**FHOD proteins in actin dynamics—a formin’ class of its own**

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**Abbreviations:** AML-1B, acute myeloid leukemia transcription factor; DAD, diaphanous auto-regulatory domain; DID, diaphanous inhibitory domain; DRF, Diaphanous-related formins; FH1, formin homology 1; FH2, formin homology 2; FH3, formin homology 3; GBD, GTPase-binding domain; Dia, Diaphanous related formin; FHOD, FH1/FH2 domain-containing protein; SRE, serum response element.

Eukaryotic cells have evolved a variety of actin-binding proteins to regulate the architecture and the dynamics of the actin cytoskeleton in time and space. The Diaphanous-related formins (DRF) represent a diverse group of Rho-GTPase-regulated actin regulators that control a range of actin structures composed of tightly-bundled, unbranched actin filaments as found in stress fibers and in filopodia. Under resting conditions, DRFs are auto-inhibited by an intramolecular interaction between the C-terminal and the N-terminal domains. The auto-inhibition is thought to be released by binding of an activated RhoGTPase to the N-terminal GTPase-binding domain (GBD). However, there is growing evidence for more sophisticated variations from this simplified linear activation model. In this review we focus on the formin homology domain-containing proteins (FHOD), an unconventional group of DRFs. Recent findings on the molecular control and cellular functions of FHOD proteins in vivo are discussed in the light of the phylogeny of FHOD proteins.

### Introduction

Formins are highly conserved multidomain proteins that control the formation of diverse cytoskeletal structures required for many cellular functions such as cell shape, adhesion, cytokinesis and cell motility (For a review see refs.1-5). The common defining features for this protein family are the formin homology domains 1 and 2 (FH1 and FH2). The proline-rich FH1 domain delivers profilin-bound actin monomers to the growing barbed end and the homodimeric FH2 domain is thought to induce actin polymerization by stabilizing actin dimers. Hence the formins stay attached to the growing barbed end and thereby prevent binding of capping proteins during actin elongation. A subgroup of formins, the Diaphanous-related formins (DRF) contain 2 additional conserved domains: the N-terminal diaphanous inhibitory domain (DID, also referred to as FH3) and the C-terminal diaphanous auto-regulatory domain (DAD), which interact with one another in order to keep the protein in an inactive state. In some DRF proteins, the auto-inhibition can be released by RhoGTPase binding. However, there is growing evidence that points to distinct regulatory mechanisms rather than a simple linear activation pathway. The FH1/FH2 domain-containing FHOD proteins share many features in the domain architecture with other DRF family members, but their molecular activities and the mechanism of activation seem to be distinct from other DRFs.

**FHOD proteins evolved in unicellular relatives of metazoans**

Most evolutionary analyses of formins focused either on the characterization of the formin complement of defined species and taxons or on the relationship of different formin families to each other.⁸ ⁹ Here, we trace the history of FHOD, i.e. one formin family, over its whole evolutionary life (Fig. 1). We calculated a phylogeny of orthologs identified in completely sequenced eukaryotes. The most basal species with an identifiable FHOD ortholog is the filasterean Capsaspora owczarzaki. Besides the choanoflagellates, where no FHOD was found, this species is the closest known unicellular relative of metazoans. Thus, the emergence of FHOD predates the evolution of metazoa and parallels the history of Diaphanous, another formin family.¹⁰ Whereas most species contain only a single member of this protein family, at least 2 are found in vertebrates. Even in the most basal vertebrate, the lamprey Petromyzon marinus, clearly defined orthologs of FHOD1 and FHOD3 are present. The genome of the urochordate Ciona intestinalis also contains 2 homologs of FHOD, but these are fragmentary and therefore could not be placed with certainty in the phylogenetic tree. Thus, a duplication before the split of urochordates and chordates cannot be excluded. Following this duplication, FHOD1 evolved significantly faster than FHOD3 (p < 0.001), indicating either a relaxed selective pressure or the emergence of new functions. Finally, within teleost...
fishes, the gene coding for FHOD3 duplicated again resulting in 3 FHOD genes in this clade (Fig. 1). The Drosophila melanogaster ortholog of FHOD, *knittrig* distinguishes itself from the other FHODs by an N-terminal extension present in some splice variants. The function of this additional large exon is unclear. However, a short *knittrig* isoform lacking this extension substantially rescues the observed phenotypic traits of *knittrig* mutants including late-pupal lethality. Analysis of further arthropod genomes revealed a similar extension in the pea aphid Acyrthosiphon pisum. Although no extension is annotated for other completely sequenced insects, inspection of adjacent genes revealed homologous sequences in the silk moth Bombyx mori, the bee Apis mellifera and the beetle Tribolium castaneum. Thus it can be assumed that within these species the FHOD genes are not correctly annotated and that they also encode an extended gene. As no traces of this extension was found in the body louse Pediculus humanus, this modification of the FHOD gene seems to be specific for Neoptera. The extension contains a repetitive structure with a conserved Trp-Trp repeat and its function is so far unknown. Still, an

