

# Characterization and differential expression of a human gene family of olfactomedin-related proteins

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## Summary

Olfactomedin-related proteins are secreted glycoproteins with conserved C-terminal motifs. Olfactomedin was originally identified as the major component of the mucus layer that surrounds the chemosensory dendrites of olfactory neurons. Homologues were subsequently found also in other tissues, including the brain and in species ranging from *Caenorhabditis elegans* to *Homo sapiens*. Most importantly, the TIGR/myocilin protein, expressed in the eye and associated with the pathogenesis of glaucoma, is an olfactomedin-related protein. The prevalence of olfactomedin-related proteins among species and their identification in different tissues prompted us to investigate whether a gene family exists within a species, specifically *Homo sapiens*. A GenBank search indeed revealed an entire human gene family of olfactomedin-related proteins with at least five members, designated hOlfA through hOlfD and the TIGR/myocilin protein. hOlfA corresponds to the rat neuronal AMZ protein. Phylogenetic analyses of 18 olfactomedin-related sequences resolved four distinct subfamilies. Among the human proteins, hOlfA and hOlfC, both expressed in brain, are most closely related. Northern blot analyses of 16 human tissues demonstrated highly specific expression patterns: hOlfA is expressed in brain, hOlfB in pancreas and prostate, hOlfC in cerebellum, hOlfD in colon, small intestine and prostate and TIGR/myocilin in heart and skeletal muscle. The link between TIGR/myocilin and ocular hypertension and the expression of several of these proteins in mucus-lined tissues suggest that they play an important role in regulating physical properties of the extracellular environment. Future studies can now assess whether other members of this gene family, like TIGR/myocilin, are also associated with human disease processes.

## 1. Introduction

Olfactomedin was identified originally as the primary structural component of the lower mucus layer of the olfactory neuroepithelium of *Rana catesbeiana*, where its specific expression in olfactory glands and supporting cells and its close association with chemosensory cilia of olfactory receptor neurons suggested a role in maintenance and/or differentiation of the apical dendrites of olfactory neurons (Anholt *et al.*,

1990; Snyder *et al.*, 1991; Bal & Anholt, 1993; Yokoe & Anholt, 1993). Olfactomedin was purified from frog olfactory tissue by lectin affinity chromatography (Snyder *et al.*, 1991) and molecular cloning of this extracellular matrix protein together with biochemical studies generated a model in which olfactomedin molecules form glycosylated polymers through intermolecular disulfide bonds (Yokoe & Anholt, 1993).

Following the characterization of amphibian olfactomedin, a homologue of olfactomedin was discovered in the rat brain (Danielson *et al.*, 1994). This neuronal protein contains an endoplasmic reticulum localization signal, is produced by neurons and expressed throughout the central nervous system and in the pituitary and adrenal glands (Danielson *et*

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*al.*, 1994). A mouse homologue of this protein was also isolated from an olfactory cDNA library (Karavanich & Anholt, 1998).

Subsequent searches through GenBank uncovered human and teleost homologues and even homologues from *Caenorhabditis elegans* (Karavanich & Anholt, 1998). Phylogenetic analyses showed that olfactomedin evolved from an early ancestral gene before the origin of the vertebrates. During its evolution the C-terminal region (the 'olfactomedin homology domain') was conserved and evolved slowly through point mutations, whereas fewer constraints were apparent on the evolution of the N-terminal region of the molecule, characterized by large sequence insertions and deletions (Karavanich & Anholt, 1998).

The first link between an olfactomedin-related protein and human disease came with the discovery of the TIGR/myocilin protein, expressed in trabecular meshwork cells in the anterior segment of the eye (Polansky *et al.*, 1997), the ciliary body (Ortego *et al.*, 1997) and the connecting cilia of photoreceptor cells (Kubota *et al.*, 1997). Mutations in this protein are closely associated with primary open angle glaucoma (Sarfarazi, 1997; Stoilova *et al.*, 1997; Stone *et al.*, 1997; Adam *et al.*, 1997; Polansky & Nguyen, 1998; Morissette *et al.*, 1998; Alward *et al.*, 1998; Richards *et al.*, 1998), a disease characterized by ocular hypertension and progressive degeneration of the optic nerve that affects 1 out of 100 individuals over the age of 40 years (for reviews see Shields *et al.*, 1996 and Sarfarazi, 1997). The TIGR/myocilin protein has a distinct olfactomedin homology domain (Ortego *et al.*, 1997; Kubota *et al.*, 1997; Adam *et al.*, 1997; Morissette *et al.*, 1998) and linkage studies showed that polymorphisms that predispose to the development of – especially juvenile onset – glaucoma are localized primarily in this olfactomedin homology domain (Sarfarazi, 1997; Stoilova *et al.*, 1997; Stone *et al.*, 1997; Adam *et al.*, 1997; Polansky & Nguyen, 1998; Morissette *et al.*, 1998; Alward *et al.*, 1998; Richards *et al.*, 1998).

