Profilin 1 is required for abscission during late cytokinesis of chondrocytes

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Profilins are key factors for dynamic rearrangements of the actin cytoskeleton. However, the functions of profilins in differentiated mammalian cells are uncertain because profilin deficiency is early embryonic lethal for higher eukaryotes. To examine profilin function in chondrocytes, we disrupted the *profilin 1* gene in cartilage (*Col2pfn1*). Homozygous *Col2pfn1* mice develop progressive chondrodysplasia caused by disorganization of the growth plate and defective chondrocyte cytokinesis, indicated by the appearance of binucleated cells. Surprisingly, *Col2pfn1* chondrocytes assemble and contract actomyosin rings normally during cell division; however, they display defects during late cytokinesis as they frequently fail to complete abscission due to their inability to develop strong traction forces. This reduced force generation results from an impaired formation of lamellipodia, focal adhesions and stress fibres, which in part could be linked to an impaired mDia1-mediated actin filament elongation. Neither an actin nor a poly-proline binding-deficient profilin 1 is able to rescue the defects. Taken together, our results demonstrate that profilin 1 is not required for actomyosin ring formation in dividing chondrocytes but necessary to generate sufficient force for abscission during late cytokinesis.

The EMBO Journal (2009) 28, 1157–1169. doi:10.1038/emboj.2009.58; Published online 5 March 2009

Subject Categories: cell & tissue architecture; development

Keywords: chondrocytes; cytokinesis; knockout mice; profilin; traction force

Introduction

Profilins, the most versatile actin monomer-binding proteins, are abundantly expressed in all eukaryotic cells from plants

and fungi to mammals. Mammals have four profilin family members; profilin 1, which is ubiquitously expressed, and profilins 2–4, which have a restricted expression pattern (Witke *et al.*, 1998; Di Nardo *et al.*, 2000; Obermann *et al.*, 2005). Genetic and cell biological studies have implicated profilins in many cellular processes such as cell migration, cytokinesis, endocytosis and transcription regulation (reviewed in Witke, 2004). Among these different processes, the role of profilin in actin-driven cellular motility has been most thoroughly studied, and has been very conclusively linked to the biochemistry of profilins with their main ligand, actin (reviewed in Pollard and Borisy, 2003; Witke, 2004). Profilin enhances the rate of actin filament turnover through two molecular mechanisms: profilin-bound ADP-G-actin is rapidly recharged with ATP (Mockrin and Korn, 1980; Goldschmidt-Clermont *et al.*, 1992), and profilin-ATP-actin complexes can be formed from the non-polymerizable thymosin-actin pool to associate productively with free barbed ends (Pollard and Cooper, 1984; Pantaloni and Carlier, 1993).

The function of profilins in cytokinesis, though extensively studied, is less clear. Profilin 1 depletion in flies, worms and mice is embryonic lethal. Consequently, studies on cell division in the absence of profilin in higher eukaryotes have mainly been performed at very early stages of embryogenesis, where null alleles cause cell division defects of varying severity (Verheyen and Cooley, 1994; Witke *et al.*, 2001; Severson *et al.*, 2002). In many cases, these defects occur during early cytokinesis and are associated with malformed or non-constricting actomyosin rings. A similar phenotype was reported for profilin deletions in *Schizosaccharomyces pombe* (Balasubramanian *et al.*, 1994). Profilins are therefore generally considered as a part of the core cytokinesis machinery that is required for actomyosin ring formation (Glotzer, 2005). However, some protists can bypass profilin deficiency by alternative cleavage mechanisms (*Dictyostelium discoideum*; Haugwitz *et al.*, 1994) or show no requirement of profilin for actomyosin ring assembly (*Tetrahymena thermophila*; Wilkes and Otto, 2003).

In animal cells, contractile ring assembly is mediated by the GTPase RhoA and its effector proteins, formins and Rho-associated kinase (ROCK), which regulate actin nucleation and myosin activation, respectively (Glotzer, 2005). Actin and myosin are two major components of the contractile ring that generate the force for cleavage furrow ingression. ROCK activates myosin II by phosphorylation of the regulatory myosin light chain (MLC) and by inhibition of myosin phosphatase activity (Glotzer, 2005). Profilin function during cytokinesis is connected with the activity of formins, a family of actin regulatory proteins, which have emerged as key ligands for profilins (reviewed in Goode and Eck, 2007). Formins nucleate actin filaments and associate with barbed ends, where various formins slow elongation from as little as 10% to more than 99%. Profilins bind to proline-rich motifs within the formin homology 1 (FH1) domain (Chang *et al.*, 1997) and thereby increase the actin filament elongation rates

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Received: 5 December 2008; accepted: 11 February 2009; published online: 5 March 2009

of formins (Romero *et al*, 2004; Kovar *et al*, 2006). The genetic interaction between formins and profilins is well documented for a wide range of organisms (Chang *et al*, 1997; Watanabe *et al*, 1997; Severson *et al*, 2002). However, it is still unclear whether formins require profilins for actin polymerization in mammalian cells.

To examine the role of profilin in cytokinesis and formin function in somatic mammalian cells *in vivo*, we have generated a mouse strain with a profilin-deficient cartilage (*Col2pfn1* mice). Cartilage is an attractive model tissue for studying profilin function because (i) cartilage is composed of only one cell type, which can be readily isolated, cultured and studied *in vitro* (Aszodi *et al*, 2003); (ii) dynamic F-actin reorganization has an essential function in arranging chondrocytes into characteristic stacks or columns, which are required for the longitudinal growth of bones and (iii) chondrocytes express a single profilin, which is profilin 1. We report that *Col2pfn1* mice are grossly normal at birth but later on develop a progressive chondrodysplasia caused by defects in chondrocyte proliferation, actin cytoskeleton organization and the formation of growth plate columns. Furthermore, we found that chondrocytes do not employ profilin for mitosis or actomyosin ring formation and contraction in early cytokinesis, but they require profilin to generate sufficient forces for abscission during late cytokinesis.

