelial cells that express autoactivated MMP3 under the control of the tetracycline promoter (8) were cultured in DMEM:F12 supplemented with 2% FBS (Atlanta Biologicals), 10 μg/mL insulin, 50 μg/mL gentamicin, and 125 μg/mL geneticin (AG Scientific). To repress MMP3 expression, a 4 mg/mL stock solution of tetracycline (Sigma-Aldrich) was added directly to the culture medium at a 1:800 dilution; tetracycline was removed from the medium to induce expression of MMP3. NMuMG cells (ATCC) were cultured in DMEM:F12 supplemented with 10% FBS, 10 μg/mL insulin, and 50 μg/mL gentamicin. MCF10A human mammary epithelial cells (ATCC) were cultured in DMEM:F12 supplemented with 5% horse serum (Fisher Scientific), 20 ng/mL EGF (Sigma), 0.5 mg/mL hydrocortisone (Fisher Scientific), 100 ng/mL cholera toxin (Sigma), 10 μg/mL insulin, and 5 mg/mL gentamicin. All cell lines were authenticated by short tandem repeat genotyping (ATCC), tested for *Mycoplasma* contamination (Lonza), and used before passage 35 (SCp2 cells), 20 (NMuMG cells), or 25 (MCF10A cells). The following reagents were added directly to fresh culture medium 24 hours after plating cells: H_2O_2, 25 μmol/L; N-acetyl-cysteine (NAC, Sigma Aldrich), 10 mmol/L; recombinant TGFβ1 (R&D Systems), 10 ng/mL. Cells were exposed to H_2O_2 or NAC for 72 hours and to TGFβ1 for 48 hours. To increase proliferation of SCp2 cells on soft substrata, 100 ng/mL of EGF was added to the culture medium for 48 hours.

Synthetic substrata

Polyacrylamide (PA) substrata were generated as described previously (7, 14). Briefly, PA gels were polymerized directly on 31-mm-diameter glass coverslips as follows: 12.5% (vol/vol) acrylamide was mixed with bis-acrylamide in water at either 0.5% (vol/vol) or 17.5% (vol/vol). Polymerization was initiated by adding 10% ammonium persulfate (Bio-Rad) at a 1:200 dilution and N,N,N′,N′-tetramethylethylenediamine (Sigma Aldrich) at a 1:2,000 dilution. Thirty-six μL of the mixture was sandwiched between coverslips for 40 minutes at room temperature. PA gels were stored in PBS at 4°C and characterized as previously described (7).

The surfaces of the PA gels were then functionalized with 200 μg/mL fibronectin (BD Biosciences) as described previously using the heterobifunctional crosslinker Sulfo-SANPAH (Thermo Scientific; ref. 14). Before plating cells, gels were washed three times with PBS and incubated with culture medium for 30 minutes at 37°C. To achieve a final density of approximately 15,000 cells/cm^2, we initially seeded 8×10^5, 4×10^5, or 1.5×10^5 cells onto soft or stiff gels or into empty wells of a 6-well plate.

Transfections and viral transductions

SCp2 cells were transfected or transduced in medium containing tetracycline, with the exception of the MMP3 ± NAC and MMP3 ± shSnail experiments, which were performed under tetracycline withdrawal. pMK782 Septin6-GFP was obtained from AddGene (plasmid # 38296). pEYFP-C1 was obtained from BD Biosciences (catalog #6005-1). Plasmids were transfected using FuGENE HD Transfection Reagent (Promega). Briefly, FuGENE and DNA were mixed in Opti-MEM medium (2:1 ratio for SCp2 cells; 3:1 for NMuMG cells) and added directly to culture medium, which was changed 20 hours later. Cells were fixed and analyzed 24 hours later.

Recombinant adenoviruses encoding YFP-Rac1b, YFP-Rac1b-SAAX, YFP-Rac1b-myr, Aurora A kinase, shRNA against Snail (shSnail) or GFP were obtained from Vector BioLabs (7). Recombinant adenovirus encoding GFP-Snail was a gift from Paul Wade (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Adenovirus was added directly to culture medium at an MOI of 100, and medium was changed 24 hours after transduction. Cells were fixed and analyzed 24 hours later.

Quantitative reverse transcriptase PCR

Gene expression was assessed by qRT-PCR 72 hours after inducing expression of MMP3 or 48 hours after exposing cells to all other conditions. RNA was extracted using TRIzol reagent (Invitrogen), followed by cDNA synthesis using a Verso cDNA Synthesis Kit (Thermo Scientific). Transcript levels were measured using an Applied Biosystems Step One Plus instrument and iTaq Supermix with SYBR Green chemistry. Amplification was followed by melt-curve analysis to verify the presence of a single PCR product. Primers (Supplementary Table S1) were designed using PrimerQuest (Integrated DNA Technologies) and determined to be specific by BLAST. The expression level of each mRNA was normalized to that of 18S in the same sample.

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Immunofluorescence staining

Samples were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature, followed by three washes with PBS. To label E-cadherin, MKLP1, or Ki67, samples were blocked with 10% (v/v) goat serum (Sigma Aldrich) in 0.3% Triton-X-100 in PBS (PBST) for 4 hours, and incubated overnight at 4°C with rabbit anti-E-cadherin antibody (Cell Signaling Technology), rabbit anti-MKLP1 antibody (Abcam), or mouse anti-Ki67 antibody (Cell Signaling Technology), respectively. Samples were washed 6 times with PBST for 30 minutes each time, and incubated with Alexa 594 goat anti-rabbit secondary antibody (Invitrogen) overnight at 4°C. To label nuclei, samples were incubated with a 1:1,000 dilution of Hoechst 33342 (Invitrogen) for 20 minutes at room temperature and washed three times with PBS for 10 minutes each time.

To label centrosomes, samples were fixed with 100% methanol for 20 minutes at −20°C, followed by three washes with PBS. Samples were blocked as described above and incubated with rabbit anti-γ-tubulin (Santa Cruz Biotechnology) overnight at 4°C. Samples were washed and incubated with secondary antibody as described above.

To assess proliferation rate in response to treatment with EGF, samples were incubated with 10 μmol/L EdU for 20 hours before fixation, permeabilization, and EdU staining, using the Click-iT EdU Alexa Fluor 594 Imaging kit (Thermo Scientific) according to the manufacturer's instructions.

Metaphase spreads

Metaphase spreads were prepared following a standard protocol using colcemid and KCl. Briefly, cells were treated with 0.5 μg/mL colcemid for 18 to 20 hours. Cells in metaphase were collected by treatment with trypsin for 5 minutes, followed by centrifugation in culture medium for 5 minutes at 2,500 rpm. Pellets were resuspended by adding 0.075 mol/L KCl dropwise and then incubated at 37°C for 20 minutes. Cells were centrifuged for 5 minutes at 2,500 rpm, resuspended in methanol:acetic acid (3:1), and incubated on ice for 20 minutes. To wash, cells were centrifuged and resuspended in fixative 3 more times. The third time, cells were resuspended in 250 μL of fixative, and 20 μL of the suspension was dropped on the center of a microscope slide held at a 45° angle. Drops were dried in a prehumidified chamber for 15 minutes at 37°C. Spreads were mounted using Fluoromount-G with DAPI (Southern Biotech) and sealed with clear nail polish before imaging.

Immunoblotting analysis

Samples were lysed in RIPA lysis buffer (Thermo Scientific) supplemented with protease inhibitors (Roche) and protein concentrations were measured using the Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific). Samples were then mixed with Laemmli sample buffer, boiled at 95°C for 5 minutes, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were then blocked in 5% milk and incubated overnight at 4°C in blocking buffer containing antibodies specific for septin-6 (Lifespan Biosciences) or GAPDH (Cell Signaling Technology).

