Adenovirus E4orf4 Hijacks Rho GTPase-dependent Actin Dynamics to Kill Cells: A Role for Endosome-associated Actin Assembly[□] [▽]

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The adenovirus early region 4 ORF4 protein (E4orf4) triggers a novel death program that bypasses classical apoptotic pathways in human cancer cells. Deregulation of the cell cytoskeleton is a hallmark of E4orf4 killing that relies on Src family kinases and E4orf4 phosphorylation. However, the cytoskeletal targets of E4orf4 and their role in the death process are unknown. Here, we show that E4orf4 translocates to cytoplasmic sites and triggers the assembly of a peculiar juxtanuclear actin-myosin network that drives polarized blebbing and nuclear shrinkage. We found that E4orf4 activates the myosin II motor and triggers de novo actin polymerization in the perinuclear region, promoting endosomes recruitment to the sites of actin assembly. E4orf4-induced actin dynamics requires interaction with Src family kinases and involves a spatial regulation of the Rho GTPases pathways Cdc42/N-Wasp, RhoA/Rho kinase, and Rac1, which make distinct contributions. Remarkably, activation of the Rho GTPases is required for induction of apoptotic-like cell death. Furthermore, inhibition of actin dynamics per se dramatically impairs E4orf4 killing. This work provides strong support for a causal role for endosome-associated actin dynamics in E4orf4 killing and in the regulation of cancer cell fate.

INTRODUCTION

The study of viral proteins and the cellular pathways they perturb is a powerful strategy for identifying critical functions that regulate cell growth and survival. The human adenovirus type 2 early region 4 ORF4 (Ad2 E4orf4) is a multifunctional early viral gene product that is not essential for adenovirus infection, but it seems to play several functions to facilitate viral replication (Branton and Roopchand, 2001; Ben-Israel and Kleinberger, 2002; O'Shea *et al.*, 2005).

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Abbreviations used: Ad2 E4orf4, adenovirus type 2 early region 4 open reading frame 4; SFK, Src family kinase; ROCK, Rho-kinase; PP2A, protein phosphatase 2A; MLC, myosin-II light chain; pCRIB, Cdc42/Rac interactive binding domain of Pak; wCRIB, N-Wasp CRIB; rRBD, Rho binding domain of ROCK; rhotRBD, RBD of Rhotekin; ScarVCA, Verprolin homology, Central and Acidic domains of Scar1; MYPT, target subunit of MLCP; cytoD, cytochalasin D; EV, empty vector; mRFP, monomeric red fluorescent protein; GalT, β -1,4-galactosyl transferase; BFA, brefeldin A; Tf, transferrin; TfR, transferrin receptor; GPI, glycosylphosphatidylinositol; RE, recycling endocytic compartment.

However, when expressed alone in transformed and tumor cells, E4orf4 induces a p53-independent cell death program that shares several apoptotic hallmarks, including phagocytosis (Lavoie *et al.*, 1998; Shtrichman and Kleinberger, 1998; Champagne *et al.*, 2004). Nonetheless, the mechanisms involved clearly differ from those associated with apoptosis. E4orf4 killing proceeds in absence of classical caspase activation in most cancer cell types and resists to acute overexpression of Bcl-2 and caspase inhibitors (Lavoie *et al.*, 1998; Livne *et al.*, 2001; Robert *et al.*, 2002). Furthermore, mitochondria depolarization is a late event and a consequence rather than a cause of cell death. Thus, E4orf4 activates a novel death pathway, which may involve calcium signaling and calpains (Robert *et al.*, 2002). Evidence also suggests that E4orf4 killing is tumor selective, raising a great interest for the mechanisms involved (Kleinberger, 2004).

Two major cellular targets are involved in the regulation of E4orf4 killing: the protein phosphatase 2A (PP2A) and the Src family of nonreceptor tyrosine kinases (Shtrichman *et al.*, 1999; Lavoie *et al.*, 2000; Marcellus *et al.*, 2000). The Src-regulated death activity of E4orf4 that we call the cytoplasmic death pathway, requires a physical interaction with Src family kinases (SFK) and subsequent E4orf4 phosphorylation on tyrosine residues, which promotes its cytoplasmic accumulation (Gingras *et al.*, 2002; Champagne *et al.*, 2004). In most tumor cell lines, Ad2 E4orf4 first accumulates in the cell nucleus in a way that relies on a multifunctional Arg-rich motif, which also mediates E4orf4 binding to SFK (Champagne *et al.*, 2004; Miron *et al.*, 2004). Induction of the

Figure 1. The cytoplasmic translocation of E4orf4 is associated with dynamic changes in actin. (A and B) MCF7 cells were transfected with E4orf4-mRFP alone (A) or with GFP-Actin (B). Twenty-four hours after transfection ($t = 0$), cells were treated with $75 \mu\text{M}$ cycloheximide and were observed by time-lapse microscopy for a 3-h period at 10-min intervals. (A) Arrowheads indicate the cytoplasmic translocation of E4orf4. (B) The microscope was refocused

cytoplasmic death pathway correlates with E4orf4 accumulation to the cytoplasm and membranes; furthermore, the cytoplasmic and membrane localization of E4orf4 is both necessary and sufficient for triggering the Src-regulated death pathway (Robert *et al.*, 2002). It was suggested that SFK binding to the Arg-rich motif inhibits E4orf4 nuclear transport and promotes interaction with some cytoskeletal components, through the recruitment of specific targets of SFK, including cortactin and p62Dok (Gingras *et al.*, 2002; Champagne *et al.*, 2004). Indeed, cell death induction is typified by early changes in actin that precede the onset of nuclear condensation and drive the induction of dynamic cell blebbing. Actin disruption with cytochalasin D (cytoD) not only blocks cell blebbing but also interferes with nuclear condensation, suggesting a key role in death signaling (Lavoie *et al.*, 2000). Thus, the current evidence indicates that E4orf4 acts as a modifier of SFK-dependent phosphorylation to hijack some cytoskeletal functions, the nature of which is unknown.

The present study seeks to identify the cytoskeletal targets of E4orf4 and their relationships with cell death induction in transformed and cancer cells. Strong evidence is provided that a cytoplasmic pool of E4orf4 orchestrates the activation of distinct Rho GTPases pathways to promote endosome-associated actin dynamics and assembly of a juxtanuclear actomyosin network. It is the ensuing imbalance in perinuclear actin-myosin dynamics that seems to engage the death machinery in cancer cells.

MATERIALS AND METHODS

Expression Vectors

The following expression vectors were described previously: Ad2 E4orf4, Flag-E4orf4, Flag-E4orf4 (6R-A), (R81/F84) and (F84A) (Champagne *et al.*, 2004); E4orf4-GFP (Robert *et al.*, 2002); GFP-N-Wasp (Moreau *et al.*, 2000); GFP-actin (Choidas *et al.*, 1998); myc-Rac1, myc-Cdc42, and myc-RhoA (Lamarche *et al.*, 1996); GFP-rhotRBD (Avalos *et al.*, 2004); Verprolin homology, Central and Acidic domains of Scar1 (myc-ScarVCA) (Machesky and Insall, 1998); ROCK2 CAT KD (Amano *et al.*, 1997); MLC (AA) (Iwasaki *et al.*, 2001); Vav2 (Marignani and Carpenter, 2001); GFP-Arp3 (Welch *et al.*, 1997); GST-pCRIB (Sander *et al.*, 1998); histone H2A-GFP (Perche *et al.*, 2000); and GFP-glycosylphosphatidylinositol (GPI) (Sabharanjak *et al.*, 2002). Monomeric red fluorescent protein (mRFP) was a kind gift from Dr. R. Y. Tsien (University of California, La Jolla, CA). E4orf4-mRFP was produced by PCR amplification using the primers 5'-CAG CTC GAG GCT AGC GCC TCC TCC GAG GAC GTC-3' and 5'-TTC GAA TTC TTA GGC GCC GG-3' and pRSETB-mRFP as template. The DNA fragment was subcloned into the XhoI/EcoRI sites of Flag-E4orf4-GFP (Robert *et al.*, 2002). YFP-pCRIB was created by subcloning amino acids 65–150 of human Pak1 into the EcoRI/BamHI sites of pEYFP-C1 (BD Biosciences, San Jose, CA). A single nucleotide was added after the EcoRI site to keep the construct in-frame with yellow fluorescent protein (YFP) using the following primers: 5'-CT AGG AAT TCC AAT AAA AAG AAA GAG AAA GAG CGG-3' and 5'-TCG AAA TGT CTA TTC AGT CGA CCT AGG CTA G-3'. The β -1,4-galactosyl transferase-green fluorescent protein (GalT-GFP) was produced by subcloning the BamHI/NotI fragment of GFP from pEGFP-N1 (Clontech, Mountain View, CA) into the BamHI/NotI sites of GalT-CFP (amino acids 1–60 of Galactosyltransferase; Nichols *et al.*, 2001). The RFP-Rab11 construct was produced by subcloning the PinA1/BglII frag-