Results

Cartilage-specific deletion of the profilin 1 gene

For tissue-specific deletion of profilin 1, we generated a mouse strain in which the promoter and exon 1 of the *profilin 1* (*pfn1*) gene were flanked by *loxP* sites (*pfn1^{fl/fl}*). The validity of the targeting strategy was tested by mating *pfn1^{fl/fl}* mice with Cre deleter mice (Betz *et al*, 1996). *pfn1^{-/-}* mice died during the pre-implantation stage, as shown previously (data not shown; Witke *et al*, 2001). To obtain mice lacking profilin 1 in chondrocytes, *pfn1^{fl/fl}* mice were crossed with transgenic mice carrying a *collagen II* promoter-driven Cre recombinase transgene, which deletes floxed genes at the time of chondrogenic differentiation (E11.5–E13) (Sakai *et al*, 2001) (*Col2a1-cre⁺/pfn1^{fl/fl}*, also called *Col2pfn1*). Western blot analysis of primary rib chondrocytes isolated from *Col2pfn1* E15 embryos and newborn mice confirmed the loss of profilin 1 (data not shown and Figure 1A). No other known profilin isoform was detectable in either control or *Col2pfn1* cartilage by western blotting for profilin 2 (Figure 1A) or northern blotting for testis-specific *profilin 3* and 4 (Figure 1B). These results demonstrate that *Col2pfn1* cartilage does not express detectable levels of known profilin isoforms.

Col2pfn1 mice develop progressive chondrodysplasia with abnormal F-actin distribution in chondrocytes

Col2pfn1 mice were born at the expected Mendelian ratio, had an intact skeleton (Figure 1C) and were viable. Although 75% of the *Col2pfn1* mice had a normal lifespan, 25% of *Col2pfn1* mice died within the first 10 days. They developed severe kyphoscoliosis and were significantly weaker and smaller as their wild-type and heterozygote littermates (data not shown). Although the external appearance of newborn *Col2pfn1* mice was grossly normal, the length of some

Figure 1 Morphology of *Col2pfn1* mice. (A) Western blot analysis of profilin 1 and 2 expressions in total protein lysates from newborn control brain, newborn control and *Col2pfn1* cartilage. (B) Northern blot analysis of total RNA from control testes, control cartilage and *Col2pfn1* cartilage. (C) Whole mount Alcian blue/Alizarin red staining of control and *Col2pfn1* mouse skeletons. Newborn *Col2pfn1* mice are indistinguishable from controls but are shorter at 4 weeks of age. (D) Length of long bones of control and *Col2pfn1* mice at birth and at 4 weeks of age (mean $\pm$ s.d., $n = 8/8$ newborn, $n = 5/5$ P28; * $P \leq 0.01$, ** $P \leq 0.05$ analysed by Mann-Whitney test). (E) Body length of control and *Col2pfn1* mice from birth to 16 days of age ($n = 6/6$). (F) Body weight of control and *Col2pfn1* mice from birth to 18 days of age ($n = 5/5$).

long bones such as the femur and humerus was moderately reduced in mutants compared with control animals (Figure 1C and D). At later stages, *Col2pfn1* mice progressively developed dwarfism as a consequence of reduced growth of the long bones. *Col2pfn1* mice (4 weeks old) have 20–30% shorter long bones compared with control littermates (Figure 1C and D), leading to a corresponding reduction in body length and weight (Figure 1E and F). As the base of the skull also develops through cartilaginous intermediates, the skull

growth was also affected in the *Col2pfn1* mice (data not shown).

To characterize the skeletal phenotype of *Col2pfn1* mice, we studied the cartilage structure during the formation of long bones of the appendicular skeleton. At E15.5, mutant bones showed no gross histological abnormalities (data not shown). At the newborn stage, some *Col2pfn1* growth plates exhibited an increased height of the hypertrophic zone accompanied by a disorganization of the columnar structure of the proliferating zone and a more rounded morphology of the normally flat proliferating chondrocytes (Figure 2A and B). The columnar arrangement of these cells was disturbed, indicating a defect in chondrocyte polarity and/or movement (Aszodi *et al*, 2003) (Figure 2B). In 4-week-old *Col2pfn1*

mice, this phenotype became more pronounced (Figure 2C). The height of the proliferative zone was reduced to a few cells, whereas many chondrocytes in the hypertrophic zone were unusually enlarged and appeared binucleated (Figure 2D). Interestingly, already at newborn stage 1.7% of the cells in profilin-deficient growth plates were binucleated compared with 0.45% of control chondrocytes (Figure 2E). Although the number of binucleated cells in control growth plates remained at a low level, the percentage of multinucleated profilin-deficient chondrocytes increased to 15% at 4 weeks of age.

The decreased size of the proliferative zone in the post-natal growth plate as well as the presence of binucleated cells pointed to a proliferation defect in *Col2pfn1* cartilage. To

Figure 2 Growth plate cartilage defects in *Col2pfn1* mice. (A) Haematoxylin/eosin (H/E)-stained sections of the knee joint region of newborn control and *Col2pfn1* mice. (B) H/E-stained sections of the proliferative zone of the tibial epiphyseal growth plate of newborn control and *Col2pfn1* mice. (C) H/E-stained sections of the knee joint region of 4-week-old *Col2pfn1* and control mice. (D) H/E-stained sections of the tibial epiphyseal growth plate of 4-week-old control and *Col2pfn1* mice. Arrows point towards binucleated cells. (E) Quantification of binucleated cells in newborn and 4-week-old growth plates of control and *Col2pfn1* mice (mean $\pm$ s.d., $n = 3/3$, $*P < 0.05$, $**P < 0.001$ analysed by Student's *t*-test). (F) Quantification of BrdU incorporation (mean $\pm$ s.d., $n = 2/3$ newborn, $n = 3/4$ P28, $*P \leq 0.01$ analysed by Mann-Whitney test). (G) Sections from the growth plate of 10-day-old control and *Col2pfn1* mice stained with fluorescently labelled phalloidin to visualize F-actin. Confocal sections and projections are shown. Abbreviations: r, resting zone; p, proliferative zone; h, hypertrophic zone.

analyse chondrocyte proliferation in *Col2pfn1* cartilage, we performed a bromodeoxyuridine (BrdU) incorporation assay to label cells in the S phase of the cell cycle. At the newborn stage, no differences were detectable in the proportion of proliferating chondrocytes in the proliferative zone between control and *Col2pfn1* cartilage. However, in 4-week-old *Col2pfn1* growth plates, significantly less chondrocytes proliferated compared with control growth plates (Figure 2F). Importantly, we found no indication for an increase in chondrocyte apoptosis in growth plates of newborn or 2-week-old *Col2pfn1* mice or for altered expression of chondrocyte differentiation markers in the different growth plate zones at E16.5 (Supplementary Figure 1A and B).

