Cdc42 is required for PIP_2-induced actin polymerization and early development but not for cell viability

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Background: Cdc42 and other Rho GTPases are conserved from yeast to humans and are thought to regulate multiple cellular functions by inducing coordinated changes in actin reorganization and by activating signaling pathways leading to specific gene expression. Direct evidence implicating upstream signals and components that regulate Cdc42 activity or for required roles of Cdc42 in activation of downstream protein kinase signaling cascades is minimal, however. Also, whereas genetic analyses have shown that Cdc42 is essential for cell viability in yeast, its potential roles in the growth and development of mammalian cells have not been directly assessed.

Results: To elucidate potential functions of Cdc42 mammalian cells, we used gene-targeted mutation to inactivate *Cdc42* in mouse embryonic stem (ES) cells and in the mouse germline. Surprisingly, Cdc42-deficient ES cells exhibited normal proliferation and phosphorylation of mitogen- and stress-activated protein kinases. Yet Cdc42 deficiency caused very early embryonic lethality in mice and led to aberrant actin cytoskeletal organization in ES cells. Moreover, extracts from Cdc42-deficient cells failed to support phosphatidylinositol 4,5-bisphosphate (PIP_2)-induced actin polymerization.

Conclusions: Our studies clearly demonstrate that Cdc42 mediates PIP_2-induced actin assembly, and document a critical and unique role for Cdc42 in this process. Moreover, we conclude that, unexpectedly, Cdc42 is not necessary for viability or proliferation of mammalian early embryonic cells. Cdc42 is, however, absolutely required for early mammalian development.

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Received: **27 March 2000**
Revised: **23 May 2000**
Accepted: **23 May 2000**

Published: **15 June 2000**

Current Biology 2000, 10:758–765

0960-9822/00/$ – see front matter

Background

The Rho GTPases are a subgroup of the Ras superfamily of 20–30 kDa GTP-binding proteins that includes Rho, Rac and Cdc42. These proteins are ubiquitously expressed from yeast to humans, conserved in primary structure and 50–55% homologous to each other. Rho GTPases act as binary molecular switches by cycling between inactive GDP-bound and active GTP-bound forms to regulate various cellular functions [1–3]. Microinjection and overexpression studies in mammalian cells have revealed roles for Rho, Rac and Cdc42 in actin cytoskeleton remodeling in response to extracellular stimuli [1,2]. Increasing evidence has also suggested that Rho GTPases have important roles in diverse cellular processes such as transcriptional regulation, cell-cycle progression, membrane trafficking, chemotaxis and axonal guidance [1,3–12]. Furthermore, potential developmental roles of Rho GTPases have been implicated from genetic analyses of *Drosophila* and *Caenorhabditis elegans*, and from transgenic mouse studies [13].

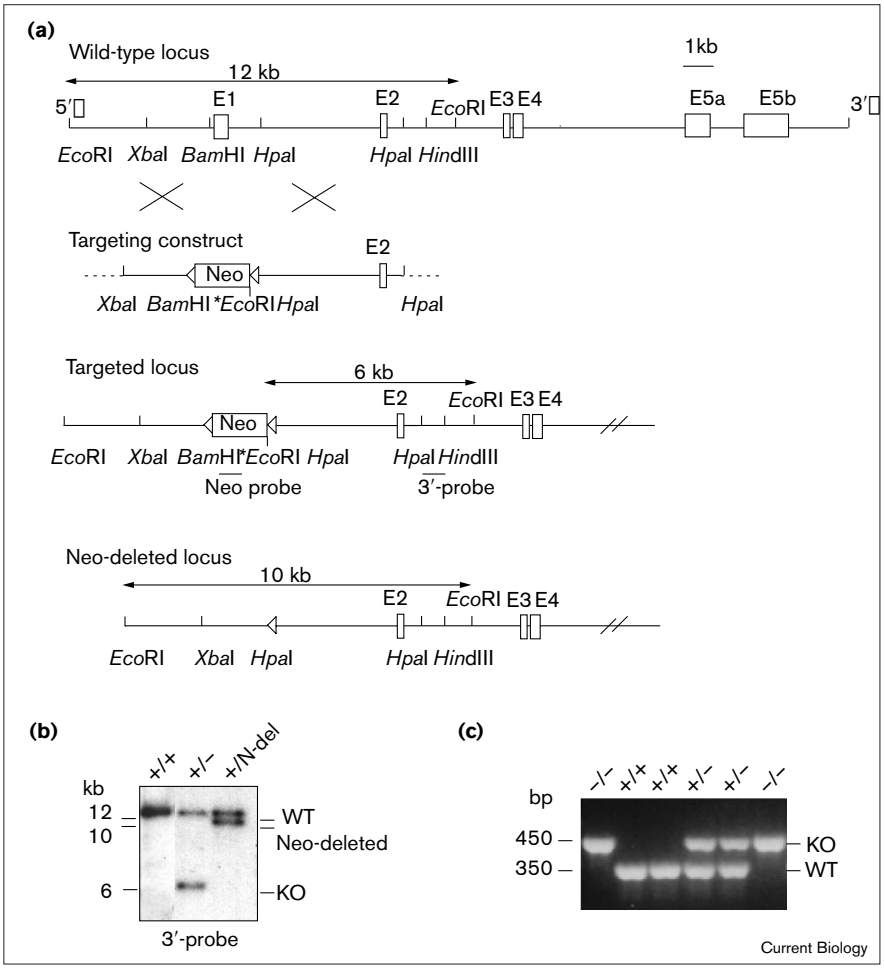
Cdc42 was discovered as an essential gene in *Saccharomyces cerevisiae* that is required for budding and establishment of cell polarity [14–16]. In fibroblasts, overexpression of activated Cdc42 leads to filopodia formation, whereas activated Rac and Rho induce lamellipodia and stress fibers respectively [17–19]. A hierarchical relationship has been proposed in which Cdc42 is a proximal mediator that signals to Rac [1,19]. Recently, N-WASP, a homolog of the Wiskott–Aldrich syndrome protein (WASP), has been shown to provide a critical link between Cdc42 signaling and actin polymerization [20–25]. Information on upstream signals and components that regulate Cdc42 activity with respect to actin assembly is limited, however. Furthermore, whereas Cdc42 and Rac, but not Rho, have been implicated in the stress-activated protein kinase signaling cascades, the physiologic significance of these findings has not been assessed [3,7,26,27].

