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Physiological and pathophysiological functions of Swiprosin-1/EFhd2 in the nervous system

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Abstract

Synaptic dysfunction and dysregulation of Ca²⁺ are linked to neurodegenerative processes and behavioural disorders. Our understanding of causes and factors involved in behavioural disorders and neurodegeneration, especially Alzheimer's disease, a tau-related disease, is on the one hand limited and on the other hand controversial. Here, we reviewed recent data about the links between the Ca²⁺-binding, EF-hand containing cytoskeletal protein Swiprosin-1/EFhd2 (EFhd2) and neurodegeneration. Specifically, we have summarized the functional biochemical data obtained *in vitro* with use of recombinant EFhd2 protein, and integrated them with *in vivo* data in order to interpret the emerging role of EFhd2 in synaptic plasticity and in the pathophysiology of neurodegenerative disorders, particularly involving the tauopathies. We have also discussed its functions in actin remodeling through cofilin and small GTPases, thereby, linking EFhd2, synapses and the actin cytoskeleton. Expression data and functional experiments in mouse and in man have led to the hypothesis that downregulation of EFhd2, especially in the cortex, is involved to dementia.

Keywords

Actin; Cofilin; Alzheimer's disease; axonal transport; GTPase; Swiprosin-1/EFhd2; tauopathy

Expression of EFhd2 in the nervous system and links to neurodegenerative diseases

Many genes and proteins have been identified in one tissue type only to be found that they have equally important signaling roles in others. Indeed, activated B cells involved in T cell dependent immune reactions within germinal centers, express many hitherto thought to be neuron-specific genes and reveal long extensions reminiscent of neurites [1]. Hence, although of different lineage, immune and nerve cells appear to share common signaling pathways. Though swiprosin-1/EFhd2 was first identified in the immune system [2], it has

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become increasingly apparent that EFhd2 has a significant role in the mammalian nervous system, and that its function can be linked to several neurological disorders.

Initial studies showed that EFhd2 is most strongly expressed in the brain [3]. Later, EFhd2 could be identified from immunoprecipitation studies of the microtubule associated protein tau, from tauopathy transgenic mouse brains [4]. Western blot analysis indicated that EFhd2 is expressed in all parts of the brain (brain stem, cerebellum, amygdala, striatum, hippocampus, cortex and prefrontal cortex) and at similar levels [4]. It was also reported that the tau - EFhd2 interaction was dependent on the stage of neurodegeneration of the tauopathy transgenic mouse model (JNPL3), being enhanced as the animals were showing more tauopathy; preliminary data also indicated that this could be the case in single cases of Alzheimer's disease (AD) and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) [4], though follow on work with larger number of AD brains, confirmed that EFhd2 and tau could be found in aggregates [5].

Our own work has extended our knowledge of where EFhd2 is expressed in the mammalian brain and how it changes expression in dementias [6, 7]. Specifically, Brachs et al. generated an EFhd2 knockout (KO)/*lacZ* reporter gene knockin mouse (EFhd2KO) by blastocyst injection of commercially available ES cells in which the *efhd2* gene had been replaced with an in frame *lacZ* reporter cassette through homologous recombination [8] EFhd2KO mice reveal a dose-dependent increase of *lacZ* compared to heterozygous mice carrying only one mutated allele (EFhd2HT), concomitant with a dose-dependent decrease of EFhd2 expression. This indicated that *efhd2* expression is haplo-insufficient, at least in this system, and that for full *efhd2* expression, both alleles need to be active [7]. This mouse strain next allowed for the detailed characterization of EFhd2 expression in the mouse brain by detecting the presence of β -galactosidase activity, which was confirmed by the use of specific EFhd2 antibodies [9] and immunostaining of sections of the brain. EFhd2 expression was observed in the pyramidal layers of the cortex, the dentate gyrus, and the CA1-CA2 regions of the hippocampus [7]. These findings confirmed previous *in situ* hybridizations described in the Allen brain atlas which had indicated strong *efhd2* mRNA expression in neuronal rich areas of the brain. White matter regions such as the corpus callosum do not show EFhd2 expression, again confirming the Allen brain atlas *in situ* hybridisation and microarray studies. In addition, our studies indicated that the EFhd2 protein is more abundant in the adult than in the embryonic brain, where it is expressed early in the developing cortex, hippocampus and thalamus [7].

