# Isolation and characterisation of DOCK8, a member of the DOCK180-related regulators of cell morphology

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**Abstract In a yeast two-hybrid system screen for Cdc42-interacting proteins, we identified a protein with similarity to the CrkII-binding protein DOCK180. A cDNA clone of this protein, designated DOCK8, encoded a gene-product of 1701 amino acid residues with a molecular mass of 190 kDa. Immunofluorescence staining showed that transiently transfected HA-tagged DOCK8, as well as endogenous DOCK8, was present at the cell edges in areas undergoing lamellipodia formation. Transient transfection of a C-terminal fragment of DOCK8 resulted in the formation of vesicular structures. Interestingly, these vesicles also contained filamentous actin. These data suggest an involvement of DOCK8 in processes that affect the organisation of filamentous actin.**



*Keywords:* Cdc42; Rac1; DOCK180; Actin

## 1. Introduction

The Rho GTPases constitute a multi-member group of proteins, which are pivotal for the morphogenic and migratory properties of eukaryotic cells [1–3]. These proteins are related to the proto-oncogenes of the Ras family of small GTPases and share a substantial amount of the biophysical properties with Ras [4]. The Rho GTPases bind and hydrolyse GTP and in this process they cycle between inactive, GDP-loaded, and active, GTP-loaded, conformations [1]. This ability to alter conformation is instrumental for the ability of the Rho GTPases to function as signal transducers and the process is strictly regulated by guanine-nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) [5–7]. Three members of the Rho GTPases, Cdc42, Rac1 and RhoA, have been extensively studied and shown to have important roles in regulating the actin filament system [2]. Current observations suggest the presence of 22 Rho GTPases in the human genome and a majority of the additional family members also participate in cytoskeletal control [3]. In addition, the Rho GTPases have been shown to regulate critical aspects of cell signalling, cell growth, cell division and cell survival [8].

A large number of proteins, which bind to the Rho GTPases, have been identified during the last decade [9,10]. The yeast two-hybrid system and affinity chromatography have been two successful tools for the identification of binding partners. In search for Cdc42-interacting proteins, employing the yeast two-hybrid system, we were able to identify seven Cdc42-binding proteins [11]. In this article, we describe one of them that showed extensive homology to the Ced-5/DOCK180/Myoblast City (CDM) family of proteins [12]. This protein was expressed in a relatively ubiquitous manner in human tissues. During the course of this study, the CDM-like proteins identified by the human genomic sequencing project were given the name DOCK8, for which reason we refer to this CDM family member as DOCK8 [13]. Ectopically expressed DOCK8, as well as endogenous DOCK8 was present in lamellipodia and we propose that DOCK8 has a role during the reorganisation of the actin filament system.

## 2. Materials and methods

### 2.1. DNA work and yeast two-hybrid system screen

The *Saccharomyces cerevisiae* strain Y190 was transformed with cDNAs encoding Rho GTPases, as depicted in Fig. 3A and B, fused to the GAL4 DNA-binding domain (GAL4DB) in the pYTH6 vector. The GAL4DB-Rho GTPase-expressing yeast strains were transformed with a plasmid encoding a C-terminal fragment of DOCK8 (DOCK8:1044–1701) fused to the GAL4 activation domain (GAL4AD) in the pACT vector following the previously described procedure [11].

### 2.2. DNA work

A human Burkitt lymphoma Daudi cell cDNA library (BD Biosciences) was screened with a DNA fragment derived from the C-terminus of DOCK8. The probe was labelled with Rediprime TM II Random Prime Labelling System (Amersham Biosciences) according to the protocol provided by the manufacturer. Hybridisation of the filters (Hybond-N+, Amersham Biosciences) followed standard methods as described in [14]. Clones potentially encoding DOCK8 were subcloned into the pBluescript-SK vector. A second round of screening was performed with an N-terminal probe. The N-terminus of DOCK8 was amplified from the Daudi cell cDNA library as a PCR product using a 5 prime $\lambda$ and an internal DOCK8 primer. The PCR product was subcloned using the PCR-Script Amp Cloning Kit (Stratagene). The full-length DOCK8 cDNA was subcloned into a HA-tagged pcDNA3 mammalian expression vector. The DNA sequence analysis was performed on an ABI Prism 310 Genetic Analyzer.

### 2.3. Northern blot analysis

A 0.8 kb DNA fragment encoding the C-terminal of DOCK8 was labelled with [$^{32}$P]-CTP employing the Rediprime labelling kit (Amersham Biosciences). The probes were thereafter hybridised to hybridisation-ready Northern blots (Human Multiple Tissue Northern Blot, BD Biosciences) according to the ExpressHyb (BD Biosciences)

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protocol. The Northern blot was visualised employing a Fuji BAS2000 Bioimager.

*2.4. GST fusion-protein production and GST pull-down assay*

GST fusion proteins of Cdc42, Rac1 and RhoA were purified as before and the proteins remained bound to the glutathione–Sepharose beads [11]. For GST pull-down assays, Cos7 cells were transfected with plasmids encoding Myc-tagged fragment encoding amino acid residues 495–826 of Dbl, including the DH and PH domains (a generous gift from M. Olson, The Institute of Cancer Research, London, UK) or a HA-tagged fragment encoding amino residues 1179–1701 of DOCK8. GST pull-down assay conditions were essentially adopted from [11,13,14]. The precipitated material was subjected to Western blotting.

