INTRODUCTION

The group of intermediate filaments (IFs) comprise numerous different subtypes with keratin filaments (KFs) being characteristic for epithelia. The functions of KFs vary from signaling to coping mechanisms for cell stress and their mere abundance and big surface area might facilitate even more, yet, unrecognized roles in the cell. However, KFs' classic role as stretch resistant mechanical stabilizers of the cell remains keratin's most outstanding feature. KFs' tensile resistance is founded on a filament architecture with non-globular subunits providing ample space for overlap between subunits and enabling a rope-like suprastructure. Briefly, a

Abstract

Keratin filaments (KFs) comprise the intermediate filaments of epithelial cells and are well known for their cytoprotective properties and their mechanical resilience. Although, several studies have demonstrated KFs' remarkable tensile properties relatively little is known about acute implications of mechanical stretch on KFs in living cells. This includes structural effects on the KFs and their higher level assembly structures as well as posttranslational response mechanisms to possibly modify KFs' properties. We subjected simple epithelial A549 lung cells to 30% unidirectional stretch and already after 10 seconds we observed morphological changes of the KF-network as well as structural effects on their desmosomal anchor sites—both apparently caused by the tensile strain. Interestingly, the effect on the desmosomes was attenuated after 30 seconds of cell stretch with a concomitant increase in phosphorylation of keratin8-S432, keratin18-S53, and keratin18-S34 without an apparent increase in keratin solubility. When mimicking the phosphorylation of keratin18-S34 the stretch-induced effect on the desmosomes could be diminished and probing the cell surface with atomic force microscopy showed a lowered elastic modulus. We conclude that the stretch-induced KF phosphorylation affects KFs' tensile properties, probably to lower the mechanical load on strained desmosomal cell-cell contacts, and hence, preserve epithelial integrity.

KEYWORDS
desmosome, mechanical resilience, phosphorylation, strain

1 INTRODUCTION

The group of intermediate filaments (IFs) comprise numerous different subtypes with keratin filaments (KFs) being characteristic for epithelia. The functions of KFs vary from signaling to coping mechanisms for cell stress and their
keratin monomer is built of a central alpha helical rod domain, flanked by a head and tail domain. A type I and a type II keratin monomer form a dimer, two dimers assemble to a tetramer, and several tetramers align to form an individual KF with a typical diameter of ca. 10 nm. Many such KFs are usually organized into thicker KF-bundles (or even prominent strands), which typically engulf the nucleus while at the same time extending radially in the cell body. In the cell periphery, these bundles successively diverge into thinner bundles, which can-again-merge with other bundles for a certain distance. This (seemingly) promiscuous assembly behavior forms an irregular mesh of interwoven KF-bundles. Bundles that extend centripetally from the mesh to the cell border usually insert into desmosomes, and hence, connect the meshes of neighboring cells. During mechanical extension of an epithelial layer, it is the desmosomes and the inserting KF-bundles that convey tensile force to the entire KF-mesh.

Despite keratin’s important mechanical role during cell stretch, only little is known how KFs/KF-bundles tolerate longitudinal extension and how interactions of with adjacent KFs/KF-bundles contribute to the extensibility of the entire keratin network. Yamada et al demonstrated the high extensibility of isolated IF subunits and stretch experiments demonstrated the remarkable extensibility of IFs. However, only few studies addressed the acute effects of mechanical stress in general or even specifically for cell stretch on the KF-arrangement in living cells.

Interestingly, few studies show that mechanical stress also leads to KF phosphorylation. Phosphorylation is probably the best characterized posttranslational modification of KFs and a known response to various kinds of cell stress. It is a rapid process, which occurs mostly in the head and tail domains of the proteins and there are several keratin phosphorylation sites that are highly conserved among different keratin isoforms. Considering the massive effect of keratin phosphorylation, an impact on the mechanical properties of the KFs is not surprising and has been suggested for KFs or other IFs. However, these studies partly use indirect reasoning or experiments performed in cell free systems.

The present study is an attempt to address several of the above aspects: Besides (i) identifying stretch-induced effects on the KF(-mesh) and the desmosomes within the first minute after stretch, we (ii) include the alongside effects of cell stretch on keratin phosphorylation and keratin solubility, and (iii) perform biomechanical measurements to investigate effects of keratin phosphorylation on the mechanical properties of KFs. A custom made unidirectional cell stretch device was used to stretch A549 cells. This alveolar simple epithelial cell line forms a single layer in cell culture, provides an uncomplicated model for cell stretch with good microscopic access, and expresses keratin 8 (K8) and keratin 18 (K18), two type I/II keratin isoforms with well characterized phosphorylation sites. Briefly, our results show that cell stretch leads to a fast structural reorganization of the KF-network and we observed phosphorylation of several serine (S) residues in K8 and K18 within 30 seconds, two of them mediated by extracellular-signal regulated kinases 1/2 (ERK1/2). Our data also show that phosphorylation affects the mechanical properties of KFs and suggest that this mechanism reduces the tensile strain on desmosomes under cell stretch. We hypothesize that stretch-induced keratin phosphorylation has evolved as a protective mechanism that preserves the structural integrity of a desmosome under tensile stress.

2 MATERIALS AND METHODS

2.1 Plasmid DNA and antibodies

pECFP-N1-K8-wild-type (K8-CFP-wt in text and figures), pEYFP-C1-K18-wt (K18-YFP-wt), pECFP-N1-K8-S432E (K8-CFP-S432E), pEYFP-C1-K18-S53A (K8-YFP-S53A), pEYFP-C1-K18-S53E (K8-YFP-S53E) were provided by T. Busch (Ulm University, Ulm, Germany). pECFP-N1-K8-S74A (K8-CFP-S74A), pECFP-N1-K8-S74D (K8-CFP-S74D), pECFP-N1-K8-S74E (K8-CFP-S74E), pECFP-N1-K8-S432A (K8-CFP-S432A), pECFP-N1-K8-S432D (K8-CFP-S432D), pEYFP-C1-K18-S34A (K18-YFP-S34A), pEYFP-C1-K18-S34D (K18-YFP-S34D), pEYFP-C1-K18-S34E (K18-YFP-S34E), pEYFP-C1-K18-S53D (K18-YFP-S53D), were made from wt plasmids by mutagenesis with In-Fusion HD Cloning Plus (Clontech Laboratories/Takara, Mountain View, USA) and the following primers: K8-S74A: forward 5'-CCTGCTGGCCCCCCTTTGCTGGAGTGGG-3', reverse 5'-AGGGGGCCACGAGGCTCTGTGTTGACC-3'; K8-S74D: forward 5'-CCTGCTGGCCCGCCTTTGCTGGAGTGGG-3', reverse 5'-AGGGGGCCACGAGGCTCTGTGTTGACC-3'; K8-S74E: forward 5'-CCTGCTGGCCCCCCTTTGCTGGAGTGGG-3', reverse 5'-AGGGGGCCACGAGGCTCTGTGTTGACC-3'; K8-S432A: forward 5'-CCTCACAGCCCCCAGCTAGCTACA-3', reverse 5'-CCGGGGCTGTGAGGCCCCCA-3'; K8-S432E: forward 5'-CCTCACAGCCCCCAGCTAGCTACA-3', reverse 5'-CCGGGGCTGTGAGGCCCCCA-3'; K8-S432D: forward 5'-CCTGCGCCGGCGCTGCTGGAGAGTGG-3', reverse 5'-AGGGGGCCACGAGGCTCTGTGTTGACC-3'; K18-S74A: forward 5'-CCTCACAGCCCCCAGCTAGCTACA-3', reverse 5'-CCGGGGCTGTGAGGCCCCCA-3'; K18-S74E: forward 5'-CCTCACAGCCCCCAGCTAGCTACA-3', reverse 5'-CCGGGGCTGTGAGGCCCCCA-3'; K18-S74D: forward 5'-CCTGCGCCGGCGCTGCTGGAGAGTGG-3', reverse 5'-AGGGGGCCACGAGGCTCTGTGTTGACC-3'; K18-S432A: forward 5'-CCTGCGCCGGCGCTGCTGGAGAGTGG-3', reverse 5'-AGGGGGCCACGAGGCTCTGTGTTGACC-3'; K18-S432D: forward 5'-CCTGCGCCGGCGCTGCTGGAGAGTGG-3', reverse 5'-AGGGGGCCACGAGGCTCTGTGTTGACC-3'; K18-S432E: forward 5'-CCTGCGCCGGCGCTGCTGGAGAGTGG-3', reverse 5'-AGGGGGCCACGAGGCTCTGTGTTGACC-3'.
CAC-3'. All plasmid sequences were verified by sequencing.