As indicated above the increased association of EFhd2 to tau was first postulated to be a link with this Ca^{2+} binding protein and AD, albeit the mouse model that has been used carries a mutated human tau protein (tau P301L) which is found in the rare neurodegenerative disease FTDP-17 [4]. Initially, FGM's group's interest in EFhd2 came from proteomic studies to identify proteins that changed expression in mitochondrial dysfunction models of AD and specifically to identify synaptic proteins that changed expression (reviewed in [10]). Other studies have also indicated potential links of EFhd2 to diseases associated with synaptic dysfunction; for example, changes in EFhd2 protein levels have been noted in the prefrontal cortex of patients with schizophrenia [11], suicide victims [12], and a mouse model for amyotrophic lateral sclerosis/motor neuron disease [13]. Interestingly, in a rat model for AD,

low frequency electromagnetic fields partially improved cognitive and pathologic symptoms correlating with up-regulation of EFhd2 in the hippocampus [14]. On the other hand, the antiinflammatory drug Ibuprofen led to down-regulation of EFhd2 in the hippocampus of mice [15]. However, to date the most systematic and largest study (n = 48 brains) that has linked EFhd2 expression directly to human dementia, was described in Borger et al. 2014, which revealed a significant down-regulation of EFhd2 both at the mRNA and protein level in the cortex of AD brain and other dementias including various different types of frontotemporal lobar degeneration (FTLD) including FTDP-17 and FTLD-tau with Pick bodies (Pick disease), which are all classified as tauopathies [6]. This down-regulation of EFhd2 did not appear to correlate with the extent of pathologic tau phosphorylation. Other tantalizing potential links between EFhd2 and neurodegeneration have also come from studies which have shown that it can bind to the Parkinsonian associated protein LRRK2 [16], and it has been shown to be downstream and phosphorylated by another Parkinsonian strongly associated protein, PINK1 [17]. The significance of this is as yet unknown, but it is interesting to note that both LRRK2 and PINK1 are linked to mitochondrial dysfunction and cytoskeletal reorganization [18], which as indicated above was how EFhd2 was also identified from proteomic screens in AD models.

With respect to regulation of EFhd2 in AD, there have been reported differences, for example, whereas Borger et al. revealed a specific down-regulation of EFhd2 in the cortex, but not the hippocampus, of human dementia brains from different pathological traits, Ferrer-Acosta showed that EFhd2 was up-regulated at the protein level in frontotemporal cortices of human AD patients [5]. The discrepancy between these two studies might be due to different extraction protocols. But Borger et al., revealed in addition to protein data also a transcriptional down-regulation of EFhd2 [6], thereby, supporting their protein biochemical data. However, it cannot be excluded that there are some forms of AD where EFhd2 is upregulated. Larger study cohorts and even more comprehensive studies than have been performed [6] might be feasible in the future.

Whereas the concomitant down-regulation of EFhd2 mRNA and protein specifically in the cortex of dementia patients suggests a tissue-specific transcriptional mechanism of *efhd2* gene regulation, additional post-translational mechanisms of EFhd2 regulation cannot be excluded. As mentioned above, in mice, the *efhd2* locus is haplo-insufficient. Thus, one mechanism of regulation of *efhd2* RNA expression could be heterozygously inherited or somatically acquired alterations of gene-regulatory elements in the *efhd2* promoter/enhancer system. There might also be other mechanisms. For instance, neurodegeneration is associated with neuroinflammation [19, 20] and EFhd2 has been shown to be down-regulated under proinflammatory conditions in peripheral blood monocytes (PBMC) from rheumatoid arthritis patients [21]. PBMC have recently been used to characterize neurodegenerative disorders [22, 23] and Brachs et al. established a flow cytometric protocol to analyze EFhd2 expression in PBMC [9]. Tentatively, the regulation of *efhd2* mRNA expression in neurodegeneration could be linked to inflammatory mediators targeting the as yet to be identified promoter/enhancer elements of the *efhd2* locus. Indeed, *efhd2* has been shown to be regulated by the PKC β /NF- κ B pathway, with NF- κ B being a classical pro-inflammatory stimulus, in mast cells [24]. To add another level of complexity, microRNAs (miRNAs) that regulate mRNA stability have been linked to amyloid production, tau

phosphorylation, and inflammation in AD [25]. Nothing is known so far about miRNAs that could potentially regulate *efhd2* expression during dementia. It is also not clear yet whether the reduction of *efhd2* expression during neurodegeneration is reversible, causative, compensatory or functionally irrelevant. Inducible *Efhd2* deletion or over-expression in a mouse model for neurodegeneration could address this question.