*2.5. Cell cultivation, transfection, immunofluorescence*

Porcine aortic endothelial cells expressing the human PDGF β-receptor (PAE/PDGFRβ cells), NIH 3T3 and Cos7 cells were cultured as described before [3,14]. Transfection with LipoFECTAMINE PLUS (Invitrogen life technologies) and immunofluorescence microscopy analysis was performed as described before [3]. A peptide encompassing amino acid residues 1679–1693 was coupled to KLH and used to immunise a rabbit to generate a polyclonal DOCK8 antiserum.

## 3. Results

*3.1. Cloning of DOCK8*

In previous studies, we have reported on a yeast two-hybrid screen, which identified seven Cdc42-interacting proteins [11]. A number of independent clones from this screen encoded a DOCK180-related protein, preliminarily designated DOCK8. A full-length DOCK8 cDNA of 8 kb, generated by screening a Daudi cell cDNA library and by PCR amplification, encoded a protein of 1701 amino acid residues (Fig. 1A). Database searches indicated the presence of alternatively spliced DOCK8 cDNAs and subsequent sequence analysis showed that at least one EST clone encoded a DOCK8 cDNA with an extended 5′ end resulting in extra 330 amino acid residues at the N-terminal part of the protein (Fig. 1B). Previous work has shown two areas in the CDM proteins with higher degree of similarity, called CZH-1 and CZH-2 domains (or DHR-1 and DHR-2) [13,15], the positions of these domains in DOCK8 are schematically outlined in Fig. 1C. The human CDM family has 11 members, however most of them are still only known from partial sequences in the public sequence databases. A previous study divided the CDM proteins in to 4 subfamilies designated DOCKA, B, C and D [13]. The proteins of DOCKA (DOCK180, KIAA0299) and B (KIAA0209 (MOCA), KIAA1706 and DOCK5) are very similar to each other. The proteins of the DOCKC (DOCK8, KIAA1395 and KIAA1771) and DOCKD (Zizimin1, DOCK10 and DOCK11) are also very similar to each other and the similarity is relatively high all over the amino acid sequence (Fig. 1D). However, the members of DOCKA/B are only marginally similar to the members of DOCKC/D and the similarity is restricted to

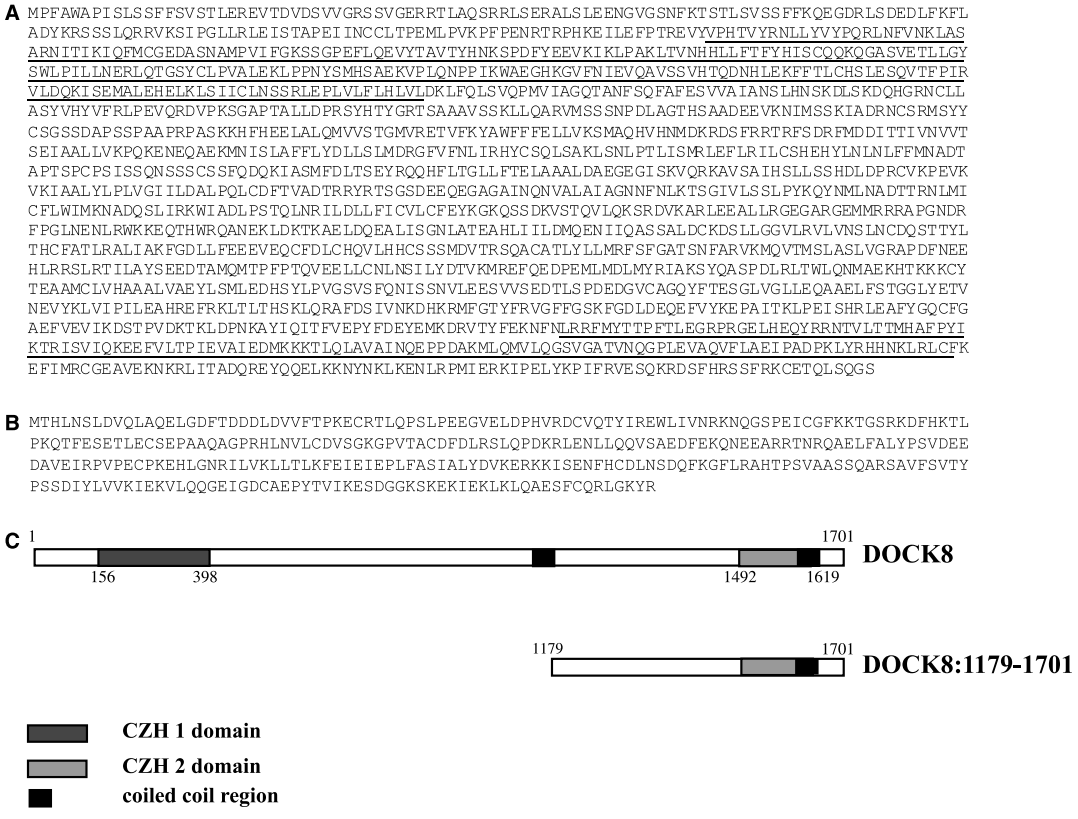


Fig. 1. Primary structure of DOCK8. (A) Amino acid sequence of DOCK8 encompassing 1701 amino acid residues. The first underlined sequence marks the CZH1 domain and the second underlined sequence marks the CZH2 domain. (B) An N-terminal extension in an alternatively spliced DOCK8, encompassing additional 330 amino acid residues. (C) Domain organisation of the full-length DOCK8 and the C-terminal fragment of DOCK8. (D) Phylogenetic tree representation of the relationship between all CZH2 domains of the human DOCK family using the ClustalW method with the PAM250 residue weight table (numbers represent % divergence). (E) Alignment of the CZH2 domain of all members of the human DOCK family.

