The Spir actin organizers are involved in vesicle transport processes

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The p150-Spir protein, which was discovered as a phosphorylation target of the Jun N-terminal kinase, is an essential regulator of the polarization of the *Drosophila* oocyte [1, 2]. Spir proteins are highly conserved between species and belong to the family of Wiskott-Aldrich homology region 2 (WH2) proteins involved in actin organization. The C-terminal region of Spir encodes a zinc finger structure highly homologous to FYVE motifs [2]. A region with high homology between the Spir family proteins is located adjacent (N-terminal) to the modified FYVE domain and is designated as "Spir-box." The Spir-box has sequence similarity to a region of rabphilin-3A, which mediates interaction with the small GTPase Rab3A [3]. Coexpression of p150-Spir and green fluorescent protein-tagged Rab GTPases in NIH 3T3 cells revealed that the Spir protein colocalized specifically with the Rab11 GTPase, which is localized at the *trans*-Golgi network (TGN), post-Golgi vesicles, and the recycling endosome [4]. The distinct Spir localization pattern was dependent on the integrity of the modified FYVE finger motif and the Spir-box. Overexpression of a mouse Spir-1 dominant interfering mutant strongly inhibited the transport of the vesicular stomatitis virus G (VSV G) protein to the plasma membrane. The viral protein was arrested in membrane structures, largely colocalizing with the TGN marker TGN46. Our findings that the Spir actin organizer is targeted to intracellular membrane structures by its modified FYVE zinc finger and is involved in vesicle transport processes provide a novel link between actin organization and intracellular transport.

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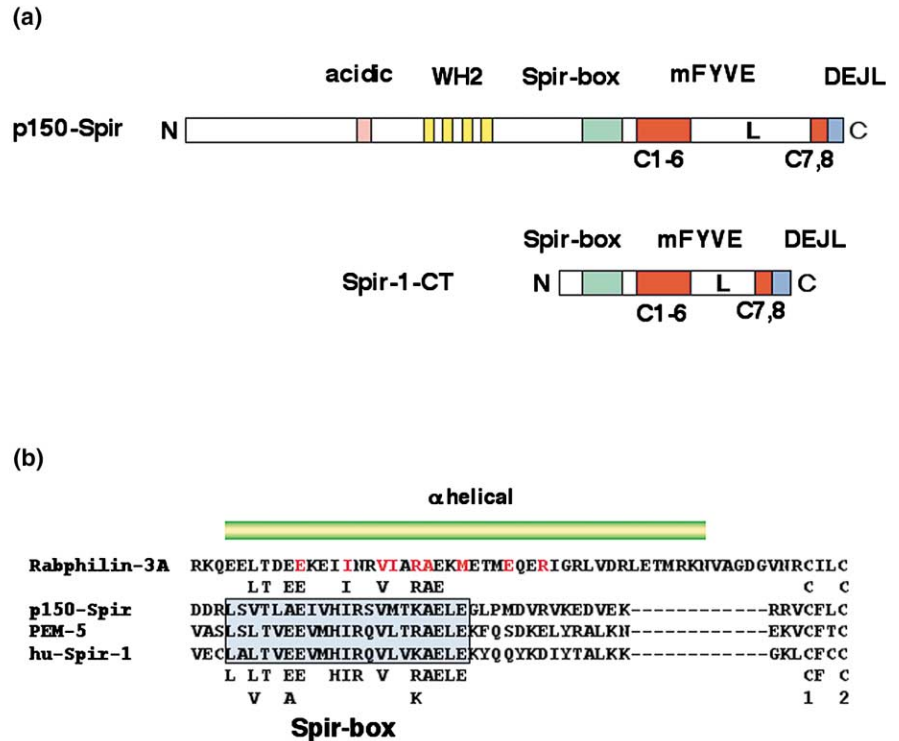
Results and discussion

The *Drosophila spire* gene encodes a protein containing an acidic domain, a cluster of four WH2 domains, and a C-terminal zinc finger structure (p150-Spir) (Figure 1a) [2]. A database search revealed that the Spir proteins are highly conserved between species. Spir has a close homolog in *Ciona savignyi*, determined as PEM-5 [1, 2]. The human *spir*-1 gene is located on chromosome 18 (EMBL database, accession: AJ277587). The assembly of overlapping expressed sequence tags (EST clones) combined with exon predictions from the chromosomal sequences resulted in an open reading frame of 620 codons encoding the acidic, the four WH2 domains, the zinc finger structure, and the C-terminal stop codon. The very N-terminal sequences are still missing.