Results and discussion

Cdc42 deficiency results in early embryonic lethality

To directly assess the physiological consequences of Cdc42 deficiency, we inactivated *Cdc42* in the mouse germline. The murine and human *Cdc42* genes contain six coding exons (Figure 1a) [28,29]. The first *Cdc42* coding exon

Figure 1

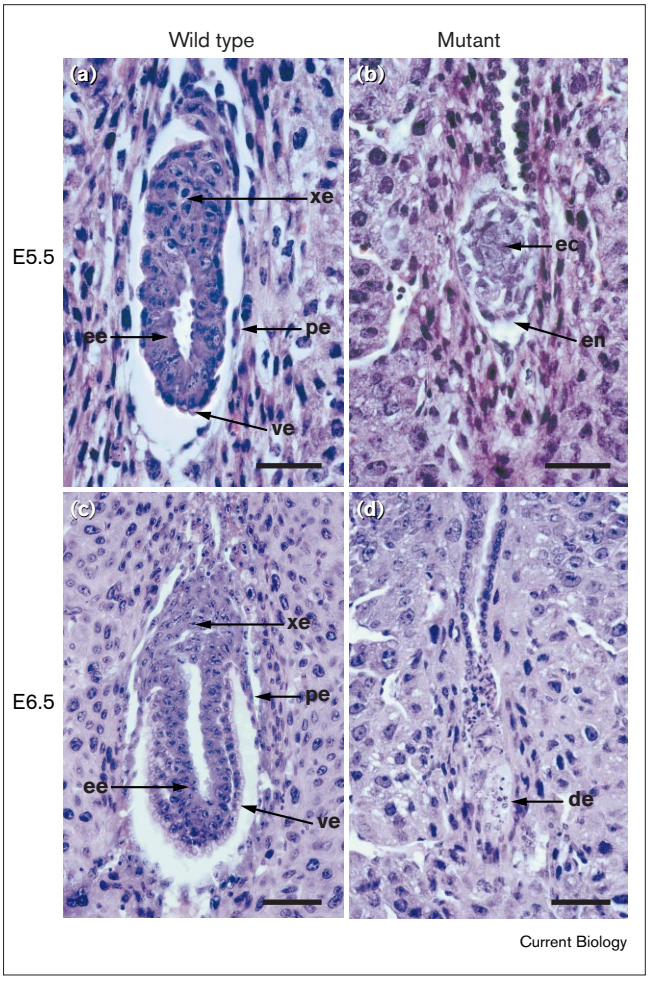


Targeting of murine *Cdc42*. **(a)** The genomic locus of the murine *Cdc42* gene is shown at the top; open boxes indicate exons identified. The knock-out vector to replace the first coding exon of *Cdc42* contains a PGK–Neo cassette flanked by *loxP* sites (triangles), a 4.5 kb 3′-homology region (*Hpa*I–*Hpa*I fragment) and a 2.5 kb 5′-homology region (*Xba*I–*Bam*HI fragment). Note that both *Eco*RI sites in the wild-type locus lie outside the homologous recombination region. **Eco*RI indicates a new restriction site brought in upon gene targeting and is used to distinguish the knock-out allele from the wild-type allele. The targeted alleles with (targeted locus) or without (Neo-deleted) the Neo marker (before or after Cre-deletion) are shown at the bottom. Also shown are two probes used for Southern analysis: the 3′-probe (*Hpa*I–*Hin*dIII) is outside the 3′-homology region; the Neo probe (*Pst*I–*Bam*HI) is inside the coding region of the neomycin-resistance gene. **(b)** Southern analysis (*Eco*RI digest, 3′-probe) of tail DNA from mice carrying *Cdc42* wild-type (WT, +/+), single knock-out (KO, +/−) and Neo-deleted (+/N-del) alleles. **(c)** PCR assay for genotypes of individual blastocysts derived from intercrosses of heterozygous *Cdc42* knock-out mice.

encodes the amino-terminal domain of the Cdc42 protein, which binds GTP and the catalytic magnesium ion. This domain is essential for GTPase activity and is also responsible for the interactions between Cdc42 and target proteins [30–32]. To generate a null mutation of *Cdc42*, we used homologous recombination to replace the first coding exon with a *loxP*-flanked PGK-Neo cassette in TC1 ES cells. The *loxP* sites ensure that the neomycine-resistance selection marker (Neo) can be deleted via Cre-mediated recombination to generate a 'clean' deletion [33]. Multiple independent $Cdc42^{+/-}$ ES clones were identified by Southern analyses and three were used to generate chimeric mice that were bred for germline transmission.

Crosses of $Cdc42^{+/-}$ mice revealed that Cdc42 deficiency causes early embryonic lethality. Thus, no $Cdc42^{-/-}$ offspring were born and no $Cdc42^{-/-}$ embryos were recovered as early as embryonic stage 7.5 (E7.5) (Figure 1b, Table 1). Intercrosses between Neo-deleted $Cdc42^{+/-}$ mice also failed to yield homozygous mutant embryos (data not shown). Histological analyses of all uterine decidua recovered at E5.5 from timed matings of $Cdc42^{+/-}$ mice revealed that approximately 25% of the embryos were smaller than normal, disorganized in structure and largely lacking embryonic primary ectoderm (Figure 2a,b). By E6.5, a similar proportion had largely degenerated (Figure 2c,d). Statistically, the defective embryos are likely to be *Cdc42* deficient as analyses of embryos derived from intercrosses of the wild-type mice with the same genetic background revealed that less than 5% were abnormal (Figure 2, and data not shown). Recently, Rac1 was also found to be required for embryonic development, but lethality occurred at a slightly later stage [34]. We note, however, that our developmental findings do not rule out the possibility that absolute Cdc42 deficiency could result in even earlier lethality, as it is conceivable that maternal Cdc42 might fulfill early developmental functions in $Cdc42^{-/-}$ embryos. In fact, given that the *Cdc42* null mutation is cell lethal in yeast, and that only one mammalian homolog has been reported, it seemed possible that complete absence of Cdc42 might even cause lethality of murine cells.

