JNK-mediated Phosphorylation of Paxillin in Adhesion Assembly and Tension-induced Cell Death by the Adenovirus Death Factor E4orf4

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The adenovirus type 2 Early Region 4 ORF4 (E4orf4) protein induces a caspase-independent death program in tumor cells involving changes in actin dynamics that are functionally linked to cell killing. Because an increase in myosin II-based contractility is needed for the death of E4orf4-expressing cells, we have proposed that alteration of cytoskeletal tension is part of the signals engaging the death pathway. Yet the mechanisms involved are poorly defined. Herein, we show that the Jun N-terminal kinase JNK is activated in part through a pathway involving Src, Rho, and ROCK (Rho kinase) and contributes to dysregulate adhesion dynamics and to kill cells in response to E4orf4. JNK supports the formation of atypically robust focal adhesions, which are bound to the assembly of the peculiar actomyosin network typifying E4orf4-induced cell death and which are required for driving nuclear condensation. Remarkably, the dramatic enlargement of focal adhesions, actin remodeling, and cell death all rely on paxillin phosphorylation at Ser-178, which is induced by E4orf4 in a JNK-dependent way. Furthermore, we found that Ser-178-paxillin phosphorylation is necessary to decrease adhesion turnover and to enhance the time residency of paxillin at focal adhesions, promoting its recruitment from an internal pool. Our results indicate that perturbation of tensional homeostasis by E4orf4 involves JNK-regulated changes in paxillin adhesion dynamics that are required to engage the death pathway. Moreover, our findings support a role for JNK-mediated paxillin phosphorylation in adhesion growth and stabilization during tension signaling.

Cellular and viral gene products that display a so-called “tumor cell-selective killing activity” were recently discovered. These include HAMLET (human α-lactalbumin made lethal to tumor cells), MDA-7/interleukin-24, the avian virus derived apopin, and the adenovirus death factor early region 4 ORF4 (E4orf4) (1–6). Although these gene products seem to have little toxicity in normal cells, they all share the ability to engage potent cell death programs in transformed and cancer cells, which often bypass the classical caspase pathways, but whose mechanisms are poorly understood. It seems that they might target crucial oncogenic pathways upon which cancer cells have become addicted, having devastating effects on the cancer cells while sparing normal cells (7). It is becoming clear that new antitumor therapy should exploit nonapoptotic forms of programmed cell death to circumvent the apoptotic resistance of tumor cells. Yet this requires a deeper knowledge of nonapoptotic death programs at the molecular level. Therefore, any advance in our understanding of the mechanisms of killing these tumor cell-selective killers is of major interest.

The adenovirus E4orf4 death factor triggers different death effector pathways (caspase-dependent and -independent), whose contributions to cell killing is determined by the genetic background (8–10). Yet, in most transformed and cancer cell lines, E4orf4 triggers a p53- and caspase-independent death pathway that resists to Bcl-2 overexpression. Notwithstanding, this death pathway is associated with several apoptotic features, including cellular shrinkage and chromatin condensation, and it can be abrogated by perturbing intracellular signaling pathways, most notably, those controlling actin dynamics (9, 11–13). In fact, one major hallmark of this nonapoptotic death program is the early assembly of a peculiar and highly organized actomyosin network at the cell juxtanuclear region. Large-scale actin remodeling by E4orf4 relies on its ability to stimulate myosin II by enhancing the phosphorylation of the myosin light chain, thus generating high motor forces in the perinuclear region of the cell (14). Induction of a dramatic cell blebbing

[1] This work was supported by the Canadian Institutes of Health Research Operating Grant MOP-49450 (to J. N. L), a maintenance Grant for the Cell imaging core facility (to J. N. L.), and by the National Cancer Institute of Canada (to P. E. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[2] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

[3] The abbreviations used are: E4orf4, early region 4 ORF4; Ad2 E4orf4, adenovirus type 2 early region 4 open reading frame 4; AP-1, activator protein 1; EV, empty vector; FA, focal adhesion; FAK, FA kinase; FRAP, fluorescence recovery after photobleaching; GAP, GTPase-activating protein; GFP, green fluorescent protein; MAP, mitogen-activated protein; siRNA, small interfering RNA; PIPIES, 1,4-piperazinediethanesulfonic acid; MES, 4-morpholinoneethanesulfonic acid; JNK, c-Jun N-terminal kinase; mRFP, monomeric red fluorescent protein; ROCK, Rho kinase; N-Wasp, Tam67, dominant-negative transactivation deletion mutant of c-Jun; WT, wild-type; ERK, extracellular signal-regulated kinase; ROI, region of interest; Mf, mobile fraction; p-, phospho-. 

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process is preceded by the sudden collapse of this contractile
network with or without cell detachment and marks the onset
of apoptotic-like nuclear condensation. Because inhibition of
myosin II can provide a marked protection against E4orf4 kill-
ing, we have proposed that some orderly lesions caused by
increased cytoskeletal tension might contribute to engage the
cell death pathway given that myosin II is the major motor
protein responsible for the generation of internal cell tension
(14, 15). In this scenario, assembly of the peculiar juxtaparanuclear
contractile network would be a consequence of the initial and
lethal changes in actin dynamics rather than a cause of cell
death.

The effects of E4orf4 on the actin cytoskeleton are complex
and poorly understood at the molecular level. The lethal
changes in actin dynamics are controlled by several pathways
involving Rho GTPase signaling downstream of Src-family
kinases (14). E4orf4 associates with Src-family kinases and pro-
motes the tyrosine phosphorylation of a subset of targets having
a common ability to regulate actin dynamics (13, 16, 17). As a
consequence, E4orf4 favors Src-dependent activation of the
Rho GTPases Rac1, Cdc42, and RhoA over that of key survival
pathways, which rather show decreased Src-dependent activa-
tion. This is the case for focal adhesion kinase (FAK) and ERK,
two pathways providing critical survival signals in tumor cells
(13, 17, 18). Based on these findings, we have proposed that the
resulting unbalance in Src signals might trigger the death of
transformed and tumor cells expressing E4orf4. This presup-
poses that acute changes in actin dynamics induced by Src can
commit tumor cells to a nonapoptotic death program when uncoupled from survival signals. Yet, these Src signaling path-
ways have remained enigmatic.