Finally, we analysed F-actin distribution in chondrocytes of the different growth plate zones in tissue sections. Overall, *Col2pfn1* chondrocytes had a less intense and more punctated F-actin staining and the membrane protrusions of *Col2pfn1* chondrocytes appeared shorter compared with control cells (Figure 2G). This defect was most pronounced in the prehypertrophic and hypertrophic zones, suggesting a deficiency of these cells in efficient F-actin assembly. In line with the *in vivo* results, rib chondrocytes isolated from newborn *Col2pfn1* mice expressed around 75% of total cellular actin compared with chondrocytes of control littermates (data not shown) and the F/G actin ratio was reduced to 60% in profilin-deficient cells compared with control cells (data not shown).

In summary, analysis of *Col2pfn1* cartilage indicates a role for profilin 1 in proliferation, arranging chondrocytes into columns and actin cytoskeleton organization, whereas chondrocyte differentiation and survival do not require profilin.

***Col2pfn1* chondrocytes assemble actomyosin rings but fail to complete abscission**

To determine whether the proliferation defects in *Col2pfn1* chondrocytes is caused by a failure in mitosis and/or cytokinesis, we observed the division of isolated primary cells by video microscopy. The sum of mitosis and cytokinesis duration measured as the time between nuclear envelope breakdown and abscission was increased in *Col2pfn1* chondrocytes (Supplementary Figure 2A). Therefore, we analysed the succession of mitotic stages to identify possible mitotic defects in *Col2pfn1* chondrocytes. The duration of prophase and metaphase was unchanged. The combined interval of anaphase and telophase measured as the time between the onset of sister chromatid separation and nuclear envelope reformation was not significantly longer in *Col2pfn1* chondrocytes compared with control cells (Supplementary Figure 2B), indicating a defect during cytokinesis. We also analysed mitotic spindle formation by immunofluorescence but found no obvious defects in *Col2pfn1* chondrocytes (Supplementary Figure 2C). Thus, profilin appears to be dispensable for mitosis in chondrocytes but critically involved in cytokinesis.

Time-lapse video microscopy showed that primary *Col2pfn1* chondrocytes were less spread but displayed equatorial furrowing and midbody formation on the same time-scale as control chondrocytes (Figure 3A–C; Supplementary Videos S1–S3). However, most *Col2pfn1* chondrocytes remained rounded up, failed to form and extend lamellipodia, and showed a marked delay in abscission (Figure 3B; Supplementary Video S2), whereas some fused following

constriction (Figure 3C; Supplementary Video S3). In line with this finding, 20.1% of *Col2pfn1* chondrocytes isolated from newborn growth plates and kept for 4 days in culture were binucleated compared with 4.9% of primary control chondrocytes. Similarly, 2.8% of immortalized control chondrocytes were binucleated, whereas around 12% of immortalized profilin-deficient cells were binucleated (data not shown). To verify that the observed cell division in profilin-deficient cells was actomyosin based, we expressed Lifeact, a 17-amino-acid peptide fused to GFP, in immortalized control and *Col2pfn1* chondrocytes, which stains F-actin structures in eukaryotic cells without interfering with actin dynamics (Riedl *et al*, 2008). In both cell types, Lifeact is equally distributed around the cell cortex at late metaphase and becomes enriched at the cell equator after anaphase onset. As cytokinesis proceeds, Lifeact localizes strongly to the constricting furrow (Figure 3D; Supplementary Videos S4 and S5). Despite an increase in blebbing during cytokinesis in *Col2pfn1* chondrocytes, the furrow diameter of the cortical actin ring and the timing of its constriction were similar to control cells (Figure 3D and E; Supplementary Videos S4 and S5). The unchanged localization of F-actin and contractile ring markers RhoA and anillin in control and profilin-deficient chondrocytes were confirmed in fixed cells (Supplementary Figure 3). In line with these observations, cytokinesis in *Col2pfn1* chondrocytes was still sensitive to myosin II inhibition by blebbistatin and to Latrunculin B treatment, an actin-sequestering agent (data not shown). Taken together, these results suggest that equatorial furrowing in *Col2pfn1* chondrocytes is caused by an actomyosin ring and is not obviously altered by the lack of profilin.

Prolonged and failed cytokinesis can be frequently linked to an abnormal midbody structure (Gromley *et al*, 2005; Zhao *et al*, 2006). Therefore we analysed the midbody of control and *Col2pfn1* chondrocytes by immunofluorescence for known midbody proteins and by electron microscopy. Midbodies of primary *Col2pfn1* chondrocytes show normal MKLP-1 and Aurora B localization (Supplementary Figure 4A). Electron microscopy images indicate similar morphology of midbody from control and profilin-deficient chondrocytes (Supplementary Figure 4B–E). Thus, defects in midbody structure do not account for the abscission defects in *Col2pfn1* chondrocytes.

