# Olfactomedin Domain-Containing Proteins: Possible Mechanisms of Action and Functions in Normal Development and Pathology

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## Abstract

A family of olfactomedin domain-containing proteins consists of at least 13 members in mammals. Although the first protein belonging to this family, olfactomedin, was isolated and partially characterized from frog olfactory neuroepithelim almost 20 years ago, the functions of many family members remain elusive. Most of the olfactomedin domain-containing proteins, similar to frog olfactomedin, are secreted glycoproteins that demonstrate specific expression patterns. Other family members are membrane bound proteins that may serve as receptors. More than half of the olfactomedin domain-containing genes are expressed in neural tissues. Data obtained over the last several years demonstrate that olfactomedin domain-containing proteins play important roles in neurogenesis, neural crest formation, dorsal ventral patterning, cell-cell adhesion, cell cycle regulation, tumorigenesis and may serve as modulators of critical signaling pathways (Wnt, BMP). Mutations in two genes encoding myocilin and olfactomedin 2 were implicated in glaucoma and growing number of evidence indicate that other genes belonging to the family of olfactomedin domain-containing proteins may contribute to different human disorders including psychiatric disorders. In this review, we summarize recent advances in understanding of possible roles of these proteins with special emphasis on the proteins that are preferentially expressed and function in neural tissues.

## Introduction

Olfactomedin was first described in 1991 as a novel 57 kDa glycoprotein that was exclusively expressed in the frog olfactory neuroepithelim [1]. Olfactomedin undergoes posttranslational modifications and is able to form homodimers and high molecular weight aggregates via intermolecular disulfides. High levels of olfactomedin expression and deposition at the chemosensory surface of the olfactory epithelium suggested that this protein plays a role in chemoreception [1]. Cloning of cDNA encoding olfactomedin and analysis of its expression pattern confirmed that olfactomedin was expressed only in olfactory neuroepithelium of frogs and that its amino acid sequence showed no homology to any known protein [2]. Subsequent experiments by many laboratories over the following 15 years demonstrated that olfactomedin contains a domain in its C-terminal part that is present in many proteins in species ranging from reef-building coral *Acropora millepora* (the phylum Cnidaria) to *H. sapiens* [3–8]. This

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domain has a length of about 250 amino acids and was named the "olfactomedin domain". There are at least 13 proteins containing the olfactomedin domain in mammals and these proteins form a family [5,9].

Olfactomedin domain-containing proteins, similar to some other signaling molecules like Wnt proteins [10], have been identified only in multicellular organisms indicating that they are essential for cell-cell interaction and cell-cell signaling. Most of the olfactomedin domain-containing proteins, similar to frog olfactomedin, are secreted glycoproteins that demonstrate specific expression patterns. Other family members are membrane bound proteins that may serve as receptors. While knockdown mice have been produced for several genes encoding olfactomedin-domain containing proteins, the genetically modified animals demonstrate only very moderate or no phenotypes [11–14]. At the same time, mutations in some of these genes lead to profound pathologies in humans [15–17]. Although the biological functions of olfactomedin domain-containing proteins remain for the most part elusive, a growing body of evidence indicates that these proteins may play very important roles in normal development and pathology.

In this review, we briefly describe known functions of this family of biologically active proteins with special emphasis on the proteins that are preferentially expressed and function in neural tissues.

## Phylogenetic classification of olfactomedin domain-containing proteins

The nomenclature of olfactomedin domain-containing proteins is not well established. In many cases, orthologous olfactomedin domain-containing proteins were independently isolated by several laboratories and were given different names. In this review, we will use accepted genetic nomenclature where possible. The most extensive classification of the family of olfactomedin domain-containing proteins was performed by Zeng and co-workers [5]. They presented almost identical phylogenetic trees based on comparison of the 68 full-length sequences, the olfactomedin domain sequences, or full sequences without the olfactomedin domains from different species [5]. The family of olfactomedin domain-containing proteins segregated into seven subfamilies and members with similar domain organization and biochemical properties all fell into appropriate subfamilies [5] (Fig. 1). Later identification of four additional olfactomedin domain-containing proteins in the sea urchin led to a small modification of the phylogenetic tree shown in Fig. 1 [18]. One sea urchin protein, named colmendin, was placed in the subfamily VI, while four other proteins (amassins) were more similar to themselves than to any other group and formed their own subfamily [18]. Olfactomedin domains belonging to different subfamilies are rather divergent. Human olfactomedin domain sequences for example show 24–40% identity (38–62% similarity). Since the orthologous olfactomedin domain sequences from divergent species are, as a rule, significantly more conserved (see below), this may indicate that the formation of the main subfamilies of olfactomedin domain-containing proteins occurred early in evolution or that proteins belonging to different subfamilies evolved to perform different functions.

## Olfactomedin 1

Olfactomedin 1 (Olfm1) is also known as noelin in chicken and *Xenopus* [19,20], pancortin in mice [21], olfactomedin-related glycoprotein in rats [22], and hOlfA in humans [6]. In the human genome the *OLFM1* gene is located at chromosome 9q34.3, contains 8 exons, and spans about 46 kb. There is a single *Olfm1* gene in most studied species and two *olfm1* genes in zebrafish [23] which appeared as a result of partial duplication of the zebrafish genome. Four structurally different mRNAs, named AMY, BMY, AMZ, and BMZ are known to be produced from the *Olfm1* gene [19,22,23]. These mRNAs share a common central region (M) and have two different 5'-regions (A and B) transcribed from separate promoters, and two different 3'-

regions (Y and Z) produced by alternative splicing of corresponding mRNA (Fig. 2) [22]. The olfactomedin domain is encoded by the last two 3'-exons found in the AMZ and BMZ forms. The AMY and BMY forms encode shorter forms of Olfm1 that lack the olfactomedin domain. The longer forms of Olfm1, AMZ and BMZ, contain 457 and 485 amino acids, respectively, while the shorter forms, AMY and BMY, contain 125 and 153 amino acids, respectively. Olfm1 is a conserved protein showing 98% and 84% identities of amino acid sequences between mouse and human and mouse and zebrafish, respectively, possibly indicating conservation of function among different species. Among other family members, Olfm1 protein shows the highest identity to Olfm3 (66 %) and Olfm2 (53 %). These three genes, *Olfm1*, *Olfm2* and *Olfm3*, also show overlapping expression patterns (see below) and encoded proteins form a distinct subfamily (subfamily I) on a phylogenetic tree (Fig. 1) [5].

Olfm1 forms dimers and oligomers; cysteine residues in the central M part of the protein are critical for this process [24]. Although it is generally accepted that Olfm1 is a secreted protein, data involving secretion of different Olfm1 forms are somewhat controversial, possibly owing to difficulties in obtaining reliable antibodies against Olfm1 (as well as against many other family members). For that reason, Olfm1 is often marked by different protein tags to facilitate detection. However, it is important to remember that these tags could modify properties of Olfm1. The AMZ and BMZ forms of Olfm1 contain the carboxy terminal sequence SDEL, which is similar but not identical to the consensus sequence KDEL essential for retention of proteins in endoplasmic reticulum [25]. It was reported that the AMY form of Olfm1 was more robustly secreted than AMZ and BMZ forms in *Xenopus* [20,26], although our experiments indicate that both the mammalian AMZ and BMZ forms are also efficiently secreted (Nakaya and Tomarev, unpublished). The absence of comprehensive information about posttranslational modifications of Olfm1 also impedes our understanding of its secretion. Although it was shown that Olfm1 is glycosylated in different species [19,20,22,27], the nature of other possible modifications, in particular lipid modifications, has not been studied.

The expression patterns of *Olfm1* are similar across the species studied although some differences exist. Chicken embryos showed the highest neural crest expression, while mouse embryos showed an intermediate level of neural crest expression, and the frog embryos showed no expression in the neural crest [28]. In general, *Olfm1* is preferentially expressed in neurogenic tissues during development [19,20,23,24,26]. Its expression in *Xenopus* neural tissues is positively regulated by neurogenin and NeuroD, but input from other genes or inducers may be required for high levels of *Olfm1* expression [20]. Postnatally, *Olfm1* is highly expressed in the brain cortex including olfactory bulb and hippocampus [21,24] with different forms of *Olfm1* showing overlapping but not identical expression patterns [23,24,26].

