

ORIGINAL RESEARCH ARTICLE

Apical cell protrusions cause vertical deformation of the soft cancer nucleus

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Abstract

Breast cancer nuclei have highly irregular shapes, which are diagnostic and prognostic markers of breast cancer progression. The mechanisms by which irregular cancer nuclear shapes develop are not well understood. Here we report the existence of vertical, apical cell protrusions in cultured MDA-MB-231 breast cancer cells. Once formed, these protrusions persist over time scales of hours and are associated with vertically upward nuclear deformations. They are absent in normal mammary epithelial cells (MCF-10A cells). Microtubule disruption enriched these protrusions preferentially in MDA-MB-231 cells compared with MCF-10A cells, whereas inhibition of nonmuscle myosin II (NMMII) abolished this enrichment. Dynamic confocal imaging of the vertical cell and nuclear shape revealed that the apical cell protrusions form first, and in response, the nucleus deforms and/or subsequently gets vertically extruded into the apical protrusion. Overexpression of lamin A/C in MDA-MB-231 cells reduced nuclear deformation in apical protrusions. These data highlight the role of mechanical stresses generated by moving boundaries, as well as abnormal nuclear mechanics in the development of abnormal nuclear shapes in breast cancer cells.

KEYWORDS

breast cancer, cancer nucleus, lamin A/C, microtubule, nuclear shape, protrusions

1 | INTRODUCTION

The nucleus is irregularly shaped in cancer that has been known since the 1860s (Beale, 1860). In breast cancers, nuclear surface abnormalities are graded and correlate with clinical aggressiveness and prognosis (Bussolati, Marchio, Gaetano, Lupo, & Sapino, 2008; Elston & Ellis, 1991). Abnormal nuclear morphologies have strong diagnostic and prognostic importance (Bussolati, Asioli, Annaratone, Sapino, & Marchio, 2014; Giardina et al., 1996; Haroske et al., 1996). Diagnostically useful features in breast cancer include nuclear lobulation, grooves, clefts, or folds in the envelope, changes in nuclear size, alterations in chromatin organization (Imbalzano et al., 2013; Zink, Fischer, & Nickerson, 2004), and changes in number or appearance of nucleoli. Although cancer diagnostics depends

critically on microscopic evaluation of patient biopsies, the molecular mechanisms for the abnormal nuclear shapes in cancer are not fully understood (Denais & Lammerding, 2014).

Abnormalities in nuclear morphologies may occur in part due to modifications of higher order chromatin structure and associated alterations in gene expression that are present during breast cancer progression (Barutcu et al., 2016; Fritz et al., 2018). Consistent with this notion, increasing the amount of euchromatin or decreasing heterochromatin through modification of histone modification states mechanically softens the nucleus and causes nuclear blebbing (Stephens, Liu, et al., 2017). Similarly, knockdown of the chromatin remodeling enzymatic subunit BRG1 in breast epithelial cells induces nuclear lobulations and invaginations (Imbalzano et al., 2013). In addition to chromatin alterations, loss of nuclear lamins, a common

occurrence in breast cancer, can also induce abnormal nuclear morphologies (Capo-chichi et al., 2011).

The nucleus in cells is constantly acted upon by mechanical stresses (Lele, Dickinson, & Gundersen, 2018). One potential source of mechanical stresses on the nuclear surface is the motion of cell boundaries proximal to the nuclear surface, which produce local deformations in the cell nucleus (Alam et al., 2015; Li et al., 2015). We have shown previously that the process of cell spreading amplifies nuclear shape abnormalities in MDA-MB-231 breast cancer cells, but not in nonmalignant human mammary epithelial cells (MCF-10A; Tocco et al., 2018). These findings suggest the hypothesis that cell protrusions may contribute, at least in part, to local abnormal nuclear deformations in breast cancer cells. In this paper, we report the existence of vertical, apical cell protrusions in cultured MDA-MB-231 breast cancer cells that are absent in MCF-10A cells. These protrusions were associated with vertically upward nuclear deformations. Dynamic imaging of cell spreading confirmed that nuclear deformations develop as a result of dynamic cell protrusion. Taken together, these results highlight the importance of cell-generated mechanical stresses in the development of abnormal nuclear shapes in breast cancer.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transfection

MDA-MB-231 cells (ATCC HTB-26) were cultured in an incubator maintained at 37°C with 5% CO₂ under full growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% donor bovine serum (Gibco), 1% penicillin-streptomycin (Gibco), 1% nonessential amino acids (Gibco), and 1% L-glutamine (Gibco). MCF-10A cells were cultured in full growth medium consisting of DMEM/F12 (Gibco) with 5% Donor horse serum, 20 ng/ml epidermal growth factor (Corning, Tewksbury, MA), 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, and 1% penicillin-streptomycin. Transfections of GFP-Lamin A, GFP-H1.1 or RFP-lifeact (ibidi, Martinsreid, Germany) were performed with Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) in Opti-MEM serum-free media (Thermo Fisher Scientific) following the manufacturer's suggested protocols. Transduction of Ad-NLS-GFP (a gift from Dr. Daniel Conway, originally from Vector Biosystems Inc., Malvern, PA) was performed with polybrene at final concentration of 5 µg/ml in normal culture medium.