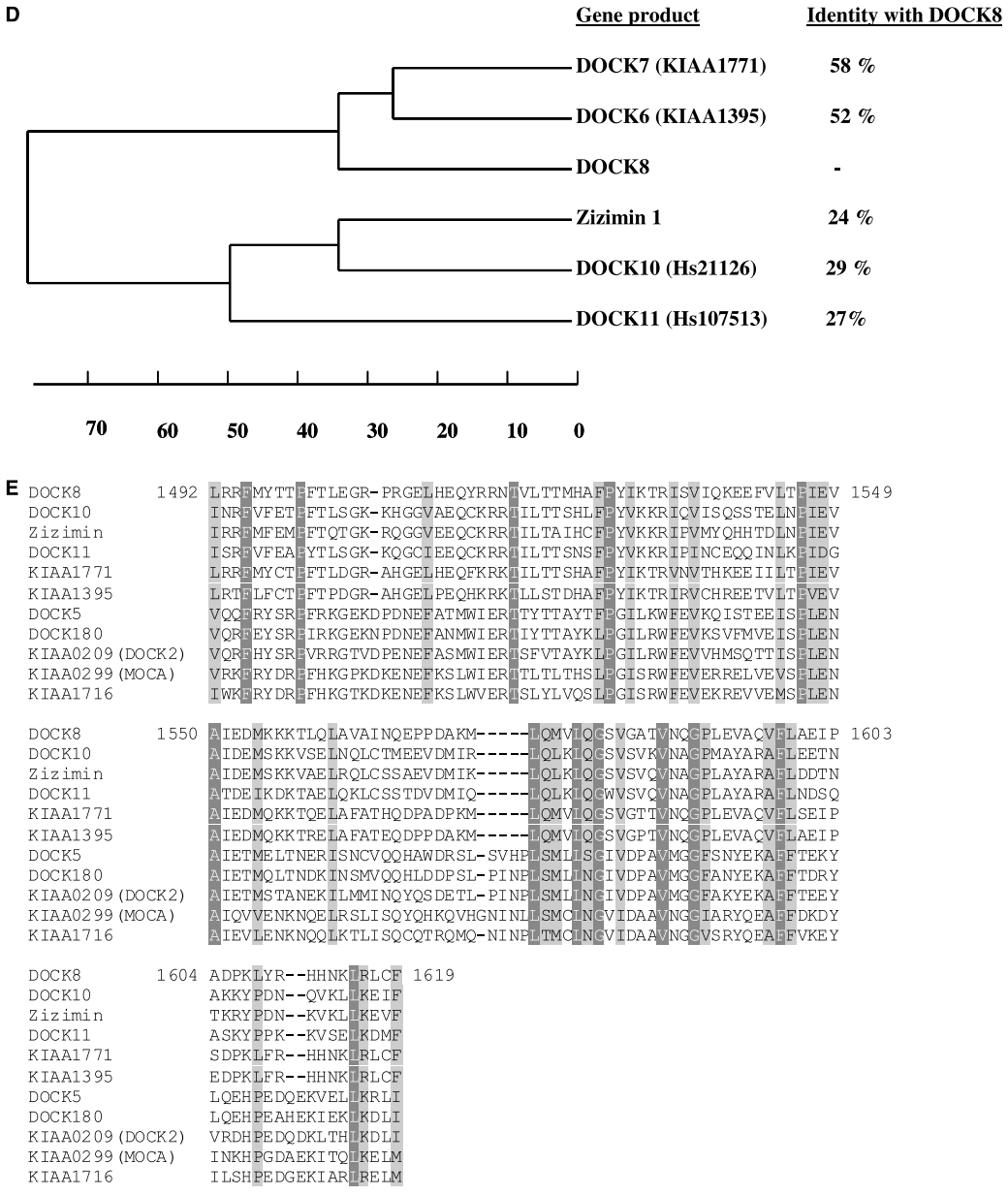


Fig. 1 (*continued*)

the CZH-1 and CZH-2 domains (Fig. 1D shows the core of the CZH-2 domain with all family members aligned side-by-side).

### 3.2. DOCK8 tissue distribution

Northern blot analysis with a 0.8 kb fragment of the 3′ portion of the coding region of DOCK8 showed the expression of the protein, detected as a single band of 8 kb, in placenta, lung, kidney and pancreas (Fig. 2A). Low expression of DOCK8 was furthermore detected in heart, brain and skeletal muscle. These data implicated that DOCK8 is expressed in a relatively ubiquitous manner. We also performed Northern blots with a 5′ fragment encoding the N-terminal extension (see Fig. 1B). In this case, a very weak band of 9 kb, indicative of a low abundance mRNA, was detected in placenta, lung and kidney (data not shown).

