A Functional Interplay between the Small GTPase Rab11a and Mitochondria-Shaping Proteins Regulates Mitochondrial Positioning and Polarization of the Actin Cytoskeleton Downstream of Src-Family Kinases

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Running Title: Rab11a regulates Drp1 -mediated mitochondrial dynamics

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Background: Mitochondrial dynamics are integrated within signaling systems through ill-defined mechanisms.

Results: During cytoskeletal rearrangements by Src-family kinases (SFK), Rab11a modulates mitochondrial dynamics, which in turn, influence actin assembly.

Conclusion: Redistribution of mitochondria near actin-rich structures is mediated by SFK and Rab11a and facilitates polarization of the cytoskeleton.

Significance: A new functional connection is uncovered between membrane traffic and mitochondrial dynamics during cellular remodeling.

ABSTRACT

It is believed that mitochondrial dynamics is coordinated with endosomal traffic rates during cytoskeletal remodeling, but the mechanisms involved are largely unknown. The adenovirus early region 4 ORF4 protein (E4orf4) subverts signaling by Src-family kinases (SFK) to perturb cellular morphology, membrane traffic and organellar dynamics and to trigger cell death. Using E4orf4 as model, we uncovered a functional connection between mitochondria-shaping proteins and the small GTPase Rab11a, a key regulator of polarized transport via recycling endosomes. We found that E4orf4 induced dramatic changes in the morphology of mitochondria along with their mobilization at the vicinity of a polarized actin network typifying E4orf4 action, in a manner controlled by SFK and Rab11a. Mitochondrial remodeling was associated with increased proximity between Rab11a and mitochondrial membranes, changes in fusion-fission dynamics and mitochondrial realocalization of the fission factor dynamin-
related protein 1 (Drp1), which was regulated by the Rab11a effector protein FIP1/RCP. Besides, knockdown of FIP1/RCP or inhibition of Drp1 markedly impaired mitochondrial remodeling and actin assembly, involving Rab11a-mediated mitochondrial dynamics in E4orf4-induced signaling. A similar mobilization of mitochondria near actin-rich structures was mediated by Rab11 and Drp1 in viral Src-transformed cells and contributed to the biogenesis of podosome rosettes. These findings suggest a role for Rab11a in the trafficking of Drp1 to mitochondria upon SFK activation and unravel a novel functional interplay between Rab11a and mitochondria during reshaping of the cell cytoskeleton, which would facilitate mitochondria redistribution near energy-requiring actin-rich structures.

Mitochondrial dynamics, including changes in shape and distribution, have emerged as crucial determinants of cell fate regulation. Processes involving extensive cellular remodeling from mitotic division, cell death, to senescence have been linked to changes in the morphology and position of mitochondria, which appear to cluster at many sites of high ATP requirement in several cell types (1). Cycles of membrane fusion and fission seem to be coordinated with cytoskeleton-based transport of mitochondria and allow the exchange of damaged mitochondrial constituents with those of healthy mitochondria to maintain network function and respond to cellular energy demand (2). Accordingly, mitochondrial dynamics have been found to influence chemotaxis (3), cell cycle progression (4-6), Ca²⁺-signaling and signaling at the immune synapse (7,8), generation of reactive oxygen species (9), migration of cancer cells (10), neurotransmission (11) and apoptotic signaling (12), perhaps by promoting spatial compartmentalization and polarization at the single-cell level. Indeed, relocalization of mitochondria could foster the asymmetric segregation of signaling complexes and lipid domains, by providing the ATP required for the proper functioning of motors, kinases and GTPases controlling membrane-cytoskeletal dynamics. For instance, synaptic mitochondria appear to feed the myosin ATPase required to mobilize reserve vesicles at Drosophila neuromuscular junctions (13).

Mitochondrial distribution is regulated by their movement along microtubules and is coordinated with shape transitions, allowing division of the mitochondrial network into smaller organelles that can be transported by motor proteins (14,15). Several large GTPases control mitochondrial shape transitions and form the core morphogenesis machinery (16). While dynamin-related protein Drp1 is the main regulator of fission (17), the Mitofusins (Mfn1 and Mfn2) and OPA1 catalyze fusion of the outer and inner mitochondrial membrane, respectively (18,19). Activation and trafficking of the cytoplasmic fission factor Drp1 is emerging as a crucial regulatory step. This is modulated by several posttranslational modifications that influence Drp1 localization to the mitochondrial outer membrane and its higher-order assembly into helical oligomers circumscribing mitochondrial tubules at fission foci (20). Thus mitochondrial shape and networks are a result of precise balancing of fusion and fission events that appear to have a direct impact on key cellular functions. Yet the mechanisms whereby mitochondrial dynamic changes are integrated to a variety of cellular processes are incompletely understood and are likely to involve a repertoire of Drp1 interacting proteins within key inter-organelle signaling pathways.

While it is generally accepted that reshaping of the cell membranes and cytoskeleton require a concerted dialog between actin, vesicular transport and organelar dynamics, little is known on the mechanisms promoting communication between the mitochondria and other organelles. Rather, it is generally assumed that mitochondria are completely disconnected from vesicular transport routes. Still, recent evidence suggests that mitochondria can make close contacts with organelles other than the ER, including Golgi membranes and endo-lysosomal vesicles, which are functionally relevant (21,22). For instance, an early coalescence of organelles to a perinuclear region has been found to promote organelle membrane “mixing” between mitochondria, Golgi and endosomes upon stimulation
of death receptors and could favor the transfer of death-promoting factors between organelles (23,24). A growing number of classic vesicular components are found at mitochondria, where they could regulate some form of inter-organellar communication and perhaps, cargo exchanges between mitochondria and other membrane compartments. In support of this notion, vesicular transport routes from mitochondria to peroxisomes and lysosomes have been reported, one of which involves the retromer complex classically associated with endosome-to-Golgi membrane traffic (25-28). Thus far, evidence suggests that cross-talks between mitochondria and other organelles are regulated by trafficking proteins and the mechanisms involved have emerged as a fundamental issue.

Infection with human viruses instigates extensive remodeling of the host cells. Many viral proteins have evolved to subvert core elements of the eukaryotic cell machinery that control actin dynamics and membrane trafficking to optimize viral infection (29-31). Studying such interactions of viral factors has provided major advances in our knowledge of the role of the cytoskeleton in key biological function. Human adenovirus type 2 early region 4 ORF4 (hereafter named E4orf4) is a 14-kDa early viral protein that has been shown to manipulate actin dynamics in many cell types and is believed to facilitate the exodus and spreading of newly assembled virions by contributing to the late disruption of infected cells (32,33). Ectopic expression of E4orf4 outside the viral context induces a nonclassical, caspase-independent form of tumor cell-selective death typified by dramatic changes in actin dynamics (34-37). Not only the distinctive changes in actin dynamics typify killing by E4orf4, but they also contribute to cell killing per se by engaging the death machinery. Indeed, drugs that inhibit myosin II ATPase or actin polymerization strongly impair E4orf4-induced cell death (38,39). Early cell polarization is observed concomitantly with assembly of a peculiar, juxtanuclear actin-myosin network upon E4orf4 expression. These cytoskeletal changes are associated with a chronic increase in cell tension, cellular blebbing and nuclear condensation (39,40). It has been further shown that E4orf4 perturbs Rho GTPase signaling and polarized membrane trafficking via Rab11a-positive recycling endosomes, leading to an impairment of organelar structure and integrity (39,41). For instance, a chronic increase in the retrograde transport of Rab11a-endosomes to the Golgi has been linked to Golgi membrane scattering and caspase-independent cell death in response to E4orf4, but also in response to the general apoptotic trigger staurosporine in tumor cells (33,41). These findings illustrate the value of using E4orf4 as a probing tool to uncover signaling mechanisms controlling non-canonical death pathways and inter-organellar communication of potential significance for tumor cell survival.

Two major signaling systems engaged during cellular transformation are involved in E4orf4 killing: the Src-family kinases (SFK) (37,38) and the protein phosphatase 2A (PP2A) (36,42). E4orf4 physically interacts with the heterotrimeric PP2A and with SFK via distinct domains and both interactions are required for optimal induction of cell death in cultured mammalian cells (43). E4orf4 binding to the kinase domain of Src promotes its tyrosine phosphorylation and increases the phosphorylation of a subset of proteins having a common ability to regulate actin dynamics (37,40,43,44). Besides, cellular transformation by v-Src can sensitize cells to E4orf4-induced killing, suggesting that E4orf4 hijacks key effectors of Src signaling in cellular transformation (unpublished data). An elegant study in Drosophila has provided evidence that both the E4orf4-PP2A and the E4orf4-SFK interaction also contribute to the induction of a distinctive, caspase-independent mode of cell death upon expression of E4orf4 in whole organism (45). Notwithstanding, it appears that E4orf4 simultaneously inhibits classic apoptotic pathways in whole organism, suggesting that damage to normal cells might be minimized in normal cellular contexts where canonical apoptotic pathways are functional. Thus the mechanistic underpinnings of E4orf4-induced signaling may be significant for cancer therapy.

In the present study, we show that the cytoskeletal rearrangements leading to cell death in response to E4orf4 depend upon changes in mitochondrial shape and distribution. We have
examinined a functional connection between mitochondrial dynamics and the small GTPase Rab11a, which mediates SFK-dependent polarized membrane trafficking from recycling endosomes in response to E4orf4. We have further explored this connection during cellular transformation by RSV v-Src. We provide evidence for a conserved pathway activated by SFK, which could coordinate changes in endocytic recycling with mitochondrial dynamics to regulate cytoskeletal-membrane dynamics, and perhaps, influence tumor cell invasive properties.

**EXPERIMENTAL PROCEDURES**

**Expression vectors**—FLAG-E4orf4-mCherry was produced by PCR amplification using the primers 5'-CAG CTC GAG GCT AGC GTC TCT AAG GGC GAG GAA -3' and 5' - CTC GGA TCC GAA TTC TTA TTT GTA CAG TTC ATC-3' and mCherry-pJ1 as a template (obtained from DNA2.0, Inc.). The DNA fragment encoding mCherry was subcloned into the XhoI / BamHI sites of the previously described FLAG-E4orf4 construct into pCDNA 3.1 (37). The adenovirus vector AdFLAG-E4orf4-mCherry was generated by Welgen Inc, Worcester, MA, USA, by subcloning cDNA for FLAG-E4orf4-mCherry into the pENTCMV-teto shuttle vector that contains a bacterial tetracycline resistance operon (TetO) sequence close to the CMV promoter. The resulting recombinant adenovirus was amplified in HEK293V cell line that expresses the tetracycline repressor and inhibits E4orf4 expression to prevent its toxic action and allow efficient replication of the virus (a generous gift from Philip E Branton, McGill University). The following DNA constructs were described before: FLAG-Ad2E4orf4-mRFP (WT, 6R-A, and 4Y-F) (39); FLAG-Ad2E4orf4 (43); OCT-YFP/ OCT-PAGFP, YFP-Drp1 and CFP-Drp1 (K38E) (46); Mfn2 (1-703)-YFP (47); GFP-Rab11a (WT/S25N) (48); GFP-FIP1/RCP; GFP-FIP5/Rip11; GFP-FIP3/ GFP-FIP4 (50); ts72 v-Src (51). The GTP mutant of GFP-Rab11a (Q70L) was generated by PCR-mediated site-directed mutagenesis using GFP-Rab11a as template and the following primers designed to replace a glutamine residue at the position 70 with a leucine: 5'-GGG ACA CAG CAG GGC TAG AGC GAT ATC GAG CT-3' and 5'-AGC TCG ATA TCG CTC TAG CCC TGT GTC CC-3'; boldface indicates nucleotide substitution. All DNA constructs were verified by DNA sequence analyses.