2.2 | Drug treatment and cell fixation

MDA-MB-231 or MCF-10A cells were cultured on glass-bottom dishes (WPI, Sarasota, FL) coated with 5 µg/ml fibronectin overnight at 4°C. After the cells were allowed to grow for >24 hr, the cell medium was replaced with full growth medium containing 5 µM nocodazole (Sigma-Aldrich, St. Louis, MO), 50 µM blebbistatin (EMD Millipore, Billerica, MA), both 5 µM nocodazole and 50 µM blebbistatin, 100 nM cytochalasin D, or 0.3% dimethyl sulfoxide were used as a control. The cells were incubated for 1 hr in 37°C at 5% CO₂ concentration, after which the cells were fixed by the following steps:

Simultaneous fixation and extraction by a solution of 0.5% glutaraldehyde, 0.8% formaldehyde, and 0.5% Triton X-100 in phosphate-buffered saline (PBS), fixation for a further 10 min by 1% formaldehyde in PBS, incubation for 10 min with freshly prepared 1% (w/v) sodium borohydride (NaBH₄, Thermo Fisher Scientific) in PBS, and blocking with 1% bovine serum albumin (BSA) in PBS for 30 min. The cells were rinsed with sterile PBS in between each step, and all steps were performed at room temperature. To stain for microtubules, cells were incubated with 2 µg/ml polyclonal anti-alpha-tubulin from rabbit (Abcam, Cambridge, MA) in 1% BSA for 1 hr, followed by incubation with an Alexa Fluor 594 goat antirabbit secondary antibody at 4 µg/ml for 1 hour, also in 1% BSA. F-actin and the nucleus were stained simultaneously by room temperature incubation of the cells with phalloidin-Alexa Fluor 488 (Life Technologies, Grand Island, NY) at a dilution of 1:40 and Hoechst 33342 (Life Technologies) at 1:200 dilution in 1% BSA in PBS.

2.3 | Partial trypsinization and fixation

Cells were partially trypsinized to round the cells in the absence of an agent depolymerizing microtubules. Cell culture medium was removed, and the cells were rinsed once with 37°C PBS. The PBS was removed and 0.25% trypsin (Corning) at 37°C was added for 30 s, at which point 37°C full growth cell medium was added to the dish to inactivate the trypsin. All liquid was then removed from the dish, and the samples were fixed with 4% formaldehyde for 15 min. The samples were blocked in 1% BSA and stained with Hoechst 33342 and phalloidin-Alexa Fluor 488, diluted 1:200 and 1:40 respectively, in PBS with 1% BSA.

2.4 | Apical protrusion analysis and statistical analysis

Image stacks of fixed cells were visually analyzed in FIJI, a distribution of ImageJ (NIH). Cells were categorized by whether an apical protrusion as defined in Section 3 was visible in a profile view. Cells in which the entire nucleus was in a protruding part were not considered as having apical protrusions and were not counted as such.

2.5 | Live cell imaging

MDA-MB-231 cells were seeded on glass-bottom dishes and transfected with GFP-histone H1.1 and/or RFP-Lifeact to visualize the nucleus and F-actin, respectively. In some experiments, MDA-MB-231 cells were transduced with Ad-NLS-GFP to visualize the nucleus. Invitrogen™ Fast Dil™ (D-7756) lipophilic tracer was used to visualize the cell membrane. The transfected cells were allowed to spread for >24 hr. The cells were imaged on a Nikon TE2000/Ti2 microscope (Nikon, Melville, NY) with a Nikon A1 confocal unit using a ×60 oil immersion objective. The confocal images were collected at 1-micron step-size in the z-direction. The Z-stacks were used to reconstruct x-y or x-z cross-sections to visualize the dynamics using the Nikon elements software.

3 | RESULTS

Using confocal microscopy, we imaged vertical nuclear shapes in MDA-MB-231 cells (referred to henceforth as MB-231 cells) that are a triple-negative breast cancer cell line with a mesenchymal-like phenotype (Charafe-Jauffret et al., 2006; Chavez, Garimella, & Lipkowitz, 2010; Lacroix & Leclercq, 2004). In two-dimensional culture, single nonmalignant mammary epithelial MCF-10A cells that are considered to have a near-normal phenotype (Imbalzano, Tatarikova, Imbalzano, & Nickerson, 2009; Merlo, Basolo, Fiore, Duboc, & Hynes, 1995; Miller et al., 1993; Soule et al., 1990) exhibited a typical vertical shape (x-z profile) with the cell being tallest over the nucleus and becoming gradually flatter farther away from the nucleus (Figure 1) consistent with our previous observations (Li et al., 2015; Lovett, Shekhar, Nickerson, Roux, & Lele, 2013; Neelam, Hayes, Zhang, Dickinson, & Lele, 2016). The nucleus resembled a flattened ellipsoid in these cells. While around 90% of

MB-231 cells exhibited a similar x-z profile, in the rest of the MB-231 cells, a cell membrane protrusion was present at the apical surface (hereafter called apical cell protrusion, Figure 1). In all MB-231 cells with apical protrusions, the apical surface of the nucleus was deformed into the apical cell protrusion. The deformed nuclei displayed a negative curvature near the opening to the apical cell protrusion mimicking the negative curvature in the cell protrusion (marked by white arrows in Figure 1). Notably, in the x-y plane, cells with such apical protrusions were well-spread, which gave a “Mexican-hat” type appearance to cells in the x-z plane.