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**Figure 1.** The evolutionary history of fhod gene. Candidate orthologues to D. melanogaster *knittrig* in selected species were extracted from ensembl. As an outgroup to metazoans, the genomes of C. owczarzaki and Monosiga brevicollis were searched for candidate orthologues by reciprocal blastp. Protein sequences of the longest splice variant were aligned using muscle and an evolutionary model was estimated with protTest. The phylogenetic tree was calculated with phylip using the approximate likelihood ratio as estimate for robustness. To compare the evolutionary rates of fhod1 and fhod2, a subset containing vertebrate sequences and the sea urchin Stronglylocentrotus purpuratus as outgroup was aligned. Following a tree calculation with phylip, 2 models were tested using codeml from the PAML package. In the first, a single evolutionary rate for the whole tree was assumed, whereas in the second the rate of the fhod1 clade was given as additional free parameter. According to a Chi2 test with one degree of freedom, the second model fit the data significantly better ($P < 0.001$).
evolutionary conservation for more than 370 million years implies its functional importance.

**FHOD proteins differ from other DRFs in their activation mode**

Initial studies by Westendorf and colleagues already revealed that FHOD1 is primarily auto-inhibited by an intramolecular interaction between N- and C-terminal residues. However, unlike other DRFs, FHOD1 associates with the RhoGTPase Rac1 in a guanine nucleotide-independent manner in vitro. The N- and C-terminal regions of FHOD proteins differ significantly in overall composition and length of the particular domains (Fig. 2A). This suggests a different auto-inhibitory mechanism compared to so far known mDia1-RhoGTPase interactions. In vitro binding studies further confirmed that the N-terminal DAD-interacting FH3 domain does not overlap with the proposed Rac1-binding domain. Thus, a competitive binding and subsequent activation by Rac1 seems unlikely. The auto-inhibitory interaction can be released by removal of the C-terminal DAD domain resulting in a stimulation of transcription from the serum response element (SRE) as well as in a strong induction of stress fibers. Gasteier and colleagues provided first evidence that FHOD1-induced stress fiber formation depends on the activity of Rho-associated kinase Rock/Rok. Further cell culture studies showed that Rock/Rok directly phosphorylates mammalian FHOD1 at highly conserved C-terminal residues, specifically at serine 1131, serine 1137 and threonin 1141. Rock/Rok-induced phosphorylation seems to be a conserved activation mechanism in vivo. Serine phosphorylation of Knitrig, the only member of the FHOD family in Drosophila, is likewise enhanced upon Rock co-expression that promotes stress fiber formation in macrophages. Thus, FHOD formins are likely downstream effectors of Rock/Rok signaling. While Rho-Rok function seems to be important for the activation, Rac1 signaling is thought to be required for the subcellular localization and recruitment of FHOD formins.

Structural analysis of the N-terminal region of mammalian FHOD1 provided further evidence for such a model of FHOD regulation. The deletion of the Ras-like GBD domain does not result in an activation of FHOD1, but on the contrary, results in a complete loss of the biological activity. In contrast, mutation of a central valine at position 228 within the FH3 domain results in a constitutive active mutant. Thus, FHOD1 contains two distinct N-terminal regulatory modules. The GBD domain is required for the biological activity and the localization mediated by Rac whereas the adjacent FH3 domain provides the surface for the intramolecular interaction with the DAD domain, which

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**Figure 2.** (A) Domain architecture of FHOD proteins and known signals/interactors linked to indicated FHOD domains. GBD: GTPase binding domain; FH3: formin homology 3 domain; FH1: formin homology 1 domain; FH2: formin homology 2 domain; DAD: Diaphanous autoregulation domain. (B) Proposed model of how different signaling pathways differentially control the localization/recruitment and the activation of FHOD proteins. Please compare text. (C) Schematic illustration of the two complementary activities at the trailing and leading edge of migrating macrophages as previously proposed.
is controlled by Rho-Rok dependent phosphorylation. How opposing Rac- and Rho-dependent signaling on FHOD1 function are integrated in vivo still remains unknown (Fig. 2B). Given that the expression of active FHOD1 interfered with Rac1-induced lamellipodia formation, it has been proposed that Rho/Rock activity may be fine-tuned by simultaneous inhibition of Rac.22 Such a scenario might be relevant in migrating cells. However, in vaccinia virus induced actin-based motility Rac1 suppression phenocopied effects of FHOD1 depletion suggesting that FHOD1 is the most highly expressed human formin in haematopoietic cells.23 The physiological relevance of this interaction was previously isolated as a yeast-2-hybrid binding partner of the acute myeloid leukemia transcription factor AML-1B from a B cell library.24 The physiological relevance of this interaction has never been proven although FHOD1 has been described to be abundantly expressed in spleen, B cells and erythroid leukemia cells.24 A recent quantitative real-time approach however revealed that FHOD1 is the most highly expressed human formin in hematopoietic as well as in non-hematopoietic cell types.25 In contrast, the FHOD3 protein, which is most related to FHOD1, is the least expressed formin with a specific expression pattern in skeletal and cardiac muscle.25

FHOD proteins play a major role in the formation of contractile actin bundles regulating cell migration and sarcomeric actin filament organization

Our current understanding of the cellular functions of FHOD proteins is largely based on cell culture studies although recent studies in Drosophila and C. elegans provided first insights into the physiological functions in vivo.

