Emerin Deregulation Links Nuclear Shape Instability to Metastatic Potential

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Abstract

Abnormalities in nuclear shape are a well-known feature of cancer, but their contribution to malignant progression remains poorly understood. Here, we show that depletion of the cytoskeletal regulator, Diaphanous-related formin 3 (DIAPH3), or the nuclear membrane–associated proteins, lamin A/C, in prostate and breast cancer cells, induces nuclear shape instability, with a corresponding gain in malignant properties, including secretion of extracellular vesicles that contain genomic material. This transformation is characterized by a reduction and/or mislocalization of the inner nuclear membrane protein, emerin. Consistent with this, depletion of emerin evokes nuclear shape instability and promotes metastasis. By visualizing emerin localization, evidence for nuclear shape instability was observed in cultured tumor cells, in experimental models of prostate cancer, in human prostate cancer tissues, and in circulating tumor cells from patients with metastatic disease. Quantitation of emerin mislocalization discriminated cancer from benign tissue and correlated with disease progression in a prostate cancer cohort. Taken together, these results identify emerin as a mediator of nuclear shape instability in cancer and show that destabilization of emerin can promote metastasis.

Significance: This study identifies a novel mechanism integrating the control of nuclear structure with the metastatic phenotype, and our inclusion of two types of human specimens (cancer tissues and circulating tumor cells) demonstrates direct relevance to human cancer.

Graphical Abstract: http://cancerres.aacrjournals.org/content/canres/78/21/6086/F1.large.jpg.

Introduction

Despite many advances that have identified molecular alterations in cancer cells, nuclear structure remains the “gold standard” for cancer diagnosis (1). Histologic features such as nuclear size, shape, the number of nucleoli, and chromatin texture are considered clinically relevant; however, the functional significance of these alterations in the context of disease progression remains difficult to interpret (1). Recent studies have identified nuclear envelope ruptures during cancer cell migration. Nuclear envelope blebs and breaks occur in nonmitotic, migratory cancer cells, facilitating their passage through small gaps, suggesting that stability of the nuclear envelope is important functionally in aggressive...
malignancies (2–4). Specific nuclear envelope proteins regulate the nuclear shape of immune cells, which are particularly migratory (5, 6). Extensive nuclear deformations facilitate immune cell passage through intestinal gaps (5, 7). Many similarities between immune cells and cancer cell motility have been described (8, 9).

A primary mechanism of metastatic dissemination is the transient epithelial-to-mesenchymal transition (EMT) of migratory carcinoma cells. However, recent evidence has emerged showing that EMT is not necessarily a prerequisite for metastasis, suggesting that other cellular strategies may also play a role in cancer progression (10, 11). Cancer cells can also exhibit an 'amoeboid' migratory phenotype that resembles immune cell movement, in which the ability of cells to undergo extensive cellular deformations plays a critical role (7, 9). Amoeboid phenotypes are associated with cancer progression (12). A key regulator of amoeboid behavior is Diaphanous-related formin 3 (DIAPH3/mDia2), a cytoskeletal protein frequently deleted in metastatic breast and prostate cancer (13). DIAPH3-depleted breast and prostate cancer cells exhibit an amoeboid phenotype, characterized by altered microtubule dynamics, increased motility, shedding of atypically large extracellular vesicles (EV) known as "large oncosomes," and increased metastatic potential (13–15). The molecular basis of these wide-ranging effects arising from loss of DIAPH3 has not been resolved.

In this study, we show that amoeboid, DIAPH3-depleted cells, which are highly migratory and metastatic, display defects in nuclear shape, including nuclear envelope blebbing, deregulation of the nuclear envelope protein emerin, and secretion of EV that contain genomic material. Emerin depletion recapitulates these nuclear features and promotes metastasis in mouse models, suggesting that emerin deregulation can play a role in malignant transformation. In drug-resistant prostate cancer, lamin A/C, which stabilizes emerin at the nuclear envelope, is downregulated. Quantitative analyses in patients using tissues and circulating tumor cells (CTC) suggest that immunolocalization of emerin can identify tumor cells with nuclear shape instability. This study provides a direct link between nuclear instability, deregulation of emerin, and malignant potential, and identifies emerin as a clinically relevant biomarker of tumor aggressiveness.

Materials and Methods

Antibodies and reagents

Emerin (4G5, Leica Biosystems); β-actin (AC-74, Sigma-Aldrich); caveolin-1 (N-20, Santa Cruz Biotechnology); GAPDH (D16H11, Cell Signaling Technology); p-Thr202/Tyr204-ERK-1/2 (D13.14.4E, Cell Signaling Technology); ERK1/2 (Cell Signaling Technology); pThr18/Ser19-MLC2 (Cell Signaling Technology); lamin A/C (4C11; Cell Signaling Technology); p-Thr202/Tyr204-ERK-1/2 (Thermo Fisher Scientific); Cholera toxin B (Sigma-Aldrich); Crystal Violet (Becton Dickinson); CellMask Orange (Thermo Fisher Scientific); Matriigel (Corning); Collagen (BD Biosciences); Cholera toxin B (Sigma-Aldrich).