Comparison of the Spir sequences to the consensus sequence of zinc finger structures revealed that the Spir zinc finger motif is most closely related to FYVE domains (Cys_8). A hallmark of FYVE domains is the cluster of basic amino acids between cysteines 2 and 3 of the consensus sequence, mediating the interaction with phosphatidylinositol 3-phosphate [5, 6]. The Spir zinc finger motif is a modified version of the FYVE domains (mFYVE), lacking the basic cluster between cysteines 2 and 3 and having a loop insertion between cysteines 6 and 7 (Figure 1a). FYVE domains are membrane binding modules [7]. Similar to Spir, the rabphilin-3A protein possesses a zinc finger motif closely related to FYVE structures [3, 8].

A region with high homology between p150-Spir, Spir-1, and PEM-5 is located adjacent (N-terminal) to the modified FYVE motifs (Figure 1a,b) and has been designated as "Spir-box" (p150-Spir aa 705–725). The Spir-box shows sequence similarity to an α-helical domain of rabphilin-3A [3] also located adjacent to its FYVE finger-related zinc finger motif (Figure 1b). The structure of the rabphilin-3A/Rab3A complex has been resolved by X-ray diffraction [3]. The rabphilin-3A α helix N-terminal to its FYVE-related zinc-finger motif mediates the interaction with GTP loaded Rab3A by contacting the switch I and II regions of the Rab3A GTPase. Five of eight rabphilin-3A amino acids contacting the Rab3A GTPase effector loop are identical to those in the Spir-box (Figure 1b,