Imaging and quantification

Still images were acquired using a Hamamatsu Orca CCD camera attached to a Nikon Ti-U inverted fluorescence microscope at ×20 magnification in air (or ×60 magnification in oil for metaphase spreads). Phase contrast and fluorescence images were merged using ImageJ. For multinucleation experiments, cells with two or more nuclei were counted and recorded as the percentage of the total number of cells in each image. The number of images needed to accurately quantify an experimental condition was determined using a running average. Briefly, the total percentage of multinucleated cells was adjusted as each additional image was analyzed. When the percentage of multinucleation stabilized, no further images were analyzed. Similar methodology was used to quantify centrosome amplification and multipolar mitosis. For metaphase spreads, total chromosomes per cell were counted for 30 to 36 cells. Ploidy was represented as the number of chromosomes per cell normalized to the average number of chromosomes per cell in a population.

Timelapse movies were acquired using a Hamamatsu C4742-95 camera attached to a Nikon Ti-U inverted microscope and fitted with an environmental chamber held at 90% humidity, 37°C, and 5% CO_2. Images were acquired at ×10 magnification every 3 minutes for a total of 8 hours, and stacked in ImageJ.

Histological analysis of tissue samples

Breast cancer biopsies were derived from waste surgical material from de-identified patients, and were formalin-fixed and paraffin-embedded, as per approval by the Mayo Clinic Institutional Review Board. Tissue sections (4 or 10 μm) were deparaffinized by placing them into three changes of xylene and rehydrated in a graded ethanol series. The rehydrated tissue samples were rinsed in water and sections were subjected to heat antigen retrieval as described by the manufacturer (DAKO) using citrate buffer pH 6.0. Slices were incubated with each primary antibody for 1 hour at room temperature. Sections were then rinsed with TBS/Triton-X-100 (TBST) wash buffer, and incubated with each secondary antibody for 30 minutes. For fluorescent detection, tissue sections were rinsed 3 times for 5 minutes each with PBS containing 1.43 μmol/L 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). Sections were incubated with primary antibody (Snail or septin-6), rinsed with TBST wash buffer, and incubated with secondary antibody (DAKO Envision anti-rabbit, HRP for 30 minutes (Snail) or anti-goat HRP (Biocare) for 30 minutes (septin-6)). Tissue sections were rinsed with TBST wash buffer and then incubated in 3,3′-diaminobenzidine (DAB+; DAKO), and counterstained with Gills I hematoxylin. Each antibody and its corresponding fluorescent secondary antibody used were: Snail (rabbit polyclonal, Bioworld Technology #BS1853) detected by Alexa488-conjugated donkey anti-rabbit IgG (Invitrogen, #A21202); septin-6 (goat polyclonal, Lifespan #LS-B9200) detected by Alexa594-conjugated donkey anti-goat IgG (H+L; Invitrogen, A11058).

Whole-slide digital images of each breast cancer sample were captured with the Aperio Scanscope AT2 slide scanner (H&E, Snail, septin-6) and the Aperio Scanscope FL slide scanner (fluorescent images) using a ×20 objective. For quantification of multinucleated cells, serial sections of breast cancer biopsy samples were stained with hematoxylin and eosin (H&E) and Snail. The IHC-stained images were used as guides to demarcate areas digitally of high and low Snail expression in a thick (10 μm) H&E-stained section. The regions were of equal size (153680.90 um^2) and were spread among the areas of low ($n = 10$) or high ($n = 10$) Snail expression. Multinucleated cells were identified and counted in each selected area.

Statistical analysis

Data represent mean ± SEM of at least three independent experiments. Statistical analysis was conducted using a Student *t* test or a one- or two-way ANOVA followed by Bonferroni post-tests. For the patient samples, data represent mean ± SEM of 10 regions of stained sections, and statistical significance was tested with the Mann–Whitney test. A P value of <0.05 was considered to represent a significant difference between conditions.

Results

MMP3 promotes genomic instability by causing multinucleation

In addition to inducing EMT in mammary epithelial cells, MMP3 has been found to induce genomic instability. We characterized MMP3-induced genomic instability using a derivative of SCp2 cells with a tet-inducible auto-activated MMP3 (8). These cells lack functional p53, which is required for survival of multinucleated cells (15). Consistently, metaphase spreads revealed that exposure to MMP3 induced aneuploidy (Fig. 1A and B). Aneuploid progeny often result from cells with an elevated number of centrosomes, a phenotype commonly associated with multinucleation. Amplified centrosomes can cause multipolar mitoses or cluster at opposite poles and give rise to merotelic attachments and lagging chromosomes (16). We sought to determine whether the chromosomal aberrations observed in MMP3-treated cells were downstream of multinucleated intermediates.

Staining for γ-tubulin and DNA revealed that treatment with MMP3 resulted in centrosome amplification (Fig. 1C and D) and multipolar and clustered mitoses (Fig. 1E and F). Similarly, staining for E-cadherin and DNA revealed a higher fraction of multinucleation in cells expressing MMP3 (Fig. 1G and H). MMP3-induced genomic instability was previously found to occur downstream of ROS (8). Consistently, we found that MMP3-induced centrosome amplification and multinucleation were ameliorated by treatment with the ROS scavenger N-acetylcysteine (NAC; Fig. 1D and H). These data suggest that MMP3-associated genomic instability arises, in part, as a consequence of multinucleation.

MMP3-induced multinucleation is regulated by substratum stiffness

We previously found that EMT-associated signaling downstream of MMP3 is regulated by the mechanical stiffness of the microenvironment (7). To determine whether MMP3-induced multinucleation is similarly regulated by matrix stiffness, we cultured cells on polyacrylamide gels of various stiffness in the presence or absence of MMP3 (Fig. 2A). Substrata with Young's moduli of 130 Pa and 4020 Pa mimicked the mechanical properties of physiologically normal breast tissue and tumor tissue, respectively, and are hereafter referred to as "soft" and "stiff" (7). We found that treatment with MMP3 induced multinucleation on stiff but not soft substrata (Fig. 2B).

MMP3 induces the expression of the Rac1 splice variant Rac1b, which on stiff substrata localizes to the plasma membrane and associates with NADPH oxidase to produce ROS, thus leading to upregulation of Snail (7). To determine if this MMP3-induced signaling pathway regulates multinucleation (Fig. 2C), we paired overexpression and knockdown experiments for Rac1b, ROS, and Snail. Ectopic expression of Rac1b induced multinucleation on stiff substrata independently of exposure to MMP3 (Fig. 2D and E). Forcing Rac1b to localize to the membrane by expressing a mutant that contains an additional membrane-targeting myristoylation sequence (Rac1b-myr) further increased multinucleation on stiff substrata. In contrast, preventing Rac1b membrane localization by expressing a mutant that blocks prenylation (Rac1b-SAAX) reduced multinucleation on stiff substrata (Fig. 2D and F). Similarly, increasing ROS in the absence of MMP3 by treating cells with H_2O_2 promoted multinucleation on stiff substrata (Fig. 2G), whereas quenching ROS by treating with NAC decreased the ability of MMP3 to induce multinucleation on stiff substrata (Fig. 2H). Finally, ectopic expression of Snail induced multinucleation on stiff substrata independently of exposure to MMP3 (Fig. 2I) or cell doubling time (Supplementary Fig. S1A and S1B). Depleting Snail by using short hairpin RNA (shRNA) decreased MMP3-induced multinucleation on stiff substrata (Fig. 2J). These data suggest that on stiff substrata, MMP3 induces multinucleation by signaling through Rac1b, ROS, and Snail. Importantly, a soft microenvironment appears to protect cells from becoming multinucleated in response to this pathway. Cells on soft substrata were impervious to becoming multinucleated even when their proliferation rate was elevated by treatment with exogenous growth factor (Supplementary Fig. S1C and S1D).

Snail induces multinucleation by increasing midbody persistence

Ectopic expression of Snail did not induce multinucleation on soft substrata (Fig. 2I). Thus, we reasoned that in addition to influencing the localization of Rac1b, substratum stiffness must modulate signaling downstream of Snail. We therefore sought to understand the mechanism by which Snail could promote multinucleation.