**Antibodies and Chemicals**—The following antibodies and drugs were used: anti-ß-actin (AC15 or AC-74), anti-FLAG (M2), anti-Myc (9E10), anti-v-Src (Ab1), mouse monoclonal anti-Drp1 (anti-MCN1L clone 3B5) and rabbit polyclonal anti-FIP5/Rip11 were obtained from Sigma-Aldrich; monoclonal anticalreticulin, monoclonal anti-cytochrome c (7H8.2C12), monoclonal anti-cytochrome c (6H2.B4), monoclonal anti-Fyn (clone 25), monoclonal anti-Drp1 (clone 8/DLP1), monoclonal anti-GM130 (clone 35) and monoclonal anti-paxillin were obtained from BD Biosciences; rabbit polyclonal anti-Cdc42 (P1) and rabbit polyclonal anti-TOM20 (FL-145) were obtained from Santa Cruz Biotechnologies; monoclonal anti-GAPDH (clone 6C5) was obtained from Fitzgerald Industries International; monoclonal anti-GFP (3E6) was obtained from Molecular Probes/ EMD Millipore Corporation; anti-HA (Ha.11) was obtained from Jackson Laboratories; monoclonal anti-phosphotyrosines (PY20) was obtained from ICN/MPI Biochemicals; rabbit polyclonal anti-Rab11a and monoclonal anti-TIR (clone H68.4) were obtained from Thermo Fisher Scientific; rabbit polyclonal anti-pSrc (416) was obtained from Cell Signaling Technology. The rabbit polyclonal anti-FIP1/RCP was a kind gift from Rytis Prekeris (University of Colorado, Denver) and was described previously (52). DMSO, oligomycin, Mdivi-1 and gelatin were obtained from Sigma-Aldrich; Blebbistatin, SKI-1 and PP2 were obtained from EMD Millipore Corporation; Alexa Fluor ® 488 phalloidin, Texas Red phalloidin, Alexa Fluor ® 647 phalloidin, Mitotracker Deep Red and Gelatin from pig skin, Oregon Green ® 488 were obtained from Molecular Probes/ Thermo Fisher Scientific. For experiments on E4orf4-expressing cells, chemical inhibitors SKI-1 (10 µM), PP2 (10 µM), Blebbistatin (50 µM), Mdivi-1 (50 µM), Oligomycin (5 µM) or appropriate vehicle was added to the culture medium during transfection before the onset of E4orf4 expression, or otherwise as indicated in
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Cell Culture, Transfection and siRNA experiments—HeLa (53) and MCF7 (54) cell lines were maintained in α-minimal essential medium (MEM) and 10% fetal bovine serum (FBS), MDA-MB-231 cells (55) were cultured in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% FBS and 293T cells (56) were grown in Dulbecco’s modified Eagle’s medium (DMEM) and 10% FBS. The MDCK ts-v-Src clonal cell line Pi34 stably expresses a temperature sensitive v-Src mutant ts72-v-Src and was described before (57). These cells were cultured at 40.5 °C (restrictive temperature) or 35°C (permissive temperature) in DMEM supplemented with 10% new born calf serum. The HeLa-(GFP)Rab11a cell line stably expresses GFP-Rab11a to near endogenous level and was obtained by selecting stable transfected cells with G418 (400 ug/ml) for a 3-week period. All cell lines were grown in a humidified atmosphere with 5% CO₂. MCF7 and HeLa cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s recommendations. 293T cells were seeded on poly-L-lysine (Sigma-Aldrich) coated dishes for transfection by the calcium-phosphate method in the presence of chloroquine (25 μM; 6 h) and were analyzed 24 h after transfection (37). MDA-MB-231 cells were infected with a control adenovirus AdLacZ or with AdE4orf4-mCherry at a multiplicity of infection of 75 plaque-forming units per cell. For knockdown experiments HeLa were transfected with 50 nM siRNA duplexes overnight using the calcium-phosphate method, split for subsequent transfection 48 h later and analyzed 60 to 72 h later by western blot or immunofluorescence. The siRNA duplexes were based on human sequences and were purchased from QIAGEN (HPP grade siRNA) or from Thermo Fisher Scientific (standard A4 grade). Sequences of the sense strands are as follow: Rab11a #1 (5’-UGU CAGACAGACCGGAAA-3’), QIAGEN (41,50); Rab11a #3 (5’-GGCAUUGUA GAGAUCUGAATT-3’), QIAGEN (Hs_ RAB11A_2); Rab11a #4 (5’-GAGAUAC AGUGAGAGGUAAUU-3’), Thermo Fisher Scientific siRab11-11; FIP1/RCP #12 (5’- GGUUAAUGAUAAACAUAAATT-3’), QIAGEN (Hs_RAB11FIP1_12); FIP1/RCP #14 (5’-CGCACUCGCAUAAUACGUUTT-3’), QIAGEN (Hs_RAB11FIP1_14); FIP5/Rip11 #5 (5’-CCAUGCAGUCGC_ GCAATT-3’), QIAGEN (Hs_RAB11 FIP5_5); and FIP5/Rip11 #6 (5’-GGAACGCGGCGA GAUUGAATT-3’), QIAGEN (Hs_RAB11 FIP5_6).

Cell Fractionation and Immunoprecipitation—For isolation of heavy/light membranes, cells were suspended at 50 X 10⁶ cells/ml in sucrose buffer (20 mM HEPES, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 15 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM PMSF, 1 mM Na₃VO₄), swollen on ice for 1 h and forced through a 27-gauge needle 50-60 times (39). PNS were pelleted at 700 x g (P1) and further fractionated by serial centrifugation (heavy membranes [P2], 8000 x g; light membranes [P3], 170 000 x g). To obtain a 10-30% linear gradient of Opti-Prep (iodixanol), the homogenized cells were centrifuged at 700 x g and PNS (1 ml) were mixed with Opti-Prep (1.5 ml of 50% solution) to reach a final concentration of 30% iodixanol. The mixtures were layered under 1.3 ml of 20% iodixanol and 1.2 ml of 10% iodixanol, respectively, as described (58). The gradient was spun at 360 000 x g for 3 h at 4°C and collected into 20 fractions. Equal volumes of fractions were loaded on SDS-PAGE gels and western blots were performed as described previously (37). Protein concentrations were determined with the Bio-Rad DC Protein Assay and densitometric analyses were performed from FluorS MAX Multimager-captured images using Quantity 1D software version 4.5.0 (Bio-Rad, Hercules, CA). For immunoprecipitation analyses, cells from 10-cm plates were transferred to ice, washed with ice-cold PBS and scraped into 0.5 ml of native immunoprecipitation buffer (0.1 M MES-NaOH pH 6.5, 1 mM magnesium acetate, 0.5 mM EGTA, 200 μM sodium vanadate, 1% (w/v) digitonin with protease inhibitors, as described (59). Cell lysates were transferred into a 1.5-ml tube on ice, centrifuged at 10,000g for 5 min and transferred to a fresh tube. Cell lysates were incubated for 60-min with 8 μg per 1ml of lysate of anti-GFP (mouse monoclonal clone 3E6; Molecular Probes/ Thermo Fisher Scientific, Ottawa, On. CA) on ice, followed by
addition of 40 µl of Dynabeads® Protein G (Invitrogen™/Thermo Fisher Scientific, On, CA) per 1 ml of lysate and incubation at 4°C for an additional 30-min period. Immune complexes were collected using a magnetic stand (EMD Millipore Corporation, Billerica, MA, USA), washed three times in lysis buffer, and transferred to a fresh tube. Equal amounts of immune complexes were resolved on SDS-PAGE, transferred onto nitrocellulose, and processed for immunoblotting as described before (43). Please note that two different anti-Drp1 antibodies were used for immune complex analyses: the mouse monoclonal anti-Drp1 from BD Biosciences (clone 8/DLP1) and the mouse monoclonal anti-DNM1L from Sigma-Aldrich (clone 3B5).

Immunofluorescence and Image Processing and Analyses—DNA was stained with cell-permeable Hoechst prior to cell fixation. Cell fixation was performed in 3.7% formaldehyde in Luftpig buffer (0.2 M sucrose, 35 mM PIPES, pH 7.4, 5 mM EGTA, 5 mM MgSO4) for 20 min at 37°C and fixation-induced fluorescence was quenched with 50 mM NH4Cl for 15 min at room temperature. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min or with 0.4% saponin in PBS for 30 min. Immunolabelling was performed as described previously (37) using the indicated primary antibodies followed by Alexa Fluor® goat anti-rabbit or goat anti-mouse antibodies and Alexa Fluor® phalloidin for F-actin labeling (Molecular Probes/Thermo Fisher Scientific, Ottawa, On. CA). The cellular phenotypes were monitored routinely by at least two independent investigators by visual inspection of fixed specimens using a 60x or 100x objective lens. Polarized remodeling of the mitochondrial network was estimated based on the relocation of short, discontinuous mitochondrial units (representing >50% of the mitochondrial units) to a juxtanuclear region. These cells were showing a very distinctive early polarization phenotype, i.e. nuclear translocation to one pole of the cell next to the remodeled mitochondrial network, along with stress fiber formation (typical of E4orf4 expression (39)). Mitochondrial remodeling was estimated in MDCK tsv-src by scoring the number of cells that exhibited mitochondrial clustering at the vicinity of podosome rosettes detected by F-actin. Typical remodeling of focal adhesions and actin was estimated from fixed specimens seeded on fibronectin-coated dishes, by scoring the number of E4orf4-expressing cells exhibiting loss of perinuclear paxillin vesicular staining along with increased paxillin staining at focal adhesions and/or showing a juxtanuclear actin network and/or blebbing, as described before (39,40), or by scoring the number of MDCK tsv-src cells exhibiting loss of stress fibers along with assembly of podosome rosettes. The Volocity software versions 5.0/6.0 (Quorum Technologies) and ImageJ 1.43 (National Institute of Health) were used for processing of entire images before cropping to emphasize the main point of the image; processing was limited to background subtraction and brightness/contrast adjustments, unless otherwise indicated.

