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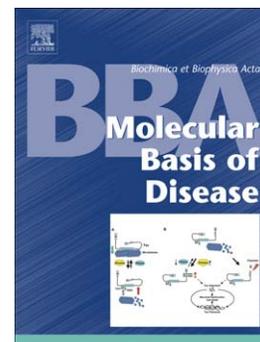
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Reduced expression of A-type lamins and emerin activates
extracellular signal-regulated kinase in cultured cells

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Key words: nuclear envelope, nuclear lamina, lamin, emerin, Emery-Dreifuss muscular
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Abstract

Background: Mutations in genes encoding A-type lamins and emerin cause cardiomyopathy and muscular dystrophy. We previously showed activation of the extracellular signal-regulated kinase (ERK) branch of the mitogen-activated protein kinase (MAPK) cascade in hearts of mice with mutations in these genes. Here, we tested the hypothesis that reducing A-type lamins and emerin in cultured cells activate ERK signaling. *Methods:* We used siRNA to knockdown A-type lamins and emerin in HeLa and C2C12 cells. Activation of ERK was assessed by immunoblotting and immunofluorescence microscopy with antibodies against phosphorylated protein and by using real-time RT-PCR to measure RNAs encoded by genes for transcription factors stimulated by ERK. *Results:* Knockdown of A-type lamins and emerin in HeLa and C2C12 stimulated phosphorylation and nuclear translocation of ERK as well as activation of genes encoding downstream transcription factors. A MAPK/ERK kinase (MEK) inhibitor reduced ERK phosphorylation in cells with reduced expression of A-type lamins and emerin. *Conclusions:* These results provide proof for the hypothesis that altered expression of emerin and A-type lamins activates ERK signaling, which in turn can cause cardiomyopathy. *General Significance:* ERK is a potential target for the pharmacological treatment of cardiomyopathy caused by mutations in the genes encoding emerin and A-type lamins.

1. Introduction

The nuclear envelope is composed of the nuclear membranes, the nuclear pore complexes and the nuclear lamina. The nuclear lamina is a meshwork of intermediate filament proteins called lamins and is primarily associated with the inner aspect of the inner nuclear membrane [1]. Clinical investigations over the past several years have shown that mutations in the genes encoding lamins and associated proteins of the inner nuclear membrane cause diverse diseases often referred to as laminopathies or nuclear envelopopathies [2,3].

Emery-Dreifuss muscular dystrophy is characterized by skeletal muscle weakness and wasting in a humeroperoneal distribution, joint contractures and dilated cardiomyopathy with conduction defects [4,5]. The same clinical condition is inherited in an X-linked and an autosomal manner [4,5]. Mutations in *EMD* are the cause of X-linked Emery-Dreifuss muscular dystrophy [6]. *EMD* encodes emerin, an integral protein of the inner nuclear membrane that is absent or has reduced expression in most cases of X-linked Emery-Dreifuss muscular dystrophy [6-8]. Mutations in *LMNA* cause autosomal dominant Emery-Dreifuss muscular dystrophy and more rare recessive cases [9,10]. *LMNA* encodes A-type nuclear lamins [11]. Most *LMNA* mutations causing Emery-Dreifuss muscular dystrophy generate single amino acid substitutions or deletions in A-type lamins but haploinsufficiency can also cause the disease [9,12-14]. While *EMD* and *LMNA* mutations were initially shown to cause the Emery-Dreifuss phenotype, the same mutations in these genes can cause cardiomyopathy with different, minimal or no apparent skeletal muscle involvement [12-18].