Plasmids

Lentiviral shRNA targeting human emerin and lamin A/C and lentiviral nontargeting control vector was purchased from Dharmacon. Emerin shRNA #1 (Oligo ID: TRCN0000083010) hairpin sequence: 5′-AACCCAGGGGCTGCTTGGAAAAG-3′; emerin shRNA #2 (Oligo ID: TRCN0000083011) hairpin sequence: 5′-AACCCAGGGGCTGCTTGGAAAAG-3′; lamin A/C shRNA #1 (Oligo ID: TRCN0000061836) hairpin sequence: 5′-AAAACCCGGGCTTGGAAAAG-3′; lamin A/C shRNA #2 (Oligo ID: TRCN0000061837) hairpin sequence: 5′-AAAACCCGGGCTTGGAAAAG-3′. The emerin lentiviral expression vector (Clone ID: csshBroad304_00501) was purchased from Dharmacon and the control vector (pLX304) was a gift from Dr. David Root (Addgene, plasmid #25890; ref. 16).

Cell culture

DU145, LNCaP, PC3, and BT-549 cells were obtained from ATCC. HMEC and HMEC-HRASV12 were a kind gift from Dr. Robert A. Weinberg (Whitehead Institute, Cambridge, MA) and were maintained as described previously (13). Cell lines stably transfected with DIAPH3 shRNA, emerin shRNA, and lamin A/C shRNA were maintained in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS (GE Healthcare Life Sciences), 2 mmol/L 1-glutamine, 100 U/mL of penicillin, 100 µg/mL streptomycin, and 2 µg/mL puromycin (Thermo Fisher Scientific). HMEC cells were maintained in DMEM/F12 as described previously (13). DIAPH3 shRNA and DIAPH3-GFP and DIU145 DIAPH3-GFP were maintained in DMEM 10% FBS supplemented with 400 µg/mL G418 (Thermo Fisher Scientific) as described previously (13). All cell lines were authenticated by short tandem repeated profiling (Laragen), and were negative for Mycoplasma. The cell lines were periodically tested with Mycoplasma Detection Kit (Lonza). All cell lines were used for no more than 5 passages after thawing.

Parallel microfiltration

The parallel microfiltration (PMF) device was assembled using polycarbonate membranes (Isopore membrane filter, EMD Millipore) of 10-µm pore diameter. Wells were loaded with 1/3 w/w BSA solution (Thermo Fisher Scientific) for 1 hour at 37°C, and then emptied and air dried at least 1 hour before each experiment. A cell suspension of 5 × 10⁵ cells/mL in a final volume of 750 µL was loaded into each well. Well-defined air pressure of 2.1 kPa was applied using a custom-built manometer and monitored using a pressure gauge (Noshok Inc.). Percentage of retention was determined by collecting the sample suspension remaining in the top well and measuring the mass using a precision balance (Northeast Scale Inc.). An automated cell counter (TC20, Bio-Rad) was used to measure cell number and size distributions. This method enables simultaneous measurements of multiple samples using a uniform air pressure to drive cell suspensions through porous membranes. The relative deformability of a cell sample is quantified by the fraction of sample retained above the membrane (17).

Nuclear circularity measurements

For the analysis of nuclear circularity, cells were trypanspinned and fixed with 2% paraformaldehyde (PFA). The nuclei were stained with DAPI. Images of the cells were taken at 100× using...
fluorescence microscopy (Eclipse 90i, Nikon). The nuclei were identified as DAPI-positive areas and the nuclear circularity was calculated with the morphometry package in NIS-Element software (Nikon).

**Immunofluorescence**

Cells were grown on 15 mm microscope cover glasses stained with 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (Thermo Fisher Scientific) for 30 minutes, washed, fixed with 4% PFA, permeabilized in 0.1% Triton X, blocked in 5% BSA, and incubated with the primary antibody overnight at 4°C. For emerin and lamin A staining, the cells were fixed and permeabilized with methanol/acetone (1:1). Cells were then washed, incubated with the antimouse conjugate with FITC (Thermo Fisher Scientific) 1:1,000 for 45 minutes at 1 hour at room temperature, further washed and mounted in cover slips with Prolong Gold (Thermo Fisher Scientific). An Axiosplan 2 microscope (Zeiss) equipped with AxioCam was used for fluorescence imaging.