Following antibodies for In-Cell ELISA, Western blot and immunolabeling were used: Pan-keratin (4545S, Cell signaling technology, Danvers, USA), Cytokeratin 8 (ab668), Cytokeratin 18 (ab9023), K8 pS74 (ab32579), K8 pS432 (ab59434), K18 pS53 (ab63393), Vimentin (ab73159) all from abcam (Cambridge, UK); K18 pS34 (Orb159737, Bioburt Ltd., Cambridge, UK), Desmoglein 2 (PA5-21444, Invitrogen, Life Technologies Corporation, Carlsbad, USA), Desmoglein-2 (MAB947, R&D systems, Minneapolis, USA); Alexa Flour 488 goat anti-chicken (A11039), Alexa Flour 488 goat anti-mouse (A11029), Alexa Flour 568 goat anti-mouse (A11004), Alexa Flour 568 donkey anti-rabbit (A10042), Alexa Flour 568 donkey anti-mouse (A10037), Alexa Flour 647 goat anti-mouse (A21235) all from Invitrogen (Life Technologies Corporation, Carlsbad, USA); Goat Anti-Mouse IgG (H + L)-HRP conj., Goat Anti-Rabbit IgG (H + L)-HRP conj. both from Jackson ImmunoResearch Europe Ltd (111-035-144; Ely, UK).

2.2 Cell culture, cell stretch, and transfection

A549 human lung adenocarcinoma cells (ATCC, Manassas, USA) were grown at 37°C and 5% of CO2 in DMEM/Ham’s F-12 (Biochrom GmbH/Merck, Berlin, Germany) supplemented with 10% heat inactivated fetal bovine serum, 500 U/mL Penicillin+ 500 µg/mL Streptomycin, and 20 mM L-Glutamine (all: Gibco/Fisher Scientific GmbH, Schwerte, Germany). For cell stretch, the cells were seeded on sterilized and fibronectin-coated (10 µg/mL in PBS for 4 hours at 4°C; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) polydimethylsiloxane (PDMS) membranes (SMI specialty manufacturing inc., Saginaw, USA). After 24 hours the membranes with the attached cells were unidirectionally stretched by 30% for 10 seconds, 30 seconds, 5 minutes, 1 hour, or 3 hours. Mounting of customized membrane holders and handling of cell stretching device is described elsewhere.23 For expression of wt and mutant keratin, the cells were one day prior to the respective experiment transiently transfected with Lipofectamine LTX (Invitrogen Life Technologies Corporation, Carlsbad, USA) according to manufacturer’s protocol (0.6 µg plasmid DNA/membrane, holding 1 mL medium).

2.3 Kinase inhibitors

Kinase inhibitors were dissolved in DMSO and diluted 1:1000 in cell culture medium to replace the culture medium 1 hour prior to cell stretch (consistent with 25-28). An equal amount of DMSO was added to cell culture medium as control. Cells remained in the inhibitor-/DMSO-medium during stretching until fixation for the In-Cell ELISA. Following substances and concentrations were used: extracellular-signal regulated kinases 1/2 (ERK1/2)-inhibitor FR180204 (3706) 10 µM; Protein kinases C (PKC) (α, β, γ, δ, ε, ζ, μ)-inhibitor Goe6983 (2285) 20 µM; c-raf kinase-inhibitor GW5074 (1381) 50 nM; c-raf kinase-inhibitor ZM336372 (1321) 100 nM; c-Jun N-terminal kinase (JNK)-inhibitor TCS JNK60 (3222) 2.5 µM. All inhibitors were purchased from Tocris (Bio-Technne GmbH, Wiesbaden, Germany).

2.4 Detection of keratin phosphorylation

For detection of keratin phosphorylation with an In-Cell ELISA Colorimetric Detection Kit (Thermo Fisher Scientific, Waltham, USA) manufacturer’s recommendations were followed. Briefly, the cells on the PDMS membranes were fixed after the cell stretch with 4% of formaldehyde in TBS for 15 minutes, permeabilized with a Triton-X-100 containing TBS buffer, and then, incubated for 20 minutes with a H2O2 solution to quench endogenous peroxidase. After blocking for 30 minutes the cells were incubated with one of the above mentioned phosphorylation site-specific antibodies (1:1000 in a 50/50 mixture of blocking buffer/wash buffer) overnight at 4°C, followed by an incubation with a horse radish peroxidase-conjugated secondary antibody (1:400 in wash buffer) for 30 minutes at RT. Between steps all required washing steps were performed. Then, 500 µL TMB substrate was added, developed, and eventually stopped by addition of 500 µL TMB stop solution. Finally, 200 µL of the solution were transferred to a 96-well plate in triplicate and absorbance at 450 nm was measured with a plate reader (Tecan Infinite 200, Tecan Austria GmbH, Grödig, Austria). Meanwhile the membrane was washed and Janus Green Whole-Cell stain was added for an estimate of the cell amount. After two other washing steps 500 µL of elution buffer was added, incubated for 10 minutes and absorbance was measured at 615 nm to determine the amount of cells. Values of individual experiments were normalized relative to the respective unstretched membrane within each experiment.