Figure 2

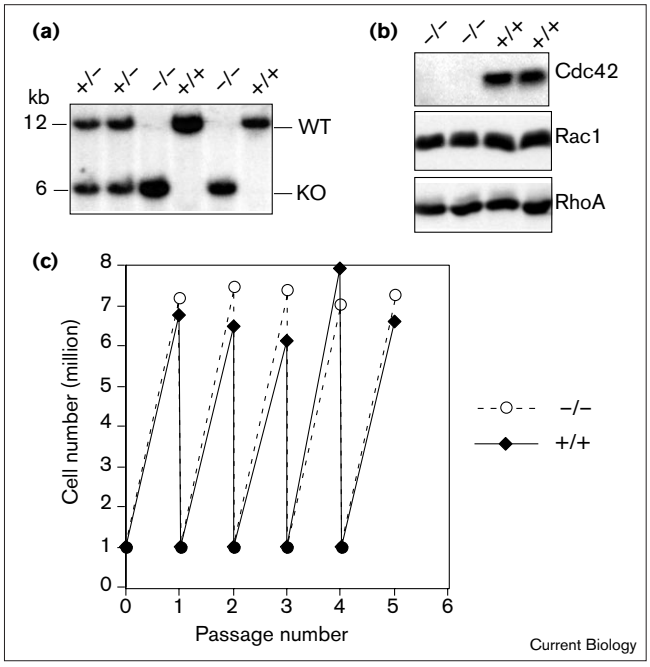


Histological examination of *in utero* embryos from matings of *Cdc42*$^{+/-}$ mice. The uteri of female *Cdc42*$^{+/-}$ mice were dissected at **(a,b)** E5.5 and **(c,d)** E6.5 after mating with *Cdc42*$^{+/-}$ males. Sagittal sections of 6–7 μm thickness were taken for all uterine decidua from each litter, followed by staining with hematoxylin and eosin (HE). Four litters were examined at E5.5; 7 out of 32 embryos were phenotypically abnormal as shown in the column labeled Mutant (presumptive *Cdc42*$^{-/-}$ mutants). Four litters were examined at E6.5; 6 out of 27 embryos were presumptive *Cdc42*$^{-/-}$ mutants. (a,c) Presumptive wild-type and heterozygous *Cdc42* embryos. ee, Primary embryonic ectoderm; pe, parietal endoderm; ve, visceral endoderm; xe, primary extra-embryonic ectoderm. Note the distinct differentiated cell layers and polarized organization of ee. (b,d) Presumptive *Cdc42*$^{-/-}$ mutant embryos. de, Dead cells. Note the initial differentiation of primary ectoderm (ec) and primary endoderm (en), but the disorganized primary ectoderm (at E5.5) and degeneration of the embryos (at E5.5 and E6.5). The scale bars represent 50 μm.

Cdc42 is not required for viability, proliferation or MAP kinase activation in ES cells

To address the cell lethality question, we first attempted to generate *Cdc42*$^{-/-}$ ES cells by culturing *Cdc42*$^{+/-}$ ES cells in increased concentrations of G418 and by re-targeting the second allele after Cre deletion of the Neo marker

Figure 3



Generation and characterization of *Cdc42*$^{-/-}$ ES cells. **(a)** Southern hybridization (*Eco*RI digest, 3′-probe) of ES cell lines derived from individual *Cdc42*$^{+/+}$, *Cdc42*$^{+/-}$ and *Cdc42*$^{-/-}$ blastocysts. **(b)** Western blotting analysis of *Cdc42*$^{+/+}$ and *Cdc42*$^{-/-}$ ES cell lines. Anti-Cdc42 (Santa Cruz Biotechnology) is a rabbit polyclonal antibody specifically against a peptide mapping near the carboxyl terminus of Cdc42, whereas anti-Rac (clone 23A8, Upstate Biotechnology) and anti-RhoA (clone 26C4, Santa Cruz Biotechnology) are both mouse monoclonal antibodies. **(c)** Growth rate of ES cell lines: *Cdc42*$^{+/+}$ (solid line with filled diamonds) and *Cdc42*$^{-/-}$ (dashed line with open circles). The early-passage ES cells were grown in complete ES medium containing LIF. One million cells were plated in each well of a six-well plate with feeder cells. Two independent cell lines of *Cdc42*$^{+/+}$ and *Cdc42*$^{-/-}$ were used, and three wells were plated in parallel for each cell line. At each passage (every 3 days when the culture reached 70–80% subconfluency) ES cells were trypsinized and counted. Data represent one of two independent experiments with similar results.

from *Cdc42*$^{+/-}$ ES cells (data not shown). Despite extensive efforts, however, no *Cdc42*$^{-/-}$ ES cells could be derived by these approaches. Next, we used a PCR-based genotyping strategy to demonstrate that *Cdc42*$^{-/-}$ blastocysts were viable and visually normal at E3.5. Moreover, when individually cultured on gelatin-coated dishes, *Cdc42*$^{-/-}$ blastocysts grew and differentiated *in vitro* in a manner similar to that of wild-type blastocysts (Figure 1c, Table 1). The viability of *Cdc42*$^{-/-}$ blastocysts suggested the possibility of deriving *Cdc42*$^{-/-}$ ES cells directly [35]. By culturing single blastocysts on feeder cells, we succeeded in obtaining *Cdc42*$^{-/-}$ ES cells, as confirmed both by Southern (Figure 3a) and western blotting analyses (Figure 3b). As predicted for a null mutation, the *Cdc42*$^{-/-}$ ES expressed no detectable Cdc42 protein, whereas levels of several other Rho GTPases tested for, including Rac1 and RhoA, remained comparable to those of wild-type ES cells

Table 1

Genotypes of neonates and embryos derived from *Cdc42*$^{+/-}$ intercrosses.

