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# Expression of multiple formins in adult tissues and during developmental stages of mouse brain

Priyanka Dutta, Sankar Maiti*

*Department of Biological Sciences, Indian Institute of Science Education and Research Kolkata, Mohanpur, 741246 Nadia, West Bengal, India*

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ABSTRACT

Formins are highly conserved heterogeneous family of proteins with several isoforms having significant contribution in multiple cellular functions. Formins play crucial role in remodelling of actin cytoskeleton and thus play important role in cell motility. Formins are also involved in many cellular activities like determining cell polarity, cytokinesis and morphogenesis. Formins are multi domain protein with characteristic homodimeric formin homology 2 (FH2) domain. It nucleates the actin filaments and its activity is regulated by the presence of characteristic formin homology 1 (FH1) domain. In higher mammals like human and mouse fifteen different formin isoforms are present. However the function and expression pattern of each and every formin in different adult tissues are not well characterized. Here we have found that multiple formins are expressing in each adult tissue of mouse, irrespective of their origin from the germ layer. Formins are also expressing from early stage of development to the adulthood in brain. The expression of many formins in a single tissue of adult mouse indicates that regulation of actin cytoskeleton dynamics by formins may be crucial for physiological processes like wound healing, tissue repairing, exocytosis, endocytosis, synapse formation and maintenance. Expression of FMNL2 and Fhdc1 are high in adult mouse brain as compare to embryonic stages. Higher expression of FMNL2 and Fhdc1 indicates that FMNL2 and Fhdc1 might be very important for the adult brain functions.



## 1. Background

In the early 90's formins are discovered for the first time (Schönichen and Geyer, 2010). Formin are highly conserved single polypeptide multi domain proteins possessing formin homology-2 (FH2) and formin homology-1 (FH1) domains. Formins belong to the class of actin binding proteins involved in cell division, filopodia formation, stress fibre formation, neurite initiation and gastrulation (Evangelista et al., 2003; Paul and Pollard, 2009; Higgs, 2005; Breitsprecher and Goode, 2013). The first formin is named after the protein product of *limb deformity* gene in mouse; mutation of which failed to form limbs in mouse (Woychik et al., 1990). The *Drosophila* formin, Diaphanous, and Bni1, the formin from *Saccharomyces cerevisiae* have been identified four years later (Castrillon and Wasserman, 1994). Progressively other formins having FH2 domain were discovered in different organisms. Simple organism like budding yeast has 2 formins and fission yeast has 3 formins (Goode and Eck, 2007). Whereas higher mammals like mouse and human have 15 members of formin family protein and they are divided into 7 major subgroups (Higgs, 2005).

The FH2 domain of formins is mainly responsible for formation of long actin filaments (Zigmond, 2004; Goode and Eck, 2007; Kevin and Copeland, 2010). They are composed of approximately 400 amino acids and are capable of *in vitro* nucleation, bundling and severing of actin filaments (Chesarone et al., 2009). The crystal structure of FH2 domain has shown that it is functional in dimeric form and remains attached to the barbed end of the actin filaments to produce branchless linear filaments (Xu et al., 2004). During the filament growth FH2 domain remains attached to the barbed end of the filament and act as a leaky capper (Schönichen and Geyer, 2010; Baarlink et al., 2010) by allowing progressive elongation of actin filaments (Pruyne et al., 2002; Sagot et al., 2002). FH2 domain has very high affinity for the barbed end of actin filaments and therefore it can remove other capping proteins from the barbed end.

The FH1 domain is present at the N-terminus adjacent to the FH2 domain and is composed of about 100–150 amino acids. The formin homology-1 domain is made up of consecutive proline repeats and interacts with profilin (Kovar et al., 2003). Profilin-actin

* Corresponding author.
*E-mail addresses:* pd_july30@yahoo.co.in (P. Dutta), spm@iiserkol.ac.in (S. Maiti).

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complex supplies the actin monomer for FH2 domain which controls activities like efficient nucleation and elongation of actin filaments (Romero et al., 2004; Kovar et al., 2006). Besides FH1 and FH2 domain some formins have other domains like GBD (GTPase binding domain), DID (Diaphanous inhibitory domain), DD (Dimerization domain), CC (Coiled coil), PDZ domain (PSD-95/Disc large/ZO-1) and C-terminal DAD domain (Diaphanous autoregulatory domain) (Alberts, 2001; Nezami et al., 2006; Miyagi et al., 2002).

DAD (Diaphanous autoregulatory domain) is present C-terminus to the FH2 domain and plays major role in influencing the activity of mDia, FRL FHOD and Daam formins. DAD domain consist of 25–30 amino acids and binds to the DID domain present N-terminal to the FH1 and FH2 domain in mDia, Daam and FRL formins. It inhibits the activity of the formins in an auto inhibitory manner (Gould et al., 2011; Otomo et al., 2005; Wallar et al., 2006). The binding of Rho GTPase to the GBD domain prevents the auto inhibition of diaphanous formins having DAD and DID domains (Watanabe et al., 1999).

Some formins like Delphilin, Inverted formin, FMN has neither GTPase binding domain nor regulated by auto inhibitory mechanisms. Delphilin has PDZ domain at its amino terminus region which binds to glutamate delta 2 receptor in the post synaptic region (Matsuda et al., 2006). The Inverted formins possess a WH2 domain, in addition to FH2 domain and present towards the C-terminal end (Chabbra and Higgs, 2006; Young et al., 2008; Brown et al., 2010).

The role of formins in regulating the structure and function of actin cytoskeleton and thereby controlling cell shape, motility and polarity is well established. Studies have shown that formins are necessary for embryonic development in higher mammals. They are also important for bud formation in yeast (Sagot et al., 2002; Nakaya et al., 2004). However, the exact function of each and every formin in higher mammals is not clear. It also remains to be known the tissue specific expression pattern of the formins.