For detection of keratin phosphorylation levels via western blot, membranes were washed with 1 mL cold PBS immediately after stretch and kept on ice. About 200 µL of Phospho-Lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1% of Triton X-100, 50 mM NaF, Pierce Protease and Phosphatase inhibitors [88668, Thermo Fisher Scientific, Waltham, USA]) were added and cells harvested with a cell scraper. After addition of Laemmli sample-buffer (with DTT), the cell lysate was sonicated, and boiled for 5 minutes. Proteins were separated in 4%-12% of Bis-Tris gels, transferred to nitrocellulose membranes, and probed with the
above listed antibodies against the phospho-serines of K8 and K18. The membranes were stripped with Restore Western blot stripping buffer (21059, Thermo Fisher Scientific, Waltham, USA) for 15 minutes at 37°C, blocked and reprobed with antibodies against total K8 and K18.

2.5 | Keratin fractionation

Keratin fractionation of stretched cells was performed as previously described8,29 with few adjustments. In brief, the medium in the membranes was replaced immediately after stretch with 200 µL Triton X-100 buffer (5 mM EDTA, 1% of Triton X-100, 50 mM NaF, 1 mM PMSF, Pierce Protease and Phosphatase inhibitors [88668, Thermo Fisher Scientific, Waltham, USA] in PBS, pH 7.4). The cells were scraped from the membrane and incubated on ice for 10 minutes. After centrifugation (16 000 g, 4°C, 10 minutes) the supernatant was collected, mixed with 4x Laemmli buffer, and boiled for 5 minutes. This fraction was considered the TX-100 soluble fraction. The pellet was homogenized in 200 µL of 10 mM Tris-HCl, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA, 0.5% of Triton X-100, a.m. protease/phosphatase inhibitors, and incubated for 30 minutes on ice. After centrifugation (16 000 g, 4°C, 10 minutes), the pellet was homogenized in 200 µL of a buffer containing 5 mM EDTA and a.m. protease/phosphatase inhibitors in PBS and again centrifuged (16 000 g, 4°C, 10 minutes). The resulting pellet, representing the TX-100 insoluble fraction, was dissolved in 200 µL 1x Laemmli sample-buffer (with DTT), sonicated and boiled for 5 minutes. Proteins from both fractions were separated by electrophoresis as already described and probed for either K8 or K18. The membranes were developed in an Odyssey FC Imaging System (LI-COR, Lincoln, USA), with secondary antibodies coupled to HRP and Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, USA) and the band intensities were quantified with ImageJ.

2.6 | Immunocytochemistry, image acquisition, and electron microscopy

For immunofluorescence, the cells were fixed immediately after stretch on the stretched membranes with 4% of paraformaldehyde in PBS for 15 minutes followed by a washing step with PBS. Then, cells were permeabilized with 0.2% of saponin in blocking buffer for 5 minutes and incubated with one of the above mentioned primary antibodies (1:250 in 0.2% of saponin blocking buffer) either overnight at 4°C or 1 hour at RT. After washing, the secondary fluorescence antibody (1:400 in 0.2% of saponin blocking buffer) was added and incubated light protected for 1 hour at RT. After several washing steps, focal Z-stacks of the cells on the membranes were acquired (Zeiss AxioImager M1, Zeiss, Oberkochen, Germany) with VisiView software (Visitron, Munich, Germany). The most appropriate focal planes of the Z-stacks were chosen for analysis. In all light microscopic images of stretched cells, the stretch-direction is strictly horizontal.

For electron microscopy the cells on the stretched membranes were fixed with 2.5% of glutaraldehyde (Plano, Wetzlar, Germany)/1% of sucrose (Sigma/Merck, Darmstadt, Germany) in PBS followed by postfixation with 2% of OsO4 (Chempur, Karlsruhe, Germany) for 1 hour. After dehydration the cells were enblock stained with saturated uranyl acetate in ethanol for 30 minutes at 37°C, embedded in EPON (Fluka/Honeywell, Seelze, Germany), and hardened 48 hours at 60°C. Small pieces were cut with a handsaw from the EPON block and reembedded to either achieve vertical sections (cut parallel to stretch direction) for measurement of bundle diameter or horizontal sections for keratin mesh morphology. Ultrathin sections (70 nm) were cut on a Reichert-Jung ultramicrotome (Leica microsystems, Wetzlar, Germany) and observed in a Jeol 1400 (JEOL USA, Peabody, USA) equipped with a Veleta camera (EMSIS, Münster, Germany).

2.7 | Image analysis

All measurements including grey value analysis were performed in Fiji/ImageJ30 and further processed in Excel (Microsoft Corporation, Redmond, USA). For quantifying, the distinctiveness of KF-bundles (Figure 1B) the range between minimum and maximum grey values of a 20-30 µm² region (including both perinuclear and peripheral parts) of the cell was determined and expressed as percentage of the mean grey value of this region. Regions with blurry, weakly distinguishable bundles resulted in lower percentages. For measuring bundle fluorescence intensity (Figure 2B), the fluorescence intensities of peripheral KF-bundles along a linear grey value profile perpendicular to the bundle (see also Figure 2A, right panel) were determined. For further processing, the grey value profiles of different bundles were aligned by using the highest grey value (= center of the bundle) as reference. To determine the spatial distribution of K-phosphorylation (Figure 3G), cells were labeled with the specific phospho-serine antibodies and the ratios of mean grey values between the peri-/nuclear region (= nuclear cage including ca. 5 µm perinuclear surrounding) and the remaining peripheral region were determined. To determine the width of the desmoglein fluorescence signal (Figure 5B) desmosomes clearly distinguishable from neighboring desmosomes were arbitrarily chosen in the samples from successfully transfected cells recognizable by the fluorescence protein labeled KFs. The distance along a linear intensity profile through the fluorescence signal perpendicular to the cell border was determined that comprised desmoglein fluorescence intensities
FIGURE 1 Comparison of vimentin IF and KF in A549 cells and the effect of cell stretch on the KF-mesh structure. A, Immunostainings demonstrate vimentin's less clearly defined bundles with lesser extensions to the cell border, as compared to keratin (see inserts from slightly different focal planes). B, Immunostainings for K18 show a crisp and well-defined network of KFs in unstretched A549 cells (left panel), whereas 30% stretch (duration: 10 seconds in middle and 30 seconds in right panel) leads to a loosened integrity of the network. Quantification of the grey value ranges confirms these observations. Comparing the ultrastructure of unstretched cells (C, E) with stretched cells (D, F) confirms the findings of the immunostainings: KF-bundles (possibly also vimentin) in unstretched cells form prominent bundles/threads (arrowheads) typically encompassing the nucleus (n) and reaching out to the periphery. The cutouts in C and D (dotted boxes) shows several thin KF-bundles in the periphery that merge and eventually insert into a desmosome (des). The IF-bundles (arrowheads) in stretched cells (D, F) are less defined with seemingly fainter bundles.
above half of the peak intensity value. For all the above described grey value analyses, the grey value from a region without cells was subtracted as background before further processing. Bundle diameter in EM images were measured in vertical sections that were cut parallel to the stretch direction. Only bundles with a reproducible diameter for a considerable length (see example in horizontal section in Figure 2C) were used for analysis.