**Three-dimensional imaging**

Three-dimensional (3D) reconstructions to allow rotation of the images were rendered with a blending software custom made by Dr. Kola Wawrowsky (Cedars-Sinai Medical Center, Los Angeles, CA; ref. 18).

**Western blot analysis**

Whole-cell lysates were suspended in RIPA buffer (Thermo Fisher Scientific) supplemented with protease inhibitors (Sigma-Aldrich) and a phosphatase inhibitor cocktail (Thermo Fisher Scientific). Next, samples were boiled at 95°C for 3 minutes. Twenty to 40 µg of protein (determined with Protein Assay dye reagent, EMD Millipore) in a tank transfer unit were loaded. Gels were run on a Mini-PROTEAN III (Biorad) and transferred to a PVDF membrane (Millipore) for 1 hour at 4°C. For detection of the sample, 5% and 10% 60% SDS-PAGE were used. The 10,000 g pellets were mixed with 1,100 µl of lysis buffer (19). A total of 1,000 cells were plated per well in 6-well plates. Control cells and cells treated with 200 ng/mL TRAIL for 5 hours were collected, washed, and stained with annexin V and propidium iodide, whereas EVs with intact membranes stained positive only for Hoechst.

**Time-lapse microscopy**

For real-time imaging movies, cells were plated in 25-mm coverslips and, after overnight attachment, incubated with Cell-Mask Orange (Invitrogen). Images were taken at 15-second intervals with a Yokogawa Spinning Disk confocal system attached to a Nikon Ti-E microscope, using a 100× Pan Apo 1.4 NA objective. Images were collected with an Andor iXon 897 EMCCD camera and processed with Andor iQ software (Andor Technology).

**EV isolation**

EVs were isolated by differential centrifugation (SW28 rotor, Beckman) from EV-free medium or serum-free medium as described previously (19). Briefly, cells and debris were eliminated by centrifugation at 2,800 × g for 10 minutes. The supernatant was then centrifuged at 10,000 × g for 30 minutes to precipitate large EVs. For discontinuous centrifugation gradient, 60%, 50%, 40%, 30%, 25%, 15%, 10%, and 5% solutions from OptiPrep (60% aqueous iodixanol, Sigma-Aldrich) in 0.25 mol/L sucrose/0.9 mol/L NaCl/120 mmol/L HEPES, pH 7.4 were used. The 10,000 × g pellets were mixed in the bottom layer and the following solutions carefully layered. Centrifugation was performed at 100,000 × g for 3 hours and 50 minutes at 4°C. Eight individual fractions were collected, washed with PBS, and after centrifugation at 100,000 × g for 1 hour at 4°C, the pellet from each fraction was resuspended in either PBS or lysis buffer (19).

**DNA quantification in EV**

EVs were isolated using differential centrifugation as described above. EVs were resuspended in PBS and treated with DNase I and exonuclease III for 30 minutes at 37°C, to eliminate external DNA attached to the EVs. The DNases were then inactivated by heating the sample at 75°C for 10 minutes. EVs were then treated with proteinase K (DNaseasy Blood and Tissue Kit, Qiagen) and RNase A (10 mg/mL) for 2 minutes at room temperature. DNA isolation was performed per manufacturer’s instruction (DNaseasy Blood & Tissue Kit, Qiagen), and retrieved DNA then quantified using a Qubit fluorometer (Thermo Fisher Scientific).

**Determination of EV membrane permeability based on DNA content**

As described above, EVs were pretreated with DNases to eliminate external DNA. Next, EVs were stained with 1 µg/mL PI for 30 minutes at 37°C and subsequently with 0.5 µg/mL Hoechst. Events between 1 and 10 µm were gated and analyzed by flow cytometry. The gate was established using calibration beads as described previously (20). EVs with compromised membrane integrity stained positive for both Hoechst and propidium iodide, whereas EVs with intact membranes stained positive only for Hoechst.

**Analysis of DNA versus RNA content in EV**

The EVs were pretreated with DNases and RNases as described above. To measure internal DNA, EVs were stained with 0.5 µg/mL of Hoechst for 30 minutes at 37°C and subsequently with 0.5 µg/mL Pyronin Y (PY), which stain the RNA. Events between 1 and 10 µm were gated and analyzed by flow cytometry. The gate was established using calibration beads as described previously (20).

**Apoptosis quantification**

The induction of apoptosis by TRAIL (Thermo Fisher Scientific) was evaluated using annexin V and propidium iodide double staining (FITC Annexin V/Dead cell Apoptosis Kit, Invitrogen). DU145 cells (3 × 10⁵) were plated in 6-well plates. Control cells and cells treated with 200 ng/mL TRAIL for 3 hours were collected, washed, and stained with annexin V and propidium iodide, according to the manufacturer’s instructions. Cells were gated by FSC-A × SSC-A to exclude debris and then by FSC-H × FSC-W following SSC-H × SSC-W to exclude cell doublets. Cells were analyzed using a flow cytometer and quantified using the FlowJo Software.