Here we have investigated the expression profile of different formins in normal adult tissues of mouse originated from different germ layers. Interestingly it is observed that in every adult tissue whether it is lung or brain or kidney, are expressing majority of the formins. This may indicate important cellular function of formins in various tissues. Expression analysis of formins from early stage of development to adulthood is done in mouse brain which shows differential expression for few formins and these may be related to age specific function.

## 2. Materials and methods

### 2.1. Sample collection

Different tissue samples were collected from C57BL/6 adult mice and from C57Bl/6 mice brain at different stages of development [prebirth (E15), Zero day pups, 15day pups, 1month adult and adult mice 8 weeks old]. The tissues were cleaned with 1X sterile PBS and stored in RNA Later (Qiagen) at −80 °C.

### 2.2. RNA preparation

50 mg of tissue was taken from each sample and total RNA was isolated using TRIzol reagent (Invitrogen). The tissues were homogenized in 1 ml TRIzol using tissue homogenizer. Thereafter 0.2 ml chloroform was added for phase separation and the RNA was precipitated with isopropanol. The RNA pellet was washed with 70% molecular grade ethanol, dried and finally dissolved in DEPC treated water (Hyclone, Thermo Scientific). The quality of the RNA and its quantification was done in Thermo Scientific Nano Drop 2000 spectrophotometer.

### 2.3. DNase treatment of total RNA

One microgram of RNA with 1 μl of buffer containing $MnCl_2$ and DEPC treated water was treated with 1 U of DNase I RNase free (Thermo Scientific) as per the manufacturer's protocol. The reaction was inactivated by addition of 1 μl of 50 mM EDTA at 65 °C for 10 min. This DNase I treatment was done twice for complete removal of genomic DNA traces from the total RNA. The integrity of the RNA was checked by running the RNA from each organ in agarose-formaldehyde gel electrophoresis. The RNA quality was further checked by setting up a PCR reaction with GAPDH primers at 57 °C.

### 2.4. cDNA preparation and RT PCR

For each sample 1 μg RNA was used for cDNA synthesis. RNA was incubated at 65 °C for 5 min with 2.5 μM oligo(dT) and 0.5 mM dNTPs. Thereafter the RT buffer, 5 mM $MgCl_2$, 0.01 M DTT, 40U of RNase OUT and 200U of Superscript III reverse transcriptase (Invitrogen) were added. The reaction was set at 50 °C for 50 min then terminated at 85 °C for 5 min and finally treated with 1 μl of RNase H at 37 °C for 20 min. The cDNA was stored in −80 °C for future use. The housekeeping gene GAPDH was used as a positive control in each case.

Primers were designed for FH2 domain and in some cases for both the FH2 and DAD domain of different formins and performed RT-PCR and products were checked in 1% agarose gel. The primers were aligned in CLUSTAL Omega (Sievers F et al., 2011) and the alignment result is provided as Supplementary Fig. S2.

FMN1: (fwd-CGGGATCCCGTAAACCAGCCATTGAGCCC,
rev- CCGCTCGAGTTAACTTTCTTCTCTGATGTCAGTTTGCT);
FMN2: (fwd-CGGGATCCATGAATCAGGACAGAGTGGCTAG,
rev- CCCAAGCTTTCAGTGATTTTCCTTTCTTCTGCCT);
Daam1: (fwd GCGGATCCAAGAAGAATATTCCCCAGCCC,
rev- CCCAAGCTTTTAAATGCGTTTCCGGTTCCG);
Daam2: (fwd-CCGGATCCAAAAAGCGCATTCCCCAGC,
rev- GCCCTCGAGTTACTTCCGGTTGCGCTTGAA)
mDia1: (fwd-GCGGATCCACCCCCAAAAAAGTTTATAAG,
rev- CCGCTCGAGTTACCTGTTGACCTGCCGGG);
mDia2: (fwd-CGGGATCCCCAAAGAAAGAATTTAAGCCTG,
rev- CCGCTCGAGTTAGCTGAGACTCTGCCGAATATC);
mDia3: (fwd CGGGATCCGGAATGAAGCAGAAAAAATT,
rev- CCGCTCGAGTTATGGATTTCTTGGAATTCGC);
FHOD1: (fwd-GCGGATCCGACGGCCCAAGGCACCCC,
rev- CCCAAGCTTTCAGCGACCACGGGTCTTGTTGC);
FHOD3: (fwd-GAGGTACCCCCAGGGGTCAGCCAGCG,
rev- GCTCTAGATTAGTTGGCCCTGGAACGCTTTC);
FMNL1: (fwd-CGGGATCCCCAAGAAACCCATCCAGACC,
rev- CCGCTCGAGTTATGGGGGTCCAGGGGGGC);
FMNL2: (fwd-5′CCGCTCGAGAAGAAGCCAATCAAGACGAA,
rev- GAGGTACCTTAATCCTGCTGCTCCATCAG);
FMNL3: (fwd-CGGGATCCATCAAGAAACCTATCAAGACCA,
rev- CCGCTCGAGTTAGTTCCGCTGGGATGGAGTC);
FHDC1: (fwd-GCGGATCCCCAGGACTCCCCTCAGTTTC,
rev- GCTCTAGATTACCCGAGTTCCCCAGTTGACC);
INF2: (fwd-CGGGATCCCGCAGAGTGAATCCACCCAC,
rev- GGAATTCTTACCTCCGAGCCTCCTCCTCC).

### 2.5. Quantitative PCR for expression

Quantitative PCR (qPCR) was conducted using cDNA product of