2.8 Atomic force microscopy

A549 cells were seeded on sterilized and fibronectin-coated (10 µg/mL in PBS; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) 24 mm cover glass slides in 35 mm cell culture dishes (both: VWR International GmbH, Darmstadt, Germany) and cultivated overnight (see above). The day after, cells were transfected with plasmid DNA (see above) coding for fluorescence protein labeled K8 or K18 (wt or mutant). Atomic force microscopy (AFM) was carried out 24 hours after transfection. Only successfully transfected cells recognizable by a fluorescent keratin network were considered for measurements. AFM images and Young’s modulus of the cells where obtained at room temperature in bicarbonate-free cell culture medium with a NanoWizard 4 (JPK BioAFM, Bruker Nano GmbH, Berlin) mounted on an inverted optical microscope (IX73, Olympus Europa SE & Co. KG, Hamburg, Germany) using Quantitative Imaging measuring mode. A biosphere B500-CONT with gold reflective coating was used (NanoAndMore GmbH, Wetzlar, Germany) with a nominal spring constant of 0.2 N/m and a high-density spherical carbon tip with a nominal tip radius.

FIGURE 2 Effect of cell stretch on individual keratin bundles. A, Pan-keratin (pan-K) immunofluorescence image showing examples of KF-bundles for measuring perpendicular fluorescence intensity profiles (arrowheads in insert; example for a profile is indicated by the line between two opposing arrowheads). B, Mean grey value intensities (± std) along profiles. The loss of fluorescence intensity at the center of the bundle (0 nm on x-axis) complies with the expected decrease after 30% extension and confirms a stretch of these bundles by the nominal stretch amplitude. C, Electron micrograph of peripheral parts of two cells (des = desmosome). Pairs of arrowheads show examples of bundles that were used for diameter measurement as they traverse the EM section in a flat angle without tangential cut-off. D, The frequency histogram shows a peak bundle diameter in the 30-40 nm range in unstretched cells. About 10 seconds cell stretch leads to thinner bundle diameters with a peak in the 20-30 nm range.
FIGURE 3  Stretch-induced phosphorylation of KFs. A-D, The fold increase in phosphorylation relative to unstretched cells of (A) K8-S74, (B) K8-S432, (C) K18-S34 and (D) K18-S53 after different stretch durations is shown. All four sites are rapidly phosphorylated and show a maximum after 30 seconds. Phosphorylation of K8-S432 and K18-S34 is sustained for hour(s) whereas phosphorylation of K8-S74 and K18-S53 shows a decline to basal levels after 5 minutes. Points in diagram represent independent measurements, black lines indicate the mean. P-values relative to the unstretched cells below .05 (or close to) are displayed above the time points. E, Immuno-blotting of whole cell lysates demonstrating the stretch-induced phosphorylation of the same four serine residues shown above. F, Effect of cell stretch on KF solubility. Triton X-100 soluble and insoluble fractions of keratin were immuno-blotted for K8 and K18, quantified and normalized to unstretched controls. Except small deviations, the solubility of the KF-network remained unaltered. G, Spatial distribution of keratin phosphorylation in unstretched and stretched A549 cells, as analyzed by image analysis of immunostainings with the respective phosphorylation-specific antibody in the peri-/nuclear (= nuclear cage including ca. 5 µm perinuclear surrounding) compared to peripheral regions of the cell. K18-S53 in stronger phosphorylated in the cell periphery, whereas no difference could be detected for the other phosphorylation sites.
of 500 nm. A maximum indentation force of 0.5 nN was set. Cantilever approach and retraction speed where 55 µm/s with a sample rate of 100 kHz. With a scan area of 30 × 30 µm and 32 × 32 pixel, a resolution of 0.9375 µm/Pixel was reached. By using the Hertz model for spherical intenders as implemented in the JPK BioAFM data processing software the Young’s modulus and the height of the measured cells were fitted.

2.9 Statistical analysis

Statistical analysis was performed in Prism 6 (GraphPad, San Diego, USA). Values were tested for Gaussian distribution (D’Agostino & Pearson omnibus normality test). For comparison with a normalized control, a One Sample t test (Gaussian distribution) or a Wilcoxon Signed Rank test (no Gaussian distribution) was performed. Otherwise, an unpaired t test (Gaussian distribution) or a Mann-Whitney test (no Gaussian distribution) was conducted. P-values in figures are only shown if close to or smaller .05. Mean ± standard deviation is presented in the results section and in the figure.

3 RESULTS

Despite their epithelial origin, it was previously reported that A549 cells express not only keratin, but also vimentin IF and co-stainings of pan-K and vimentin confirm these findings (Figure 1A). Both keratin and vimentin IF form a prominent cage around the nucleus but vimentin not only forms less clearly distinguishable bundles reaching out to the desmosome at the cell border, but also appears in general to be less abundant in the in the cell periphery. The vimentin bundles look thinner and more branched and rarely overlap with KF-bundles.

The immunostainings for K18 in Figure 1B compare the overall KF-mesh of unstretched A549 cells with cells subjected to 10 seconds (middle panel) and 30 seconds (right panel) cell stretch. In unstretched cells, a crisp network of KF-bundles (maybe even individual filaments) can be distinguished (Figure 1B, left panel including insert). It forms a complex network with typical architectural features shared by other epithelial cell types and keratin isoforms. Massive KF-strands encompass the nucleus, form a cage around it, and eradiate in the cell periphery, forming successively more delicate (and fainter) bundles. In stretched cells, the main components of the network are still discernable but the lack of the pronounced contrast between cytosol and KF-bundles and poorly defined individual bundles is obvious (30%—10 seconds, insert in middle panel). This “blurry” appearance was observed after 10 seconds (middle panel in A) as well as 30 seconds cell stretch (right panel), the KF-mesh seemed to have regained a slightly more defined structure after 30 seconds of 30% stretch though. This observation was confirmed by image analysis, which also shows a decrease in the distinctiveness (defined as relative grey value range) of keratin bundles after 10 seconds (unstr.: 148%±40 vs 10 seconds—30%: 81%±29; P < .001) and to a lesser extent after 30 seconds of 30% cell stretch (101%±29; P < .001). N_cells = unstr.: 137, 10 seconds: 99, 30 seconds: 109. The data also suggest a slight recovery after 30 seconds. The ultrastructure of unstretched and stretched cells in electron microscopy (EM) agrees with the immunostainings although we cannot exclude that some of the observed filaments/bundles also include vimentin IF. Filaments of unstretched cells (Figure 1C,E) form massive (electron dense) strands in the perinuclear region whereas cell stretch seems to reduce their thickness. In stretched cells, the bundles are stronger lacerated, leading to branching of finer bundles and/or eventually resulting in individual filaments separating laterally from bundles or from bundle endings (Figure 1D,F). No apparent difference in the ultrastructure after 10 seconds or 30 seconds stretch could be noticed. Taken together the findings in the EM correspond well with the observed changes in the fine network structure in the immunostainings and indicate an overall loosening of the KF-mesh after cell stretch.