High levels of *Olfm1* expression in developing and adult neural tissues implies that this protein may play an important role in their development and function. Indeed, over-expression of Olfm1 using recombinant retrovirus caused an excess of neural crest emigration and extended the time that the neural tube is competent to generate and regenerate neural crest [19]. Over-expression of the AMY form of Olfm1 in *Xenopus* first caused expansion of the neural plate at the expense of neural crest and epidermis, and later led to enlargement of the neural tube and retina [26]. In zebrafish, over expression of full-length Olfm1, and to a greater extent its BMY form lacking the olfactomedin domain, increased the thickness of the optic nerve and produced a more extended projection field in the optic tectum compared with control embryos [27]. Inhibition of Olfm1 expression by *olfm1*-specific morpholino oligonucleotides reduced the eye size, inhibited optic nerve extension, and increased the number of apoptotic cells in the retinal ganglion cell and inner nuclear layers [27]. These data suggested that zebrafish Olfm1 may play roles in the early eye determination, differentiation, optic nerve extension, and branching of the retinal ganglion cell axon terminals [27].

Elimination of the central (M region) part of Olfm1 using a Cre-Lox system in mice produced animals that had a normal lifespan, but did not mate well [12]. This relatively mild phenotype may be explained by the possibility that elimination of the central part of Olfm1 might still lead to the production of partially functional protein or that other olfactomedin-domain proteins (Olfm2 or Olfm3) having high similarity to Olfm1 and showing a similar expression pattern may perform the functions of Olfm1 in a redundant manner. Cortical neurons in Olfm1 knockout mice were more protected against ischemic injury indicating that Olfm1 is a mediator of ischemia-induced apoptosis of neurons in the adult cerebral cortex [12]. Focal ischemic stroke induced the formation of a protein complex that included the BMY (but no other) form of Olfm1, WAVE1, and the anti-apoptotic protein Bcl-xL [12]. WAVE1 is the actin-modulating protein containing region of homology to the Wiskott-Aldrich proteins (WASPs) in its C-terminal region [29]. WAVE1 is enriched in neural tissues and is a key regulator of dendritic spine morphology [30]. Olfm1, WAVE1 and Bcl-xL form a mitochondria-associated complex that promotes the interaction and apoptotic function of Bax with mitochondria. This results in cytochrome c release and apoptosis [12] (see panel A in Fig. 5).

There is a growing number of cases showing that interacting proteins may be involved in similar phenotypes when mutated and that protein-protein interactions may be used to identify new candidate genes for diseases [31]. WAVE1 and Bcl-xL are not the only proteins interacting with Olfm1 (see Table 1). It was reported that Olfm1 is also able to interact with β-dystrobrevin [32]. β-dystrobrevin is a component of the dystrophin-associated protein complex that links the actin cytoskeleton to the extracellular matrix and may serve as a scaffold for signaling proteins [33] (see panel D in Fig. 5). β-dystrobrevin is abundantly expressed in brain, lung, kidney and liver. In the brain, its expression in the hippocampus, olfactory bulbs [34] overlaps with that for Olfm1. Another β-dystrobrevin binding partner, dysbindin [35], is a probable susceptibility gene for schizophrenia [36,37]. It is interesting to note that yeast two-hybrid screens identified Olfm1 as a possible partner of DISC1, a protein encoded by a schizophrenia risk gene [38]. The expression pattern of the *Disc1* gene overlaps with that for *Olfm1* throughout mouse brain development [39]. DISC1 alternative transcripts produce 4 predicted protein isoforms in humans that are localized to many cellular compartments [40] and may have multiple roles in different locations. Various data indicate that DISC1, similar to WAVE1 and β-dystrobrevin, may interact with the cytoskeleton. In hippocampal neurons, DISC1 is located in growth cones [40]. Knockdown of Disc1 in rat PC12 cells inhibited neurite outgrowth, while over expression of DISC1 had the opposite effect [40]. Finally, it has been shown that Olfm1 may interact with the secreted antagonist of Wnt signaling pathway, Wif-1 [27] (see panel A in Fig. 5). This data led to a suggestion that Olfm1, similar to another family of olfactomedin domain-containing proteins, myocilin (see below), serves as a modulator of Wnt signaling [27].

Although no mutations in the *OLFM1* gene leading to pathology in humans have been discovered yet, high expression of Olfm1 in hippocampus and cortex as well as its possible interaction with DISC1 and β-dystrobrevin implies that disruptions of OLFM1 functions may lead to psychiatric illness. Further studies of the available Olfm1 knockout as well as development and investigation of new *Olfm1* knockout lines may clarify possible involvement of Olfm1 in psychiatric disorders.

## Olfactomedin 2

The human *OLFM2* gene is located at chromosome 19p13.2. Bioinformatic analysis indicated that the coding sequence of the *OLFM2* gene contains 6 exons spanning 82 kb of the genomic sequence [9]. It appears that mouse *Olfm2* mRNAs are transcribed from three different promoters. They encode 478, 456, and 448 amino acid long proteins. However, there is no data describing alternative splicing of *OLFM2* mRNA leading to forms that encode proteins without

the olfactomedin domain, as in the case of Olfm1. Similarly to Olfm1, Olfm2 is a conserved protein showing 96% and 74% identities of amino acid sequences between human and mouse and human and zebrafish, respectively. Olfm2 protein shows the highest 53% and 60% identity to Olfm1 and Olfm3 proteins, respectively. Although direct experimental data are not available, it may be suggested that Olfm2, similar to Olfm1 and Olfm3, is a secreted glycoprotein that is able to form dimers and heterodimers.

There is only one *olfm2* gene in zebrafish and its expression has been studied in detail [41]. In the course of zebrafish development, expression of *olfm2* was observed in the central nervous system including branchial motor nuclei, midbrain, hindbrain, inner nuclear layer and ganglion cell layer of the retina. Outside on the CNS, expression of *olfm2* was observed in the developing pharyngeal arches [41]. Knockdown of Olfm2 protein expression by morpholino oligonucleotides produced a highly penetrant phenotype, including perturbation of the formation of axonal projections from branchiomotor neurons, disruption of anterior head and CNS development including severe defects on development of olfactory pits, eyes and optic tectum. The absence of most cartilaginous structures in the pharyngeal arches indicated that the craniofacial phenotype may be due to defects in differentiation of cranial neural crest [41]. The *olfm2* expression pattern and knockdown phenotype are very similar to those described for the *olfm1* genes in zebrafish [23].

Genetic data indicate that mutation in the *OLFM2* gene in humans leading to Arg144Gln substitution in the protein sequence is a possible disease-causing mutation in Japanese patients with open-angle glaucoma. Moreover, common polymorphisms in *OLFM2* and another glaucoma-associated gene, optineurin, may interactively contribute to the development of open-angle glaucoma [16]. Although the connection between mutations in another olfactomedin-domain containing gene, myocilin, and glaucoma is well established (see below), this was the first demonstration that mutations in other olfactomedin domain-containing genes may lead to a human disease.

## Olfactomedin 3 (Optimedin)

Olfm3 was originally identified in a cDNA library produced from combined rat eye tissues involved in aqueous humor production and outflow and was named optimedin [42,43]. The human *OLFM3* gene is located on chromosome 1p21.2 and spans about 206.5 kb. The mouse *Olfm3* gene contains 8 exons and has a length of about 215 kb [42]. The first intron in the mouse gene comprises 62% of the total gene length. Two major *Olfm3* mRNA are transcribed from two different promoters and encode secreted glycoproteins which are 458 and 478 amino acids long [42]. Although the *Olfm1* and *Olfm3* genes have very similar exon-intron structure, there is no reported experimental evidence demonstrating alternatively spliced forms encoding Olfm3 without the olfactomedin domain. Similar to Olfm1 and Olfm2, Olfm3 is a very conserved protein showing 99% and 83% identity of amino acid sequences between human and mouse and human and zebrafish, respectively. Olfm3 forms homodimers and the N-terminal part of the protein sequence is critical for this process.