The occurrence of a neuronal olfactomedin-related protein, similar to that found in the rat brain and the TIGR/myocilin protein together with the observation that two different olfactomedin-related proteins can be identified in the genome of *C. elegans*, suggested the existence of a human gene family of olfactomedin-related extracellular matrix proteins. This notion and the importance of the TIGR/myocilin protein for glaucoma, a progressive human disease of high incidence, motivated us to pursue the characterization of this new gene family. Here we document the existence of at least five distinct olfactomedin-related proteins in the human genome and their phylogenetic relationships. Furthermore, a survey of 16 human tissues reveals highly selective expression of each of these proteins. Our observations underscore the notion

that olfactomedin-related proteins are an important new class of secreted extracellular matrix components.

## 2. Methods

### (i) Identification of olfactomedin-related sequences

To identify human olfactomedin-related proteins we searched the GenBank data base using the BLAST programs (Altschul *et al.*, 1997). This resulted in the identification of 32 EST sequences. After consolidating duplicate entries five distinct gene products with clearly defined olfactomedin homology domains were selected for detailed analysis. These sequences were classified as hOlfA (U79299), hOlfB (AA447264), hOlfC (AA324877), hOlfD (W53028) and TIGR/myocilin (AF001620), where 'h' designates the species, *Homo sapiens*. GenBank accession numbers are indicated in parentheses.

### (ii) Phylogenetic analyses of olfactomedin-related sequences

Amino acid sequences for 18 proteins containing olfactomedin homology domains from different species were chosen for phylogenetic analyses. These proteins were consolidated from multiple, partial or overlapping entries in the data base and reflect the diversity of proteins containing olfactomedin domains. They ranged in size from 1512 amino acids in latrophilin 3 to an 84 residue fragment for hOlfD. These sequences were aligned using the ClustalX and Dialign-2 multiple sequence alignment programs (Thompson *et al.*, 1997; Morgenstern, 1999). Unrooted phylogenetic trees were estimated using the PAM 250 distance and the neighbour-joining tree-building algorithm (Saitou & Nei, 1987). Bootstrap values based upon 1000 iterations provide estimates of statistical support for the trees.

To align the five human olfactomedin-related proteins separately, multiple sequence alignment was carried out using ClustalX with manual improvement by eye. A neighbor-joining phylogenetic tree of the sequences was obtained using the *p*-distance (percent difference) to estimate pair-wise relationships. Several other distance metrics gave the same tree topology. The tree was bootstrapped 500 times to provide estimates of statistical support for the topology.

### (iii) Expression patterns of olfactomedin related proteins

To characterize the expression patterns of the five olfactomedin-related proteins, non-crosshybridizing antisense oligonucleotides (Life Technologies, Gaithersburg, MD) specific for each sequence were designed as follows: hOlfA, 5'-CTTGACAGTCA-CGGGGTCACTGATGCCCGTCAACTTCCC;

hOlfB, 5'-TTGTATACCACGTGGCCTGTGCCG-ATCCAGCTGTACGGG; hOlfC, 5'-CACAGAGC-GCGAGCGGAAGTGGTATTTGACCACCACG-TT; hOlfD, 5'-TAGTAAAAAATCTCTTCTGTT-CTGGTGTTCCATAGTACG; and TIGR/myocilin, 5'-GGATTCATTGGGACTGGCCACACTGAA-GGTATACTGGCA. To verify specificity of the primers, each of the antisense oligonucleotides was used in combination with a specific sense primer to amplify polymerase chain reaction (PCR) fragments from human genomic DNA. Amplification products of the expected sizes were obtained, sequenced and verified to correspond to the expected olfactomedin-related EST sequences. The antisense oligonucleotides were 5'-end labelled with radioactive  $^{32}\text{P}$ - $\gamma$ ATP (Amersham Life Science, Arlington Heights, IL) using T4 polynucleotide kinase (Amersham Pharmacia Biotech, Piscataway, NJ) and used to probe multiple tissue Northern blots from ClonTech Laboratories, (Palo Alto, CA) containing 2  $\mu\text{g}$  of human mRNA per lane from heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate gland, testis, ovary, small intestine, colon and peripheral blood leucocytes. Hybridizations were performed in ClonTech's ExpressHyb hybridization solution according to the manufacturer's protocol at 56 °C for hOlfA, hOlfB and TIGR/myocilin, at 57 °C for hOlfC, at 49 °C for hOlfD and at 50 °C for an actin probe as a control. The membranes were exposed in a phosphorimager cassette for 3–5 days prior to image analysis.