Swiprosin-1/EFhd2 at the protein level

At the protein level, EFhd2 consists of an N-terminal region of low complexity with an alanine stretch, a functional SH3 binding motif [26], two EF hands [27] and a C-terminal coiled-coil domain (CC) [27] (see also Figure 1). The unstructured N-terminal part has been shown to contribute to the strong thermal stability of recombinant EFhd2 [28]. The proline rich domain entailing amino acids 70 -79 is in fact a functional binding partner for the SH3 domain of the src-like protein tyrosine kinase fgr [26]. Interestingly this binding depends on phosphorylation of EFhd2 and there are two serine residues in this region, S74 and S76, that have been described to be phosphorylated [29]. S74 has been shown to be phosphorylated by cdk5 [29] (for a comprehensive overview of EFhd2's phosphorylation we refer to <http://www.phosphosite.org>). The SH3 domains of the src kinase lyn but not that of yes, and the SH3 domain of PLC γ bind EFhd2 but their binding sites in EFhd2 are not yet known [26]. EFhd2 has also been shown to bind Ca²⁺ [4]. Single amino acid mutations at critical polar amino acids predicted to be important in EF hands for Ca²⁺ binding due to their conserved positions (E116A, E152A, D105A, D141A), have demonstrated that both EF-hands are functional [28, 30]. Of note, by determining the affinity of EFhd2's EF-hands by equilibrium centrifugation it has become clear that EFhd2 binds Ca²⁺ cooperatively, but with an affinity of only 68 μ M [30]. Thus, Hagen et al. have proposed that EFhd2 is more like a Ca²⁺ sensor protein as it could bind Ca²⁺ only at higher concentrations, but then release it quickly. It is also possible that EFhd2 buffers strong Ca²⁺ transients temporarily. Hence, EFhd2 could modulate the spatiotemporal information of the second messenger Ca²⁺ (Figure 1). This notion is important as dysregulation of Ca²⁺ is involved in synaptic dysfunction in neurodegenerative and behavioural disorders [31, 32]. In that sense, phosphorylation of EFhd2 at S74 (potentially by cdk5) [29] and a functional single nucleotide polymorphism (SNP), which is associated with AD (F89L) [28], have both been shown to reduce EFhd2's ability to bind Ca²⁺ and could exert effects on EFhd2's quaternary structure: The C-terminal coiled-coil domain mediates self-interaction of EFhd2 [5, 33], in a Ca²⁺ - dependent manner [33]. Ca²⁺ dependency for dimerization has, however, only been shown in one study [33]. This discrepancy might be due to different methods: whereas Ferrer-Acosta et al. used an ELISAlite solid phase interaction assay [5], Kwon et al. tested the dimerization capacity in solution and by co-immunoprecipitation from extracts of transfected cells [33]. In conclusion, EFhd2 might interact weakly in the absence of Ca²⁺, which could be detected in solid phase binding assays, and more strongly in the presence of Ca²⁺. However, as the affinity of EFhd2 for Ca²⁺ is low, the dimeric forms of EFhd2 are expected to form at a low rate or only transiently. A very similar CC domain is also present in the EFhd2 orthologue EFhd1 [27], but so far there is no reported evidence for hetero-dimerization of EFhd2 and EFhd1 by coimmunoprecipitation analysis of endogenously expressed proteins [9]. The CC domain has also been reported to mediate the interaction of EFhd2 with a mutant form of the

microtubule associated protein (MAPT) tau (tauP301L) in tau transgenic brain extracts [5]. Here, it is not clear whether dimerization of EFhd2 through the CC domain exposes a tau binding domain of EFhd2 or whether EFhd2 binds mutant tau directly through its CC domain. Dimerization of EFhd2 through its CC domain is mediated by a lysine-rich stretch [33]. Mutant tau isoforms, such as tauP301L, are usually highly phosphorylated [34]. One possibility could be that the positively charged Lysine rich stretch in the CC domain of EFhd2 binds negatively charged tau residues. If Ca^{2+} induces EFhd2 dimers via the CC domain, it could be possible that the EFhd2-tau interaction site is blocked. Thus, Ca^{2+} induced dimerization could regulate the observed tau-EFhd2 interaction negatively. In the future, it will be interesting to know whether monomeric or dimeric EFhd2 binds to wildtype tau or tau P301L, especially with respect to the proposed amyloid structure of recombinant EFhd2 [5]. EFhd2 binds also directly to F-actin, in a Ca^{2+} independent manner, and has three F-actin binding regions [aa 69-84, aa 96-163, aa 163-199] [marked in red in Figure legend 1].