**Colony assay**

A total of 1,000 cells were plated per well in 6-well plates. After 2 weeks, the colonies were fixed with 4% PFA for 10 minutes at room temperature, and then stained with crystal violet for 15 minutes at room temperature. Wells were scanned and the average value was represented in the figures.

**Patient blood samples**

Human studies were approved by and under the oversight of Cedars Sinai Medical Center Institutional Review Board.
according to protocols 00030191 and 00030350, in compliance with the declaration of Helsinki. All subjects provided written informed consent for research blood donation. Patient samples were obtained from the Urologic Oncology Program and the Cedars-Sinai BioBank.

**CTC enrichment using NanoVelcro chips**

Venous blood was collected in acid-citrate-dextrose–containing vacutainers (BD Biosciences) and processed within 4 hours of collection. CTCs were isolated using NanoVelcro Chips as described previously (21). Briefly, 1.0 mL of blood was subjected to standard density gradient centrifugation with Ficoll-Paque solution (Sigma-Aldrich). The peripheral mononuclear cells were harvested and incubated with a capture agent (biotinylated goat anti-hEpCAM antibody; R&D Systems). After washing carefully, the sample was loaded into the NanoVelcro Chip by an automated fluid handler that consists of a streptavidin-coated NanoVelcro substrate and an overlaid polydimethylsiloxane chaotic mixer, at a consistent flow rate of 0.5 mL/hour. The captured cells immobilized on the NanoVelcro substrates were fixed using 2% PFA (Electron Microscopy Sciences), and then subjected to immunocytochemical staining with DAPI, anti-pan cytokeratin (Life Technologies and Abcam), anti-emerin (Leica Biosystems Novocastra), Alexa Fluor 488–conjugated antirat, and Alexa Flour 555–conjugated antimouse (Life Technologies). Alexa 647–conjugated antirat (Life Technologies). Subsequent microscopic imaging was performed to identify the CTCs.

**CTC imaging**

NanoVelcro Chips were imaged using an upright fluorescence microscope (Eclipse 90i and TI2 imaging systems; Nikon Inc.) and accompanied NIS-Element imaging software (Nikon). An automatic scan over the NanoVelcro chip was performed by the imaging system under ×40 and ×100 magnification with the channels corresponding to nuclear, CK, CD45, and emerin staining, respectively. CTCs were identified at ×100 magnification, and individually imaged at ×200 and ×400 magnification for subsequent morphologic analysis.

**3D CTC imaging**

CTCs for 3D imaging were captured by NanoVelcro Chips as mentioned above. After fixation, the CTCs immobilized on the NanoVelcro substrates were stained with DAPI, anti-pan cytokeratin (Life Technologies and Abcam), anti-emerin (Leica Biosystems Novocastra), Cholera toxin B (Sigma-Aldrich), Alexa Fluor 555–conjugated antimouse (Life Technologies), and Alexa Fluor 647–conjugated antirat (Abcam). An automatic scan over the NanoVelcro chip was performed by the ImageXpress Ultra Confocal High-Content Analysis System (Molecular Devices) under 40× and 100× magnifications. CTCs were identified at 100× magnification, and individually imaged with Z-stack confocal imaging protocol with an interval of 0.5 μm. The resulting image series were reconstructed into 3D animations using MetaMorph software (version 7.7.2.0; Molecular Devices).

**Experimental metastasis**

Cedars-Sinai Medical Center Institutional Animal Care and Use Committee approved the animal studies. Four- to 5-week-old male C.B-1gh-1b/GbmsTac-Prkdcscid-Lystbg N7 SCID/Beige mice were purchased from Taconic and maintained under specific pathogen-free conditions. A total of 1 × 10⁶ cells were injected via intracardiac route into 6- to 8-week-old SCID/Beige mice. Prior to injection, the DU145 control, emerin-depleted, and lamin A/C–depleted cells were transduced with luciferase-containing lentiviral particles (Genetarget Inc.) and selected with 400 μg/mL neomycin (Thermo Fisher Scientific) for two weeks. Bioluminescence imaging was assessed weekly to monitor tumor metastasis using a Xenogen IVIS Spectrum Imaging System (PerkinElmer). Mice were sacrificed and the organs collected and fixed in 10% formalin for further histologic analyses. Animals with tumor growth around the heart were excluded.

**Migration and invasion assays**

Boyden chambers were coated with collagen for migration assays, or with Matrigel for invasion. Briefly, 1 × 10⁵ cells were resuspended in 500 μL of serum-free medium and added to the top compartment of a transwell chamber (pore size of 8.0 μm, Corning). Five-hundred microliters of medium supplemented with 10% FBS were added to a 12-well plate. The transwell was placed on the plate for 12 hours (migration) or 24 hours (invasion). Cells were fixed and stained with crystal violet. The cells that remained in top of the chamber were discarded. The number of cells that migrated or invaded were quantified by imaging using 5 randomly chosen fields. The cell counter from ImageJ was used for quantification.

