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Filamin A Protects Cells against Force-Induced Apoptosis
By Stabilizing Talin and Vinculin-Containing Cell Adhesions

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List of Non- Standard Abbreviations

BSA- bovine serum albumin

DAPI- 4',6-diamidino-2-phenylindole

H & E – hemotoxylin and eosin

FITC - Fluorescein isothiocyanate

FLNa – Filamin A

FLNa CKO – filamin A conditional knockout

FLNa KD - filamin A knockdown

FLNa WT - filamin A wildtype

PCR – polymerase chain reaction

PSR – picrosirius red

PL – periodontal ligament

SDS/PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

TIRF – total internal reflection fluorescence

Abstract

In mechanically loaded tissues such as weight-bearing joints, myocardium and periodontal ligament, pathophysiological forces can disrupt cell-matrix contacts, which in turn induce cell death leading to tissue and organ dysfunction. Protection against force-induced cell death may be mediated by filamin A (FLNa), an actin-binding protein that regulates $\beta 1$ integrin-mediated cell adhesion. We examined collagen distribution, cell densities and cell death in periodontal ligament of filamin A wildtype and fibroblast conditional-knockout mice. Deletion of FLNa in fibroblasts was associated with 2-fold increase of acellular areas in the periodontal ligament and a 7-fold higher proportion of apoptotic cells. In cultured fibroblasts with FLNa knockdown, we examined the impact of supra-physiological forces ($1 \text{ pN}/\mu\text{m}^2$ cell area; applied through the $\beta 1$ integrin) on recruitment of talin and vinculin to focal adhesions and on apoptosis. Compared with wildtype, FLNa knockdown cells exhibited 3-fold increases of floating cells after overnight force application and a 2-fold increase of cell detachment. Force induced time-dependent reductions ($p < 0.05$) of the numbers of activated $\beta 1$ integrin, talin and vinculin-stained adhesions in FLNa knockdown compared with wildtype cells. We conclude that FLNa protects against apoptosis in cells subjected to mechanical forces and this protection is mediated by enhanced formation and maturation of matrix adhesions.

Keywords: filamin A, mechanical force, talin, vinculin, focal adhesions, periodontal ligament

Introduction

Supra-physiological mechanical forces can induce apoptosis and loss of normal tissue function in bone (1), brain (2) and cardiac tissues (3, 4). In physiologically functioning periodontal ligament (PL) there are higher proportions of apoptotic fibroblasts at sites subjected to increased mechanical load compared to sites with lower loads (5), but proliferation of local fibroblast populations balances cell death and maintains cell populations in steady state conditions (6). In PL subjected to mechanical over-load, fibroblasts exhibit increased apoptosis (7-9), a process that can result in cell deletion and the formation of acellular sites in the PL (10), as well as inappropriate remodeling of the extracellular matrix in response to high amplitude exogenous force (11). The mechanism by which mechanical forces induce cell death is not defined but one possible process may involve anoikis (12) or detachment-induced cell death, which involves disruption of the structure and function of cell-matrix adhesions in anchorage-dependent cells leading to cell death (13).

In cultured cells, focal adhesions are aggregates of cytoskeletal, signaling and adhesion receptor proteins that mediate and control connectivity of the cytoskeleton to matrix proteins (14). Direct transfer of mechanical forces applied through matrix proteins to cells occurs at focal adhesions enriched with receptors such as integrins, which on their cytoplasmic faces, bind to various adaptor proteins (15). These adaptor proteins link adhesions to the actin cytoskeleton, therefore enabling the transmission of exogenously applied forces and the generation of mechanically-induced signals that may promote or protect cells against force-induced death (16). The control systems that mediate force-induced signals and protect or promote cell death are not defined.

Cells subjected to tensile forces resist detachment and detachment-induced cell death in part due to adhesion-associated molecules like FLNa and talin, actin binding proteins that associate with the cytoplasmic tail of $\beta 1$ integrin (17). The binding of talin to $\beta 1$ integrin not only provides a mechanical linkage between the extracellular matrix and the cytoskeleton but is also required for integrin activation, which promotes matrix ligand binding (18). As a result of talin binding to integrins, the recruitment of other actin binding proteins like vinculin to adhesions is enhanced. Vinculin may in turn act as a force-transducing protein to mediate reorganization of cell polarity and adhesion structures to adapt to more stressful conditions (19).

FLNa is thought to inhibit integrin-mediated activation and adhesion by a 'displacement' mechanism in which FLNa competes with talin for binding to the cytoplasmic tail of $\beta 1$ integrin (20, 21). The ability of talin to displace FLNa binding to $\beta 1$ integrin may enhance integrin activation and as a result, cell adhesion (22). More recent data indicate that FLNa is required for $\beta 1$ integrin activation at the outset of cell spreading on collagen, possibly by promoting the trafficking and cell surface expression of $\beta 1$ integrin (23, 24). Notably, we found that FLNa is involved in the prevention of forced-induced apoptosis through the Rac/Pak/p38 signalling pathway (25) and may play a mechanoprotective role in response to applied tensile forces. Currently, the

mechanism by which FLNa regulates cell adhesion to affect the viability of cells subjected to increased mechanical loads is not defined.

In this study we investigated the mechanoprotective roles of FLNa in the PL of mice and in cultured fibroblasts. For the study of mechanoprotection in intact animals, the PL provides an excellent model system since the effects of high amplitude mechanical forces on connective tissue structure can be readily examined (26) and there are tightly regulated homeostatic systems that preserve PL cell populations, structure and function in the face of high amplitude mechanical loading (27). In cultured cells subjected to tensile forces, we examined the recruitment of $\beta 1$ integrin, talin and vinculin to focal adhesions. Our data show that in the PL of intact mice, FLNa protects fibroblasts against apoptosis. In cultured fibroblasts, FLNa helps to preserve talin and activated $\beta 1$ integrins at cell adhesions, thereby preserving cell attachment to matrix substrates and enhancing the viability of cells subjected to applied mechanical force.