Oncogenic induction of multinucleation has been found to occur through at least two physical mechanisms. Timelapse imaging of malignant cells in culture has suggested that multinucleation results from a failure of cytokinesis (17). Cancerous mutations in *BRCA2* are linked to multinucleation by causing a failure of cytokinesis upon improper regulation of the midbody (18). Conversely, it is well recognized that cell–cell fusion can promote tumor progression. For example, oncogenic fusion can result from dysregulated expression of fusogenic proteins such as CD44 (19) or syncytin (20). We investigated both physical mechanisms in the context of Snail signaling.

Previous microarray data revealed that Aurora A kinase (AURKA) is upregulated in mammary epithelial cells in response to MMP3 exposure (21). Overexpression of AURKA is common in several cancers and linked to multinucleation by causing a failure of cytokinesis (22). In contrast to previous studies, we found that ectopic expression of AURKA did not induce multinucleation in cells cultured on soft or stiff substrata (Supplementary Fig. S2A). In addition, neither fusogenic protein CD44 nor syncytin was identified as a new target of Snail that could lead to multinucleation (Supplementary Fig. S2B and S2C).

Since Snail did not appear to induce multinucleation through cell–cell fusion, we turned to timelapse microscopy to visualize the cell division process in response to ectopic expression of Snail. Phase contrast images suggested that multinucleation in cells overexpressing Snail was frequently preceded by a persistent midbody, which appeared as a phase-dense structure between two daughter cells (Fig. 3A; Supplementary Movies S1 and S2). Midbodies are organelles created by compaction of the mid-zone of the mitotic spindle, a dense collection of overlapping

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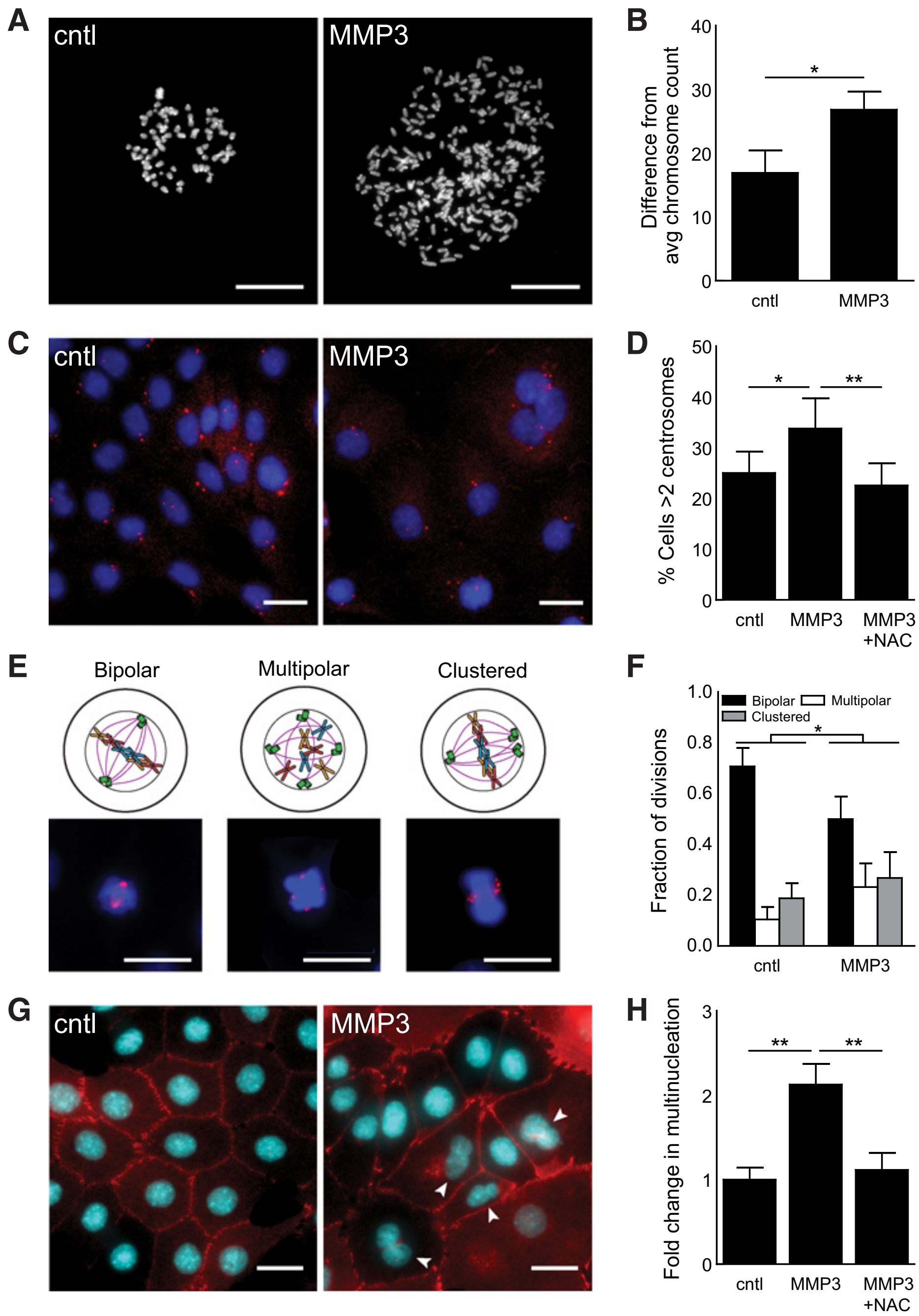

Figure 1.
MMP3 induces chromosomal instability, centrosome amplification, mitotic abnormalities, and multinucleation. **A** and **B,** Metaphase spreads of SCp2 mouse mammary epithelial cells treated with or without MMP3. **C** and **D,** MMP3-induced ROS causes centrosome amplification. Red, γ-tubulin; blue, DNA. **E** and **F,** Multipolar and clustered mitoses in cells treated with or without MMP3. Red, γ-tubulin; blue, DNA. **G** and **H,** MMP3 induces multinucleation. Red, E-cadherin; cyan, DNA. Cells were exposed to MMP3 for 72 hours. Arrowheads, multinucleated cells. Scale bars, 25 μm. Shown are mean ± SEM for 3–7 experiments ($n > 30$ cells for **B**; $n > 400$ cells for **D**; $n > 30$ cells for **F**; $n > 150$ cells for **H**). *, $P < 0.05$; **, $P < 0.01$ (**B**, paired *t* test; **D** and **H**, one-way ANOVA; **F**, two-way ANOVA).

microtubules that serves to spatially orient the cleavage furrow during cytokinesis (23). The final stage of cytokinesis is midbody abscission, a complex orchestration involving hundreds of proteins, during which, the cytokinetic bridge is severed on one or both sides of the midbody. The resulting structure, referred to as the midbody descendent (MB^D), is either pulled by its tether into the cytoplasm of the attached daughter cell or released into the intercellular space and subsequently engulfed and degraded by one of the daughter cells (24, 25). Midbody persistence can result in abscission failure and multinucleation, and is evidenced here by accumulation of MB^Ds.