We further investigated stretch-induced effects on individual KF-bundles in the periphery (Figure 2A), where the abundance of bundles is lower and observation is not impaired by KFs in the vicinity. Figure 2B compares fluorescence profiles perpendicular to individual KF-bundles before and after stretch to determine their actual extension by the decrease of the immunofluorescence signal. Since the decrease in immunofluorescence intensity to 74.2% at 30% stretch (from 2118 ± 738 to 1572 ± 410, P = .02) at the center of the bundle deviated only marginally from the expected 77% ((100/130) × 100), we deduce that the bundles were extended by the nominal stretch amplitude of 30%. The relatively high standard deviation is also caused by the broad range of different bundle intensities. N_cells = 20+ in each condition, N_bundles = unstr.: 27, 10 seconds: 29, 30 seconds: 27. To investigate if bundle diameters are affected by their extension, we determined diameters in vertical EM sections cut parallel to the direction of the cell stretch, where stretched bundles can be followed along their longitudinal course. For clearer depiction, a horizontal section is shown in Figure 2C. Figure 2D shows the frequency distribution of bundle diameters in unstretched and stretched cells. Interestingly, unstretched cells displayed surprisingly narrow defined bundle diameters of 41.9 ± 13.8 nm. After 10 seconds cell stretch the peak of the distribution was shifted to smaller diameters (34.9 ± 17.6, P = .02). This effect was less pronounced after 30 seconds stretch. N_cells = 40+ in each condition, n_bundles = unstr.: 52, 10 seconds: 61, 30 seconds: 63.
Since cell stress is a known trigger for keratin phosphorylation,\textsuperscript{17} we determined the phosphorylation of four well established serine phosphorylation sites: K8-S74, K8-S432, K18-S34, and K18-S53 (numerical data in Table S1, supplemental information). As depicted in Figure 3A-D, where the fold increase relative to unstretched cells is shown, 30 seconds of cell stretch was sufficient to clearly induce phosphorylation in three sites. K8-S74 had the highest variability, the highest $P$-values, and lowest mean increase in phosphorylation of all observed sites. For K8-S432 and K18-S34, the phosphorylation remained elevated for one or even several hours. Phosphorylation of K18-S53 was transient, disappeared after 5 minutes and again rose after 1 hour. Interestingly, for K18-S53, we observed a decrease of the phosphorylation after 10 seconds.

To confirm the above results from In-Cell ELISA experiments, we performed immuno-blotting of whole cell lysates with the same antibodies against phosphorylated S74/S432 of K8 and S34/S53 of K18. The representative blots in Figure 3E further underline the validity of our findings.

Since keratin phosphorylation affects the solubility of KFs in the cell, we investigated whether the increase in keratin phosphorylation after stretch, also affected the pool of soluble keratin proteins and oligomers by performing biochemical fractionation. Therefore, keratin proteins were separated into a TritonX-100 (TX-100) soluble (single keratin proteins and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption[Pharmacological inhibition of stretch-induced phosphorylation relative to unstretched w/o inhibitor.]{Pharmacological inhibition of stretch-induced phosphorylation relative to unstretched w/o inhibitor. Different kinase inhibitors were applied to investigate the role of different signaling pathways in the stretch-induced phosphorylation after 30 seconds stretch by In-Cell ELISA. Cells were treated with agents inhibiting (A) extracellular-signal regulated kinases 1 and 2 (ERK1/2), (B) c-Jun N-terminal kinase (JNK), (C) and (D) c-raf kinase, (E) protein kinases C (PKC). Treatment with ERK-inhibitor FR180204 significantly lowered the stretch-induced increase in phosphorylation of the two K8 residues S74 and S432. Inhibition of c-raf activity by ZM336372 reduced phosphorylation of K18 S53 after stretch. $P$-values below .05 (or close to) are displayed above the bars.}
\end{figure}
oligomers) and an insoluble (filamentous keratin) fraction, followed by immuno-blotting for K8 or K18. Stretch-induced phosphorylation did not significantly change the amount of the soluble and filamentous keratin protein pool (Figure 3F).

To determine if stretch-induced phosphorylation is stronger in certain parts of the cells, we compared the fluorescence intensity ratio between the peri-/nuclear region (= nuclear cage including ca. 5 μm perinuclear surrounding) and the cell periphery before and after stretch for all four phosphorylation sites (Figure 3G; numerical data in Table S2, supplemental information). Only K18-S53 showed a marked difference ($P < .001$) with stronger stretch-induced phosphorylation in the cell periphery.

**FIGURE 5** Stretch-induced desmosomal widening reflecting tensile strain of inserting KFs. A, Immunofluorescence staining for desmoglein-2 (Dsg2) and pan-keratin (pan-K) in stretched cells. Width of desmosomes was determined perpendicular to cell border. B, Diagram showing the increase of desmoglein fluorescence width after 30%—10 seconds stretch compared to unstretched cells and an attenuation of this effects toward unstretched values after 30%—30 seconds stretch (horizontal lines mark mean). C, Immunostaining for K18 in unstretched and stretched cells with inserts highlighting the KF-bundles emanating from desmosomes. D, Diagram showing that the distance between the bundle endings (measured as indicated by green lines in (C) markedly increases after 30%—10 seconds stretch and remains increased after 30%—30 seconds stretch. E, When comparing the relative width of the desmoglein-2 (Dsg2) fluorescence after transfection with different phosphorylation mimicking (S → E) mutants only K18-YFP-S34E abolished the stretch-induced widening after 30%—10 seconds.
Several kinases and pathways have already been identified for the phosphorylation sites examined in our study. To investigate which of those candidates could be involved in the fast, stretch-induced increase in keratin phosphorylation, we used pharmacological inhibitors and determined the effects on the phosphorylation levels after 30 seconds—30% stretch (Figure 4; numerical data in Table S3, supplemental information). By inhibiting the activity of extracellular-signal regulated kinases 1 and 2 (ERK1/2) (Figure 4A), the phosphorylation of the K8 residues S74 and S432 after 30 seconds 30% stretch clearly differed from untreated cells after cell stretch (S74: $P = .0286$; S432: $P = .0286$) and no significant increase in phosphorylation of S74 and S432 between inhibitor-treated unstretched cells and inhibitor-treated 30 seconds stretched cells was observed. Hence, we consider ERK1/2 to be involved in phosphorylation of S74 and S432. However, we can of course not exclude that other kinases within the ERK1/2 regulated network (or affected by it) account for the effect. Blocking ERK1/2 had no strong effect on the increase in phosphorylation at S34 and S53 of K18 after 30 seconds 30% cell stretch. Inhibiting c-Jun kinase (JNK) activity (Figure 4B) had no significant effect on the short-time stretch-induced phosphorylation of any of the four serine residues. Furthermore, we inhibited c-raf kinase activity with two different inhibitors, GW5074 and ZM336372 (Figure 4C,D, respectively) but—except for K18 S53 after ZM336372 treatment—we saw no significant change in phosphorylation after cell stretch. Inhibiting protein kinase C (PKC) with Go6983 (Figure 4E) did not prevent the rapid stretch-induced phosphorylation of any of the investigated phosphorylation sites. Some of the inhibitors are likely to have an effect on the basal keratin phosphorylation, as we observed a decrease in the phosphorylation levels in unstretched inhibitor-treated cells vs unstretched control cells.

