The Ste20-like kinase SvkA of Dictyostelium discoideum is essential for late stages of cytokinesis

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Summary
The genome of the social amoeba Dictyostelium discoideum encodes ~285 kinases, which represents ~2.6% of the total genome and suggests a signaling complexity similar to that of yeasts and humans. The behavior of D. discoideum as an amoeba and during development relies heavily on fast rearrangements of the actin cytoskeleton. Here, we describe the knockout phenotype of the svkA gene encoding severin kinase, a homolog of the human MST3, MST4 and YSK1 kinases. SvkA-knockout cells show drastic defects in cytokinesis, development and directed slug movement. The defect in cytokinesis is most prominent, leading to multinucleated cells sometimes with >30 nuclei. The defect arises from the frequent inability of svkA-knockout cells to maintain symmetry during formation of the cleavage furrow and to sever the last cytosolic connection. We demonstrate that GFP-SvkA is enriched at the centrosome and localizes to the midzone during the final stage of cell division. This distribution is mediated by the C-terminal half of the kinase, whereas a rescue of the phenotypic changes requires the active N-terminal kinase domain as well. The data suggest that SvkA is part of a regulatory pathway from the centrosome to the midzone, thus regulating the completion of cell division.

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Key words: Severin kinase, Ste20 kinases, Cytokinesis, Actin cytoskeleton, Severin, Dictyostelium, Midbody

Introduction
Ste20-like kinases are ubiquitous Ser/Thr kinases subdivided into two families, the p21-activated kinases (PAKs) numbering six and the germinal centre kinases (GCKs) numbering 27 representatives in humans (Manning et al., 2002). GCKs harbor the catalytic domain close to the N-terminus, whereas the PAK family is characterized by a C-terminal kinase domain and an N-terminal region that facilitates interaction with Rho-related GTPases by means of PDB or CRIB domains (Hofmann et al., 2004). By contrast, the role of the highly diverse C-terminal region of GCKs is not well understood. There are, however, indications that the C-terminal domain regulates activity, localization or intermolecular interactions (Arasada et al., 2006; Dan et al., 2001).

Kinases from both families are believed to be upstream regulators of mitogen-activated protein kinase (MAPK) cascades (Raman and Cobb, 2003). In addition, GCKs play important roles as activators and represors of apoptosis (Hippler and Cohen, 2004; Huang et al., 2005; McCarty et al., 2005). Well characterized is the role of Hippo, the Drosophila melanogaster homolog of human MST2, that plays a fundamental role during the development of the eye (reviewed in Edgar, 2006). Further functions for GCKs are the regulation of ion transport and cell volume (reviewed in Gagnon et al., 2006; Strange et al., 2006) and the response to various cellular stresses (Lehtinen et al., 2006; Pombo et al., 1996; Taylor et al., 1996; Tsutsumi et al., 2000). Several GCKs interact with cytoskeletal proteins (Eichinger et al., 1998; Mitsopoulos et al., 2003; Nakano et al., 2003; Storbeck et al., 2004). A thoroughly studied example for the regulation of cytoskeletal components is the D. melanogaster kinase Misshapen, and its vertebrate homolog Nck-interacting kinase. These kinases regulate the formation of filopodia (Ruan et al., 2002) and the localization of dynein in flies (Houallia et al., 2005), as well as recruitment of actin and myosin II during fish epiboly (Köppen et al., 2006) or cell migration in mammalian embryos (Xue et al., 2001). They are also involved in the formation of lamellipodia upon stimulation by growth factors (Baumgartner et al., 2006).

The GCK-III (or YSK) subfamily is one of eight GCK subfamilies and has three representatives in mammals: YSK1/SOK1 (Osada et al., 1997; Pombo et al., 1996), MST3 (Schinkmann and Blenis, 1997) and MST4 (Lin et al., 2001; Qian et al., 2001). The role of these kinases in MAPK signaling cascades is a matter of debate (Lin et al., 2001; Pombo et al., 1997; Qian et al., 2001; Schinkmann and Blenis, 1997), but recent data suggest very clearly a regulatory function for MST4 in the ERK pathway (Ma et al., 2007). YSK1/SOK1 was demonstrated to influence cell migration by phosphorylating the dimeric adaptor protein 14-3-3ζ (Preisinger et al., 2004). In addition, YSK1/SOK1 is involved in organization of the Golgi and shuttles between cytosol and Golgi, modulated by an interaction with the Golgi matrix protein GM130 (Preisinger et al., 2004). An RNAi screen in D. melanogaster showed that the knockdown of the only fly GCK-III representative (CG5169) causes changes in the actin cytoskeleton (Kiger et al., 2003). This correlates well with data from Dictyostelium discoideum, where one member of the GCK-III subfamily was purified to near homogeneity and shown to phosphorylate the gelsolin-like F-actin-fragmenting protein severin (Eichinger et al., 1998). This severin kinase (SvkA) has an overall identity...
of 50% to the stress-dependent human GCK-III kinase YSK1/SOK1. Furthermore, the kinase domains are 74% identical.