Figure 1



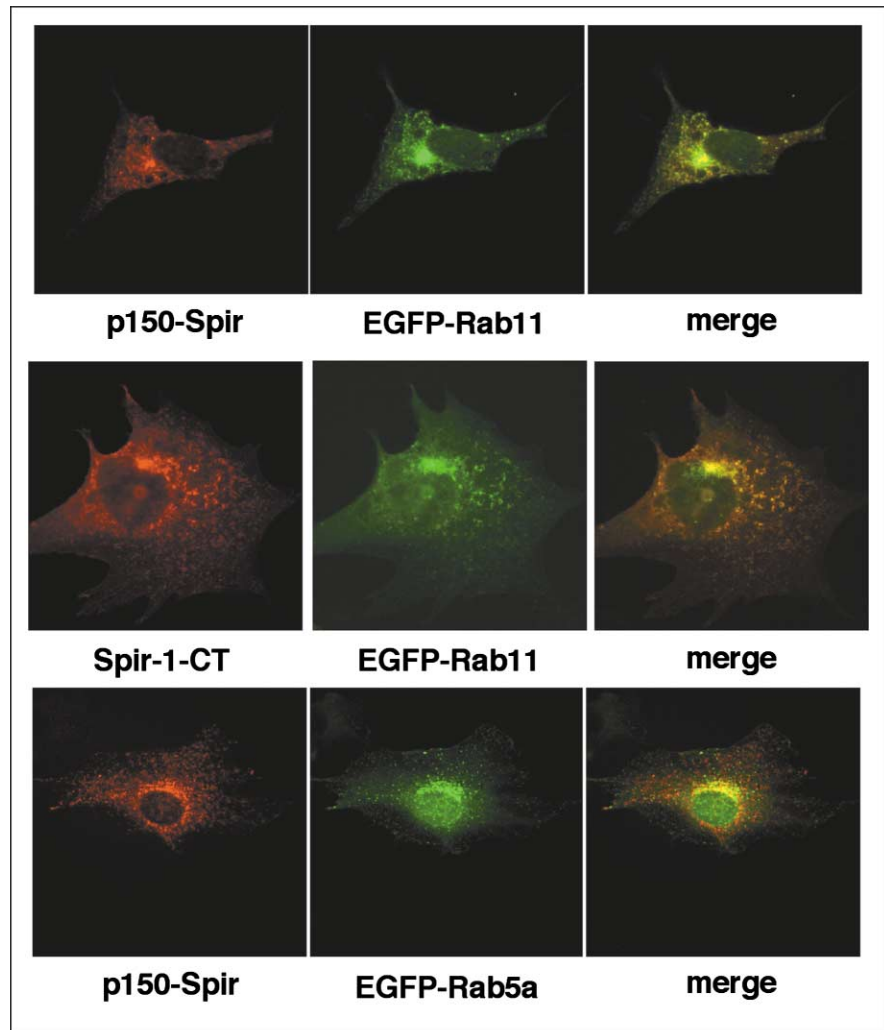
Structure of Spir proteins. **(a)** The N-terminal region of the *Drosophila* p150-Spir protein encodes an acidic domain and four WH2 domains which mediate the interaction of the protein with monomeric actin [1, 2]. A zinc finger structure, highly homologous to FYVE zinc finger motifs, is located in the C-terminal part of the protein (modified FYVE domain, mFYVE). The modification is clearly seen in the insertion (loop, L) between cysteine 6 (C6) and cysteine 7 (C7) of the consensus FYVE structure. A region located at the N-terminal end of the mFYVE motif is highly conserved and designated "Spir-box." At the most C-terminal region, Spir proteins contain a docking site for Erk and JNK, LxL (DEJL) motif. In addition, the structure of the isolated C-terminal end of the human and mouse Spir-1 protein (Spir-1-CT) is shown. **(b)** The Spir-box is a highly conserved region between the Spir family proteins and is an N-terminal extension of the mFYVE domain. An alignment of the Spir-box sequences of the *Drosophila* p150-Spir, the ascidian PEM-5, and the human Spir-1 proteins are shown. The beginning of the mFYVE domain is marked by cysteines 1 and 2 (C1, C2). The Spir-box encompasses the p150-Spir amino acids 705 to 725 and is highlighted in blue. The Spir-box exhibits sequence similarity to the α-helical N-terminal extension of the rabphilin-3A FYVE finger-related structure, which mediates the interaction of the protein with the switch I and II region of the Rab3A GTPase [3]. The amino acids of rabphilin-3A mediating the contact to the GTPase are shown in red.

highlighted in red). The switch I and switch II regions of the Rab family proteins and other small GTPases are very similar to each other. The homology of the Spir-box and the rabphilin-3A α helix suggested a role for the Spir-box in mediating the association of the Spir actin organizers with a small GTPase and most likely with a Rab GTPase.

Rab GTPases regulate vesicle transport processes of the endocytotic and exocytotic pathways [9]. To test whether Spir proteins colocalize with specific Rab GTPases, we coexpressed the Myc-epitope-tagged p150-Spir protein together with green fluorescent protein-tagged Rab5, Rab8, and Rab11 GTPases in NIH 3T3 mouse fibroblasts (Figure 2; data not shown). The p150-Spir proteins were localized by immunodetection. These experiments revealed that p150-Spir specifically colocalized with the Rab11 GTPase. The proteins had an identical localization pattern characterized by an accumulation in perinuclear regions and a punctate cytoplasmic distribution (Figure 2). Rab11 localizes to the TGN, post-Golgi vesicles, and the recycling endosome [4, 10–12]. The specific localization pattern of the p150-Spir protein was dependent on the integrity of the Spir-box and the modified FYVE domain. p150-Spir protein, mutated in either of these domains, showed an even cytoplasmic distribution (Figure S1 in the Supplementary material available with this article online). The C-terminal region of the mouse Spir-1 protein encoding the Spir-box and the modified FYVE domain (Spir-1-CT) was sufficient to direct the specific localization (Figures 1a and 2). The subcellular localization of Rab5, Rab8, and the *trans*-Golgi network marker γ-adaptin [13, 14] displayed a partial overlap in the perinuclear region but were otherwise different from the p150-Spir localization (Figure 2; data not shown).