to allow changes in actin at the ventral face and in the middle-top part of the cell to be followed simultaneously. Key frames from the two focal planes recorded at the indicated time after 24 h of E4orf4 expression are shown. Red dashed lines delineate the nucleus revealed by labeling with cell-permeable Hoechst, which condenses as the cell retracts and enters the blebbing phase (2 h). Arrowheads designate the juxtanuclear network of GFP-actin-coated vesicles enriched in E4orf4-mRFP and arrows show stress fibers at the cell ventral face. (C) Confocal images of 293T transfected with the vector only (EV) or with Flag-E4orf4 after immunostaining of E4orf4 (anti-2420) and F-actin (phalloidin). Arrows, perinuclear actin ring. N, nucleus. Bars, $10 \mu\text{m}$.

Figure 2. E4orf4 induces myosin II activation in juxtanuclear regions and polarized blebbing. (A) Confocal images of mono-p-MLC staining (p-MLC [Ser19]) in 293T transfected with the vector-only (EV) counterstained with fluorescent phalloidin (F-actin), compared with cells transfected with E4orf4-mRFP in the early stages of E4orf4 expression (early) and in the late blebbing stage (late). Arrowheads indicate p-MLC accumulation in juxtanuclear regions. The white dashed line delineates the cell nucleus. (B) 293T cells were transfected with the vector only (EV) or with mutant E4orf4 constructs displaying alanine substitutions on the indicated residues: E4orf4, wild type; 6R-A, mutant defective in Src binding; R81/F84A and F84A, mutants defective in PP2A binding. Equal amounts of total cell extracts were analyzed by Western blot using anti-p-MLC (Ser19) and anti-p-MLC (Thr18/Ser19). The levels of E4orf4 proteins were revealed using anti-Flag (M2) and β -actin levels are shown as loading controls. Right, 293T were treated with the Src family kinase inhibitor SU6656 (5 μ M) or with the vehicle (dimethyl sulfoxide [DMSO]) during and after transfection and p-MLC was analyzed by Western blot 24 h after transfection. (C) Confocal images of F-actin (phalloidin) and myosin heavy chain IIb (anti-MHC IIb) in MCF7 transfected with the vector, compared with cells expressing E4orf4-mRFP, in cells treated with DMSO versus 50 μ M blebbistatin for 1 h before cell fixation. Arrowhead shows the colocalization of F-actin and MHC IIb in E4orf4 cell, which are organized into a juxtanuclear contractile ring from which prominent stress fibers radiate to the cell periphery. The white dashed lines delineate the cell nucleus (N). Bars, 10 μ m. Graph shows the ratios of the average intensity of E4orf4-mRFP in the nucleus over that in the cytoplasm in E4orf4 cells treated with DMSO versus blebbistatin (***) $p < 0.0001$. Data are the means $\pm$ SD of at least 30 cells (n). (D) Crude cell fractionation of control (EV) versus cells transfected with Flag-E4orf4. Equal amounts of cellular fractions were analyzed by Western blot using anti-p-MLC (Ser19), anti-histone H3 (nuclei), anti-GM130 (Golgi membranes), anti-TOM20 (mitochondria) and anti-Flag (E4orf4). P1, nuclei and tightly associated membranes; P2, heavy membranes; P3, light membranes; C, cytosol. CytoD, cells were treated with cytoD for 30 min before cell fractionation. W/O, without cytoD treatment. (E) Phase contrast micrographs from time-lapse analyses of the blebbing dynamics of E4orf4-expressing 293T cells, compared with 293T treated with nocodazole (100 ng/ml; 16 h). Arrows show the direction of the protruding/retracting blebs and the white dashed lines indicate the polarized axes of blebbing. See Supplemental Video 1.

ment of pmRFP-C1 into the PinA1/BglIII sites of GFP-Rab11 (Hunyady *et al.*, 2002). The GFP-RhoA construct was produced by subcloning the BamHI/EcoRI fragment of pRK5-myc-RhoA (Lamarche *et al.*, 1996) into the BglIII/EcoRI sites of pEGFP-C1 vector (Clontech).

Antibodies and Chemicals

The following antibodies and drugs were used: anti-myc (9E10) and anti-hemagglutinin (HA) (Ha.11; BAbCO, Richmond, CA); anti-Flag (M2), anti-

Rac1-2-3 (23A8), and anti- β -actin (AC-74) (Sigma-Aldrich, St. Louis, MO); anti-pMYPT1 (Thr696), anti-Cdc42(P1), and anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA); anti-p-myosin light chain (MLC) (Ser19) and anti-p-MLC (Thr18/Ser19) (Cell Signaling Technology, Beverly, CA); anti-RhoA, anti-MYPT1, anti-calreticulin, and anti-GM130 (BD Biosciences Transduction Laboratories, Lexington, KY); anti-TOM 20 (a gift from Gordon Shore, McGill University, Montreal, Canada); anti-Histone H3 (a gift from A. Ruiz-Carillo, Laval University); anti-E4orf4 (2419) and (2420) (Lavoie *et al.*, 2000); anti-Ki-67

Figure 3. E4orf4 induces de novo actin polymerization in the perinuclear region. MCF7 or 293T cells were transfected as indicated and fixed after a 5-min incubation with fluorescent G-actin in saponin-permeabilization buffer. Where indicated, 5 μ M cytoD was added during the in situ polymerization assay. (A) Epifluorescence images of MCF7 transfected with the vector (EV) or E4orf4-mRFP and fixed after G-actin incorporation. The nuclei were labeled with Hoechst, and the remaining E4orf4-mRFP staining in the nucleus and the cytoplasm (insoluble) is shown in red. Dashed circle lines show examples of the juxtannuclear regions used for the quantitative analyses presented in B. (B) Average intensity of pixels was measured over the entire cell or in the juxtannuclear regions from the original confocal images using the MetaMorph software version 4.5 (** $p < 0.0001$, ** $p < 0.001$, and * $p < 0.01$). Data are the means $\pm$ SD of 15–58 cells (n) from at least three independent experiments. E4orf4(6R-A), mutant defective in Src binding; R81/F84A, mutant defective in PP2A binding. (C) Confocal images of 293T transfected with EV (top) or E4orf4-GFP (bottom) and fixed after a 5-min G-actin incorporation. F-actin staining was performed after cell fixation using fluorescent-labeled phalloidin to compare the preexisting actin network with the newly formed dynamics microfilaments that incorporate G-actin. Arrows and arrowheads indicate dynamic sites of actin nucleation at the juxtannuclear ring displaying insoluble E4orf4 protein. N, nucleus. Bars, 10 μ m.

(MIB-1; Dako Denmark A/S, Glostrup, Denmark); nocodazole, brefeldin A (Sigma-Aldrich); and SU6656, cytoD, BocD-fmk, blebbistatin, wiskostatin, NSC23766, and Y-27632 (Calbiochem, San Diego, CA).

Cell Culture and Transfection

293T, MCF7, and HeLa cells were derived from human embryonic kidney cells (Graham *et al.*, 1977), from human mammary adenocarcinoma (Soule *et al.*, 1973), and from human cervix adenocarcinoma (Jones *et al.*, 1971), respectively. 293T cells were maintained in DMEM, whereas MCF7 and HeLa cells were maintained in α -minimal essential medium (MEM), and both culture media were supplemented with 10% fetal bovine serum. Transfection of 293T cells was performed as described previously (Lavoie *et al.*, 2000). MCF7 and HeLa cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendations.