The social amoeba *D. discoideum* is a valuable model organism for investigating the influence of Ste20-like kinases in cytoskeletal reactions because it is highly mobile as single cell and during multicellular development, it is amenable to molecular genetics, and data on the complete genome are available (Eichinger et al., 2005). The amoeba contains four PAK proteins, 13 GCK proteins that represent the subfamilies GCK-II, -III and -VI (Arasada et al., 2006) and several *D. discoideum*-specific GCK subfamilies (Goldberg et al., 2006). An investigation of the Ste20-like kinase Krs1, homologous to Hippo and MST2, revealed an inhibitory function of the C-terminal domain. Furthermore, Krs1 is necessary for direct cell migration during chemotaxis (Arasada et al., 2006; Muramoto et al., 2007).

Here, we characterize the role of the Ste20-like SvkA protein during cytokinesis in *D. discoideum*. The disruption of the gene impairs cell division and leads to multinucleated cells. In addition, knockout of *svkA* affects directed slug movement and the formation of fruiting bodies, suggesting an important role for this kinase not only in single cells but also during development.

**Results**

**SvkA** knockout and rescue

To examine the cellular activities affected by SvkA, we disrupted the *svkA* gene by homologous recombination (Fig. 1A). The *svkA* gene disruption of several independent clones was confirmed by PCR and immunoblots (Fig. 1B,C). Expression of a full-length (FL) kinase fused to GFP under the control of the actin 15 promoter was used to rescue the *svkA*-null phenotype (Fig. 1C,D). Despite the good reaction of the polyclonal antiserum in immunoblots, its specificity in immunoprecipitation could not be demonstrated. Western blotting was poor. Therefore, we used mainly GFP-fusion constructs to study the cellular localization of SvkA. Partially purified GFP-SvkA-FL phosphorylated the already described in vitro substrates domains 2 and 3 of severin (DS211C) or myelin basic protein (MyBP) (Eichinger et al., 1998), which showed that the fusion to GFP did not abolish SvkA function (Fig. 1D).

Phenotype of the *svkA* mutant

The *svkA*-knockout strain had a slight growth defect in shaking culture and on lawn of *Klebsiella aerogenes*. Phagocytosis, adhesion and random motility were normal. The most dramatic phenotypes were a developmental and a cytokinesis defect, as discussed below.

In wild-type cells, SvkA is expressed at about equal levels throughout the developmental cycle (Fig. 2A). Interestingly, multinucleated *svkA*-knockout cells (Fig. 2B) separated into smaller cells within 30-60 minutes after transfer to low-salt phosphate buffer and remained connected by cytoplasmic bridges, sometimes for several hours (Fig. 2C,D, supplementary material Movie 1). A direct result of this behaviour was an uncoordinated movement towards a cAMP source and the appearance of multiple leading fronts (supplementary material Movie 2). Consequently, the formation of normal streams and early aggregates was delayed by several hours (Fig. 3A). The fruiting bodies were smaller and, in particular, the stalks were broader and distorted. Expression of a full-length kinase restored the normal phenotype (Fig. 3B), whereas expression of a kinase-dead mutant could not rescue the phenotype (data not shown). Mixtures of *svkA*-knockout and wild-type cells improved the morphology of the fruiting body gradually, but it required a composition of >50% wild-type cells for development into essentially normal fruiting bodies (data not shown).

A transient step in the developmental cycle is the slug stage in which the strongly polarized multicellular aggregate moves in a positively thermotactic and phototactic fashion. The knockout of *svkA* produced drastic changes in phototactic motility, and the defect could be rescued again by expression of full-length SvkA (Fig. 3C). The overall motility of *svkA*-knockout slugs was reduced, but aggregates moved on a straight path towards the light source. This indicates that reception of the light signal in mutant slugs is still intact.