On the basis of these observations, we hypothesized that *Col2pfn1* chondrocytes could be impaired in developing the traction forces required for daughter cell separation. This hypothesis is further supported by the analysis of F-actin distribution in control and *Col2pfn1* chondrocytes. Actin filament polymerization and reorganization are important factors for traction force generation (reviewed in Wang and Lin, 2007). Although control cells exhibit a defined actin cytoskeleton with stress fibres during later stages of cytokinesis, stress fibres are largely absent in *Col2pfn1* chondrocytes (Figure 4A). To analyse traction forces semiquantitatively, we performed traction force microscopy (TFM) on dividing immortalized control and profilin-deficient cells cultured on elastic polyacrylamide substrates (Dembo and Wang, 1999). Similar to primary *Col2pfn1* chondrocytes (Figure 3A–C), immortalized profilin-deficient chondrocytes show also a strongly reduced re-spreading during cell division and failure to form lamellipodia (Figure 4B). Two representative deformation diagrams determined over 40 min

Figure 3 Profilin-deficient cells assemble contractile rings. (A–C) Montage of phase-contrast video of mitotic primary control and *Col2pfn1* chondrocytes; (A) control chondrocyte, (B) dividing *Col2pfn1* chondrocyte and (C) fusing *Col2pfn1* chondrocyte. Bar, 10 μm. (D) Montage showing Lifeact localization to the cleavage furrow region of dividing immortalized control and profilin-deficient cells. Selected time points were taken from time-lapse recordings of live cells. Upper panel, confocal section; lower panel, pseudocoloured intensity profile. Time (in minutes and seconds) starts at the onset of anaphase. Bar, 10 μm. (E) Plots of furrow diameter over time after anaphase onset. The furrow diameter is shown for three representative examples of control and profilin-deficient cells.

following complete cleavage furrow constriction are shown in Figure 4B; raw data to this figure are provided in Supplementary Videos S6 and S7. Active substrate deformation by the control cell occurred predominantly at the leading edges of the lamellipodia of the two daughter cells, whereas relaxation took place in the area comprising the contracting rear ends and the midbody. In contrast, deformation forces were smaller and less directional in profilin-deficient cells. We next measured the marker displacement amplitude in a defined perimeter around the cells as an indicator for the total deformation force over the observation period. We found that the displacement amplitude was shifted to significantly lower values in profilin-deficient cells (Figure 4C). Quantification of the mean displacement amplitude showed that this parameter is three times as large for control cells as for profilin-deficient cells (Figure 4D). Thus, *Col2pfn1* chondrocytes can assemble and constrict actomyosin rings, but fail to generate sufficient traction force to achieve a timely daughter cell separation.

Profilin is required for mDia-mediated actin polymerization

Traction force generation requires actin filament dynamic and myosin activation, and critically depends on focal adhesions (FAs) that link the actin cytoskeleton to the ECM substrate and thereby transmit the traction force to the ECM (reviewed in Wang and Lin, 2007). Therefore, we compared cellular processes dependent on actin and FA dynamics including spreading, migration and polarity between control and *Col2pfn1* chondrocytes adhering to fibronectin. Primary as well as immortalized profilin-deficient chondrocytes failed to form lamellipodia, were unable to spread to the same extent as control cells (Figure 5A–C) and were less motile as apparent by the analysis of single migrating cells (Figure 5D and E). They were also incapable of polarizing as profilin-deficient cells were impaired in orienting the microtubule-organizing centre in scratched monolayers towards the scratch (Supplementary Figure 5). Spreading and migration

Figure 4 *Col2pfn1* chondrocytes fail to complete abscission during late cytokinesis. (A) Control and *Col2pfn1* chondrocytes during late cytokinesis stained with an antibody against tubulin and fluorescently labelled phalloidin to visualize F-actin. Bar, 10 μ m. (B) Representative deformation maps of control and profilin-deficient cells on a flexible polyacrylamide substrate during cytokinesis. Arrow direction and colour indicate deformation direction and magnitude (blue<green<yellow<red). Bar, 10 μ m. (C) Relative distribution of maximal deformation marker displacement around dividing control and profilin-deficient cells (mean $\pm$ s.d., $n = 7/7$, ** $P = 0.0047$ analysed by Mann–Whitney test). (D) Quantification of mean maximal deformation marker displacement around dividing immortalized control and *Col2pfn1* cells (mean $\pm$ s.d., $n = 7/7$, ** $P = 0.0047$ analysed by Mann–Whitney test).

failure were a consequence of the absence of profilin, as re-expressing wild-type profilin 1 in profilin-deficient cells rescued both defects (Figure 5C and E).

The examination of FA and actin stress fibre formation revealed that both structures critically depend on profilin. Although FAs formed 3 h after seeding on fibronectin in control cells, they could only be observed after 12 h in *Col2pfn1* chondrocytes. Stress fibres in *Col2pfn1* chondrocytes were reduced in numbers, were short and thick, and oriented in a random manner (Figure 6A). In contrast, wild-type chondrocytes developed a robust stress fibre system that was connected to large FAs at the cell edges and smaller FAs in the centre of the cell (Figure 6A). Similarly, Cre-mediated deletion of profilin 1 in immortalized fibroblasts induced the disassembly of stress fibres and FAs followed by a rounding up and de-attachment of the fibroblasts (data not shown). In summary, profilin-deficient cells exhibit severe defects in actin organization, cell polarity and the formation of matrix adhesions.

RhoA and its effectors ROCK and mDia1 are central regulators of matrix adhesion maturation and the formation of contractile actomyosin structures (Watanabe *et al*, 1997).

As RhoA regulates actin nucleation through formins, and myosin II activation through ROCK-dependent phosphorylation of MLC-2 (Amano *et al*, 1996), we examined their expression levels and activities in immortalized *Col2pfn1* chondrocytes. Control and profilin-deficient cells expressed RhoA pathway members at comparable levels (Figure 6B). RhoA GTP loading was increased in profilin-deficient cells (Figure 6C), and MLC-2 was hyperphosphorylated on serine-19 and threonine-18 (Figure 6D). Thus, increased RhoA activity in profilin-deficient cells correlates with increased myosin II activity. In contrast to RhoA, we did not detect significant changes in the activity of Rac1 and Cdc42 between control and profilin-deficient cells (data not shown).