Pull-Down Assays, Cell Fractionation, Immunoprecipitation, and Western Blot

All biochemical assays were performed 24 h posttransfection. Pull-down assays were done as described in Ren *et al.* (1999) with minor modifications. Briefly, cells were transfected with Flag-E4orf4 alone or with minimal amounts of myc-Rac1/Cdc42. Cells were harvested, rinsed in phosphate-buffered saline containing 0.5 mM Na_3VO_4 , and lysed in ice-cold radioimmu-

noprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 10 mM MgCl_2 , 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 0.5 mM Na_3VO_4 , 15 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Cell lysates were incubated with glutathione S-transferase (GST)-pCRIB beads at 4°C for 45 min. Beads were washed three times with ice-cold washing buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 10 mM MgCl_2 , 1% Triton X-100, 15 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin A, and 0.1 mM PMSF), resuspended in SDS sample buffer, boiled, and processed for SDS-PAGE and Western blot. Densitometric analyses were performed from FluorS MAX Multimager-captured images using Quantity One software (Bio-Rad, Hercules, CA). For fractionation analyses, cells were resuspended in sucrose buffer at 50×10^6 cells/ml (20 mM HEPES, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 15 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 1 mM PMSF, and 1 mM Na_3VO_4), swelled on ice for 1 h, and disrupted by forcing cells through a 27-gauge needle 40–50 times. Cellular fractions were obtained by serial centrifugation (P1, 700 $\times$ g; P2, 8000 $\times$ g; and P3, 170,000 $\times$ g), and protein concentrations were determined using the Bio-Rad DC Protein Assay. Equal amounts of proteins from each fraction were analyzed by Western blot. Immunoprecipitations were performed as described previously (Champagne *et al.*, 2004), after cell lysis in modified RIPA buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl_2 , 1 mM EGTA, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.5% SDS, 10% glycerol,

Figure 4. E4orf4-induced actin nucleation is not associated with Golgi membranes. (A) MCF7 or HeLa cells transfected with the Golgi marker GalT-GFP alone (EV) or with E4orf4-mRFP were fixed after a 5-min incubation with Alexa-647-labeled G-actin in saponin-permeabilization buffer and analyzed by confocal microscopy. Representative images of HeLa cells show the juxtanuclear position of the Golgi complex (EV) and the absence of overlap between Golgi membranes (GalT-GFP) and the dynamic juxtanuclear actin ring in E4orf4 expressing cell. (B) Transfected HeLa cells were incubated with DMSO or 5 μ M BFA to disrupt the Golgi complex, either before E4orf4 expression for a 4-h period (chronic treatment) or after the onset of actin assembly for a 30-min period (acute treatment), followed by in situ G-actin incorporation. Confocal images and quantification of de novo actin polymerization show that BFA treatment effectively disrupts the Golgi complex, without interfering with the formation or maintenance of the actin ring. Juxtanuclear actin nucleation was quantified by measuring the average intensity of pixels from the original cross-section images using the MetaMorph software version 4.5. Data are the means $\pm$ SD of at least eight individual cells (n). ***p < 0.0001. Dashed lines delineate the nucleus (N). Bars, 10 μ m.

50 mM NaF, 10 mM β -glycerophosphate, 1 mM Na_2VO_4 , 15 μ M leupeptin, 5 μ M aprotinin, and 1 μ M pepstatin A). Western blot analyses were performed as described previously (Lavoie *et al.*, 2000).

Immunofluorescence, Transferrin Labeling, and In Situ Actin Polymerization Assay

Twenty-four hours after transfection, 293T and MCF7 cells were fixed with 3.7% formaldehyde, and immunostaining was performed using 4,6-diamidino-2-phenylindole (DAPI), Hoechst, Alexa-488-, Alexa-594-, Texas Red-, and Alexa-647-labeled goat-anti-rabbit, goat-anti-mouse, and phalloidin (Invitrogen) as described previously (Lavoie *et al.*, 2000). Incorporation of fluorescent G-actin in situ was performed as described previously (Symons and Mitchison, 1991). Briefly, cells were incubated with 0.4 μ M Alexa-594-conjugated G-actin together with 1 mM ATP in permeabilization buffer (0.2 mg/ml saponin, 20 mM HEPES, pH 7.4, 138 mM KCl, 4 mM MgCl_2 , and 3 mM EGTA) for 5 min at room temperature before cell fixation. CytoD (5 μ M) was added to cap G-actin and prevent incorporation. Where indicated, G-actin incorporation was preceded by steady-state labeling of transferrin to visualize the endocytic compartments. Cells were serum starved for 1 h in α -MEM containing 0.1% bovine serum albumin and 10 mM HEPES, pH 7.4, and incubated with 5 μ M Alexa-647-conjugated transferrin (Invitrogen) for 45 min in complete culture media. Where indicated, 50 μ M wiskostatin, 100 μ M NSC23766, or 10 μ M Y-27632 were added during transferrin and G-actin incorporation.

Small Interfering RNA (siRNA) to Rho GTPases, DNA Condensation, and Cell Killing Assays

The following sense and antisense oligonucleotides corresponding to the cDNA sequences of *Homo sapiens* Cdc42, Rac1, RhoA, and GFP were purchased from QIAGEN (Valencia, CA): 5'-GUG UCG GCA UCA UAA AdTdT-3' (Cdc42#1) and 5'-CAG CAA UGC AGA CAA UUA AdTdT-3' (Cdc42#4); 5'-GGU UGG UAU UAU CAG GAA AdTdT-3' (Rac1#1) and 5'-GAC AUA ACA UUG UAC UGU AdTdT-3' (Rac1#3); and 5'-CCC AGA UAC CGA UGU UAU AdTdT-3' (RhoA#1). A siRNA to GFP labeled in 3' with rhodamine 5-carboxytetramethylrhodamine was used as negative control (GCA AGC UGA CCC UGA AGU UCA U (Caplen *et al.*, 2001)). MCF7 cells were grown to 60–80% confluence and transfected with individual siRNAs, or a mix of two different siRNAs to a given Rho GTPase for 24 h using the TransIT-TKO reagent (Mirus Bio Corporation, Madison, WI), according to the manufacturer's recommendations. 293T cells were grown to 25% confluence and transfected with siRNAs by the calcium-phosphate method for 8 h as described previously (Lavoie *et al.*, 2000). MCF7 cells were split 24 h after

transfection and seeded for subsequent transfection with E4orf4 plasmids cDNA. Seventy-two hours after transfection with siRNAs, cells were processed for in situ actin polymerization assay, immunofluorescence, or Western blot. DNA condensation was assessed 24 h after transfection of the indicated plasmids cDNAs by DNA labeling with DAPI, and colony-forming assays were performed as described previously (Gingras *et al.*, 2002).

Microscopy and Image Processing

Live cell imaging of GFP-actin and E4orf4-RFP and epifluorescence analyses were performed using a Nikon TE-2000 inverted microscope equipped with a humidified 5% CO_2 /thermoregulated chamber and a 40 $\times$ 0.6 numerical aperture (NA), or 60 $\times$ oil 0.6–1.25 NA objectives. Time-lapse acquisitions used shuttered illumination and neutral density filters to protect cells from light damage. Images were captured as 16 bit TIFF files with a CoolSNAP FX cooled charge-coupled device camera (-30°C ; Photometrix, Melbourne, Australia) driven by the MetaMorph software version 6.2.2 (Molecular Devices, Sunnyvale, CA). Binning was set to 2 by 2 for lowering exposure times to limit photobleaching and damage. Selected images were subjected to two-dimensional deconvolution using AutoDeblur version 9.3 (AutoQuant Imaging, Silver Spring, MD) to remove the "glow" from far-from-focused regions of the cells and were converted to 8 bit TIFF images with the MetaMorph software version 4.5. Confocal microscopy was performed using a Bio-Rad MRC 1024 mounted on Nikon TE-200 inverted microscope with a 60 $\times$ oil 1.4 NA objective. Fluorescence live cell imaging of vesicle-associated actin comets tails was performed using a Bio-Rad Radiance 2001 multiphoton system mounted on a Nikon TE-300 inverted microscope equipped with a thermoregulated chamber and a 60 $\times$ oil 1.4 NA objectives. Dual channel fluorescence live cell imaging of GFP-actin and RFP-Rab11 was performed using a Quorum WAVE FX spinning disk confocal mounted on Leica DMIRE2 inverted microscope equipped with an environmental chamber and a 63 $\times$ oil 1.4 NA objective. MetaMorph software version 4.5 and Photoshop software version 7.0 (Adobe Systems, Mountain View, CA) 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, brightness/contrast adjustments, pseudocolors, and images merged. Colocalization images are from confocal analyses. Acquisitions were taken on separate channels with appropriate restrictive filters. For quantitative fluorescence analyses including G-actin incorporation, analyses of p-MLC, and quantification of cytoplasmic/nuclear E4orf4, confocal images were acquired using the same parameters (gain and laser power), exported in TIFF format, and analyzed with the MetaMorph software version 4.5 using the Measure Average Intensity function, calculated from a selected area corresponding to the entire cell or to the