The molecular mechanism underlying how deficiency in emerin or A-type lamins causes striated muscle diseases is poorly understood. We previously identified abnormal activation of the extracellular signal-regulated kinase (ERK) and c-jun-N-terminal kinase (JNK) branches of the mitogen-activated protein kinase (MAPK) signaling pathway in hearts of *Lmna* H222P “knock in” mice, a model of autosomal Emery-Dreifuss muscular dystrophy [19]. We also observed activation of the ERK in hearts of emerin-deficient *Emd*^{ly} mice, a model of X-linked Emery-Dreifuss muscular dystrophy [20]. These studies suggested a connection between abnormalities in proteins of the nuclear envelope and a cell signaling pathway implicated in the pathogenesis of cardiomyopathy in tissues. However, data from model cellular systems have been limited to the demonstration that ERK and JNK are activated in transfected cultured cells overexpressing lamin A variants in striated muscle diseases [19]. Experiments showing that reducing expression of A-type lamins or emerin in cultured cells directly activates a MAPK are critical, as positive results would support the hypothesis that MAPK activation is a primary event rather than a secondary one occurring as a consequence of cardiomyopathy. We therefore tested the hypothesis that reducing A-type lamins and emerin in cultured cells activate ERK.

2. Materials and methods

HeLa cells and C2C12 cells were maintained in a 5% CO₂ atmosphere at 37°C. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf bovine serum and 0.1% gentamicin. One day before transfection, HeLa and C2C12

cells were trypsinized, diluted with fresh medium without antibiotics and transferred to 24-well plates. Transient transfection of siRNAs (50 pM final concentration) was carried out using Oligofectamine (Invitrogen) as recommended by the manufacturer. Cells were assayed 72 hours after transfection for HeLa cells and 48 hours after transfection for C2C12 cells. Reduction of expression of targeted genes was confirmed in at least 3 independent experiments.

For measuring gene expression in cultured cells, media was removed from cultures reaching 80% and total RNA was extracted using the Rneasy isolation kit (Qiagen) according to the manufacturer's instructions. Adequacy and integrity of extracted RNA were determined by gel electrophoresis. Concentrations were measured by ultraviolet absorbance spectroscopy. For real-time RT-PCR, RNA was reverse transcribed using SuperScript First-Strand Synthesis System according to the manufacturer's instructions (Invitrogen). Real-time RT-PCR was performed as previously described [19,20]. Specificity of amplification was checked by melting-curve analysis. Relative levels of mRNA expression were calculated according to the $\Delta\Delta C_T$ method, normalized by comparison to *Gapdh* mRNA expression [21].

For immunoblotting, proteins were extracted from cells as previously described [19,20], separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with primary antibodies against ERK1/2 (Santa-Cruz), pERK1/2 (Cell Signaling), lamin A/C (Santa-Cruz), emerin (Novocatra), β -actin (Santa-Cruz) and *Gapdh* (Santa-Cruz). Secondary antibodies were horseradish peroxidase-conjugated (Amersham). Recognized proteins were visualized by enhanced chemiluminescence (ECL-Amersham) and visualized using Hyperfilm ECL (Amersham). Signal generated using

antibody against β -actin was used as an internal control to normalize amounts of protein between immunoblots. Band densities were calculated using Scion Image software (Scion Corporation) and normalized to the appropriate total extract to control for protein loading. Data are reported as means \pm standard deviations and compared with respective controls using a two-tailed t test.

For immunofluorescence microscopy, HeLa and C2C12 cells were grown on coverslips and washed with phosphate-buffered saline. Cells were fixed for 10 minutes in methanol at -20°C . HeLa and C2C12 cells were then incubated at room temperature with antibody against phosphorylated ERK (pERK) (Cell Signaling). Cells were then washed with phosphate-buffered saline and incubated with Texas Red conjugated goat anti-rabbit antibody in PBS (Molecular Probes). Cells were washed with phosphate-buffered saline and slides mounted in Mowiol (Santa-Cruz) with $0.1\ \mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole. Immunofluorescence microscopy was performed using an Axiophot microscope (Carl Zeiss). Micrographs were processed using Adobe Photoshop 6.0 (Adobe Systems).