Materials and Methods

Antibodies and reagents

Mouse monoclonal antibodies (talin 1/2-clone 8d4; vinculin-clone hVIN-1) were purchased from Sigma-Aldrich (Oakville, ON). Rat monoclonal antibody against activated mouse $\beta 1$ integrin (clone 9EG7) was obtained from Pharmingen (Mississauga, ON). Rabbit FLNa polyclonal antibody (for immunofluorescence) was a gift from Dr. F. Nakamura (Harvard University, Boston, MA). Rabbit monoclonal antibodies (β -actin, FLNa) were purchased from Epitomics (Burlingame, CA). Horseradish peroxidase-conjugated goat IgG was purchased from Sigma-Aldrich. Plasmids containing the sequence of full length-FLNa cloned into dsRED- pcDNA3 vector were a gift of Miguel Del Pozzo (Melchor Fernández Almagro, Madrid, Spain). Collagen was obtained from BD Sciences (Bedford, MA, USA). Fibronectin was purchased from Sigma-Aldrich. FITC-annexin and TUNEL kits were obtained from Roche (Montreal, QC).

Filamin A conditional mice and genotyping

As deletion of FLNa is embryonic lethal (E12) (28), we used the Cre-Lox system to generate conditional knockouts in which FLNa is deleted from fibroblasts and osteoblasts. We obtained collagen 1 Cre mice from Dr. B. Kream (University of Connecticut, Farmington, CT) in which the Cre-recombinase is expressed under control of the collagen $\alpha 1(I)$ promoter (3.6 kb), a construct that is expressed in connective tissues but not in other tissues (29). The floxed FLNa allele mouse was obtained from D. Kwiatkowski (Harvard University). For the production of conditional mice we performed crosses to obtain ideal breeders (males-FilA^cCol-3.6^{cre/+} and females- FilA^{c/+}Col-3.6^{cre/+}) who produced wild-type, heterozygous or null mice.

PCR lysis reagent (Viagen Biotech, CA) was used to prepare mouse tails for PCR genotypic analysis according to the manufacturer's instructions. A primer pair (forward 5'-CTGCATTACCGGTGCGATGCA-3'; reverse 5'-ACGTCCACCGGCATCAACGT-3) was used to detect the present of LysM-Cre (product

size 300 bp). A primer pair (forward 5'-TCTTCCTCTTTCAGCTGG-3'; reverse 5'-ACAACTGCTGCTCCAGAG-3') were used to amplify the conditional (floxed) (product size- 200-300 bp) and wildtype (100 bp) FLNa alleles.

Histology

Mandibles were dissected and fixed in formalin prior to decalcification. Jaws were trimmed, paraffin-embedded, sectioned sagittally and processed for Masson's trichrome, hematoxylin and eosin or picrosirius red. Processing and staining were performed at the Mount Sinai Hospital histology service (Toronto, ON).

Cell Culture

Explant cultures were prepared by dissecting mandibles and maxilla from FLNa WT and KO mice. Jaws were washed three times in antibiotics (146 units/ml penicillin G, 50 µg/ml gentamicin and 0.25 µg/ml amphotericin) for 10 minutes, minced and the explant pieces were cultured on fibronectin-coated glass coverslips in 37°C in α MEM supplemented with 10% fetal bovine serum and antibiotics. NIH 3T3 cells that express constitutively FLNa or that were transfected with short hairpin RNA for FLNa (obtained from David Calderwood, Yale University, New Haven, CT) were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (146 units/ml penicillin G, 50 µg/ml gentamicin and 0.25 µg/ml amphotericin). NIH 3T3 cells stably transfected with short hairpin RNA against FLNa were grown in the presence of puromycin (1 µg/ml).

Force application

Tensile forces were applied to cells as described (30). Briefly, magnetite beads (Fe_3O_4 , modal diameter = 5 µm; Sigma-Aldrich, Oakville, ON) were incubated with 1 mg/ml collagen, neutralized to pH 7.4 and rinsed with PBS to induce fibril formation. Cells were plated in equal numbers on fibronectin-coated plates (10 µg/ml) and allowed to spread for 3 hours. Collagen beads were incubated on the dorsal surfaces of cells for 1 hour prior to force application. Magnetic fields were applied with a ceramic permanent magnet (Jobmaster, Mississauga, ON) to generate perpendicular tensile forces on cells. The magnet pole face was orientated parallel with the culture dish surface at a distance of 20 mm from the surface. At these distances, single cells of $\sim 250 \mu\text{m}^2$ surface area are exposed to tensile forces of 160 pN (30). For all experiments involving force application, controls involved incubation of cells with collagen beads, but without force application.

Immunoblotting and immunostaining

Cells were lysed and proteins were separated by SDS/PAGE and transferred to nitrocellulose membranes. Protein concentrations of cell lysates were determined using the BCATM protein assay (Pierce, Rockford, IL). Equal amounts of protein were loaded in individual lanes. Nitrocellulose membranes were probed for the indicated antibody, followed by incubation with anti-mouse or anti-rabbit FITC-conjugated secondary

antibodies (Mandel, Guelph, ON). The LiCor Odyssey (Mandel) and Image Studio software (Mandel) were used for detection and analysis.

For immunostaining, cultured cells were washed and fixed with 4% buffered paraformaldehyde after force application. Cells were permeabilized with 0.2% PBS Triton-X and blocked for 20mins with 0.2% BSA. Cells were incubated with primary antibodies at 4°C overnight followed by incubation with FITC-conjugated secondary for 1h at room temperature.

Collagen adhesion-associated proteins

Following force treatment, cells were washed with ice-cold 1xPBS as described (31). Cells with bound collagen beads were scraped from culture dishes with cytoskeletal extraction buffer (0.5% Triton X-100, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride and 10 mM PIPES, at pH 6.8, with the Sigma Protease inhibitor cocktail at a ratio of 1:50). Lysates were homogenized and using a side-pull magnet, focal adhesion and non-focal adhesion fractions were isolated. Beads were re-suspended in 1x Laemmli buffer and boiled for 10 mins to release bead-associated proteins. Equal amounts of proteins were loaded and probed for FLNa and talin 1/2.