To determine whether signaling downstream of Snail causes multinucleation by increasing midbody persistence, we quantified MB^Ds in cells that ectopically expressed Snail. MB^Ds were identified as puncta in cells stained for the midbody marker mitotic kinesin-like protein (MKLP1; Fig. 3B). Quantification of MKLP1-positive puncta revealed that a significantly higher fraction of multinucleated cells had MB^Ds than did mononucleated cells (Fig. 3C). MB^Ds were previously found to accumulate in cancer cells, which contributes to tumorigenicity in culture (26). We hypothesized that as multinucleated cells continued to divide, persistent midbodies would accumulate. Accordingly, we found

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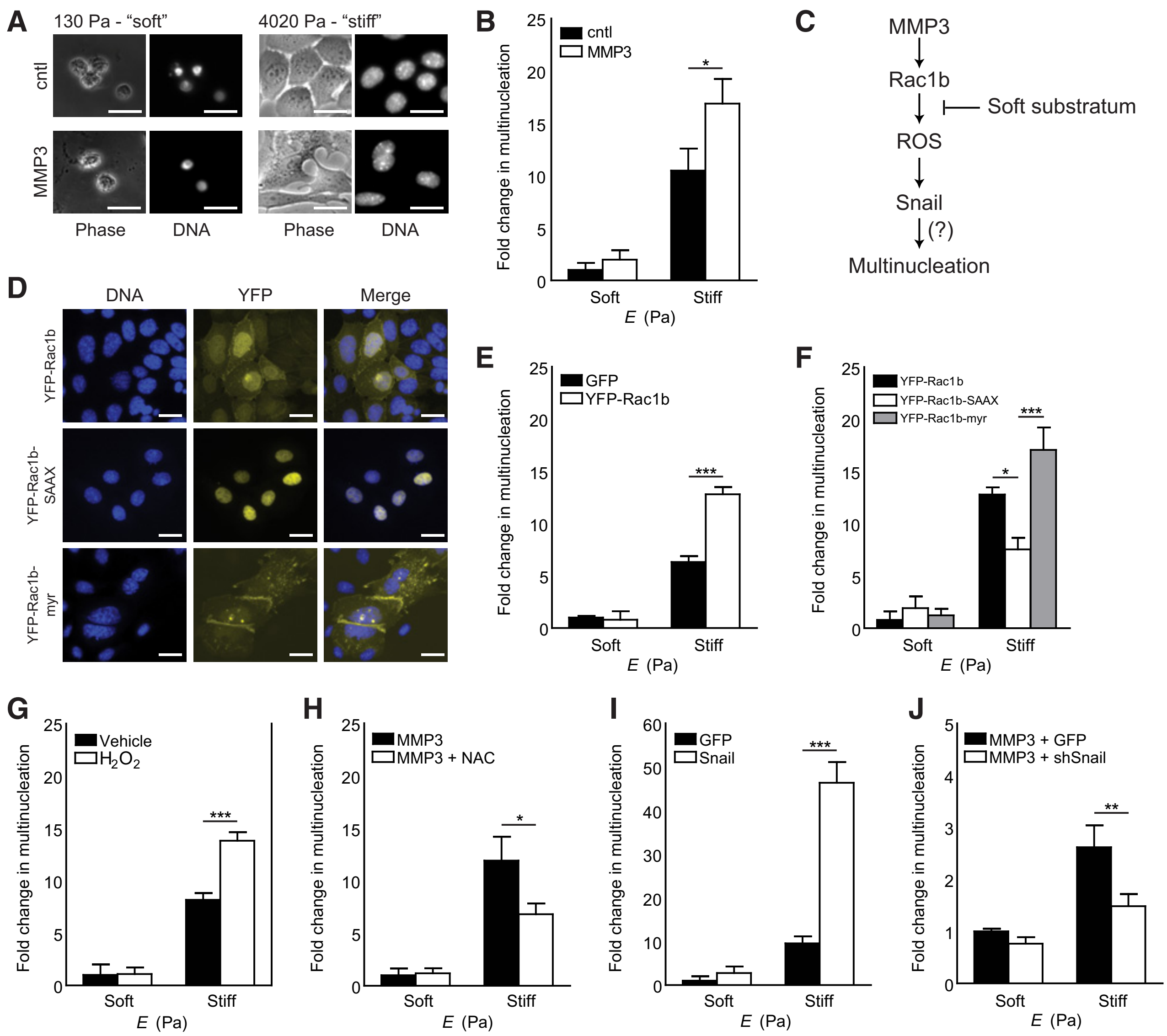

Figure 2.

Substratum stiffness regulates multinucleation downstream of MMP3, Rac1b, ROS, and Snail. **A,** SCp2 mouse mammary epithelial cells were cultured on soft or stiff substrata in the presence or absence of MMP3. **B,** MMP3 induces multinucleation on stiff substrata only. **C,** MMP3 induces the expression of Rac1b, causing an increase in production of ROS and elevated expression of Snail, resulting in multinucleation. **D–F,** Multinucleation on stiff substrata depends on localization of Rac1b to the membrane. **G–J,** ROS and Snail induce multinucleation on stiff substrata. Blocking these signals reduces multinucleation on stiff substrata. Culture on soft substrata prevents multinucleation. Cells were exposed to MMP3, ROS, or ROS+NAC for 72 hours (**B**, **G**, and **H**), or expressed Snail or Rac1b variants for 48 hours (**E, F**, **I**, and **J**); scale bars, 25 μm. Shown are mean ± SEM for three to six experiments ($n > 80$ cells for **B**; $n > 100$ cells for **E**; $n > 100$ cells for **F**; $n > 100$ cells for **G**; $n > 50$ cells for **H**; $n > 80$ cells for **I**; $n > 200$ cells for **J**). *, $P < 0.05$; ***, $P < 0.001$ (two-way ANOVA).

that a significantly higher fraction of multinucleated cells than mononucleated cells in the control population had accumulated at least two MB^{D}s. This difference was exacerbated in cells ectopically expressing Snail (Fig. 3D). Together, these data suggest that Snail increases midbody persistence, which prevents or delays abscission and results in multinucleation.

MKLP1 is a kinesin-like protein that is necessary for cytokinesis (27) and stabilizes the interaction between the midbody and the cortex (28). We therefore asked whether dysregulation of MKLP1 or other kinesins caused errors in abscission or induced multinucleation downstream of Snail. We found that ectopically expressing Snail did not change the levels of MKLP1 (Supplementary Fig. S2D) or other kinesin family members (Supplementary Fig. S2E), suggesting that these proteins are not responsible for Snail-induced multinucleation.

Snail and substratum stiffness elevate septin-6 to increase midbody persistence

How does Snail enhance midbody persistence and accumulation, and subsequently, multinucleation? Previous microarray data showed that centromere-associated protein E (CENP-E), which localizes to the midbody and is degraded before abscission

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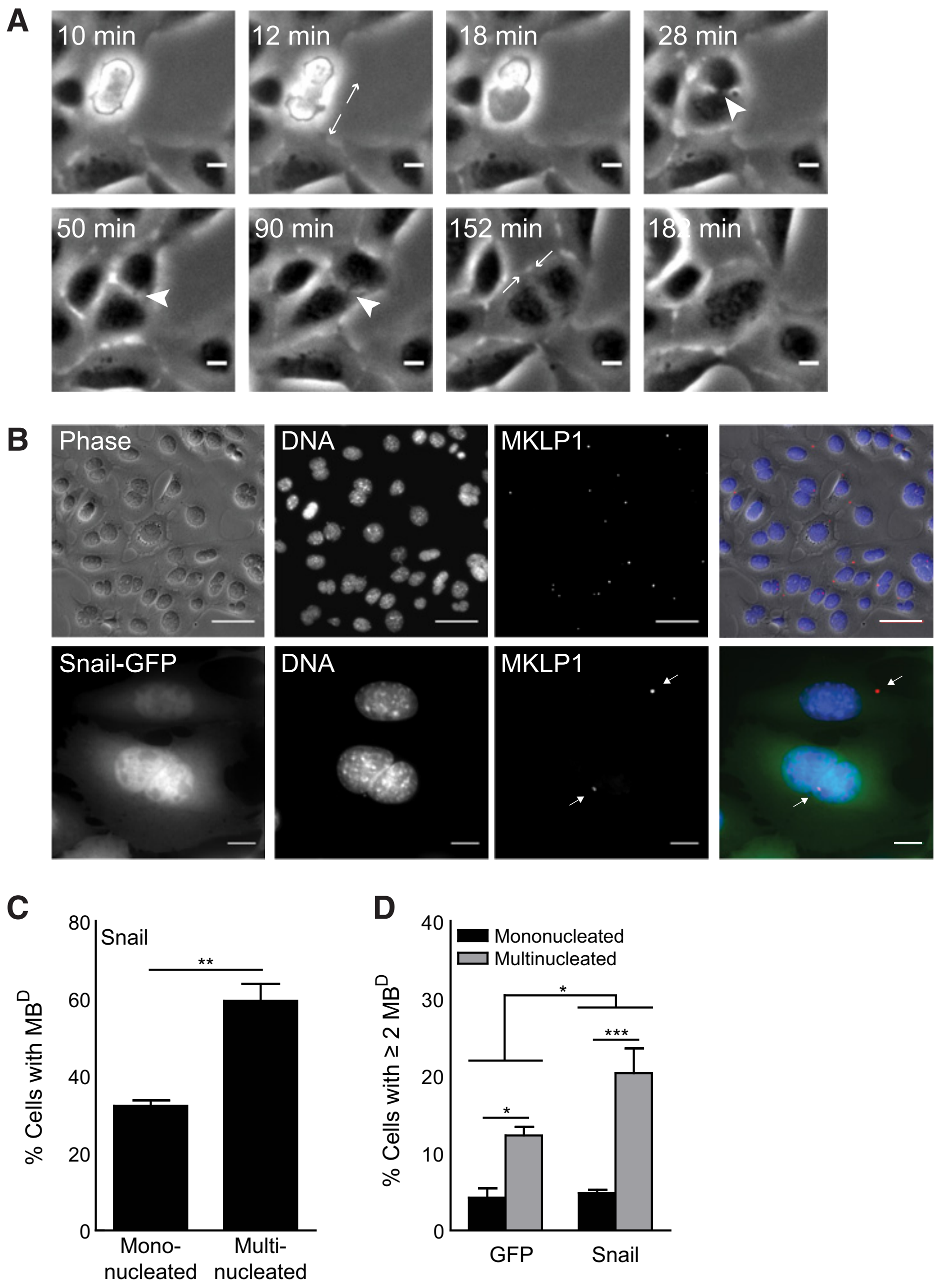