We have shown that E4orf4–Src signaling induces a selective
and sustained activation of Jun N-terminal kinases (JNKs), sug-
uggesting that the JNK mitogen-activated protein kinase pathway
might transduce some Src pro-death signals (17). This is of
particular interest given that Drosophila Rho and JNK orthologs have been recently implicated in the migration and
apoptotic elimination of clonal patches of cells displaying acti-
vated Src kinases (19). Yet the functional relevance of JNK acti-
vation in response to E4orf4 has not been addressed, and
whether it is activated downstream of Rho GTPases is
unknown. JNK proteins have a clear role in stress-induced apo-
ptosis by virtue of their function in gene expression and of their
ability to phosphorylate several members of the Bcl-2 family of
proteins controlling the intrinsic mitochondrial apoptotic
pathway (20, 21). Nonetheless, the JNK pathway also pro-
motes cell proliferation, survival, and migration, depending
on the physiological context and spatiotemporal mode of
activation. It is becoming clear that JNK is a common down-
stream target of signaling pathways controlling cell move-
ment; notably, Rho GTPases and JNK activation is correlated
with an increase in cell migration in several systems (22, 23).
Evidence indicates that paxillin, an adaptor protein involved
in focal adhesion (FA) organization, is an important media-
tor of JNK signaling in cell migration. How exactly JNK-
mdivated phosphorylation of paxillin regulates adhesion
dynamics and whether it contributes to the pro-death func-
tion of JNK remains unknown.

Mechanical forces generated by myosin II-based contractility
inside the cell have a key role in the regulation of basic cel-
lar functions including cell proliferation and survival, and
these forces are sensed at cell-matrix adhesion sites (15, 24).
Because E4orf4 killing depends on the activation of myosin II,
we have postulated that deregulation of adhesion dynamics
might be among the initial lesions engaging the death pathway
in tumor cells. Herein, we show that JNK is activated in part
through a pathway involving Src-Rho-Rho kinase (ROCK) in
response to E4orf4 and is required for induction of the initial
cytoskeletal changes triggering the death of tumor cells. Strong
evidence is presented that paxillin phosphorylation at Ser-178
by JNK promotes FA stabilization, leading to the formation of
atypically enlarged adhesions that support E4orf4-induced cell
tension and killing in MCF7 cells. We propose a model whereby
E4orf4 perturbs tensional homeostasis in tumor cells by hijack-
ing a Src-Rho-ROCK pathway that controls cell tension and
adhesion dynamics via the concerted action of myosin II and
JNK/paxillin.

EXPERIMENTAL PROCEDURES

Expression Vectors—The following expression vectors were
described previously: Ad2 E4orf4, FLAG E4orf4 (13); FLAG-
E4orf4-GFP (9); FLAG-E4orf4-mRFP (14); GFP-p190RhoGAP
(25); myc-RhoAN19 (26); c-Jun dominant-negative TAM67
(27); GFP-paxillin WT and S178A (28) and GFP-vinculin (29).
The adenovirus vectors for expression of FLAG-Ad2 E4orf4 or
FLAG-GFP were constructed by subcloning cDNA for FLAG-
E4orf4 or for FLAG-GFP into T-Rex™ system (Invitrogen) to
produce AdTRexFLAG-E4orf4 (referred here to AdE4orf4) or
AdTRexFLAG-GFP (referred here to AdGFP).

Antibodies, Chemicals, and siRNAs—The following antibod-
ies were used: anti-phospho-Paxillin (Ser-178) (BIOCOURSE,
Camarillo, CA, or Bethyl, Montgomery, TX); anti-E4orf4
(2419) (13); anti-phospho-c-Jun (Ser-63), anti-phospho-SAPK
(stress-activated protein kinase)/JNK(Thr-183/Tyr-185), anti-
JNK2 (specific to the p54 JNK isoform) (Cell Signaling Tech-
nology, Beverly, CA); anti-myc (9E10), anti-β-actin (AC-74),
and anti-FLAG (M2) (Sigma-Aldrich); anti-c-Jun, anti-JNK1
(FL) and anti-GFP (B-2) (Santa Cruz Biotechnology, Santa
Cruz, CA); anti-calreticulin and anti-paxillin (BD Transduction
Labs). Alexa Fluor-coupled secondary antibodies and phalloidin
were purchased from Molecular Probes (Carlsbad, CA). The drugs SU6656, Y-27632, and NSC23766 were from Calbiochem/EMD (Gibbstown, NJ), and SP600125 was from
Sigma-Aldrich. Secramine A was synthesized by Bo Xu, and
G. B. Hammond (University of Louisville) and was kindly pro-
duced Dr. T. Kirchhausen (Harvard Medical School) and Dr.
G. B. Hammond (University of Louisville). The HP Validated
siRNA were from Qiagen (Valencia, CA); human JNK1
(HS_MAPK8_13_HP and HS_MAPK8_12_HP, referred here
to JNK1a and JNK1b, respectively), human JNK2
(HS_MAPK9_5_HP and HS_MAPK9_7_HP, referred here to
JNK2a and JNK2b, respectively), and the All Stars Negative
Control and GFP-22 (3’-labeled with rhodamine 6-carboxytet-
ramethylrhodamine) siRNAs, which were used as controls in
siRNA experiments.
JNK Regulates FA Assembly and Cell Killing by E4orf4

Cell Culture and Biochemistry—293T and MCF7 cells were derived from human embryonic kidney cells and from human mammary adenocarcinoma, respectively (30, 31). 293T were maintained in Dulbecco’s modified Eagle’s medium and MCF7 in α-minimum Eagle’s medium, both supplemented with 10% fetal bovine serum. MCF7 were transfected using Lipofectamine 2000 (Invitrogen) for plasmid DNA or using the TransIT-TKO reagent for siRNA (Mirus Bio Corp., Madison, WI) or were infected with AdGFP or AdE4orf4 at a multiplicity of infection of 75–100 plaque-forming units per cell. 293T were transfected using the calcium phosphate method as described (13). For immunoprecipitation analyses, cells from 10-cm plates were lysed in 0.5 ml of modified radioimmunoprecipitation assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 10 mM β-glycerophosphate, 1 mM Na₃VO₄, 15 μg/ml leupeptin, 0.1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 μg/ml pepstatin) then diluted with 0.5 ml of HNTG buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1% Triton X-100, 10% glycerol, 10 mM β-glycerophosphate, 1 mM Na₃VO₄, 15 μg/ml leupeptin, 0.1 μg/ml aprotinin, 1 μg/ml pepstatin) and processed as described previously (17). Western blot analyses were done as described (13), and protein concentrations were determined previously (17). Western blot analyses were done as described (13), and protein concentrations were determined using the Bio-Rad DC Protein Assay. Densitometric analyses were performed from FluorS MAX Multimager-captured images using the Quantity 1-D software version 4.6.0 (Bio-Rad). Activator protein-1 (AP-1)-dependent transcription was measured using chloramphenicol acetyltransferase (CAT) assays (32). The reporter 3AP-1Col was generated by cloning the AP-1 sequence of the collagenase promoter upstream of thymidine kinase minimal promoter of the TK-CAT vector (33). Transfections were normalized using a Luciferase reporter at a plasmid DNA ratio 1:10.