For colorimetric analysis of ERK1/2 phosphorylation, cells were cultured for 24 hours in the presence of PD98059 ($45\ \mu\text{M}$). ERK1/2 phosphorylation was measured using an enzyme linked immunosorbent assay (SuperArray CASE, ERK1/2 Kit) as per the manufacturer's protocol. Signal intensities for pERK1/2 or total ERK1/2 were measured at an optical density (OD) of 450 nm and relative cell number was assayed in each well (OD of 595 nm). To determine ERK1/2 phosphorylation, we normalized the pERK1/2 signal ratio ($\text{OD}_{450\text{nm}}/\text{OD}_{595\text{nm}}$) to the total ERK1/2 signal ratio

(OD_{450nm}/OD_{595nm}). Data are reported as means \pm standard deviations and compared with respective controls using a two-tailed t test.

3. Results

To investigate if reduction of A-type lamins and emerin lead to activation of ERK signaling, we used a human epithelial cell line (HeLa cells) and a mouse myogenic cell line (C2C12 cells) and knocked down targeted genes using siRNA. Total RNAs and proteins were extracted from HeLa cells cultured without siRNA treatment (mock) or treated with *Gapdh*, *Emd* and *Lmna* siRNAs. When *Gapdh*, *Emd* and *Lmna* siRNAs were used, corresponding mRNAs (Fig. 1A) and proteins (Fig. 1B) were reduced by approximately 50%. In C2C12 cells, total RNA and proteins were extracted after mock treatment or treatment with *Gapdh*, *Emd* and *Lmna* siRNAs. When *Gapdh*, *Emd* and *Lmna* siRNAs were used, corresponding mRNAs (Fig. 1C) and proteins (Fig. 1D) were reduced by approximately 50%. Treatment with *Lmna* siRNA also led to modest but reproducible decreases in cellular emerin (Fig. 1C,D).

To determine if siRNA treatment lead to ERK activation, we first evaluated phosphorylation of ERK isoforms 1 and 2 (ERK1/2). Immunoblotting with anti-pERK1/2 antibody demonstrated an increase in pERK1/2 in HeLa cells treated with *Emd* and *Lmna* siRNAs, whereas no significant increase was observed in mock treated cells or cells treated with *Gapdh* siRNA (Fig. 2A). pERK1/2 activates a series of downstream target genes, including those encoding c-Jun, Elk1 and Elk4. We analyzed expression of these transcripts using real-time quantitative RT-PCR. While these individual genes

were not found to be significantly differentially expressed in mock treated cells or cells treated with *Gapdh* siRNA (Fig. 2B), treatment with *Emd* and *Lmna* siRNAs lead to 2-fold to 3-fold increased expression of *c-Jun*, *Elk1* and *Elk4* (Fig. 2B). Abnormal ERK1/2 activation was also observed in C2C12 cells treated with *Emd* or *Lmna* siRNAs (Fig. 2C). We also found 1.5-fold to 2-fold up-regulation of *c-Jun* when C2C12 cells were treated with *Emd* and *Lmna* siRNAs (Fig. 2D). In C2C12 cells, expression of *Elk1* and *Elk4* was increased approximately 1.5-fold only when treated with *Lmna* siRNA but not *Emd* siRNA (Fig. 2D).

Translocation of pERK1/2 from cytoplasm to nucleus is necessary for activation of downstream genes. In mock treated HeLa cells and HeLa cells treated with *Gapdh* siRNA, pERK was weakly or not detectable and only approximately 2% of HeLa cells had detectable nuclear pERK (Fig. 3A). In contrast, treatment with *Emd* and *Lmna* siRNAs induced detectable levels of pERK in the nucleus in significantly more cells (Fig. 3A, arrowheads). Approximately 8% of HeLa cells treated with *Emd* siRNA and 10% of HeLa cells treated with *Lmna* siRNA had detectable nuclear pERK (Fig. 3A). In mock treated C2C12 cells and C2C12 cells treated with *Gapdh* siRNA, pERK was detectable in less than 1% of nuclei (Fig. 3B). When C2C12 cells were treated with *Emd* or *Lmna* siRNAs, there was a significant increase of cells (approximately 6%) with detectable nuclear pERK (Fig. 3B, arrowheads). Hence, knocking down expression of *Emd* and *Lmna* induces phosphorylation of ERK, nuclear translocation of pERK and activation of downstream targets.