Regulation of Actin dynamics by EFhd2

a Cofilin

EFhd2 has been shown to regulate the accessibility of the actin severing protein cofilin to F-actin and it induces F-actin bundling [33, 35]. Brachs et al. showed that in B cells, genetic knockout of EFhd2 enhanced the dephosphorylation of cofilin at S3, i.e. its activation, after B cell receptor stimulation of resting B cells [8]. Hence, deletion of EFhd2 is expected to have a profound effect on local actin stability by regulating the activity of cofilin at several levels: firstly, by competing with it for F-actin binding [35]; secondly, by regulating its dephosphorylation on S3 and therefore its activity, at least in B cells [8]. The dephosphorylation of cofilin is regulated by increases in the intracellular Ca^{2+} concentration; for instance after B cell receptor (BCR) [36] or epidermal growth factor receptor (EGFR) activation, leading to activation of the phospholipase-inositol-tris-phosphate (PLC-IP3) pathway. EGF stimulation induced phosphorylation of EFhd2 on S138 in transfected 293 cells [35]. Another group has shown that EGF can reversibly dephosphorylate EFhd2 on Y83 in HeLa cells with the same kinetics as proteins involved in actin dynamics, namely gelsolin and arp2/3 [37]. Increased intracellular Ca^{2+} elicited by the PLC-IP3 pathway activates two protein phosphatases, calcineurin and slingshot, which dephosphorylate cofilin [38]. Enhanced dephosphorylation of cofilin in BCR-activated EFhd2-deficient B cells [8] and putatively other cell types after stimulation with other agonists might be explained by a locally increased Ca^{2+} concentration because EFhd2's buffering capacity is missing, leading to increased phosphatase activity. Interestingly, EGF activates the calcineurin signaling pathway followed by a transient increase of cofilin at the leading edge allowing for neurite outgrowth [39–41]. Modulation of EGF elicited cofilin activity by EFhd2 might therefore play a role in neurite outgrowth of primary cortical neurons [42]. Deduced from the published data, one would assume that as soon as the local Ca^{2+} concentration is high enough (in the micromolar range; [8]), F-Actin bound EFhd2 could dimerize, bundle F-actin and prevent cofilin from actin severing (Figure 1). This is expected to be a dynamic process due to the low affinity of EFhd2 for Ca^{2+} but could allow for the stabilization of actin-dependent signalling or membrane complexes, thereby, impacting longer lasting responses

mediated by, for instance, growth factors or synaptic Ca^{2+} oscillations. Whereas wildtype EFhd2 or a S138A mutation inhibit cofilin activity *in vitro* and in transfected B6F10 melanoma cells, the phospho-mimetic mutant S138E does not. Hence, EGF leads to local de-stabilization of F-actin through a phosphorylation of S138 in EFhd2 [35]. The EGF-induced phosphorylation of EFhd2 on Y83 and Y104, with Y104 being in the first EF-hand domain, appear to be irrelevant for this process [35]. However, both S138A and S138E mutations disturbed the localization of cofilin at the leading edge of EGF-elicited lamellipodia, and lamellipodia dynamics. Hence, a dynamic equilibrium of EFhd2 phosphorylation regulates likely cofilin activity and localization, which is required to provide a pool of G-actin endowing dynamic assembly of F-actin. F-actin assembly and branching are important for formation of filopodia and lamellipodia and are up-stream of cofilin, generally controlled by a family of small Ras-like GTPases comprising Rho, Ras, Rab, Arf, Sar and Ran proteins [43]. In particular the Rho and Ras members play critical roles during all stages of axonogenesis [44]. EFhd2 is expressed in dendrites, axons and synapses and knock-down of Efhd2 increases pre-synaptic densities [6, 7]. It has to be noted that at first glance and macroscopically, brains of EFhd2KO mice appear to be normal. However, deletion of EFhd2 might have very specifically localized effects on one of these GTPases - Cdc42, Rac and Rho - within the neuron or even within specific subtypes *in vivo*, which might explain the effects on axonal transport ([7]; see below) or presynaptic plasticity [8]. In the following section, we will therefore discuss the published involvement of EFhd2 in the regulation of small GTPase activity and put it into the context of axonogenesis.

b Small GTPases and Axonogenesis

Axonogenesis is important during brain development and regeneration after traumatic or disease induced injury, such as in neurodegenerative disorders (for review please see [45, 46]). Of note, axonogenesis is a differentiation process. It is likely that there is a coordinate regulation between migration of neuronal stem cells during development and regeneration, and their differentiation [46]. Axonogenesis does require a close interplay between the actin and microtubule (MT) systems. Both migrating and differentiated neurons are polarized, with the MT organizing center (MTOC; also called centrosome) directed into the direction of movement. Directed movement is dictated by the growth cone, that in itself, becomes instructed by axon guidance signals and is a dynamic region rich in F-actin [46]. The actin cytoskeleton plays a major role in pathfinding. The MT cytoskeleton, on the other hand, provides structure to the axon shaft and MT-actin interactions are fundamental for axon extension. Specifically, the plus end of MT is attached to actin structures in the growth cone, which also influence MT growth mechanically. In particular, MT extension occurs preferentially along filopodia [45]. Interestingly, the filopodia are composed of bundled F-actin and are sensors of guidance cues [47]. It remains to be determined whether and where EFhd2 exhibits F-actin bundling activity in neurons. One possibility could be within filopodia, particularly in light of EFhd2's regulation of Cdc42 activity (see below). Whereas filopodial bundles attract the plus ends of MT and also transport MT, the growth cone contains, in addition, transverse actin bundles (also called actin arcs) that restrain MT from attaching [45]. It has been proposed that the distal MT position is controlled by the rate of plus end assembly and retrograde actin flow [48]. Besides mechanical mechanisms there is also a plethora of proteins that interact specifically with both the MT and the actin