Transfection

For co-localization of exogenous FLNa with focal adhesions, FLNa knockdown cells were transfected with dsRED- FLNaWT using Polyjet transfection reagent (FroggaBio, Toronto, ON, Canada) according to the manufacturer's instructions.

Cell death

After force application (applied to dorsally-loaded magnetite beads on cells), we used permanent magnets placed under the dish to maintain cells *in situ* on the dishes for subsequent preparatory steps. To isolate floating cells that had detached from the bottom of the dishes and with attached magnetite particles, medium was collected and cells were isolated with a side-pull magnet and re-suspended in 95% ethanol. All cell fractions were air-dried on 1% gelatin-coated slides. Cells were stained with FITC-annexin to identify dead cells. For analysis of cell death *in vivo*, sections of mouse periodontium were stained for TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling).

Microscopy

Analysis of immunostaining for talin, vinculin, FLNa and β 1 integrin was performed on the ventral surfaces of attached cells (fibronectin-coated glass) by TIRF as described (24).

Statistics

For all experiments, separate assays were repeated at least three times. For quantitative data, mean $\pm$ S.D. values were computed. Comparisons of multiple samples were analyzed with ANOVA followed by analysis with Tukey's test. Statistical significance was set at $p < 0.05$.

Results

Mouse characterization

As a global FLNa knockout is embryonic lethal in mice (28), we developed a conditional FLNa knockout mouse (CKO) under the control of the collagen α I promoter (29) in which deletion of FLNa expression was restricted to fibroblasts and osteoblasts (Fig. 1A). Confirmation of FLNa deletion in fibroblasts was assessed by immunofluorescence staining for FLNa of cultures of PL fibroblasts from FLNa WT and CKO mandibular molar root explants (Fig. 1B). We conducted morphometric analyses of periodontal structures in sections of mandibles from 2-month old FLNa WT and CKO, which were stained for hematoxylin and eosin, Masson's trichrome or picrosirius red (Fig. 1C). The orientation and organization of collagen fibers in the intact PL was not apparently different between WT and CKO mice but there was increased frequency of ankylosis, sites where there was in-growth of bone into the PL space and which are associated with isolated PL cell death (32, 33). From sections of the furcation region of the mandibular first molar, a site of high mechanical loading as a result of physiological masticatory function (34), cell densities, PL widths and relative collagen densities were measured at sites where there was no ankylosis or loss of cellularity. At these sites there were no significant differences of cell densities between FLNa WT and CKO mice ($p>0.2$; Fig. 1D) nor was there an effect of FLNa on PL width ($p>0.2$, Fig. 1E), an indicator of intact homeostatic mechanisms in the PL (5). With picrosirius red staining we analyzed the effect of FLNa on fibrillar collagen. Comparisons of measurements from the various regions of the periodontal ligament within genotypes indicated no differences in collagen density (data not shown) but we observed increased amounts of collagen staining in the PL of the furcation in the FLNa CKO mice relative to the FLNa WT mice (Fig. 1F). We also quantified the areas of PL in which there were acellular zones or hyalinization (35). FLNa CKO mice exhibited 2-fold increased areas of hyalinization compared with FLNa WT mice ($p<0.05$; Fig. 1G)

Cell death

We reported earlier that FLNa protects cells from apoptosis after application of supra-physiological tensile forces *in vitro* (25). To determine whether this protection was detectable *in vivo*, TUNEL staining of sections of mandibular molar periodontium, which contains the PL, was performed (Fig. 2A). The PL, which is primarily populated by fibroblasts (5), showed an ~7-fold increase of the proportion of apoptotic cells in FLNa CKO mice compared with WT mice (Fig. 2B; $p<0.001$).

Mechanical force application to cultured cells can stimulate cell death (36) but it is not known whether this process involves anoikis, a form of apoptosis caused by cell detachment from the extracellular matrix (37). To investigate the effect of FLNa on protecting cells against force-induced anoikis, we cultured NIH 3T3 cells with wildtype levels of FLNa expression (FLNa WT) or with FLNa knockdown (FLNa KD; by short hairpin RNA) (38) (Figs. 3A,B). Cells were plated on fibronectin-coated surfaces and collagen-coated beads were

attached to the dorsal cell surface, an intentional strategy that reduces the likelihood of adhesion receptor competition by using different matrix ligands on the ventral and dorsal cell surfaces. After 24h-force application, FLNa WT and KD cells were stained with DAPI to quantify the number of cells that remained adherent to dishes after tensile force application (Fig. 3C). Compared with FLNa WT cells, FLNa KD cells subjected to tensile force exhibited ~2-fold increases of the number of detached cells from the fibronectin substrate (Fig. 3D) and ~40% reduction of the numbers of cells that remained attached to the substrate (Fig. 3E; $p < 0.001$).

We determined whether the detached cells were apoptotic by measuring FITC-annexin binding to cells, an early marker of apoptosis (39). Detached FLNa KD cells exhibited annexin binding whereas adherent FLNa KD cells were unstained (Fig. 3F). In cells subjected to mechanical force, FLNa WT cells showed very little FITC-annexin binding whereas FLNa KD cells were frequently bound by FITC-annexin (Fig. 3G).

Filamin A in cell adhesions

We investigated the role of FLNa in protecting against force-induced anoikis by assessing the spatial distribution of FLNa after force application to cultured cells. In cells with attached collagen beads that were not subjected to force, immunostaining showed that endogenous FLNa was diffusely distributed throughout the cytoplasm. After force treatment, FLNa was more enriched at sites of force application (Fig. 4A). We also examined the localization of FLNa in cell adhesions to collagen beads by immunoblot analysis of collagen bead-associated proteins (40) (Fig. 4B). Consistent with the immunofluorescence data of FLNa in adhesions on the ventral cell surface, there was increased abundance of FLNa in the protein fractions prepared from collagen-coated magnetite beads that were attached to the dorsal cell surface. We found no difference in the abundance of FLNa in the remaining proteins that did not bind to collagen beads (data not shown).