Stage	Total	+/+	+/−	−/−	Resorbed
Full term	86	37	49	0	–
E13.5	36	9	15	0	12
E9.5	30	8	15	0	7
E8.5	21	5	11	0	5
E7.5	30	6	14	0	10
E3.5					
Blastocyst	43	9	24	10	–
Outgrowth	29	7	17	5	–

Timed breeding of heterozygous Cdc42 mice was set up in a pathogen-free facility. Neonates and embryos were harvested at indicated time. Blastocyst, freshly isolated E3.5 embryos; Outgrowth, blastocyst cultured on gelatin-coated dishes. Genotypes were assayed by Southern analysis or by PCR (E3.5).

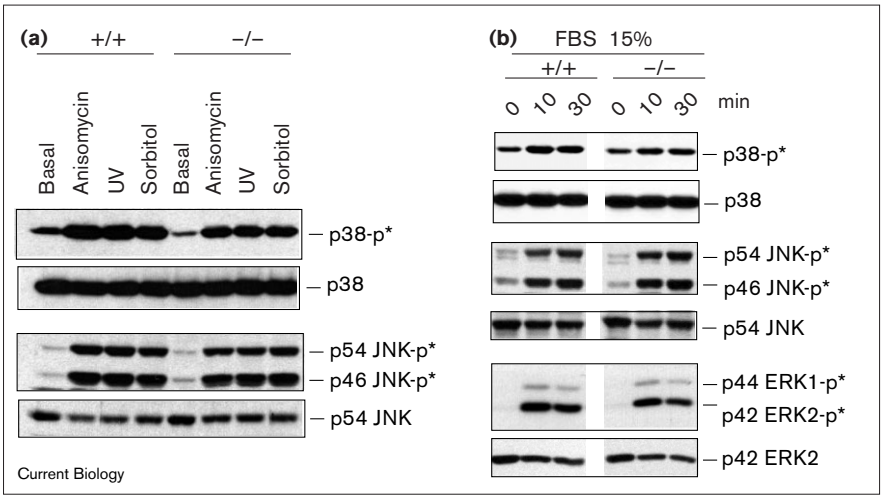
(Figure 3b, and data not shown). In subsequent studies, we focused on two ES cell lines of each genotype derived from littermate E3.5 embryos. Strikingly, we observed no gross proliferation defects of *Cdc42*$^{-/-}$ ES cells upon culture in complete ES medium for multiple continuous passages (Figure 3c).

Earlier studies in which mutant forms of Cdc42 were overexpressed in cell lines suggested a specific role for this protein in selective induction of the mitogen-activated protein kinase (MAP kinase) cascades [3,7,26,27]. To test directly for the requirement for Cdc42, we assayed *Cdc42*$^{-/-}$ ES cells for the ability to phosphorylate specific members of these cascades, including c-Jun N-terminal/stress-activated protein kinase (JNK/SAPK) and p38 as well as both isoforms of extracellular signal-regulated kinase (p44 ERK1 and p42 ERK2). Following treatment with anisomycin, UV irradiation or sorbitol, phosphorylation of both JNK and p38 kinases in *Cdc42*$^{-/-}$ ES cell lines was induced at comparable levels to that of wild-type cells (Figure 4a, and Supplementary material). We also found that JNK, p38 and both isoforms of ERK were similarly rapidly phosphorylated upon stimulation of *Cdc42*$^{-/-}$ ES cells with serum (Figure 4b, and Supplementary material). Our findings therefore clearly establish the existence of Cdc42-independent mechanisms for activation of these MAP kinase pathways.

Cdc42 is essential for PIP_2-induced actin polymerization

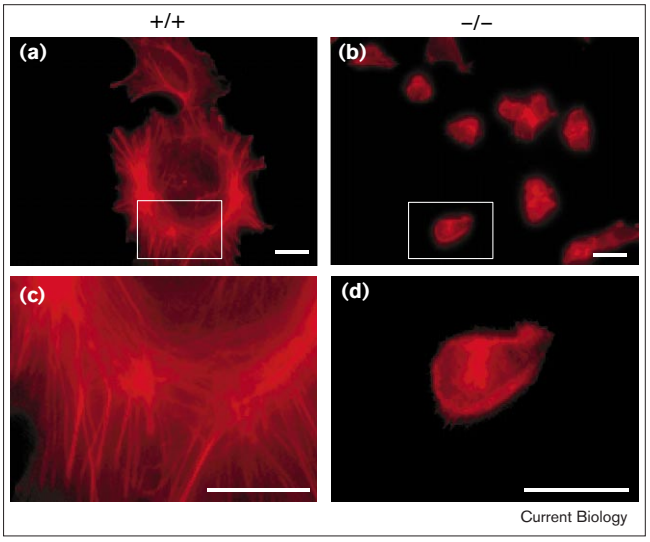
The actin cytoskeleton is a dynamic structure that cells maintain by tightly regulating temporal and spatial actin assembly in response to extracellular signals. When grown on fibronectin-coated coverslips, wild-type ES cells attached well and showed a rich mixture of actin