Figure 3.
Snail-induced multinucleation follows midbody persistence. **A,** Persistent midbodies (arrowheads) are visible between sister SCp2 mouse mammary epithelial cells that later merge to form a multinucleated cell. Images extracted from timelapse microscopy. Scale bars, 10 μm. See also Supplementary Movie S1 and S2. **B,** MB^Ds in cells ectopically expressing Snail are quantified by MKLP1-positive puncta. Red, MKLP1; green, Snail-GFP; blue, DNA. Top row, scale bars, 50 μm. Bottom row, scale bars, 10 μm. Arrows in bottom row, MB^Ds. **C,** A higher fraction of multinucleated cells ectopically expressing Snail have MB^Ds than mononucleated cells expressing Snail. **D,** Multinucleated cells accumulate more MB^Ds than do mononucleated cells. This difference is exacerbated in cells expressing Snail as compared with those expressing GFP alone. Cells expressed GFP or Snail for 48 hours. Shown are mean ± SEM for three experiments (n > 500 cells for **C** and **D**). *, $P < 0.05$; ***, $P < 0.001$ (**C**, paired t test; **D**, two-way ANOVA).

(29), is upregulated in response to MMP3 (21). Similarly, degradation of Aurora B kinase (AURKB) at the midbody is necessary for complete abscission, and its dysregulation results in tetraploidy (30). However, neither CENP-E nor AURKB levels were affected by ectopic expression of Snail (Supplementary Fig. S2F,G).

We therefore examined the role of septins, a family of filament-forming GTPases. The actomyosin contractile ring is anchored to the midbody by septin–anillin complexes, which also aid the recruitment of abscission machinery to the midbody (31). In doing so, and in direct interactions with microtubule-binding proteins such as MAP4 (32), septins facilitate microtubule depolymerization and the completion of cytokinesis. Dysregulation of septins can result in multinucleation, developmental errors, and embryonic lethality (33–35).

We assessed the levels of septins in Snail-expressing cells and found that Snail caused an increase in the core filament-forming septin, septin-6 (Fig. 4A), but not the other core septins-2 or -7 (Supplementary Fig. S2H–S2I). Similarly, we found no change in the level of septin-9, previously associated with errors in midbody abscission and multinucleation (33), or septin-4, previously found to be downregulated in response to MMP3 (Supplementary Fig. S2J,K; ref. 21). Consistently, expression of a septin6-GFP fusion protein induced midbody persistence (Fig. 4B), MB^D accumulation (Fig. 4C and D), and multinucleation (Fig. 4E) in the absence of ectopic Snail. We also found that septin6-GFP

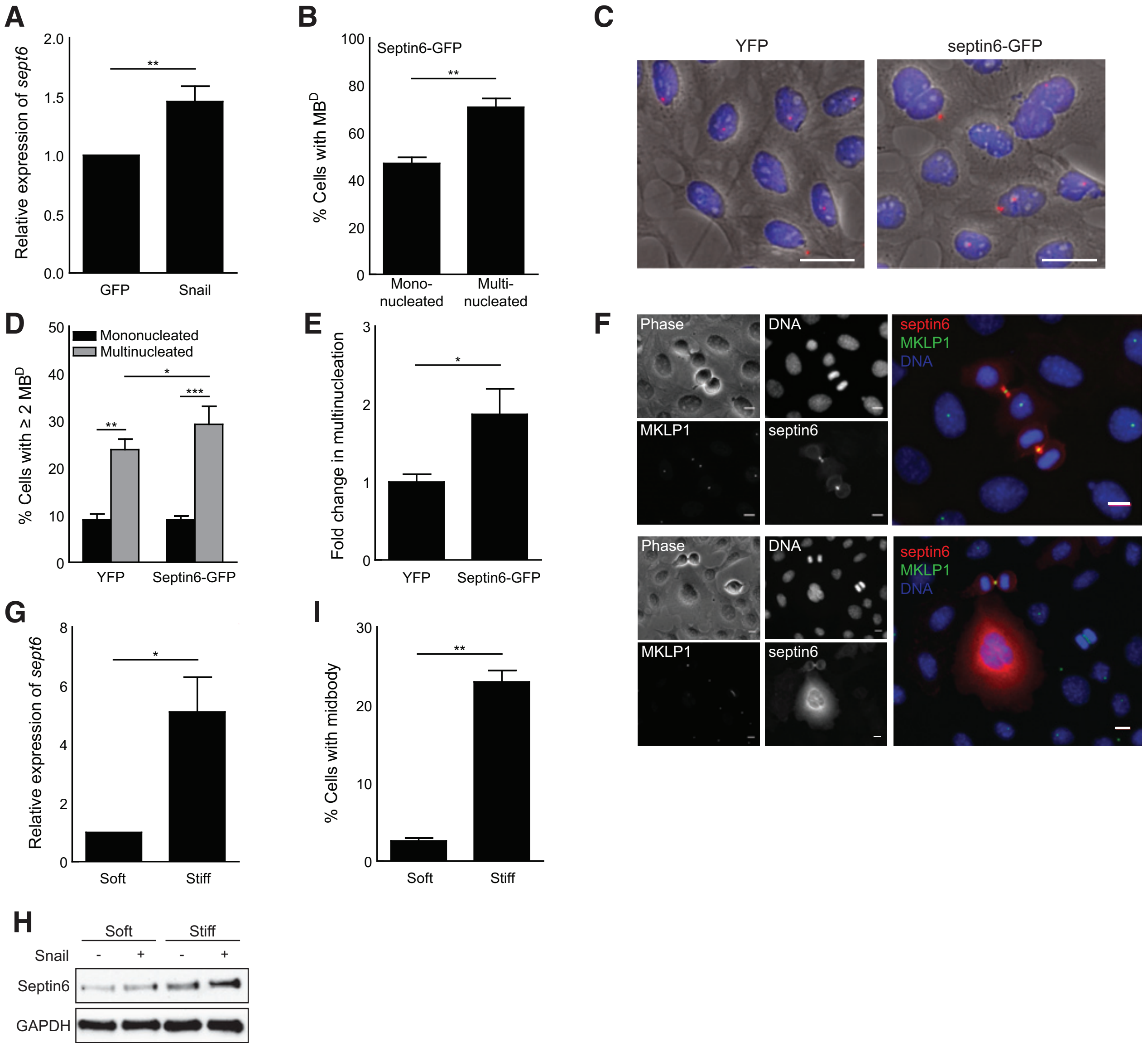

Figure 4.