Immunofluorescence, Microscopy, and Image Processing—Microscopic analyses and DNA condensation assays were performed 24 h after transfection. DNA was labeled with cell-permeable Hoechst (33423) before cell fixation, and cells were fixed in 3.7% formaldehyde in Luftig buffer (0.2 M sucrose, 35 mM PIPES, pH 7.4, 5 mM EGTA, 5 mM MgSO₄) for 20 min at 37 °C (34). When indicated in the figure legends, cells were extracted in situ before fixation in cytoskeleton extraction buffer (0.02% Triton-X-100, 10 mM MES, pH 6.1, 138 mM KCl, 3 mM MgCl₂, 2 mM EGTA, 0.32% sucrose, 1 μg/ml phalloidin) for 8 min at 4 °C to reveal proteins associated with the actin cytoskeleton (35). Fixation-induced fluorescence was quenched with 50 mM NH₄Cl for 15 min at room temperature, and immunostaining was performed as described (14). Fixed cells were mounted in a 50:50 mix of glycerol and glycline buffer pH 8.6 (0.2 M glycine 0.3 M NaCl, 0.1% NaCl). Confocal microscopy of fixed specimen was performed using a Bio-Rad MRC 1024 mounted on a Nikon TE-200 inverted microscope with a 60× oil 1.4 NA objective. Acquisitions were taken on separate channels with appropriate restrictive filters. The Metamorph 4.5 software (Molecular Devices, Union City, CA) and Photoshop CS3 software (Adobe) were used for processing of the overall images before cell cropping to emphasize the main point of the image. Processing was limited to background subtraction and brightness/contrast adjustments. For quantitative fluorescence analyses of endogenous paxillin, confocal images were acquired using the same parameters (gain and laser power), exported in TIFF format, and analyzed with the ImageJ 1.38 software (NIH) using the Measure Average Intensity function, calculated from a selected area corresponding to the entire cell. For quantitative average area of FA, the Measure Area function was used, calculated from a selected area corresponding to the FA. An average area value was calculated for each cell, and then, an average area value was calculated for each treatment. For quantitative analyses of paxillin p-Ser-178 intensity ratios, cells were imaged using a Bio-Rad MRC 1024 mounted on a Nikon TE-200 inverted microscope with a 20× air 0.5 NA objective, with the pinhole completely open. Then fields were selected on the coverslips containing both E4orf4-mRFP-positive cells and negative cells (non-transfected cells). To minimize the variability due to differences in cell size (late stage blebbing cells), only cells whose area is 1000 ± 200 μm² (our estimated average area of MCF7 cells) were included. All the images were taken with identical acquisition parameters optimized for the fluorescent signals to be in the dynamic range. Under those conditions, the amount of p-Pax(Ser-178) is proportional to the total fluorescence, which was analyzed using the ImageJ 1.38 software (NIH). A ratio of intensity of p-Pax-(Ser-178) in E4orf4-expressing cells was calculated relative to E4orf4-negative cells for each individual experiment (sample) to minimize the variability due to differences in staining intensity.

FRAP and Time-lapse Analyses—For live microscopy, cells were incubated in media with 20 mM HEPES, pH 7.4, and imaged using an Olympus FV1000 confocal equipped with a 60× NA 1.4 objective and a temperature-controlled chamber driven by the FluoView software (Olympus, Markam, ON, Canada). FRAP analyses were performed 24 h after transfection according to the method previously described (36, 37). Images were acquired every 0.7 s for 90 s. Five images were acquired before photobleaching of a region of interest (ROI) with the 405-nm laser using the tornado setting during 1 frame. To directly compare recovery curves from different treatments, data were normalized for background fluorescence (F bgd) and loss of fluorescence during the bleach using $F(t)_{\text{norm}} = 100 \times (F(t)_{\text{ROI}} - F_{\text{bgd}})/(F_{\text{cell}} - F_{\text{bgd}})/F(t)_{\text{ROI}} - F_{\text{bgd}}$, where $F(t)_{\text{ROI}}$ is ROI intensity, $F_{\text{cell}}$ is total cell intensity at any given time point ($t$), $F_{\text{ROI}}$ is initial intensity of the ROI, and $F_{\text{cell}}$ is initial intensity of the entire cell. The resulting normalized data were then averaged for different cells, and the associated S.D. were calculated with Excel 11.3.8 software (Microsoft). The mobile fraction (Mf) was calculated according to Mf = 100 × ($F_{\text{bgd}}$)/($F_{\text{cell}} - F_{\text{ROI}}$), where $F_{\text{bgd}}$, $F_{\text{cell}}$, and $F_{\text{ROI}}$ are the normalized fluorescence intensities at the asymptote, determined immediately after the bleach and before the bleach, respectively. Nonlinear regression (curve fit) was used to calculate the half-time of recovery ($t_{1/2}$) using the R freeware 2.5.1 software (Boston, MA). Time-lapse acquisitions used low power illumination to protect cells from light damage and limit photobleaching.
Statistical Analyses—For statistical analyses, a one-way analysis of variance test was used with p values of <0.05 considered significant (p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)). The Prism 5.0a software (GraphPad Software, San Diego, CA) was used to compare mean values of individual experiments, whereas the SAS/STAT 9.1 software (SAS Institute, Cary, NC) was used to compare all single cell measurements from each treatment of individual experiments.