cytoskeleton, regulating their dynamic interaction, such as cofilin and tau and now also EFhd2 (Figure 1), which are all involved in controlling axon growth [49, 50]. MT extending with their plus ends towards the growth cone are linked there to the dynamic actin structures through complexes of linker proteins, such as the PAFAH1B1-CLIP170-Cdc42-Rac complex [46]. Proteins such as PAFAH1B1 promote both MT and actin dynamics, thereby, connecting MT to newly formed protrusions in the growth cone of the axon [46]. Attracting or repelling extracellular signals, such as elicited through the Reelin pathway, Semaphorins or NoGo transmit signals via specific receptors, such as LRP8, to ultimately control activation of small GTPases, such as Cdc42/Rac/Rho [46].

Small GTPases cycle between an active, GTP bound state and an inactive, GDP bound state. GTP exchange factors (GEFs) activate GTPases by catalyzing the exchange of GDP for GTP, whereas GTPase activating proteins (GAP) inactivate these small GTPases by increasing their GTPase activity. GTP-loaded GTPases bind many down-stream effector proteins of various signalling pathways, amongst them effectors regulating actin and microtubule assembly, such as PAK (p21 activated kinase) or WAVE proteins [43]. The Rho and Ras members are involved in initiation, elongation, guidance and branching of axons [44]. Their main function is to regulate the interaction of the growing axon with other cells and extracellular matrix. They also control the delivery of cargo to the axon through the exocytic machinery, and they are involved in the internalization of membrane and proteins at the leading edge of the growth cone through endocytosis [44]. Asymmetric transport of proteins to the axon is important for maintenance of neuronal polarity. Cdc42 is important for neuronal polarization and Rac regulates pathways that regulate microtubule growth in the developing axon [51]. Conditional deletion of Cdc42 reveals normal initial neurite sprouting but axon formation was strongly impaired [52]. As in many systems, Rho appears to be antagonistic in axon specification towards Cdc42 and Rac by activating ROCK (Rho activated coiled-coil kinase) [53]. In contrast to axon initiation, axon elongation can, however, also be positively regulated through the SDF1- α /Rho/mDia pathway [54]. Interestingly, SDF1- α elicited spreading of Jurkat cells overexpressing an EFhd2-GFP fusion protein is enhanced, indicating that EFhd2 can amplify SDF-1 α signals through an yet to be identified pathway [33]. The regulation of the Cdc42, Rac and Rho pathways appears to be involved in nerve regeneration as well. For instance, TC10, a close relative of Cdc42, was initially identified in neuronal cells as a gene that was dramatically re-expressed after axotomy of motor neurons in the hypoglossal nuclei [55]. Both Rho and Pak (which is downstream of Rac) can inhibit cofilin through Lim kinase 1 [44]. Thus, Rac may limit axon growth through this pathway, but may also activate it through another effector, STEF [56]. In any case, it is clear that cofilin phosphorylation and dephosphorylation are important in axonal outgrowth. Moreover, Rho and ROCK often, but not always, play antagonistic role in axon outgrowth and guidance towards Rac and Cdc42, which provide attractive cues and forward protrusion [44].

Plating CHO-K1 cells on fibronectin revealed that overexpression of an EFhd2-GFP protein enhances basal and fibronectin-induced Rac activity [33]. Likewise, B6F10 melanoma cells stably over-expressing an EFhd2-GFP fusion protein revealed basally increased Rac and Cdc42 activity but reduced Rho Activity. In contrast, shRNA mediated silencing of EFhd2 in B6F10 cells increased Rho activity [33]. Thus, EFhd2 appears to be antagonistic towards