The expression pattern of the *Olfm3* gene overlaps with those of the *Olfm1* and *Olfm2* genes. It is expressed in different brain regions and the ganglion cell and inner nuclear layers of the retina in all species studied [42]. Expression of *Olfm3* was also detected in the eye drainage structures, eye lens and lung [42]. *Olfm3* expression is temporally regulated in the medial ganglionic eminence implicating *Olfm3* in the formation of hippocampal GABAergic interneurons [44]. The *Olfm3* gene is directly regulated by Pax6 in both brain [45] and lens [46]. Over-expression of Olfm3 in PC12 rat pheochromocytoma cells increased their growth rate and attachment to collagen extracellular matrix [47]. After stimulation with nerve growth factor, Olfm3-over-expressing PC12 cells demonstrated elevated levels of N-cadherin, β-

catenin, α-catenin and occludin as compared with stimulated, control cells. Expression of Olfm3 inhibited neurite outgrowth and induced $Ca^{2+}$-dependent aggregation of NGF-stimulated cells [47]. It was suggested that expression of Olfm3 stimulates the formation of adherens and tight junctions and modulates cytoskeleton organization, cell-cell adhesion and cell migration in the brain and retina [47]. Torrado et al. demonstrated that Olfm3 may interact with another olfactomedin domain-containing protein, myocilin, and that the C-terminal olfactomedin domain is essential for this interaction [42]. These data suggest that Olfm3, similar to Olfm2 and myocilin, may be involved in eye disorders involving the anterior segment of the eye and the retina. It is interesting to note that the human *OLFM3* gene is located close to the known translocation breakpoint associated with a malignant ependymoma [48].

## Olfactomedin 4

*OLFM4*, also known as *GW112* [49], *hGC-1* [50], *pDP4* [51], and *hOlfD* [6] in mammals is located on chromosome 13q21.1 in humans and consists of 5 exons spanning over 23 kb [50]. Olfactomedin domain-containing protein isolated from the frog olfactory epithelium [1] and the *Xenopus* protein tiarin [52] show 42% and 49% identities with mammalian Olfm4, which is higher than their identities with any other olfactomedin domain-containing protein and most probably are frog orthologues of mammalian Olfm4. These proteins form subfamily V of a phylogenetic tree (Fig. 1) [5]. Olfm4 protein is less conserved than Olfm1–3 proteins as identity between human and mouse is only about 66%. Similar to other family members, OLFM4 forms dimers and oligomers with cysteine 226 being critical for oligomer formation [53].

Although bullfrog olfactomedin is expressed in the olfactory neuroepithelium and Xenopus tiarin is expressed in nonneural ectoderm adjacent to the anterior neural plate during Xenopus development [52], mammalian OLFM4 genes are preferentially expressed in non-neuronal tissues. The main sites of OLFM4 expression in humans are prostate, small intestine, colon, bone marrow, and stomach [50,51]. Available data suggest [54] that only one promoter is used to transcribe *Olfm4* mRNA, unlike *Olfm1–3* mRNAs. Granulocyte colony-stimulating factor induces *OLFM4* expression and this induction is regulated by the transcription factor NF-κB [54]. The ETS-family transcription factor PU.1 binds to a functional site in the *Olfm4* promoter and regulates its expression in myeloid cells [51].

Biological activity of Olfm4 was studied in several experimental systems. OLFM4 is up-regulated or selectively expressed in gastric, colon, breast and lung cancer tissues [55–58] and promotes S-phase transition in proliferation of pancreatic cancer cells [59]. Olfm4 is also considered to be a novel marker of intestinal stem cells expressing G protein-coupled receptor, Lgr5 [60]. OLFM4 enhanced spreading and attachment of NIH3T3 and HEK293 cells [53]. It is able to interact with the potent apoptotic inducer, GRIM-19 [49] and binds to cadherin and lectins. Its interaction with cadherin requires the C-terminal olfactomedin domain [53]. In *Xenopus*, tiarin functions as a patterning signal affecting the dorsalization of the neural tube [52]. Overexpression of tiarin in *Xenopus* embryos caused expansion of dorsal markers and suppression of ventral markers. In the eye-forming field of the diencephalon, tiarin induced retinal markers (*Rx*, *Pax6*) and repressed optic stalk markers (*Vax2*, *Pax2*). In the double axis formation assay, tiarin did not enhance activities of dorsally-expressed Wnts (Wnt1, and Wnt3a), although interaction of Wnt and tiarin could not be excluded in other contexts [52]. Indeed, recent data indicate that *Xenopus* tiarin is a target for Wnt-pathway-regulated degradation [61]. It was hypothesized that the presence of olfactomedin domain in both tiarin and seven-transmembrane receptors, the latrophilins (see below), is analogous to the case of the Frizzled seven-transmembrane receptors and binding antagonists, sFRPs [62]. However, the expression patterns of latrophilin 1 and latrophilin 3 in the brain and retina are more similar

to those of Olfm1–3 [43,63] and these family members are probably better candidates for interactions with latrophilins than Olfm4.

## Olfactomedin-like 1

The human olfactomedin-like 1 (*OLFML1*) gene encodes a secreted glycoprotein belonging to subfamily VII on a phylogenetic tree (sequence Hs-AAQ88954 in Fig. 1 [5]) and is located on chromosome 11p15.4 [64]. The *OLFML1* gene comprises of 3 exon and spans 25.8 kb. It encodes moderately conserved protein with a length of 402 amino acids also known as ONT2 in mice. Human Olfml1 protein shows 90% and 57% identities with mouse and zebrafish proteins, respectively. The human *OLFML1* gene was highly expressed in small intestine, liver, lung, heart and spleen and was not expressed in brain [64]. Available data suggest that OLFML1 protein enhanced human cancer cell proliferation *in vitro* via accelerating the entry into S phase [64].

## Olfactomedin-like 2A and Olfactomedin-like 2B

The olfactomedin-like 2A (*OLFML2A*) and olfactomedin-like 2B (*OLFML2B*) genes encode secreted glycoproteins also known as photomedin-1 and photomedin-2 in mice [65]. The human *OLFML2A* and *OLFML2B* genes are located on chromosomes 9q33.3 and 1q23.3, respectively. *OLFML2A* and *OLFM2B* comprise of at least 8 exons and are spanning 37.7 kb and 40.7 kb, respectively. The encoded two proteins are more closely related to each other than to other members of the family and form subfamily IV on a phylogenetic tree (Fig. 1) [5]. They demonstrate 69% identity of the olfactomedin domains, contain the unique Ser/Thr-rich region preceding the olfactomedin domain which is absent in other family members [65] and form a separate subfamily of olfactomedin domain-containing proteins [5]. Similarly to other family members, Olfml2 proteins are able to form disulfide-bonded homodimers and oligomers, and the N-terminal parts of the proteins are critical for this process. Olfml2a is proteolytically cleaved after secretion from cells and the resulting C-terminal fragment containing the intact olfactomedin domain was unable to form dimers. Olfml2b appeared to be not cleaved in the central part [65]. Although the *Olfml2a* gene is not expressed in neuronal tissues and the *Olfml2b* gene shows only low level of expression in the brain, both of these genes are expressed in the adult retina where they showed mutually exclusive expression patterns. Olfml2a was predominantly detected in the photoreceptor layer, while Olfml2b was present in ganglion cell and inner nuclear layers, the inner segment of photoreceptor layer and retinal pigmented epithelium [65]. Olfml2 proteins preferentially bind to chondroitin sulphate-E and heparin among extracellular matrix components tested [65]. The functions of Olfml2 proteins are still not clear.

## Olfactomedin-like 3

The olfactomedin-like 3 (*OLFML3*) gene encodes a secreted glycoprotein, also known as hOLF44 [66] and HNOEL-iso in humans, ONT3 in mice [13] and ONT1 in chicken and Xenopus [67,68]. These proteins form subfamily VII together with OLFML1 on a phylogenetic tree (Fig. 1). The human gene is located on chromosome 1p13.2, spans about 2.8 kb, and comprised of 3 exons similar to the *OLFML1* gene. Human and mouse Olfml3 proteins are 97% identical, while human and *Xenopus* proteins show 64% identity. Human *OLFML3* is preferentially expressed in placenta. Its expression was also detected in other adult tissues including liver and heart [66]. Expression in brain is very low. In the eye tissues tested, the *OLFML3* gene is more strongly expressed in the sclera and iris than in trabecular meshwork or retina [43]. In early chick embryos, *Olfml3* expression is first detected at Hensen's node and subsequently in the axial and paraxial mesoderm [67]. A similar pattern of *Olfml3* expression was observed in *Xenopus* [68].