MAPK/ERK kinase (MEK) is immediately upstream of ERK in this branch of the MAPK signaling cascade. MEK phosphorylates ERK to activate it. We analyzed the

effect of the MEK inhibitor PD98059 on ERK1/2 activity in HeLa and C2C12 cells treated with siRNA against *Emd* and *Lmna*. Cells were cultured with or without PD98059 at a concentration of 45 μ M for 24 hours. Immunoblotting with anti-pERK1/2 antibody demonstrated that the increase in pERK in HeLa and C2C12 cells treated with *Emd* and *Lmna* siRNAs was reduced with PD98059 added to the culture medium (Fig. 4A and 4B). Results from an enzyme-linked immunosorbent assay also confirmed that PD98059 inhibited activation of ERK1/2 by in the siRNA-treated cells (Fig. 4B).

4. Discussion

Our previous studies have shown that a missense mutation in *LMNA* encoding A-type lamins and loss emerin activate MAPKs in the hearts of genetically modified mice [19,20]. We have analyzed affected and unaffected tissues in *Lmna*^{H222P/H222P} mice and found abnormal activation of genes downstream of ERK only in cardiac and to a more limited extent in skeletal muscle [19]. We have similarly demonstrated abnormal activation of ERK and downstream genes in hearts of emerin-deficient mice [20]. We have also demonstrated previously activation of MAPK signaling in transfected cultured cells overexpressing lamin A variants with amino acid substitutions found in subjects with Emery-Dreifuss muscular dystrophy [19]. Here we showed an aberrant increase of ERK activation and downstream transcription factors in siRNA-treated HeLa and C2C12 cells with decreased emerin and A-type lamin expression. It is unclear why in *Lmna*^{H222P/H222P} mice ERK activation was observed only in cardiac and to a more limited extent in skeletal muscle while in the present study reduction of A-type lamins and

emerin activated ERK in both myogenic and epithelial cell lines. This could potentially be explained by the fact the ERK activation is influenced by the composition of the extracellular matrix [22-24].

The present results show that decreasing the expression of A-type lamins and emerin activate MAPKs. We have also previously shown that expressing lamin A variants that cause cardiomyopathy in transfected cultured cells activate ERK [19]. These results, along with evidence of MAPK activation in hearts of *Lmna*^{H222P/H222P} mice prior to the presence of clinically detectable cardiomyopathy [19], strongly suggest that abnormalities in nuclear envelope proteins are a cause of rather than a consequence of cardiomyopathy. Induced activation of ERK in hearts of transgenic mice induces a hypertrophic response and sustained hypertrophy is a leading predictor of heart failure [25]. Hence, the present results provide proof for the hypothesis that nuclear envelope abnormalities resulting from alterations in emerin and A-type lamin expression activate ERK that in turn contributes to the development of cardiomyopathy.

While most of the human *LMNA* mutations causing cardiomyopathy and skeletal muscle disease are missense, some patients carry nonsense mutations leading to haploinsufficiency [9,12-14]. Therefore, it was physiologically relevant to show that reductions in A-type lamins, as well as expression lamin variants [19], can activate ERK. While most mutations in *EMD* lead to a loss of emerin, some that cause Emery-Dreifuss muscular dystrophy lead to stable expression of emerin with amino acid substitutions [6-8,26,27]. It would be interesting to determine in future studies if expression of rare emerin variants with amino acid substitutions found in patients with Emery-Dreifuss muscular dystrophy similarly activate ERK.