RESULTS

JNK Activation in Response to E4orf4 Involves a Src-Rho-ROCK Signaling Axis—The ability of E4orf4 to associate with and dysregulate Src-family kinase signaling causes the activation of several Rho GTPase pathways that comes along with increased JNK activity (14, 17). To determine the functional relationship between Rho GTPases and JNK activation in response to E4orf4, we took advantage of chemical inhibitors to block E4orf4-activated RhoGTPase pathways for short periods of time in 293T or MCF7 cells, two cell lines highly responsive to the Src-dependent death activity of E4orf4 (14, 17). 293T were transfected with E4orf4, whereas MCF7 cells were infected with adenovirus vectors expressing either E4orf4 as a single product (AdE4orf4) or GFP as a control (AdGFP). Cells were exposed to drugs that inhibit Rac1 (NSC23766) (38), Cdc42 (Secramine A) (39, 40), or ROCK (Y-27632) for 1–2 h before lysis. The latter was used because RhoA connects to JNK through ROCK (41). Activation of endogenous JNK1 and JNK2 was monitored by Western blot using a phosphospecific antibody to the dually phosphorylated Thr-183/Tyr-185 or a phosphospecific antibody that recognized phospho-c-Jun. Consistent with previous work (17), E4orf4 increased the level of phospho-JNKs by ~2.5-fold in 293T relative to control cells transfected with the vector only (EV) (Fig. 1A) and by ~2.0-fold in MCF7 cells relative to cells infected with AdGFP (Fig. 1B). However, in both cell lines, only the Rho/ROCK signaling axis appeared to significantly contribute to E4orf4-induced JNK activation. Although inhibition of Rac1 had no detectable effect on the level of E4orf4-induced phospho-JNKs, inhibition of Cdc42 either potentiated (MCF7) or did not affect E4orf4-induced JNK activation (293T). In contrast, short-term inhibition of ROCK reproducibly decreased phospho-JNK levels in E4orf4-expressing cells by ~40% (Fig. 1, A and B). Under such conditions, the basal level of phospho-JNKs in control cells was not consistently affected, supporting a specific effect on E4orf4-induced JNK activation. Long term inhibition of RhoA by expression of a dominant-negative RhoA (N19) or p190 RhoGAP, a Rho-selective GAP that negatively regulates Rho GTPases (42), also interfered with E4orf4-mediated phospho-c-Jun (supplemental Fig. S1A, ~50% reduction), just like a dominant-negative ROCK (data not shown), corroborating the involvement of RhoA/ROCK. Under these conditions, the basal level of phospho-c-Jun was also reduced in control 293T cells, indicating that Rho is an important regulator of the JNK pathway in this cell line (supplemental Fig. S1A). Finally, because c-Jun phosphorylation in response to E4orf4 was similarly impaired by the Src inhibitor (SU6656) (supplemental Fig. S1B) and because Rho/ROCK are activated downstream of Src (14), we concluded that the ability of E4orf4 to promote a sustained and efficient activation of the JNK pathway relied, at least in part, on a Src-Rho-ROCK signaling axis. However, JNK activation in response to E4orf4 appeared to require the contribution of another signaling pathway distinct from Rac1 and Cdc42.

JNK Contributes to E4orf4-induced Actin Remodeling and Cell Death—To next determine whether JNKs contribute to the lethal cytoskeletal alterations induced by E4orf4, we used two distinct siRNA sequences to delete JNK1 or JNK2 individually, as both isoforms are activated by E4orf4 (Fig. 1, A and B). Transfection of siRNAs was performed 48 h before transfection of E4orf4-mRFP in the more adherent MCF7 cells, where the typical changes in actin are easily detected by F-actin staining in fixed cells. The siRNAs achieved at least 65% reduction of the protein level of a single JNK isoform without affecting the level of the other (Fig. 1C). As shown before, transfection of the control siRNA did not affect the assembly of the juxtanuclear actin network typically seen in E4orf4-expressing cells before the onset of nuclear condensation (Fig. 1D, siControl). This peculiar and highly organized cytoskeletal structure is assembled through the concerted action of Cdc42/N-Wasp (Neural Wiskott-Aldrich syndrome protein) and Rho/ROCK/myosin II and is composed of a juxtanuclear ring of actin-myoosin II (Fig. 1D, first row, arrows) anchored at the cell cortex by prominent actin cables (arrowhead) (14). Likewise, this structure was found to be associated with the generation of high motor forces in the juxtanuclear region of the cell, as revealed by the polarized distortions of the nucleus close to the sites of activated myosin II (Fig. 1D, first row, white dashed line) (14). Remarkably, depletion of JNK1 led to a consistent ~45% reduction in the number of E4orf4-expressing cells displaying the juxtanuclear actin network (Fig. 1D, siJNK1a, Fig. 1E, siJNK1a, siJNK1b). A similar reduction of nuclear condensation in response to E4orf4 was observed (~50% inhibition), consistent with previous findings supporting a functional link between actin changes and cell death. In contrast, depletion of JNK2 did not have a strong impact on E4orf4-induced actin remodeling (~15% inhibition) and nuclear condensation (~10% inhibition) in MCF7 cells (Fig. 1D, siJNK2a, Fig. 1E, siJNK2a, siJNK2b). Thus, the results indicated that JNK contributed to the lethal changes in actin dynamics in response to E4orf4 and suggested that JNK1 was selectively involved, at least in the context of MCF7 cells.

JNK Contributes to E4orf4-mediated FA Enlargement, Stress Fiber Formation, and Tension-induced Cell Death through Paxillin Phosphorylation at Ser-178—The JNK proteins generally contribute to the induction of apoptosis through their regulation of the mitochondrial intrinsic pathway (via phosphorylation of the Bcl-2 family of proteins) or through their stimulation of nuclear gene expression (via the AP-1 transcription factor) (43). However, we have shown that E4orf4 killing requires neither p53 nor caspase activation or cytochrome c release and resists the anti-apototic activity of Bcl-2 (9, 11). Likewise, it seems that AP-1-dependent transcription is not involved either. Although we found that JNK activation by E4orf4 did increase AP-1-dependent transcription (supplemental Fig. S2A), inhibition of AP-1-dependent transcription did not impair E4orf4-induced nuclear condensation. This was revealed by overexpression of a dominant-negative c-Jun mutant defective in transcriptional activation (TAM67) (sup-
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To get some hints into the identity of substrates for JNK potentially involved in E4orf4 killing, we looked at the subcellular distribution of activated JNKs in E4orf4-expressing cells, using the phospho-JNK antibody. To better visualize cytoskeletal proteins, immunolocalization of endogenous JNKs was performed after a mild extraction with low detergent in a cytoskeletal stabilization buffer in MCF7 cells. As expected, E4orf4-expressing cells displayed stronger phospho-JNK staining relative to control cells (Fig. 2A). In E4orf4-positive cells, active JNKs were predominantly localized at FAs, as revealed by the striking co-distribution of phospho-JNKs and paxillin, a bona fide component of FAs (Fig. 2A, middle row). In marked contrast, JNKs activated by a classical inducer anisomycin, essentially accumulated in the cell nucleus (Fig. 2A, third row). This suggested that JNK activation by E4orf4 could specifically affect adhesion dynamics. Incidentally, we have shown previously that E4orf4 leads to a redistribution of FAs along with a decrease in Src-FAK-dependent signaling (13). Yet, the exact impact on adhesion dynamics has not been addressed.