A DOCK8 specific antiserum was produced by immunising a rabbit with a peptide derived from the C-terminal of DOCK8. In order to test the specificity of the antiserum, different cell-lines were $^{35}$S-labelled and DOCK8 was immunoprecipitated with the DOCK8 specific antiserum. As a control, pre-immune serum was employed for immunoprecipitation. A band with a molecular mass of around 190 kDa was present in both Burkitt lymphoma Daudi cells as well as in human promyelocytic leukaemia HL-60 cells, consistent with a protein of 1701 amino acid residues (Fig. 2B, arrow). HA-tagged DOCK8 was transfected into Cos7 cells, the cells were $^{35}$S labelled and HA-DOCK8 was precipitated with the DOCK8 antiserum. A band with a molecular mass of around 190 kDa, representing ectopically expressed DOCK8, was visible (Fig. 2B). No corresponding band was detected in the material subjected to immunoprecipitation with the pre-immune serum.

For reasons unknown, the mouse monoclonal anti-HA antibody worked poorly in immunoprecipitation. Taken together, these observations suggest that the 1701 amino acid residue protein is the predominant form of DOCK8.

### 3.3. Interaction of DOCK8 with Rho GTPases

To study the GTP-dependency of the DOCK8–Cdc42 interaction, one of the plasmids isolated in the yeast two-hybrid screen (encoding GAL4-AD:DOCK8/1044–1701) was trans-

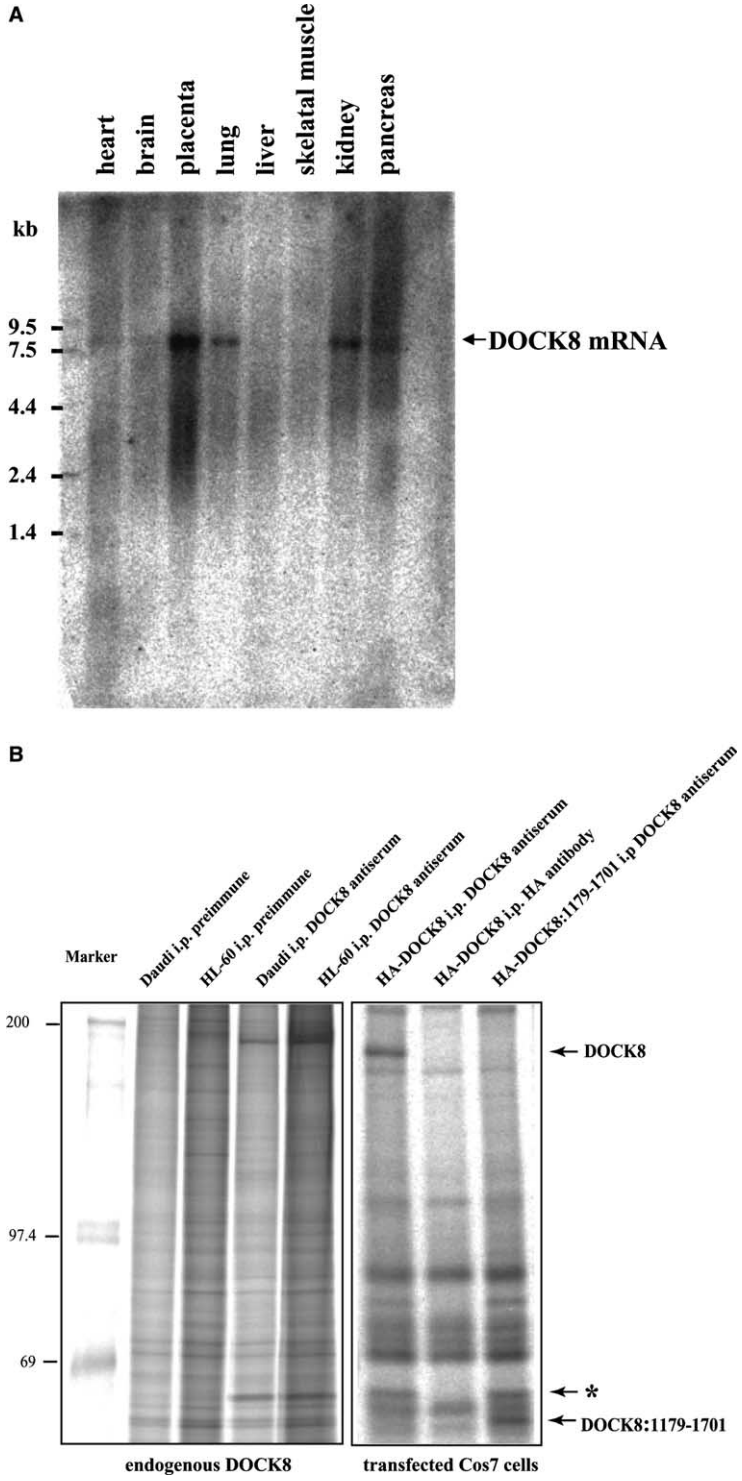


Fig. 2. Tissue distribution of DOCK8. (A) Northern blot analysis of DOCK8 mRNA in human tissues, as indicated in the panel. The arrow marks the DOCK8 mRNA of 8 kb. (B) DOCK8 was precipitated with the DOCK8-specific antiserum from lysates of $^{35}$S-labelled Daudi, HL-60 cells or Cos7 cells transfected with HA-tagged DOCK8 or DOCK8:1179–1701. The arrow marks the position of the band possibly representing endogenous, as well as transfected DOCK8. For unknown reasons, the HA antibody did not work in immunoprecipitation. The asterisk marks the position of an additional band of 60 kDa in Daudi, HL-60 and Cos7 cells, representing either a DOCK8-interacting protein or a splice-variant of DOCK8.