Snail and substratum stiffness induce midbody persistence via septin-6. **A,** Ectopic expression of Snail causes an increase in the levels of septin-6 in SCp2 mouse mammary epithelial cells. Expressing a septin6–GFP fusion protein induces midbody persistence (**B**), midbody accumulation (**C** and **D**), and multinucleation (**E**). Red, MKLP1; blue, DNA. Scale bars, 50 μm. **F,** SCp2 mouse mammary epithelial cells expressing septin6-GFP show that septin-6 colocalizes with the midbody marker MKLP1. Red, septin6-GFP; green, MKLP1; blue, DNA. Scale bars, 10 μm. **G,** Cells cultured on stiff substrata express a higher level of septin-6 than cells on soft substrata. **H,** Ectopic expression of Snail (+) in SCp2 mouse mammary epithelial cells induces the expression of septin-6 protein on stiff but not soft substrata. Control cells (−) were transduced with GFP alone. **I,** A higher fraction of cells cultured on stiff substrata have MB^{D}s than cells on soft substrata. Cells were transfected with plasmid 48 hours before analysis. Shown are mean ± SEM for 3–10 experiments ($n > 800$ cells for **B** and **D**; $n > 800$ cells for **E**; $n > 200$ cells for **I**). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (**A**, **B**, **E**, **G**, **I**, paired *t* test; **D**, two-way ANOVA).

colocalized with the developing midbody as cells progressed through mitosis (Fig. 4F). These data suggest that septin-6 is specifically involved in Snail-induced multinucleation.

To test the hypothesis that substratum stiffness modulates signaling downstream of Snail, we asked whether stiffness affects the expression of septin-6 and induction of midbody persistence. As predicted, we found higher levels of septin-6 transcript (Fig. 4G) and protein (Fig. 4H) in cells cultured on stiff substrata than in those on soft substrata. In addition, quantification of MKLP1-positive puncta showed that a higher fraction of cells cultured on stiff substrata had MB^{D}s than cells cultured on soft substrata (Fig. 4I).

Stiffness regulates multinucleation through EMT signaling pathways

Because MMP3 leads to both EMT and multinucleation in a stiffness-dependent manner, we hypothesized that multinucleation might be a general response to EMT inducers, which commonly elevate the expression of Snail. To test this hypothesis, we treated cells with the EMT-inducer TGFβ (Fig. 5A) and quantified

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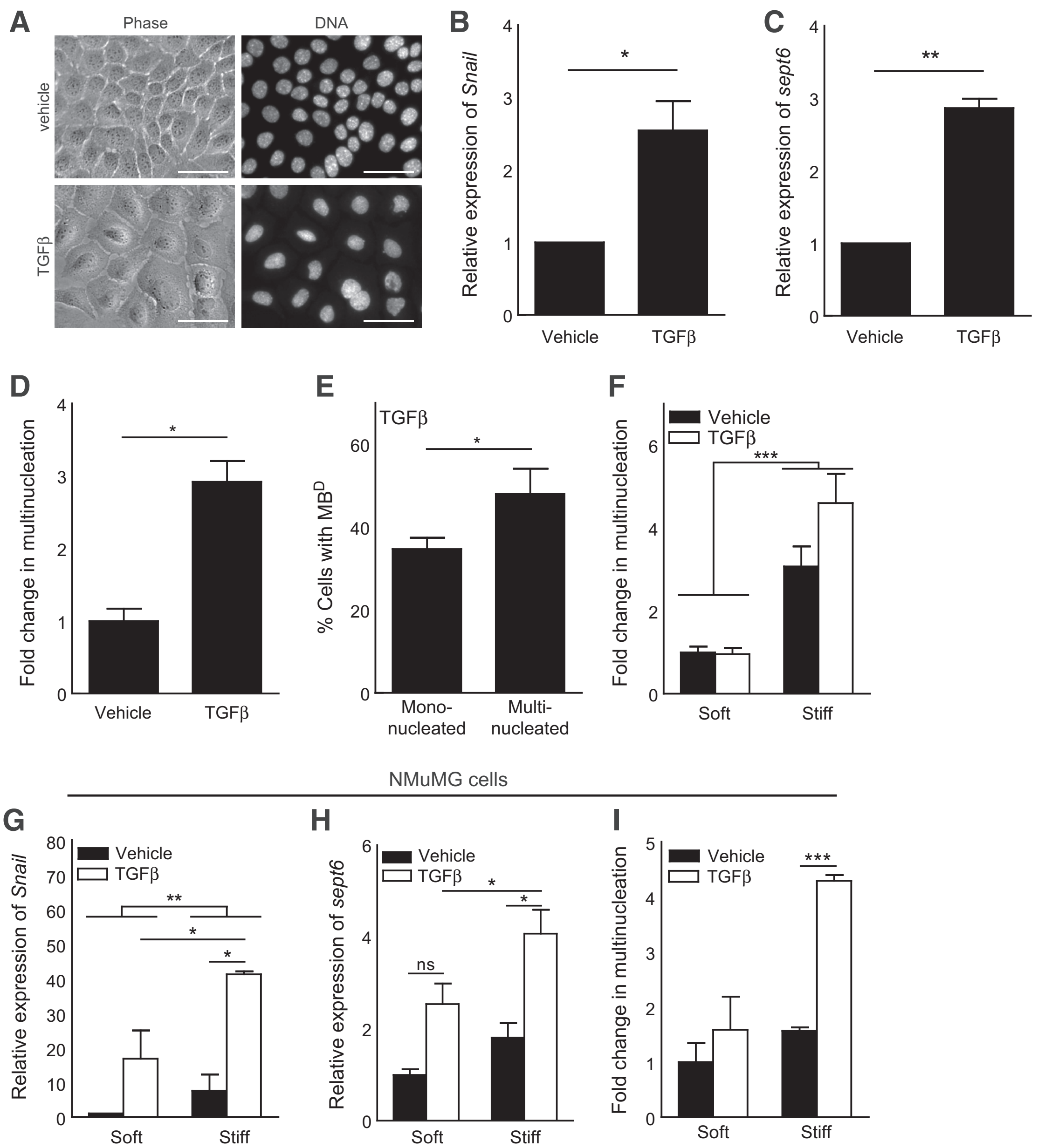

Figure 5.

TGFβ induces Snail, increases septin-6, and leads to multinucleation. **A,** SCp2 mouse mammary epithelial cells were cultured on soft or stiff substrata in the presence or absence of TGFβ. Treatment with TGFβ increases the levels of Snail (**B**) and septin-6 (**C**), and induces multinucleation (**D**). **E,** A higher fraction of multinucleated cells had MB^Ds than did mononucleated cells. **F,** TGFβ-induced multinucleation is blocked in cells cultured on soft substrata. Treatment with TGFβ increases the expression of Snail (**G**), septin-6 (**H**), and multinucleation (**I**) in NMuMG mouse mammary epithelial cells cultured on stiff substrata. Cells were exposed to TGFβ for 48 hours. Scale bars, 50 μm. Shown are mean ± SEM for 3–6 experiments ($n > 500$ cells for **D**; $n > 30$ cells for **E**; $n > 170$ cells for **F**). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (**B–E**, paired t test; **F–H**, two-way ANOVA).

subsequent levels of multinucleation. Similar to signaling downstream of MMP3, treatment with TGFβ enhanced the expression of Snail (Fig. 5B) and septin-6 (Fig. 5C). Furthermore, treatment with TGFβ resulted in significantly more multinucleated cells (Fig. 5D) than controls, and staining for MKLP1 revealed that a significantly higher fraction of multinucleated cells had MB^Ds than did mononucleated cells (Fig. 5E). As with MMP3, TGFβ-induced multinucleation was inhibited on soft substrata (Fig. 5F). Similarly, we found that treating NMuMG mouse mammary epithelial cells with TGFβ significantly enhanced the expression of Snail (Fig. 5G) and septin-6 (Fig. 5H) and increased multinucleation (Fig. 5I) only on stiff substrata. Altogether, our data suggest that the increased levels of Snail observed during the EMT program drive multinucleation on stiff substrata by upregulating septin-6, which increases midbody persistence and leads to abscission failure.