FHOD1, one of the 2 members of the mammalian FHOD family, was originally isolated as a yeast-2-hybrid binding partner of the acute myeloid leukemia transcription factor AML-1B from a B cell library.23 The physiological relevance of this interaction has never been proven although FHOD1 has been described to be abundantly expressed in spleen, B cells and erythroid leukemia cells.24 A recent quantitative real-time approach however revealed that FHOD1 is the most highly expressed human formin in hematopoietic as well as in non-hematopoietic cell types.25 In contrast, the FHOD3 protein, which is most related to FHOD1, is the least expressed formin with a specific expression pattern in skeletal and cardiac muscle.25

FHOD proteins have been best characterized for their role in stimulating stress fiber formation upon over-expression of C-terminal truncated mutant proteins. A recent detailed cell culture study further demonstrated a more differential role of FHOD1 in regulating the formation and turn over of transverse arcs into mature ventral stress fibers.22 This prominent function of FHOD1 on contractile actin bundles seems to be essential for proper cell spreading, cell adhesion and cell migration.14,22 More recent RNAi-mediated knockdown experiments further revealed that FHOD1 assembles F-actin at integrin adhesions to drive cell spreading and migration by coordinating traction stress and adhesion maturation.26 The ability of FHOD1 in forming actin bundles might also play an important physiological function in nuclear movement in migrating cells as previously observed in fibroblasts.27

The role of FHOD in cell spreading and cell migration seems to be conserved in Drosophila.11 Loss of knirtrig function in flies results in strong cell spreading defects of isolated primary macrophages. More importantly, mutant macrophages show strong defects in directional wound-induced cell migration in vivo.11 Live imaging studies further revealed a remarkably dynamic accumulation of FHOD at the cell rear of migrating macrophages.11 Consistent with this distinct localization, an active FHOD protein lacking the DAD region promotes the formation of actin bundles at the cell rear in a Rok-dependent manner (Fig. 2C). Based on these findings a model has been proposed in which FHOD acts downstream of Rok coordinating actin assembly and actomyosin-based retraction in the trailing edge of migrating macrophages.11 However, it remains unclear how Drosophila FHOD is recruited to the cell rear in vivo while the same EGFP-tagged fusion localized throughout the cytoplasm in cultured macrophages, which might be an artifact due to the culturing of isolated cells. Targeting of human FHOD1 to adhesion sites depends on Src kinase phosphorylation on tyrosine Y99 that enables subsequent activation by Rok.26 Drosophila FHOD contains several conserved tyrosine residues within its N-terminus, thus it might also be a downstream target of Src. Future genetic studies are required to rule out if the localization of FHOD is affected in src mutant macrophages. A positive activity on cell migration has recently been observed for human FHOD1, whose expression is up-regulated during epithelial-mesenchymal transition promoting cancer cell migration.28 Consistently, overexpression of human FHOD1 in melanoma and breast cancer cells promotes cell migration and cell invasion.29 The physiological function of mammalian FHOD1 in cell migration has not yet been addressed in a knockout model.

A knockout mouse model has recently been established to analyze fhod3 function in vivo.30 The phenotypic analysis of mutant mice revealed an essential role in cardiogenesis by organizing myofibrillogenesis.30 FHod3 deficient mice are embryonic lethal with severe defects at early stages of heart development. These data confirmed previous findings obtained from RNAi-mediated depletion of FHOD3 in cultured cardiomyocytes that shows a reduction of filamentous actin and disruption of the sarcomeric organization.31,32 Together, these studies document a differential requirement of FHOD1 and FHOD3, which is already reflected by their distinct expression pattern. A function of FHOD in Drosophila myogenesis has not yet been found. Loss of knirtrig function affects trachea and wing morphology.11 Analysis of the only fhod-1 ortholog in C. elegans revealed more functional similarities to mammalian FHOD3. Fhod-1 is present in sarcomeres of developing striated body wall muscles and worms lacking fhod-1 showed modest morphological and functional muscle defects.33 A recent RNAi screen in C. elegans identified an additional essential function of fhod-1 in organizing...
actin stress fibers in contractile lateral epidermal cells.\(^3,4\) Unlike FHOD3 function in mammals it remains unclear if FHod-1 acts downstream of Rho/Rok signaling. Future studies should also provide insights into a possible conserved function of C. elegans FHod-1 in cell migration.

Taken together, recent studies advance substantially our understanding about the cellular functions of the FHOD proteins. Findings from different model systems underline a major role in the formation of contractile actin bundles that are required for cell migration and sarcomeric actin filament organization. The phylogeny of FHOD suggests that these 2 important cellular functions were first adopted by a single gene. During evolution both function were split between 2 genes in vertebrates (fhod1 and fhod3). Many questions about the exact mechanisms of how FHOD proteins promote actin bundles and how different signals control their in vivo activity in time and space remain important challenges for future research.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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