**Prostate cancer tissue microarray**

A prostate cancer tissue microarray (TMA) was constructed using semiautomated robotic strategy as described previously (22). TMA cores were taken from formalin-fixed, paraffin-embedded tissue obtained at the time of prostatectomy for patients treated at the Vanderbilt University Medical Center (Nashville, TN: 2000–2012). Each patient was represented by two cores of tumor and two cores of adjacent benign tissue. Two cores of lymph node tissue were included when available. A total of 80 patients were evaluated for this study.

**TMA immunofluorescent staining**

A 4-6 μm section of the TMA was stained with fluorescently labeled antibody after dewaxing in xylene, rehydrating in PBS, and antigen retrieval in a boiling water bath using pH 6 citrate buffer (Dako) for 30 minutes. The tissue sections were stained using a two-step cycling staining strategy, in which the first cycle included antibodies to cytokeratin 18, emerin, and Histone H3. For the second cycle, a mouse anti-smooth muscle actin antibody (clone 1A4, Abcam) was used. Conventional fluorescent anti-mouse secondary antibodies were used to visualize staining with anti-cytokeratin 18 and emerin antibody. Histone H3 and smooth muscle actin were detected with antibodies enabled with photo- cleavable fluorescent labels. SYTOX Green (Thermo Fisher Scientific) was used to stain the nuclear DNA. Whole-slide scanning (Aperio) was used to digitize the slide. Images of individual cores were extracted and processed for further analysis.

**TMA quantitative analysis of emerin particles**

An integrated image analysis workflow for the identification and quantitation of emerin particles was created in Konstanz Information Miner (KNIME, 10.1145/1656274.1656280, Supplementary Fig. S1). After images for individual fluorescent channels were loaded into the workflow (Supplementary Fig. S1A) and
shape stability. Initially, we measured the deformability of prostate cancer cells. Here, and in agreement with the proteomic findings, we observed that DIAPH3 colocalizes with emerin in the nuclear envelope (24) and has a functional role inside the nucleus. Analyses were performed with Prism 6 software (GraphPad) and MATLAB (Mathworks Inc.) Data were plotted as mean ± SD.

Results

DIAPH3-depleted, amoeboid cells display irregular nuclear shapes and secrete nonapoptotic EVs with nuclear material

Although DIAPH3/mDia2 is well-characterized in the nucleation of actin filament polymerization (23), recent evidence suggests that DIAPH3 interacts with protein complexes at the nuclear envelope (24) and has a functional role inside the nucleus (25, 26). In a previous report describing the DIAPH3 interactome (14), the nuclear envelope protein, emerin, was detected (Supplementary Fig. S1E and S1F) on the basis of their size, shape, and emerin content. Segmentation models were improved through an iterative training process until ≥95 concordance was achieved with manual counting. Application of the epithelial mask was subsequently used to capture only emerin particles present within the prostate epithelium. The epithelial mask, segmented nuclei, and emerin particles were mapped back to the original images for visualization and grouped (summed) by tissue to quantify the number observed in tumor and adjacent benign tissue for each patient (Supplementary Fig. S1G and S1H).

Statistical analyses

Student t test (two-tailed) was used if the data were normally distributed. Wilcoxon rank-sum test was performed to test differential expression of LMNA between the prostate cancer subgroups. Kaplan–Meier analysis and Cox proportional hazard regression analysis were performed to examine association between biochemical recurrence and LMNA expression. Analyses were performed with Prism 6 software (GraphPad) and MATLAB (Mathworks Inc.). Data were plotted as mean ± SD.

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To examine whether emerin plays a role in cancer cell aggressiveness and nuclear shape instability, we enforced emerin expression in DIAPH3-depleted cells. A reported consequence of DIAPH3 knockdown in amoeboid models is hyperactivation of the ERK pathway (13). Consistent with this, we found ERK activation and motility enhanced in DU145 DIAPH3-depleted cells, but ERK activation and motility were suppressed when expression of emerin is enforced (Fig. 2A–C). These results suggest that emerin intersects with the ERK pathway.

Emerin downregulation recapitulates features seen with DIAPH3-loss

To further analyze the functional consequences of emerin loss, we depleted emerin from DU145, PC3, LNCaP prostate, and BT-549 breast cancer cells (Supplementary Fig. 4A). Emerin-depleted cells exhibited increased phosphorylation of both ERK1/2 (32) and myosin light chain (MLC2), phenocopying DIAPH3 silencing (Supplementary Fig. 4A; ref. 13). Depletion of emerin also resulted in irregular nuclear shape,
visualized by immunofluorescence imaging of lamin A or the lipid probe, CellMask Orange (Fig. 2D–F; Supplementary Fig. S4B and S4C). Image analysis of nuclei further confirmed significantly lower circularity in emerin-depleted cells (Fig. 2G).