The data described above indicate that FLNa may protect cells from detaching from their underlying fibronectin-coated substrate (Fig. 3D,E). In this model system, tensile forces are applied to collagen beads on the dorsal surface of cells while cell attachment to the culture dish is mediated through ventrally-located adhesion receptors that bind fibronectin (Fig. 4C). We next examined proteins in ventrally-located adhesions that provide protection against anoikis in response to tensile forces. FLNa KD cells were transfected with full-length FLNa cloned into a dsRED expression plasmid. By immunostaining for vinculin (as a focal adhesion marker) and application of TIRF microscopy for assessing ventrally-located focal adhesions, we found that in WT cells, FLNa co-localized with vinculin (Fig. 4D; Pearson's coefficient $r = 0.73$).

Quantification of focal adhesions

We sought to examine the effect of FLNa on focal adhesion formation at ventral cell surfaces. First we tested the validity of a measurement system for quantifying adhesion formation. FLNa WT and KD cells were plated on defined concentrations of fibronectin in the coating buffer. Cells spread for 5 h prior to immunostaining for

talins and vinculin and ventral adhesions were imaged by TIRF microscopy (Fig. 5A and C, respectively). The number of focal adhesions per cell in FLNa WT cells increased as the coating concentration of fibronectin was raised while in FLNa KD cells there was no difference in the number of focal adhesions, independent of fibronectin coating concentration. At fibronectin coating concentrations of $\geq 10 \mu\text{g/ml}$, the number of talin-stained focal adhesions was higher (by 2-4 fold) in FLNa WT cells compared with FLNa KD cells (Fig. 5B; $p < 0.05$). We found a similar pattern of the number of focal adhesions stained for vinculin (Fig. 5C) although at very high coating concentrations of fibronectin ($100 \mu\text{g/ml}$) the difference in the number of focal adhesions per cell for FLNa WT and KD cells was not different (Fig. 5D; $p > 0.2$). As these data of focal adhesion number varied as a function of matrix ligand concentration, we subsequently used focal adhesion number as a surrogate measure of cell adhesion formation.

As we noted above, dorsal force application causes cells to detach from the ventral substrate (Figs. 3C-E), particularly those where FLNa is knocked down. Also, cells plated on a fibronectin-coating concentration of $10 \mu\text{g/ml}$ was associated with the largest difference of the number of focal adhesion per cell between FLNa WT and KD cells (Fig. 5B). Accordingly, we plated cells on fibronectin at $10 \mu\text{g/ml}$, followed by incubation with dorsally-loaded collagen beads and applied force for 1 hr or 12 hrs. We then assessed the correlation between the presence of FLNa and focal adhesion formation, as well as factors that prevent cell detachment.

With this model system we first immunostained force-treated cells with 9EG7, a neo-epitope antibody that recognizes activated $\beta 1$ integrins. Quantification of the mean number of focal adhesions per cell in TIRF images showed that there were more activated $\beta 1$ integrin-stained adhesions in FLNa WT cells compared with FLNa KD cells after 12 hrs of force (Fig. 6A; $p < 0.05$).

From images acquired by TIRF microscopy of talin-stained cells, we quantified the number of talin-stained focal adhesions. FLNa WT cells exhibited no change of the number of talin-stained focal adhesions per cell with or without force treatment at 1 and 12 hrs after force application. In contrast, FLNa KD cells that were treated with or without force for 1 hr showed $< 40\%$ of the number of adhesions as FLNa WT cells. After 12 hrs of force, FLNa KD cells showed no increase of focal adhesion numbers compared with 1 hr (Fig. 6B; $p > 0.2$). These patterns of lower numbers of talin-stained focal adhesions in the FLN KD cells compared with wildtype cells was consistent with data on talin-stained focal adhesion length and area (Suppl. Fig. 1A-C). By means of immunoblotting, we determined that the relative amounts of talin in whole cell lysates were unchanged in the presence or absence of FLNa and were independent of force application (Suppl. Fig. 1D), indicating that talin was not preferentially degraded in the FLNa KD cells. Consistent with the data of talin in ventral adhesions, the abundance of talin in collagen bead-associated fractions was also reduced after force application in the FLNa KD cells (Suppl. Fig. 1D).

We assessed whether FLNa expression or force application affected the formation of more mature focal adhesions. In cells subjected to force for 1 hr or 12 hrs, immunostained for vinculin and examined by TIRF, there was enhanced staining of ventral adhesions after 12 hrs but this staining was diminished in FLNa KD cells subjected to force (data not shown). Quantification of ventral adhesions showed increased numbers of vinculin-stained adhesions per cell after 12 hrs compared with 1 hr ($p<0.05$; Fig. 6C). FLNa KD cells exhibited reduced numbers of ventral adhesions after 12 hr, especially after application of force ($p<0.05$). There were reduced length and area of vinculin-stained adhesions in FLNa KD cells after 1hr force (Suppl. Fig. 2A-C), which was less marked at 12 hr.

Discussion

Supra-physiological forces applied to cells can stimulate signaling pathways that lead to anoikis, which is a type of detachment-induced cell death (36, 41). As a result of cell death, mechanically over-loaded tissues with limited adaptation to high amplitude applied forces may exhibit pathological loss of structure and function, which is seen in cardiac pressure overload, osteoarthritis and the PL of teeth subjected to occlusal trauma. We have focused here on the PL, which is an excellent model for examining force-induced cell death: sites of increased cell death co-localize with sites of high mechanical loading and these sites can be defined and examined in detail (26, 27). Although mechanical force-induced alterations of PL structure have been described in general terms (42), the mechanisms by which forces affect PL cell function and how PL cells survive force application are not defined. Our data show that FLNa may be mechanoprotective *in vivo* as indicated by increased area of acellular zones and increased death of PL fibroblasts in FLNa conditional knockout mice compared with FLNa wild-type mice. Consistent with these data on fibroblasts in intact tissues in mice, we found that in cultured fibroblasts subjected to tensile forces, FLNa prevented anoikis by promoting the formation of ventral adhesions, which manifested as increased numbers of ventral adhesions stained for activated $\beta 1$ integrin, talin and vinculin. Collectively these data suggest that FLNa mechanoprotects cells in force-loaded tissues by promoting the formation of integrin adhesions bound to matrix ligands.