The mechanism responsible for the activation of ERK in cells with abnormalities in A-type lamins and emerin remains to be determined experimentally. A-type lamins and emerin interact and depletion of A-type lamins leads to a redistribution from the inner nuclear membrane to the bulk endoplasmic reticulum [28-31]. Expression of some lamin A variants that cause cardiomyopathy and muscular dystrophy also leads to redistribution of some emerin from the nuclear envelope to the bulk endoplasmic reticulum [32,33]. Redistribution of emerin to the bulk endoplasmic reticulum is further associated with proteasome-mediated degradation of emerin [34] and cellular emerin levels were modestly reduced in the present study in cells treated with *Lmna* siRNA. Hence, loss of at least some emerin from the nuclear envelope along with decreased steady-state cellular levels may be common phenomena in striated muscle diseases caused by *LMNA* and *EMD* mutations.

Our results have potential practical implications because small molecule drugs can be used to inhibit ERK. The inhibitor we used, PD98059, is a selective inhibitor of MEK [35]. It mediates its inhibitory effects on ERK signaling by preventing phosphorylation of ERK. In this study, we showed that PD98059 reduces ERK1/2 activity in HeLa and C2C12 cells with reduced A-type lamins and emerin. We have recently shown that systemic administration of PD98059 prevents or delays the development of cardiomyopathy in *Lmna*^{H222P/H222P} mice [36]. Hence, pharmacological inhibition of ERK signaling may also be a potential treatment of cardiomyopathy caused by mutations inducing A-type lamin haploinsufficiency or emerin loss in human subjects.

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List of abbreviations

ERK, extracellular signal-regulated kinase; JNK, c-jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; OD, optical density

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Figures legends

Fig. 1. Knockdown of emerin and A-type lamins using siRNA. (A) Expression of mRNA encoded by *Gapdh*, *Emd* and *Lmna* in HeLa cells transfected with siRNA duplexes, using real-time quantitative RT-PCR. Bars indicate fold overexpression of the indicated mRNA. Values are means \pm standard deviations for n=4 samples (* p <0.05). (B) Immunoblot showing expression of GAPDH, emerin and lamin A/C in HeLa cells transfected with siRNA duplexes. Antibody against actin was used as a loading control. (C) Expression of mRNA encoded by *Gapdh*, *Emd* and *Lmna* in C2C12 cells transfected with siRNA duplexes, using real-time quantitative RT-PCR. Bars indicate fold overexpression of the indicated mRNA. Values are means \pm standard deviations for n=3 samples (* p <0.05). (D) Immunoblot showing expression of GAPDH, emerin and lamin A/C in C2C12 cells transfected with siRNA duplexes. Antibody against actin was used as a loading control.

Fig. 2. Knockdown of emerin and A-type lamins leads to phosphorylation of ERK and increased expression of downstream genes. (A) Representative immunoblot showing expression of total ERK1/2 and pERK1/2 in HeLa cells transfected with siRNA duplexes. (B) Expression of downstream genes in ERK pathway in HeLa cells transfected with siRNA duplexes. Real-time RT-PCR results for *c-Jun*, *Elk1* and *Elk4* are shown. Bars indicate the fold overexpression of the indicated mRNA normalized to *Gapdh*. Values are means \pm standard deviations for n=4 samples per group (* p < 0.05). (C) Representative immunoblot showing expression of total ERK1/2 and pERK1/2 in

C2C12 cells transfected with siRNA duplexes. (D) Expression of downstream genes in ERK pathway in C2C12 cells transfected with siRNA duplexes. Real-time RT-PCR results for *c-Jun*, *Elk1* and *Elk4* are shown. Bars indicate the fold overexpression of the indicated mRNA normalized to *Gapdh*. Values are means \pm standard deviations for n=4 samples per group (* $p < 0.05$).