We next determined whether the hyperphosphorylation of MLC-2 in profilin-deficient cells was sensitive to the ROCK inhibitor Y-27632. ROCK inhibition reduced MLC-2 phosphorylation to control levels, suggesting that increased ROCK activity contributes to MLC-2 hyperphosphorylation in immortalized *Col2pfn1* chondrocytes (Figure 6E). However, the increased ROCK activity does not explain the phenotype of *Col2pfn1* chondrocytes, as inhibiting ROCK with Y-27632 led to a further disassembly of stress fibres and FAs and failed

Figure 5 Cell spreading and migration are strongly affected in *Col2pfn1* chondrocytes. **(A)** Phase-contrast images of primary newborn control and *Col2pfn1* chondrocytes seeded on fibronectin. Selected time points were taken from time-lapse recordings. *Col2pfn1* chondrocytes display a spreading defect. **(B)** Quantification of spreading area of isolated primary control and *Col2pfn1* chondrocytes (mean $\pm$ s.d., summary of four independent control-*Col2pfn1* pairs, $n > 100$ cells, *** $P \leq 0.001$ analysed by Mann-Whitney test). **(C)** Quantification of spreading area of the indicated immortalized cell lines seeded on fibronectin for 24 h (mean $\pm$ s.d., $n > 100$ cells, NS = not significant, *** $P \leq 0.001$ analysed by Mann-Whitney test). **(D)** Trajectories of individual primary control and *Col2pfn1* cells from the frame-by-frame analysis of time-lapse recordings during a 90-min observation period. The migration velocities of the respective primary cells are indicated (mean $\pm$ s.d., migration data of over 110 cells from four independent control-*Col2pfn1* pairs). **(E)** Quantification of cell migration velocity of the indicated immortalized cell lines (mean $\pm$ s.d., migration data of over 140 cells for each cell line from three independent experiments were pooled, NS = not significant, *** $P \leq 0.001$ analysed by Mann-Whitney test). Rescue of profilin-deficient cells with wild-type profilin 1 restores the migratory behaviour.

to increase cell spreading (data not shown). We also examined the phosphorylation levels of cofilin, a key regulator of actin filament dynamics, which is regulated by ROCK through an LIM kinase-dependent pathway (Maekawa *et al*, 1999). However, *Col2pfn1* chondrocytes displayed normal cofilin phosphorylation (Figure 6B). In summary, although profilin-deficient cells are defective in FA formation and stress fibre assembly, they paradoxically show increased RhoA, ROCK and myosin II activity.

To better understand the molecular basis underlying these surprising results, we next overexpressed constitutively active RhoA pathway members in control and profilin-deficient chondrocytes (Figure 6F). Overexpression of a constitutively

active RhoA (RhoA Q63L) induced stress fibres in both cell types (Figure 6F, upper row), demonstrating that factors downstream of RhoA can at least partly compensate for the loss of profilin in RhoA-mediated stress fibre formation. We hypothesized that ROCK could function as one of these factors by causing increased actin bundling and force generation through MLC-2 phosphorylation. In line with this, constitutively active ROCK1 (ROCK $\Delta 4$) induced stellate stress fibres both in control and in profilin-deficient cells (Figure 6F, middle row). Thus, both RhoA and ROCK can function in a profilin-independent way to induce stress fibre formation. Independently of ROCK, RhoA can activate the formin mDia1, which is critical for the assembly of long unbranched actin

Figure 6 Profilin function in RhoA-induced actomyosin assembly. (A) Primary control and *Col2pfn1* chondrocytes were allowed to spread on fibronectin for 3 h and 12 h and stained with a vinculin antibody (red) and fluorescently labelled phalloidin to visualize F-actin (green). Nuclei were counterstained with DAPI (blue). Confocal sections are shown. Bar, 20 μ m. (B) Western blot analysis of total protein lysates from immortalized control and *Col2pfn1* cells for the expression of indicated RhoA pathway members. (C) Quantification of RhoA GTP loading in control and profilin-deficient cell lines measured by ELISA (mean $\pm$ s.d., $n = 3/3$). (D) Western blot analysis of total protein lysates from control and profilin-deficient cell lines for the level of myosin light chain phosphorylation. (E) Western blot analysis of total protein lysates from immortalized *Col2pfn1* cells after treatment with the indicated concentrations of ROCK inhibitor Y-27632. (F) Control and profilin-deficient cells were transiently transfected with the indicated constitutively active RhoA pathway members and stained after 16 h with a myc antibody (red) and fluorescently labelled phalloidin to visualize F-actin (green). Nuclei were counterstained with DAPI (blue). Confocal sections are shown. Bar, 20 μ m.

filaments. To test whether mDia1 requires profilin *in vivo* for rapid and efficient filament elongation, we overexpressed constitutively active mDia1 (mDia1FH1FH2) and observed a

striking difference between profilin-deficient and control cells (Figure 6F, lower row). Although mDia1FH1FH2 induced long, parallel stress fibres similar to the ones obtained with

RhoA Q63L in control cells, it had no effect when over-expressed in profilin-deficient cells. Taken together, profilin is not required for F-actin bundling and contraction through ROCK/MLC-2, but is important for increased stress fibre formation induced by activated mDia1.

Actin- and poly-proline binding contribute to profilin 1 function in chondrocytes

To determine the role of profilin for mDia1 function and to investigate the relative contribution of actin and non-actin ligands for profilin 1 activity, we transduced primary *Col2pfn1* chondrocytes with retroviral constructs for different profilin cDNAs, including wild-type profilin 1, an actin binding-deficient point mutant profilin 1 R74E (Korenbaum *et al*, 1998) as well as two profilin 1 point mutants (Y6D and H133S) with >98-fold reduced affinities for poly-proline (Björkegren-Sjögren *et al*, 1997; Kovar *et al*, 2006; Ezezika *et al*, 2009). It is known from studies in fission yeast that only profilin mutants with a considerable actin or poly-proline binding of >10% of wild-type profilin can restore viability (Lu and Pollard, 2001).