To investigate the effect of E4orf4 on paxillin adhesions and the role of JNK, we examined the distribution and the size of FAs by paxillin staining in MCF7 cells expressing E4orf4, as compared with control cells and cells transfected with siRNAs to the individual JNK proteins. In cells expressing E4orf4, regardless of the presence of the control siRNA, there was a striking 5-fold increase in the mean size of FAs that clustered in patches around the cell periphery and in more central regions joining the juxtanuclear actomyosin ring to the cell cortex (Fig. 2B, arrowhead, and C). The atypically enlarged paxillin-positive FAs served as convergence sites for robust actin fibers, often crossing intracellularly at multiple sites along the cell ventral face (Fig. 2D, second row). Contrastingly, control cells displayed a more organized network of thin peripheral actin fibers converging to smaller paxillin-contain-

**FIGURE 1.** Activation of the JNK pathway in response to E4orf4 involves Rho/ROCK and is required for triggering the lethal cytoskeletal changes. A and B, Western blots of equal amounts of total cell extracts from 293T cells harvested 24 h after transfection with the EV or with FLAG-E4orf4 (A) or from MCF7 cells harvested 40 h after infection with AdGFP or AdE4orf4 (B). Cells were exposed to drugs that inhibit Rac1 (NSC23766, 10 μM), Cdc42 (Secramine A, 10 μM), or ROCK (Y-27632, 10 μM) for 1–2 h. JNK1 and JNK2 activation was detected using anti-phospho-SAPK (stress-activated protein kinase)/JNK (Thr-183/Tyr-185). C, The atypically enlarged paxillin-positive FAs served as convergence sites for robust actin fibers, often crossing intracellularly at multiple sites along the cell ventral face (Fig. 2D, second row). Contrastingly, control cells displayed a more organized network of thin peripheral actin fibers converging to smaller paxillin-contain-

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To investigate the effect of E4orf4 on paxillin adhesions and the role of JNK, we examined the distribution and the size of FAs by paxillin staining in MCF7 cells expressing E4orf4, as compared with control cells and cells transfected with siRNAs to the individual JNK proteins. In cells expressing E4orf4, regardless of the presence of the control siRNA, there was a striking 5-fold increase in the mean size of FAs that clustered in patches around the cell periphery and in more central regions joining the juxtanuclear actomyosin ring to the cell cortex (Fig. 2B, arrowhead, and C). The atypically enlarged paxillin-positive FAs served as convergence sites for robust actin fibers, often crossing intracellularly at multiple sites along the cell ventral face (Fig. 2D, second row). Contrastingly, control cells displayed a more organized network of thin peripheral actin fibers converging to smaller paxillin-contain-
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Because paxillin is a substrate for JNK, we hypothesized that paxillin phosphorylation could contribute to FA remodeling in response to the increase in actomyosin contractility induced by E4orf4-Src signaling. Indeed, we have shown that E4orf4 activates myosin II through a Src-Rho-ROCK pathway, and myosin II is the major motor protein responsible for the generation of cytoskeletal tension, which is directly proportional to adhesion strength (14, 15, 28). This prompted us to monitor paxillin phosphorylation in E4orf4-expressing cells using phospho-antibodies that recognize the JNK phospho-site Ser-178. These antibodies reacted to the wild type GFP-paxillin but not to a paxillin mutant in which Ser-178 was mutated to Ala (S178A) (data not shown). Western blot analyses using both antibodies revealed that the level of Ser-178-paxillin phosphorylation (p-Ser-178) was increased in immune complexes of endogenous paxillin (Fig. 3A) and in total cell lysates (Fig. 3B) of 293T cells transfected with E4orf4 compared with control cells (EV). This increase in Ser-178 phosphorylation was lost upon depletion of JNK1 before E4orf4 transfection, consistent with a role for JNK (Fig. 3, A and B, siJNK1a). Because cell infection with adenovirus vectors altered paxillin phosphorylation at Ser-178 independently of E4orf4 expression (data not shown), we analyzed the level of endogenous p-Ser-178 in single MCF7 cells transfected with E4orf4 using a quantitative immunofluorescence assay, as described under “Experimental Procedures.” We measured a reproducible ∼2-fold increase in the fluorescence ratio intensity of paxillin p-Ser-178 in E4orf4-expressing cells calculated relative to negative cells from the same sample (Fig. 3C). Furthermore, in E4orf4-expressing cells, inhibition of ROCK with Y-27632 reduced paxillin p-Ser-178 ratio intensity by ∼35%, consistent with the contribution of ROCK to JNK activation in response to E4orf4 (Fig. 1, A and B). Thus, the results suggested that paxillin was phosphorylated at Ser-178 downstream of JNK in response to E4orf4, in part as a result of RhoA/ROCK activation.

To next address the functional relevance of Ser-178-paxillin phosphorylation, E4orf4-mRFP was coexpressed with wild type GFP-paxillin (GFP-PaxWT) or with a Ser-178 to Ala mutant of GFP-paxillin (GFP-PaxS178A), and the changes in FA and actin were examined in fixed cells. In MCF7 cells, FA size was not with the indicated siRNA 48 h before E4orf4 transfection; Ctrl, control siRNA. D, confocal images of transfected MCF7 cells showing the representative phenotypes observed upon depletion of JNK1 versus JNK2 in E4orf4-mRFP-positive cells. Note that the presence of robust actin fibers converging to enlarged paxillin-positive adhesions is not affected by the control siRNA (second row) or by JNK2 depletion (fourth row) but is completely inhibited by depletion of JNK1 (third row). The insets show high magnifications (2.5×) of the co-distribution of actin fibers and FAs displaying a patch-like morphology in E4orf4-positive cells (siCtrl, siJNK2a) but not in control (EV) cells or in E4orf4-positive cells depleted in JNK1 (siJNK1a). N, cell nucleus; Bars, 10 μm.
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Significantly different between cells expressing GFP-PaxWT and GFP-PaxS178A, as seen before in Swiss 3T3 cells, and no major change in the size and distribution of FAs was observed relative to cells transfected with the vector only (Figs. 3E and 2B, EV) (28). However, in the presence of E4orf4 there was a striking difference in FA size and morphology between cells expressing GFP-PaxWT and those expressing similar levels of GFP-PaxS178A. Although GFP-PaxWT accumulated high levels at hypertrophic FAs and along the ventral tensile fibers (Fig. 3D, arrows), GFP-PaxS178A displayed a more modest accumulation to smaller adhesion sites at the cell periphery (Fig. 3D, arrowhead). Importantly, the Ser-178 to Ala mutant of paxillin abrogated FA enlargement in cells expressing similar levels of E4orf4 (Fig. 3, D and E). This was associated with a 50% reduction in the number of cells displaying the characteristic actomyosin network and nuclear condensation, an effect similar to JNK1 depletion (Fig. 3E). Together the results indicated that Ser-178-paxillin phosphorylation mediates the JNK-dependent reorganization of FAs required for E4orf4 killing in cancer cells.