Figure 4



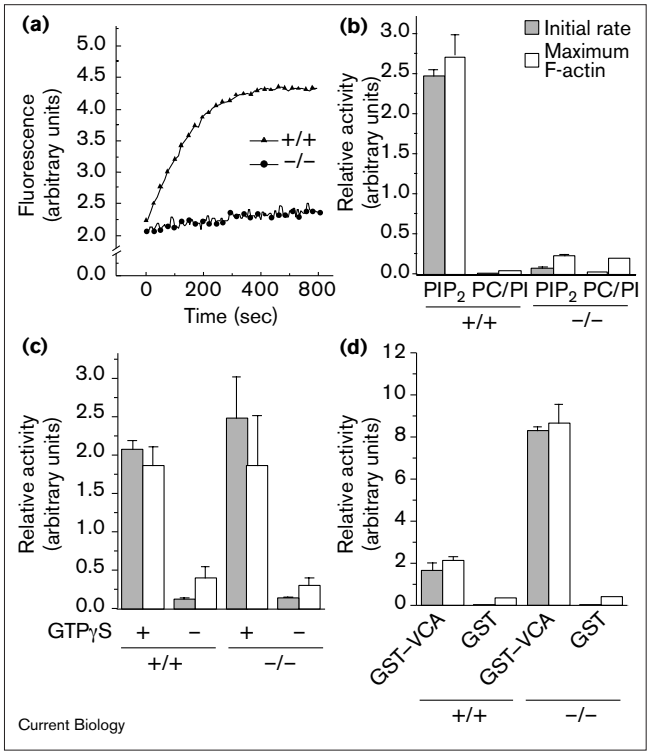
Activation of JNK, p38 and ERK in *Cdc42*$^{-/-}$ ES cells. ES cells were grown on gelatin-coated plates without feeder cells for at least two passages before being used in these experiments. All assays were conducted within five passages and two independent cell lines of *Cdc42*$^{+/+}$ and *Cdc42*$^{-/-}$ were used in parallel. Antibodies used in western blotting specifically detect dually phosphorylated isoforms of JNK (Thr183/Tyr185), p38 (Thr180/Tyr182) or p44/p42 ERK (Thr202/Tyr204). Three independent experiments were performed and one representative experiment is shown. Quantitative analysis of results can be found in the Supplementary material. **(a)** Stress-induced activation of JNK and p38. Subconfluent *Cdc42*$^{+/+}$ and *Cdc42*$^{-/-}$ ES cells were used in western blotting analysis after the following treatments: anisomycin at 20 μg/ml for 30 min; UV irradiation for 80 J/m^2 and recovery for 30 min in tissue culture incubator (UV); and sorbitol at 250 mM for 30 min. **(b)** Serum-induced activation of JNK, p38 and ERK. Subconfluent ES cells were starved in ES medium containing LIF and 0.5% FBS for 25–30 h, stimulated with 15% FBS for the indicated time (0, 10 and 30 min) and lysed for western blotting.

Figure 5



Cdc42 is required for actin cytoskeletal organization. Rhodamine–phalloidin staining of the actin cytoskeleton of *Cdc42*$^{+/+}$ and *Cdc42*$^{-/-}$ ES cells. Note the smaller size and rounded morphology of *Cdc42*$^{-/-}$ cells. Boxed regions in **(a,b)** are enlarged in **(c,d)**. Scale bars represent 10 μm.

Figure 6



Cdc42 is essential for PIP_2-induced actin polymerization *in vitro*. **(a)** Real-time measurement of pyrene actin polymerization in extracts of *Cdc42*$^{+/+}$ and *Cdc42*$^{-/-}$ ES cells stimulated by phosphatidylinositol-4,5-biphosphate (PIP_2). Filled triangles, +/+; filled circles, −/−. **(b)** Initial rate of pyrene actin polymerization (filled bars) and maximum F-actin (open bars) in response to PIP_2, phosphatidylcholine (PC) or phosphatidylinositol (PI) calculated from the type of data shown in Figure 4a as described in [25,38]. **(c)** The initial rate (filled bars) and maximum F-actin (open bars) for actin polymerization stimulated by GTPγS-charged wild-type Cdc42. **(d)** The initial rate (filled bars) and maximum F-actin (open bars) for actin polymerization stimulated by N-WASP carboxy-terminal fragment VCA.

cytoskeleton structures that included stress fibers, lamellipodia and microspikes of different sizes (Figure 5a,c). In contrast, more than 80% of the *Cdc42*$^{-/-}$ ES cells had a rounded morphology and were smaller in size. In addition, the actin structures present in the *Cdc42*$^{-/-}$ ES cells were limited to diffuse and disorganized cytoplasmic actin, concentrated actin in cortical areas of the cells, and a few short microspikes (Figure 5b,d). Given the fact that *Cdc42*$^{-/-}$ ES cells exhibited an abnormal actin cytoskeleton, it seemed likely that these mutant cells may also have motility and/or adhesion defects. Assaying for such defects in ES cells is problematic, however, as ES cells tend to form colonies and, in general, are not motile. To further address this issue, we cultured ES cells in differentiating medium free of lymphocyte inhibitory factor (LIF) to form embryoid bodies [35] and derived 'fibroblast-like cells'. Preliminary analyses of these *Cdc42*$^{-/-}$ cells suggested defective adhesion and migration of cells in the context of various extracellular matrix proteins including fibronectin, collagen and laminin (F.C. and F.W.A., unpublished data).

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a signaling intermediate that interacts with several actin-binding proteins [36,37]. Both the GTP-bound activated form of Cdc42 and PIP_2 can stimulate actin polymerization in *Xenopus* egg extracts and dominant-negative Cdc42 inhibits PIP_2-induced actin assembly [38–40]. To examine directly the functional relationship of PIP_2 and Cdc42 in actin assembly, we assessed the requirement for Cdc42 in PIP_2-induced actin polymerization. Cell extracts were prepared from wild-type and *Cdc42*$^{-/-}$ cells and pyrene actin assays were performed *in vitro* as previously described [38,41,42]. Whereas PIP_2 was able to induce robust actin assembly in extracts from wild-type cells, it failed to stimulate any actin polymerization in *Cdc42*$^{-/-}$ extracts. Negative controls using only the carrier lipids phosphatidylcholine (PC) and phosphatidylinositol (PI) validated the specific activity of PIP_2 (Figure 6a,b).