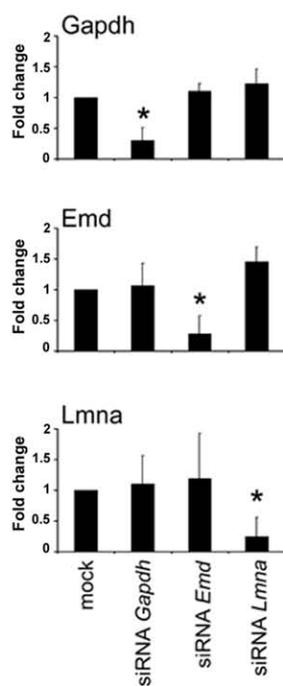
Fig. 3. Knockdown of emerin and A-type lamins leads to enhanced nuclear translocation of pERK. (A) Effect of siRNAs on nuclear translocation of pERK in transfected HeLa cells. Representative photomicrographs are shown for mock transfected cells, cells transfected with siRNA against *Gapdh* (siRNA *Gapdh*), *Emd* (siRNA *Emd*) and *Lmna* (siRNA *Lmna*). Top panels show labeling of nuclei with 4',6-diamidino-2-phenylindole (dapi) and bottom panels labeling with antibodies against pERK1/2. Arrowheads show enhanced nuclear localization of pERK in cells transfected with *Emd* and *Lmna* siRNAs. Bar: 10 μ m. Bar graph shows percentages of HeLa cells with pERK primarily in the nucleus (see arrowheads for example). Values are means \pm standard deviations for n=200 cells per group (* $p < 0.05$). (B) Effect of siRNAs on nuclear translocation of pERK in transfected C2C12 cells. Representative photomicrographs are shown for mock transfected cells, cells transfected with siRNA against *Gapdh* (siRNA *Gapdh*), *Emd* (siRNA *Emd*) and *Lmna* (siRNA *Lmna*). Top panels show labeling of nuclei with 4',6-diamidino-2-phenylindole (dapi) and bottom panels labeling with antibodies against pERK1/2. Arrowheads show enhanced nuclear localization of pERK in cells transfected with *Emd* and *Lmna* siRNAs. Bar: 10 μ m. Bar graph shows percentages of C2C12 cells with pERK primarily in the nucleus (see

arrowheads for example). Values are means \pm standard deviations for n=150 cells per group (* p <0.05).

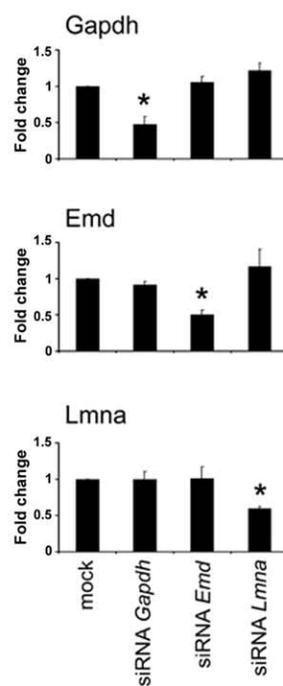
Fig. 4. MEK inhibitor PD98059 blocks ERK activation in cells with reduced emerin and A-type lamins. (A) Immunoblot showing effect of the MEK inhibitor PD98059 on the expression of total ERK1/2 and phosphorylated ERK1/2 in HeLa cells transfected with siRNAs against Gapdh, *Lmna* and *Emd*. (B) Upper part is an immunoblot showing effect of the MEK inhibitor PD98059 on the expression of total ERK1/2 and phosphorylated ERK1/2 in C2C12 cells transfected with siRNAs against Gapdh, *Lmna* and *Emd*. Lower part shows results of enzyme-linked immunosorbant assay showing effect of the MEK inhibitor PD98059 on the expression of total ERK1/2 and phosphorylated ERK1/2 in C2C12 cells transfected with siRNAs against Gapdh, *Lmna* and *Emd*. Bar graph shows the relative phosphorylation of ERK1/2. Values are means \pm standard deviations for n=3 samples per group (* p <0.05 when compared to mock treatment, # p <0.05 when compared C2C12 cells with or without addition of PD98059).