### 3. Results

#### (i) Identification of a family of olfactomedin-related proteins in the human genome

Our previous studies showed that olfactomedin-related proteins occur ubiquitously in many vertebrate and invertebrate species (Karavanich & Anholt, 1998). In addition, we found a human homologue of the rat neuronal AMZ protein (Karavanich & Anholt, 1998). These observations together with the discovery of the glaucoma-associated TIGR/myocilin protein, which contains an olfactomedin homology domain (Sarfarazi, 1997; Stoilova *et al.*, 1997; Stone *et al.*, 1997; Adam *et al.*, 1997; Polansky & Nguyen, 1998; Morissette *et al.*, 1998; Alward *et al.*, 1998; Richards *et al.*, 1998), suggested that a family of proteins with olfactomedin homology domains may exist in the human genome. This realization motivated us to search the GenBank data base for olfactomedin-related sequences and led to the discovery of five distinct genes encoding olfactomedin-related proteins, including the TIGR/myocilin gene. Amino acid alignments of the C-terminal regions, containing the olfactomedin homology domains, are presented in

Fig. 1. The sequence of hOlfA shows 98% amino acid identity to the neuronal rat AMZ protein (Danielson *et al.*, 1994). The sequences of hOlfB, hOlfC and hOlfD have not been recognized previously as olfactomedin-related sequences.

Protein sequences in the olfactomedin homology domains show 36–63% amino acid identity among the five olfactomedin-related proteins. Many of the conserved motifs 2, 3, 4, 5 and 6, previously identified as characteristic of the olfactomedin homology domain (Karavanich & Anholt, 1998), are readily identifiable in the hOlf sequences, shown in Fig. 1. Motif 2 is found in hOlfA, hOlfC and TIGR/myocilin, whereas hOlfB is divergent in this region. Motif 3, however, the most prominent conserved domain, is found in all five sequences. Characteristic motifs 4 and 5 are observed in all sequences except for motif 5 in the truncated sequence of hOlfD. Motif 6 can be identified in hOlfA, hOlfB, hOlfC and TIGR/myocilin. The presence of these characteristic motifs clearly identifies each of these gene products as a member of this new family of olfactomedin-related proteins.

#### (ii) Phylogenetic relationships among olfactomedin-related proteins

To assess the evolutionary interrelationships between these human olfactomedin related proteins, we performed phylogenetic analyses on 18 proteins that contain olfactomedin homology domains and that are documented in GenBank. Because these 18 proteins varied significantly in length, we have provided neighbour-joining trees for both the entire protein sequences and for the olfactomedin homology domains. The aligned olfactomedin homology domains for these 18 proteins are provided in Fig. 2 and neighbour-joining trees for the entire protein sequences and for the olfactomedin homology domains are shown in Fig. 3A and B, respectively. When the complete sequences are considered, olfactomedin-related proteins appear to cluster in four groups. Olfactomedin-related proteins that are expressed in the brain form one distinct group, including hOlfA and its rat and mouse homologues, mouse pancortin (Nagano *et al.*, 1998), which is closely related to the AMZ protein (Danielson *et al.*, 1994) and hOlfC. The original olfactomedin from *Rana catesbeiana* (Snyder *et al.*, 1991; Yokoe & Anholt, 1993) is most closely related to human hOlfD. hOlfB and TIGR/myocilin appear to be most similar and are the closest relatives of the two olfactomedin-related proteins from *C. elegans*. Interestingly, latrophilin and related proteins also form a distinct cluster (Fig. 3A). Latrophilin is the synaptic black widow spider venom (latrotoxin) receptor; it is a member of the superfamily of G-protein coupled receptors and