JNK-mediated Ser-178-paxillin Phosphorylation Enhances Its Recruitment to FA from an Internal Pool and Stabilizes Adhesions by Increasing the Time-Residency of Paxillin at FA—Ser-178-paxillin phosphorylation by JNK was reported to regulate cell movement, supporting a role in adhesion assembly/disassembly (28, 45–47). However, the significance of Ser-178 phosphorylation for paxillin dynamics remains unclear. Overexpression of GFP-PaxS178A was shown to increase FA size and number in one specific cell line but not in other cell types (28) (Fig. 3, D and E). Hence, we took advantage of E4orf4 to clarify the impact of Ser-178-paxillin phosphorylation on adhesion dynamics. Because Ser-178-paxillin phosphorylation is required for FA enlargement in response to E4orf4, we reasoned that it might modulate paxillin affinity for FA components. Indeed during measurements of FA size, we observed that endogenous and GFP-PaxWT had a more intense FA pattern in E4orf4-expressing cells, suggesting that paxillin p-Ser-178 might enhance paxillin targeting to FA. To ascertain whether this was so, a paxillin intensity ratio was obtained by measuring the intensity of paxillin staining at FA over that of paxillin staining in the perinuclear region (P) in unextracted fixed cells (Fig. 4A). Although E4orf4 expression did not affect the overall level of endogenous paxillin, it triggered a 3-fold increase in the FA/perinuclear paxillin ratio (Fig. 4, B and C). This increase in paxillin recruitment was significantly impaired by the JNK inhibitor SP600125 or by siRNA to JNK1, but not by siRNA to JNK2, supporting a specific role for JNK1 and Ser-178-paxillin phosphorylation in paxillin targeting to FA. To ascertain whether this was so, a paxillin intensity ratio was obtained by measuring the intensity of paxillin staining at FA over that of paxillin staining in the perinuclear region (P) in unextracted fixed cells (Fig. 4A). Although E4orf4 expression did not affect the overall level of endogenous paxillin, it triggered a 3-fold increase in the FA/perinuclear paxillin ratio (Fig. 4, B and C). This increase in paxillin recruitment was significantly impaired by the JNK inhibitor SP600125 or by siRNA to JNK1, but not by siRNA to JNK2, supporting a specific role for JNK1 and Ser-178-paxillin phosphorylation in paxillin targeting to FA. To ascertain whether this was so, a paxillin intensity ratio was obtained by measuring the intensity of paxillin staining at FA over that of paxillin staining in the perinuclear region (P) in unextracted fixed cells (Fig. 4A). Although E4orf4 expression did not affect the overall level of endogenous paxillin, it triggered a 3-fold increase in the FA/perinuclear paxillin ratio (Fig. 4, B and C). This increase in paxillin recruitment was significantly impaired by the JNK inhibitor SP600125 or by siRNA to JNK1, but not by siRNA to JNK2, supporting a specific role for JNK1 and Ser-178-paxillin phosphorylation in paxillin targeting to FA.

FIGURE 3. JNK-mediated paxillin p-Ser-178 is necessary for FA enlargement, actin remodeling, and nuclear condensation in response to E4orf4. A, Western blot of paxillin p-Ser-178 (p-Pax S178) in paxillin immune complexes (Pax IP) isolated from 293T cells transfected with control siRNA (siCt) or siRNA to JNK1 (siJNK1) 48 h before transfection with FLAG-E4orf4 or the vector only (EV) using anti-phospho-Pax(Ser-178) from BioSource. B, Western blot of paxillin p-Ser-178 (p-Pax S178) in total cell extracts from 293T cells transfected as indicated using anti-phospho-Pax(Ser-178) from Bethyl. The -fold increase in Ser-178 phosphorylation in E4orf4-transfected cells (Fold) was estimated by densitometric analysis and is representative of 3 independent experiments (A) or 1 experiment (B). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, quantitative analysis of paxillin p-Ser-178 in MCF7 cells transfected with E4orf4-mRFP using a microscopy-based assay described under “Experimental Procedures.” Phospho-paxillin was immuno-labeled with anti-phospho-Pax(Ser-178) from BioSource. The graph shows the average ratios of total fluorescence intensity of phospho-paxillin in E4orf4-expressing cells over that in E4orf4-negative cells from the same samples. Data are the means ± S.D. of 5 independent experiments for untreated cells (w/o) or of 2 experiments for cells exposed to the ROCK inhibitor Y-27632, 10 μM (1.5 h), Bar, 30 μm. D and E, MCF7 cells were transfected with GFP-PaxWT or with GFP-PaxS178A together with E4orf4-mRFP or the vector alone (EV) and processed for staining of F-actin (phalloidin) and DNA (Hoechst 33342) after a brief extraction in cytoskeletal stabilization buffer containing 0.02% Triton X-100. D, confocal images showing the representative phenotypes of E4orf4-positive cells expressing GFP-PaxWT as compared with GFP-PaxS178A. Arrows point to the distribution of GFP-PaxWT in dramatically enlarged FAs at the cell center and extending along the tensile fibers. In contrast, the arrowhead shows the more modest distribution of GFP-PaxS178A in peripheral small adhesion sites in a cell devoid of the typical tensile fibers induced by E4orf4. Bar, 10 μm. E, the average focal adhesion area was quantified from confocal images and the number of cells expressing GFP-PaxWT or GFP-PaxS178A with or without E4orf4-mRFP that displayed a typical perinuclear actin network, and nuclear condensation was scored over the total number E4orf4-positive cells (n). Data are the means ± S.D. of three independent experiments. ***, p < 0.001; **, p < 0.01.
paxillin p-Ser-178 modulates its mobility and affinity for binding partners at FA. To study the mobility of wild type GFP-Pax as compared with GFP-PaxS178A in MCF7 cells, FRAP experiments were performed to measure paxillin exchange dynamics at cell-substrate adhesions on fibronectin. After the rapid photobleaching of a small region of wild type GFP-Pax at FA, fluorescence recovery was imaged at 0.7-s intervals using low power laser illumination. Single exponential fits were used to estimate the halftime of recovery \( (t_{1/2}) \) and the mobile fraction (Mf), as reported before by others with similar probes (48). In control cells there was no difference in the rates and percentages of fluorescence recovery of the wild type GFP-Pax and GFP-PaxS178A over the 90-s duration of experiments \( (t_{1/2} \text{ of } 3.6 \pm 0.8 \text{ and Mf of } 66.5\% \pm 2.7 \text{ for WT as compared with } t_{1/2} \text{ of } 3.5 \pm 1.2 \text{ and Mf of } 66.6\% \pm 10.8 \text{ for [S178A])} \) (Fig. 5, A and D). This was consistent with the lack of difference in the organization of paxillin adhesions in control cells (Fig. 3D). The fast rates of fluorescence recovery were within the range reported for GFP-Pax in other cell types, and the incomplete recovery of fluorescence supported the existence of two distinct pools of paxillin; a dynamic pool that rapidly turned over and a more stable pool that did not turn over within 90 s (49, 50). A striking 3-fold increase in halftime of recovery \( (t_{1/2} \text{ of } 11.5 \pm 3.8) \) and a 1.8-fold decrease in the mobile fraction of GFP-PaxWT (Mf of 37.4% \pm 3.1) were measured in cells expressing E4orf4 compared with control cells (EV) (Fig. 5, B–D). In marked contrast, the halftime of recovery and mobile fraction of the GFP-PaxS178A mutant were not significantly affected by E4orf4 \( (t_{1/2} \text{ of } 4.8 \pm 2.4; \text{ Mf of } 51.3 \pm 10.5) \) (Fig. 5, C and D). This suggested that paxillin p-Ser-178 modifies its affinity for binding partners at FA, resulting in an increased stability of paxillin within FAs.