To ensure that extracts from *Cdc42*$^{-/-}$ cells had intact downstream actin assembly components, purified wild-type Cdc42 was used in reconstitution experiments. We demonstrated that activated Cdc42 (GTPγS charged) triggered actin polymerization in the *Cdc42*$^{-/-}$ extracts to an extent indistinguishable from that observed with wild-type extracts (Figure 6c). A critical link between Cdc42-dependent signaling and actin assembly has been proposed to be a ubiquitously expressed WASP family

protein, N-WASP [23–25,43]. N-WASP contains a domain that binds PIP_2, a Cdc42-binding (GBD) domain, a proline-rich region, a G-actin-binding verprolin homology (V) domain, a cofilin homology (C) domain and a carboxy-terminal acidic segment (A). A carboxy-terminal fragment of N-WASP containing the V, C and A domains interacts with the Arp2/3 complex and dramatically stimulates its actin nucleation ability [25,44–47]. Correspondingly, the N-WASP VCA fragment stimulated actin polymerization in *Cdc42*$^{-/-}$ extracts, but, unexpectedly, at an even higher rate than in wild-type extracts (Figure 6d). This enhanced stimulation of actin polymerization by the VCA domain in *Cdc42*$^{-/-}$ extracts might reflect the more ready availability of downstream components, such as the Arp2/3 complex, in *Cdc42*$^{-/-}$ extracts because of the presumably inactive state of endogenous N-WASP in the absence of Cdc42. Alternatively, it is conceivable that an inhibitory regulator of actin nucleation reaction in wild-type cells is downregulated in the *Cdc42*$^{-/-}$ cells to compensate for loss of positive regulation via Cdc42. In any case, our findings firmly establish that PIP_2-induced actin assembly is mediated by Cdc42.

Conclusions

In yeast, Cdc42 is essential for cell viability, and in mammalian cells, studies of dominant-negative mutants in established cell lines have implicated Cdc42 and Rac as essential for cell-cycle progression and Ras transformation [6,7,9,10]. Thus, it is striking that our genetic studies unequivocally show that Cdc42 is not required for ES cell viability or proliferation, although we do not exclude possible roles in other cell types. The differential requirements for Cdc42 between yeast cells and mammalian ES cells might be attributed to differences in cell physiology, for example with respect to the mechanisms used for cell division. On the other hand, such differences may also reflect the presence of redundant factors in mammalian cells compared to yeast. In this regard, we show that *Cdc42*$^{-/-}$ ES cells exhibit activation of the JNK, p38 and ERK pathways upon appropriate stimulation, providing direct evidence for redundant functions of different Rho GTPases and/or other factors in these MAP kinase signaling cascades. A related possibility would be the occurrence of compensatory increases in the expression of another Rho GTPase in *Cdc42*$^{-/-}$ ES cells, resulting in overlapping activity with Cdc42. At the expression level, we did not find such increases in Rac1 and RhoA in *Cdc42*$^{-/-}$ ES cells. We have not, however, ruled out increased expression of other Cdc42 homologs such as TC10 [48–50] or Chp [51], for which there are no readily available antisera.

Our studies clearly show that Cdc42 is absolutely required for the early stages of murine development and for normal actin cytoskeleton organization in ES cells. Moreover, our assays of extracts made from Cdc42-deficient ES cells provide direct evidence that PIP_2-induced actin polymerization in mammalian cells is mediated by Cdc42. In this context, local concentrations of plasma membrane PIP_2 have been proposed to regulate the local adhesion between the actin-based cortical cytoskeleton and plasma membrane and, therefore, to control cell shape and dynamic membrane functions [52,53]. Given that Cdc42 is not required for ES cell proliferation or activation of the MAP kinase cascades, we speculate that a major factor contributing to defective post-implantation development of Cdc42$^{-/-}$ embryos could be an inability to properly form and reorganize actin-based cellular structures crucial for further gastrulation.

Materials and methods

Generation and genotype analysis of Cdc42 knock-out mice and embryos

The *Cdc42* knock-out mice were generated by standard methods. To PCR genotype single blastocysts, timed breeding of *Cdc42*$^{+/-}$ mice was set up, E3.5 embryos were harvested individually into 20 μl lysis buffer (50 mM Tris pH 8.0, 0.5% Triton X-100, proteinase K to 1 mg/ml) and incubated at 50°C, overnight. The lysate was heat inactivated at 95°C for 5 min before PCR reaction with following primers: common forward primer: 5′-ATATCGGTCACTGTTCTACTTTG-3′; knock-out reverse primer: 5′-CCTTCTTGACGAGTTCTTCTGAGG-3′; wild-type reverse primer: 5′-AGTTGGTACATATTCCGATG-3′.

ES cell culture

ES cells were derived following protocols described in [28]. The early-passage *Cdc42*$^{+/+}$ and *Cdc42*$^{-/-}$ ES cell lines were cultured on gelatin-coated plates in DMEM (Gibco-BRL) containing 15% fetal bovine serum (FBS, Sigma), 2 mM L-glutamine (Gibco-BRL), 0.1 mM nonessential amino acids (Gibco-BRL), 0.1 mM β-mercaptoethanol (Sigma), 100 units/ml penicillin–streptomycin (Gibco-BRL), and 1000 units/ml lymphocyte inhibitory factor (LIF, Gibco-BRL) at 37°C and 5% CO_2.

MAP kinase analysis

Following the indicated treatment, ES cells were lysed in KLB buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% glycerol) supplemented with 1 mM PMSF, 1% aprotinin, 1 mM DTT and 0.1 mM H_2O_2-activated sodium pervanadate. Rabbit polyclonal antibodies against phospho-JNK, phospho-p38 and phospho-ERK (New England Biolabs) were used for western blotting. Protein normalization was done using antibodies against p54 JNK, p38 (New England Biolabs) and ERK2 (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies (Pierce) were used in an enhanced chemiluminescence detection method (Amersham).

Actin cytoskeleton staining

Coverglasses were coated with human plasma fibronectin (Gibco-BRL) at 37°C for 2 h and washed with PBS before use. 5×10^4 ES cells are seeded on fibronectin-coated coverglasses in a 24-well plate for 20 h and fixed in 4% paraformaldehyde. Cells were then permeabilized and stained with rhodamine-conjugated phalloidin.