Cell morphology, actin cytoskeleton organization and FA formation were rescued by transducing wild-type profilin 1 (Figure 7A and data not shown). Similar results were obtained with immortalized *Col2pfn1* chondrocytes. Retroviral transduction of either profilin 1 or profilin 2a cDNAs rescued the abnormalities in cell morphology, total mitosis duration and random motility (Supplementary Figure 6). As expected, the actin binding-deficient mutant R74E did not change the mutant phenotype (Figure 7A). Interestingly, the two poly-proline binding-deficient mutants analysed differed in their ability to rescue *Col2pfn1* chondrocytes.

Expression of profilin 1 H133S in *Col2pfn1* chondrocytes restored the wild-type cell morphology and actin cytoskeleton organization, whereas profilin 1 Y6D failed to rescue these defects (Figure 7A). Actually, profilin 1 H133S showed weak binding to poly-proline sepharose, whereas the Y6D mutant lacked detectable poly-proline binding (Figure 7B). Taken together, these data suggest that poly-proline and actin binding are important for profilin 1 function.

The profilin-actin interaction is thought to execute several biochemical activities that are crucial for the function of actin. They include the ability of profilin to lower the critical actin concentration by making thymosin-bound actin available for polymerization (Pantaloni and Carlier, 1993; Kang *et al*, 1999), to accelerate the exchange of G-actin-bound nucleotide (Goldschmidt-Clermont *et al*, 1992) and to facilitate nuclear export of actin (Stüven *et al*, 2003). Interestingly, the ratio of total ATP to ADP actin was similar between immortalized control and *Col2pfn1* chondrocytes as determined chromatographically in fractionated cell extracts (Supplementary Figure 7A). Furthermore, we were unable to detect a significant difference in the ratio of nuclear and cytoplasmic actin between control and *Col2pfn1* chondrocytes by cellular fractionation (Supplementary Figure 7B).

Finally, we analysed whether profilin regulates the availability of thymosin-bound actin for polymerization. Thymosins are highly expressed, G-actin-sequestering proteins in many cell types (Pollard and Borisy, 2003). We detected very low mRNA levels of *thymosin-β4* and *thymosin-β10* by real-time PCR in mouse cartilage when compared with brain or thymus, and neither of the two thymosins became upregulated after profilin 1 deletion (Supplementary Figure 7C and D). In line with the low thymosin

Figure 7 Profilin 1 requires actin and poly-proline binding in chondrocytes. (A) Primary *Col2pfn1* chondrocytes after viral infection with the indicated profilin 1 variants were allowed to spread on fibronectin for 3 h, fixed and stained with fluorescently labelled phalloidin to visualize F-actin (red). Nuclei were counterstained with DAPI (blue). GFP expression indicates profilin 1-expressing cells. Bar, 20 μ m. (B) Western blot analysis of poly-L-proline affinity precipitation from total cell lysates of profilin-deficient cells and cells expressing the indicated profilin 1 variants (profilin 1 wild-type (wt1), profilin 1 H133S (H133S), profilin 1 Y6D (Y6D)).

levels in chondrocytes, we detected only actin but no thymosin- β 4-actin complexes in cell extracts from primary mouse chondrocytes using native PAGE (Supplementary Figure 7E). This finding points to a critical role of profilin 1 as G-actin-sequestering protein in mouse chondrocytes to prevent spontaneous actin nucleation and to maintain the pool of polymerizable actin.

Discussion

Gene deletion studies in early embryos have suggested that profilins are essential housekeeping genes, as they are key regulators of actin polymerization and critically required for cell division and F/G-actin homeostasis (Verheyen and Cooley, 1994; Witke *et al*, 2001; Severson *et al*, 2002). However, it is unknown whether they have similar functions in differentiated mammalian cell types and which biochemical activities are crucial for their function.

We have generated a mouse strain that expresses none of the known profilin isoforms in cartilage, allowing to perform a loss-of-function analysis of this important protein class in a differentiated mammalian cell type *in vivo* and *in vitro*. Although chondrocyte survival and differentiation proceed normally, functions associated with rapid actin cytoskeleton rearrangements are severely impaired resulting in a chondrodysplasia. Prominent defects are alterations in proliferation accompanied with frequent binucleation of *Col2pfn1* chondrocytes.

Profilin function in chondrocyte cytokinesis

Our analysis of the proliferation of *Col2pfn1* chondrocytes demonstrates defects during cytokinesis, whereas mitosis was unaffected. Interestingly, we uncovered an important role for profilin in late cytokinesis instead of actomyosin ring formation. This is surprising as profilins are generally considered to be linked with formin activity in contractile ring formation (Glotzer, 2005). However, the actual cytokinetic phenotypes of *profilin* gene deletions show a more heterogeneous picture. *Cdc3*, the *S. pombe* profilin, is absolutely essential for contractile ring formation and cytokinesis (Balasubramanian *et al*, 1994). In contrast, profilin-deficient *T. thermophila* ciliates form and constrict contractile rings (Wilkes and Otto, 2003). *Dicystostelium* amoebae can bypass profilin deficiency by a furrowless, actomyosin-independent cell division (Haugwitz *et al*, 1994). In higher eukaryotes, null alleles of the *Drosophila* profilin gene *chickadee* allow development of fly embryos until the larval stage, likely due to perdurance of maternally supplied profilin (Verheyen and Cooley, 1994), whereas worm embryos do not complete the first meiotic cytokinesis when *PFN-1* RNA is depleted from oocytes (Severson *et al*, 2002).