We used a cell culture model and TIRF imaging (43, 44) to enable measurements of the number, area and length of ventral adhesions attached to fibronectin and how these adhesions are altered by exogenous tensile forces (25). There was reduced adhesion formation in cells plated on substrates with lower fibronectin concentrations, demonstrating the ability of the system to model the expected relationship between substrate ligand concentration, integrin activation and maturation of adhesions. Our data also showed the effect of FLNa on cell survival as cells with low levels of FLNa expression were less able to form ventral adhesions and were more susceptible to detachment-induced cell death after application of tensile forces. While other reports have described the mechanics of intermolecular interactions in adhesions responding to force (45, 46), we used our

model to measure the effect of applied forces on adhesion formation in whole cells and their impact on cell death.

Application of mechanical forces to cells can cause a conformational ‘opening’ of the FLNa molecule (47). This allosteric modification enables the release of FilGAP, a FLNa-binding GTPase that activates Rac and stimulates downstream signaling pathways that control actin assembly (48). We showed earlier that FilGAP plays a central role in mediating cell survival in response to force application to cells (38). In addition to the disassociation of FilGAP from FLNa, mechanical force application to FLNa may expose cryptic integrin binding sites (49, 50), a notion which is consistent with our data of enhanced $\beta 1$ integrin activation in FLNa wildtype cells exposed to tensile force. Taken together, these processes may enable FLNa to promote the activity of FilGAP and to enhance FLNa binding to integrins (47).

Focal adhesion formation requires the recruitment, assembly and ordered interaction of a large number of structural and adaptor proteins that enables $\beta 1$ integrin binding to matrix ligands (51). Talin may compete with FLNa for binding to $\beta 7$ integrin and possibly $\beta 1$ integrin cytoplasmic tails (21). Indeed, we observed markedly decreased numbers of ventral adhesions and a reduction in the area and length of adhesions in force-treated cells with FLNa knockdown. These data indicate that FLNa may not compete with talin; instead the data suggest that talin recruitment to adhesions is dependent on FLNa in cells subjected to tensile forces.

Focal adhesion maturation involves recruitment of vinculin to adhesions and the interaction of vinculin with other adhesion proteins such as talin (52). These processes facilitate cell anchorage to substrates, which may be important for the survival of cells subjected to detachment forces. As vinculin associates with talin sequestered in mature focal adhesions (19), we determined whether the number of vinculin-stained adhesions was affected by FLNa or force. After 12 hr of force application in cells with FLNa knockdown, there was a very large reduction of the number of vinculin-stained adhesions. These data underscore the role of vinculin assembly in focal adhesion formation and the importance of these adhesions in the susceptibility of cells to anoikis (46). Previous reports have shown that in cells subjected to force, talin is required for the recruitment of vinculin to focal adhesions where it amplifies adhesion signals from activated $\beta 1$ integrin (19, 21, 46, 53-56).

In the current study we found that FLNa localizes to ventral cell adhesions, sites that are critical for anchorage of cells exposed to tensile forces. We propose that FLNa acts to promote focal adhesion maturation, thereby preventing cell deletion by anoikis (57). However in addition to the role of FLNa in regulating adhesion formation, we note that FLNa also protects cells from force-induced cell death initiated by the Rac/Pak1/p38 pathway (25). The protection provided by FLNa may involve the recruitment of FilGAP to sites of force application and there contribute to the regulation of p38-induced apoptosis (25).

Fibroblasts in the PL of mouse molar teeth provide a good model to study force-induced cell death because apoptotic cells are found at sites of high mechanical load (5), such as furcations. PL fibroblasts are

renewal cell populations in which cell death and cell migration are balanced by cell proliferation to maintain cell and tissue homeostasis (5, 6, 58). In agreement with these reports we found that in areas of intact PL, the width of the PL, cell density and collagen fiber abundance were not significantly altered in FLNa CKO mice compared with FLNa WT mice. However, the FLNa CKO mice exhibited much larger acellular zones than the FLNa WT mice, which is consistent with the greatly increased percentage of apoptotic cells in the PL of the FLNa CKO mice. These data emphasize the importance of FLNa in maintaining PL fibroblast viability, which is in turn important for the preservation of the structure and function of this force-loaded tissue.

Figure Legends

Figure 1. Characterization of conditional FLNa knockout mice

(A) Mice were genotyped by PCR to determine homozygous floxed FLNa product of 200-300bp compared to the wildtype mouse FLNa amplicon of 100 bp in addition to LysM-Cre. (B) FLNa deletion confirmed by immunofluorescence of cells cultured from mandible explants from FLNa wildtype (FLNaWT) and conditional knockout mice (FLNa CKO). Scale bar = 0.5 μ m (C) Hematoxylin and eosin (H&E), picrosirius red (PSR) staining and Masson's trichrome of the furcation region of the first molar from FLNa WT and CKO mice. Scale bar = 1 μ m (D) Quantification of cell density from H&E staining. Measurements were made using ImageJ software where eight measurements were obtained for each mouse (total of three mice for each genotype; $p < 0.05$). (E) Quantification of periodontal ligament width from Masson's trichrome staining. Data representative of 24 measurements per mouse; total of three mice for each genotype). (F) Relative collagen density quantified from PSR staining. Data representative of 24 measurements from each mouse for a total of four mice for each genotype ($p < 0.05$). (G) Quantification of hyalinized regions in furcation of first molar of FLNa WT and CKO mice measured in relation to total furcation area. Data representative of 24 measurements from each mouse for a total of three mice for each genotype ($p < 0.05$)

Figure 2. Apoptosis and cell loss in FLNa CKO

(A) Representative images from FLNa WT and CKO tissues assessed for apoptosis by TUNEL. Scale bar = 0.5 μ m (B) Quantification of TUNEL-stained cells. Data are TUNEL-positive cells as a ratio over total cell numbers. Data are mean $\pm$ SEM from three individual experiments ($p < 0.001$).