To determine whether the slower dynamics was a property of paxillin or of the adhesion as a whole in E4orf4-expressing cells, we measured the mobility of GFP-vinculin, which is considered as a key player in the regulation of FA stability (49). A striking
2.7-fold increase in halftime of recovery and 1.6-fold decrease in the mobility fraction of GFP-vinculin were measured in E4orf4-expressing cells compared with control cells, being consistent with inhibition of FA turnover in E4orf4-expressing cells (Fig. 5E). To corroborate FRAP analyses, the dynamics of GFP-PaxWT-positive adhesions were imaged in cells expressing E4orf4-mRFP and compared with the dynamics of adhesions formed by the mutant GFP-PaxS178A. In control cells (EV), the
small GFP-PaxWT-positive adhesions underwent assembly and disassembly over a 15-min period (Fig. 6A). Not surprisingly, the large GFP-PaxWT-positive adhesions displaying a patch-like morphology in E4orf4-expressing cells were more stable and did not disassemble over a similar period of time (Fig. 6B). In contrast, the turnover of adhesions formed by the mutant GFP-PaxS178A in E4orf4-expressing was more similar to paxillin adhesions in control cells (Fig. 6C). This further confirmed that paxillin p-Ser-178 was required for FA stabilization in response to E4orf4. To determine whether a sustained activation of the ROCK-JNK pathway was necessary for promoting the maturation and stabilization of FAs, the dynamics of GFP-PaxWT-positive adhesions in E4orf4-expressing cells were imaged after exposure to the ROCK inhibitor (Y-27632) or the JNK inhibitor (SP600125). Exposure to Y-27632 for 60 min led to a reorganization of adhesions that adopted a more pointed morphology at the cell edges (Fig. 6D). Importantly, ROCK inhibition increased adhesion turnover in E4orf4-expressing cells, as GFP-PaxWT adhesions showed a faster rate of disassembly. Such was the case also in cells exposed to the JNK inhibitor (SP600125) (Fig. 6E). Based on the overall data, we concluded that JNK-mediated phosphorylation of paxillin at Ser-178, which is mediated in part by a Src-RhoA-ROCK signaling axis, is required to functionally link FA stabilization with actin remodeling during tension-induced cell death by E4orf4.

**DISCUSSION**

We have shown previously that the ability of E4orf4 to upregulate actomyosin contractility depends on its binding to and deregulation of Src kinases and contributes to tumor cell killing (14). However, the discrete lesions engaging the death pathway have remained enigmatic. Herein, we provide strong evidence that dysregulation of adhesion dynamics downstream of a Src-Rho-ROCK-JNK pathway is an important component of the death signaling pathway, which appears to involve a chronic increase in internal force generation.

A chief mechanism for inducing FA assembly involves local mechanical forces generated by myosin II; indeed the level of cytoskeletal tension inside the cell is directly proportional to adhesion strength (15). Because adhesion overgrowth and stress fiber formation were functionally linked to the propagation of E4orf4 death signal, the forces generated by increased cytoskeletal tension might directly contribute to turn on the death pathway. In this scenario, the chronic increase in cytoskeletal tension generated by the co-assembly of robust adhesions and tensile actin fibers would ultimately cause the breakage of adhesions and the sudden collapse of the contractile perinuclear actin network, which precedes the onset of cell blebbing in E4orf4-expressing cells (14). This is supported by experiments showing a marked inhibition of nuclear condensation following depletion of JNK1 or inhibition of paxillin phosphorylation at Ser-178, which similarly impaired FA growth, stress fiber formation, and cell shrinkage. Based on our findings, it is reasonable to think that one of the major lesions induced by E4orf4 in tumor cells is the generation of a high level of internal cell tension combined with deregulation of the cell traction forces, which would be driven by the concerted action of myosin II and JNK downstream of ROCK. Indeed, we have shown before that ROCK is responsible for myosin II activation in response to E4orf4 (14), and here we show that ROCK also contributes to JNK activation and the ensuing changes in adhesion dynamics. Hence, we propose that the key function of ROCK in mechanotransduction is exploited by E4orf4 to kill cancer cells and involves two arms of ROCK; first, its ability to control actomyosin contractility, and second, its ability to pro-
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Cell tension is known to influence basic cellular functions, including cell proliferation, differentiation and apoptosis, but the detailed mechanisms of force transduction and how it impacts on cell behavior have yet to be identified (15). Recent studies have shown that phosphorylated signaling and particularly the early activation of Src-family kinases is involved in force sensing and regulation of adhesion dynamics (53). Importantly, transformed cells have an altered rigidity/tensional homeostasis, and early observations have linked transformation to profound alterations in cell shape and migration that depend on oncogenic tyrosine kinase signaling (54, 55). In transformed cells, stress fibers are usually absent, and mature FAs can be replaced by more invasive structures, such as podosomes that typify v-Src-transformed cells (56). Thus, transformed cells generally develop weaker traction forces (decreased cell adhesion on the outside) and display increased adhesion dynamics that rely in part on deregulated Src-FAK signaling (18). Such an altered tensional homeostasis is believed to promote unregulated motility and anchorage independence, allowing tumor cells to bypass apoptotic cell death upon detachment (anoikis) (57). Actually, normal nonhematopoietic cells require adhesion to a rigid substrate to survive. Anoikis results from unopposed cell tension in the inside of the cell and is understood mainly in terms of loss of integrin-dependent outside-in survival signals. It seems that tumor cells can bypass anoikis by up-regulating the survival proteins FAK, Src, Akt, Ras, and ERK (58–60). Thus, the ability of E4orf4 to uncouple Src-dependent survival signaling from Src-dependent tension signaling in tumor cells by inhibiting FAK and ERK while up-regulating RhoA-ROCK-JNK is likely relevant to its antitumor activity (13, 14, 17).