Rho, and to activate Cdc42 and Rac. In accordance, increased membrane ruffling induced through overexpression of EFhd2-GFP could be reduced by simultaneous expression of dominant-negative Rac and conversely, reduction of membrane ruffling by shRNA against EFhd2 was reverted by dominant-negative Rac [33]. Interestingly, silencing of EFhd2 prevented transwell invasion of B6F10 melanoma cells induced *in vitro* through constitutively active Rac and enhanced invasion prevented through constitutively active Rho [33]. These results indicate that EFhd2 can function upstream of Cdc42, Rac and Rho, but can also modulate the effector functions of these small GTPases. This mechanism may relate to modulation of cofilin activity by EFhd2 but other mechanisms cannot be excluded. In total, EGF-induced phosphorylation of EFhd2 resulted in loosening of F-actin bundles. Thereby, cofilin gained more access to F-actin at the leading edge, and cofilin activity led to the generation of short actin filaments with free barbed ends [33]. This may alter actin dynamics. In light of these data it is intriguing that actin and cofilin mediated actin remodelling appear to be important for synaptic plasticity [57, 58] .

In summary, we conclude from biochemical data that EFhd2 has the potential to transmit Ca^{2+} transients into the regulation of actin stability (Figure 2). This function of EFhd2 could have profound pleiotropic effects in many systems. It is not clear which receptors and GEFs are up-stream of EFhd2-regulated GTPase activation and effector function within the brain, but SDF-1 α or EGF might play a role. As EFhd2 appears to regulate F-actin bundling, too, it might well exert pleiotropic effects. For instance, Semaphorin3A leads to the activation of Rho and ROCK, and attenuates actin polymerization, while promoting intra-axonal F-actin bundling and myosin II-mediated force generation [59]. More specifically, as EFhd2 is downregulated in Nogo-A knockout neurons, which reveal an increased regeneration potential [60], it could mediate the inhibitory effects of Nogo. The Nogo receptor mediates the inhibitory effects of Nogo-A, MAG, and OMgp on neurite outgrowth [61] by activating Rho and decreasing Rac activity. Loss of EFhd2 could shift this equilibrium. Down-regulation of EFhd2 in the cortices of dementia patients could therefore be a rescue pathway, by blocking the function of inhibitory receptors.

Function of EFhd2 in primary neurons: synapses and intracellular transport

EFhd2 is expressed in cortical neurons and its expression appears to be biphasic when neurons are allowed to culture *in vitro* [7]. At the sub-cellular level, Efhd2 has a discrete punctate distribution which was shown to co-stain along neurites with MAP2 and tau, potentially hinting at it being involved in vesicle transport, and it was partially located at synaptic regions co-staining with synapsin 1a/b and closely associated with PSD95. The specific and significant change in the EFhd2 protein levels in dementia leads to ask the question what does EFhd2 do and is this down-regulation of expression important for the biochemistry and physiology of the neuron? Recent studies have now indicated that EFhd2 may have roles in both synapses and intracellular transport.

The synaptic presence of EFhd2 was confirmed by biochemical analysis of isolated synaptosomes [6, 7]. As described above, physiologically in various types of dementia there is a decrease in the amount of both protein and mRNA levels of EFhd2 [6], though intriguingly this only appears to occur in the cortex regions and not the hippocampus. It is

also of note that there is also no difference in EFhd2 levels whether the dementia has underlying tau pathology [6]. The decrease in the protein levels of synaptogenic EFhd2 would suggest a potential phenotypic change in neuronal behaviour. Initially and possibly surprisingly, the removal of EFhd2 appeared to have no effect on the activity of synapses as there were no reported differences in electrophysiologically stimulated release of synaptophluorin from transfected primary neurons derived from EFhd2KO as compared to wildtype mice [7]. However, there was a significant difference in the number of synapses that could be formed, i.e. a decrease in EFhd2 expression led to the production of more synapses [6]. Given the ability of the frontal cortex to respond to the onset of dementia by neuronal reorganization [62], the regulation of EFhd2, be it on the RNA or protein level, could allow a challenged cortical neuronal circuit system to cope with damage. It is tempting to speculate that these hypothetical remodeling mechanisms function through EFhd2's functions in the Cdc42/Rac/Rho/Cofilin pathways (as outlined above); or its function in kinesin-mediated microtubule gliding (outline below) [7]; or in a dynamic exchange between the actin and MT system (Figure 3).