Col2pfn1 chondrocytes divide in an actomyosin-dependent manner with equatorial furrowing and constriction that proceed on a normal timescale, indicating that those processes can apparently be achieved through profilin-independent pathways in mouse chondrocytes. Both *de novo* nucleation by formins and F-actin recruitment are known to be involved in actomyosin ring formation, and their relative contribution appears to differ between species and cell types (reviewed in Wang, 2005). For instance, although incorporation of pre-existing filaments into the ring is negligible in *S. pombe* (Pelham and Chang, 2002), this pathway clearly contributes

to actomyosin ring formation in NRK cells (Cao and Wang, 1990). It is proposed that profilins function together with formins to generate the furrow actin. Yet, formins (besides fission yeast *Cdc12p*) elongate actin filaments without profilin *in vitro* and rely on profilins to accelerate elongation rates (Romero *et al*, 2004; Kovar *et al*, 2006). As formins and ATP-actin are present in *Col2pfn1* chondrocytes, the basal filament elongation rate of formins without profilin might be sufficient to generate a functional actomyosin ring in chondrocytes.

Hypothetically, profilin deficiency may be compensated by an increased recruitment of pre-existing filaments to the cleavage furrow. However, incorporation of pre-made F-actin alone seems not to be sufficient for actomyosin ring ingression as shown by a similar sensitivity of control and *Col2pfn1* chondrocytes to Latrunculin B treatment (RTB and RF, unpublished observation). This finding suggests that actin polymerization is a prerequisite for early stages of cytokinesis in the absence of detectable levels of profilins.

Although early steps of cytokinesis have been extensively analysed over the past years, it is less clear what mechanisms and proteins contribute to later cytokinesis events including abscission of the daughter cells. Abscission appears critically affected in our model system. The abscission failure coincides with lower and less oriented traction forces in profilin-deficient daughter cells. Evidence that traction forces can contribute to thinning of the intercellular bridge and its cleavage came from mutant amoebae devoid of conventional myosin (De Lozanne and Spudich, 1987; Fukui, 1993) and direct measurements of traction forces of dividing Swiss 3T3 cells (Burton and Taylor, 1997). Cell traction force is generated by the cell's contractile apparatus consisting of an actin stress fibre system and myosins that contracts the cell body (reviewed in Wang and Lin, 2007). Alternatively, cells generate traction forces by actin polymerization at the leading edge that pushes the leading front forward. The tension is relayed to the ECM substrate by FAs, which connects the actin cytoskeleton with the ECM.

Besides the severely impaired ability of profilin-deficient chondrocytes to form lamellipodia and to assemble FAs, a dysfunctional RhoA-induced actomyosin assembly of the stress fibre system contributes to the traction force defect in the absence of profilin. Here, an important open question is to explain what additional function of profilin is required for the assembly of functional stress fibres compared with the actomyosin ring. One explanation could be the involvement of different formins or mDia isoforms for actin nucleation/elongation in different cellular processes. Although stress fibre formation has been linked to mDia1 function (Watanabe *et al*, 1997), actomyosin ring formation in NIH3T3 and C2C12 cells is dependent on mDia2 (Watanabe *et al*, 2008). Our data show that an activated mDia1 requires profilin function for effective stress fibre formation *in vivo*. *In vitro*, the F-actin elongation rate of distinct mDia isoforms differently depends on profilin function (Romero *et al*, 2004; Kovar *et al*, 2006) and formins require specific profilin isoforms to enhance actin filament elongation (Neidt *et al*, 2009). It is tempting to speculate that the F-actin elongation rate of each mDia isoform differently depends on profilin function *in vivo* as it does *in vitro*.

Despite a failure to form a stress fibre system, we find that RhoA activity is increased and myosin II is hyperphosphorylated in a ROCK-dependent manner in profilin-deficient cells.

Interestingly, in this case increased ROCK activity appears to be funnelled specifically towards myosin phosphorylation because cofilin phosphorylation, which is also regulated by ROCK through an LIM kinase-dependent pathway (Maekawa *et al*, 1999), is unaffected. Increased RhoA activity could result from a feedback mechanism in response to the contractile state of the cell. Conversely, profilin-deficient cells might attempt to compensate for the loss of formin function by increased activation of RhoA and other effectors.

In addition to traction force generation, our data indicate defects in transmission of the traction force to the substratum as FA assembly and maturation are defective in profilin-deficient chondrocytes. An important role of profilin in these processes is consistent with previous findings where profilin 1 overexpression caused increased substrate adhesion (Roy and Jacobson, 2004), whereas silencing profilin 1 in HUVECS caused loss of actin stress fibres and suppressed FA maturation (Ding *et al*, 2006). FA formation initiated through integrin clustering requires both ECM binding and contractility. It has been demonstrated that the formin mDia1 is critically involved in FA maturation in a Rho- and ROCK-independent manner (Riveline *et al*, 2001). A likely explanation would be that an impaired formin function causes this defect in profilin-deficient chondrocytes.

Mechanism of the *in vivo* role of profilin for actin dynamics

Despite extensive biochemical literature, it is unclear to which extent binding to ligands other than actin contributes to profilin function *in vivo*. Overexpression experiments performed with point-mutated profilins have consistently demonstrated the importance of actin binding for profilin function, but provided conflicting data on its interaction with other ligands (Mimuro *et al*, 2000; Wittenmayer *et al*, 2004; Lambrechts *et al*, 2006). An elegant study by Lu and Pollard (2001) revealed that only profilin mutants with a residual poly-L-proline binding of >10% of wild-type profilin can restore viability in fission yeast. We took advantage of our *Col2pfn1* chondrocytes and expressed different profilin isoforms or point mutants. Actin binding is crucial for proper profilin function in chondrocytes. Interestingly, one of the two poly-proline binding-deficient profilin 1 mutants analysed was still able to rescue cell morphology, spreading, actin cytoskeleton organization and FA assembly. Human profilin 1 Y6D and H133S mutants bind actin with comparable dissociation equilibrium constants (Ezezika *et al*, 2009). In contrast, the affinity of Y6D profilin for poly-proline was 28 times lower than H133S profilin and 1200-fold lower than wild-type profilin 1 (Ezezika *et al*, 2009). We cannot rule out that small differences in actin binding contribute to the observed differences or that another important biological ligand of profilin specifically fails to bind the profilin 1 Y6D mutant. Still, the residual weak poly-proline-binding ability of profilin 1 H133S can explain the different abilities of the two analysed profilin 1 variants to rescue the mutant phenotype. This suggests that poly-proline binding is important for profilin function in chondrocytes. The poly-proline-binding site of profilin mediates the interaction with a variety of proteins involved in actin-driven mechanisms, including formins, Arp2, Ena/VASP, N-WASP and WAVE (Witke, 2004). However, our results also indicate cell type-dependent differences to the necessary extent of poly-proline ligand