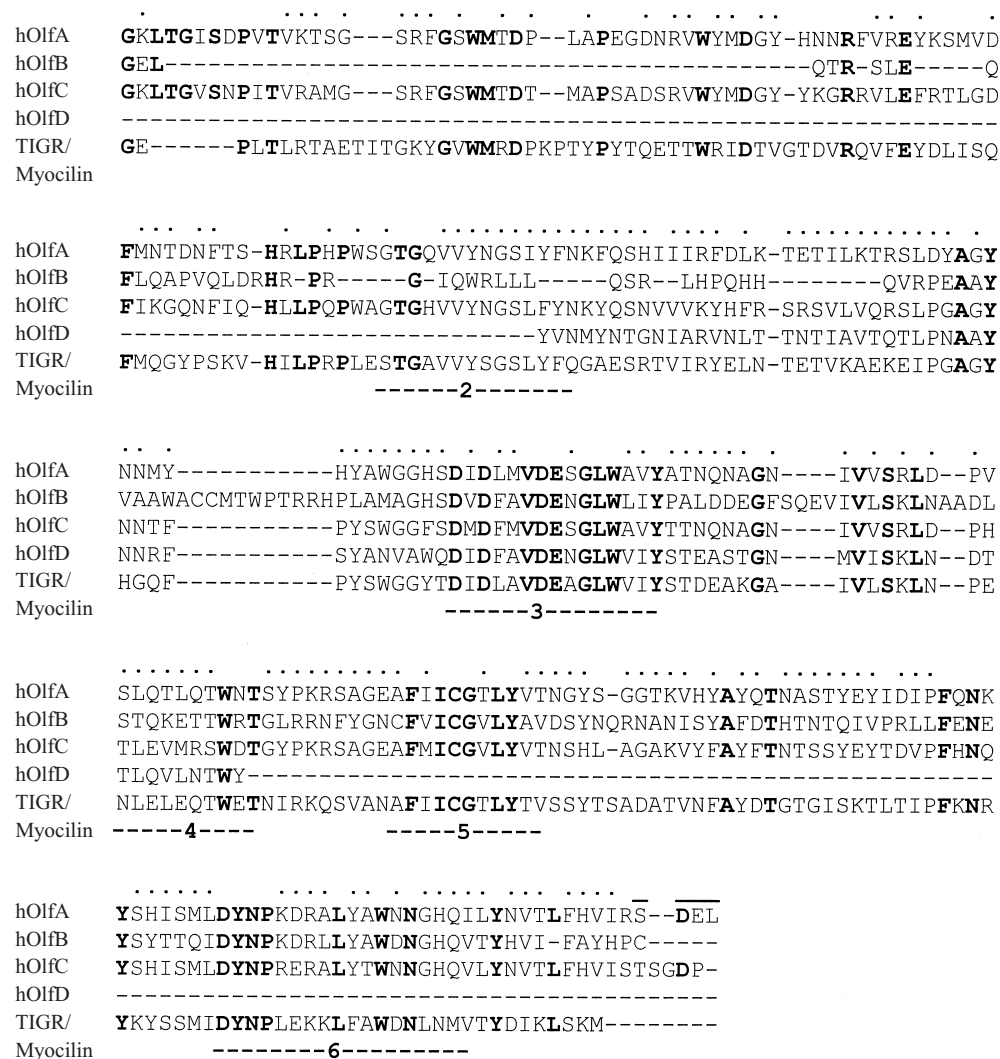


Fig. 1. Amino acid sequence alignments of the olfactomedin homology domains of five human olfactomedin-related proteins. Multiple sequence alignment was produced using ClustalX with manual improvement by eye. Amino acids shared between two or more sequences are indicated with a dot. Amino acids that are invariant among all olfactomedin-related proteins are in bold print. Sequences with dashed underlines correspond to phylogenetically conserved motifs characteristic of the olfactomedin homology domain (Karavanich & Anholt, 1998). The endoplasmic reticulum targeting signal (SDEL) at the C-terminus of hOlfA is overlined. The aligned sequences correspond to the region between glycine 252 and the C-terminal methionine 504 of TIGR/myocilin (Ortego *et al.*, 1997; Kubota *et al.*, 1997).

contains an extracellular olfactomedin homology domain (AJ131581; Lelianova *et al.*, 1997).

A neighbour-joining tree based only on the olfactomedin homology domains shows similar relationships, except that in this tree the olfactomedin homology domain of the TIGR/myocilin protein stands by itself and appears to be most closely related to the neuronal olfactomedin-related proteins and latrophilins (Fig. 3B).

A simplified tree that considers only the olfactomedin homology domains of the five human olfactomedin-related proteins based on the sequence alignments of Fig. 1 is shown in Fig. 4. This tree agrees with the more comprehensive trees shown in Fig. 3 and shows again that hOlfA is most closely related to hOlfC; hOlfA and hOlfC show the smallest

proportion of amino acids that are different in a pairwise comparison, i.e. 0.358 (SE =  $\pm 0.030$ ). The next smallest difference is between hOlfA and hOlfD with a value of 0.560 ( $\pm 0.054$ ).