Neurofibrillary tangles (NFT) but not senile plaques parallel duration and severity of AD [63]. Hence, a critical issue appears to be the amount and phosphorylation status of the tau protein: even moderate overexpression of wildtype tau can induce neurodegeneration, possibly by interfering with kinesin-mediated transport of mitochondria, vesicles and endoplasmic reticulum [64, 65]. It does so by limiting distance travelled by cargo and also a reduction in multiple-kinesin velocity [66]. Hyperphosphorylated tau that relocates to the somatodendritic compartment disrupts normal axonal transport massively by trapping the kinesin adapter-molecule JIP1 (Jun N-terminal kinase interacting protein 1; now called MAPK8IP1) in the soma [67, 68]. JIP1 would normally interact with Doublecortin (DCX), thereby, also controlling actin dynamics in the growth cone [69]. Thus, tau malfunction impacts also the actin cytoskeleton. A physiologic function of tau, however, appears to be the stabilization of microtubules to control axonal transport, which is negatively regulated by phosphorylation, but reversible and controlled phosphorylation of tau are as well be important for synaptic plasticity [34, 70]. Interestingly, alterations in intracellular endo-lysosomal vesicle transport as well as autophagy appear even to precede Amyloid beta (A β) and tau pathology [71]. In light of these findings, it is therefore intriguing that we have previously shown that kinesin (KIF5A)-induced MT gliding was decelerated by the addition of a GST-EFhd2 fusion protein to this system, indicating a dose-dependent functional interaction between EFhd2 and KIF5A [7]. Therefore, we proposed that a potential mechanism of the interference with kinesin activity towards MTs could be inhibition of MT binding to kinesin by EFhd2 (Figure 2). Of note, neurite outgrowth and axonal re-generation of *Drosophila* neurons have been proposed to be mediated by kinesin-mediated microtubule gliding [72, 73].

Thus, down-regulation of EFhd2 could foster synaptic delivery through kinesin-mediated transport, an idea that is supported by Borger et al.'s finding of increased presynapse formation in EFhd2 knock-down neurons [6]. It is therefore reasonable to assume that EFhd2 is linked to intra-neuronal transport and dementia, which would support hypotheses of alterations in the endo-lysosomal vesicle transport system as being involved in neurodegeneration [74–76]. Given the reported interaction of EFhd2 and tauP301L [4] it is

striking that both proteins inhibit kinesin-mediated transport independently [7, 77]. It is even more striking that both EFhd2 and tau exhibit F-actin bundling activity [78], that actin dynamics are altered in tauopathies [77] and that EFhd2 was found in synapses [6, 7]. A locally increased concentration of EFhd2 in synapses could thus serve to stop axonal transport and to place cargo correctly – in synapses (Figure 3). On the other hand, a local Ca^{2+} transient, for instance induced through an action potential, could foster synaptic stabilization by aiding Ca^{2+} -controlled EFhd2 dimerization and F-actin bundling – putatively in filopodia or arcs composed of bundled F-actin. If the EFhd2 concentration was limiting, kinesin-mediated transport could then resume, thereby, replenishing used-up cargo. Moreover, there might be pools of EFhd2 shuttling between the microtubule and actin system, depending perhaps on the local Ca^{2+} concentration (Figure 3). It is thus tempting to speculate that loss of EFhd2 could modulate long term potentiation by modulating delivery of kinesin-transported cargo and by orchestrating local actin dynamics in a dynamic equilibrium.

Putative amyloid structures of EFhd2 and their possible function

Intracellular transport along microtubules and axon formation under control of actinmembrane dynamics is required for delivery of vesicles and mitochondria to synapses. Vice versa, this system is also required for intracellular waste disposal and recycling. The hallmark of many dementias is the occurrence of intra-and extracellular aggregates of misfolded proteins. Especially deposits of tau have been intensively characterized for many years [79].

Two main degradation pathways for tau exist: autophagy and the proteasome [80]. Both degradation pathways appear to be possible targets for the treatment of AD [81, 82]. Autophagy is a lysosomal survival pathway and regulates degradation and recycling of damaged organelles and cytoplasmic proteins, which appears to be specifically important in neurodegenerative diseases [83, 84]. In chaperone mediated autophagy (CMA), Hsc70 binds proteins with exposed KFERQ motifs and delivers them to lysosomes through LAMP-2A (Lysosom-associated membrane protein type 2A) [85]. The tau complexes Vega et al. had purified from JNPL3 mice did not only contain EFhd2 but also Hsc70 [4], which controls CMA [85]. Hence, EFhd2 could be involved in CMA and removal of pathologic tau isoforms. On the other hand, EFhd2 co-localizes with hyperphosphorylated tau in AD brain, with $\text{A}\beta$ -bearing neurons and recombinant EFhd2 can be rendered an amyloid protein itself as shown by electron microscopy [5]. In addition, Thioflavin S binding in the presence and absence of Heparin has revealed extensive β -barrel sheets in EFhd2 [5]. Whereas the authors interpreted their finding as evidence for amyloid EFhd2 being putatively dangerous, EFhd2 as a component of insoluble tau or $\text{A}\beta$ aggregates could as well be a harmless or even beneficial “off pathway” side-product, perhaps from CMA, as tau-tangle bearing neurons can also be long-lived [86].