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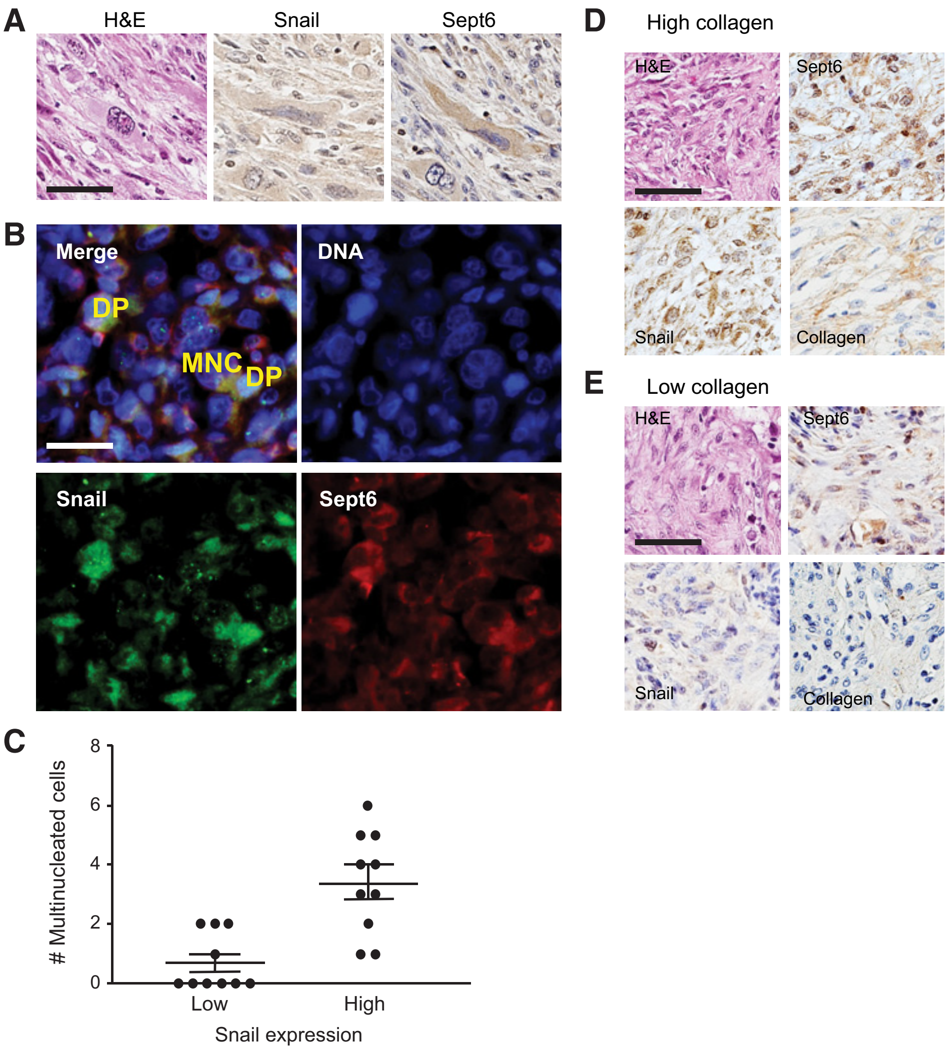

Figure 6.
Multinucleation is elevated in regions of high Snail expression in metaplastic carcinoma. **A,** IHC analysis for Snail and septin-6 in human breast metaplastic carcinoma. Scale bar, 100 μm. **B,** Immunofluorescence analysis of human breast metaplastic carcinoma reveals multinucleation (MNC) in cells double-positive (DP) for Snail and septin-6. Scale bar, 50 μm. **C,** Quantification of multinucleated cells in regions of high and low Snail expression. $P = 0.007$ (Mann–Whitney test). IHC analysis of human breast metaplastic carcinoma reveals elevated expression of Snail and septin-6 in regions of high collagen (**D**) compared with low collagen (**E**). Scale bars, 100 μm.

We also found that expression of Snail induced multinucleation in MCF10A human mammary epithelial cells (Supplementary Fig. S3A), a spontaneously immortalized line derived from fibrocystic breast tissue that is generally considered normal because the cells are not malignant (36). Consistent with our observations of the mouse mammary epithelial cell lines, we found that Snail only induced multinucleation of MCF10A cells when they were cultured on stiff substrata (Supplementary Fig. S3A) and that the multinucleated cells were associated with an increase in MB^Ds (Supplementary Fig. S3B). Curiously, culture on stiff substrata elevated the expression of septin-6 as well as septin-2 and -7 (Supplementary Fig. S3C), suggesting the possibility of a tighter co-regulation of the core septins in these cells.

Multinucleation is elevated in regions of high Snail expression in metaplastic carcinoma

To evaluate the relationship between EMT signaling, abscission failure, and multinucleation *in vivo*, we examined the expression of Snail and septin-6 and quantified multinucleation in human breast cancer samples. Histological analysis revealed Snail and septin-6 double-positive tumor cells in a metaplastic carcinoma sample, a rare cancer characterized by the presence of both epithelial- and mesenchymal-like cells, in which EMT is believed to be active (Fig. 6A; Supplementary Fig. S4A; refs. 37, 38). In regions containing high levels of Snail, nearly 4% of cells were multinucleated, compared with less than 1% of cells in regions with low levels of Snail (Fig. 6B and C). Relative collagen content is highly correlated with stiffness in mammary cancers (39, 40), and we found high expression of Snail and septin-6 specifically in tumor regions with high collagen (Fig. 6D and E). Consistently, the level of septin-6 expression correlates with degree of multinucleation (Supplementary Fig. S4B). These data are consistent with our experiments in culture, and suggest that EMT signaling, through Snail and septin-6, promotes multinucleation in stiff microenvironments.

Discussion

Mechanical signaling is known to regulate the cell cycle. For example, the orientation of the mitotic spindle is dictated by retraction fibers and the subcortical actin network in HeLa cells (12). Similarly, the spatial distribution of ECM proteins can