Figure 5. E4orf4 promotes the nucleation of dynamic actin particles associated with endosomes motility. Confocal images of MCF7 transfected with the vector (EV) or E4orf4-mRFP and fixed after incorporation of Alexa-647-labeled transferrin for 45 min, followed by a 5-min incorporation of Alexa-488-labeled G-actin. Arrows indicate the region in insets showing high magnifications of the asymmetric association of endosomal vesicles and the tubular-vesicular actin structures in juxtanuclear regions. Note that endosomes were massively recruited to the dynamic actin ring. (B) Confocal images of MCF7 transfected with E4orf4-mRFP and GFP-GPI, stained with Alexa-647-labeled phalloidin (F-actin) after cell fixation. High magnification of the juxtanuclear region show the clear overlapping patterns of the

juxtannuclear region of a single cell. Statistical analyses were performed using the StatView software version 4.51 (Abacus Concepts, Berkeley, CA).

RESULTS

Dynamic Actin Changes Mediated by E4orf4

Actin changes, which require E4orf4 accumulation to the cytoplasm and membranes, are typical hallmarks of E4orf4 proposed to transduce the death signal (Lavoie *et al.*, 2000; Robert *et al.*, 2002). However the dynamics of E4orf4 localization, the exact actin changes, and their relationships with cell death induction are largely unknown. To address these issues, we used 293T and MCF7 cells as main model systems. 293T cells are transformed with adenovirus proteins and provide a system to study dominant functions of E4orf4 by mimicking the adenovirus context. In the more adherent human breast tumor MCF7 cells, changes in actin dynamics occur several hours before the onset of nuclear condensation, providing a larger time frame to assess functional relationships. In both cell lines, E4orf4-induced apoptotic-like cell death bypasses the classical caspase pathways (Robert *et al.*, 2002).

It has been shown that E4orf4 displays a preferential nuclear localization (Miron *et al.*, 2004). To determine whether the cytoplasmic accumulation of E4orf4 is a regulated process, or whether it is simply a consequence of high expression levels, the dynamic changes in E4orf4 localization were monitored by fluorescence live cell imaging of E4orf4-mRFP or E4orf4-GFP that behave like the wild-type E4orf4 (Robert *et al.*, 2002; Champagne *et al.*, 2004). Cycloheximide was added to the cell culture 30 min before cell imaging to inhibit the novo synthesis of E4orf4 and allow the changes in localization to be followed. Twenty-four hours after transfection, E4orf4-expressing cells display various phenotypes ranging from nuclear- to cytoplasmic-enriched E4orf4, as the onset of E4orf4 expression is asynchronous and the levels vary within individual cells. However, as shown previously, cells in the early stage of E4orf4 expression display a clear nuclear accumulation and these were recorded for a 3-h period (Figure 1A, $t = 0$). We observed that E4orf4 progressively translocated to the cytoplasm and stably accumulated to perinuclear vesicles and cortical sites forming membrane protrusions (Figure 1A, arrowheads). This supports the notion that the cytoplasmic distribution of E4orf4 is regulated and relevant to its primary function.

We next examined the progression of actin changes by recording cells coexpressing GFP-actin. We observed that E4orf4 accumulated to tubular-vesicular structures enriched in GFP-

actin in the juxtannuclear region (Figure 1B, arrowheads) and to a lesser extent to actin fibers at the ventral face (Figure 1B, arrows). The juxtannuclear actin structures seemed connected to the actin fibers, forming a basket-like actin network anchored at the nuclear periphery. Later, this peculiar actin network collapsed to form a perinuclear actin ring and cells entered the blebbing phase (late phase, 2–3 h). Coincidentally, the nucleus shrunk and chromatin condensed, as revealed by labeling with cell-permeable Hoechst (Figure 1B, dotted lines). A similar juxtannuclear actin network was detected in 293T cells expressing E4orf4, in the absence of coexpressed GFP-actin (Figure 1C). We conclude that the cytoplasmic translocation of E4orf4 mediates cytoskeletal changes, which involve some vesicular membrane compartment and clearly precede the onset of blebbing and nuclear condensation.

E4orf4 Activates Myosin II

Cell blebbing is widely caused by an increase in myosin II motor activity driven by phosphorylation of the myosin light chain (p-MLC), which promotes interaction of myosin II with actin and contractility (Tan *et al.*, 1992; Bresnick, 1999; Mills *et al.*, 1999; Coleman and Olson, 2002). We used phospho-specific anti-MLC antibodies to assess the status of myosin II activation in E4orf4-expressing cells. Confocal microscopy and Western blot analyses revealed a marked increase in p-MLC, which mainly accumulated in juxtannuclear regions (Figure 2A, arrowheads, and B). Activation of myosin II by E4orf4 relied on Src kinases, as the Src inhibitor SU6656 markedly impaired E4orf4-dependent p-MLC. Furthermore, a mutant unable to interact with Src kinases (E4orf4 [6R-A]) was completely defective for MLC activation (Figure 2B). In contrast, mutants defective for PP2A binding behaved like the wild type (E4orf4[R81/F84A]; [F84]), in agreement with their respective cytoplasmic death activity (Champagne *et al.*, 2004). The myosin heavy chain IIb (MHC IIb) was also recruited to the juxtannuclear region forming the heart of the actin network and colocalized with a subset of actin filaments organized into a peculiar actomyosin ring, which radiated prominent stress fibers to the cell periphery (Figure 2C, arrowhead). Remarkably, the myosin II inhibitor blebbistatin (Straight *et al.*, 2003) triggered the disassembly of the actin ring and the nuclear translocation of E4orf4. This was revealed by a twofold increase in the ratio of nuclear over cytoplasmic E4orf4 (Figure 2C). The results suggest that a subfraction of E4orf4 is associated with and regulates a pool of F-actin through the activation of a myosin II motor, which in turn promotes the stable accumulation of E4orf4 in the cytoplasm.

Crude biochemical cell fractionation further revealed that most of the p-MLC induced by E4orf4 was recovered in a P1 fraction containing nuclei and tightly associated membranes (Figure 2D, W/O). Depolymerization of F-actin before cell fractionation increased the proportion of p-MLC recovered in a P2 fraction containing endosomes, Golgi, endoplasmic reticulum membranes, and mitochondria (Figure 2D, CytoD). This suggests that the interaction of myosin II with a stable pool of organelle-based actin filaments reorganizes the perinuclear actin cytoskeleton. In sharp contrast, the basal level of p-MLC in control cells (EV) was recovered in the cytosolic fraction and presumably dissociated from peripheral actin fibers during cell fractionation. Transient and polarized distortions of the nucleus close to the sites of activated myosin II further indicated the presence of high motor forces in perinuclear regions (Figure 2A, dotted line). Moreover, time-lapse analyses revealed that E4orf4-dependent blebbing is clearly different from the typical membrane blebbing induced by various stresses affecting actin dynamics. E4orf4

Figure 5 (cont). GPI-positive endosomes with F-actin tails. (C) Multiphoton time-lapse imaging of GFP-actin in MCF7 cells transfected with E4orf4-mRFP and GFP-actin. The time series at 30-s intervals show the association of GFP-actin comet tails with large vesicles (arrowheads) that fused with one another. Note that some vesicles were totally covered with GFP-actin (arrow) and some others were associated with trailing actin tails (arrowhead). (D) Confocal time-lapse imaging of GFP-actin and RFP-Rab11 in cells expressing Flag-E4orf4, showing the recruitment of Rab11-positive endosomes to the forming actin network. High magnifications of the region delineated by the white box show dynamics vesicular structures decorated with RFP-Rab11 and associated with small tails of GFP-actin (arrow). These motile vesicles were recruited to the perinuclear actin fibers and displayed a rocket-like movement reminiscent of actin comets (see Supplemental Video 2). The red track in the last panel indicates the movement of the pointed vesicle over a 48-s period (arrow). Dual channel acquisitions were performed at an interval of 2 s/frame. Bars, 10 μ m.