Concluding remarks

The observed down-regulation of EFhd2 in dementia might have opposing functions and its mechanism is unknown. Thus, the function of EFhd2 in neurodegeneration has to be further

elucidated, probably with inducible knockout or transgenic EFhd2 mouse models in a neurodegenerative background. It will also be important to analyse more dementia patients across different intercontinental cohorts for EFhd2 expression. One facilitation in this process might be the analysis of PBMC for EFhd2 expression. As EFhd2 has been shown to modulate F-actin bundling, cofilin activity and Cdc42, Rac and Rho activity, a special interest lies on the investigation of these pathways in primary neurons, specifically in synaptic plasticity. In addition, nothing is known about the growth factors, intercellular signals, receptors and GEFs that are up-stream of EFhd2's ability to regulate small GTPases. Amongst others, Nogo-A, EGF or SDF-1 α might, however, be interesting candidates as they are involved in small GTPase signaling, neurite development and plasticity. Another system to focus on is kinesin-mediated transport along microtubules, which is partly also organized by small GTPases. Taken together, these investigations will help to understand the significance of the regulation of EFhd2 in the cortex of dementia patients.

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Abbreviations

AD	Alzheimer's disease
Aβ	Amyloid beta
BCR	B cell receptor
CMA	chaperone mediated autophagy
CC	coiled coil
EGFR	epidermal growth factor receptor
FTDP-17	frontotemporal dementia with parkinsonism linked to chromosome 17
KO	knockout
FTLD	frontotemporal lobar degeneration
GAP	GTPase activating protein
GEF	GTP exchange factor
IP3	inositol-tris-phosphate
LAMP-2A	Lysosome-associated membrane protein type 2A
MT	microtubule
MAPT	microtubule associated protein
MTOC	microtubule organizing center

miRNAs	microRNAs
NFT	neurofibrillary tangle
PBMC	peripheral blood monocytes
Pak	p21 activated kinase
PLC	Phospholipase
ROCK	Rho activated coiled-coil kinase
SNP	single nucleotide polymorphism

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Figure Legend 1.

Schematic of the proteins and molecules represented in Figures 1-3. Proteins and Ca²⁺ ions are not drawn to scale. The F-Actin binding domains of EFhd2 are highlighted in red. EF, EF hand; CC, coiled-coil domain. G-Actin, globular actin.

Figure 1. Transmission of Ca^{2+} signals and regulation of F-Actin stability through EFhd2
 Depending on the local Ca^{2+} concentration EFhd2 shuttles between monomeric and dimeric states. Due to low affinity of EFhd2 for Ca^{2+} this is expected to be a dynamic process. Enhancement of Rac and Cdc42 activity through EFhd2 leads to local Actin polymerization. F-Actin bound by EFhd2 through its F-Actin binding domains (marked in red) will be bundled at higher Ca^{2+} concentrations. EFhd2 mediated F-Actin bundling prevents access of Cofilin to F-Actin which stabilizes F-Actin locally. F-Actin polymerization and bundling

through EFhd2 could stabilize pre-synaptic structures. The signals leading to EFhd2 mediates Rac and Cdc42 activation in neurons remain to be identified.

Figure 2. Regulation of kinesin mediated transport along microtubules by EFhd2.

Recombinant EFhd2 inhibits kinesin-mediated microtubule gliding in vitro. Knock-out of EFhd2 increases the velocity of kinesin mediated transport of a synaptic protein, synaptophysin in primary neurons. Knock-down of EFhd2 in primary neurons increases the formation of pre-synaptic densities. Thus, the local concentration of EFhd2 may regulate controlled delivery of cargo. Either overexpression or loss of EFhd2 may lead to displaced presynaptic cargo.

Figure 3. Bidirectional regulation of kinesin mediated transport and actin dynamics through EFhd2 in presynaptic compartments

- a) In the absence of EFhd2, a local increase in Ca^{2+} might be less translated into F-Actin stabilization. Concomitantly, kinesin-mediated transport increases and gets delivered into a relative flexible pre-synaptic compartment.
- b) When the local Ca^{2+} concentration increases in the presence of EFhd2, F-Actin becomes bundled by EFhd2 and, thereby, stabilized. Whether this affects transport of cargo along microtubules is not known, but a pool of EFhd2 might be stabilized at F-Actin and be less accessible at microtubules.

c) In the presence of EFhd2 and with low intracellular Ca^{2+} kinesin-mediated transport on microtubules occurs normally, F-Actin is accessible to Cofilin, not bundled by EFhd2 and therefore more flexible.