The mitochondrial aspect ratio (ARmit) was estimated by a computer-assisted morphometric analysis of confocal images using the “Object” plugin in ImageJ software (Amsterdam University, The Netherlands; http://simon.bio.uva.nl/object/). Images were processed with a median filter to remove point noise and objects were defined manually to determine the length and the width of individual mitochondria. ARmit values (length/width) were calculated for 30–40 individual mitochondria within a cell and averaged to obtain a mean ARmit per cell. The coincidence between fluorescent subcellular structures within a cell was analyzed using confocal image stacks, or single plane images that have been selected to include the highest density of Rab11a-labelled structures, by 2 different approaches. Confocal images were cleared of background using a region of interest (ROI) outside cells with the “substract background from ROI” function and filtered with a median filter to remove point noise. The “colocalisation threshold” plugin of ImageJ or Volocity Software (Quorum Technologies) were used to estimate single channel specific threshold-adjusted Mander’s coefficients (tM), which may even be used if the intensities in both channels are different from one another (60). As a complementary approach, we used the Volocity 5.0/6.0 (Quorum Technologies) to perform an object-based analysis as described (41,61). Briefly,
fluorescent objects in both channels were found by applying a threshold set at 800 a.u. or greater and objects were delineated using a segmentation procedure based on a watershed algorithm. The intersecting area between objects with a size volume \( \geq 3 \) voxels was determined and was expressed as a ratio in percent over the total area of voxels within individual channels (% overlap). A similar object-based analysis was performed to estimate the number of mitochondrial Drp1 foci/\( \mu \text{m}^2 \) from a fixed ROI (40 \( \mu \text{m}^2 \)) that had been selected to include the area within a cell showing the highest density of Drp1 foci. Single plane images showing the highest Drp1 staining were chosen for every cell and were cleared of background. Fluorescent objects in both channels (Drp1-YFP and mitochondrial cytochrome c in red) were found by applying a threshold set at 150 a.u. or greater. Drp1 objects with a size \( \geq 3 \) pixels intersecting with mitochondrial staining were scored and divided by the area. To measure mitochondrial connectivity, HeLa cells were transfected with siRNAs and then with E4orf4-mRFP or the vector together with OCT-PAGFP, and cells were imaged 24 h after transfection of E4orf4 using a FV1000 confocal laser scanning microscope driven by FluoView software (Olympus). Quantitative assessment of OCT-PAGFP dilution was performed according to (62). Briefly, region of perinuclear mitochondria within a fixed ROI were stimulated by irradiation at 405 nm (scanning mode: SIM Tornado, ROI: 45 pixels by 45 pixels, laser: 5%, 12.5 \( \mu \text{s/pixel} \)), then fluorescence emission at 488 nm was measured at 10-min intervals by acquiring confocal z-sections covering the entire cell, and the entire mitochondrial network was finally stimulated by irradiation at 405 nm. Image analyses were performed using ImageJ.

**3D/ 4D Confocal Microscopy and Statistical Analyses**—Confocal microscopy of live and fixed cells was performed with an Olympus FV1000 confocal (100x oil 1.4 NA) driven by FluoView software (Olympus), or with a Perkin Elmer UltraVIEW Spinning Disc Confocal (100x oil 1.4 NA, 60x oil 1.4 NA, or 40x oil 1.3NA with 1.5x optovar), equipped with an EMCCD cooled charge-coupled camera at -50°C (Hamamatsu Photonics K.K) and driven by Volocity software version 6.01. Both systems were equipped with a humidified 5% CO/ thermoregulated chamber. For quantitative cellular imaging, acquisitions were taken on separate channels using the same parameters (gain and laser power) optimized to keep fluorescent signals in the dynamic range. For live cell imaging, HeLa(GFP)-Rab11a cells were seeded on fibronectin-coated glass dishes (MatTek Corporation, USA), transfected with E4orf4-mRFP or the vector, and incubated with 25 nM MitoTracker Deep Red for 20 min at 37°C before imaging using spinning disc confocal microscopy and 60x oil 1.4 NA objective. Confocal z-sections (5-8 z-steps of 0.5 \( \mu \text{m} \)) were acquired on separate channels (green, Rab11a-GFP; far red, MitoTracker) at 7.5 sec intervals for a 3-min period. The numbers of Rab11a-mitochondrial interactions at hotspots were estimated by visual inspection of 3D-time sequences of deconvolved image stacks using the Volocity 5.0 software, by scoring contacts between Rab11a-GFP-positive structures and mitochondria only if they met two criteria: first, if they occurred more than once at the same site (region) on a defined mitochondrial tubule over the 3-min period; and second, if they were associated in time and space with remodeling of the mitochondrial tubule (i.e. stretching/constriction, or contact/merging with another mitochondrial unit). Mitochondrial motility was estimated from 3D-time sequences of MitoTracker-labeled mitochondria using a method adapted from (63). Briefly, two single plane confocal images acquired 7.5-s apart were overlaid, the yellow pixels were subtracted and the number of green and red pixels were scored to estimate the number of pixels changing over time (disappearing or appearing into the focal plane). The mean mitochondrial movement per cell was estimated from 8 time points.. One-way analysis of variance (ANOVA) tests were used with p-values of <0.05 considered significant (*\(p<0.05\); **\(p<0.01\); ***\(p<0.001\)). Prism 5.0 software (GraphPad Software) was used to compare mean values of individual experiments whereas SAS/STAT 9.1 software (SAS Institute) was used to compare all single cell measurements. Two-way ANOVA tests were performed using Prism 6.0 to compare measures from single cells within distinct groups and independent experiments, with p-values of <0.05
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RESULTS

E4orf4 hijacks a pathway involving SFK and Rab11a for recruiting mitochondria at the vicinity of a forming actin network—While studying the cytoskeletal transitions that promote cell killing in response to E4orf4, we were intrigued by the recurrence of dramatic changes in mitochondrial network organization that followed recruitment of Rab11a-positive recycling endosomes to the juxtanuclear region. To characterize these mitochondrial changes, HeLa cells were cotransfected with E4orf4-mRFP and OCT-YFP, a marker of the mitochondrial matrix, or were processed for staining of Tom20, a marker of the outer mitochondrial membrane. Mitochondria from cells transfected with the vector only were interconnected and distributed homogenously, showing a tubular and elongated shape (Fig. 1A, Z view; 1C,Ctl). In contrast, E4orf4-expressing cells showed a very distinctive, polar redistribution of mitochondria next to the nucleus that was typically relocated to one side of the cell before the onset of nuclear condensation (polarization at the single cell level). In these cells, mitochondria adopted more heterogeneous, punctate shapes and accumulated at the vicinity of the juxtanuclear actin ring (Fig. 1A, E4orf4-mRFP, Z view), where Rab11a-positive recycling endosomes were also recruited (Fig. 1B, red arrow). Such a phenotype was observed in ~35% of E4orf4-expressing cells early after transfection (Fig. 2B, polarized remodeling, E4orf4 WT) and was associated with mitochondrial fragmentation (Fig. 1C, insets 2.5x). This was reflected by a significant decrease of the aspect ratio of individual mitochondria in E4orf4-expressing cells (ARmit = length/width, ~2.0-2.5-fold; Fig. 1D) and by a marked loss of the population of long mitochondria, as seen by electron microscopy (unpublished data). However in contrast to classic apoptotic changes and in agreement with our previous work, we found that mitochondrial fragmentation was not accompanied by outer membrane permeabilization and cytochrome c release and could not be prevented by overexpression of Bcl-2 in E4orf4-expressing cells (data not shown) (38). Rather, these cells exhibited a progressive, late loss of mitochondrial transmembrane potential that predominated within the pool of smaller mitochondria being recruited at the juxtanuclear region (data not shown). This was consistent with previous work showing that the caspase-independent death activity of E4orf4 is associated with a late disruption of mitochondrial bioenergetics (34,41).

Similar changes in mitochondrial shape and position were observed in MCF7 and 293T cells expressing E4orf4 (data not shown), two additional cell lines for which a key role for SFK in E4orf4-mediated cytoskeletal transitions has been well established (37,39,41). To explore the dependence of mitochondrial changes on the SFK-dependent activity of E4orf4, we first used E4orf4 mutant proteins that are unable to subvert SFK-signaling (E4orf4[4Y-F], E4orf4[6R-A]) (38,43,44). Alternatively, cells expressing wild type E4orf4 were incubated in the presence of SFK inhibitors (SKI-1, PP2). As shown in Fig. 2A-C, E4orf4 mutants were severely defective in induction of mitochondrial remodeling, just as wild type E4orf4 in the presence of SFK inhibitors, indicating that changes in organelar dynamics were linked to SFK-dependent signaling. We next explored the role of Rab11a that is required downstream of SFK to mobilize the traffic of recycling endosomes, remodel actin and trigger cell death in response to E4orf4 (39,41). To that end, we used siRNA sequences achieving >75% depletion of Rab11a in HeLa cells (Fig. 2E). Remarkably, depletion of Rab11a reduced the ability of E4orf4 to translocate and reorganize the mitochondrial network by ~55-60%, mimicking the block induced by SFK inhibitors (Fig. 2A, 2B; right graph). Under these conditions, E4orf4-induced mitochondrial fragmentation was completely inhibited (Fig. 2C; ARmit, red bars) (41). In marked contrast, control siRNA had no effect on E4orf4-induced changes in mitochondrial shape and distribution (Fig. 2B, right graph; 2C). The mitochondrial phenotype was inhibited by 3 distinct siRNA sequences targeting Rab11a, while it was rescued by cotransferring a siRNA-resistant Rab11a-GFP, ruling out off-target effects (Fig. 2A, right panels; Fig. 2B, right graph; 2E, Rab11a-GFP). Further quantitative assessment of the mitochondrial fission-fusion balance using
mitochondrial photoactivatable GFP (OCT-PAGFP) (62) revealed a marked decrease in the dilution of OCT-PAGFP fluorescence within the remodeled network that reflected a loss of mitochondrial connectivity; such decrease was alleviated by transfection of Rab11a-specific siRNA (Fig. 2D). Together, the data strongly suggested that E4orf4 could alter the mitochondrial fission/fusion balance by exploiting some novel functions of Rab11a activated downstream of SFK.

**Increased proximity between Rab11a and mitochondria membranes at sites of fission/fusion dynamics correlates with enhanced mitochondrial motility**—We reasoned that the dependence of mitochondrial remodeling on Rab11a could reveal a novel interaction between the trafficking factor and mitochondria. To determine whether this was so, we first examined the cellular distribution of YFP-Rab11a or GFP-Rab11a relative to mitochondria probed by immunofluorescence with anti-Tom20 or by cotransfection of OCT-Cherry in cells transfected with E4orf4-mRFP or the vector only, by quantitative analyses of the degree of co-localization using two different methods (60,61). While there was little overlap between markers in control HeLa cells (<10%), E4orf4 markedly promoted the co-distribution of punctated Rab11a structures with mitochondrial markers, which was increased ∼2.6-3.1-fold relative to control cells (Fig. 3A, 3B). Notably, only wild type Rab11a, but not GDP or GTP mutants of Rab11a (S25N or Q70L), was relocalized at, or close to mitochondrial membranes in response to E4orf4 (Fig. 3C). This was observed in another set of experiments in which different forms of GFP-Rab11a were expressed alone, or together with E4orf4, for a short period of time to avoid toxicity induced as a result of deregulated Rab11a-dependent trafficking. This suggested that mitochondrial relocalization of Rab11a and changes in mitochondrial shape requires cycling of Rab11a between its GTP-bound and GDP-bound states, just as Rab11a-mediated endosomal recycling through various pathways (64-66). Besides, overexpression of GDP or GTP mutants of Rab11a interfered with E4orf4-induced polarization of recycling endosomes and mitochondrial shape changes (data not shown).