Figure 1

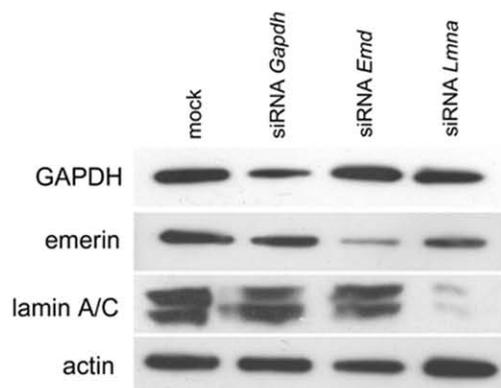
A HeLa cells



C C2C12 cells



B HeLa cells



D C2C12 cells

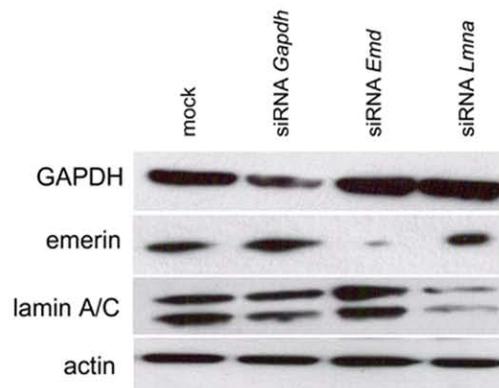
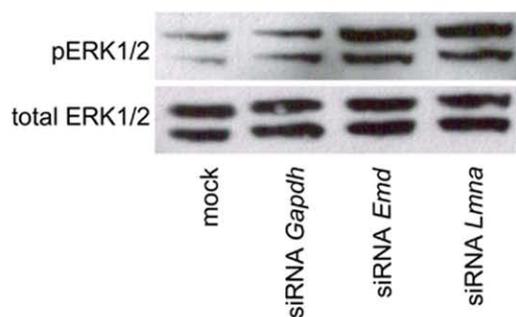
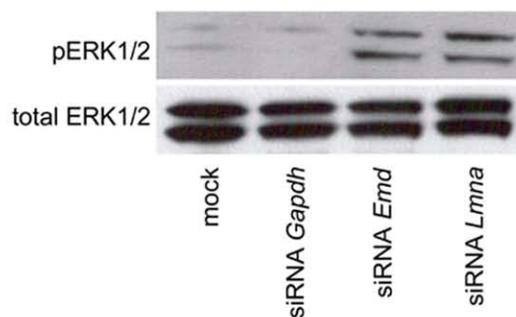