The Rab11 GTPase is required for TGN to plasma membrane transport [15]. The vesicular stomatitis virus (VSV) G protein is a membrane protein which is transported to the cell surface along the exocytotic pathway. A mutant Rab11 protein (Rab11S25N) blocks exocytosis of the VSV G protein and causes the protein to accumulate in the Golgi [15]. Expression of isolated C-terminal sequences (Spir-1-CT) (Figure 1a), sufficient to mediate the localiza-

Figure 2

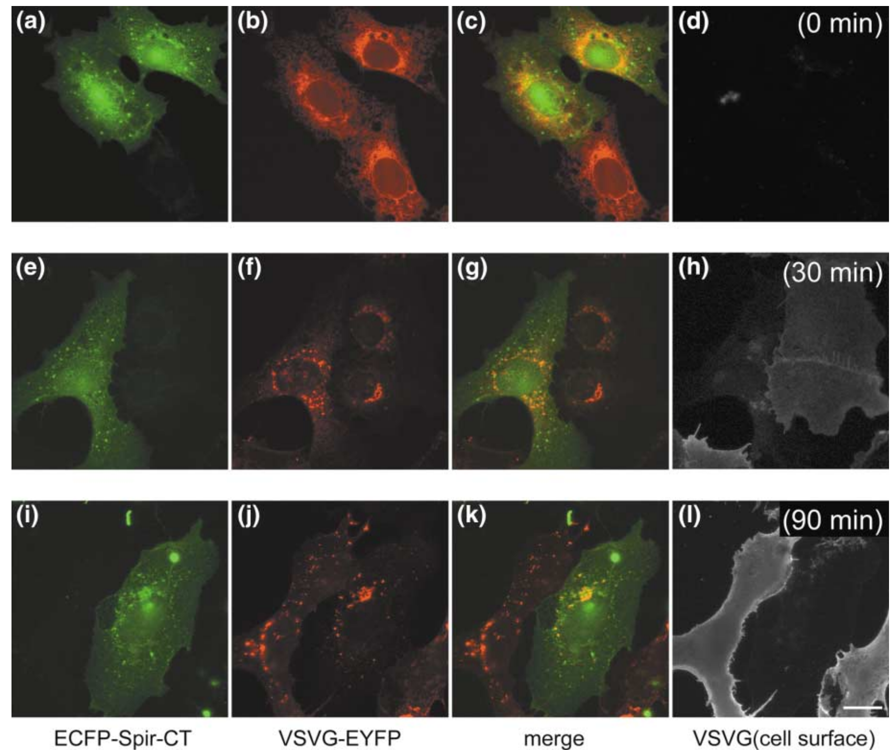


Colocalization of Spir proteins with the Rab11 GTPase. NIH 3T3 fibroblasts were transiently transfected with expression vectors directing the expression of the Myc epitope-tagged p150-Spir protein (p150-Spir) or m-Spir-1-CT protein together with vectors encoding green fluorescent protein-tagged Rab11 (EGFP-Rab11) or Rab5a (EGFP-Rab5a) GTPases [4]. The subcellular localizations of the Myc-tagged proteins were determined by immunostain experiments (red fluorescense), and the localization of Rab GTPases was detected by autofluorescence (green fluorescence).