To further analyze the impact of E4orf4 on the distribution of endogenous Rab11a and mitochondria, post-nuclear supernatants from 293T cells transfected with E4orf4 were fractionated using a self-generated Opti-Prep gradient. As shown previously, E4orf4 promoted the translocation of Rab11a, along with transferrin receptor-positive endosomes, to fractions of intermediate density relative to control cells (41). This phenomenon likely reflected the enhanced retrograde transport of Rab11a-endosomes to the Golgi in E4orf4-expressing cells. We observed that mitochondrial markers (Tom20 and cytochrome c), but not Fyn, also shifted to these intermediate density fractions upon E4orf4 transfection and showed greater co-distribution with Rab11a (Fig. 3D, fractions circumscribed by red dashed lines). This was consistent with increased proximity between Rab11a and mitochondrial membranes, and fragmentation of the organelles as seen in our single-cell analyses of E4orf4-expressing cells.

**Inspection of 3D-reconstructions of image stacks** revealed that Rab11a were frequently localized at sites of mitochondrial constriction (Fig. 3E insets, arrowheads) or between two mitochondria in an end-to-end configuration (Fig. 3E insets, arrows), suggesting that Rab11a could be involved in mitochondrial fission/fusion dynamics. To follow the behavior of Rab11a relative to mitochondrial dynamics, we performed 4D microscopic analyses in HeLa-GFP-Rab11a cells transfected with E4orf4-mRFP after labeling mitochondria with MitoTracker, by using spinning disk confocal microscopy. The precise outcome of perinuclear GFP-Rab11a dynamics on mitochondrial morphology could not be determined, given the co-clustering of markers and the dynamicity of mitochondria. Nonetheless, we observed what appeared as recurring contacts between more peripheral mitochondrial tubules and GFP-Rab11a at defined sites, which were spatially and temporally associated with remodeling of the mitochondrial tubules at, or proximal to, the interaction sites (Fig. 3F). Remarkably, such transient and recurrent “contacts” between Rab11a and mitochondria occurred at what appeared as preferential sites (hotspots) on
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Mitochondrial movement is thought to be highly coordinated with changes in their shape (fission/fusion dynamics) for producing more “movable” mitochondria (14). Indeed, we noticed that small mitochondrial fragments often budded from the remodeled network and showed long distance movement (Fig. 4A, arrowheads in frames 0:30-1:30). To further evaluate mitochondrial motility in cells undergoing organelle remodeling, time-sequences of MitoTracker-labeled mitochondria were analyzed using a method adapted from (63). Two images obtained 60 sec apart from each other were colored green and red, respectively, and were subsequently overlaid to visualize mitochondria that maintained their position (yellow pixels) and those that moved (green and red pixels) (Fig. 4B). Image subtraction allowed for visualization of the sites of movement (t=60−t=0; Fig. 4, bottom panels). In control cells, green and red pixels were mostly side-by-side and reflected lateral, wiggling movements of the organelles (Fig. 4B, insets la, lb). Longitudinal movements of the organelles and single green and red pixels were more abundant in E4orf4-expressing cells and likely manifested directional movement into or out of the focal plane (Fig. 4B, insets 2, 3). Assessment of mitochondrial movement over a 1-min time period by subtraction of sequential images (7.5-s interval) revealed that the overall motility of the mitochondrial network was increased ~1.5-fold in E4orf4-expressing cells (% mitochondrial movement=motile pixels/total pixels; Fig. 4C). Together, the results suggested a functional connection between the mitochondrial localization of Rab11a and changes in fission-fusion dynamics that would promote mitochondrial redistribution.

Changes in mitochondrial dynamics rely on Rab11a-regulated recruitment of Drp1— The above-described mitochondrial dynamics associated with Rab11a were reminiscent of transient fusions, which have been characterized as the mitochondrial version of the “kiss and run” phenomenon (67). These events appear to promote mitochondrial motility and rely on both fission (Drp1) and fusion (Mfn2, OPA1) factors that can interact at the fusion/fission sites to regulate the pairing of fusion/fission events (67-69). Actually, we found that transfection of either a dominant-negative Drp1 construct lacking GTPase activity (CFPDrp1K38E) (70,71), or of a mutant Mfn2(1-703)YFP lacking the C-terminal coiled-coil domain essential for mitochondrial fusion (47,72), markedly inhibited the distinctive rearrangement of mitochondria in E4orf4-expressing cells along with early nuclear polarization (Fig. 5A, ∼53% to ∼58% inhibition, respectively). In agreement with a role for mitochondrial division, co-transfection of dominant-negative Drp1 inhibited mitochondrial fragmentation and restored the aspect ratio of individual mitochondria in E4orf4-expressing cells to near control value (Fig. 5A, ARmit). The Drp1 inhibitor Mdivi-1 also reduced the number of E4orf4-expressing cells displaying polarized mitochondrial remodeling (Fig. 5A, ∼62% inhibition) and inhibited the E4orf4-mediated increase in mitochondrial motility (Fig. 4C), further supporting a strong dependence on mitochondrial division (73). Thus the results were consistent with a role for mitochondria-shaping proteins.

The mitochondrial recruitment of Drp1 is a key event in the regulation of mitochondrial dynamics (17,67,74). Cytoplasmic dynein regulates the correct targeting of Drp1 to the outer mitochondrial membrane and it has been proposed that Drp1 itself, or Drp1-decorated
membrane vesicles, are cargos for dynein (14). Given the spatiotemporal dynamics of Rab11a at or close to mitochondria and the strong dependence of mitochondrial changes on Drp1, we postulated that Rab11a could modulate the mitochondrial recruitment of Drp1 (directly or via regulation of vesicle trafficking). Owing to the asynchronous onset of E4orf4 expression, we first performed single cell analyses to visualize the localization of YFP-Drp1 cotransfected with E4orf4-mRFP so as to focus our attention on the early steps of the remodeling process. We observed that E4orf4 induced an early decrease in diffuse, cytoplasmic YFP-Drp1 along with an increase in membrane-associated punctate Drp1 foci (Fig. 5B, siCtl + E4orf4; 5C, no siRNA) that were enriched at sites of mitochondrial constriction/fission (Fig. 5D, arrows). A similar impact on Drp1 localization was visualized at the level of the endogenous protein (data not shown). Importantly, while control siRNA did not perturb the mitochondrial recruitment of Drp1, which was increased ~1.5-fold in the early stages of E4orf4 expression, Rab11a-specific siRNAs ablated the E4orf4-dependent increase in mitochondrial Drp1 foci (Fig. 5C, E4orf4 + siRab11a).

A Rab11a-FIP1/RCP-targeting complex promotes Drp1 recruitment and remodeling of the mitochondrial network—Rab11-family-interacting proteins (Rab11-FIP1s, hereafter named FIPs) are effectors of Rab11 GTPases, which regulate their different functions in endosomal recycling by assembling mutually exclusive targeting complexes on recycling endosomes (75,76). We next sought to explore whether the Rab11a-dependent mitochondrial recruitment of Drp1 could involve specific FIPs. To first analyze the impact of E4orf4 on Rab11/FIPs-targeting complexes, native immunoprecipitations of FIPs were performed in HeLa cells coexpressing various FIPs fused to GFP and E4orf4-mRFP using a MES-digitonin lysis buffer, as described (59,77,78). As expected from a protein that mobilizes Rab11a-regulated trafficking, E4orf4 was detected in immune complexes of several FIPs, in particular with FIP1C/RCP, FIP4 and FIP5/RIP11 (Fig. 6A). Single cell analyses further revealed a marked recruitment of FIP1C/RCP(GFP) and FIP5/RIP11(GFP)-labeled vesicles to the juxtanuclear region, where Rab11a and typical recycling cargos (transferrin receptor, TfR) accumulate upon E4orf4 expression (Fig. 6C, arrows) (41). This suggested that E4orf4 could perturb the polarized trafficking of Rab11a-vesicles by diverting FIPs, in particular FIP1C/RCP and FIP5/RIP11 (33,41).

To next determine whether Rab11a-mediated recruitment of Drp1 could involve a specific FIP, we assessed the presence of Drp1 in FIP immune complexes. Anti-Drp1 antibodies detected bands of ~100 kDa and ~160 kDa only within FIP1C/RCP(GFP)-immune complexes and furthermore, these bands were more abundant in E4orf4-containing complexes (Fig. 6A, 6B, red asterisk), suggesting that a specific interaction between FIP1C/RCP and Drp1 was enhanced by E4orf4. Since monomeric Drp1 essentially migrated as a doublet of ~80 kDa (Fig. 6A, 6B, TL), the Drp1 species associated with FIP1C/RCP might represent modified forms (SUMO1- or ubiquitin-modified forms) and/or dimers of Drp1 incompletely disassembled by SDS-PAGE, respectively. All forms were observed previously along with Drp1 mitochondrial targeting and organelle fission (46,79-81). A complex picture is emerging regarding the multiple covalent modifications of Drp1 controlling its localization, proteins interactions, higher order assembly and GTPase activity, which are likely to influence one another dynamically (20). Notwithstanding the uncertainty regarding specific modification(s), the identity of the ~100 kDa band in FIP1C/RCP-immune complexes was validated as Drp1 using two distinct anti-Drp1 antibodies (compare Fig. 6A and 6B, asterisks) and similar results were obtained using 293T cells (unpublished data). As expected, Rab11a was also detected in FIP1C/RCP-immune complexes but not in GFP-immune complexes. We further found that a fraction of FIP1C/RCP(GFP) was localized to mitochondria or in vesicles tightly associated with mitochondria, the proportion of which was significantly increased in E4orf4-expressing cells (~1.5-1.8-fold; Fig. 6D, 6E). In these cells, FIP1C/RCP(GFP)-punctae could be observed at discrete sites along mitochondrial tubules (Fig. 6F). Together, the data were consistent with a potential role for FIP1C/RCP in E4orf4-mediated, Rab11a-dependent recruitment of
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To directly address the functional relevance of FIP1C/RCP and FIP5/RIP11, we used 4 different siRNA sequences that depleted FIP1/RCP or FIP5/RIP11 by ~60-75% or ~85-90% respectively, in HeLa cells (Fig. 7A). Depletion of FIP1/RCP or FIP5/RIP11 inhibited the number of cells exhibiting polarized recruitment of Rab11a-positive endosomes in response to E4orf4 by ~48-60% (Fig. 7B). However, only FIP1/RCP knockdown could prevent the mitochondrial targeting of Drp1 (Fig. 7C, siFIP1/RCP) that remained unaffected by transfection with FIP5/RIP11-specific siRNAs (Fig. 7C, siFIP5/RIP11). Likewise, FIP1/RCP siRNAs, but not FIP5/RIP11 siRNAs, reduced mitochondrial remodeling by ~37-57% in response to E4orf4 (Fig. 7D), consistent with a specific interaction between FIP1C and Drp1. Under such conditions Golgi membrane scattering, which is a result of a chronic increase in the transport of Rab11a-endosomes to the Golgi (41), was also inhibited by ~60% (data not shown). This was in agreement with a recently described role for FIP1C/RCP in retrograde transport via the early/recycling endosomes-to-TGN pathway (82). In marked contrast, silencing either FIP1/RCP or FIP5/RIP11 similarly impaired actin assembly in E4orf4-expressing cells (Fig. 8A, arrowhead). We concluded that while FIP1/RCP could regulate organelle dynamics, in part by controlling Drp1 trafficking, both FIP1/RCP and FIP5/RIP11 contributed to the cytoskeletal changes induced by E4orf4.