Simple epithelial A549 cells form a continuous flat cell layer in cell culture with neighboring cells laterally attached and connected by desmosomes (Figure 5A). When measuring the width of the fluorescence immunostaining for the desmosomal cadherin desmoglein perpendicular to the cell border (Figure 5B), we found that the width of 311 ± 18 nm in unstretched cells ($n = 49$) increased by 23.2% to 384 ± 40 nm after 10 seconds cell stretch ($n = 45$, $P < .01$). Interestingly, this effect was attenuated after stretch durations of 30 seconds to 344 ± 24 nm ($n = 50$). In agreement with this finding, also the distance between two KF-bundle endings inserting into the same desmosome was increased (Figure 5C,D) from 324 ± 165 nm ($n = 45$) in unstretched cells to 548 ± 138 nm ($n = 25$) after 10 seconds of stretch, although here the widening effect was not markedly attenuated after 30 seconds cell stretch. Both findings show that 10 seconds cell stretch increased the pull of the inserting KFs and widens inner as well as outer portions of the desmosome. The attenuated effect on desmoglein fluorescence after 30 seconds cell stretch points at a rapid reduction of the tensile force within this time span, however, apparently not sufficient to also allow the KF-bundle endings to return to their initial distance before the stretch.

Since the lowered pulling effect after 30 seconds coincided with keratin phosphorylation of several phosphorylation sites in K8/K18 (see Figure 3), we tried to ascertain a potential effect of phosphorylation on the tensile properties of the KFs. Cells were transfected with different K8/K18 mutants, where K8-S74, K8-S432, K18-S34, or K18-S53 were replaced with glutamate (E) to mimic phosphorylation. Assuming a tension-releasing effect of phosphorylated KFs, a reduction of the desmoglein fluorescence widening after 10 seconds cell stretch was expected. It should be noted that in the transfected cells, the ratio of endogenous to introduced wild-type/mutant keratin protein remains unknown, hence the results represent a mixture of both keratin types. Figure 5E shows the width of the desmoglein fluorescence after 10 seconds stretch relative to equally transfected but unstretched cells (numerical data in Table S4, supplemental information). For microscopic selection of successfully transfected cells, wt and mutant keratins were tagged with fluorescence proteins (K8-CFP, K18-YFP). Although expression of both wt keratins already reduced the widening of the desmoglein fluorescence signal after cell stretch (increase of 11.9% and 11.3% vs 23.2% in untransfected cells), most phospho-mutants still lead to a marked stretch-induced widening of the desmoglein fluorescence. However, mimicking phosphorylation of K18-S34 with K18-YFP-S34E almost abolished the widening, demonstrating the importance of this phosphorylation site for lowering the tensile stress on KFs.

To verify the altered mechanical properties with mimicked phosphorylation of K18-S34 (K18-YFP-S34E), we used AFM to determine the elastic modulus (E-modulus) in cells after transfection with the same phospho-mimicking mutants used for desmosomal width experiments (Figure 6A,B). Additionally, we also measured cells transfected with another phosphorylation-mimicking keratin mutant, where the respective serine was replaced with aspartic acid (S to D), and mutants with abolished phosphorylation by replacing serine with alanine (S to A). Since we expected the vertically depressing AFM probe to be highly biased in parts of the cell with a mechanically interfering structure closely underneath (eg, surface of culture dish in the lowermost or nucleus in the uppermost parts of the cell), we analyzed and compared the E-moduli of different cell height ranges separately (Figure 6A; numerical data in Table S5, supplemental information). Comparing the E-moduli in a range of $z = 1.5$-2.5 μm above growth substrate between wild-type (K18-YFP-wt) and mimicked phosphorylation of K18-S34, both mutant keratins showed a clear difference.
from 1919 ± 712 Pa to 1070 ± 583 Pa (P = .0137) for K18-YFP-S34E and 1162 ± 677 Pa (P = .0549) for K18-YFP-S34D. Abolishing phosphorylation of K18-S34 (K18-YFP-S34A) did not affect the E-modulus. Surprisingly, abolished phosphorylation of K8-S432 (K8-CFP-S432A) lowered the E-modulus extensively, from 2579 ± 990 Pa to 952 ± 1039 Pa (P = .0065), despite any effect of the two phosphorylation-mimicking mutants at this site. We did not observe noticeable differences in the E-moduli of cells transfected with S74 mutants and S53 mutants in comparison to their respective wild-types. Figure 6C,D shows that CFP- or YFP-tagged wt keratins become properly incorporated into the endogenous keratin network and that all keratin variants form proper keratin networks.

**FIGURE 6** Elastic modulus of cells with mimicked keratin phosphorylation. A, Example of a topographic AFM scan (upper panel) and the corresponding E-moduli (lower panel). Black line in topography panel indicates the approximate region used for analysis. B, E-moduli of cells transfected with different fluorescence protein-labeled phosphorylation-mimicking (S → E and S → D) or phosphorylation-abolishing (S → A) keratin mutants and the corresponding control with the fluorescence protein-labeled wt keratins. Both mutants mimicking the phosphorylation of K18-S34 (K18-YFP-S34E and K18-YFP-S43D) showed a marked lowering of the E-moduli as well as the abolished phosphorylation of K8-S432 (K8-CFP-S432A). C, twofold panels: Cells transiently transfected with K8-CFP-WT (upper image) and co-stained with antibody against K8 (lower image). Sixfold panels: Cell transiently transfected with different K8 mutants. D, twofold panels: Cells transiently transfected with K18-YFP-WT (upper image) and co-stained with antibody against K18 (lower image). Sixfold panels: Cell transiently transfected with different K18 mutants. C and D, Co-stainings in twofold panels demonstrate proper incorporation of transiently transfected, fluorescent keratin into the endogenous KF-network. Sixfold panels show that all transiently transfected, fluorescent keratin mutants form together with the endogenous keratin intact networks in the cells.

**4 | DISCUSSION**

Many tissues are exposed to tensile stress and skin or lung are probably only the most striking examples where KFs experience this kind of mechanical stress. On a cellular level, it is the network of IFs that has to withstand cell stretch. Although the high tensile resistance of IFs is known for several decades and remained their most outstanding feature, surprisingly little is known about IFs’ behavior in living cells.

IFs—or keratin in the case of epithelia—consist of elongate monomers (unlike actin filaments or microtubules) that assemble to form the classic 10 nm filaments, which in turn assemble into thicker bundles, engulfing the nucleus while at the same time extending radially in the cell body. The simple
epithelial cell line A549 was derived from a human alveolar cell carcinoma, and expresses not only keratin IFs, but also vimentin IFs as well. As shown in Figure 1A, both IF type networks share a massive nuclear cage but differ already in the perinuclear region and vimentin shows weaker (if any) defined bundles reaching out to the cell border. Vimentin binds to α6β4 integrin in A549 cells and binding to β4 is required for proper vimentin filament formation, as vimentin filaments in β4 integrin knockdown showed only perinuclear localization in contrast to the well spread vimentin networks of control cells. Since α6β4 integrins are highly selective laminin-binding integrins, we speculate that our culture conditions (coating with fibronectin only) might account for the mainly perinuclear distribution of vimentin. The vimentin network certainly contributes to the mechanical properties of the entire cell, however, the lower amounts of vimentin in the cell periphery as compared to keratin with its apparently strong desmosomal anchorage, points at KFs to be the determining factor of A549's tensile properties and resistance to mechanical stress.