To determine why apical cell protrusions form in MB-231 cells, we treated cells with cytoskeletal inhibitors. Upon 1 hr of treatment with 5 μ M nocodazole to depolymerize the microtubule network, apical cell protrusions were substantially enriched in MB-231 cells, occurring in over 50% of the treated cells compared with only about 10% in untreated cells (Figure 2a and Table 1). Apical cell protrusions were also enriched in MCF-10A cells upon nocodazole treatment but

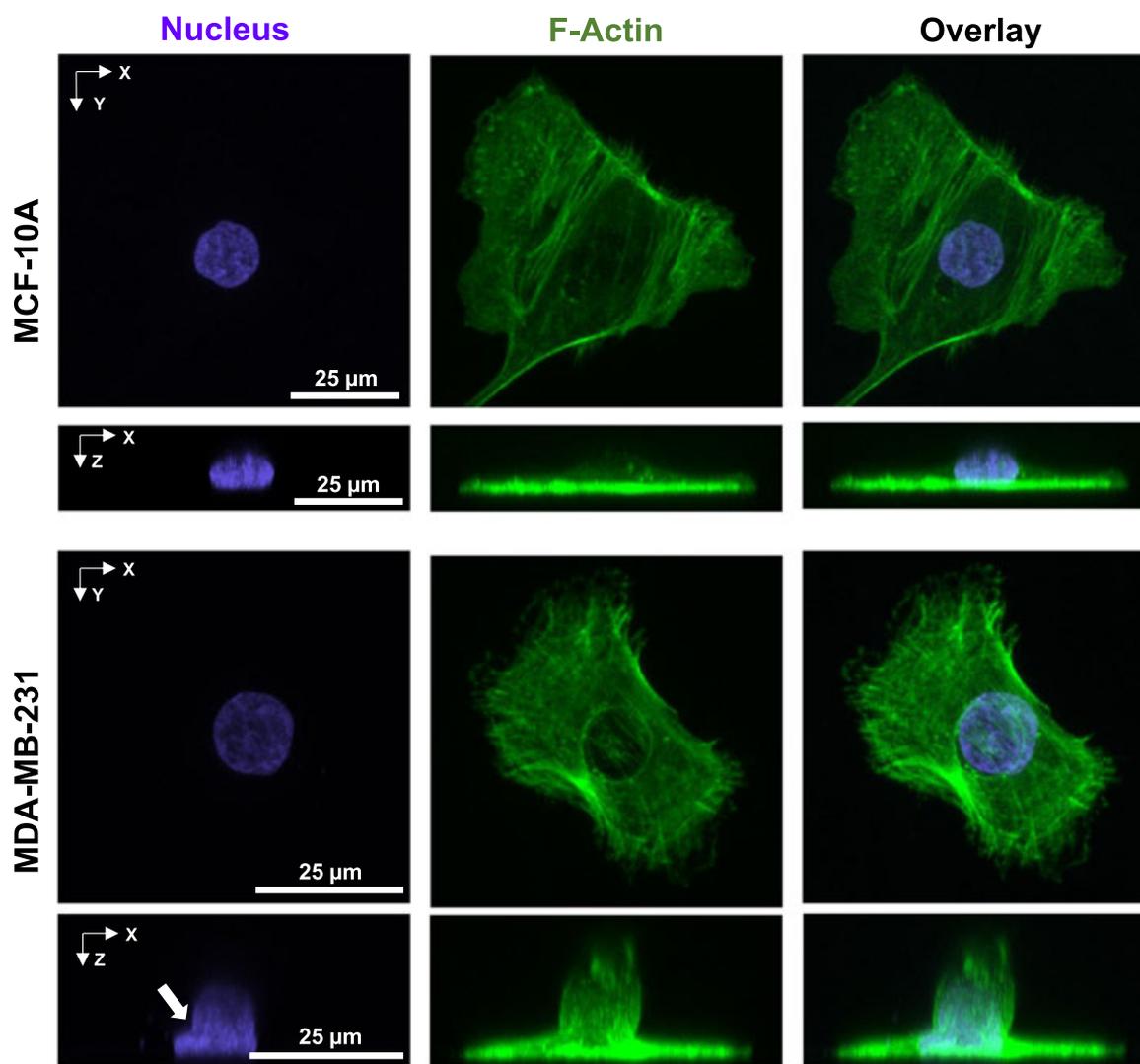


FIGURE 1 The presence of apical cell protrusions with associated nuclear deformation in MB-231 cells. Images show representative fluorescent images of x-y and x-z cross-sections of an MCF-10A cell (top) and MB-231 cells (bottom). The MB-231 cell has a “Mexican-hat” type appearance due to the local apical cell protrusion in an otherwise well-spread cell [Color figure can be viewed at wileyonlinelibrary.com]