Gene disruption of *Olfml3* in mice by introduction of the lacZ reporter after the initiator methionine of Olfml3 did not produce a clear phenotype. The homozygous mutants were born normal and fertile [13]. It was suggested that the function of the *Olfml3* gene is dispensable and possibly compensated by other redundant members of the family of olfactomedin domain-containing proteins [13]. Although a convincing demonstration of the absence of functional Olfml3 protein fragments in knockout animals is still missing, these results are somewhat similar to those for the *Olfm1* knockout [12]. Unlike mouse Olfml3, Xenopus protein (ONT1) is indispensible for fine-tuning BMP signaling in the axial tissue [68]. Xenopus Olfml3 stabilizes axial formation by restricting chordin activity on the dorsal side. Olfml3 binds chordin and BMP1/Tolloid-class proteinase (B1TP) via olfactomedin and coiled-coil domains, respectively. It acts as a secreted scaffold that enhances B1TP-mediated Chordin degradation. It was proposed that Xenopus Olfml3, together with dorsally expressed BMPs, plays an indispensable role in Chordin activity regulation and ensures stable dorsal-ventral patterning in the embryo [68]. These results raise an interesting possibility that other olfactomedin domain-containing proteins also may serve as scaffolds for different enzymes and substrates.

## Myocilin

The Myocilin (*MYOC)* gene, also known as *TIGR*, *GLC1A*, and *JOAG1*, probably has been studied more extensively than all other genes encoding olfactomedin domain-containing proteins combined and several reviews summarizing existing knowledge about *MYOC* have been published recently [69–71]. The main reason for this interest is that mutations in the *MYOC* gene are found in more than 10% of juvenile open-angle glaucoma cases and in 3–4% of patients with adult onset primary open-angle glaucoma [15,17,72–74]. Glaucoma is one of the leading causes of irreversible blindness in the world and primary open angle glaucoma is the most common form of glaucoma. It will affect more than 60 million and blind about 4.5 million people worldwide by the year 2010 [75]. Myocilin, which was originally named TIGR (trabecular meshwork glucocorticoid inducible), was identified as a protein that was highly inducible by glucocorticoids in human trabecular meshwork cell lines [76]. The human *MYOC* gene is located on chromosome 1q23-q24 and is comprised of 3 exons. The human MYOC has a length of 17 kb and encodes a secreted glycoprotein protein with a length of 504 amino acids which belongs to subfamily III on a phylogenetic tree (Fig. 1). Myocilin is less conserved than Olfm1–3 proteins: identity between human and mouse proteins is about 82 % [77,78]. If gliomedin may represent the most ancient member of the family (see below), myocilin is probably one of the youngest, since clear *orthologues* of myocilin are not found in zebrafish and frogs.

Myocilin is proteolytically cleaved at the C-terminus of Arg$^{226}$ by calpain II producing two stable protein fragments [79,80]. The N-terminal fragment contains a leucine zipper which is part of two coiled-coil domains, while the C-terminal fragment contains the olfactomedin domain. Myocilin, similar to many other family members, is able to form dimers and multimers and the N-terminal region of myocilin is critical for dimerization [42,81–83]. Secretion of myocilin was investigated by several laboratories [84–88]. Recent data suggest that secretion of myocilin from trabecular meshwork may occur through an unconventional mechanism, likely associated with exosome-like vesicles [89] and that the coiled-coil domain, not the putative signal sequence, is responsible for the targeting of myocilin to intracellular membranes [90]. At the same time, the C-terminal olfactomedin domain-containing fragment without the coiled-coil region is also secreted [79,91].

*MYOC* is highly expressed in the trabecular meshwork, iris, ciliary body, sclera, retinal pigmented epithelial cells and sciatic nerve, with lower levels of expression observed in skeletal muscle, mammary gland, thymus and testis [15,17,42,77,92]. The proximal promoter of the

human *MYOC* gene contains several potential binding sites for different transcription factor with the E-box being critical for basal promoter activity [93].

Although *MYOC* is highly expressed in the trabecular meshwork, the absence of open-angle glaucoma in an elderly woman homozygous for the Arg46Stop mutation [94] as well as absence of glaucoma in people hemizygous for *MYOC* [95] suggests that the loss of functional myocilin is not critical for the development of glaucoma or for normal eye functioning. These observations were supported by data in mice with targeted disruption of the *Myoc* gene. Mice heterozygous and homozygous for a targeted null mutation in *Myoc* do not have a detectable eye phenotype [14]. Although some data indicate that the levels of myocilin may be elevated in the trabecular meshwork and aqueous humor of patients with open angle glaucoma [71, 96], a fifteen fold increase in the levels of normal myocilin in the eyes of transgenic mice does not lead to the elevation of intraocular pressure (IOP) or glaucoma [97]. This may indicate that elevation of myocilin in primary open angle glaucoma is a secondary effect and not a cause of IOP elevation. Another possible explanation is that there might be a significant difference in the reaction of the human and mouse eye to expression of myocilin.

A glaucoma phenotype in humans appears to be dependent upon expression of mutated myocilin protein in the eye tissues. More than 70 glaucoma-causing mutations were identified and greater than 90% of them are located in the region encoding the olfactomedin domain (Fig. 3). Mutations causing severe glaucoma phenotypes lead to the retention of myocilin in the endoplasmic reticulum and prevent its secretion [98,99]. Moreover, secretion of wild-type myocilin is impeded in the presence of mutated myocilin protein [84,99–101]. Accumulation of mutated myocilin in endoplasmic reticulum may be deleterious for cells and lead to cell death [88,102]. Secretion of some myocilin mutants from transfected cells *in vitro* may be partially restored by culturing cells at 30° C [87,88,103] or in the presence of chemical chaperones [104,105]. Such treatments increased viability of cells expressing mutated myocilin *in vitro* and were suggested for correction of glaucoma phenotype in human [88]. The presence of certain myocilin mutants leads to severe juvenile open-angle glaucoma with high penetrance. For example, patients with the Tyr437His mutation were diagnosed at 20 years of age on average and had a mean maximum IOP of 44 mm Hg versus IOP less than 20 mm Hg in control population [73]. At the same time, expression of the same mutated human or corresponding mutated mouse myocilins in the eye drainage structures of transgenic mice led to only moderate (about 2 mm Hg diurnal and 4 mm Hg nocturnal) elevation of IOP and progressive degenerative changes in the peripheral retinal ganglion cell layer and optic nerve with normal organization of the drainage structures in animals that were older than one year [106,107]. In a separate study, the expression of mutated myocilin allele (Tyr423His) specifically in the iridocorneal angle did not lead to IOP elevation and did not produce any degenerative changes in the retina. [108]. These differences might be explained by differences in the levels of mutated myocilin expression as well as differences in genetic background. Data obtained with expression of mutated myocilin in mice reinforce the suggestion that mouse and human eyes may react differently to myocilin expression.

Multiple attempts have been undertaken to identify proteins interacting with myocilin. Several candidate proteins belonging to different functional classes (extracellular matrix, cytoskeleton, cell signaling and metabolism, membrane proteins) have been identified (see [71,109] for recent reviews). In many cases, interaction of myocilin with other proteins could not be confirmed by independent techniques and more work should be done to confirm the specificity of these interactions.

Although is now well established that some mutations in the *MYOC* gene may lead to a severe glaucoma phenotype, the functions of wild-type myocilin are still not very clear. Recent data demonstrate that myocilin can interact with cystein-rich domains of several frizzled receptors

and secreted frizzled-related proteins as well as with Wnt inhibitory factor WIF-1 [91]. The affinities of myocilin binding to the CRD domains or to WIF-1 may be similar to those for some Wnt proteins. Myocilin modulates the organization of actin cytoskeleton, stimulating the formation of stress fibers through components of Wnt signaling pathway and this may be essential for the contractility of the trabecular meshwork and the regulation of intraocular pressure (Fig. 4). These data suggest that myocilin may serve as a modulator of Wnt signaling and that the absence of a glaucoma phenotype resulting from myocilin null mutation in the eye may be explained by the compensatory action of Wnt proteins [91].