KFs in A549 cells form a robust mesh, which is shown in the immunostainings and micrographs in Figure 1. In our experiments, cell stretch immediately (10 seconds—30% stretch) lead to less distinct KF-bundles (Figure 1B) and smaller, clearly defined filament-bundles in EM (Figure 1C-F), what we interpret as longitudinal splitting of KF-bundles (or strands). The stretch-induced effect on the lateral interaction would, therefore, be important for the required net increase of the keratin mesh length during cell stretch. Nevertheless, our findings also show that individual KF-bundles are affected by tensile forces, since we observed a mean distension of filament bundles by the nominal stretch amplitude in the cell periphery, tensile forces, since we observed a mean distension of filament bundles by the nominal stretch amplitude in the cell periphery, tensile forces, since we observed a mean distension of filament bundles. The stretch-induced effect on the lateral interaction would, therefore, be important for the required net increase of the keratin mesh length during cell stretch. Nevertheless, our findings also show that individual KF-bundles are affected by tensile forces, since we observed a mean distension of filament bundles by the nominal stretch amplitude in the cell periphery, tensile forces, since we observed a mean distension of filament bundles. The stretch-induced effect on the lateral interaction would, therefore, be important for the required net increase of the keratin mesh length during cell stretch. Nevertheless, our findings also show that individual KF-bundles are affected by tensile forces, since we observed a mean distension of filament bundles by the nominal stretch amplitude in the cell periphery, tensile forces, since we observed a mean distension of filament bundles. The stretch-induced effect on the lateral interaction would, therefore, be important for the required net increase of the keratin mesh length during cell stretch. Nevertheless, our findings also show that individual KF-bundles are affected by tensile forces, since we observed a mean distension of filament bundles by the nominal stretch amplitude in the cell periphery, tensile forces, since we observed a mean distension of filament bundles. The stretch-induced effect on the lateral interaction would, therefore, be important for the required net increase of the keratin mesh length during cell stretch. Nevertheless, our findings also show that individual KF-bundles are affected by tensile forces, since we observed a mean distension of filament bundles by the nominal stretch amplitude in the cell periphery, tensile forces, since we observed a mean distension of filament bundles. The stretch-induced effect on the lateral interaction would, therefore, be important for the required net increase of the keratin mesh length during cell stretch. Nevertheless, our findings also show that individual KF-bundles are affected by tensile forces, since we observed a mean distension of filament bundles by the nominal stretch amplitude in the cell periphery, tensile forces, since we observed a mean distension of filament bundles. The stretch-induced effect on the lateral interaction would, therefore, be important for the required net increase of the keratin mesh length during cell stretch. Nevertheless, our findings also show that individual KF-bundles are affected by tensile forces.

We further observed an effect on bundle diameters in EM, which supports an effect on individual bundles, likely caused by their longitudinal extension. KF-bundle diameters are not unequivocally defined in literature for various reasons, and even the diameter of individual KFs seems negotiable. The present study also revealed diameters of several 100 nm (without regularly sized smaller bundles within) as well as minute diameters practically limited by the dimensions of single filaments. However, the remarkable abundance of KF-bundles with a diameter of 41 nm in the cell periphery points at an energetically preferable bundle size—at least in unstretched A549 cells—and we interpret the stretch-induced thinning as a deformation caused by the tensile force. It remains speculative how to explain the thinner bundle diameters after stretch in our experiments, but Wagner et al suggested that uncoiling of keratin helices explain keratins high flexibility (bending) and Qin et al demonstrated the same effect with isolated IF subunits, and even report a thinning of stretched IFs.

Despite the compliance of the KF-mesh resulting from lateral or longitudinal mesh responses, desmosomes still experience a mechanical load under tensile strain, detectable by an extension of desmosomal desmoplakin. Our study seems to confirm these findings. Figure 5B shows a robust increase of the desmosomal width after 10 seconds stretch when measuring the dimension of desmoglein fluorescence signal from individual desmosomes, hence demonstrating effects on the outer (ie, close to the cell membrane) components of the desmosome. Also, the innermost parts of the desmosome (ie, furthest away from the membrane) experience a dramatic extension since the distance between the two opposing KF-bundles of a desmosome dramatically increases after 10 seconds (Figure 5D). Interestingly, after 30 seconds of cell stretch the width of the desmoglein fluorescence returns to values about half in between unstretched and 10 seconds stretch whereas the innermost part of the desmosome remains extended (Figure 5D). Since too little is known about stretch-induced effects on the entire desmosomal architecture or domain unfolding of proteins within, we can only hypothesize about the molecular cause of this transient widening. Focusing on the possible role of keratin in this respect, these findings could be the result of a drop of the tensile force—however, not entirely subsided to unstretched values—and by different “recoil” forces in the inner and the outer parts of the desmosome. Since mutated keratin affected the stretch-induced widening of the desmoglein fluorescence in our experiments (see below), we consider the pulling forces of keratin indeed a contributing factor and we exploited this phenomenon as a read-out for keratin's tensile force.

Besides the effects of cell stretch on KF structure and the desmosomal width, we also determined phosphorylation levels of four well-known K8 and K18 serine residues, which have already been described to undergo phosphorylation during mitosis or as a response to heat or chemical, osmotic, microbial, and mechanical (shear) stress (reviewed in 16, 17). Upon stretch, we observed an increase in phosphorylation of at least three of the four investigated phosphorylation sites. K18-S34, with the most robust increase in phosphorylation in our experiments, has already been shown to be phosphorylated by shear stress and during mitosis, which both require a high cell deformability, and therefore, a flexible and rearrangeable KF-network. K8-S74 showed the least conclusive result with a high variability in the phosphorylation levels. Since K8-S74 has been shown to be phosphorylated as a consequence of chemical stress, long-term shear stress and also during apoptosis, we assume that the mechanical stress in our experiments was not appropriate or not sufficiently high to elicit unequivocal phosphorylation at K8-S74.

Although previous studies demonstrate degradation of hyperphosphorylated keratins as a consequence of mechanical stress, our experiments do not indicate a disassembly of the KF-network or an increase of the soluble keratin pool (Figure 3F). This discrepancy may have several reasons. In our experiments, only K18-S34 showed a marked and prolonged increase in phosphorylation whereas already after
5 minutes the phosphorylation levels of the other serine residues had at least temporarily returned to values close to unstretched controls and had completely declined to basal levels after 3 hours (Figure 3A). Studies that describe the disassembly and degradation of phosphorylated KFs as a consequence of mechanical stress used several hours (4-48 hours) of high degree (30 dyn/cm²) shear stress. Moreover, the nature of mechanical stress seems to be crucial, as Ridge et al describe that even long-term (24-72 hours) 20% cyclic stretch did not result in changes of keratin solubility, whereas shear stress did. Furthermore, these studies show that keratin degradation upon mechanical stress involves K8-S74 hyperphosphorylation, which showed the weakest response and high variability in phosphorylation upon stretch (Figure 3A).