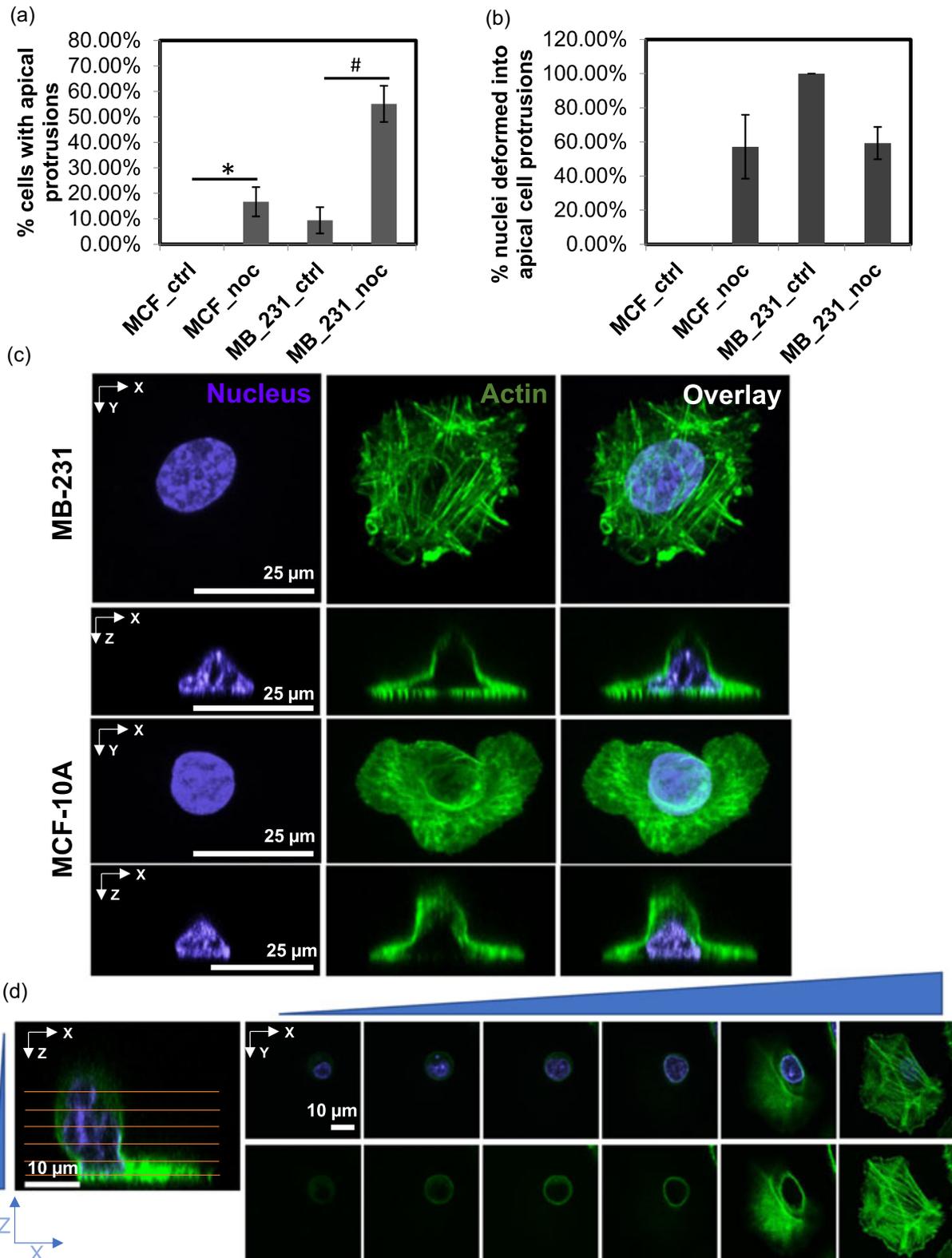


FIGURE 2 Nocodazole treatment enriches apical cell protrusions in MB-231 cells. (a) Percentage of fixed cells with apical protrusions under various. The χ^2 test was used to test statistical differences. *or # $p < 0.05$. $N > 30$ cells for each condition (MCF_ctrl: untreated MCF-10A cells; MB_231_ctrl: untreated MB-231 cells; MCF_noc and MB_231_noc: MCF-10A cells and MB-231 cells, respectively, treated with 5 μ M nocodazole). Error bars indicate SEM. (b) Percentage of cells with an apical cell protrusion in which the nuclear shape was vertically deformed into the protrusion. Error bars are SEM. (c) Representative fluorescent images of an MB-231 and MCF-10A cell treated with nocodazole for 1 hr, showing the apical cell protrusion and associated vertical nuclear deformation. (d) Fluorescent images show confocal images at different planes (marked by horizontal lines) of an MB-231 cell treated with nocodazole. The apical protrusion is present in an otherwise well-spread cell. SEM: standard error of the mean [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Occurrence of apical cell protrusions and associated nuclear shape changes