Figure 3. Force-induced FLNa redistribution *in vitro*

(A) Immunoblot characterization of FLNa WT and FLNa KD NIH 3T3 cells. Lanes were loaded with equal amounts of protein and probed for FLNa. β -actin was used as a loading control. These data are representative of three independent and separate experiments. (B) FLNa WT and FLNa KD NIH 3T3 cells were allowed to spread and immunostained for FLNa. Scale bar = 0.5 μ m (C) Representative DAPI images of FLNa WT and FLNa KD NIH 3T3 cells plated on 10 μ g/mL fibronectin and subjected to 12hrs force application. Scale bar = 1 μ m (D) Quantification of proportional floating cells with force treatment normalized to their no force controls. These data are means $\pm$ SEM for three individual experiments. (E) Quantification of proportional adherent cells with force treatment as normalized to their no force controls. These data are means $\pm$ SEM for three individual experiments. (F) Representative images of annexin-stained cells from adherent and floating fractions of FLNa WT cells after force-treatment as previously described in (C). Scale bar = 0.5 μ m (G) FLNa WT and FLNa KD cells were plated, subjected to force (as described in (C)) and were stained for annexin. Scale bar = 1 μ m

Figure 4. Force-induced apoptosis

(A) FLNa WT NIH 3T3 cells were plated on 10µg/mL fibronectin for 3hrs prior to 30mins force application and immunostained for FLNa. BF- Bright field. Scale bar = 0.5 µm (B) FLNa WT cells were subjected to force as previously described in (A). Focal adhesion (FA) preparations were isolated as described under ‘Materials and Methods’. Equivalent amounts of each fraction were loaded and immunoblotted for FLNa, using β-actin as a loading control. (C) Schematic diagram of model system for cell stretching cells *in vitro*. As described in the “Materials and Methods” section, cells were subjected to force and imaged using TIRF microscopy. Encircled region indicates targeted area for TIRF microscopy. ER = Endoplasmic reticulum. (D) FLNa KD cells were transfected with a dsRed-tagged full length FLNa-WT vector, immunostained for vinculin and imaged using TIRF microscopy. Enlargements are of boxed regions. Scale bar = 25 µm

Figure 5: Effect of Fibronectin Concentration on Focal adhesion formation

(A) FLNa WT and KD cells were plated on increasing concentrations of fibronectin, allowed to spread for 5 hrs and immunostained for talin. Images were obtained using TIRF microscopy. Scale bar = 25 µm. (B) Number of focal adhesions per cell was quantified using MetaMorph software. Data are mean ± SEM for 30 individual cells ($p<0.05$). (C) Cells were allowed to spread as described in (A) and immunostained for vinculin. Scale bar = 25 µm and quantified for (D) Number of focal adhesions per cell. Data are mean ± SEM for 30 individual cells ($p<0.05$).

Figure 6. Effect of force and FLNa on activated β1 integrin, talin and vinculin-stained adhesions

FLNa WT and FLNa KD cells were plated on 10 µg/mL fibronectin, subjected to no force, or force for 1 hr or 12 hrs force, and immunostained. Using TIRF imaging and MetaMorph software, cells from each treatment group were quantified for the mean number of focal adhesions per cell quantified from (A) activated β1 integrins, (B) talin and (C) vinculin immunostaining. These data are mean ± SEM for 30 individual cells ($p<0.05$).

Supplementary Figure 1: Effect of force and FLNa on talin-stained adhesions

Using images obtained by TIRF microscopy and Metamorph software, talin-stained cells were quantified for (A) mean focal adhesion length/cell, (B) mean focal adhesion area/cell, and (C) mean cell area. Data are mean ± SEM for 30 individual cells. ($p<0.05$) (D) FLNa WT and FLNa KD cells were treated as in (A). Focal adhesion (FA) preparations and non-focal adhesion fractions were isolated as described under ‘Materials and Methods’. Equivalent amounts of each fraction were loaded and immunoblotted for talin with β-actin as a loading control.

Supplementary Figure 2: Effect of force and FLNa on vinculin-stained adhesions

Using TIRF imaging and MetaMorph software, cells from each treatment group were quantified for (A) mean focal adhesion length/cell, (B) mean focal adhesion area/cell and (C) mean cell area. Data are mean ± SEM for 30 individual cells ($p<0.05$)