## Gliomedin

The *Gliomedin* (*GLDN*) gene [110] encodes a protein also known as collomin and CRG-L2 in mammals [111,112] and colmedin in C. elegans [7] and in sea urchin [18]. These proteins form subfamily VI on a phylogenetic tree (Fig. 1). *GLDN* is located on chromosome 15q21.2 in humans and is comprised of 10 exons [111]. The mouse gliomedin gene has a length of more than 59 kb [111], while the human gene is 66.5 kb. Gliomedin is less conserved than Olfm1– 3 proteins: human and mouse proteins are 86% identical, while human and zebrafish proteins shows 42% identity. Unlike other olfactomedin domain-containing proteins that were discussed so far and that are secreted proteins, gliomedin is a type II transmembrane protein with a length of 551 amino acids containing a short cytoplasmic tail at its amino terminus and a C-terminal extracellular region. The extracellular region included two collagen domains and the olfactomedin domain at the C-terminus [110]. The collagen domains are essential for the formation of gliomedin trimers [113]. However, gliomedin exists not only as a transmembrane protein but also as a soluble form that is shed from the cell surface in a furin-dependent manner. Gliomedin can be further proteolytically processed by bone morphogenic protein 1/Tolloid-like enzymes, releasing the olfactomedin domain from association with the collagen domains [113,114]. The same class of enzymes form complexes with *Xenopus* Olfml3 through its coiled-coil domain [68] (see above). The cleaved olfactomedin domain of gliomedin may form insoluble molecular aggregates [114].

In mammals, the gliomedin gene was first described as a liver cancer-related gene, which displays high expression in murine and human hepatocellular carcinomas [111]. It became clear later that gliomedin is also expressed in neuronal tissues including spinal cord, brain, and sciatic nerve, among tissues tested [110]. Expression of gliomedin in rat sciatic nerve demonstrated a dramatic increase in myelinated Schwann cells during the first postnatal week and coincides with the initial period of active myelination in the peripheral nervous system [110]. Moreover, it was shown that gliomedin is a glial ligand for neurofascin and NrCAM, two axonal immunoglobulin cell adhesion molecules that are associated with $Na^+$ channels at the nodes of Ranvier [110] (see panel B in Fig. 5). Elimination of gliomedin expression by gliomedin RNAi abolished node formation. The multimerized olfactomedin domain of gliomedin was essential for interaction with neurofascin and NrCAM [113], and oligomers of the cleaved olfactomedin domain of gliomedin may stabilize mature nodes of Ranvier [114]. It was proposed that gliomedin provides a glial cue for the formation of peripheral nodes of Ranvier [110]. Gliomedin together with neurofascin may serve as novel immune targets in experimental allergic neuritis. It was shown that gliomedin and neurofascin were selectively reduced at the nodes of Ranvier prior to demyelination in a model of acute inflammatory demyelination induced in the Lewis rats by immunization against peripheral myelin [115].

Among invertebrate homologues of gliomedin, only one, unc-122, was characterized in detail. It is a postsynaptic protein located at neuromuscular junctions that may participate in maintaining a structural microenvironment that is necessary for efficient neuromuscular signaling [7].

## Latrophilins

Latrophilin 1 (LPHN1), also known as CIRL1, LEC2, and CL1, was isolated from brain extracts using affinity chromatography on α-latrotoxin from black widow spider venom [116,117]. Further experiments demonstrated that there are three closely related *LPHN* genes in vertebrates [118,119]. The human *LPHN1*, *LPHN2*, and *LPHN3* genes are located on chromosomes 19p13.2, 1p31.1, and 4q13.1, respectively. *LPHN1* contains 24 exons and spans about 60 kb. *LPHN1* and *LPHN3* are preferentially expressed in neuronal tissues including brain and retina [43,118,119], while *LPHN2* is more actively expressed in other tissues [118, 119]. *LPHNs* encode unusual G-protein-linked receptors having the same domain structure that form subfamily II on a plylogenetic tree (Fig. 1). LPHN1 has a molecular weight about 185 kDa and contains a long N-terminal glycosylated extracellular domain and a long intracellular domain. The olfactomedin domain is located in the extracellular part of LPHNs. LPHN1 is localized in presynaptic terminals. It is cleaved in the endoplasmic reticulum and this cleavage is necessary for receptor delivery to the plasma membrane [120]. On the plasma membrane, N-terminal and C-terminal fragments behave as separate membrane proteins. However, agonist binding to the N-terminal fragment induces re-association of fragments and triggers signal transduction via the C-terminal fragment [120]. The C-terminal fragment of LPHN1 can bind Shank, an ankyrin repeat-containing protein of postsynaptic density [63, 121]. Knockout mice that lack *LPHN1* are viable and fertile [11]. This may indicate that other LPHNs, and LPHN3 in particular, may compensate for the lack of LPHN1. Natural ligands for LPHNs have not been identified yet.

## Evolution of olfactomedin domain-containing proteins

The founder of olfactomedin domain-containing family of proteins, olfactomedin or Olfm4 by the genetic nomenclature, was identified in frogs [1]. Proteins containing the olfactomedin domain were later found in species belonging not only to Chordata but also to several other major phyla of multicellular organisms including Arthropoda (*Drosophila*), Nematoda (*C. elegans*), and Echinodermata (*Strongylocentrotus purpuratus*). Recent sequencing of the coral larval (*Acrospora millepora*) transcriptosome using next-generation sequencing technology, 454 sequencing, led to the identification of the olfactomedin domain-encoding mRNA in Cnidaria. Cnidarians are considered to be the simplest metazoans with a nervous system and neuroendocrine activity and genes encoding proteins associated with practically all major intracellular signaling pathways have been identified in *Acrospora millepora* [8]. The sequenced region of olfactomedin domain-encoding mRNA from *A. millespora* is relatively short (240 nucleotides) and nothing is known about its tissue distribution. The encoded protein sequence showed the highest identity with the olfactomedin domain of Atlantic salmon (*Salmo salar*) Olfml2a of and mouse myocilin (46% and 45%, respectively). The lowest identity (26%) was observed with Olfm4. Only one class of olfactomedin-domain containing proteins has been identified in Cnidaria so far. Since only a partial sequence is available, it is not clear whether the olfactomedin domain-containing protein from *A. millepora* is a secreted or membrane-bound protein. Elucidation of a complete sequence of this protein may lead to better understanding of the evolutionary history of the family of olfactomedin domain-containing proteins.

Only few olfactomedin domain-containing proteins have been identified in invertebrates: one in *Drosophila melanogaster* (NP_573262) and two in *Caenorhabditis elegans*. Many invertebrate olfactomedin domain-containing proteins (colmedin, unc-122, *Drosophila melanogaster* protein) can be grouped together with vertebrate gliomedin into subfamily VI on a phylogenetic tree [5,18]. This subfamily contains members from four animal phyla: Nematoda, Arthropoda, Chordata and Echinodermata and is the only subfamily representing both protostomes and deuterostomes [18]. It was suggested that this subfamily is the most

ancient subfamily of olfactomedin domain-containing proteins and that further diversification of the olfactomedin domain-containing proteins has occurred only within deuterostomes [18]. The formation of different subfamilies of olfactomedin domain-containing proteins might have occured relatively early in evolution before the radiation of vertebrates. It was also suggested that olfactomedin domain and flanking non-olfactomedin domain regions have co-evolved and may be functionally interdependent, since the phylogenetic tree topologies that were build on the basis of comparison of different domains were almost identical [5]. It is remarkable that in analyzed invertebrate species (*C. elegans*, sea urchin), olfactomedin domain-containing proteins were found in neuronal tissues implying that the nervous system was the primary site of their action in evolution.