Cell and Condition	% cells with apical protrusions
MCF_ctrl	0%
MCF_noc	17% ± 6%*
MB_231_ctrl	9% ± 5%
MB_231_noc	55% ± 7%##
MB_231_bleb	0%
MB_231_bleb_noc	15% ± 6%
MB_231_tryp 30 s	11% ± 5%
MB_231_CytoD	17% ± 6%
MB_231_CytoD_noc	39% ± 8%###
MB_231_GFPLA_noc	81% ± 5%##

Note. All statistical comparisons (χ^2 test with Bonferroni correction) are with respect to corresponding control for a given cell type). bleb: blebbistatin; ctrl: control; CytoD: cytochalasin D; GFPLA: GFP-Lamin A; noc: nocodazole; SEM: standard error of mean; tryp 30 s: trypsinization for 30 s. $N > 30$ cells for each condition. Values represent mean ± SEM.

* $p < 0.05$

$p < 0.01$

$p < 0.001$

to a much smaller extent (Figure 2a and Table 1). In both MB-231 cells and MCF-10A cells, apical cell protrusions were associated with a vertically upward nuclear deformation (Figure 2b,c) in about 60% of cells with apical protrusions.

We have previously shown that vertical nuclear flattening correlates inversely with cell spreading area in fibroblasts (Li et al., 2015), with rounded cells displaying rounded vertical nuclear shapes. Therefore, one possibility is that the presence of apical cell protrusions in a fraction of the MB-231 cell population is due to cells which have not reached a steady state in the cell spreading process after mitosis. Furthermore, nocodazole treatment might result in cell rounding, thereby creating apical cell protrusions and hence deformation of the apical nuclear surface. We, therefore, compared the degree of cell spreading between control and nocodazole-treated cells (Figure S1). Although nocodazole treatment severely rounded up some MB-231 cells in culture, we could observe several well-spread cells which had apical cell protrusions and associated nuclear deformation (an example of a confocal series of images is shown in Figure 2d). There was no statistically significant difference between the area of cell spreading between control and nocodazole-treated MB-231 cells that displayed apical protrusions. This result argues against the hypothesis that enrichment of apical cell protrusions and vertical nuclear deformation in nocodazole-treated cells necessarily requires cell rounding. As trypsinization of cells can also cause rounding of the vertical nuclear cross-section (Li et al., 2015), we further challenged the above hypothesis by causing partial rounding of cells through trypsinization for 30 s before fixation. Partial trypsinization of cells did not induce the development of apical cell protrusions in MB-231 cells, nor deformation of the apical nuclear surface. Trypsinization

also did not change the frequency of apical cell protrusions in MB-231 cells (Table 1).

Cell protrusions in the x-y plane of the cell proximal to the nuclear surface cause local nuclear deformations in the direction of protrusions (Alam et al., 2015; Tocco et al., 2018). Relaxation of cell protrusions causes relaxation of nuclear deformations. To test if vertical apical protrusions precede vertical nuclear deformation, we imaged the dynamic formation of apical cell protrusions and the vertical shape of the NLS-GFP expressing nucleus using confocal fluorescence microscopy. In these experiments, we treated MB-231 cells with nocodazole to enrich apical cell protrusions.

Figure 3a shows the dynamic formation of an apical protrusion upon nocodazole addition at time $t = 0$ min, and the corresponding x-z nuclear profiles tracked by staining lipid membranes with Dil (a lipophilic tracer used to visualize the cell membrane). The nucleus can be seen to round up vertically with changes to the vertical cellular shape. At around 20 min, a small apical protrusion is seen to develop that subsequently becomes large and a major portion of the nucleus is drawn into the protrusion. These results demonstrate that the apical protrusions form first, followed by vertically upward deformation of the nucleus.

We also tracked the F-actin network using RFP-Lifeact during the development of the vertical protrusion (Figure 3b), observed similar behavior where the vertical shape of the nucleus roughly mimicked the vertical shape of the cell. Extension of the apical surface of the nucleus extended the apical surface, whereas relaxation caused the apical nuclear surface to relax back. No stress fibers were visible in these cells on top of the nucleus. Inward movement of the boundaries in the basal plane and their outward movement in the apical plane correlate with changes in the shape of the nucleus.

Remarkably, the lifetime of the protrusions was several hours (Figure S2) and they were observed to move across the cell surface in a seemingly random fashion, sometimes changing in size. In response, the apical surface of the nucleus stayed deformed for time scales of hours before collapsing back due to loss of the protrusion (Figure S2).