References

1. Verborgt, O., Tatton, N. A., Majeska, R. J., and Schaffler, M. B. (2002) Spatial distribution of Bax and Bcl-2 in osteocytes after bone fatigue: complementary roles in bone remodeling regulation? *J Bone Miner Res* **17**, 907-914
2. Shreiber, D. I., Bain, A. C., Ross, D. T., Smith, D. H., Gennarelli, T. A., McIntosh, T. K., and Meaney, D. F. (1999) Experimental investigation of cerebral contusion: histopathological and immunohistochemical evaluation of dynamic cortical deformation. *J Neuropathol Exp Neurol* **58**, 153-164
3. Shyu, K. G. (2009) Cellular and molecular effects of mechanical stretch on vascular cells and cardiac myocytes. *Clin Sci (Lond)* **116**, 377-389
4. Cheng, W., Li, B., Kajstura, J., Li, P., Wolin, M. S., Sonnenblick, E. H., Hintze, T. H., Olivetti, G., and Anversa, P. (1995) Stretch-induced programmed myocyte cell death. *J Clin Invest* **96**, 2247-2259
5. McCulloch, C. A., Barghava, U., and Melcher, A. H. (1989) Cell death and the regulation of populations of cells in the periodontal ligament. *Cell Tissue Res* **255**, 129-138
6. McCulloch, C. A., and Melcher, A. H. (1983) Cell density and cell generation in the periodontal ligament of mice. *Am J Anat* **167**, 43-58
7. Zhong, W., Xu, C., Zhang, F., Jiang, X., Zhang, X., and Ye, D. (2008) Cyclic stretching force-induced early apoptosis in human periodontal ligament cells. *Oral Dis* **14**, 270-276
8. Hao, Y., Xu, C., Sun, S. Y., and Zhang, F. Q. (2009) Cyclic stretching force induces apoptosis in human periodontal ligament cells via caspase-9. *Archives of oral biology* **54**, 864-870
9. Ritter, N., Mussig, E., Steinberg, T., Kohl, A., Komposch, G., and Tomakidi, P. (2007) Elevated expression of genes assigned to NF-kappaB and apoptotic pathways in human periodontal ligament fibroblasts following mechanical stretch. *Cell Tissue Res* **328**, 537-548
10. Kurol, J., and Owman-Moll, P. (1998) Hyalinization and root resorption during early orthodontic tooth movement in adolescents. *Angle Orthod* **68**, 161-165
11. Von Bohl, M., Maltha, J., Von den Hoff, H., and Kuijpers-Jagtman, A. M. (2004) Changes in the periodontal ligament after experimental tooth movement using high and low continuous forces in beagle dogs. *Angle Orthod* **74**, 16-25
12. Taddei, M. L., Giannoni, E., Fiaschi, T., and Chiarugi, P. (2011) Anoikis: an emerging hallmark in health and diseases. *J Pathol* **226**, 380-393

13. Verderio, E. A., Telci, D., Okoye, A., Melino, G., and Griffin, M. (2003) A novel RGD-independent cell adhesion pathway mediated by fibronectin-bound tissue transglutaminase rescues cells from anoikis. *J Biol Chem* **278**, 42604-42614
14. Wehrle-Haller, B. Structure and function of focal adhesions. *Curr Opin Cell Biol* **24**, 116-124
15. Geiger, B., and Bershadsky, A. (2001) Assembly and mechanosensory function of focal contacts. *Curr Opin Cell Biol* **13**, 584-592
16. Janmey, P. A., and Weitz, D. A. (2004) Dealing with mechanics: mechanisms of force transduction in cells. *Trends Biochem Sci* **29**, 364-370
17. Kim, C., Ye, F., and Ginsberg, M. H. (2011) Regulation of integrin activation. *Annu Rev Cell Dev Biol* **27**, 321-345
18. Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O., and Ginsberg, M. H. (1999) The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. *J Biol Chem* **274**, 28071-28074
19. Carisey, A., Tsang, R., Greiner, A. M., Nijenhuis, N., Heath, N., Nazgiewicz, A., Kemkemer, R., Derby, B., Spatz, J., and Ballestrem, C. (2013) Vinculin regulates the recruitment and release of core focal adhesion proteins in a force-dependent manner. *Curr Biol* **23**, 271-281
20. Kim, C., Ye, F., and Ginsberg, M. H. Regulation of integrin activation. *Annu Rev Cell Dev Biol* **27**, 321-345
21. Kiema, T., Lad, Y., Jiang, P., Oxley, C. L., Baldassarre, M., Wegener, K. L., Campbell, I. D., Ylanne, J., and Calderwood, D. A. (2006) The molecular basis of filamin binding to integrins and competition with talin. *Mol Cell* **21**, 337-347
22. Das, M., Ithychanda, S. S., Qin, J., and Plow, E. F. (2011) Migfilin and filamin as regulators of integrin activation in endothelial cells and neutrophils. *PLoS One* **6**, e26355
23. Kim, H., Sengupta, A., Glogauer, M., and McCulloch, C. A. (2008) Filamin A regulates cell spreading and survival via beta1 integrins. *Exp Cell Res* **314**, 834-846
24. Kim, H., Nakamura, F., Lee, W., Hong, C., Perez-Sala, D., and McCulloch, C. A. (2010) Regulation of cell adhesion to collagen via beta1 integrins is dependent on interactions of filamin A with vimentin and protein kinase C epsilon. *Exp Cell Res* **316**, 1829-1844

25. Shifrin, Y., Pinto, V. I., Hassanali, A., Arora, P. D., and McCulloch, C. A. (2012) Force-induced apoptosis mediated by the Rac/Pak/p38 signalling pathway is regulated by filamin A. *Biochem J* **445**, 57-67
26. Naveh, G. R., Brumfeld, V., Shahar, R., and Weiner, S. (2012) Tooth periodontal ligament: Direct 3D microCT visualization of the collagen network and how the network changes when the tooth is loaded. *J Struct Biol* **181**, 108-115
27. Trombetta, J. M., and Bradshaw, A. D. (2010) SPARC/osteonectin functions to maintain homeostasis of the collagenous extracellular matrix in the periodontal ligament. *J Histochem Cytochem* **58**, 871-879
28. Feng, Y., Chen, M. H., Moskowitz, I. P., Mendonza, A. M., Vidali, L., Nakamura, F., Kwiatkowski, D. J., and Walsh, C. A. (2006) Filamin A (FLNA) is required for cell-cell contact in vascular development and cardiac morphogenesis. *Proc Natl Acad Sci U S A* **103**, 19836-19841
29. Liu, F., Woitge, H. W., Braut, A., Kronenberg, M. S., Lichtler, A. C., Mina, M., and Kream, B. E. (2004) Expression and activity of osteoblast-targeted Cre recombinase transgenes in murine skeletal tissues. *Int J Dev Biol* **48**, 645-653
30. Glogauer, M., Ferrier, J., and McCulloch, C. A. (1995) Magnetic fields applied to collagen-coated ferric oxide beads induce stretch-activated Ca²⁺ flux in fibroblasts. *Am J Physiol* **269**, C1093-1104
31. Wang, Q., Herrera Abreu, M. T., Siminovitch, K., Downey, G. P., and McCulloch, C. A. (2006) Phosphorylation of SHP-2 regulates interactions between the endoplasmic reticulum and focal adhesions to restrict interleukin-1-induced Ca²⁺ signaling. *J Biol Chem* **281**, 31093-31105
32. Atrizadeh, F., Kennedy, J., and Zander, H. (1971) Ankylosis of teeth following thermal injury. *J Periodontal Res* **6**, 159-167
33. Lindskog, S., Pierce, A. M., Blomlof, L., and Hammarstrom, L. (1985) The role of the necrotic periodontal membrane in cementum resorption and ankylosis. *Endod Dent Traumatol* **1**, 96-101
34. Jeon, P. D., Turley, P. K., Moon, H. B., and Ting, K. (1999) Analysis of stress in the periodontium of the maxillary first molar with a three-dimensional finite element model. *Am J Orthod Dentofacial Orthop* **115**, 267-274
35. Rygh, P. (1974) Hyalinization of the periodontal ligament incident to orthodontic tooth movement. *Nor Tannlaegeforen Tid* **84**, 352-357