The finding that emerin loss is sufficient to cause nuclear shape instability is consistent with previous results in muscular dystrophic fibroblast models (27, 33). In addition to morphologic characteristics of nuclear shape instability, emerin-depleted cells exhibited higher deformability in comparison with control cells, as demonstrated by lower retention percentage in the PMF assay (Fig. 2H). Similar to what we observed in DIAPH3-depleted cells, emerin knockdown resulted in nuclear membrane blebbing and formation of cytosolic vesicles containing DNA (Fig. 2D–F; Supplementary Fig. S4B and S4C). We also detected significantly more extracellular DNA in EVs from emerin-depleted cells (Fig. 2I). These EVs had intact membranes, consistent with a nonapoptotic origin of the particles (Supplementary Fig. S4D). Along with the morphologic features indicating nuclear shape instability, emerin-depleted cells were more migratory (Fig. 2J; Supplementary Fig. S4E), more invasive (Fig. 2K; Supplementary Fig. S4F), and efficiently grew into bigger colonies (Fig. 2L), compared with empty vector controls. These findings suggest that emerin depletion alone is not only sufficient to cause nuclear shape instability,
Figure 2.
Emerin depletion induces nuclear shape instability and increases cancer cell aggressiveness in vitro. A, DU145 DIAPH3-depleted overexpressing (OE) emerin display reduced migration in comparison with DIAPH3-depleted cells, quantified using a Boyden chamber assay. B, Representative immunoblot showing levels of emerin and β-actin in DU145 control, DIAPH3-depleted cells, or DIAPH3-depleted emerin-overexpressing cells. C, Reduction of p-ERK in DU145 DIAPH3-depleted emerin-overexpressing cells. Representative immunoblot showing levels of emerin, p-ERK, ERK, and β-actin. D, Representative images of DU145 control or emerin-depleted cells. DNA (Hoechst, blue) and lamin A (FITC, green) staining are shown. Scale bar, 10 μm. Emerin-depleted cells exhibit disrupted nuclear shape. E, Representative images of DT-549 control or emerin-depleted cells. DNA (Hoechst, blue) and lamin A (FITC, green) staining are shown. Scale bar, 10 μm. Arrows, nuclear blebs or cytosolic vesicles. F, Representative images of DU145 control or emerin-depleted stained with the lipid affinity dye, CellMask Orange. Arrows, nuclear blebs. G, Quantification of nuclear circularity in DU145 control and emerin-depleted cells (perfect circle = 1; <1 represents departure from circularity). H, DU145 emerin-depleted cells show increased filtration of cells compared with controls, as measured by PMF, indicating increased cell deformability. I, DU145 emerin-depleted cells shed EVs containing DNA. J and K, DU145 emerin-depleted cells display enhanced migration (J) and invasion (K) quantified using a Boyden chamber assay. Graphs show mean ± SD. ** P < 0.01, *** P < 0.001, unpaired t test. L, Quantification of colonies in DU145 control or emerin-depleted cells by ImageJ.
but also leads to the amoeboid phenotype in cancer cells, suggesting that emerin depletion could contribute to disease progression.

Emerin depletion promotes widespread metastasis in mouse models

To further test the hypothesis that nuclear shape instability induced by emerin depletion contributes to metastasis, luciferase-expressing DU145 control or emerin-depleted cells were introduced into SCID mice through intracardiac injection. At week 5 postinjection, bioluminescent imaging showed an increase and a wider distribution of luciferase activity in mice injected with emerin-depleted cells versus controls (Fig. 3A; Supplementary Fig. S5A and S5B). At necropsy, we found that the number of metastatic sites was significantly higher in mice injected with emerin-depleted cells compared with controls (Fig. 3B; Supplementary Table S1) as confirmed by gross examination, micro-CT, hematoxylin and eosin staining, and IHC for cytokeratin, which is frequently used as a marker to detect metastatic cells of epithelial origin (Fig. 3C). Taken together, these results show that emerin loss can lead to the development of widespread metastases.