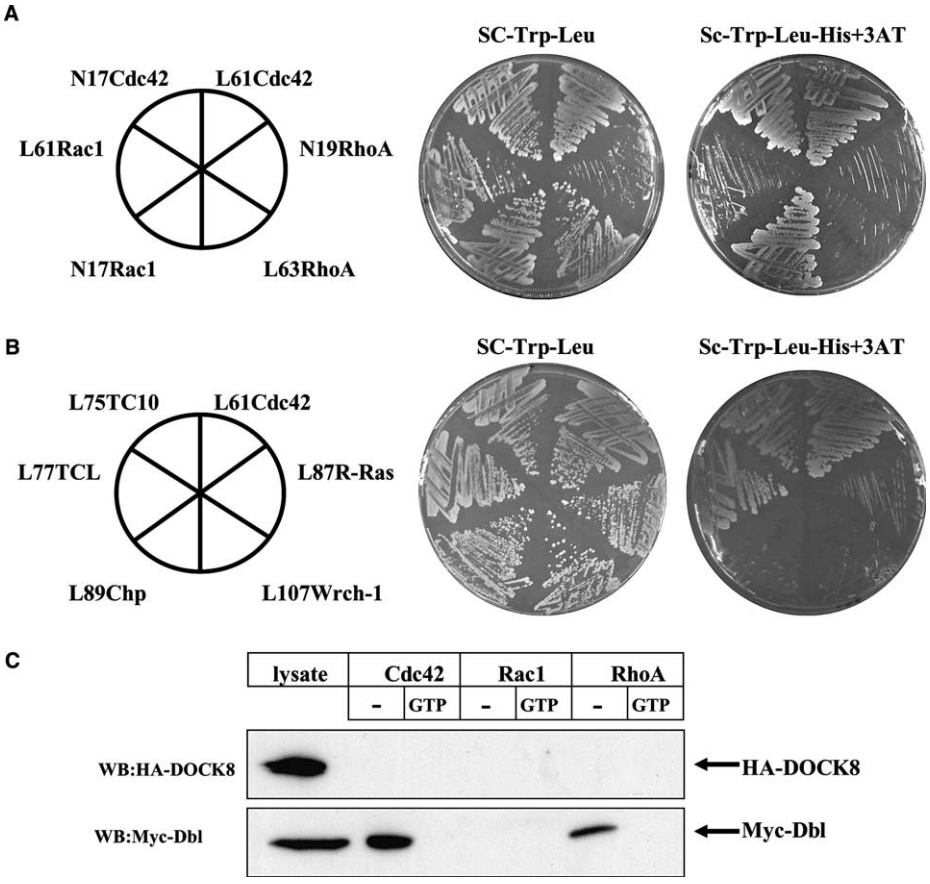


Fig. 3. Interaction of DOCK8 with Rho GTPases. (A) Interaction between GAL4AD-DOCK8:1044–1701 with GAL4DB-fusion proteins of L61Rac1, N17Rac1, L61Cdc42, N17Cdc42, L63RhoA or N19RhoA in the yeast two hybrid system. The colonies were streaked out on plates with media selecting for the plasmids (Sc–Trp–Leu) or media selecting for a positive interaction (Sc–Trp–Leu–His + 3AT). (B) Interaction between GAL4AD-DOCK8:1044–1701 with GAL4DB-fusion proteins of L61Cdc42, L75TC10, L77TCL, L89Chp, L107Wrch-1 or L87R-Ras in the yeast two hybrid system. The colonies were streaked out on plates with media selecting for the plasmids (Sc–Trp–Leu) or media selecting for a positive interaction (Sc–Trp–Leu–His + 3AT). (C) HA-tagged DOCK8:1179–1701 in precipitates of GST fusion-proteins of Cdc42, Rac1 or RhoA, either nucleotide free (–) or GTP-loaded (GTP).

formed into yeast strains encoding GAL4-DB fused to L61Cdc42 or N17Cdc42. The L61 mutant Cdc42 is constitutively in the GTP-bound conformation, whereas the N17 mutant mimics the GDP-bound conformation [16]. DOCK8 bound to L61Cdc42 and N17Cdc42 with the same apparent affinity (Fig. 3A). DOCK8 bound also to L61Rac1 and N17Rac1 but not to L63RhoA. In addition, DOCK8 bound to the Cdc42 family members TCL and TC10 (Fig. 3B). Next, DOCK8 was tested for binding to Cdc42 in a GST pull-down assay. GST-Cdc42, GST-Rac1 and GST-RhoA were either loaded with GTPγS, to generate Rho GTPases stably in the GTP-bound conformation, or treated with EDTA to obtain nucleotide-free GTPases. HA-DOCK8/1179–1701 was transiently transfected into Cos7 cells, the cells were lysed and the lysates were passed over the glutathione–Sepharose-bound GTPases. In contrast to the yeast two-hybrid system, we could not detect binding under these conditions (Fig. 3C). We also made GST fusion-protein of DOCK8/1179–1701 in *Escherichia coli*, the proteins were spotted on nitrocellulose filters and incubated in the presence of $\gamma^{32}$P-GTP-loaded GTPases as has been done for other Cdc42 binding-partners [11]. We could not find a stable binding to any of the Rho GTPases tested. Moreover, this GST fusion-protein was not active in any of the GEF (or GAP) assays performed (data not shown). These observations might reflect that the interaction between DOCK8 and Rho GTPases is too transient to allow a stable interaction in the GST pull-down assay. However, it can be noted that a similar result was obtained from studies on the DOCK8-related CDM family member DOCK7, in which a stable interaction between DOCK7 and Rho GTPases could not be detected [13].