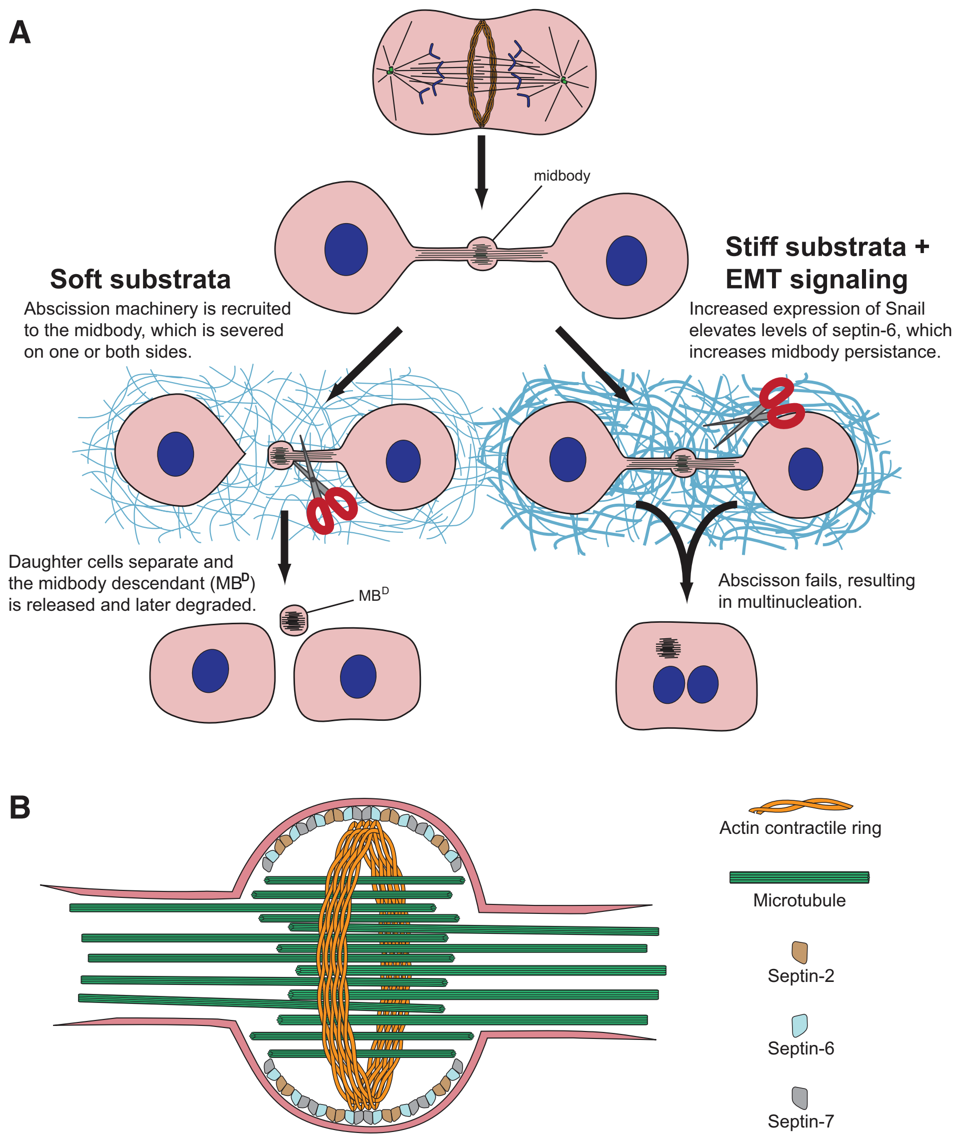

Figure 7.
Stiffness modulates multinucleation downstream of EMT signaling. **A,** EMT inducers MMP3 and TGFβ lead to increased expression of Snail, which increases expression of septin-6, causing midbody persistence, abscission failure, and multinucleation on stiff microenvironments only. **B,** Elevated levels of septin-6 downstream of Snail may disrupt the function of septin filaments, which help anchor the contractile ring to the plasma membrane.

determine the axis of division by regulating actin dynamics (13). Disruption of the cell cycle can result in cancer, but little is known about how tissue mechanics affects stability of the genome. Here, we uncovered a mechanotransduction pathway that links the mechanical properties of the microenvironment with EMT and genomic instability through disruption of the final stage of cytokinesis. Exposure to either MMP3 or TGFβ leads to increased expression of Snail, but has divergent effects on other EMT-associated transcription factors (Supplementary Fig. S5A and S5B). Snail elevates the levels of septin-6 in cells on stiff microenvironments that are characteristic of breast tumors. Septin-6 overexpression increases midbody persistence, causing a failure of midbody abscission and subsequent multinucleation (Fig. 7A).

Septin filaments help anchor the actomyosin contractile ring to the plasma membrane (31). Maintaining homeostatic levels of septins-2, -6, and -7, the core filament-forming septins, is necessary to avoid mitotic errors, aneuploidy, and multinucleation (33–35). The behavior of the core septins seems closely linked; specifically depleting one can cause loss of the others (32, 33, 35). Thus, although septin variants can perform unique roles (33, 41), concomitant loss makes this difficult to determine for the core septins. Frequent mutations and dysregulation of septins in cancer (42) necessitates continued investigation of the individual functions of the variants. It was recently determined that septins-2 and -7 contribute to both tumor suppression (43) and increased proliferation and invasion of cancer cells (44). Unique functions of septin-6 have mainly been studied in developmental contexts (45). We found that septin-6 is independently upregulated by Snail, and further, that excess septin-6, which localizes to the midbody, contributes to an increased midbody persistence, resulting in abscission failure and the eventual coalescence of the two daughter cells. These data lead us to predict that elevated levels of septin-6 downstream of Snail disrupt the balance of core septins necessary for the filament to function correctly (Fig. 7B). The specific role of septin-6 in abscission failure will be the subject of a future study.

Given that Snail most commonly behaves as a transcriptional repressor, we wondered how it might induce the expression of septin-6. We found a putative Snail-binding site (CAGGTG; ref. 46) within 1 kb upstream of the predicted transcription start sites of septin-6 in mouse and human (Supplementary Table S2). Recent studies have demonstrated that Snail can act as a transcriptional activator by interacting with Twist (47) or CREB-binding protein (48). In addition, stiff matrices enhance Twist translocation to the nucleus (49) and the expression of CREB-binding protein (Supplementary Fig. S5C). With these considerations in mind, it is tempting to speculate that on stiff substrata, Snail cooperates with another factor to drive the expression of septin-6.

Our study adds to recent compelling evidence that TGFβ can induce genomic instability by disrupting cell division (10). Traditionally, TGFβ has been considered to be a suppressor of genomic instability (50) through its signaling via SMADs to activate the DNA damage response (DDR). Nonetheless, TGFβ signaling is elevated in many malignancies and correlates with enhanced metastasis (51). Because treatment with TGFβ does not alter the expression of MMP3 in our system (Supplementary Fig. S5D), multinucleation appears to be specific to its induction of Snail. It is likely that the mechanical properties of the microenvironment play a role in orchestrating the final outcome of exposure to TGFβ, either by SMAD-mediated induction of DDR machinery or Snail-mediated induction of midbody persistence.

Metaplastic carcinoma of the breast, a rare subtype of cancer that accounts for less than 1% of breast cancer cases, is characterized by biphasic lesions containing both epithelial and mesenchymal-derived cells. These tumors are identified as rapidly growing lesions of high mammographic density (52), often contain regions of spindle-shaped, vimentin-positive cells, indicative of active EMT, and express increased reactive ECM molecules and mediators of EMT (53). In addition, metaplastic carcinomas of the breast often harbor multinucleated cells (54, 55) and mutations in p53 (56). While this tumor type is relatively rare, our findings of a link between Snail, septin-6, and multinucleation may be reflective of protumorigenic processes that occur in more limited regions of a broader range of breast cancers. Moreover, because tumors are stiffer than normal tissue, our findings suggest that therapies aiming to return tumor tissue to physiologically normal stiffness may prevent multinucleation and aneuploidy, characteristic of genomic instability. This theory has been implicated in the clinic, and chemopreventative treatment with tamoxifen has been found to lead to a reduction in mammographic density (57), which correlates with ECM density and tissue stiffness (58). Our findings suggest that cancer prevention strategies, which seek to block the earliest steps of malignant transformation, would be more effective if optimized to target the contextual cues that drive multinucleation and resultant aneuploidy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.K. Simi, D.C. Radisky, C.M. Nelson
Development of methodology: A.K. Simi, S. Zhang, T. Hsia, D.C. Radisky
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.K. Simi, A.A. Anlaş, M. Stallings-Mann, T. Hsia, M. Cichon, D.C. Radisky
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.K. Simi, A.A. Anlaş, M. Stallings-Mann, S. Zhang, T. Hsia, M. Cichon, D.C. Radisky
Writing, review, and/or revision of the manuscript: A.K. Simi, S. Zhang, D.C. Radisky, C.M. Nelson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.K. Simi, S. Zhang, M. Cichon, C.M. Nelson
Study supervision: C.M. Nelson

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A Soft Microenvironment Protects from Failure of Midbody Abscission and Multinucleation Downstream of the EMT-Promoting Transcription Factor Snail

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