In vitro *actin polymerization assay*

For the preparation of cell extracts, ES cell pellets were thawed and resuspended in an equal volume of lysis buffer (10 mM HEPES pH 7.6, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10 μg/ml chymostatin, pepstatin and leupeptin, and 0.5 mM PMSF). Cells were then broken using a probe sonicator and the lysate was centrifuged at 3000*g* for 30 min. The supernatant (low-speed ES cell extract) was carefully removed from the nuclear pellet, diluted sevenfold in lysis buffer, and centrifuged at 400,000*g* for 1 h. The clear supernatant was collected and concentrated to 0.5–1 volume of the

original low-speed extract using a Centriprep-10 spin column (Amicon Corp). The high-speed ES cell extract was then supplemented with an energy-regenerating mix (1 mM ATP, 1.25 mM $MgCl_2$, 7.5 mM creatine phosphate) and stored at –80°C. Actin polymerization was assayed in the ES cell extracts following a previously published protocol [38]. Briefly, cell extracts were diluted in the lysis buffer to the same final concentration (~10 mg/ml) and then supplemented with 1 μM pyrene-labeled rabbit skeletal muscle actin. Fluorescence was monitored in 80 μl samples of reaction mixture using a fluorospectrometer. After the basal fluorescence was stabilized within 5 min, 2–5 μl of phospholipids, Cdc42 or NWASP-VCA were added to stimulate actin polymerization. The initial rate was measured from the initial slope of fluorescence increase, and the maximum F-actin was calculated from the fluorescence difference between the peak and the baseline.

Supplementary material

Supplementary material including a table showing the activation of MAP kinases in wild-type and Cdc42-deficient ES cells is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We thank Roger Ferrini and Landy Kangaloo for technical assistance, Jeff Peterson for preparing lipid vesicles, Rajat Rohatgi for Cdc42 and Dyche Mullins (University of California San Francisco) for sharing the fluorospectrometer. We thank Rod Bronson, En Li, Zhenyu Gu and Mary Donahoe for assistance in histological analysis. F.C. is a postdoctoral fellow of the Irvington Institute for Immunological Research. This work was supported in part by NIH grants AI200047 (F.W.A.), GM26875 (M.W.K.), HL59561 (F.S.R. and F.W.A.), HL 59561-02 (T.K. and M.L.), HL56949, HL54188 (D.J.K.), and by NCI grant CA82258 (B.J.M.). F.W.A .and S.H.O. are investigators of the Howard Hughes Medical Institute.

References

1. Hall A: **Rho GTPases and the actin cytoskeleton.** *Science* 1998, **279:**509-514.
2. Ridley AJ: **Rho family proteins and regulation of the actin cytoskeleton.** *Prog Mol Subcell Biol* 1999, **22:**1-22.
3. Van Aelst L, D'Souza-Schorey C: **Rho GTPases and signaling networks.** *Genes Dev* 1997, **11:**2295-2322.
4. Alberts AS, Geneste O, Treisman R: **Activation of SRF-regulated chromosomal templates by Rho-family GTPases requires a signal that also induces H4 hyperacetylation.** *Cell* 1998, **92:**475-487.
5. Hill CS, Wynne J, Treisman R: **The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF.** *Cell* 1995, **81:**1159-1170.
6. Olson MF, Ashworth A, Hall A: **An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1.** *Science* 1995, **269:**1270-1272.
7. Lamarche N, Tapon N, Stowers L, Burbelo PD, Aspenstrom P, Bridges T, *et al.*: **Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade.** *Cell* 1996, **87:**519-529.
8. Drechsel DN, Hyman AA, Hall A, Glotzer M: **A requirement for Rho and Cdc42 during cytokinesis in Xenopus embryos.** *Curr Biol* 1997, **7:**12-23.
9. Qiu RG, Chen J, Kirn D, McCormick F, Symons M: **An essential role for Rac in Ras transformation.** *Nature* 1995, **374:**457-459.
10. Qiu RG, Abo A, McCormick F, Symons M: **Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation.** *Mol Cell Biol* 1997, **17:**3449-3458.
11. Lin R, Bagrodia S, Cerione R, Manor D: **A novel Cdc42Hs mutant induces cellular transformation.** *Curr Biol* 1997, **7:**794-797.
12. Wu WJ, Lin R, Cerione RA, Manor D: **Transformation activity of Cdc42 requires a region unique to Rho-related proteins.** *J Biol Chem* 1998, **273:**16655-16658.
13. Settleman J: **Rho GTPases in development.** *Prog Mol Subcell Biol* 1999, **22:**201-229.
14. Johnson DI: **Cdc42: an essential Rho-type GTPase controlling eukaryotic cell polarity.** *Microbiol Mol Biol Rev* 1999, **63:**54-105.
15. Johnson DI, Pringle JR: **Molecular characterization of CDC42, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity.** *J Cell Biol* 1990, **111:**143-152.
16. Richman TJ, Sawyer MM, Johnson DI: **The Cdc42p GTPase is involved in a G2/M morphogenetic checkpoint regulating the apical-isotropic switch and nuclear division in yeast.** *J Biol Chem* 1999, **274:**16861-16870.
17. Ridley AJ, Hall A: **The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors.** *Cell* 1992, **70:**389-399.
18. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A: **The small GTP-binding protein rac regulates growth factor-induced membrane ruffling.** *Cell* 1992, **70:**401-410.
19. Nobes CD, Hall A: **Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia.** *Cell* 1995, **81:**53-62.
20. Symons M, Derry JM, Karlak B, Jiang S, Lemahieu V, McCormick F, *et al.*: **Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization.** *Cell* 1996, **84:**723-734.
21. Aspenstrom P, Lindberg U, Hall A: **Two GTPases, Cdc42 and Rac, bind directly to a protein implicated in the immunodeficiency disorder Wiskott-Aldrich syndrome.** *Curr Biol* 1996, **6:**70-75.
22. Miki H, Miura K, Takenawa T: **N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases.** *EMBO J* 1996, **15:**5326-5335.
23. Miki H, Sasaki T, Takai Y, Takenawa T: **Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP.** *Nature* 1998, **391:**93-96.
24. Bi E, Zigmond SH: **Actin polymerization: Where the WASP stings.** *Curr Biol* 1999, **9:**R160-R163.
25. Rohatgi R, Ma L, Miki H, Lopez M, Kirchhausen T, Takenawa T, *et al.*: **The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly.** *Cell* 1999, **97:**221-231.
26. Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, *et al.*: **The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway.** *Cell* 1995, **81:**1137-1146.
27. Minden A, Lin A, Claret FX, Abo A, Karin M: **Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs.** *Cell* 1995, **81:**1147-1157.
28. Marks PW, Kwiatkowski DJ: **Genomic organization and chromosomal location of murine Cdc42.** *Genomics* 1996, **38:**13-18.
29. Nicole S, White PS, Topaloglu H, Beigthon P, Salih M, Hentati F, *et al.*: **The human CDC42 gene: genomic organization, evidence for the existence of a putative pseudogene and exclusion as a SJS1 candidate gene.** *Hum Genet* 1999, **105:**98-103.
30. Hoffman GR, Nassar N, Cerione RA: **Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI.** *Cell* 2000, **100:**345-356.
31. Abdul-Manan N, Aghazadeh B, Liu GA, Majumdar A, Ouerfelli O, Siminovitch KA, *et al.*: **Structure of Cdc42 in complex with the GTPase-binding domain of the 'Wiskott-Aldrich syndrome' protein.** *Nature* 1999, **399:**379-383.
32. Mott HR, Owen D, Nietlispach D, Lowe PN, Manser E, Lim L, *et al.*: **Structure of the small G protein Cdc42 bound to the GTPase-binding domain of ACK.** *Nature* 1999, **399:**384-388.
33. Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee E, *et al.*: **Efficient *in vivo* manipulation of mouse genomic sequences at the zygote stage.** *Proc Natl Acad Sci USA* 1996, **93:**5860-5865.
34. Sugihara K, Nakatsuji N, Nakamura K, Nakao K, Hashimoto R, Otani H, *et al.*: **Rac1 is required for the formation of three germ layers during gastrulation.** *Oncogene* 1998, **17:**3427-3433.
35. Robertson EJ: **Derivation and maintenance of embryonic stem cell cultures.** *Methods Mol Biol* 1997, **75:**173-184.
36. Janmey PA: **Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly.** *Annu Rev Physiol* 1994, **56:**169-191.
37. Hartwig JH, Bokoch GM, Carpenter CL, Janmey PA, Taylor LA, Toker A, *et al.*: **Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets.** *Cell* 1995, **82:**643-653.
38. Ma L, Cantley LC, Janmey PA, Kirschner MW: **Corequirement of specific phosphoinositides and small GTP-binding protein Cdc42 in inducing actin assembly in Xenopus egg extracts.** *J Cell Biol* 1998, **140:**1125-1136.
39. Ma L, Rohatgi R, Kirschner MW: **The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42.** *Proc Natl Acad Sci USA* 1998, **95:**15362-15367.