binding for profilin activity, as the H133S mutant possesses less than 10% poly-proline binding and a similar mutant failed to restore viability in fission yeast (Lu and Pollard, 2001). Such cell type- or organism-dependent differences likely reflect the different need to interact with non-actin ligands for crucial cellular processes. An alternative explanation is based on a theoretical model by Kozlov and Bershadsky (2004), which predicts that moderate pulling forces in cells are sufficient to reduce the critical actin concentration for formin-mediated barbed end polymerization (Kozlov and Bershadsky, 2004). The potential to generate such forces might vary between cell types. In cells capable of generating sufficient force, the activity of formins might therefore mainly depend on the availability of polymerizable ATP-G-actin, which is governed by profilin regardless of poly-proline binding.

Materials and methods

Mice

Generation of floxed *pfn1* mice is described in the Supplementary data. To obtain a chondrocyte-specific deletion of *pfn1*, mice carrying the *loxP*-flanked *pfn1* gene were crossed with mice carrying the *collagen II* promoter-driven *Cre* transgene (Sakai *et al*, 2001).

Skeletal analysis, histology and immunohistochemistry

Skeletal staining with Alcian blue/Alizarin red, histochemistry, immunostaining and *in situ* hybridization were carried out as described (Aszodi *et al*, 1998). Apoptotic chondrocytes were detected using the *In Situ* Cell Death Detection Kit (Roche Diagnostics). Cell proliferation was analysed using the BrdU incorporation assay (Aszodi *et al*, 1998).

Isolation of primary mouse rib chondrocytes

Chondrocytes from rib or limb cartilage were released by digestion with collagenase type II (Worthington) at 2 mg/ml in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal calf serum (FCS) (Gibco), at 37°C for 2–4 h. Freshly isolated chondrocytes were immortalized by transduction with SV40 large T antigen. Primary cells and cell lines were maintained in DMEM/10% FCS.

Immunofluorescence

For immunocytochemistry, cells were cultured on glass slides coated with 10 µg/ml bovine plasma fibronectin (Calbiochem). For the detection of aurora B, tubulin and MKLP-1, cells were fixed with methanol at –20°C for 5 min. For RhoA and anillin, cells were fixed in 10% TCA on ice for 15 min (Hayashi *et al*, 1999) followed by permeabilization with 0.2% Triton X-100. For phalloidin staining for F-actin, cells were either fixed in 4% paraformaldehyde/3% sucrose in PBS for 15 min or in 1% glutaraldehyde in modified Hank's balanced salt solution for 10 min at room temperature. After fixation and permeabilization, the cells were incubated with 3% BSA in PBS for 1 h followed by incubation with the primary antibody for 1 h at room temperature or over night at 4°C. Secondary antibodies were incubated for 1 h at room temperature. Images were collected at RT by confocal microscopy (DMIRE2; Leica, Bensheim, Germany) with 63 ×/1.4 or 100 ×/1.4 oil objectives using the Leica Confocal Software (version 2.5, build 1227) or collected with a AxioImager Z1 microscope (Zeiss, Germany) with the 63 ×/1.4 oil objective.

Video microscopy and TFM

Images of live cells were recorded at 37°C and 5% CO₂ on a Zeiss Axiovert 200M (Zeiss) equipped with 10 ×/0.3, 20 ×/0.4 and 40 ×/0.6 objectives, a motorized stage (Märzhäuser, Germany) and an environment chamber (EMBL Precision Engineering, Germany) with a cooled CCD camera (Roper Scientific, Princeton, NJ). Image acquisition and microscope control were carried out with MetaMorph software (Molecular Devices, Downingtown, PA). Cells were plated on glass bottom culture dishes (MatTek, Ashland, MA) precoated with 10 µg/ml fibronectin.

TFM was carried out as described (Dembo and Wang, 1999) with minor modifications. Briefly, a solution of 5% acrylamide and 0.1% bisacrylamide was copolymerized on aldehyde-derivatized glass bottom culture dishes (MatTek) to yield 50- to 75- μ m-thick flexible substrates that were subsequently crosslinked to fibronectin using sulfo-SANPAH (Pierce). Cells were grown and filmed on these substrates as described above. To visualize substrate deformation, polystyrene microspheres were embedded in the polyacrylamide matrix as displacement markers. Marker displacement was measured over time and integrated over the cell area to estimate total deformation forces exerted by dividing cells on the substrate.

For live-cell imaging of Lifeact localization during cell division, cells seeded on fibronectin-coated glass bottom culture dishes were kept in phenol red-free DMEM at 37°C. Images were acquired with a 40 $\times$ /0.75 or 63 $\times$ /1.4 objective on a Zeiss Observer Z1 microscope equipped with a CSU10 spinning disc scanhead (Yokogawa), Coolsnap HQ2 camera (Roper Scientific) run by MetaMorph software (Molecular Devices). The system was implemented by Visitron Systems.

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Statistics

Results are expressed as the means and SD. All statistical analysis was performed using the GraphPad Prism software (version 5.00; GraphPad Software). Mann–Whitney *U*-statistics were used for comparisons between different data sets. Asterisks indicate significant differences (**P* < 0.05, ***P* < 0.01 and ****P* < 0.005).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Art Alberts, Christoph Ampé, Brigitta Jockusch, Roger Karlsson and Guido Posern for generously sharing antibodies and constructs and for fruitful discussions and Dr Roy Zent for critically reading the paper. This study was supported by the Max Planck Society and the German–Israeli Project (DIP) programme.

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