Evidence of nuclear shape instability can be detected in vivo

The above experiments demonstrate that emerin loss leads to nuclear shape instability and increased cancer aggressiveness both in vitro and in vivo. Similar effects can result from loss of DIAPH3 (13), a protein that associates with emerin (Supplementary Fig. S2A and S2B; ref. 14). These findings suggest a critical role for emerin and emerin-associated nuclear membrane proteins in nuclear shape stability and cancer metastasis. De novo genomic alterations (mutations, deletions, and amplifications) involving the emerin gene (EMD) are rare events (<5%) in published prostate and breast cancer datasets (Supplementary Fig. S6A and S6B; ref. 34). In contrast, DIAPH3 loss has been shown to occur as a result of chromosomal deletion in 32% of prostate cancers and 46% of breast cancers (13). However, we found that LMNA, which encodes the emerin-anchoring proteins, lamins A/C (35), is frequently downregulated in castration-resistant prostate cancer (Fig. 4A). Using a transcriptomic classification system that divides prostate cancers into 3 subgroups (PCS1-3; ref. 36), we observed downregulation of lamin A/C expression most frequently in the subtype with the worst prognosis, PCS1
Reduced lamin A/C expression also correlated with shorter biochemical recurrence–free survival (Fig. 4C). These observations suggest that lamin A/C downregulation could disrupt emerin localization and lead to nuclear shape instability, as well as aggressive cancer.

To test the role of lamin A/C in emerin localization, lamin A/C was depleted in DU145 and BT-549 cells (Supplementary Fig. S7A). This manipulation induced emerin mislocalization and nuclear membrane blebbing (Fig. 4D; Supplementary Fig. S7B). Reduced lamin A/C levels also resulted in decreased nuclear circularity (Fig. 4E) and increased shedding of DNA-containing EVs (Fig. 4F). We also observed enhanced migration (Fig. 4G) and invasion of lamin A/C-depleted cells in vitro (Fig. 4H) as well as a modest increase in metastasis in vivo (Supplementary Fig. S7C–S7E; Supplementary Table S1). Lamin A/C-silenced metastatic lesions exhibited emerin mislocalization, nuclear blebs, and EV-like positive structures (Fig. 4I). Taken together these findings indicate that lamin A/C downregulation, which is frequent in drug-resistant prostate cancer, leads to nuclear shape instability, destabilization of emerin, and increased aggressiveness in vitro and in vivo.

Emerin deregulation is associated with aggressive disease in prostate cancer

Our findings identifying nuclear shape instability and concurrent emerin mislocalization suggest that emerin spatial organization might be employed to identify cells with nuclear shape instability in vivo. Emerin-positive nuclear blebs and punctate...
structures are evident in high-grade human prostate cancer tissue from the Human Protein Atlas (Supplementary Fig. S7F). To explore the reproducibility of this finding, we performed quantitative IHC on a prostate cancer TMA (n = 80) with emerin antibody. Using an integrated image analysis workflow developed in Konstanz Information Miner (KNIME; see Materials and Methods), we identified and quantified emerin-positive particles of the prostate epithelium (Fig. 5A). In normal prostate epithelium, emerin is localized to the nuclear envelope (top row, white arrows). In cancer prostate epithelium, distinct emerin-positive particles are visible (red arrows). Scale bar, 100 μm. B–E, Quantitative analysis of emerin-positive particles in human prostate cancer. B, The number of emerin-positive particles was quantified in tumor and adjacent benign prostate tissue. Pairwise analysis (C) and ratio (D) of particles in tumor and adjacent benign tissue; note an elevation of particles in tumor tissue. E, The ratio of emerin-positive particles correlates with biochemical recurrence. F, Peripheral blood specimens from prostate patients were tested for CTCs. The CTCs DAPI-positive/cytokeratin (CK)-positive/CD45-negative were stained with emerin. Membrane staining (FITC-cholera toxin B, green) was additionally used to identify CTCs with membrane blebs that were emerin-positive (middle, DAPI-positive/cytokeratin-positive/cholera toxin B). G, Emerin intensity in CTCs were compared with WBCs in the same high-power fields from the same patients (>50 fields were analyzed). H, Emerin-positive particles identified in CTCs. *P < 0.05, ***P < 0.001.

Figure 5.
Emerin is a marker of nuclear shape instability in prostate cancer. A, Detection of emerin-positive particles in prostate cancer. Immunofluorescent staining was performed for emerin, histone H3 (HH3, nuclear mask), and cytokeratin 18 (epithelial mask) on a prostate cancer TMA (n = 80). In normal prostate epithelium, emerin is localized to the nuclear envelope (top row, white arrows). In cancer prostate epithelium, distinct emerin-positive particles are visible (red arrows). Scale bar, 100 μm. B–E, Quantitative analysis of emerin-positive particles in human prostate cancer. B, The number of emerin-positive particles was quantified in tumor and adjacent benign prostate tissue. Pairwise analysis (C) and ratio (D) of particles in tumor and adjacent benign tissue; note an elevation of particles in tumor tissue. E, The ratio of emerin-positive particles correlates with biochemical recurrence. F, Peripheral blood specimens from prostate patients were tested for CTCs. The CTCs DAPI-positive/cytokeratin (CK)-positive/CD45-negative were stained with emerin. Membrane staining (FITC-cholera toxin B, green) was additionally used to identify CTCs with membrane blebs that were emerin-positive (middle, DAPI-positive/cytokeratin-positive/cholera toxin B). G, Emerin intensity in CTCs were compared with WBCs in the same high-power fields from the same patients (>50 fields were analyzed). H, Emerin-positive particles identified in CTCs. *P < 0.05, ***P < 0.001.