## Concluding remarks

Biological functions of olfactomedin domain-containing proteins are far from being well understood. Nevertheless available data demonstrate that these proteins play important roles in neurogenesis, neural crest formation, dorsal ventral patterning, cell-cell adhesion, cell cycle regulation, tumorigenesis, reorganization of cytoskeleton, and may serve as modulators of critical signaling pathways (Wnt, BMP). More than half of the olfactomedin domain-containing genes are expressed in neural tissues and were implicated in their development and function. Fig. 5 illustrates possible mechanisms of olfactomedin domain-containing protein action in neuronal cells. The search for mutations in these genes represents an important and challenging task. Slow progress in the elucidation of the functions of these proteins is partially explained by difficulties with their purification and problems with the production of good antibodies against these proteins which are essential for the elucidation of their mechanisms of action. Although it is now well established that proper lipid modification of secreted signaling proteins are critical for their secretion and activity [122], we still do not have a complete scheme of posttranslational modifications of these proteins. It was reported that Wnt proteins require a special protein, Wntless, for their secretion [123]. We can not exclude a possibility that secretion of olfactomedin domain-containing proteins also requires a special protein(s). Further work is required to understand functional differences between the same olfactomedin domain-containing proteins having different localization (intracellular versus extracellular). Several unsuccessful attempts were made to crystallize some of the olfactomedin domain-containing proteins, but only one work described successful crystallization of the olfactomedin domain from sea urchin amassin [124]. Elucidation of three-dimensional structure of the olfactomedin domain will be a significant step forward in our understanding of its interaction with other proteins. Although some possible interacting partners have been identified for several olfactomedin domain-containing proteins, it is important to understand whether other family members are able to interact with the same or similar partners. In particular, it would be interesting to determine specificity of interaction of olfactomedin domain-containing proteins with Frizzled receptors. We still do not fully understand functional differences between different isoforms of olfactomedin domain-containing proteins that are generated by alternative splicing, alternative promoter usage or proteolytic cleavage. It will be important to understand why in some experimental systems the forms without olfactomedin domain are more active than full length forms. It will be important to elucidate whether olfactomedin domain-containing proteins showing overlapping expression patterns are able to interact with each other and whether mutations in one of the interacting partners blocks the secretion of the second partner. Since single knockouts of genes encoding the olfactomedin domain-containing proteins did not produce dramatic phenotypes, double and triple knockouts of these genes may clarify the roles of these proteins in mammals. Elucidation of the molecular mechanisms by which Olfm1 and Olfm2 may regulate neurite growth and/or branching represents another important area of research. Connection of olfactomedin domain-containing proteins and stem cell maintenance and/or differentiation also deserves further studies. Mutations in two genes, *MYOC* and *OLFM2* were implicated in glaucoma and growing number of evidence indicate

that other genes belonging to this family may contribute to different human disorders including psychiatric disorders. This list may continue but there no doubts that proteins belonging to this understudied family will continue to be identified as new players in many unexpected processes and human diseases.

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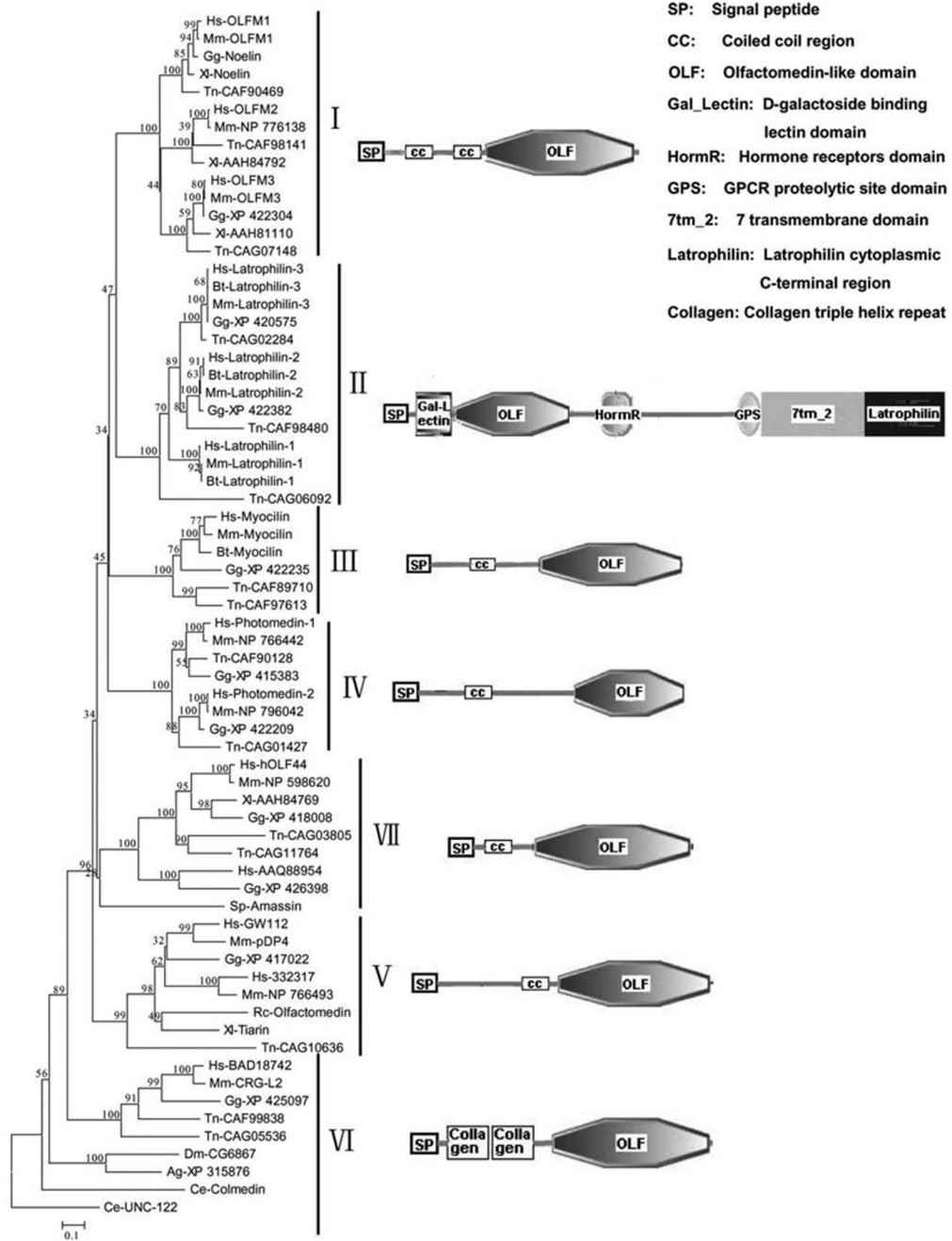**Fig. 1.**
Rooted neighbor-joining tree for the 68 full-length olfactomedin domain-containing proteins and the domain architecture of the typical human members in each subfamily. Vertical bars and Roman numerals delineate the seven subfamilies. Domain names are noted at the up right corner. Branch lengths are shown to scale. Bootstrap values based on 1000 replications are shown above the branches (Reproduced from FEBS Lett. 579 (2005) 5443-5455 [5] by permission of Dr. Wei-Jun Ma)
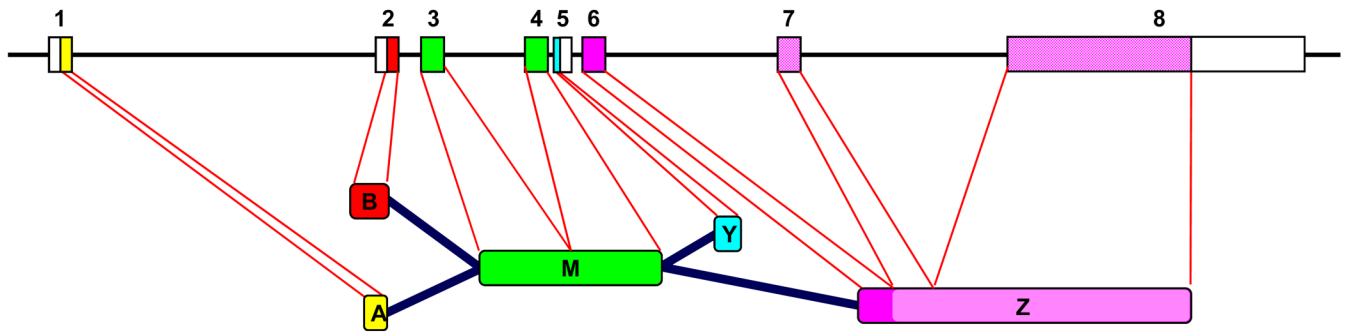
**Fig. 2.**
Schematic diagram showing different forms of Olfm1 protein that produced as results of
different promoter usage (exon 1 or exon 2) and alternative splicing (exon 5 or exons 6–8).
The central M part of Olfm1 encoded by exons 3 and 4 are common for all forms. The
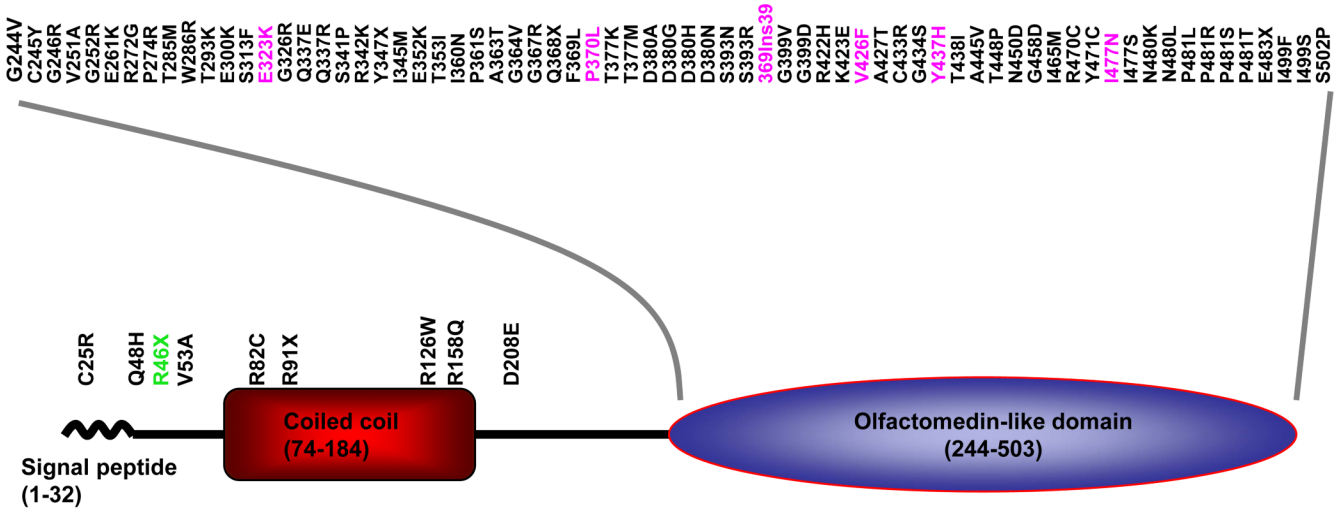olfactomedin domain is encoded by exons 7 and 8 and marked by pink color.