36. Kainulainen, T., Pender, A., D'Addario, M., Feng, Y., Lekic, P., and McCulloch, C. A. (2002) Cell death and mechanoprotection by filamin a in connective tissues after challenge by applied tensile forces. *J Biol Chem* **277**, 21998-22009
37. Frisch, S. M., and Screaton, R. A. (2001) Anoikis mechanisms. *Curr Opin Cell Biol* **13**, 555-562
38. Shifrin, Y., Arora, P. D., Ohta, Y., Calderwood, D. A., and McCulloch, C. A. (2009) The role of FilGAP-filamin A interactions in mechanoprotection. *Mol Biol Cell* **20**, 1269-1279
39. van Engeland, M., Nieland, L. J., Ramaekers, F. C., Schutte, B., and Reutelingsperger, C. P. (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* **31**, 1-9
40. Glogauer, M., Arora, P., Yao, G., Sokholov, I., Ferrier, J., and McCulloch, C. A. (1997) Calcium ions and tyrosine phosphorylation interact coordinately with actin to regulate cytoprotective responses to stretching. *J Cell Sci* **110** (Pt 1), 11-21
41. Hsieh, M. H., and Nguyen, H. T. (2005) Molecular mechanism of apoptosis induced by mechanical forces. *Int Rev Cytol* **245**, 45-90
42. Reitan, K. (1967) Clinical and histologic observations on tooth movement during and after orthodontic treatment. *Am J Orthod* **53**, 721-745
43. Rajshankar, D., Downey, G. P., and McCulloch, C. A. (2012) IL-1beta enhances cell adhesion to degraded fibronectin. *FASEB J* **26**, 4429-4444
44. Mohammad, I., Arora, P. D., Naghibzadeh, Y., Wang, Y., Li, J., Mascarenhas, W., Janmey, P. A., Dawson, J. F., and McCulloch, C. A. (2012) Flightless I is a focal adhesion-associated actin-capping protein that regulates cell migration. *FASEB J* **26**, 3260-3272
45. del Rio, A., Perez-Jimenez, R., Liu, R., Roca-Cusachs, P., Fernandez, J. M., and Sheetz, M. P. (2009) Stretching single talin rod molecules activates vinculin binding. *Science* **323**, 638-641
46. Grashoff, C., Hoffman, B. D., Brenner, M. D., Zhou, R., Parsons, M., Yang, M. T., McLean, M. A., Sligar, S. G., Chen, C. S., Ha, T., and Schwartz, M. A. (2010) Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature* **466**, 263-266
47. Ehrlicher, A. J., Nakamura, F., Hartwig, J. H., Weitz, D. A., and Stossel, T. P. (2011) Mechanical strain in actin networks regulates FilGAP and integrin binding to filamin A. *Nature* **478**, 260-263
48. Ohta, Y., Hartwig, J. H., and Stossel, T. P. (2006) FilGAP, a Rho- and ROCK-regulated GAP for Rac binds filamin A to control actin remodelling. *Nat Cell Biol* **8**, 803-814

49. Chen, H. S., Kolahi, K. S., and Mofrad, M. R. (2009) Phosphorylation facilitates the integrin binding of filamin under force. *Biophys J* **97**, 3095-3104
50. Pentikainen, U., and Ylanne, J. (2009) The regulation mechanism for the auto-inhibition of binding of human filamin A to integrin. *J Mol Biol* **393**, 644-657
51. Choi, C. K., Vicente-Manzanares, M., Zareno, J., Whitmore, L. A., Mogilner, A., and Horwitz, A. R. (2008) Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nat Cell Biol* **10**, 1039-1050
52. Wehrle-Haller, B. (2012) Structure and function of focal adhesions. *Curr Opin Cell Biol* **24**, 116-124
53. Lee, H. S., Anekal, P., Lim, C. J., Liu, C. C., and Ginsberg, M. H. (2013) Two Modes of Integrin Activation Form a Binary Molecular Switch in Adhesion Maturation. *Mol Biol Cell*
54. Galbraith, C. G., Yamada, K. M., and Sheetz, M. P. (2002) The relationship between force and focal complex development. *The Journal of cell biology* **159**, 695-705
55. Goult, B. T., Zacharchenko, T., Bate, N., Tsang, R., Hey, F., Gingras, A. R., Elliott, P. R., Roberts, G. C., Ballestrem, C., Critchley, D. R., and Barsukov, I. L. (2013) RIAM and vinculin binding to talin are mutually exclusive and regulate adhesion assembly and turnover. *J Biol Chem*
56. Lee, S. E., Chunsrivirod, S., Kamm, R. D., and Mofrad, M. R. (2008) Molecular dynamics study of talin-vinculin binding. *Biophys J* **95**, 2027-2036
57. Chiarugi, P., and Giannoni, E. (2008) Anoikis: a necessary death program for anchorage-dependent cells. *Biochem Pharmacol* **76**, 1352-1364
58. McCulloch, C. A., and Melcher, A. H. (1983) Cell migration in the periodontal ligament of mice. *J Periodontal Res* **18**, 339-352

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Supplementary Figure 1

Supplementary Figure 2

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