E4orf4-induced signaling depends upon mitochondrial dynamics—We next attempted to determine the functional relevance of mitochondrial dynamic changes to the cytoskeletal transitions driving E4orf4-induced cell death, including focal adhesions remodeling, assembly of the polarized actin network and ultimately, cellular blebbing (39-41). Remarkably, interfering with mitochondrial fission/fusion dynamics by transfection of mutant constructs of mitochondria-shaping proteins or by treating cells with the Drp1 inhibitor Mdivi-1 severely impaired paxillin recruitment to the atypically enlarged focal adhesions (Fig. 8A, arrowhead, ~53-67% inhibition), just as it inhibited assembly of the juxtanuclear actin ring in E4orf4-expressing cells (Fig. 8B, arrowhead, ~38-56% inhibition). The Drp1 inhibitor Mdivi-1 also impaired Rab11a recruitment on large tubulovesicular structures (data not shown), suggesting that polarized endosomal transport via Rab11a-endosomes might be regulated by Drp1-mediated mitochondrial dynamics. Intriguingly, all treatments that impaired mitochondrial changes in response to E4orf4 also interfered at a very early step in the process of cell polarization; this was reflected by a loss of nuclear translocation to one side of the cell as typically observed in HeLa cells (Fig. 5A, 8B-C). Furthermore, the dramatic cellular blebbing phenotype that characterizes E4orf4 tumoricidal action in breast cancer cells (MDA-MB-231) and depends upon a chronic increase in myosin II ATPase (37,39) was reverted by a short treatment with Mdivi-1 (Fig. 8C, ~75% inhibition), just as it was impaired by the mitochondrial ATP-synthase inhibitor oligomycin (Fig. 8C, oligomycin, ~71% inhibition). The results were consistent with a distinctive role for mitochondria in early E4orf4-induced signaling and reinforced long-standing evidence that E4orf4-induced cell death does not rely on a classic mitochondrial pathway involving an early loss of mitochondrial functions (38). We concluded that under these experimental conditions, the cell-demolition program induced by E4orf4 depended upon mitochondrial dynamics and bioenergetic metabolism.

Morphological transformation by v-Src also involves Rab11-regulated mitochondrial dynamics—It has been shown that E4orf4 action largely relies on its ability to subvert SFK-signaling in the context of transformed and cancer cells (37,43,44). The above-described findings suggest that E4orf4 might exploit a pathway rewired during cellular transformation for supporting cellular energy and metabolic needs, presumably to stimulate high cytoskeletal dynamics. To investigate further the relationship between oncogenic SFK-signaling, Rab11a and mitochondrial dynamics, we exploited MDCK cells expressing a thermosensitive mutant of viral Src (MDCK-tsv-Src) that are rapidly and reversibly transformed at the permissive temperature of 35°C and form actin-rich...
structures called podosomes and rosettes. These organelles are matrix-degrading adhesion structures related to the invadopodia found in some cancer cells. Such adhesive structures, collectively called “invadosomes”, are believed to regulate protease-driven invasive cell migration and represent a powerful model to study the convergence of cytoskeletal and membrane trafficking pathways (83-85).

On switching MDCK-\textit{tsv}-Src cells to the permissive temperature, cells accumulated high levels of tyrosine-phosphorylated proteins and exhibited morphological changes typical of an epithelial-mesenchymal transition along with assembly of actin-rich, dot-like podosome structures on a fibronectin matrix (Fig. 9A, white arrowheads; data not shown). These organelles were organized into polarized, circular clusters called rosettes (Fig. 9A, black arrowheads), which required sustained v-Src-signaling at 35°C (Fig. 9A, 35°C O/N + Rev) and exhibited high matrix-degrading activities (data not shown). Remarkably, on v-Src activation mitochondria exhibited a more punctate morphology and were redistributed near rosettes that formed at the vicinity of the perinuclear GFP-Rab11a-containing endosomal compartment (Fig. 9B, 35°C 2h; Fig. 9C, black arrowheads). Changes in mitochondrial shape and distribution were observed in ~70% of v-Src-transformed cells (Fig. 9D, 35°C O/N) and could be partially reverted by switching the cells back to the restrictive temperature of 40.5°C (Fig. 9B, 35°C O/N + Rev, 9D, reverted), suggesting that they were related to oncogenic Src signaling.

To assess functional relationships between Rab11a, mitochondrial dynamics and rosette biogenesis, we overexpressed dominant-negative constructs that block recycling via Rab11a-positive endosomes (Rab11aS25N-GFP) or impair mitochondrial dynamics (Drp1K38E, Mnf2[1-703]). Remarkably, Rab11aS25N-GFP could impair both the early changes in mitochondrial shape and rosette formation upon v-Src activation (Fig. 9D, 9E, ~40-64%, respectively), involving Rab11a in the biogenesis of rosettes. Importantly, mutants of mitochondria-shaping proteins drastically impaired rosette formation on v-Src activation, just like the Drp1 inhibitor Mdivi-1 (Fig. 9E, 9F, ~96-90% inhibition). The mitochondrial ATPase inhibitor oligomycin had a partial inhibitory effect on rosette formation (~46% inhibition, Fig. 9E.), suggesting that v-Src-transformed cells require mitochondrial ATP production at the vicinity of rosettes to support the high dynamicity of these organelles. Finally, a 4-h treatment with Mdivi-1 at 35°C could revert v-Src-induced rosette formation and morphological transformation to the same extent as switching the cells back to the restrictive temperature for 4 h (Fig. 9E, 35°C O/N + Mdivi-1.; O/N + reverted), indicating a strong dependence of cell transformation on mitochondrial division. We concluded that E4orf4 exploits a SFK-, Rab11a-regulated pathway that serves to coordinate mitochondrial functions with membrane trafficking during cellular transformation.

**DISCUSSION**

It is believed that E4orf4 has evolved the ability to hijack SFK-signaling for promoting a concerted dialog between actin, vesicular transport and organellar dynamics, for reasons that are not entirely clear (33). Yet, it provides a unique probing tool for studying the mechanisms involved, which appear to control a non-canonical, tumor cell-selective death pathway. The results presented in this study convey two original and interrelated messages: first, that a signaling pathway regulated by Rab11a and FIP1C/RCP makes connections with mitochondria to modulate the mitochondrial recruitment of the fission factor Drp1; and second, that the Rab11a-regulated pathway is modulated by SFK and mediates some of the signaling to mitochondria required for mobilizing these organelles near energy-requiring actin-rich structures. These findings were substantiated by taking advantage of E4orf4 as a prototype model, and of v-Src, to confirm the relevance of our findings in the context of Src-transformed cells. They have important implications in inter-organelle signaling and oncogene-induced cytoskeletal transformations that are discussed below.

The small GTPase Rab11a mainly localizes to the tubulovesicular recycling compartment and functions in the slow retrieval of internalized membranes and signaling
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molecules to the plasma membrane or to the Golgi via retrograde membrane transport (86). Recycling endosomes also appear to provide an intracellular reservoir of membranes and signaling molecules regulating actin dynamics, which would be mobilized during dynamic rearrangements of the cell (87-89). For instance, polarized membrane trafficking of Rab11 vesicles derived from recycling endosomes to the intercellular bridge plays a crucial role in the completion of cytokinesis (90,91). Likewise, polarized membrane trafficking of Rab11a vesicles is required for E4orf4-induced actin assembly and cell death (41). Though the trafficking of Rab11 vesicles has been associated with SFK-signaling (33,92), this is the first report, to our knowledge, for a role for Rab11a in mitochondrial dynamics that would be regulated by SFK-signaling. Thus Rab11a may function as a converging regulator of polarized membrane trafficking and mitochondrial dynamics during rearrangements of the cell, to help coordinating organellar functions with cytoskeletal dynamics. The detailed molecular mechanism whereby Rab11a regulates mitochondrial dynamics remains to be clarified, but evidence was obtained involving FIP1C/RCP, an effector of Rab11a in endosome-to-Golgi transport, in the mitochondrial recruitment of Drp1. While we observed a specific FIP1C-Drp1 association, it is complicated to discriminate whether such interaction is taking place before, or following their mitochondrial recruitment at fission sites. In theory, there are several possibilities for functional interactions between Rab11a-FIP1C and Drp1, since Drp1 can also be found on membrane vesicles and on the Golgi in some, but not all cell lines, where it has been proposed to regulate the apical sorting of proteins (93-97). The question of whether Drp1 is co-transported together with Rab11a-FIP1C/RCP to the mitochondria could not be resolved with the resolution achieved in our live-cell imaging analyses. Other than modulating the trafficking of Drp1, Rab11a-FIP1C could promote the stable association of Drp1 with mitochondrial membranes by contributing to the necessary membrane remodeling underlying the stable assembly of Drp1 oligomers at fission foci. It is intriguing that FIP1C was reported to bind another member of the dynamin family of proteins (82), suggesting that it may function to scaffold oligomeric complexes controlling membrane scission in various organelles (endosomes, Golgi and mitochondria). While future work and emerging super-resolution imaging techniques will be needed to elucidate the exact function of Rab11a-FIP1C at the mitochondria (see below), the current study provides a novel functional link between mitochondrial dynamics and trafficking factors controlling vesicle traffic at the endosome-Golgi interface.