**Fig. 3.**
Glaucoma-causing mutations in myocilin. More than 90% of mutations affect the olfactomedin domain. The most severe mutations (to maximum IOP higher than 40 mm Hg and mean age at diagnosis less than 30 years [71]) are marked in pink. The Arg46Stop mutation [93] that may be considered as a natural human myocilin knockout in homozygous state is marked by green color.
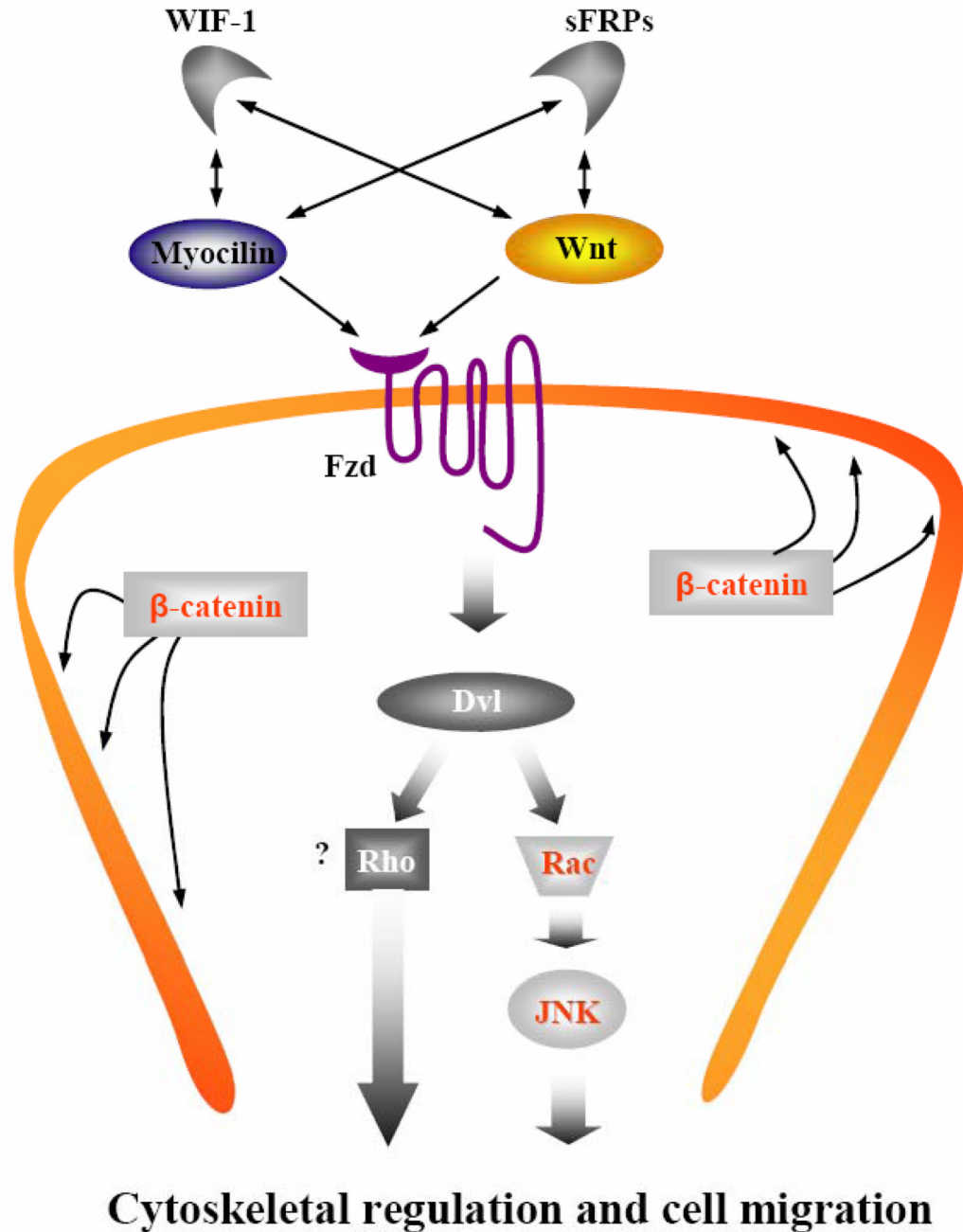
**Fig. 4.**
Schematic diagram of myocilin action. Myocilin may bind Wnt antagonists WIF-1 and sFRPs and compete with Wnt for binding to several Frizzled receptors. Proteins that are affected by myocilin treatment are indicated in red. Thin uninterrupted lines with two arrows indicate proteins that interact with each other (from Kwon et al., 2009, by permission of American Society for Microbiology).
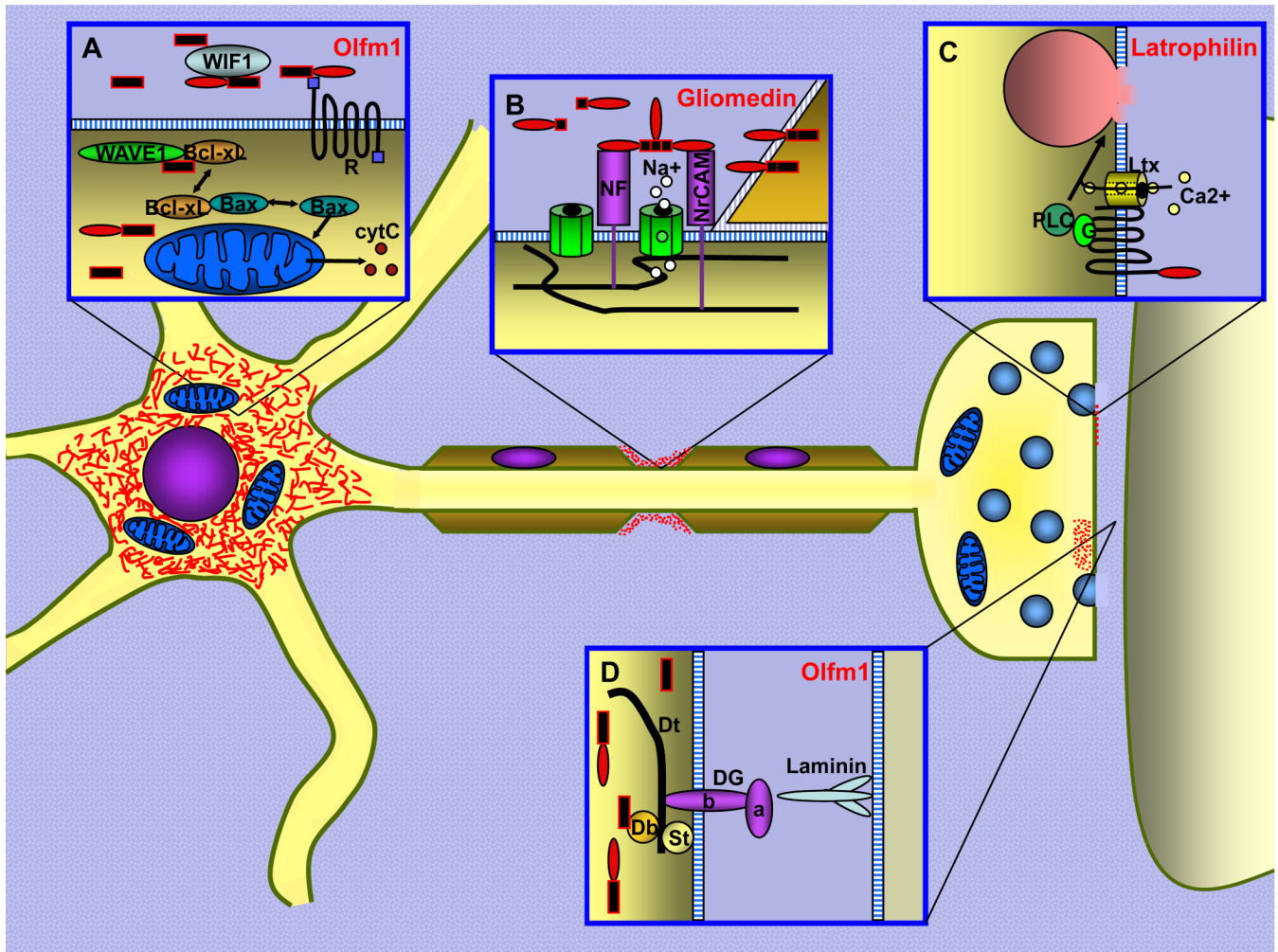
**Fig. 5.**
The localization and proposed functions of olfactomedin domain-containing proteins in neurons. Olfactomedin domains are shown as red ovals. The N-terminal parts of olfactomedin domain-containing proteins are shown as black rectangles. Three major olfactomedin containing protein groups are expressed and function in neurons. (A) Olfm1 is mainly localized in the endoplasmic reticulum and Golgi apparatus in cell soma. It translocates to mitochondria in the event of neuronal death, forms WAVE1-Bcl-xL complex, and activates proapoptotic proteins BAX and BAK that leads to a release of cytochrome c (cytC). Secreted Olfm1 binds to a Wnt inhibitory protein, WIF1, and may serve as a modulator of Wnt signaling. Interaction of Olfm1 with unknown receptors (R) may be essential for mediating the Olfm1 action. (B) At the nodes of Ranvier, gliomedin is released from adjacent Schwann cells and binds to neurofacin (NF) and NrCAM on the axonal surface. This binding induces clustering of sodium channels and involves recruitment of ankyrin G and βIV spectrin (shown as black lines). (C) Latrophillins are seven membrane-spanning receptors localizing at the presynaptic membrane. They were identified as binding targets for spider venom, latrotoxin (Ltx), which becomes embedded into the membrane and transports calcium ions to the presynaptic body. (D) Olfm1 may also exist in presynapse and bind to β-dystrobrevin (Db), regulating the synapse attachment through the distroglycan complex. Dt; Dystrophin, St; Syntrophin, DG; alpha (a) and beta (b) Dysroglycans. The figure represents a general image of neuron. The expression