### 3.4. Subcellular localisation of DOCK8

In order to examine to subcellular localisation of DOCK8, PAE/PDGFRβ cells were transiently transfected with HA-tagged DOCK8, the cells were starved and stimulated with either 100 ng/ml PGDF-BB for 10 min or 10% FCS for 5 min. The cells were fixed and HA-DOCK8 was visualised with HA-specific antibodies followed by TRITC-conjugated secondary antibodies. Filamentous actin was detected with FITC-conjugated phalloidin and the cells were examined by immunofluorescence microscopy. In non-stimulated cells, DOCK8 was present in the cytoplasm but an accumulation of DOCK8 in protrusions at the cell edges, presumably in lamellipodia, was clearly visible (Fig. 4A). The localisation to lamellipodia in-

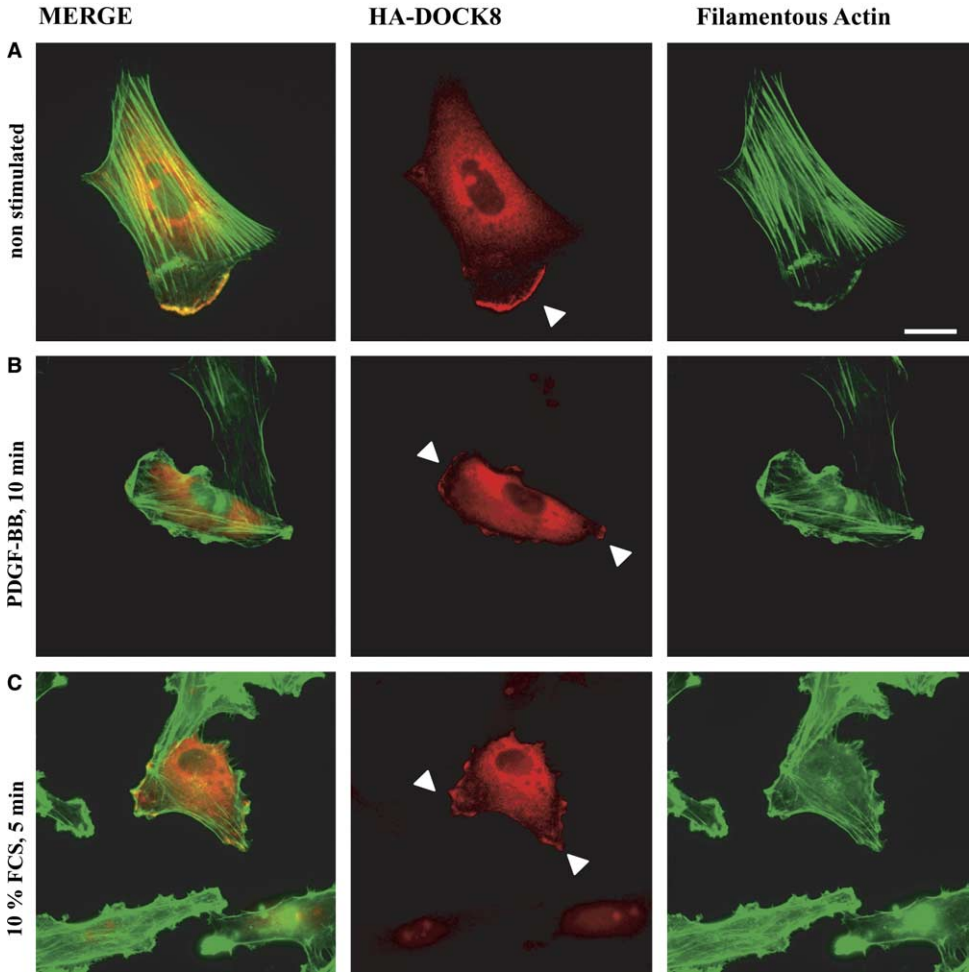


Fig. 4. Subcellular localisation of HA-tagged DOCK8. PAE/PDGFRβ cells were transiently transfected with HA-tagged DOCK8, the cells were starved and left non-stimulated (A) or treated with 100 ng/ml PDGF-BB for 10 min (B) or 10% FCS for 5 min (C). HA-tagged DOCK8 was detected by an HA-specific mouse anti-HA antibody followed by a tetramethyl rhodamine isothiocyanate (TRITC)-labelled anti mouse antibody. Filamentous actin was detected by Fluorescein isothiocyanate (FITC)-labelled phalloidin. The arrowheads mark the presence of HA-tagged DOCK8 in lamellipodia of transfected cells. The bar denotes 20 μm.

creased when the cells were treated with PDGF-BB or FCS (Fig. 4B and C).