Another unresolved question is whether Rab11a and FIP1C are directly recruited at mitochondrial fission foci, or if they may be transported on membrane vesicles to the mitochondria. Although speculative, we feel that there is sufficient evidence to support the later possibility. A precedent exists for the transport of recycling endosomes to mitochondria, which is thought to contribute to mitochondrial iron trafficking. In this paradigm, direct transfer of iron from transferrin-containing recycling endosomes to the mitochondrion is believed to occur via a “kiss and run” process that would entail molecular motors and docking complexes (98,99). The interactions between Rab11a-GFP structures bearing resemblance to endocytic tubules and mitochondrial membranes seen here by 3D live-cell microscopy, was reminiscent of a “kiss and run” phenomenon that could modulate the transfer of factors influencing fission-fusion events at the mitochondrion. This could be part of a mechanism coupling shape transitions of mitochondria to their cytoskeleton-based transport, as previously suggested (67). Such process would be consistent with transient residency of Rab11a at mitochondrial membranes, at least in the early stages of the remodeling process, which might explain the low level of co-localization measured with standard confocal imaging methods in fixed cells. Notwithstanding, we could repeatedly measure a significant increase in Rab11a localization at, or proximal to mitochondrial membranes in response to E4orf4, which was observed only with wild type Rab11a, but not with GDP or GTP mutants when expressed together with E4orf4 in similar conditions. This further supports a specific function for cycling of Rab11a at mitochondria, in the transport or
recruitment of components modulating fission-fusion dynamics. We believe that super-resolution imaging techniques such as STED or STORM will be required in the future to provide a definitive answer to this issue. Though electron microscopy analyses are in progress to attempt revealing associations between Rab11a-endosomes and mitochondria, they may not be suitable to detect “transient” interactions like those suggested by our 3D-live cell microscopy analyses. Actually, increased proximity events between Rab11a-GFP and mitochondria of short duration were frequently observed at “hotspots” on a mitochondrial tubule and could reflect the involvement of tethering and docking complexes at the interface of membranes. It was shown that mitochondrial iron trafficking involves Sec15I1, a component of the mammalian exocyst complex that interacts with Rab11a (100,101). This vesicle-tethering complex is proposed to mediate the initial recognition between secretory vesicles and the target membrane and could in principle contribute to the tethering of Rab11a-endosomes to mitochondrial membranes (102). Of much interest here, the small GTPase RalA, another regulator of polarized membrane trafficking that interacts with the exocyst, has been reported to translocate to mitochondria or to vesicles tightly associated with mitochondria at mitosis, where it modulates the recruitment of Drp1 and proper mitochondrial division (58,103). While much remains to be clarified regarding the molecular details including a potential role for RalA, it is intriguing that the Rab11a-mediated mitochondrial dynamics described here were found to rely on a Rab11a-effector protein in endosomal recycling. Whether the observed phenomenon could reflect a transport route connecting some signaling endosomes, maybe en route to the Golgi complex, and the mitochondria remains to be established in future studies.

Finally, taking advantage of both Ad2 E4orf4 and RSV v-Src, we obtained evidence for a role for Rab11a-, Drp1-mediated mitochondrial dynamics in the assembly of polarized actin-rich structures including podosome rosettes in v-Src-transformed cells, which represents a powerful paradigm to study the convergence of signaling, adhesive, cytoskeletal and polarized membrane trafficking pathways (83-85). This provides further strength to a model whereby E4orf4 would subvert oncogenic signaling by SFK, leading to an imbalance in Src morphogenic and survival functions in tumor cells (104). We propose that Ad2 E4orf4 and RSV v-Src, and possibly cancer cells, might exploit a SFK-controlled pathway which regulates a functional interplay between Rab11a-FIP1C and mitochondria-shaping proteins. Such a pathway would contribute to remodel host cells during viral infection and could modulate the invasive properties of tumor cells. Structural adaptations of mitochondria could be coordinated with metabolic reprogramming in malignant cells (the Warburg effect), a process for which SFK are though to play a role by targeting several glycolytic enzymes (105,106). Besides, Src can be observed in mitochondria of several cell types, including cancer cells, where it could modulate mitochondrial dynamics and bioenergetics (107,108). Contrary to early beliefs, mitochondria are functional in cancer cells and proliferating cells still need mitochondrial functions; actually, cancer cells appear to derive a significant fraction of their ATP through oxidative phosphorylation (105). Although the mechanism whereby SFK are targeted to the mitochondria is not completely understood, it has been linked to the traffic of receptor tyrosine kinase in some systems (109). Likewise, the activation of various SFK members and their effect on actin polymerization are related to their traffic via Rab11-endosomes, which also modulates the trafficking of receptor tyrosine kinases and signaling dynamics (92). Together, evidence suggests that SFK regulate multifunctional signaling platforms at the recycling endosome involving Rab11a, which could promote inter-organellar communication during morphogenic events (33). Based on the results here, we further speculate that mitochondria are critical for fuelling polarized transport processes in the context of cancer cells and that dysregulated mitochondrial dynamics could play a pivotal role in tumor invasion downstream of SFK-signaling. In support of this notion, a role for Drp1-mediated mitochondrial fission in lamellipodia formation and breast cancer cell migration has been recently reported (10). In fact, dysregulated mitochondrial dynamics might be a general feature of transformed cells, as several cancer cells lines
have been shown to exhibit abnormal mitochondrial morphologies (110).

In conclusion, we propose that the ability of E4orf4 to exploit SFK-signaling in key pathways controlling vesicle trafficking, cell polarity and organellar dynamics makes E4orf4 an appealing system to target crucial elements of signaling in cancer metastasis.

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FOOTNOTES

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2 The abbreviations used are: Ad2 E4orf4, Human adenovirus type 2 early region 4 open reading frame 4 protein; ARmit, Aspect Ratio of mitochondria; Ctl, control; Drp1, Dynamin-related protein 1; ER, Endoplasmic reticulum; EV, empty vector; FIPs, Rab11-family-interacting proteins; Mdivi-1, mitochondrial division inhibitor 1; Mfn1/2, Mitofusin 1 and 2; OPA1, optic atrophy type 1; PAGFP, Photoactivable GFP; PNS, post-nuclear supernatant; PP2A, Protein Phosphatases 2A; pTyr, phosphotyrosines; RaLA, RAS-Like Protein A; RE, recycling endosome; ROI: region of interest; RSV v-Src: Rous Sarcoma virus v-Src protein; SFK: Src-family kinases; SKI-1: Src Kinase inhibitor 1; TGN, trans Golgi network; TL, total lysates; tM: threshold-adjusted Manders’s coefficient; TfR: transferrin receptor; tsv-Src: thermo-sensitive mutant of v-Src; 3′-UTR: 3′-untranslated region

FIGURES LEGENDS

FIGURE 1. Fragmentation and redistribution of mitochondria towards the vicinity of actin structures follow the polarized recruitment of Rab11a-endosomes— (A) 3D reconstructions of image stacks from HeLa cells transfected with the vector only (control) or E4orf4-mRFP showing mitochondria (red, Tom20) and actin staining (green, phalloidin). Z-views of deconvolved image stacks show the polar clustering of mitochondria next to the juxtanuclear actin ring in E4orf4-expressing cells; dashed lines, z-axis; Bars, 10 µm; N, nucleus. (B-C) Single-plane images of HeLa cells transfected with mRFP or E4orf4-mRFP with or without a marker of recycling endosomes (GFP-Rab11a), showing mitochondrial staining (Tom20). Please note that polarized remodeling of mitochondria is typified by fragmentation of the organelles at the juxtanuclear region, where GFP-Rab11a-endosomes are recruited (red arrow); this phenotype could be observed as early as 9 h after transfection. (C) Enlarged views of the boxed regions show more punctuated and heterogeneous shapes of juxtanuclear mitochondria in E4orf4-expressing cells; Bars, 10 µm; N, nucleus. (D) Graph depicting the mitochondrial morphological aspects ratios (ARmit: mitochondrial length over mitochondrial width) measured from 20–40 individual mitochondria from HeLa cells transfected as indicated; mitochondria were selected from a perinuclear ROI or randomly within the overall area of a cell. Data are the means +/- SE of 3 independent experiments, n=14 cells (ROI) or n=29 cells (all cells); ***p<0.001.

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FIGURE 2. Polarized remodeling of mitochondria depends upon SFK and Rab11a— (A) Single-plane views of HeLa cells transfected with a non-phosphorylatable mutant E4orf4 (E4orf4[4Y-F]) or with wild type E4orf4 after transfection with control siRNA (E4orf4+siCtl) or Rab11a-specific siRNA (E4orf4+siRab11a), showing mitochondrial staining (OCT-YFP, mitochondrial matrix). Enlarged images of the boxed regions show that the tubular morphology of juxtanuclear mitochondria is preserved upon blocking E4orf4-mediated changes in SFK-signaling and Rab11a-mediated trafficking. Left panels: Confocal image stacks of a HeLa cell cotransfected with E4orf4 and a siRNA-resistant GFP-Rab11a after transfection of Rab11a-siRNA, showing a representative rescue of E4orf4-induced mitochondrial remodeling, which is associated with a clustering of GFP-Rab11a endosomes at the juxtanuclear region (panels on the right); Bars, 10 µm; N, nucleus. (B) Graphs depicting the percentages of cells expressing mRFP (EV) or E4orf4-mRFP or mutants E4orf4-mRFP (6R-A, 4Y-F), which have been incubated with Src inhibitors (SKI-1, PP2) (left graph) or the vehicle, or transfected with the indicated siRNAs (right graph), which show typical polarized remodeling of mitochondria; means +/- SD calculated from at least 3 independent experiments, n=600 cells, or from a representative experiment, n>160 cells (PP2). Dashed line delineates the background level of polarized mitochondrial remodeling. (C) Graph depicting the mitochondrial morphological aspects ratios (ARmit: mitochondrial length over mitochondrial width) measured from 30-40 individual mitochondria selected from a perinuclear ROI or randomly throughout cells (all cells) from HeLa cells transfected as indicated. Data are the means +/- SE of at least 3 independent experiments, n=15-32 cells. ***p<0.001; **p<0.01. (D) Confocal image stacks of HeLa cells which had been transfected with the vector only (EV) or E4orf4-mRFP together a mitochondrial photoactivatable GFP (OCT-PAGFP) 48 h after transfection with the indicated siRNAs, showing OCT-PAGFP fluorescence dilution. Confocal image stacks were acquired at 488 nm, 1 min after photoactivation of a fixed perinuclear ROI at 405 nm and then successively at 10-min intervals for a 40-min period followed by photoactivation of the overall cell (total). The graph depicts the relative percentages of dilution of OCT-PAGFP fluorescence, which were estimated from individual cells by measuring the increase in OCT-PAGFP fluorescence volume over a 40-min period over total OCT-PAGFP fluorescence volume (% signal: Δt40m – t1m / total); means +/- SD from at least 8 cells from a representative experiment; Bar, 10 µm; ***p<0.001; **p<0.01. Please note that E4orf4-expressing cells displaying typical polarized remodeling of the mitochondrial network show a ~3-fold decrease in mitochondrial fusion/connectivity, which is completely restored by silencing Rab11a. (E) Western blots of extracts of HeLa cells harvested 57 h after transfection with the indicated siRNAs with or without GFP-Rab11a. The estimated percentages of reduction of Rab11a protein levels are indicated and were obtained from titration curves established by loading increasing amounts of control extracts (not shown); ß-actin, calreticulin and transferrin receptor (TfR) levels: loading controls. Please note that numbers in panels B and E designate distinct Rab11a-specific siRNAs; the Rab11a-siRNA#4 targets the 3’UTR region and was used in rescue experiments together with GFP-Rab11a (green bar in the right graph of panel B).