40. Moreau V, Way M: **Cdc42 is required for membrane dependent actin polymerization *in vitro*.** *FEBS Lett* 1998, **427:**353-356.
41. Zigmond SH, Joyce M, Borleis J, Bokoch GM, Devreotes PN: **Regulation of actin polymerization in cell-free systems by GTPgammaS and Cdc42.** *J Cell Biol* 1997, **138:**363-374.
42. Zigmond SH, Joyce M, Yang C, Brown K, Huang M, Pring M: **Mechanism of Cdc42-induced actin polymerization in neutrophil extracts.** *J Cell Biol* 1998, **142:**1001-1012.
43. Miki H, Suetsugu S, Takenawa T: **WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac.** *EMBO J* 1998, **17:**6932-6941.
44. Zigmond SH: **Actin cytoskeleton: The Arp2/3 complex gets to the point.** *Curr Biol* 1998, **8:**R654-R657.
45. Mullins RD, Pollard TD: **Rho-family GTPases require the Arp2/3 complex to stimulate actin polymerizationin in *Acanthamoeba* extracts.** *Curr Biol* 1999, **9:**405-415.
46. Higgs HN, Pollard TD: **Regulation of actin polymerization by Arp2/3 complex and WASp/Scar proteins.** *J Biol Chem* 1999, **274:**32531-32534.
47. Machesky LM, Mullins RD, Higgs HN, Kaiser DA, Blanchoin L, May RC, *et al.*: **Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex.** *Proc Natl Acad Sci USA* 1999, **96:**3739-3744.
48. Drivas GT, Shih A, Coutavas E, Rush MG, D'Eustachio P: **Characterization of four novel *ras*-like genes expressed in a human teratocarcinoma cell line.** *Mol Cell Biol* 1990, **10:**1793-1798.
49. Neudauer CL, Joberty G, Tatsis N, Macara IG: **Distinct cellular effects and interactions of the Rho-family GTPase TC10.** *Curr Biol* 1998, **8:**1151-1160.
50. Murphy GA, Solski PA, Jillian SA, de la Ossa PP, D-Eustachio P, Der CJ, *et al.*: **Cellular functions of TC10, a Rho family GTPase: regulation of morphology, signal transduction and cell growth.** *Oncogene* 1999, **18:**3831-3845.
51. Arohneim A, Broder YC, Cohen A, Fritsch A, Belisle B, Abo A: **Chp, a homologue of the GTPase Cdc42HS, activates the JNK pathway and is implicated in recognizing the actin cytoskeleton.** *Curr Biol* 1998, **8:**1125-1128.
52. Raucher D, Stauffer T, Chen W, Shen K, Guo S, York JD, *et al.*: **Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion.** *Cell* 2000, **100:**221-228.
53. Raucher D, Sheetz MP: **Cell spreading and lamellipodial extension rate is regulated by membrane tension.** *J Cell Biol* 2000, **148:**127-136.

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