Microtubule depolymerization is known to increase actomyosin contractility through activation of the GTPase RhoA (Burakov, Nadezhkina, Slepchenko, & Rodionov, 2003; Wittmann & Waterman-Storer, 2001). We hypothesized, therefore, that the enrichment of apical protrusions in microtubule disrupted cells may be caused by nonmuscle myosin II (NMMII) mediated contraction of the cortical F-actin cytoskeleton. If apical cell protrusions are dependent on NMMII, then this would also explain their enrichment upon nocodazole treatment. F-actin was localized along the periphery of these protrusions whereas phosphorylated myosin (visualized with a phospho-specific antibody) was found to be present in a diffuse pattern in the protrusion with slight enrichment at parts of the periphery (Figure 4a). Treatment of MB-231 cells with blebbistatin to inhibit myosin activity completely suppressed apical cell protrusions (Figure 4b and Table 1). To determine if the increase in apical protrusions in microtubule disrupted cells was NMMII dependent, we treated MB-231 cells with blebbistatin and nocodazole together. The occurrence of apical cell protrusions was again greatly reduced

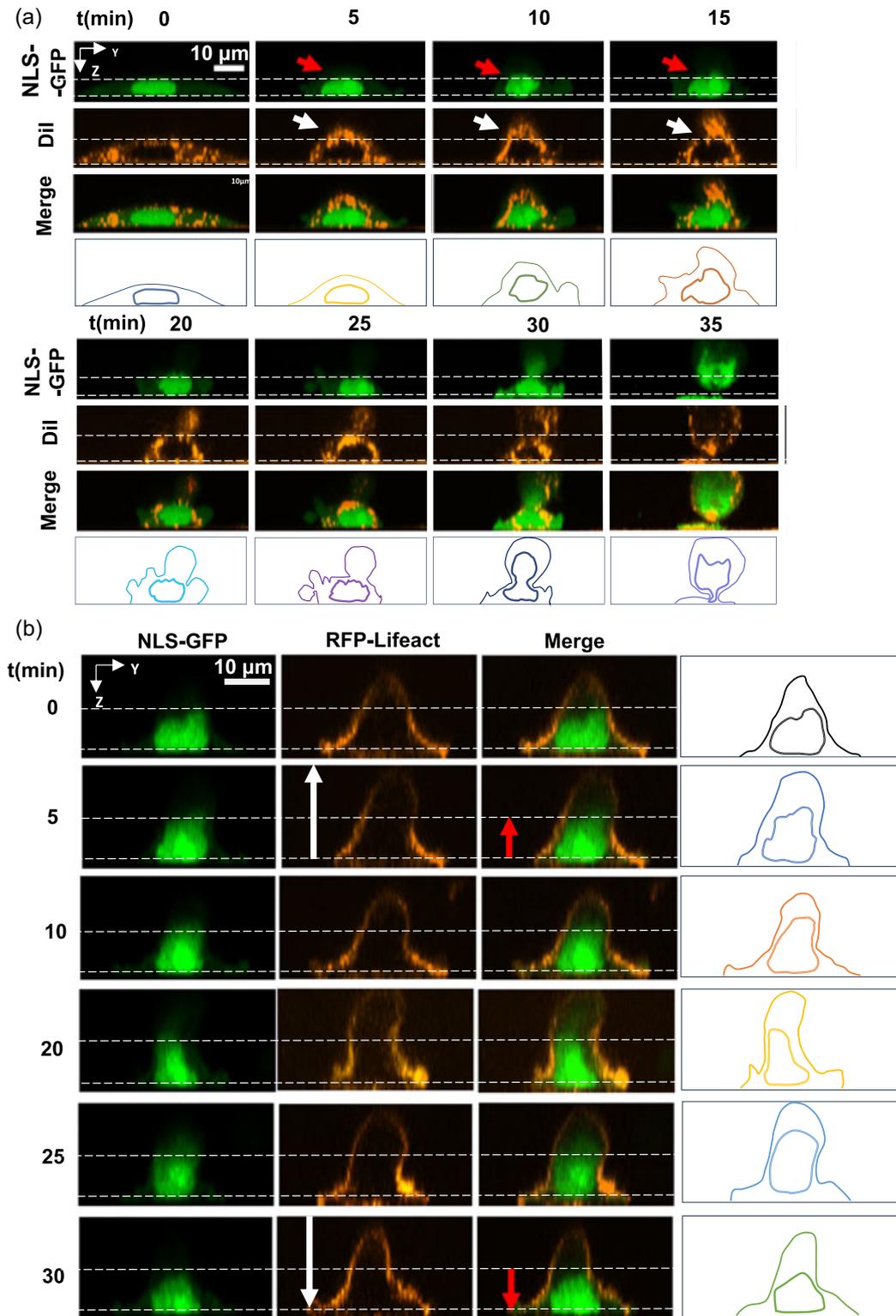


FIGURE 3 Apical cell protrusion precedes vertical nuclear deformation. (a) Reconstructed fluorescent time-lapse images of the vertical cross-section of a live cell expressing NLS-GFP and treated with lipophilic tracer Dil showing the formation of a vertical protrusion in the cell membrane near the nucleus (white arrows) and the consequent deformation of the nucleus (red arrows) after treatment with 5 μM nocodazole at time $t = 0$ min. For reference, vertical dashed lines indicate the initial position of the nucleus and cell membrane. (b) Reconstructed fluorescent time-lapse images of the vertical cross-section of a living cell expressing NLS-GFP and RFP-LifeAct showing the formation and retraction of a vertical cell protrusion near the nucleus (white arrows) and the consequent deformation of the nucleus (red arrows) after treatment with nocodazole at $t = 0$ min. Vertical dashed lines indicate the initial position of the nucleus for reference [Color figure can be viewed at wileyonlinelibrary.com]