FIGURE 3. Increased coincidence between Rab11a and mitochondrial membranes is associated with dynamic “contacts” that take place at remodeling sites on mitochondrial tubules— (A) Single-plane images of HeLa cells transfected with the vector only (control) or with E4orf4-mRFP together with GFP-Rab11a, showing GFP-Rab11a (green) and mitochondria staining (Tom20, red). Quantitative dual channel analyses of coincident pixels were performed using the “colocalisation threshold” plugin of ImageJ or Volocity Software. (B) The left graph shows the threshold-adjusted Manders’s coefficients (tM) of Rab11a over mitos or of mitos over Rab11a; means +/- SE calculated from 5 cells from one representative experiment, or from 16 cells from 3 independent experiments, respectively. The right graph depicts the relative percentages of Rab11a staining that overlaps with mitochondria, or the percentages of mitochondrial staining overlapping with Rab11a estimated from 3D deconvolved images; means +/- SE calculated from 5 cells from one representative experiment, or from 16 cells from 3 independent experiments. (C) In another set of experiments, wild type GFP-Rab11a or GDP (S25N) or GTP (Q70L) mutants of GFP-Rab11a were transfected in HeLa cells together with the vector only (EV) or with E4orf4-mRFP to compare their mitochondrial recruitment in response to E4orf4, 9h after transfection. The graphs
show that only wild type GFP-Rab11a exhibits increased co-distribution with mitochondrial markers; means +/- SE calculated from 15 to 30 cells from 4 independent experiments (GFP-Rab11a WT, S25N), or from 3 independent experiments (GFP-Rab11a Q70L). (D) PNS from 293T cells transfected with the vector (EV) or FLAG-E4orf4 (E4orf4) were fractionated using a linear 10-30% Opti-Prep gradient. Fractions (equal volumes) were analyzed by Western blot using the indicated markers to visualize the codistribution Rab11a and mitochondrial proteins (Tom 20, cytochrome c); Fyn was used as loading control. Red dashed lines delineate intermediate density fractions enriched in Rab11a and mitochondrial markers. (E) 3D reconstruction of image stacks of a HeLa-GFP-Rab11a cell transfected with E4orf4-mRFP showing mitochondrial staining (Tom20). Enlarged views of the boxed region show the localization of GFP-Rab11a-labelled spots at constriction sites on a mitochondrial tubule (arrowhead), or at the junction of two mitochondrial tubules in an end-to-end configuration (arrow). (F) Representative 3D-time sequence from confocal z-sections taken at ≥7.5 s intervals from a HeLa-GFP-Rab11a cell expressing E4orf4-mRFP which exhibits typical remodeling of the mitochondrial network as visualized by MitoTracker Deep Red staining. Please note that cycles of interactions between GFP-Rab11a tubular structures and a single mitochondrion are followed by stretching/fission of the mitochondrial tubule at those sites, or fusion between the two fragments of the original mitochondrion restoring the initial shape (arrowheads, compare frames 1:07 and 2:00; Supplemental Video). YZ views show the spatial superposition of GFP-Rab11a and MitoTracker-labelled mitochondria; dashed lines: Y axis. (G) The average incidence per min per cell of apparent contacts between GFP-Rab11a and mitochondria at a “hotspot”, which were followed by remodeling of the mitochondrial tubule at this site, was determined by analyses of 3D-time sequences from HeLa-GFP-Rab11a cells transfected with the vector only (EV) or with E4orf4-mRFP; means ± SE calculated from 12 mitochondria from 2 representative cells (EV), or from 60 mitochondria from 5 representative cells (E4orf4-cells). ***p<0.001; **p<0.01; *p<0.05.

FIGURE 4. Increased motility of the mitochondrial network in E4orf4-expressing cells— (A) 3D reconstructions of image stacks of a representative HeLa-GFP-Rab11a cells transfected with E4orf4-mRFP and stained with MitoTracker Deep Red. Enlarged images of the boxed regions show 3D-time sequences from confocal z-sections taken at ≥7.5-s intervals. Arrows point to small mitochondrial fragments at the vicinity of clusters of GFP-Rab11a-labelled structures, and arrowheads designate a small mitochondrial fragment departing from the juxtanuclear region, which shows directional movement towards peripheral clusters of Rab11a-tubulovesicular structures. N, nucleus; Bars, 10 µm. (B) Mitochondrial motility was assessed from time-sequences of MitoTracker-labelled mitochondria. Green/red overlays of single plane confocal images of MitoTracker Deep Red fluorescence, taken 60 sec apart in HeLa cells transfected with the vector only (control), or with E4orf4-mRFP treated or not with the Drp1 inhibitor Mdivi-1 (50 µM), which show motile mitochondria (green and red pixels) and those that maintained their position (yellow pixels); Bar, 10 µm. Subtracted images in lower panels delineate the sites of movement; the pattern of green/red pixels likely represents wiggling movement when positioned in a side-by-side configuration (boxed regions 1a and 1b), while a higher occurrence of single green and red pixels may indicate directional movements of mitochondria coming out of or into the focal plane in E4orf4-cells (boxed regions 2 and 3). (B) Graph depicting the percentages of movement expressed as the ratios of motile pixels over the total number of pixels, which were calculated by subtraction of sequential images at 7.5-sec intervals (8 time points). Individual points on the graph represent the mean percentage of mitochondrial movement within a single cell over a 1 min-period and a global mean +/- SE was calculated from 6 to 9 cells from a representative experiment; **p<0.01; *p<0.05.

FIGURE 5. Polarized remodeling of the mitochondrial network by mitochondria-shaping proteins and Rab11a-mediated Drp1 mitochondrial recruitment in response to E4orf4— (A) Confocal image stacks of HeLa cells transfected with E4orf4-mRFP showing typical remodeling of mitochondria (cytochrome c staining), which is prevented by addition of the Drp1 inhibitor Mdivi-1, or by expression of dominant-negative CFP-Drp1 (K38E) or mutant YFP-Mfn2 (1-703); please note that expression of YFP-Mfn2 (1-
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703) alone also induces mitochondrial fragmentation. The graphs depict the percentages of E4orf4-positive cells exhibiting mitochondrial remodeling; means +/- SD of at least 3 independent experiments, n≥ 350 cells, or 2 independent experiments (Mfn2 [1-703]), n≥ 200 cells, and mitochondrial morphological aspects ratios (ARmit) measured from >30 individual mitochondria, means +/- SD of 3 independent experiments. (B) Single-plane images of HeLa cells transfected with the indicated siRNAs before transfection with YFP-Drp1 together with the vector (EV) or E4orf4-mRFP. Enlarged images of the boxed regions show a higher density of mitochondrial Drp1 foci in a E4orf4-expressing cell in the early stage of the remodeling process (cytochrome c staining). (C) Graph depicting the numbers of Drp1 foci overlapping with mitochondrial staining per µm², which were estimated from a fixed perinuclear ROI on single plane images of individual cells as described in the Experimental procedures; means +/- SE of a representative experiment (no siRNA) or 3 independent experiments (siCtl, siRab11a), n≥12 cells. ***p<0.001; **p<0.01. (D) 3D reconstruction of image stacks of a HeLa cell transfected with E4orf4-mRFP together with YFP-Drp1 and stained for cytochrome c. Enlarged views of the boxed region show the coincidence between YFP-Drp1-labelled structures and mitochondrial tubules, which are frequently observed at constriction sites along mitochondria or at the tip of short mitochondria (arrows). Bars, 10 µm; N, nucleus.

FIGURE 6. Association of Rab11-FIP1C/RCP with Drp1 and mitochondrial membranes in response to E4orf4— (A-B) Western blots of Drp1 using two different anti-Drp1 antibodies (A and B), Rab11a and Flag-E4orf4-mRFP (anti-Flag) in immune complexes of GFP-FIPs (anti-GFP) isolated from HeLa cells which had been transfected with E4orf4-mRFP (+) or the vector only (-) together with GFP or the indicated GFP-FIPs and lysed in native immunoprecipitation buffer. Please note that E4orf4 increased the amount of Drp1 coprecipitating with FIP1C/RCP (red asterisks), but with no other FIPs despite their association with E4orf4; levels of Drp1 and E4orf4-mRFP in total cell extracts are shown (TL). (C) Single-plane views of HeLa cells transfected with the vector only (EV) or E4orf4-mRFP together with GFP-FIP1C/RCP or with GFP-FIP5/Rip11, showing the staining of transferrin receptor (TfR) that recycles via Rab11a-endosomes. Please note the recruitment GFP-FIP1C/RCP and GFP-FIP5/Rip11 to a juxtanuclear region where TfR-containing vesicles also accumulate in E4orf4-expressing cells (arrows). (D-E) Quantitative analyses of coincident pixels between GFP-FIP1C/RCP and mitochondrial staining (Tom 20) by the threshold-ajusted Manders’s coefficient (tM) method, or by an object-based analysis function of the Volocity software, showing increased coincidence in E4orf4-cells; means +/- SE calculated from 10 cells from a representative experiment; ***p<0.001. (F) 3D reconstruction of image stacks of a HeLa cell transfected with E4orf4-mRFP and GFP-FIP1C/RCP and stained for Tom20. Enlarged images of the boxed region show FIP1C/RCP spots embedded in a mitochondrial tubule (arrows). Bar, 10 µm; N, nucleus.

FIGURE 7. Silencing of FIP1/RCP, but not of FIP5/RIP11, inhibits mitochondrial remodeling, while cytoskeletal remodeling is impaired by either FIP1/RCP- or FIP5/RIP11-specific siRNAs— (A) Western blots of extracts of HeLa cells harvested 57 h after transfection with the indicated siRNAs. The estimated percentages of reduction of FIP1/RCP and FIP5/RIP11 protein levels are indicated; β-Actin levels: loading controls. Please note that anti-FIP1/RCP and anti-FIP5/Rip11 antibodies detect both ∼70 and ∼150 kDa splice isoforms of the proteins which are both silenced by the siRNAs, although the predominant ∼70-kDa forms of FIP1/RCP is mainly shown on this immunoblot. (B) Confocal image stacks of HeLa-GFP-Rab11a cells transfected with the indicated siRNAs prior to transfection with the vector or E4orf4-mRFP, showing expansion of the perinuclear Rab11a-vesicular compartment in a representative E4orf4-expressing cell (arrow), which remains unaffected by E4orf4 upon silencing FIP1/RCP or FIP5/Rip11 (arrowheads); numbers designate the percentages of transfected cells showing a robust juxtanuclear recruitment of GFP-Rab11a-labelled endosomes, means +/- SD from 2 experiments (n> 200 cells). (C) Graph depicting the numbers of Drp1 foci overlapping with mitochondrial staining per µm², which were estimated from single plane images of individual HeLa cells transfected with the indicated siRNA before transfection with YFP-Drp1 together with the vector (EV) or E4orf4-mRFP; means +/- SE, n=10 cells or
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12 cells for siCtl+E4orf4 from a representative experiment. (D) Single-plane images of HeLa cells transfected with the indicated siRNAs before transfection with E4orf4-mRFP together with the mitochondrial marker OCT-YFP. Enlarged views of the boxed regions show the punctate morphology of juxtanuclear mitochondria in E4orf4-cells, which is prevented by FIP1/RCP siRNAs but not by FIP5/RIP11 siRNAs. Bar, 10 µm; N, nucleus. The graph depicts percentages of cells treated as indicated which show typical mitochondrial remodeling; means +/- SD of at least 3 independent experiments, n≥ 450 cells. Dashed line delineates the background level of polarized mitochondrial remodeling. (E) Confocal image stacks of representative F-actin phenotypes (phalloidin staining) of HeLa cells transfected with the indicated siRNAs before transfection with E4orf4-mRFP. The arrowhead designates the juxtanuclear actin ring typically assembled in E4orf4-cells, which is absent in cells treated with either FIP1/RCP or FIP5/RIP11 siRNAs. The graph depicts the percentages of HeLa cells that have been transfected as indicated, which show the typical actin ring structure. ***p<0.001; **p<0.01; *p<0.05.