of each olfactomedin protein is specific for some type of neurons in central and peripheral nervous systems.

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**Table 1**

Olfactomedin domain-containing genes and their main properties

| Gene | Other names | Chromosome | Known protein forms | Main expression sites | Interacting partners | Affected pathways/phenotypes |
|---|---|---|---|---|---|---|
| OLFM1 | Olfactomedin1, Noelin1, pancortin, hOlfA, AMY | 9q34.3 | 125 aa, 135 aa, 457 aa, 485 aa | Cortex, hippocampus, retina | Wave1, dysntrobrevin, Wif-1, Bcl-xL, DISC1 *, lectin | Eye determination, axon growth and branching, apoptosis, Wnt signaling, neurogenesis (Xenopus), neural crest formation (chicken) |
| OLFM2 | olfactomedin2, Noelin2, OlfC | 19p13.2 | 454 aa ** | CNS, retina, pharyngeal arches | | Glaucoma |
| OLFM3 | Olfactomedin3, Optimedin | 1p21.2 | 458 aa, 478 aa | CNS, retina | Myocilin | Cell adhesion, cytoskeleton organization |
| OLFM4 | Olfactomedin4, GW112, hGC-1, pDP4, hOlfd, olfactoimedin, Tiarin | 13q14.3 | 510 aa | Prostate, small intestine, colon, bone marrow | GRIM-19, cadherin, lectins | Cell proliferation, itestinal stem cell marker, dorsalization of neural tube (Xenopus) |
| OLFML1 | Olfactomedin-like1, ONT2 | 11p15.4 | 402 aa | Small intestine, liver, lung, heart, spleen | | Cell proliferation |
| OLFML2a | Olfactomedin-like2A, Photomedin-1 | 9q33.3 | 652 aa | Lung, eye, testis, uterus | Heparin, chondroitin sulfate-E | |
| OLFML2b | Olfactomedin-like2B, Photomedin-2 | 1q23.3 | 750 aa | Broad spectrum of tissues | Heparin, chondroitin sulfate-E | |
| OLFML3 | Olfactomedin-like3, hOLF44, HNOEL-iso, ONT1? | 1p13.2 | 406 aa | Placenta, liver, heart, sclera, iris | Chordin, BMP1/Tolloid-class proteinase | Dorsal-ventral patterning (Xenopus) |
| MYOC | Myocilin, TIGR, GLC1A, JOAG1 | 1q23-q24 | 504 aa | trabecular meshwork, sclera, sciatic nerve, retinal pigmented epithelium | Fibrilin 1, fibronectin 1, flotillin, Olfm3, myosin light chain, hevin | Glaucoma, Wnt signaling |
| GLDN | Gliomedin, CRG-L2, collomin, colmendin (C. elegans) | 15q21.2 | 551 aa | spinal cord, brain, sciatic nerve | NrCAM, neurofascin | Nodes of Ranvier formation |

| Gene | Other names | Chromo some | Known protein forms | Main expression sites | Interacting partners | Affected pathways/phenotypes |
|---|---|---|---|---|---|---|
| Lphn1 | Latrophilin1, Lectomedin2, CL1, CIRL1 | 19p13.2 | 1459 aa | CNS | latrotoxin, syntaxin, SHANK2, lectins, synaptotagmin-3, SAP102, PSD95, ANKS1A | Exocytosis from neurons |
| Lphn2 | Latrophilin2, Lectomedin1, CL2, CIRL2, LPHH1 | 1p31.1 | 1403 aa | Ubiquitous, CNS | latrotoxin, lectins, SHANK2, synaptotagmin-3 | Exocytosis from neuroins and neuroendocrine cells |
| Lphn3 | Latrophilin3, Lectomedin3, CIRL3 | 4q13.1 | 1240 aa | CNS | latrotoxin | |

*
Interaction of Olfm1 and DISC1 was shown only by yeast two-hybrid screen.

**
Three protein forms (478 aa, 456 aa and 448 aa) were reported for mouse Olfm2.