Defects in cytokinesis are most pronounced in the phenotype of the mutant

The loss of SvkA resulted in cells that were up to ten times larger than average wild-type cells (Fig. 2B), with sometimes >30 nuclei and a corresponding number of centrosomes (data not shown). A comparison between subermerged and shaking

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**Fig. 1.** Disruption of the *svkA* gene encoding severin kinase. (A) Two fragments of the kinase were amplified by PCR from genomic DNA, ligated to the blastocidin-resistance cassette and electroporated into AX2 wild-type cells. (B) Blastocidin-resistant clones were tested for knockout events by PCR1 (primers 1 and 2, 1385 bp for knockout) and PCR2 (primers 1 and 3, 2038 bp as a wild-type control). Under the conditions used, PCR2 did not ever amplify ~3400 bp, including the blastocidin-resistance cassette, in the knockout, but the lack of a PCR product indicated a disruption of the *svkA* gene as well. (C) Western blot of 2×10^5 cells per lane, visualized with kinase-specific antibodies confirms the knockout at the level of SvkA protein and also shows the expression of the GFP fusion protein in the wild-type (first lane) and the mutant background (fourth lane). (D) GFP-SvkA-FL, immunoprecipitated from rescue cells, phosphorylated domains 2 and 3 of severin (DS211C, left), myelin basic protein (MyBP, right) and itself (both lanes).
cultures (150 rpm) showed that cells in shaking cultures remained multinucleated (Fig. 4A,B). At higher velocity (220 rpm), the number of large cells (more than four nuclei) was reduced in favor of mono- and binucleated cells (data not shown). Apparently, an increased shear force helps to rupture the thin cytoplasmic bridges between incompletely separated cells. In cultures grown on solid surfaces until confluence or on a lawn of *K. aerogenes*, it was noted that the cells tended to become smaller again, possibly because so-called ‘midwife cells’ assisted in cell division (supplementary material Movie 3).

Single-cell analysis of cell division
To analyze cell division at higher resolution, we recorded phase-contrast time-lapse movies of individual *svkA*-knockout cells in HL-5 medium. Frequently, dividing cells followed at first the usual sequence of events: rounding, spreading, polarization and formation of a cleavage furrow (Fig. 5A). In 10–20% of the observed cell divisions, formation of the cleavage furrow was asymmetric. Initially, the cleavage furrow appeared normal, but then further constriction progressed asymmetrically, leading to a crescent-shaped intermediate. The final cytoplasmic bridge did not sever and was sometimes still visible after more than 1 hour. In many cases, the thin connection allowed a subsequent fusion of the two pseudo-daughter cells, thus accounting for the appearance of cells with increasing numbers of nuclei. The corrupted formation of the cleavage furrows in binucleated mutant cells led to the formation of structures reminiscent of a four-part clover leaf (Fig. 5B) that either divided into mono- and multinuclear cells or frequently failed to separate altogether.

To mark key events of cell division, we transformed *svkA*-knockout cells with vectors encoding different GFP constructs (alpha-tubulin, coronin, cortexillin, histone 2B and myosin II). None of these constructs altered the aberrant cytokinesis, and the cells remained large and multinucleated. Furthermore, overexpression of the homologous Ste20-like kinase Krs1 as a GFP fusion protein (Arasada et al., 2006) or the in vitro substrate GFP-severin did not alter the phenotype. The knockout cells progressed normally through the first steps of cell division as judged by: (1) the separation of chromosomes (GFP-histone 2B), (2) the assembly and disassembly of a spindle (GFP-alpha-tubulin), and (3) the first indications of a cleavage furrow (GFP-cortexillin I). The only mislocalization in comparison with normal cell division was observed occasionally in knockout cells expressing GFP-cortexillin I (Fig. 5C; supplementary material Movie 4).