On the other hand, it seems that the activation of specific “mechanosensory” pathways is necessary for induction of anoikis in addition to disabling of survival pathways (61, 62). In that regard, the JNK pathway was involved in anoikis and in stretch-induced apoptosis (63, 64). Strong evidence is provided here that the JNK pathway also contributes to the Src-regulated death activity of E4orf4, supporting its role as a key mechanoresponsive pathway regulating cell tension and cell behavior. Nevertheless, the death process induced by E4orf4 is clearly distinct, as it does not depend on known apoptotic targets of JNK and rather relies on a cytoskeletal function of JNK; that is, the regulation of paxillin dynamics. Furthermore, although anoikis is an apoptotic death process induced after loss of cell adhesion, E4orf4 killing is caspase-independent and seems to involve a seemingly opposite mechanism defined by increased cell traction forces (increased cell adhesion to the outside) (9, 57). It could be argued that both processes led to a deregulation of the balance of forces between cell adhesion on the outside and myosin II-based contractility in the inside of the cell, which could commit cells to both apoptotic and nonapoptotic death pathways depending on the cellular context. In any case, because E4orf4 killing appears to rely on a particular context shared by transformed and tumor cells, it is tempting to speculate that part of E4orf4 antitumor activity could result from its ability to "reverse" the "altered tensional homeostasis" necessary to support the malignant phenotype by increasing tumor cell traction forces. It is possible that by up-regulating the Src-dependent JNK-paxillin signaling axis, E4orf4 could disable a key function of Src in mechanotransduction contributing to the

FIGURE 7. Working model. In light of the data, we propose that E4orf4-Src signaling perturbs tensional homeostasis in tumor cells in part by promoting the sustained activation of a Rho-ROCK pathway controlling both internal cell tension and cell traction forces (cell adhesion on the outside) through the concerted action of myosin 2 and JNK/Ser-178-paxillin phosphorylation. JNK activation by E4orf4 also involves the contribution of another unknown pathway (gray dotted line). Such chronic activation of the Rho-ROCK-JNK pathway would promote the development of atypically enlarged FAs and of robust tensile fibers, leading to a lethal increase in cytoskeletal tension. Deregulation of the balance between cell tension and extracellular matrix rigidity (ECM stiffness) that normally maintains tensional homeostasis would contribute to engage the nonapoptotic cell death pathway induced by E4orf4 through unknown mechanisms. MLC, myosin light chain; MYPT, target subunit of the myosin light chain phosphatase.

mote FA growth and maturation via the JNK-paxillin signaling axis (Fig. 7). However, despite that the Rho/ROCK-paxillin signaling axis is clearly required for efficient cell killing by E4orf4, it might not be sufficient by itself. This is supported by previous work showing a requirement for other pathways involving RhoGTPases and protein phosphatase 2A (14, 51, 52). The present data suggest that JNK activation in response to E4orf4 also involves the contribution of another unknown pathway, distinct from the RhoGTPase pathways activated by E4orf4. Our previous work indicated that JNK activation does not require E4orf4 binding to protein phosphatase 2A (17). Thus, it seems that the lethal cytoskeletal changes induced by E4orf4 rely on a complex interplay of several signaling pathways hijacked by E4orf4 whose functional relationships to each other remain to be determined. Nonetheless, the present findings clearly indicate that E4orf4 killing relies on its ability to deregulate cell tension in cancer cells.

Cell tension is known to influence basic cellular functions, including cell proliferation, differentiation and apoptosis, but...
changes in tensional homeostasis promoting and supporting the malignant phenotype (24). In doing so, E4orf4 could trigger a “differentiation process” characterized by “supermature focal adhesions” and stress fiber formation that would change the epigenetic program of the cell (65). The connection with and the nature of the nonapoptotic death machinery being engaged by deregulated cell tension are currently under study.

Another significant finding highlighted by our study concerns the influence of mechanical force on paxillin dynamics. This corroborates recent work suggesting that paxillin phosphorylation acts as a switch in adhesion assembly/disassembly in response to changes in actomyosin contractility and mechanical cues. It was proposed that mechanical force-induced paxillin targeting to FA involves the dephosphorylation of paxillin tyrosine residues, which leads to dissociation of FAK from the complex, promoting stabilization and growth of the adhesion (66, 67). Based on existing evidence, the disengagement of FAK from FA is expected to inhibit turnover and increase adhesion stability (68). We have observed previously that the Src-mediated tyrosine phosphorylation of paxillin and FAK is reduced in E4orf4-expressing cells, consistent with the marked decrease of adhesion turnover described here (13). Still, the E4orf4-dependent increase in adhesion stability and overgrowth was found to depend on S178-paxillin phosphorylation and was associated with the recruitment and stabilization of paxillin at FAs. This strongly suggests that force-induced paxillin targeting to FA involves Ser-178 phosphorylation in addition to dephosphorylation of tyrosine residues. It is not clear how exactly the phosphorylation at Ser-178 affects paxillin dynamics, but it is conceivable that Ser-178 phosphorylation induces a conformational change affecting paxillin interactions with FA components. That E4orf4 increases the time-residency of paxillin at FA in a way that relies on Ser-178 phosphorylation supports this model. Although beyond the scope of this study, it is tempting to speculate that vinculin, which also displayed a marked increase in time-residency at FA, could contribute to stabilize paxillin at FA and vice versa by inhibiting paxillin-FAK interaction. Indeed, vinculin was reported to negatively regulate adhesion dynamics, cell motility, and survival, and its ability to control the accessibility of paxillin for FAK interaction seems to play an important role (69–71). Regardless of the mechanism involved, strong evidence suggests that JNK-mediated Ser-178-paxillin phosphorylation regulates its binding to FA components and promotes stabilization of adhesion sites in response to an increase in actomyosin contractility. This model is consistent with recent findings in Drosofila, suggesting that JNK hyperactivation increases adhesiveness and might eventually block cell migration (72, 73). It is currently unclear why JNK1 was selectively involved in the context of MCF7 cells, as both isoforms were activated in response to E4orf4. The differential contribution of JNK1 and JNK2 needs to be addressed in other cancer cell lines before we can draw conclusions regarding a potential specificity of action for JNK isoforms within this pathway.

In conclusion, we propose that JNK-mediated Ser-178-paxillin phosphorylation is part of the early events in force detection downstream of Src tyrosine kinases requiring the contribution of Rho/ROCK, which can alter tensional homeostasis and affect tumor cell behavior. Because tension-induced cell death by E4orf4 is a caspase-independent death process that resists overexpression of Bcl-2, increased understanding of the molecular mechanisms engaging the death machinery may lead to identification of an entirely new class of molecular targets for anticancer therapy.

Acknowledgments—We are grateful to K. Burridge, K. Jacobson (University of North Carolina at Chapel Hill, Chapel Hill, NC), and N. Marceau (Laval University, Quebec, Canada), for providing critical molecular tools and reagents. We thank Dr. D. Allard for expertise regarding AP-1-dependent transcription reporter assays as well as A. Lorranger and C. St.-Pierre for dedicated support and assistance in microscopic analyses (Centre de Recherche en Cancérologie de l’Université Laval).

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