Figure 6. Spatial activation of Rho proteins by E4orf4. (A) 293T cells were transfected with the indicated plasmids DNA, and pull-down assays were performed using GST-pCRIB 24 h after transfection. The bound material was analyzed by Western blot and levels of the GTPases and Flag-E4orf4 in total lysates are shown (TL). The relative levels of active GTPases were estimated by densitometric analyses and are the means $\pm$ SD of three independent experiments. (B and C) Confocal or multiphoton images of live cells transfected with the indicated plasmids DNA, together with YFP-pCRIB (B) or with GFP-wCRIB (C) showing representative recruitment patterns of the reporter probes for active Cdc42 and Rac. (D) Cells expressing the indicated constructs together with the reporter probes for active Rho proteins myc-rRBD were

triggered prominent and polarized protrusions/retractions of the cell cytoplasm that displaced the cell along the axis of the protruding bleb (Figure 2E, arrows, and Supplemental Video 1, left). In sharp contrast, microtubules disruption with nocodazole induced small asynchronous membrane protrusions/retractions at the cell surface (Figure 2E, bottom, and Supplemental Video 1, right). We conclude that the juxtanuclear increase in actin-myosin-based contraction drives the peculiar dynamics of E4orf4-induced blebbing.

E4orf4 Promotes De Novo Actin Polymerization Associated with Vesicle Motility

Large-scale cytoskeletal reorganizations often require local F-actin polymerization in addition to transport and reorientation of actin filaments by a myosin II motor. The drastic juxtanuclear actin changes prompted us to investigate whether, and if so where, E4orf4 triggers de novo actin polymerization. To detect actin polymerization in situ, short-term incorporation of fluorescent monomeric actin (G-actin) was performed during gentle cell permeabilization with saponin. This assay is suitable for visualizing dynamic sites of actin nucleation inside cells. This is revealed by the rapid incorporation of G-actin (within 5 min) at the tip of lamellipodia in cells overexpressing Rac(Q61L), which was prevented by cytoD (Supplemental Figure S1A). We found that E4orf4 induces a dramatic increase in G-actin incorporation at the juxtanuclear sites forming the actomyosin ring, where insoluble E4orf4 was also localized (Figure 3A, dotted circle, and C, arrows). Although the total level of incorporated G-actin increased by 1.7- to 2.6-fold in E4orf4-expressing 293T and MCF7 cells, respectively, the level of G-actin in the juxtanuclear region was enhanced by more than 4- to 10-fold relative to control cells and was prevented by cytoD (Figure 3B). Importantly, the increase in actin nucleation required E4orf4 interaction with Src kinases (E4orf4[6R-A]) but not PP2A binding (E4orf4[R81/F84A]) (Figure 3B).

We next investigated the relationships between the juxtanuclear actin structures and the endomembranes systems. Golgi membranes have dynamics actin filaments associated with them and could play a role in directing juxtanuclear actin assembly (Musch *et al.*, 2001; Fucini *et al.*, 2002; Luna *et al.*, 2002; Matas *et al.*, 2004; Dubois *et al.*, 2005). However, in situ actin polymerization in cells coexpressing the Golgi marker GalT-GFP failed to reveal overlapping distributions between the actin ring and Golgi membranes, which were rather localized next to each others (Figure 4A). Furthermore, disassembly of the Golgi complex by treating cells with brefeldin A either before the onset of E4orf4 expression (chronic), or after assembly of the actin ring (acute), had no effect on the increase in actin nucleation, or on the stability of the actin ring, respectively (Figure 4B, BFA). Thus, it seems that assembly of the juxtanuclear actin network does not rely on an intact Golgi structure.

Figure 6 (cont). analyzed by epifluorescence microscopy after cell fixation followed by myc-rRBD staining using anti-myc (9E10). Where appropriate, a pseudocolor intensity scale was used to highlight the relative amount of membrane-associated CRIB or RBD, over the total amount of CRIB or RBD in individual cells (from black to white: low-to-high intensity). Arrows and arrowheads designate membrane vesicles and cortical protrusions, respectively, in which E4orf4 recruits the CRIB and RBD probes, respectively. Activation of Rho proteins is impaired in cells expressing the mutant E4orf4 (6R-A) defective in Src binding, as revealed by the lack of CRIB and RBD recruitment, but not in cells expressing the mutant E4orf4 (R81/F84A) defective in PP2A binding. Bars, 10 μ m.

Endosomes also carry the machinery needed for actin polymerization (Schafer, 2002; Qualmann and Mellor, 2003). To visualize the endocytic compartments, endosomes were labeled at steady state with fluorescent-labeled transferrin (Tf) before incorporation of G-actin. Tf is internalized in coat pits in association with its receptor (TfR) and returns to the cell surface after transit in the juxtanuclear endocytic recycling compartment (RE), where other endosomes carrying nonclathrin-dependent cargo such as GPI-anchored proteins also transit (Figure 5A, EV arrows, top) (Hopkins, 1983; Yamashiro *et al.*, 1984; Sabharanjak *et al.*, 2002). We observed that the Tf-positive endosomes were massively recruited to the juxtanuclear actin ring in E4orf4-expressing cells, contrasting with their more diffuse pattern in control cells (Figure 5A, E4orf4). Higher magnifications reveal the proximity of the actin structures, which mainly show an asymmetric association with endosomal vesicles (Figure 5A, insets). A marker for tubular-vesicular endosomes carrying clathrin-independent cargo, GPI-GFP, similarly displayed a striking codistribution with the juxtanuclear F-actin tails (Figure 5B). These actin structures were reminiscent of the actin "comets tails" that have been detected in association with motile endogenous vesicles, including endosomes and lysosomes, and which are triggered by several pathogens to propel themselves within the host cell (Higley and Way, 1997; Fehrenbacher *et al.*, 2003). Indeed, fluorescence live cell imaging revealed that the actin particles induced by E4orf4 are dynamic. The comets were associated with large vesicles that fused with one another (Figure 5C, arrowheads) and translocated to the forming juxtanuclear actin network, propelled by a rocket-like movement (Figure 5D and Video 2). Notably, the vesicles were often decorated with RFP-Rab11, a marker of the juxtanuclear RE (Ullrich *et al.*, 1996). Together, the results raise the intriguing possibility that E4orf4 triggers the nucleation of F-actin on the surface of endosomes to recruit them to the site of actin assembly.

Activation of Rho GTPases by E4orf4

The Rho family members of small GTPases, of which RhoA, Cdc42, and Rac1 are the best studied, control the dynamics of the actin cytoskeleton and through this, cell morphology, motility, and vesicles traffic. Classically, Cdc42 and Rac control Arp2/3-directed actin nucleation, whereas Rho proteins control stress fiber formation (Etienne-Manneville and Hall, 2002). To determine whether E4orf4 acts through Rho GTPases, activation of Rho proteins was first assessed by the biochemical pull-down assays. Cdc42 and Rac1 GTP loading were determined by binding of the active GTPases to the Cdc42/Rac interactive binding (CRIB) domain of Pak1 fused to GST (GST-pCRIB), whereas RhoA activity was assessed by binding of active Rho to the Rho-binding domain of Rho kinase (ROCK; GST-RBD) (Bagrodia *et al.*, 1998; Kranenburg *et al.*, 1999). We found that E4orf4 induced a marginal increase (1.5- to 2-fold) in myc-Cdc42-GTP or endogenous Rac-GTP relative to control (EV) (Figure 6A), and no reproducible variation of RhoA-GTP was detected (our unpublished data).