tion of the protein (Figure 2), should block the Spir function by occupying its subcellular localization compartments without being able to induce actin polymerization. We analyzed the localization of the VSV G protein in NIH 3T3 cells by immunostaining experiments in the presence of the Spir-1-CT 36 hr following transfection. Expression of the VSV G protein alone resulted in an accumulation of the virus protein at the cellular boundary and a strong staining of the plasma membrane (data not shown). Expression of the VSV G protein in the presence of the Spir-1-CT protein partially inhibited the transport to the cell surface and led to a strong accumulation of the virus protein in both the perinuclear region and more punctate peripheral structures where the Spir-1-CT protein was also localized (Figure S2, Figure 3i–l in Vero cells). Coexpression of a mFYVE or Spir-box mutant Spir-1-CT protein with the VSV G protein in NIH 3T3 cells did not influence the exocytosis of the viral protein (Figure S2; data not shown).

A crucial question regarding the function of Spir in vesicle transport was to determine in which compartment the VSV G proteins became trapped and how the proteins got there. Spir proteins colocalize with Rab11 (Figure 2), which is involved in exocytosis and recycling processes [11, 15, 16]. To distinguish if the dominant interfering Spir-1-CT mutant blocks the transport of the VSV G proteins en route to the plasma membrane or if the viral protein clusters result from endocytosed proteins, which failed to be recycled, we have performed kinetic studies employing a temperature-sensitive VSV G mutant protein (ts045) [17]. At the nonpermissive temperature (39.5°C), the VSV G (ts045G) proteins accumulate in the endoplasmic reticulum (ER). A shift to the permissive temperature (32°C) releases the viral proteins from the ER and leads to a rapid transport to the plasma membrane. For protein detection, we have used enhanced cyan fluorescent protein-tagged Spir-1-CT (ECFP-Spir-1-CT) and enhanced yellow fluorescent protein-tagged VSV G (ts045G) pro-