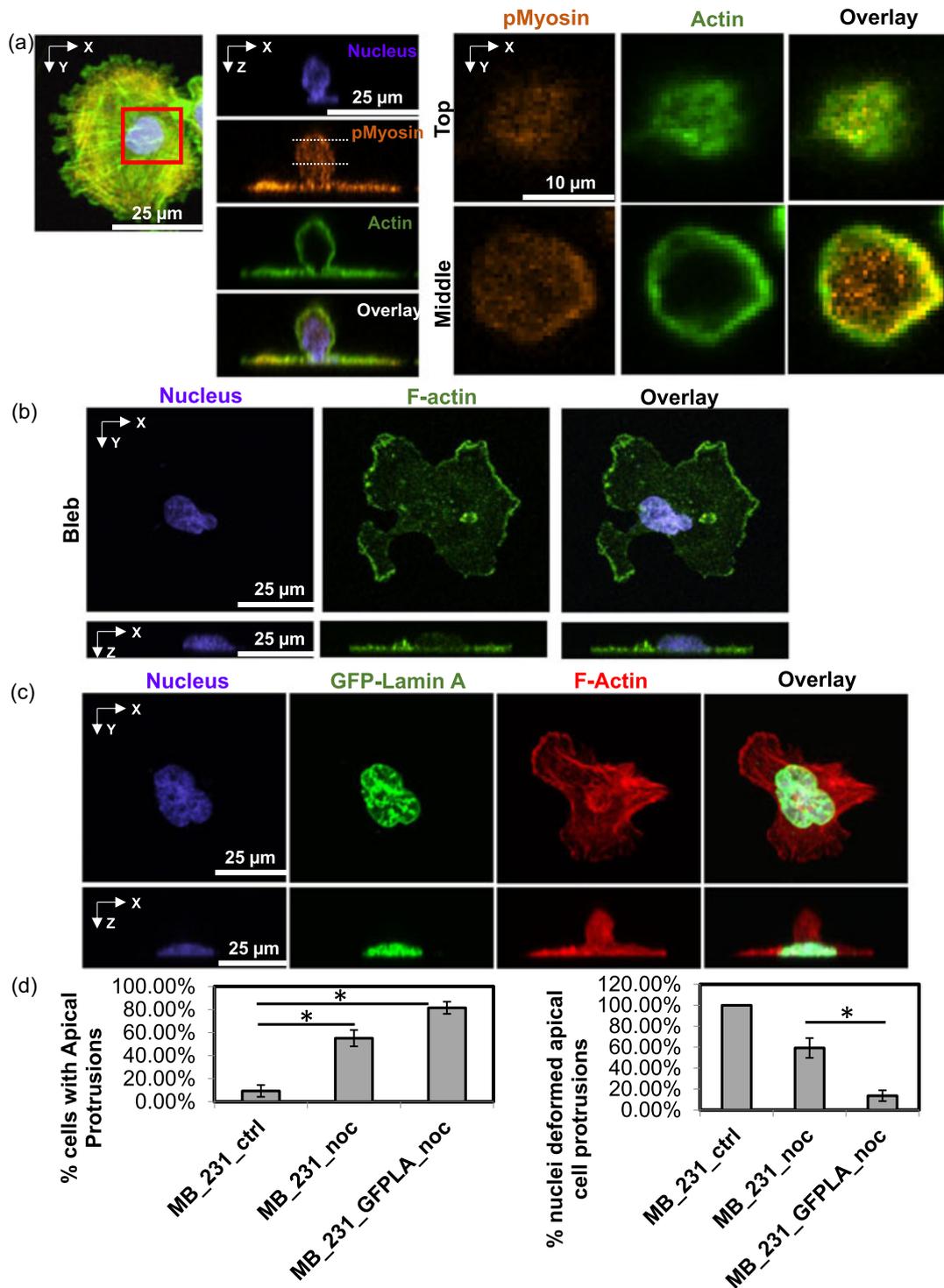


FIGURE 4 Apical cell protrusions are NMMII dependent, whereas vertical nuclear deformation is reduced upon overexpression of GFP-Lamin A. (a) An MB-231 cell treated with nocodazole for 1 hr before fixation and stained for pMyosin (orange), F-actin (green) and nucleus (blue). Inset at two z-positions (top and middle planes) shows the distribution of F-actin and pMyosin in the apical cell protrusion. (b) Fluorescent images show an example of MB-231 cell treated with blebbistatin, which abrogated the apical cell protrusion. (c) Images show a GFP-Lamin A expressing cell treated with nocodazole for 1 hr before fixation. An apical protrusion is clearly visible, but with no vertically upward deformation. (d) Bar plot shows the frequency of cells with apical protrusions of MDA-MB-231 cells (left) and frequency of nuclei deformed into the apical cell protrusions. (MB_231_ctrl: untreated MB-231 cells; MB_231_noc: MB-231 cells treated with 5 μM nocodazole; MB_231_GFPLA_noc: MB-231 cells expressing GFP-Lamin A and treated with 5 μM nocodazole). Error bar is SEM. At least 32 samples from 3 different dishes are analyzed. The χ^2 test with Bonferroni correction was used to test statistical differences. $*p < 0.05$. SEM: standard error of the mean [Color figure can be viewed at wileyonlinelibrary.com]

compared with treatment with nocodazole alone and was similar to the occurrence in untreated cells (Table 1). Collectively, these results suggest that apical cell protrusions are NMMII dependent.