Given the asynchronous onset of E4orf4 expression and actin dynamics, we used a more sensitive approach to visualize the spatial activation of Rho GTPases in cells. Moderate and noninterfering levels of reporter probes for active Rho GTPases were coexpressed with E4orf4. These include the RBD of Rho kinase, a Rho effector (Myc-rRBD) (Amano *et al.*, 1997), the CRIB domain of Pak1 fused to YFP, a Cdc42/Rac effector (YFP-pCRIB) and the CRIB domain of N-Wasp fused to GFP, and a Cdc42-specific effector (GFP-wCRIB) (Scaplehorn *et al.*, 2002). Their spatial distribution was mon-

Figure 7. Distinct contributions of Rho proteins to E4orf4-induced de novo actin polymerization. (A) Top, confocal images of N-Wasp-GFP in MCF7 cells coexpressing myc-Cdc42, after a 45-min treatment with 50 μ M wiskostatin (N-Wasp inhibitor) or DMSO. Middle and bottom, confocal images showing two focal planes of the same MCF7 cells transfected with E4orf4-mRFP. Cells were fixed after a 45-min incubation with or without wiskostatin, followed by a 5-min incorporation of fluorescent G-actin. Note that inhibition of N-Wasp blocks actin polymerization at the juxtanuclear actin ring (arrows) but not in stress fibers spanning the ventral face of the same cells. Graph, cells were treated for 45-min with DMSO, wiskostatin, 100 μ M NSC23766 (Rac1 inhibitor), or 10 μ M Y-27632 (ROCK inhibitor) before G-actin incorporation. G-actin incorporation was quantified in the juxtanuclear region versus the entire ventral face of the cells by measuring the

itored by fluorescence microscopy to visualize the membrane sites where the GTPases are activated (Kim *et al.*, 2000; Bement *et al.*, 2005; Benink and Bement, 2005). Although the pCRIB, wCRIB, and rRBD showed a diffuse distribution in control cells (EV), the reporter probes were specifically recruited to cytoskeletal-membrane structures, including lamellipodia (Figure 6B, pCRIB), perinuclear vesicles and filopodia (Figure 6C, wCRIB), and cortical protrusions (Figure 6D, rRBD), in cells expressing Rac (Q61L), Cdc42 (Q61L), and RhoA, respectively. This is consistent with their ability to report the local Rac, Cdc42, or RhoA activity, respectively.

In the presence of E4orf4, pCRIB and wCRIB were recruited to juxtanuclear vesicles reminiscent of the actin structures induced by E4orf4 (Figure 6, B and C, arrows). Strikingly, coexpression of modest levels of Cdc42 enhanced wCRIB recruitment on large perinuclear vesicles in a way that relied on E4orf4 (Figure 6C, GFP-wCRIB+Cdc42, arrows). Arp3-GFP, a member of the Arp2/3 complex that promotes actin nucleation downstream of Cdc42/N-Wasp (Stradal *et al.*, 2004), was also recruited in the juxtanuclear region (Supplemental Figure S1B). Such was also the case for the rRBD probe, which accumulated to juxtanuclear vesicles and fiber-like structures (Figure 6D, arrows). However, a major proportion of the rRBD was recruited to more peripheral sites forming small protrusions in E4orf4 cells, suggesting that Rho is independently activated at the cortical membrane (Figure 6D, arrowheads). We further found that E4orf4 is associated with and enhances the phosphorylation of the target subunit of the myosin light chain phosphatase (MYPT), a Rho kinase substrate (Supplemental Figure S1C). Rho kinase-mediated phosphorylation on Thr696 inhibits the catalytic activity of the myosin phosphatase (Kimura *et al.*, 1996; Feng *et al.*, 1999), and this could account for E4orf4-induced p-MLC. Finally, activation of Rho GTPases by E4orf4 required Src binding but not PP2A, consistent with actin polymerization and myosin II activation. This was revealed by the diffuse distribution of the reporter probes in cells expressing the mutant E4orf4(6R-A) defective in Src binding (Figure 6, C and D), contrasting with specific membrane and perinuclear recruitment in cells expressing E4orf4 (R81/F84A), which behaved like the wild-type E4orf4.

Figure 7 (cont). average intensity of pixels in each regions from the original confocal cross section and ventral face images of the same cell, using the MetaMorph software version 4.5 (right). Data are the means $\pm$ SD of at least 10 individual cells (n). (B) Western blots of total cell lysates from control and MCF7 cells transfected with 75 nM siRNAs to GFP, Cdc42, RhoA, or Rac1 48 h posttransfection. The levels of Rho proteins were revealed using the indicated antibodies and calreticulin protein levels are shown as loading control. Titration curves were established by loading increasing amounts of the control extract (25, 10, and 5 μ g) to estimate the percentage of inhibition of Cdc42, RhoA, and Rac1, -2 and -3 protein levels (italic numbers) in extracts from siRNA-transfected cells (25 μ g) by densitometric analyses. Note that the antibody against Rac1, -2 and -3 revealed >55% reduction of Rac isoforms, suggesting a potent reduction of Rac1. (C) Confocal images of MCF7 cells transfected with the indicated siRNAs and mRFP (left) or transfected with the siRNAs and E4orf4-mRFP. Seventy-two hours after siRNA transfection (24 h after E4orf4-mRFP transfection), cells were processed for labeling with fluorescent transferrin followed by G-actin incorporation. Cross sections and ventral face images of the same cells show representative phenotypes observed at the level of juxtanuclear actin nucleation and endosomes recruitment, and nucleation of stress fibers formation, respectively. The percentages of cells with actin structures showing endosome-associated actin vesicles are indicated and were estimated from two independent experiments, $n > 25$ cells. Bars, 10 μ m.

Figure 8. RhoA, but not Cdc42 or Rac1, is required for E4orf4-induced myosin II activation. (A) 293T cells transfected with E4orf4 were incubated with DMSO or with the Rho kinase inhibitor (10 μ M Y-27632) for a 2-h period 24 h after transfection or with the Rac1 inhibitor (100 μ M NSC23766) added 6 h after transfection for a 18-h period (middle), or cells were transfected with the indicated siRNAs to Rho proteins 48 h before E4orf4 transfection (right). Cells were lysed 24 h after E4orf4 transfection, and equal amounts of cell extracts were analyzed by Western blot using the indicated antibodies. (B) Confocal images of MCF7 cells transfected with the indicated siRNAs 48 h before the transfection with Flag-E4orf4 or Flag-E4orf4-GFP or treated with the Rac1 inhibitor (100 μ M NSC23766) for 16 h before E4orf4 transfection. Cells were fixed 24 h after E4orf4 transfection and processed for double immunostaining of p-MLC (Ser19) and F-actin (phalloidin). The focal plan displaying the highest p-MLC staining is shown. Flag-E4orf4 was immunolabeled using the anti-E4orf4 (2419) antibody. Arrows show p-MLC-containing stress fibers in E4orf4 cells transfected with siRNAs to Cdc42. Arrowheads indicate the filopodia-like microfilaments, which accumulate in E4orf4 cells transfected with siRNAs to RhoA or Rac1 or were treated with the Rac1 inhibitor. Bar, 10 μ m.