Next, PAE/PDGFRβ cells were fixed in 3% paraformaldehyde and endogenous DOCK8 was visualised with the rabbit polyclonal DOCK8 antibody. Endogenous DOCK8 was also found at lamellipodia, thereby confirming the observation with the transiently transfected HA-DOCK8 (Fig. 5A). DOCK8 was also visualised in NIH3T3 fibroblasts. These cells had very small lamellipodia, thus the peripheral localisation of DOCK8 was not very prominent (Fig. 5A). If the PAE/PDGFRβ cells instead were fixed in ice-cold methanol, a nuclear staining was seen in addition to the cytoplasmic staining (Fig. 5B). Pre-incubation of the DOCK8 antibody with the peptide antigen effectively quenched the signal, indicating that the staining pattern indeed represented the localisation of the endogenous DOCK8 (Fig. 5B). A HA-tagged C-terminal fragment of DOCK8 (DOCK8:1179–1701) was transfected into PAE/PDGFRβ cells. This fragment localised into ring-like structure, a staining pattern very distinct from the full-length DOCK8 (Fig. 5C). The DOCK8-induced rings also attracted filamentous actin to form ring-like structures (Fig. 5C, detail). When the cells were treated with PDGF-BB, the DOCK8 induced rings persisted, but DOCK8 also relocalised into lamellipodia.

## 4. Discussion

The first described member of the CDM family of proteins, DOCK180, was isolated during a search for proteins interacting with the proto-oncogene product c-Crk [17]. The *Caenorhabditis elegans DOCK180* orthologue *Ced-5* has been shown to control the engulfment of apoptotic cells as well as the migration of gonad cells [18]. In *Drosophila melanogaster*, the flies harbouring mutations in *Myoblast City (Mbc)* are defective in the process that result in the fusion of single cell myocytes into multinuclear myoblasts. In addition, *Mbc* mutants are defective in the migration of so-called border cells to the egg chambers [19]. This process is also dependent on the PDGF-like ligand PVF [20]. In mammalian cells, recent observations indicate that DOCK family of proteins is encoded by at least 11 genes, which can be further divided into 4 sub-

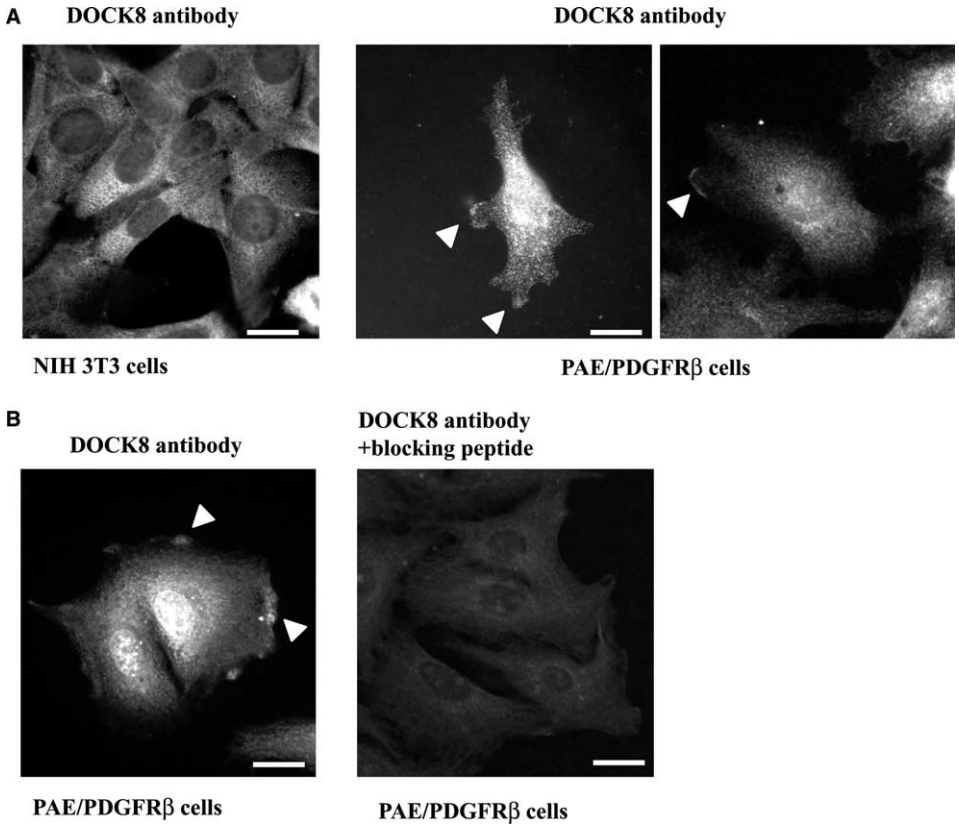


Fig. 5. Subcellular localisation of endogenous DOCK8 and the C-terminal fragment of DOCK8. (A) NIH3T3 and PAE/PDGFRβ cells were starved and fixed in 3% paraformaldehyde. Endogenous DOCK8 was detected with a rabbit polyclonal DOCK8 antibody followed by a TRITC-labelled anti rabbit antibody. (B) PAE/PDGFRβ cells were fixed in ice-cold methanol. Endogenous DOCK8 was detected with a rabbit polyclonal DOCK8 antibody followed by a TRITC-labelled anti rabbit antibody. The specificity of the DOCK8 antibody was determined by pre-incubating the antibody with the peptide antigen. The arrowheads mark the presence of DOCK8 in lamellipodia. (C) PAE/PDGFRβ cells were transiently transfected with HA-tagged DOCK8:1179–1701, the cells were starved and then either non-stimulated or treated with 100 ng/ml PDGF-BB for 5 min. HA-tagged DOCK8 C-terminal was detected by an HA-specific mouse anti-HA antibody followed by a TRITC-labelled anti mouse antibody. Filamentous actin was detected by FITC-labelled phalloidin. The bar denotes 20 μm.