Immunostaining of fixed *svkA*-knockout cells showed that dividing cells became polarized, as judged by the localization of F-actin (Fig. 6A,B) and coronin (Fig. 6G). Cortexillin I was enriched in the midzone of the cleavage furrow (Fig. 6C,E), myosin II was observed at the polar ends and the midzone of the cell as expected (Fig. 6D). DGAP1 was found in the late cleavage furrow (Fig. 6F), and alpha-tubulin was visualized at the centrosome, the mitotic spindle and in the midzone, also as expected (Fig. 6A-G).
The localization of SvkA supports its role during the late stages of cytokinesis

Fixed cells expressing GFP-SvkA-FL in the knockout background showed an overall localization of the kinase in the cytosol, to a lesser extent also in the nuclei and with a somewhat diffuse, but clear, enrichment around the centrosome (Fig. 7A). This centrosomal localization was also observed in fixed wild-type cells expressing the C-terminal half of the kinase (GFP-SvkA-CT; Fig. 7B). In dividing cells, GFP-SvkA-FL formed a cloud around the spindle and nuclei, without clear colocalization with the microtubules (Fig. 7C).

In an analysis of live cells undergoing cell division, GFP-SvkA-FL was enriched in the cleavage furrow during the late stage of cytokinesis (Fig. 8A), which is the most affected stage in the svkA knockout (Fig. 5C). The remnants of a midbody-like structure with its enriched GFP-SvkA-FL were observed for several minutes at the surface of the two daughter cells (Fig. 8A).

The localization of SvkA is a function of the C-terminal half of the kinase because a GFP-SvkA-CT construct in the svkA-null background, which could not rescue the aberrant cytokinesis phenotype, still localized at the centrosome (data not shown) and, during later phases of cytokinesis, localized to the cleavage furrow (Fig. 8B). The expression of a kinase-dead GFP-SvkA-K134A construct could not rescue the cytokinesis defect, although it localized to the midzone (Fig. 8C). This highlights the combined importance of proper localization and SvkA kinase activity for cytokinesis.

Discussion

Disruption of the svkA gene in D. discoideum led to defects in development, phototaxis and, most prominently, in cytokinesis. SvkA-null cells became multinucleated on solid surfaces as well as in shaking cultures. The appearance of multinucleated cells seemed to be the consequence of incomplete cytokinesis because svkA-null cells started to divide normally but
frequently failed to sever the final connecting bridge between the two daughter cells. This led to a frequent re-fusion of daughter cells, resulting in multinucleated cells. The enrichment of GFP-tagged SvkA at centrosomes and in the late cleavage furrow suggests that the kinase regulates the final stages of cytokinesis. All defects could be rescued by expression of a full-length GFP-SvkA kinase fusion protein.

The observed severe phenotype of the svkA mutant cannot easily be explained by absent phosphorylation of the F-actin-fragmenting protein severin. First, the phosphorylation of severin that coined the name ‘severin kinase’ is based on in vitro data only (Eichinger et al., 1998). Second, disruption of the gene encoding severin resulted in mutants with only subtle defects (André et al., 1989). Thus it is reasonable to conclude...
that severin is not the primary target of SvkA in a regulatory cascade during cell division. This would be in agreement with data from animal cells, where the later stages of cytokinesis become insensitive to the actin-depolymerizing drug latrunculin, implying that the plasma membrane is linked to the midbody by a connection that does not involve actin dynamics (Eggert et al., 2006).

This raises a question regarding the identity of other proteins that could interact with, or become phosphorylated by, SvkA. In mammalian cells, the Golgi matrix protein GM130 interacts with the SvkA homolog YSK1 and directs the kinase to the Golgi (Preisinger et al., 2004). There is no clear homolog of GM130 in *D. discoideum*, and the svkA-null cells showed no disruption of the overall Golgi structure (data not shown). Thus, it is unlikely that the functions of SvkA are similar to those of YSK1, at least as far as the organization of the Golgi is concerned. Furthermore, the only known downstream target for YSK1 is a 14-3-3 protein, and 14-3-3 proteins have been implicated in regulating cell polarity and cytokinesis (Hurd et al., 2003; Mishra et al., 2005). Another candidate is a nuclear Dbr2 related (NDR) kinase, which plays a role in cell-cycle progression and cell morphology (Hergovich et al., 2006). Also pertinent is the fact that a mammalian Ste20-like kinase (MST3), which possesses in its catalytic domain 75% sequence identity to SvkA, phosphorylates NDR2 at a hydrophobic motif (Stegert et al., 2005).