Differential Roles for Cdc42, Rac1, and RhoA

E4orf4 promotes the formation of distinct F-actin structures (stress fibers and actin comets) that likely involve different Rho GTPases pathways. To address this issue, we first took advantage of chemical inhibitors to block specific Rho GTPases pathways in E4orf4-expressing cells for a short period (45 min). We used the recently identified inhibitor of

Figure 9. E4orf4-induced actin dynamics trigger apoptotic-like cell death and cell killing. (A) 293T or MCF7 cells were transfected with Flag-E4orf4 alone or together with the indicated siRNAs or plasmid DNA to inhibit Cdc42 (CRIB-N-Wasp), Rho (RBD-Rhotekin), Rho kinase (ROCK2 CAT KD), Arp2/3 complex (Scar VCA), or myosin II (MLC-AA), or treated with the myosin II inhibitor blebbistatin (293T, 10 μM; MCF7, 50 μM), or the Rac1 inhibitor NSC23766 (100 μM). Cells were fixed 24 h after transfection with Flag-E4orf4 and processed for immunostaining of both E4orf4 and the interfering proteins, followed by DAPI labeling to visualize the nuclear morphology. The nuclear condensation (apoptotic-like nuclear shrinkage and chromatin condensation) was determined by counting the number of cotransfected cells with apoptotic-like condensation and is expressed as the percentage of inhibition relative to cells expressing E4orf4 alone that displayed at least 35 to 50% of the cell population with apoptotic-like nuclei (inset graph). Data are the mean $\pm$ SD of three independent experiments, $n > 1000$. Micrographs at the bottom left show an example of E4orf4-induced nuclear condensation, which is inhibited in cells coexpressing the Scar VCA. Bar, 10 μM. Top right, the nuclear condensation was evaluated in live cells transfected with mRFP (EV) or E4orf4-mRFP together with histone H2A-GFP and treated with vehicle (DMSO) or blebbistatin immediately after transfection, $n > 100$. (B) Colony-forming assays. 293T cells were transfected with pGKpuro together with the vector only (EV) or Flag-E4orf4 using a plasmid DNA ratio of 1:25. Other cultures were cotransfected with a nonphosphorylatable mutant MLC (AA). Where indicated, blebbistatin (1.5–3.0 μM) was added to the culture medium immediately after transfection and replaced every 72 h. Aliquots of transfected cells were kept for Western blot analyses of expression levels 24 h after transfection (middle, inset), before applying the selection for transfected cells (3 μg/ml puromycin) with or without blebbistatin for 12 d. Percentages of surviving cells were obtained by counting the number of resulting colonies (top) and are expressed relative to the total number of colonies obtained in cells transfected with the vector only and similarly processed. Data are the means $\pm$ SD of 3 independent experiments. Bottom left, immunostaining of E4orf4 in the surviving colonies showing high levels of E4orf4 only in blebbistatin-treated cells. Middle and right, double immunostaining with anti-E4orf4 and anti-Ki67 (cell proliferation marker) of a E4orf4-positive colony, compared with a E4orf4-negative colony in samples treated with blebbistatin. E4orf4-positive cells proliferate, but to a lower rate, as revealed by a twofold decrease in the size of colonies. Bar, 30 μm.

N-Wasp, wiskostatin, which stabilizes the autoinhibited conformation and effectively prevented the recruitment of N-Wasp-GFP and filopodia formation in cells expressing Cdc42 (Figure 7A, top) (Peterson *et al.*, 2004). We also used the Rac1 inhibitor NSC23766, which inhibits Rac1 binding and activation by the Rac-specific GEF Trio and Tiam1 (Gao *et al.*, 2004) and the Rho kinase inhibitor Y-27632. Remark-

ably, N-Wasp inhibition abolished the juxtanuclear increase in actin nucleation (80% reduction) and triggered the disassembly of the actin ring (Figure 7A, arrows, cross section). However, in these cells, the nucleation of fibers at the ventral face was promoted, suggesting the involvement of two distinct actin nucleation promoting factors (Figure 7A, ventral face). In sharp contrast, Rac1 inhibition reduced by 60% the

overall level of actin polymerization and triggered a similar inhibitory effect at the juxtanuclear ring and along the ventral actin fibers. Nonetheless, the overall structure of the actin network was preserved over this short inhibitory period (our unpublished data). Finally, short-term inhibition of Rho kinase had no significant effect on the rate of actin polymerization. Thus, Cdc42 and Rac1 seem to activate distinct classes of actin nucleation-promoting factors in response to E4orf4, giving rise to different microfilaments structures.

To further discriminate the roles of Rho GTPases, we used siRNAs that reduced Cdc42 protein by >70%, RhoA protein by >95%, or Rac1, -2, and -3 proteins by >55%, and the Rac1 inhibitor NSC23766 (Figure 7B and Supplemental Figure S2A) (Gao *et al.*, 2004). Transfection of a rhodamine-labeled siRNA directed to GFP had no effect on Rho GTPases protein levels, or on the cell morphology, and revealed that >95% of the cell population was effectively transfected (Figure 7B and Supplemental Figure S2B). As expected, reduction of Rho GTPases levels was associated with various morphological phenotypes in 293T and MCF7 cells (see Supplemental Figure S2, C–E). To assess the effects on E4orf4-induced actin nucleation, individual siRNAs were transfected 48h before E4orf4 transfection. Although assembly of the juxtanuclear actin network was not affected by the control siRNA to GFP (Figure 7C, top), it was severely impaired by reduction of each of the Rho GTPases. Knockdown of Cdc42 phenocopied N-Wasp inhibition and blocked actin nucleation in the juxtanuclear region together with endosomes recruitment (Figure 7C, second row). However, stress fibers formation at the cell ventral face was not affected. In sharp contrast, reduction of either RhoA or Rac1 gave similar phenotypes and inhibited stress fibers formation at the cell ventral face, without affecting actin comet formation associated with endosomes. Nonetheless, the juxtanuclear F-actin was no more organized into a ring structure (Figure 7C, third and forth rows).

We next investigated the effects on the organization and level of p-MLC. Inhibition of Cdc42 or Rac1 did not impair E4orf4-induced p-MLC per se (Figure 8A). However, p-MLC organization was markedly affected and localized along prominent stress fibers in cells with reduced Cdc42 or displayed a diffuse distribution in the perinuclear region of cells with reduced Rac1 (Figure 8B). In marked contrast, reduction of RhoA or inhibition of Rho kinase prevented E4orf4-induced p-MLC (Figure 8A and Supplemental Figure S3). Notably, RhoA and Rac1 inhibition was associated with the appearance of a network of thin actin filaments reminiscent of Cdc42-dependent filopodia structures (Figure 8B, arrowheads). Collectively, the results indicate that activation of Cdc42, Rac1, and RhoA play crucial and differential roles in the assembly of the peculiar juxtanuclear actin network induced by E4orf4.

A Causal Role for Actin Dynamics in E4orf4 Killing

To our knowledge, this is the first report of such atypical juxtanuclear-based actin network associated with endosomes dynamics. This further prompted us to investigate thoroughly the relationship with cell death induction by E4orf4. We first looked at apoptotic-like cell death by monitoring nuclear condensation (nuclear shrinkage and chromatin condensation). Inhibition of each of the Rho-GTPase pathways led to a marked reduction of nuclear condensation (Figure 9A). This was revealed by transfection of individual siRNAs to Rho proteins or by coexpression of high levels of wCRIB (Cdc42 inhibition) or rRBD (Rho inhibition). Inhibition of ROCK (ROCK2 CAT KD) also prevented nuclear

condensation (70% inhibition). Even more remarkably, inhibition of actin dynamics per se prevented apoptotic-like cell death. Indeed, inhibition of Arp2/3-dependent actin polymerization by overexpression of the VCA domain of Scar1 (Machesky and Insall, 1998) triggered a severe block of nuclear condensation (60% inhibition). Such was the case also when myosin II was inhibited by blebbistatin, or by overexpression of a nonphosphorylatable MLC mutant (MLCAA) with alanine substitutions at Thr18 and Ser19 (65–90% inhibition) (Iwasaki *et al.*, 2001). Thus, the peculiar actin dynamics induced by E4orf4 are not a consequence of death signaling, but they clearly play an active role in induction of apoptotic-like cell death.