Figure 3

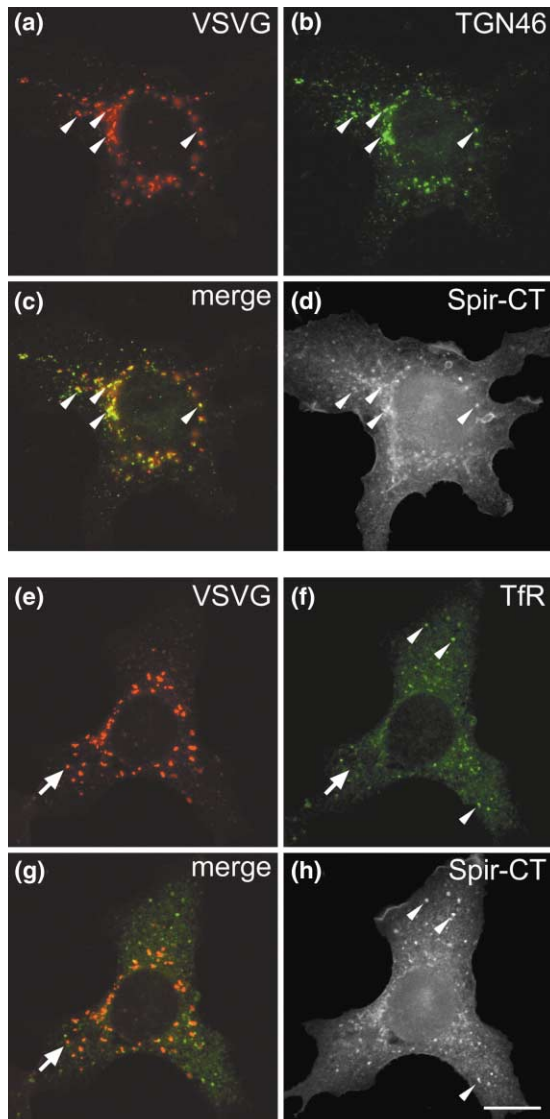


Time course of VSV G (ts045G) transport from ER to cell surface in the presence of Spir-1-CT. Vero cells were transfected with a vector expressing ECFP-Spir-1-CT, then infected with recombinant adenovirus expressing VSVG-EYFP. After infection, the cells were incubated at 39.5°C for 16 hr to accumulate the VSV G in the ER. The cells were then shifted to 32°C in the presence of cycloheximide to allow a synchronous wave of release of the VSV G. Cells were fixed in paraformaldehyde at **(a–d)** 0 min, **(e–h)** 30 min, and **(i–l)** 90 min after release, then stained for cell surface VSV G using an antibody specific to the extracellular domain of the viral glycoprotein. Panels (a,e,i) show the ECFP-Spir-1-CT (ECFP-Spir-CT) expressing cells, and panels (b,f,j) show the VSVG-EYFP within intracellular compartments. VSV G on the cell surface at the various time points is shown in (d,h,l) [VSVG(cell surface)], clearly illustrating that the VSV G is unable to reach to the cell surface in the Spir-1-CT-expressing cells compared to nontransfected cells. Scale bar represents 10 μm.

teins (VSVG-EYFP). The fluorescent protein tags did not alter the VSV G [17] or Spir-1-CT protein properties. When coexpressed in NIH 3T3 cells, the ECFP-Spir-1-CT and the Myc-Spir-1-CT proteins had an identical localization pattern, and the ECFP-Spir-1-CT protein equally inhibited exocytosis of the VSV G protein (data not shown, Figures 3, 4, and S2). The presence of the VSV G protein on the cell surface was analyzed by immunostaining using an antibody specific for the extracellular domain of the protein. Vero (monkey kidney) cells were transfected with an ECFP-Spir-1-CT expression vector and infected with adenoviruses directing the expression of the VSVG-EYFP protein. Following transfection/infection, the cells were incubated at the nonpermissive temperature. We have analyzed the localization of the ECFP-Spir-1-CT and VSVG-EYFP proteins immediately before (0 min) and 15, 30, 45, 60, and 90 min after the shift to the permissive temperature. At the nonpermissive temperature, the VSVG-EYFP proteins accumulated in the ER in cells expressing and not expressing the Spir-1-CT protein (Figure 3a–d). In cells not expressing the ECFP-Spir-1-CT protein, the VSVG-EYFP protein was first detected on the plasma membrane at 30 min after the release (Figure 3e–h). The accumulation of VSV G at the plasma membrane greatly increased up to 60 min and then only a little further by 90 min (Figure 3d,h,l; data not shown). In the presence of ECFP-Spir-1-CT, the localization of the viral protein was seen to change from a reticular pattern (ER) to more punctate structures by 30 min (Figure 3e–g); however, it was never seen to reach the plasma membrane within the 90 min of the experiment (Figure 3h,l). This indicates that a transport step within the exocytotic pathway is impaired by the dominant interfering Spir-1-CT mutant. To analyze the nature of the compartments where the VSVG-EYFP proteins accumulated, we have performed colocalization studies with the early Golgi marker ERGIC53 [18] and the *trans*-Golgi marker TGN46 [19] 90 min after the release (Figure 4a–d; data not shown). At this time point we scored VSV G-positive structures for the presence of ERGIC53 or TGN46. In ECFP-Spir-1-CT-expressing cells, VSV G was only found to colocalize with ERGIC53 in 31% of the structures, whereas, with TGN46, this degree of colocalization was increased to 57% (Figure 4a–d, arrowheads), indicating that the greatest inhibition of viral protein transport occurs at the TGN.

The transferrin receptor cycles between the plasma membrane and endosomes allowing cells to take up iron from transferrin ligand. The receptor is localized in a dispersed and punctate population of vesicles (Figure 4f) [16]. Expression of a dominant interfering Rab11 mutant causes a redistribution of the transferrin receptor into an extended tubular network of recycling endosomal origin and blocks recycling of transferrin from recycling endosomes [11, 16]. We have analyzed the localization of the transferrin receptor in Vero cells expressing the ECFP-Spir-1-CT and