We also tested the possibility that emerin levels and localization could be observed in CTCs, which are mediators of cancer metastasis (37). We isolated CTCs from patients with prostate cancer with progressing disease using a microfluidic device (21, 38) where CTCs were defined by cytokeratin(+)CD45(−)DAPI-positive cellular events (21). CTCs recovered from 15 patients were studied for emerin levels and localization. In CTCs, we identified nuclear membrane blebs, cytosolic emerin particles, and EV-like structures that appeared tethered to the cell (Fig. 5F). Emerin puncta were associated with a total of 31 CTCs (n = 70), the majority of which (n = 28) were derived from a patient with advanced disease (Fig. 5F–H). In a separate analysis, we compared emerin expression levels in CTCs versus white blood cells (WBC) in the same high-power field. We found that emerin expression is significantly reduced in CTCs (n = 63) compared with the same number of WBCs [cytokeratin(−)/CD45(−) and DAPI-positive], which typically express low levels of emerin (6). These data suggest that CTCs derived from metastatic prostate cancer patients...
express lower emerin levels than WBCs with highly deformable nuclei (Fig. 5G).

Discussion

In this study, we have shown that loss or mislocalization of the inner nuclear envelope protein, emerin, in cancer cells leads to nuclear shape instability and an aggressive phenotype that results in more metastasis in vivo. Nuclear shape instability induced by emerin depletion was phenocopied by depletion of DIAPH3 or lamin A/C, suggesting that various molecular mechanisms that destabilize nuclear shape can converge on a phenotype characterized by amoeboid behavior and increased aggressiveness. Strikingly, we observed consistent features across a range of experimental settings, including experimental models, prostate cancer patient tissue and CTCs. Nuclear shape instability is associated with disruption of the nuclear envelope, nuclear blebbing, cytosolic fragments with nuclear content, and shedding of EVs that contain nuclear material. Low levels of emerin, which can arise in part by proteolytic degradation, were a common feature seen in the experimental models, including in clinical specimens (CTCs). Depletion of emerin levels by proteolytic degradation has been reported in cells containing LMNA mutations (30). Enforced emerin expression suppressed the motility and ERK pathway activation, suggesting that depletion of emerin is a critical node in nuclear shape destabilization and amoeboid behavior.

Baarlink and colleagues (26) previously showed that DIAPH3/mDia2 has an important role regulating nuclear actin and the activity of the transcription factor, serum response factor (26). Similar functions were attributed to emerin and lamin A/C (39), suggesting a functional overlap among the proteins we focus on in our study: emerin, lamin A/C, and DIAPH3/mDia2. The functional relationship among these proteins directed our attention to emerin, whose role remains poorly understood in cancer.

Formins interact with LINC complex proteins and play a key role regulating actin dynamics (40), suggesting that formin depletion might induce cytoskeletal–nucleoskeletal destabilization. Our observations demonstrate that DIAPH3 loss destabilizes the cell nucleus, consistent with depletion of lamin A/C in models of laminopathies (41). Evidence for nuclear shape instability was obtained in human prostate cancer tissues and CTCs from patients with prostate cancer. Using mouse models and in vitro cell studies, we have demonstrated that nuclear shape instability can result independently of loss of DIAPH3, emerin, or lamin A/C. These findings suggest that nuclear shape instability is one mechanism underlying the emergence of amoeboid properties in cancer cells, and that this phenotype can be assayed in tissues and in the circulation. Beyond the potential of emerin as a biomarker, the observation that particles that contain nuclear material can be shed from cells via nuclear blebbing provides a potential mechanistic explanation for previous reports of EVs with genomic DNA cargo in the circulation of patients with cancer (42–44). In support to our findings, Coban and colleagues (45) describe the utility of using emerin IHC staining as biomarker for diagnosis and prognosis of thyroid lesions.

Collectively, these findings suggest that cancers with aggressive amoeboid features can be identified and monitored using clinically relevant methods. Studies are ongoing to determine whether evaluation of nuclear shape stability has a clinical benefit.

Disclosure of Potential Conflicts of Interest

H. Tseng has ownership interest in CytoLumina Technologies Corp. E.M. Posadas is a consultant/advisory board member for CytoLumina. No potential conflicts of interest were disclosed by other authors.

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References


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