To further determine whether actin dynamics contribute to long-term cell killing by E4orf4, it was necessary to measure cell proliferation. Not surprisingly, chronic inhibition of myosin II and Arp2/3-dependent actin polymerization were highly toxic in control cells. To overcome this problem, we took advantage of 293T cells that have a very low basal level of myosin II activity and in which E4orf4-induced actin dynamics is exquisitely sensitive to myosin II inhibition. Conditions were set up to partially block the function of myosin II with minimal cytotoxicity, using low concentrations of blebbistatin (1.5–3.0 μ M) or moderate levels of the nonphosphorylatable MLC mutant (MLCAA). Cell survival and proliferation were assessed by colony-forming assays. Amazingly, 55–75% of the cell populations transfected with E4orf4 survived when myosin II was down-modulated, whereas <20% of the cells transfected with equivalent amounts of E4orf4 survived in the absence of blebbistatin or MLCAA (Figure 9B). Immunostaining of the surviving colonies using anti-E4orf4 and anti-Ki-67, a proliferation marker (Scholzen and Gerdes, 2000), revealed that in the absence of blebbistatin, <5% of the resulting colonies were positive for E4orf4 expression and those displayed very low levels of E4orf4 restricted to the cell nucleus. Remarkably when myosin II was down-modulated, 25–40% of the resulting colonies did sustain high levels of E4orf4. (Figure 9B, left). Not surprisingly, however, cell proliferation was decreased in E4orf4-positive colonies relative to E4orf4-negative cells. This was revealed by a twofold reduction in the average size of E4orf4-positive colonies relative to that of E4orf4-negative colonies (Figure 9B, middle and right panels). This was expected given evidence supporting the existence of a distinct Src-independent toxic activity (Kornitzer *et al.*, 2001; Robert *et al.*, 2002; Miron *et al.*, 2004). We conclude that the cytoplasmic death activity of E4orf4 clearly contributes to E4orf4 killing in transformed and cancer cells and relies on its ability to imbalance actin dynamics per se.

DISCUSSION

In the present study, compelling evidence is provided that E4orf4 killing largely relies on induction of peculiar Rho GTPase-dependent actin-myosin dynamics, which not only contribute to the apoptotic-like features induced by E4orf4 but also engage the death machinery in the absence of classical caspase activation. This work suggests a more prominent role for actin dynamics in the regulation of cell fate and challenges the current model that regards cytoskeletal alterations only as a consequence of cell death.

The results indicated that the regulated assembly of the juxtanuclear actin network by E4orf4 involves a complex interplay of several Rho GTPases pathways. First, a RhoA-ROCK-regulated myosin II motor at cortical and perinuclear membranes is required, which may transport and reorganize microfilaments into juxtanuclear bundles and fibers

Figure 10. Working models. (A) Three-dimensional representation of the peculiar structure of the juxtanuclear actin network, showing the spatial contributions of Rho proteins to E4orf4-induced actin dynamics (see *Discussion* for details). We speculate that the nucleation of actin comets at the surface of endosomes (dark gray circles) drives their recruitment to the juxtanuclear region where they provide a localized input of microfilaments and actin modifiers for assembly of the contractile ring. (B) E4orf4 hijacks Rho GTPases signaling to kill cells in a way that requires interaction with Src-family kinases. Potential mechanisms include the activation of Rho-GEFs and/or inhibition of RhoGAPs through Src-mediated phosphorylation. We propose that E4orf4-induced actin dynamics lead to severe perturbations of vesicular traffic and organelle-based membrane dynamics, which trigger the activation of an endo-lysosomal-based caspase-independent death pathway.

that radiate to the cell periphery (see model in Figure 10A). Second, a Rac1-dependent actin nucleation activity that seems to cooperate with a RhoA function promotes stress fibers formation and nucleation of the perinuclear actin filaments anchored to the actin ring. The Rac-interacting DRF FHOD1 can trigger the formation of thick actin fibers in a way that requires the activity of the Rho-ROCK cascade and could provide a link for the observed functional cooperation of RhoA and Rac1 in E4orf4 cells (Gasteier *et al.*, 2003). Regardless, a formin activity likely generates the RhoA-Rac1-dependent actin fibers (Baum and Kunda, 2005). Third, a distinct Cdc42–N-Wasp–Arp2/3 complex-regulated actin nucleation activity is also required and may provide a localized input of microfilaments for assembly of the contractile ring. The Cdc42-regulated activity promotes the formation of motile actin structures reminiscent of actin comet tails, and these are associated with endosomes of the recycling compartment, which are recruited to the sites of actin assembly. Pathogenic bacteria and viruses widely exploit the Arp2/3 complex actin nucleation system to promote comet

tail formation at their surfaces. However, to our knowledge, this is the first report of a viral protein that seems to promote the nucleation of actin comets onto intracellular membranes. Evidence suggests a mechanism for endosome movement whereby Cdc42 drives recruitment of an Arp2/3 complex activator to the endosome (Fehrenbacher *et al.*, 2003). In light of our data, we speculate that E4orf4 exploits such a mechanism to nucleate the assembly of actin filaments, which are then organized into a juxtanuclear network by myosin, bundlers, and cross-linkers provided by the recycling endosomes (Figure 10A). Although it remains to be determined whether such activity is involved in some critical adenovirus-cytoskeleton interactions, the observation that low levels of cytoplasmic E4orf4 affect actin dynamics is consistent with a potential role during adenovirus infection.

Strong evidence was obtained that E4orf4 controls the spatial activation of the Rho GTPases Cdc42, Rac1, and RhoA in a way that requires interaction with SFK. This is consistent with previous work indicating that SFK activity and tyrosine phosphorylation of E4orf4 are mandatory for induction of blebbing and cell death (Lavoie *et al.*, 2000; Gingras *et al.*, 2002; Champagne *et al.*, 2004). The exact mechanisms by which E4orf4-SFK signaling controls distinct Rho GTPases pathways remain to be determined. Nonetheless, evidence suggests that the phosphorylation of E4orf4 provides a critical switch for assembling actin-remodeling complexes (Gingras *et al.*, 2002). E4orf4 is phosphorylated on three important Tyr motifs that can provide docking sites for adaptor proteins, which could recruit distinct Rho proteins signaling modules (our unpublished data). The spatial activation of a given Rho pathway is likely dictated by the subcellular distribution of the phosphorylated E4orf4 isoforms and that of Rho protein regulators and effectors. In that respect, a more thorough investigation of the mechanisms governing the dynamics of E4orf4 nuclear/cytoplasmic shuttling is needed. It has been shown that the nuclear accumulation of E4orf4 is directed by an Arg-rich motif and is impaired by tyrosine phosphorylation, but the exact effects on nuclear import and/or export are unknown (Gingras *et al.*, 2002; Miron *et al.*, 2004). Furthermore, nothing is known regarding the transport of E4orf4 out of the nucleus, which seems to be an active process rather than passive nuclear diffusion. This is supported by the observation that the myosin II inhibitor induced the nuclear translocation and retention of E4orf4. This raises the intriguing possibility that E4orf4 might exploit a putative nuclear actin-myosin transport system proposed to mediate the movement of the ND10 nuclear bodies, which are modified by many viruses replicating in the nucleus, including adenoviruses (Muratani *et al.*, 2002; Radtke *et al.*, 2006). Such intranuclear transport system was also proposed to transport the progeny nuclear capsids of herpes simplex virus to the nuclear membrane for primary budding (Forest *et al.*, 2005). Regardless, the data support a key role for actin-myosin in the regulation of E4orf4 subcellular distribution and function.

Finally, key experiments were performed in several cancer lines, including MCF7 and HeLa cells, and confirmed that deregulation of actin dynamics is a major mechanism by which E4orf4 kills cells and not a cell-type specific effect. Indeed, down-modulation of myosin II activity sufficed to promote a marked protection at the level of cell survival, and these cells could sustain high levels of nucleo-cytoplasmic E4orf4. The idea that actin dynamics regulate cell fate is not widely accepted; the current dogma regards cytoskeletal alterations as a subprogram and a consequence of caspase activation that drives the apoptotic morphological changes. However, recent evidence challenges this model and suggests

that changes in the dynamic state of actin influence cell fate. Increased F-actin turnover was associated with prolonged cell longevity and transformation, whereas decreased actin turnover seems to promote ageing and death, although the connections with the death machinery remain elusive (Gourlay *et al.*, 2004; Rao and Li, 2004). The endo-lysosomal compartment has emerged recently as a critical determinant of cancer cell survival, which is somehow connected to caspase-independent death pathways (Kroemer and Jaattela, 2005). We propose that E4orf4-induced actin dynamics trigger profound dysfunctions of membrane traffic and organelle-based membrane dynamics that lead to the activation of a specialized death machinery at the level of the secretory pathway (Figure 10B). If this is the case, E4orf4 could provide a powerful model to uncover the death effector mechanisms involved and potential cross-talk with classical apoptotic pathways.

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