As MB-231 cells have low levels of lamin A/C (Matsumoto et al., 2015) which softens the nucleus (Denais & Lammerding, 2014), we hypothesized that overexpressing lamin A/C in these cells will reduce the apical nuclear protrusions. Overexpression of GFP-lamin A/C in these cells abolished the nuclear deformations associated with apical protrusions, although the occurrence of apical protrusions themselves was increased (Figure 4c,d and Table 1). These results are consistent with the hypothesis that the MB-231 nucleus is deformed upward into the apical protrusion because it is soft due to low levels of lamin A/C (Davidson, Sliz, Isermann, Denais, & Lammerding, 2015; Neelam et al., 2015; Stephens, Banigan, Adam, Goldman, & Marko, 2017).

4 | DISCUSSION

We report the existence of vertically upward protrusions in spread breast cancer cells. These cells resemble a "Mexican-hat" in the x-z plane. The protrusions are associated with apical nuclear deformations. The enrichment of apical MB-231 cell protrusions after nocodazole treatment was abrogated upon NMMII inhibition. This suggests that enrichment of protrusions in nocodazole-treated cells may be due to the well-known upregulation of NMMII activity due to microtubule depolymerization.

Although the mechanism for the generation of apical cell protrusions is not clear, it is possible that NMMII-mediated contraction extrudes the apical cell membrane and associated cortex upward. The protrusions are dynamic and seem to change in orientation along the apical cell surface but are persistent over time scales of hours. The corresponding deformations of the apical nuclear surface correlate with the presence (or absence) of protrusions and persist over similarly long times (Figure 4 and Figure S2).

We have previously suggested that motion of boundaries away from or toward the nuclear surface can exert tensile or compressive stresses that pull or push on the nuclear surface (reviewed in Lele et al., 2018). The dynamic behaviors of the apical surface of the nucleus in response to the moving apical cell protrusions appear consistent with this model. How the apical protrusions might generate stress on the nucleus is not clear, but we have previously proposed that it is due to frictional transmission of stress between the cell boundary and the nuclear surface, likely through F-actin filaments (Li et al., 2015). Along these lines, disruption of F-actin by treatment with cytochalasin D in MB-231 cells caused severe cell rounding and a long, almost cylindrical vertically upward shape of the cell, but the vertical nuclear shape did not appear correspondingly severely deformed (Figure S3). This at least seems consistent with a role for F-actin in transmitting stress to the nuclear surface in MB-231 cells. In untreated MB-231 cells, apical protrusions were always associated with a nuclear protrusion, but 40% of apical cell protrusions in nocodazole-treated cells were not associated with

vertical nuclear deformations (Figure 2b). As nocodazole treatment altered F-actin filament organization (Figure S4), it is possible that depletion of F-actin filaments in nocodazole-treated cells reduces stress transmission from the protruding boundary to the nuclear surface, and reduces upward deformation of the nucleus in response to apical protrusions.

Cancer cells frequently have low levels of the nuclear lamins, which can also contribute to irregular nuclear shapes (Chen et al., 2018; Dahl, Ribeiro, & Lammerding, 2008; Irianto, Pfeifer, Ivanovska, Swift, & Discher, 2016; Lammerding et al., 2006; Uhler & Shivashankar, 2018). Our finding that overexpressing lamin A/C reduced the apical nuclear deformations in nocodazole-treated cells without having an effect on apical cell protrusions, supports the concept that the pliability of the cancer nucleus is an important determinant of its response to cellular mechanical stresses. The extent to which abnormal nuclear shapes can develop in breast cancer independent of cellular stresses is not clear. At least one study has suggested that the abnormal phenotypes generated after BRG1 knockdown may not depend on the cytoskeleton or alterations in lamins (Imbalzano et al., 2013). However, it is experimentally difficult to completely eliminate all cellular stresses on the nuclear surface. We propose here that studies of the mechanism of stress generation on the nuclear surface, and studies of the mechanisms underlying abnormal nuclear response to these mechanical stresses will both be required for a complete understanding of cancer nuclear dysmorphia.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTIONS

I. A. K., Q. Z., A. K., and Y. L., designed, performed experiments and interpreted results. S. P. performed experiments and interpreted results. R. B. D., and T. P. L. designed experiments and interpreted results. T. P. L. wrote the manuscript with support from I. A. K., Q. Z., A. K., Y. L., and R. B. D.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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