The localization of *D. discoideum* SvkA during the late stages of cytokinesis fits very well to reports on other Ste20-like kinases. In the fission yeast *Schizosaccharomyces pombe*, the Ste20-like kinase Nak1/Orb3, a member of the GCK-III family, is required for cell separation and late stages of cytokinesis (Leonhard and Nurse, 2005). In addition, *Ustilago maydis* mutants that lack the Ste20-like kinase Don3 fail to trigger initiation of a secondary septum necessary for cell separation after mitosis (Sandrock et al., 2006). The Ras association domain family 1A (RASSF1A) gene product binds to human MST2 and forms with additional proteins a complex that controls apoptosis and exit from mitosis (Guo et al., 2007). This complex is localized at centrosomes, spindle poles and the midbody. The distribution of SvkA is very similar, suggestive of the existence of a similar complex in *D. discoideum*. But the mammalian and insect signaling pathway relies on the formation of a complex by means of a SARAH (Salvador, RASSF1, Hippo) domain that is not present in SvkA. In *D. discoideum*, an explicit SARAH domain is only present in the Ste20-like kinase Krs1 (Arasada et al., 2006). It is intriguing, however, that the C-terminus of SvkA is essential for the distribution to the midzone in dividing cells. However, the inability of the kinase-dead construct to rescue the aberrant phenotype clearly shows that phosphorylation of a currently unknown substrate is an essential function of SvkA.

Fig. 6. Analysis of cytokinesis with marker proteins. (A-G) Confocal sections of fixed svkA-knockout cells representing different stages and the symmetrical population of SvkA-null cell division. All cells are stained for alpha-tubulin and in addition for F-actin (A,B) (the arrow in A indicates the dividing cell), cortexillin I (C,E), myosin II (D), DGAP1 (F) or coronin (G). Bars, 5 μm.
A variety of gene disruptions in *D. discoideum* lead to defects in cytokinesis (Adachi, 2001; Robinson and Spudich, 2004; Glotzer, 2005) and, according to these data, Uyeda and Nagasaki divided cytokinesis into four different types (Uyeda and Nagasaki, 2004): (1) cytokinesis A, the so called ‘purse string method’, where a ring of actin and myosin II contracts in the cleavage furrow until the two daughter cells are fully separated. Besides myosin II, several other proteins were reported as important for cytokinesis A – for example clathrin (Gerald et al., 2001; Niswonger and O’Halloran, 1997), LvsA, a protein implicated in lysosomal membrane processing (Kwak et al., 1999), possibly PAKa, whose exact function is still unclear (Chung and Firtel, 1999; Müller-Taubenberger et al., 2002), RasG (Tuxworth et al., 1997) and talin (Niewohner et al., 1997). (2) Cytokinesis B was postulated as ‘attachment-assisted mitotic cleavage’. The best examples are myosin-II-null cells, which become multinucleated and burst in shaking suspension but can divide normally on a solid surface (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Neujahr et al., 1997a; Neujahr et al., 1997b). The lack of a contractile ring in these mutants allows only a passive furrowing that is driven by opposite polar traction forces. Proteins involved in this myosin-II-independent form of cytokinesis are, among others, aggregation minus A (AmiA)
(Nagasaki et al., 2002), coronin (de Hostos et al., 1993), cortexillin I and II (Faix et al., 1996) and profilin I and II, based on evidence from gene disruption (Haugwitz et al., 1994). (3) Cytokinesis C, as classified by Uyeda and Nagasaki (Uyeda and Nagasaki, 2004), is an extension of this traction-mediated separation and rather unluckily termed ‘cytokinesis’ as it is cell-cycle independent. It describes the decreasing volume of a multinucleated cell that tears itself apart, exploiting adhesive forces and actin-driven cell migration. The resulting decrease in the number of nuclei occurs also in large and multinucleated wild-type cells that have been generated by forced cell fusion (Neujahr et al., 1997b). Thus, this type of cell separation is better described as cytofission. In the svkA-knockout cells, we observed a similar effect when we reduced the osmolarity of the medium and by this way induced cell separation. (4) Uyeda and Nagasaki (Uyeda and Nagasaki, 2004) classified cytokinesis D as a cell-cycle-dependent form of division that needs the help of midwife cells that, attracted by a stimulus, forcefully sever the remaining connection between the two emerging daughter cells (Insall et al., 2001). For Entamoeba invades, it was shown that 30% of division events rely on the activities of midwife cells (Biron et al., 2001). In svkA-null mutants, we also observed this type of cytokinesis, especially in dense cultures (supplementary material Movie 3). However, we stress the point that the remaining strings between daughter cells are the primary defect in cytokinesis D and that the mechanical separation by cells that move across these bridges is a secondary effect.