Figure 2

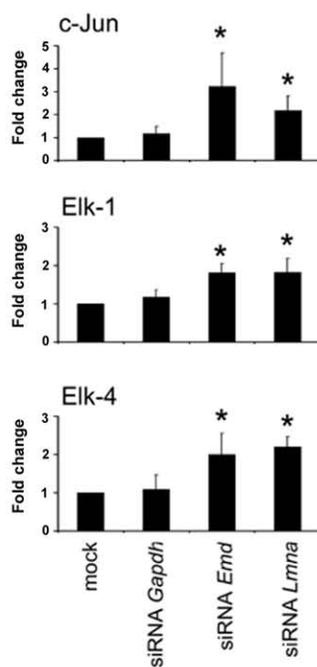
A
HeLa cells



C
C2C12 cells



B
HeLa cells



D
C2C12 cells

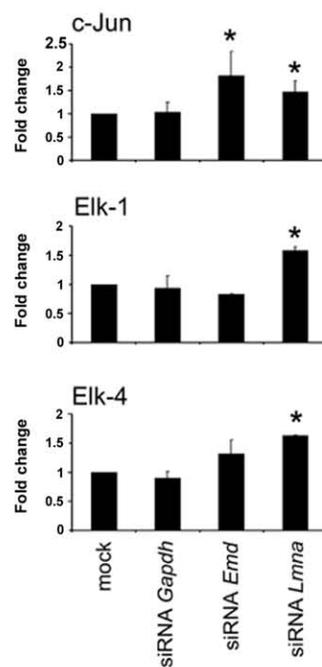
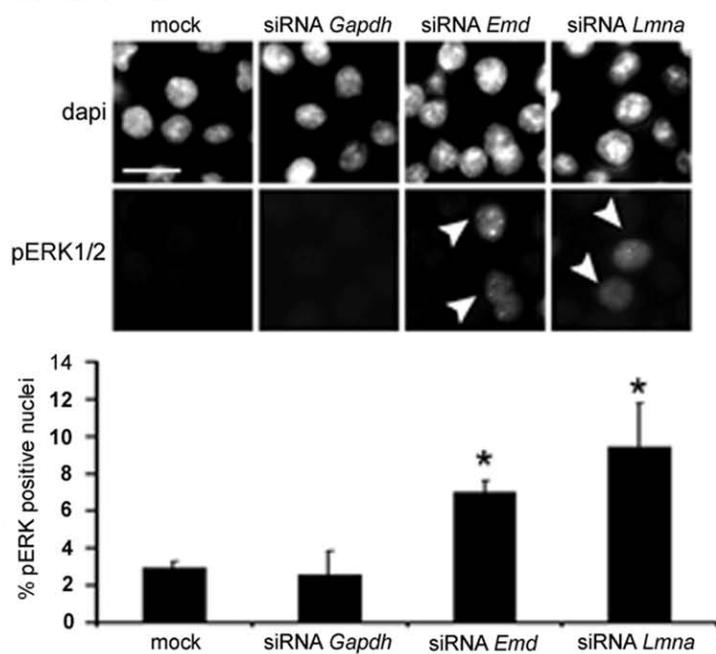


Figure 3

A

HeLa cells



B

C2C12 cells

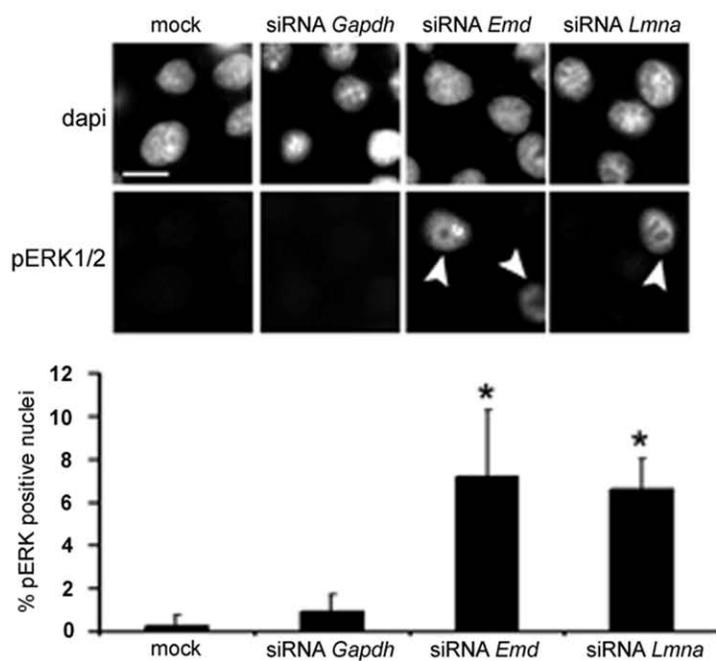
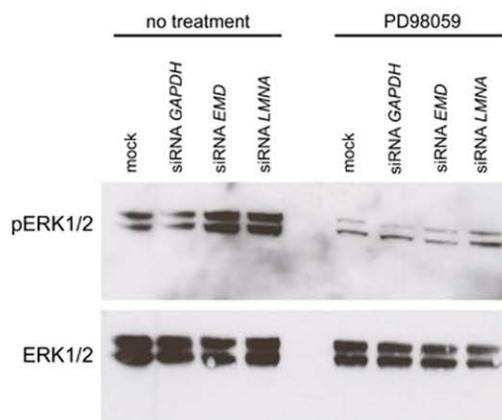


Figure 4

A

HeLa cells



B

C2C12 cells