groups [13]. So far, only a few of them have been characterised in more detail and most of our current knowledge comes from studies on the DOCK180 and DOCK2, which have roles in regulating different aspects of cytoskeletal reorganisation [17,21]. Interestingly, the DOCK180-like modifier of cell adhesion (MOCA), which was originally identified as a presenilin-binding protein, has been linked to the regulation of amyloid precursor protein metabolism [22,23]. Apart from these examples, very little is known about the DOCK family proteins and, in fact, they share little sequence homology outside the CZH1 and CZH2 domains. The data presented in this article suggest that DOCK8 is present in lamellipodia and other areas undergoing dynamic actin reorganisation. In addition, a C-terminal fragment of DOCK8, encompassing the CZH2 domain, was able to induce filamentous actin-containing vesicular structures.

In *C.elegans*, as well as in mammalian cells, DOCK signalling has been shown to involve CrkII, Rac1 and ELMO [24,25]. Interestingly, DOCK has been shown to trigger GTP-loading of Rac, through a mechanism that is independent of the classical Dbl homology domain-containing GEFs [26,27]. Some additional DOCK family members, such as Zizimin, also activate GTPases (in this case Cdc42) [15]. In contrast, we have not been able to detect a stable interaction between Cdc42 and DOCK8 in any of the immunoprecipitation or pull-down experiments performed. In addition, no major influence of DOCK8 on the GTP-loaded status of Cdc42 nor of Rac1 was detected, yet DOCK8 binds Cdc42, TCL, TC10 and Rac1 in the yeast two-hybrid system with high affinity. To this end, it is worth noticing that the yeast two-hybrid system will also allow the detection of very transient interactions. This suggests that a functional interaction between DOCK8 and Cdc42 is likely to be transient and that an additional protein, such as ELMO, might be needed in order to achieve an efficient binding or exchange activity of DOCK8 [24,25]. ELMO binds to the SH3 domain of DOCK180, a domain absent from DOCK8, making ELMO a less likely "stabiliser" of a potential DOCK8 exchange activity [25]. In addition, the difference between the pull-down experiments and the yeast two-hybrid experiments might be caused by the use of different mutants of Rac1 and Cdc42. In the yeast two-hybrid system, GTPases with mutations in the CAAX box were used, whereas in the pull-down experiments the CAAX box was intact. Zizimin was shown to trigger the exchange of a Cdc42 CAAX box mutation with a significantly higher efficiency [15]. Hopefully, future studies will resolve the nature of the DOCK8:Cdc42 interaction.

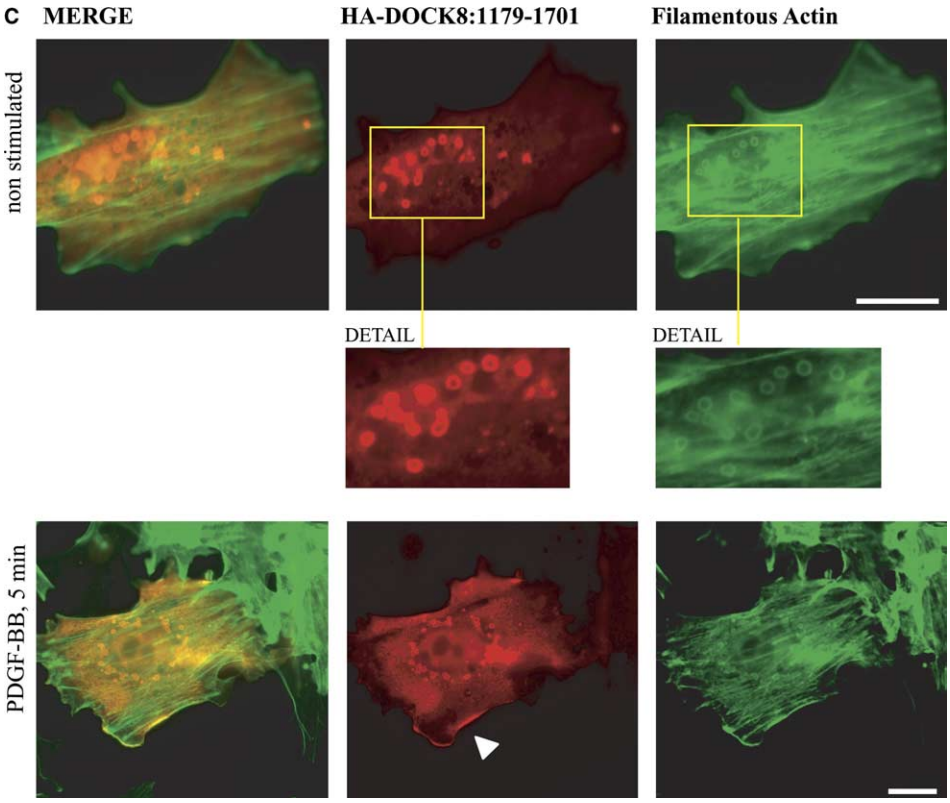


Fig. 5 (*continued*)

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