Figure 4



Colocalizations of VSV G (ts045G) with TGN46 and transferrin receptor in the presence of Spir-1-CT. Vero cells were transfected with a vector expressing ECFP-Spir-1-CT, then infected with adenovirus expressing VSVG-EYFP, as described in Figure 3. At 90 min after release of the VSV G from the ER, the cells were fixed in paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were immunostained for the **(a–d)** *trans*-Golgi marker TGN46 or **(e–h)** the transferrin receptor (TfR), each visualized with appropriate Alexa647-labeled secondary antibodies. Colocalization of VSV G with TGN46 is shown in (a–c) (arrowheads), in an ECFP-Spir-1-CT (Spir-CT)-expressing cell (d) where the VSV G was unable to reach the cell surface. Panels **(e–h)** show that the VSV G is not present in the majority of TfR-positive compartments (arrow). In contrast, the Spir-1-CT (Spir-CT) (h) largely colocalizes with the TfR (f), indicated by the arrowheads. Scale bar represents 10 μm.

VSVG-EYFP proteins 90 min after the release of the viral proteins from the ER (Figure 4e–h). We did not detect a relocalization of the transferrin receptor into a tubular network in the presence of the dominant interfering ECFP-Spir-1-CT protein. However, ECFP-Spir-1-CT and the transferrin receptor were frequently found in the same punctate structures (Figure 4f,h, arrowheads), which did not contain the trapped VSVG-EYFP protein (Figure 4e–h). Presence of the transferrin receptor in structures containing VSVG-EYFP was only seen in rare cases (18%), which further supports a TGN-level rather than an endosomal block of the viral protein transport. The localization of the transferrin receptor and the ECFP-Spir-1-CT protein in the same punctate structures indicates that Spir proteins may also be involved in receptor recycling processes. Structures containing high levels of the ECFP-Spir-1-CT often also exhibited an accumulation of the transferrin receptor (Figure 4f,h, arrowheads), indicating that the dominant interfering Spir-1-CT protein might influence transferrin receptor recycling.

Our experiments suggest a role for the Spir actin organizers in vesicle transport processes. We favor a model in which the induction of actin polymerization by Spir might drive the vesicle motor activity. Actin-dependent propulsion of cytoplasmic vesicles has been recently described in *Xenopous* eggs and in mammalian fibroblasts [20–22]. Interestingly, in fibroblasts most of the vesicles containing actin comet tails were Golgi derived [22]. Spir proteins perfectly colocalize with the Rab11 GTPase (Figure 2). The Spir-box, as a potential Rab GTPase interaction domain (Figure 1b), is necessary for the subcellular localization of the Spir proteins (Figure S1). Spir might therefore be targeted to cellular membranes by its interaction with Rab11. Preliminary studies employing bacterially expressed Spir-1-CT and Rab11 proteins indicate a GTP-dependent direct interaction of the proteins. Genetic evidence for a functional relation between Rab11 and Spir has recently arisen from discovery of the Rab11 mutant phenotype in *Drosophila* [23]. Both *spire* and Rab11 belong to the posterior group of genes, being involved in pole plasma assembly [1, 23, 24]. Similar to *spire* mutants, the *Drosophila* Rab11 mutants have defects in microtubule organization during midoogenesis [1, 23]. Future experiments may answer the question how Spir- and Rab11-regulated vesicle transport processes influence microtubule organization and if those mechanisms are regulated by external signals such as MAP kinase phosphorylation.

Supplementary material

In addition to the Materials and methods section, the Supplementary material available at http://images.cellpress.com/supmat/supmatin.htm contains experiments showing that the specific localization pattern of the p150-Spir protein is dependent on the integrity of the mFYVE motif. Mutation of the zinc complexing cysteines, the Spir-box, or two hydrophobic phenylalanines between cysteine 2 and 3 resulted in an even cytoplasmic distribution of the p150-Spir protein. A second supplementary figure shows that coexpression of the dominant interfering Spir-1-CT protein and the VSV G protein in NIH 3T3 cells partially inhibited the transport of the viral protein to the cell surface.

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