Under these assumptions, the defective cytokinesis in svkA-knockout cells can best be described as defective cytokinesis D because the essential feature of this type of cell division is the lack of final cleavage. The localization of SvkA to the midzone underscores the importance of this kinase for the regulation of this crucial step during cytokinesis.

Materials and Methods

D. discoideum strains, growth conditions, development and mutant analysis

D. discoideum strain AX2 (referred to as wild type) and transformants derived from this strain were cultivated in suspension culture with HL-5 medium (1% proteose peptone, 0.5% yeast extract (both Oxoid), 1% glucose, 3.6 mM NaHPO₄, 3.5 mM KH₂PO₄, pH 7.5) at 21°C, essentially as described previously (Urushihara, 2006). For development, cells were washed in phosphate buffer (14.6 mM KH₂PO₄, 2 mM NaHPO₄, pH 6.0), resuspended at a density of 1×10⁷ cells/ml, starved for at least 6 hours, and the movement towards a capillary filled with cAMP was observed, essentially as described previously (Mendoza and Firtel, 2006). For development on a solid substratum, growing cells were harvested, washed and transferred onto phosphate agar plates (9 cm) at a density of 1×10⁵ cells per plate (Dormann and Weijer, 2006). To analyze phototaxis of slugs, cell aggregates from the edge of a colony growing into a lawn of Klebsiella aerogenes were placed on water agar plates (9 cm) (Fisher and Annesley, 2006). Slugs were allowed to form and migrate towards light for 2 to 3 days before slugs and slime trails were transferred to nitrocellulose filters and visualized with antibodies against actin.

Gene knockout, rescue and expression of SvkA constructs

Gene knockouts were performed as described previously (Faix et al., 2004), using electroporation and homologous recombination with a blasticidin S resistance cassette flanked by two homologous fragments of the kinase gene. Genomic DNA of blasticidin-resistant clones was tested by PCR with knockout-specific primers. The blasticidin-resistance cassette was removed using the cre-loxP system, as described previously (Faix et al., 2004); the knockout mutants did not show any changes in the phenotype after removal of the resistance cassette. Full-length SvkA (GFP-SvkA-FL, amino acids 1–478) and the C-terminal domain (GFP-SvkA-CT, amino acids 278–478) were amplified from genomic DNA. The kinase-dead construct (GFP-SvkA-K134A) was generated by

Fig. 8. Live-cell recordings show SvkA in the midzone. (A) Wild-type cells expressing GFP-SvkA-FL accumulate the kinase only after the appearance of the cleavage furrow. The arrows indicate the localization of SvkA in the midzone during cell separation. (B) A svkA-knockout cell (confocal section of live cell, agar overlay) containing four nuclei (stars) and expressing the CT-terminal domain of SvkA. GFP-SvkA-CT determines localization to the midzone but is not sufficient to prevent incomplete cell division (see renewed fusion of partially separated cells in frame 11'). (C) SvkA-knockout cells expressing the dead kinase show that GFP-SvkA-K134A-FL localizes to the late cleavage furrow (arrows). (The stars indicate the approximate localization of the nuclei outside the focus plane. Bars, 5 μm.)
overlap extension PCR introducing a K134A point mutation. All constructs were cloned into the DpKO vector (Noegel, 2004). The constructs were transformed into the knockout and wild-type strains, resulting in expression of N- terminal GFP fusion proteins under the control of the actin 15 promoter.

Fluorescence microscopy
Cells were fixed with picric acid/formaldehyde as described previously (Hagedorn et al., 2006). F-actin was detected using Alexa-488- or TRITC-labeled phalloidin (Invitrogen or Sigma). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) or ToPro-3 (Invitrogen). For immunofluorescence, primary antibodies were visualized with Alexa-488-conjugated (Invitrogen) or Cy3-conjugated (Dianova) goat anti-mouse or goat anti-rat IgG. Cells were analyzed on an Axiovert 200 (Zeiss, Germany) or, for confocal microscopy, on a LSM 510 Meta (Zeiss, Germany). Live cells expressing GFP-fusion proteins were observed in phosphate buffer with 4% glucose (with 50 mM HEPES). Immunoprecipitations were performed with polyclonal antibodies against GFP as previously described (Hagedorn et al., 2006). Monoclonal antibodies were visualized with Alexa-488-conjugated (Invitrogen) or Cy3-conjugated (Invitrogen or Sigma). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI).

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