FIGURE 8. E4orf4-induced cytoskeletal transitions rely on mitochondria-shaping proteins—(A-B) Confocal image stacks of HeLa cells transfected with E4orf4-mRFP together with the indicated mutants of mitochondria-shaping proteins, or incubated with the Drp1 inhibitor Mdivi-1 (50 µM), which were fixed 9 h after transfection and processed for paxillin or F-actin staining. Open arrowheads designate the enlarged paxillin-rich focal adhesions at the juxtanuclear region (A), or the juxtanuclear actin ring (B), whose assembly is abrogated by impairing mitochondrial dynamics. Graphs depict the percentages of HeLa cells treated as indicated, which displayed paxillin recruitment to enlarged focal adhesions or the typical actin ring; means +/- SD of 3 independent experiments, n>350 cells, or 2 independent experiments, n>200 cells (Mnf2[1-703]); ***p<0.001; **p<0.01. (C) Representative confocal image stacks of MDA-MB-231 cells infected with an adenoviral vector expressing E4orf4 as a single product (Ad E4orf4-mCherry) or with a control adenovirus (Ad LacZ), which have been incubated with the vehicle, Mdivi-1 (50 µM), Oligomycin (5 µM), or blebbistatin (myosin II inhibitor, 50 µM) for 0.5 h to 2 h before being processed for F-actin staining (phalloidin). Bar, 10 µm. Open arrowheads designate the typical phenotype of E4orf4-expressing cells treated with vehicle only experiencing dramatic blebbing and cellular condensation 16 h after infection with AdE4orf4-mCherry, which is markedly abrogated upon inhibition of Drp1 or mitochondrial ATPase function. The graph depicts the percentages of control cells compared to E4orf4-mCherry-cells treated as indicated, which displayed blebbing and condensation; n>300 cells from a representative experiment. Bars, 10 µm; N, nucleus.

FIGURE 9. Polarized remodeling of the mitochondrial network in RSV v-Src-transformed cells follows rosette assembly, which depends upon mitochondrial dynamics—(A) Confocal image stacks of MDCK cells stably expressing tsv-Src, showing the reversible induction of invasive actin structures upon switching cells to the permissive temperature (35°C); arrowheads: podosomes; open arrowheads: rosettes. Note the disappearance of most podosomes and rosettes upon switching cells back to the restrictive temperature (40.5°C) for 4 h after an overnight induction period at 35°C (35°C O/N+Rev; reverted). (B) Confocal image stacks of MDCK-tsv-Src cells maintained at the restrictive temperature (40.5°C) or induced at 35°C as indicated, showing a rapid repositioning of the mitochondrial network (Tom20 staining, green) at the vicinity of rosettes (phalloidin, red) being assembled at the juxtanuclear region upon v-Src activation. Enlarged images of the boxed regions and YZ-views show the clustering of mitochondria next to actin structures (35°C 2h) and the more punctate morphology of mitochondria in RSV v-Src-transformed cells (35°C, O/N), which is partially reversed upon switching cells back to the restrictive temperature for 4 h (35°C O/N+Rev). Dashed-lines; Y-axis. (C) Confocal image stacks of MDCK-tsv-Src transfected with GFP-Rab11a before (40.5°C) and after a 2 h-period of v-Src activation at 35°C, showing the clustering of GFP-Rab11a and mitochondria (Tom 20 staining) at the vicinity of podosomes forming a rosette at the juxtanuclear region (arrowheads). YZ-views show the distribution of GFP-Rab11a-vesicles and mitochondria, while enlarged images show the co-distribution of actin structures, GFP-Rab11a and mitochondria upon v-Src activation; dashed line: y-axis. (D) Graph depicting the percentages of MDCK-
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tsv-Src cells transfected with the vector only or with dominant negative Rab11(S25N) before switching them to the permissive temperature as indicated, which show a remodeled mitochondrial network; means +/- SD calculated from 600 cells or more from 3 independent experiments (35°C, 2h), or from 250 cells or more from 2 independent experiments (35°C, 2h + GFP-Rab11aS25N), or from 125 cells or more from a representative experiment (35°C, O/N; reverted), (n>125 cells). Please note that dominant negative Rab11(S25N) inhibited the early effect of v-Src on mitochondrial reorganization (35°C, 2h; ~40% inhibition) to a level similar to that observed upon inactivation of v-Src after an overnight period of induction (O/N, reverted; ~44% inhibition). (E) Graph depicting the percentages of MDCK-.tsv-Src cells that had been transfected with Rab11(S25N) or mutants of mitochondria-shaping proteins as indicated, or pre-treated with the Drp1 inhibitor Mdivi-1 (50-100 µM) or with oligomycin (20 µM) for a 1 h-period before v-Src induction (.tsv-Src, 2h), which exhibit rosettes. In other experiments, cells were incubated at 35°C for an overnight period of v-Src induction before adding Mdivi-1 or oligomycin for an additional 4 h-period at 35°C (.tsv-Src O/N), or before switching them back to 40.5°C for 4 h (Reverted), to assess the dependence of rosettes on mitochondrial dynamics. The data are the means +/- SD calculated from 100 cells or more from a representative experiment (oligomycin, CFP-Drp1[K38E], YFP-Mfn2[1-703], YFP-Mfn2[G12V]), or from 200 cells or more from 2 independent experiments (GFP-Rab11aS25N, Reverted), or from 800 cells or more from 3 independent experiments (40.5°C, 2h; 35°C O/N +/-Mdivi-1). (F) Confocal image stacks of non-transformed MDCK-.tsv-Src cells maintained at the restrictive temperature (40.5°C) or treated with Mdivi-1 for 1 h prior to v-Src induction for 2 h at 35°C, showing the dramatic inhibitory effect of the Drp1 inhibitor on rosette formation. Bars, 10 µm; ***p<0.001; **p<0.01.
Figure 1

A

Control

F-Actin

E4orf4-mRFP

F-Actin

Tom20

Zview

Top

Bottom

B

mRFP

E4orf4-mRFP

GFP-Rab11a

GFP-Rab11a

Tom20

Tom20

C

mRFP

E4orf4-mRFP

Tom20

Tom20

Ctl

2.5x

E4orf4

2.5x

D

ARmit (mean per cell)

Ctl

E4orf4

All Cell

Perinuclear ROI

**

***
Figure 2

A

mRFP
mitochondria

E4orf4 (4YF)
OCT-YFP
N

E4orf4 + siCtb
OCT-YFP
N

E4orf4 + siRab11a
OCT-YFP

B

Polarized remodeling

| (%) cells | EV | E4orf4 |
|-----------|----|--------|
| siCtb     | 5  | 10     |
| 4Y-F      | 15 | 20     |
| SKI-1     | 20 | 25     |
| PP2       | 25 | 30     |
| 6R-A      | 30 | 35     |

C

ARmit

| (mean per cell) | EV | E4orf4 |
|-----------------|----|--------|
| siCtb           | 5  | 10     |
| 4Y-F            | 10 | 15     |
| SKI-1           | 15 | 20     |
| PP2             | 20 | 25     |
| 6R-A            | 25 | 30     |

D

OCT-PAGFP Dilution at 40 min

| 1 min | 40 min | total |
|-------|--------|-------|

E

siRab11a
Rab11a
Calreticulin
GFP-Rab11a
TFR
Figure 3

A

Control

E4orf4-mRFP

GFP-Rab11a

Tom 20

Colocalized pixels

Colocalized pixels

B

Colocalized pixels

Overlap (%)

Colocalized pixels

Overlap (%)

Rab11a:mitos mitos:Rab11a

Rab11a:mitos mitos:Rab11a

C

Colocalized pixels

Overlap (%)

WT

S25N

Q70L

Rab11a:mitos:Rab11a

WT

S25N

Q70L

D

Density

Rab11a

Tom20

CytoC

Fyn

E4orf4

EV

E4orf4

EV

E4orf4

EV

E4orf4

E4orf4-mRFP

Tom20

GFP-Rab11a

E4orf4-mRFP

GFP-Rab11a

MitoTracker

YZ

0:52

1:07

1:37

1:45

2:00

2:07

2:75x

N

2 µm

G

Rab11a-mitos "contacts" at hotspots (um/min per mitos)

EV

E4orf4

***
A

E4orf4-mRFP
GFP-Rab11a
MitoTracker

B

\( t = 0 \) sec, 60 sec

Control
E4orf4-mRFP
E4orf4-mRFP + Mdivi-1

C

Mitochondrial Movement (%)

- Control
- E4orf4
- E4orf4+Mdivi-1

Lateral movements
> single red pixels
Longitudinal movements

** *
**Figure 7**

**A**

| Condition | FIP1/RCP | β-Actin |
|-----------|----------|---------|
| siCt     | ~60-75%  | ~37     |
| siFIP1 #12 | 150-175 | ~75     |
| siFIP1 #14 | 75-100  | ~50     |

**B**

- E4orf4-mRFP
- GFP-Rab11a

| Condition | Nucleus (% of cells) |
|-----------|----------------------|
| siCt      | 1%                   |
| siFIP1    | 59%                  |
| siFIP5    | 24%                  |

**C**

- Drp1 foci/um²

| Condition | EV | E4orf4 |
|-----------|----|--------|
| siCt      | 1.0 | 1.5    |
| siFIP1 #12 | 1.0 | 1.5    |
| siFIP1 #14 | 1.0 | 1.5    |
| siFIP5 #5  | 1.0 | 1.5    |
| siFIP5 #6  | 1.0 | 1.5    |

**D**

- E4orf4-mRFP
- OCT-YFP

| Condition | Polarized Remodeling (% of cells) |
|-----------|-----------------------------------|
| siCt      | none                             |
| siFIP1    | siFIP1 #12                       |
| siFIP5    | siFIP5 #5                        |

**E**

- E4orf4-mRFP
- F-Actin

| Condition | Actin Ring (% of cells) |
|-----------|-------------------------|
| siCt      | none                    |
| siFIP1    | siFIP1 #12              |
| siFIP5    | siFIP5 #5               |
Figure 8

A

Vehicle  Mdivi-1  CFP-Drp1 (K38E)  Mfn2 (1-703)-YFP

Paxillin recruitment to FAs

% cells

Vehicle  Mdivi-1  Drp1(K38E)  Mfn2(1-703)

B

Vehicle  Mdivi-1  CFP-Drp1 (K38E)  Mfn2 (1-703)-YFP

Actin ring

% cells

Vehicle  Mdivi-1  Drp1(K38E)  Mfn2(1-703)

C

(Ad) LacZ  (Ad) E4orf4-mCherry

Blebbing

% cells

Vehicle  Mdivi-1  Oligomycin

mCherry

F-Actin
A Functional Interplay between the Small GTPase Rab11a and Mitochondria-Shaping Proteins Regulates Mitochondrial Positioning and Polarization of the Actin Cytoskeleton Downstream of Src-Family Kinases
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