When investigating the spatial distribution of K8 and K18 phosphorylation before and after 30 seconds 30% stretch (Figure 3G), we found a mostly even increase in peri-/nuclear (= nuclear cage including ca. 5 µm perinuclear surrounding) and peripheral regions of the cell except for K18-S53. Here the phosphorylation in the periphery increased stronger after stretch as compared to the peri-/nuclear region of the cell, which is in agreement with previous observations of our group in SCC25 cells.

The different phosphorylation patterns in the four investigated serine residues point on activation of different pathways responsible for their phosphorylation, and probably different functions thereof. Several kinases have already been associated with keratin phosphorylation in the past and we used specific inhibitors to study their role in rapid stretch-induced phosphorylation of K8 and K18. Inhibiting ERK1/2 (Figure 4A) showed the strongest effect and abolished the increase in phosphorylation of K8-S74 and K8-S432 after 30 seconds—30% stretch. Mechanical stimulation leading to integrin signaling and downstream ERK1/2-activation is well known. Also, phosphorylation of K8/K18 by ERK1/2 was often observed, for example, as a consequence of EGF stimulation, treatment of cells with sphingosylphosphorylcholine, and in tumor tissue. However, to our best knowledge, the present study that shows for the first time that stretch-induced activation of ERK1/2 leads to increased phosphorylation on K8-S74 and K8-S432. However, it needs to be determined, whether ERK1/2 directly acts as a kinase on K8-S74 and K8-S432 or whether indirect effects, such as other ERK1/2 regulated kinases phosphorylate K8-S74 and K8-S432 upon cell stretch. In contrast, blocking the activity of another member of the MAP kinase family, the stress-activated JNK (Figure 4B) had no significant effect on the short-time stretch-induced phosphorylation on any of the four serine residues. Even though, mechanical activation of JNK has been reported previously, JNK activation required a slow and prolonged stimulation with a different mechanical stimulus and 30 seconds of 30% cell stretch might, therefore, not be sufficient to elicit JNK activation. In the past, c-raf kinase was observed to be directly associated with K8, activated upon cell stress, and recombinant raf kinase phosphorylated K18 at S53 and to lesser extent S34. In our study, only phosphorylation of K18-S53 could be markedly inhibited when using the c-raf-specific inhibitor ZM336372, whereas another c-raf inhibitor only mildly reduced phosphorylation (Figure 4C,D, respectively), maybe caused by a slightly different biological activity of the two inhibitors. Lastly, another family of kinases often activated upon mechanical stimuli are PKCs. PKCδ, and PKCζ were found to phosphorylate K8-S74 and K18-S34, respectively, upon shear stress in A549 cells. However, the broad spectrum PKC inhibitor Go6983 (Figure 4E) could not prevent the rapid stretch-induced phosphorylation of any of the investigated serine residues in our stretch experiments. We were not able to identify potential kinases involved in the stretch-induced phosphorylation of K18-S34.

Since the peak of the increase in phosphorylation of three of the investigated KF-sites after 30 seconds coincided with the reduction of the desmoglein fluorescence width (also after 30 seconds), we tried to verify a potential mechano-modulatory effect of keratin phosphorylation. For this, we used cells transiently transfected with mutant keratin proteins mimicking phosphorylation on K18-S34, K18-S53, K8-S432, and K8-S74 and determined to what extent this mimicked phosphorylation would affect the widening of the desmoglein fluorescence signal. Among all phosphorylation sites, only mimicked phosphorylation on K18-S34 (K18-YFP-S34E) showed a clear effect and markedly diminished the widening of desmosomes (Figure 5E). This implies that phospho-mimicking of K18-S34 leads to a more compliant KF-mesh, and hence lowers the tensile force exerted on the desmosome. This is in accordance with previous findings of our group where an effect of phosphorylation on the tensile force on the desmosome upon stretch was suggested.

To confirm these findings, we performed life-cell AFM measurements in a region of the cell where the KFs are suspended between the flat cell periphery and the elevated nuclear region to avoid a potential restraint of the AFM probe by the underlying substrate. The E-moduli in our measurements were well within the range of previous studies and—despite considerable differences between cells with the same mimicked phosphorylation site—both mutants mimicking phosphorylation of K18-S34 (K18-YFP-S34E and K18-YFP-S34D) reduced the mean elastic moduli in the respective cells (Figure 6B). Interestingly, Sivaramakrishnan et al observed in their experiments an increase in cell stiffness after 1 hour of mechanical stress, however, this study used primary cells, assessed the storage modulus and shear stress instead of cell stretch was applied. Moreover, in our experiments both—the recoil of desmosomal width and the lowered E-modulus—point at K18-S34 phosphorylation as a determining factor of KF elasticity in our experiments and other studies already.
indicated that keratin phosphorylation alters the structural properties of KFs, accompanied by lowered forces, necessary to stretch cells.

The present study does not provide evidence on the mechanism of the proposed mechano-modulatory function of keratin phosphorylation, and both longitudinal effects (within keratin bundles) and altered lateral interactions (between bundles) might equally account for the phenomenon. In the ex vivo study of Deek et al., phosphomimicry mutants of K8/K18 reduced intra-connectivity of K8/18 networks and lead to mechanically weaker, more deformable networks and stronger bundle branching. An increase in bundle branching is in agreement with our own morphological findings of a less distinctive KF-mesh in the electron micrographs and immunostainings after stretch (Figure 1) and favors an effect on the interaction between bundles. However, the formation of bundles and interactions between are not yet entirely understood, which impedes interpretation. On the contrary, we observed stretch of individual filament bundles accompanied by a temporary reduction of individual KF-bundle diameter after 10 seconds cell stretch (Figure 2D). This could be the consequence of uncoiling of helices that starts—at least in stretched IF dimers—at ca. 20% stretch or subunit sliding past each other within IFs, as suggested by Wagner et al. Theoretically, both effects of phosphorylation on lateral as well as longitudinal KF interactions could account for the observed strain releasing effect of keratin phosphorylation.

Our study demonstrates that cell stretch leads to a rapid phosphorylation of several serine residues in K8/K18 and at least two of them (K8-S74 and -S432) are mediated by ERK1/2. Furthermore, our data indicate that phosphorylation of K18-S34 is capable of lowering the tensile force of the keratin network. Such a response to tensile strain might have evolved as a mechano-protective mechanism to avoid desmosomal rupture and hence to preserve the structural integrity of an epithelial layer in a mechanically challenged tissue.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

A. Lutz and E. Felder designed research; A. Lutz, D. Jung, K. Diem performed research; F. Port and K. Gottschalk contributed analytic tools; A. Lutz, D. Jung, M. Fauler and E. Felder analyzed data; A. Lutz, F. Port and E. Felder wrote the manuscript.

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