### (iii) Expression patterns of the human olfactomedin-related proteins

Despite the association of the TIGR/myocilin protein with the pathogenesis of glaucoma (Sarfarazi, 1997; Stoilova *et al.*, 1997; Stone *et al.*, 1997; Adam *et al.*, 1997; Polansky & Nguyen, 1998; Morissette *et al.*, 1998; Alward *et al.*, 1998; Richards *et al.*, 1998) and the prominent and widespread distribution of the AMZ protein, the hOlfA homologue, throughout the mammalian brain (Danielson *et al.*, 1994), little

OlfB HUMAN	1	HPQHH-----QVRPEA-AYVAAWACCMTP-----TRRH-PLAMAGHSDVDFAVDENGLWLIYPALDDEGFSQEVIVLSK
OlfD HUMAN	1	-----YVNMNNTGNIARVNLTTNTIAVTQTLPLNA-AYNNRF-----SYANVAWQDIDFAVDENGLWVIYSTEASTG----NMVISK
OlfA HUMAN	1	HPWSGTGQVVYNGSIYFNKFQSHIIIRFDLKTETILKTRSL-DYAGYNNMY-----HYAWGGHSDIDLMDVESGLWAVYATNQAG----NIVVSR
OlfI WORM	1	-----PG-AAYRGDQ-----YLYNNSMNYFDLALDENALWVMFHYQEEAF----LSVAK
TIGR/MYOCILIN	1	RPLESTGAVVYSGSLYFQGAESRTVIRYELNTEITVKAKEKEIPG-AGYHGQF-----PYSWGGYTDIDLAVDEAGLWVIYSTDEAKG----AIVLSK
PANCORTIN 3	1	HPWSGTGQVVYNGSIYFNKFQSHIIIRFDLKTETILKTRSL-DYAGYNNMY-----HYAWGGHSDIDLMDVENGLWAVYATNQAG----NIVISK
OlfA MOUSE	1	HPWSGTGQVVYNGSIYFNKFQSHIIIRFDLKTETILKTRSL-DYAGYNNMY-----HYAWGGHSDIDLMDVENGLWAVYATNQAG----NIVISK
OlfA RAT	1	HPWSGTGQVVYNGSIYFNKFQSHIIIRFDLKTETILKTRSL-DYAGYNNMY-----HYAWGGHSDIDLMDVENGLWAVYATNQAG----NIVISK
OlfC HUMAN	1	QPWAGTGHVVYNGSLFYNKYQSNVVVKYHFRSRSVLVQRSLPG-AGYNNTF-----PYSWGGFSDMDFMVDESGLWAVYTTNQAG----NIVISK
Olf2 WORM	1	YLASGTGNVVYNGSYYYHKHGTSLVRYELETGVQVEAELDPEM-SHIDCGRLPDHTFEECNATDRHWLYDRPHNYVDFAVDENGLWAIYAGADSET----MRMAK
Olf FROG	1	FAGQGAGVVHNNNLYNCFNHSHTMCRASLTSGVYQKKPLL-NA-LFNRRF-----SYAGTMFQDMDFSSTEKGLWVI FTTEKSAG----KIVVGK
CL3 RAT	1	HRVDGTGFVVYDGALEFNKERTRNIVKFDLRTRIKSGEAIIAN-ANYHDTS-----PRYWGGKSDIDLAVDENGLWVIYATEQNGG----KIVISQ
LATROPHILIN	1	NRVDGTGFVVYDGALEFNKERTRNIVKFDLRTRIKSGEAII-NTANYHDTS-----PYRWGGKTDIDLAVDENGLWVIYATEQNGG----MIVISQ
CL2BA RAT	1	NRVDGTGFVVYDGALEFNKERTRNIVKFDLRTRIKSGEAII-NTANYHDTS-----PYRWGGKTDIDLAVDENGLWVIYATEQNGG----MIVISQ
LATROPHILIN	1	HRVDGTGFVVYDGALEFNKERTRNIVKFDLRTRIKSGEAIIAN-ANYHDTS-----PYRWGGKSTIDLAVDENGLWVIYATEQNGG----KIVISQ
LATROPHILIN	1	NRVDGTGFVVYDGALEFNKERTRNIVKFDLRTRIKSGETVI-NTANYHDTS-----PYRWGGKTDIDLAVDENGLWVIYATEGNGG----RLVVSQ
LATROPHILIN	1	NRVDGTDFVVYDGALEFNKERTRNIVKFDLRTRIKSGETVI-NTANYHDTS-----PYRWGGKTDIDLAVDENGLWVIYATEGNGG----RLVVSQ
KIAA0821 H		NRVDGTGFVVYDGALEFNKERTRNIVKFDLRTRIKSGETVI-NTANYHDTS-----PYRWGGKTDIDLAVDENGLWVIYATEGNGG----RLVVSQ
OlfB HUMAN	1	LNAADLS--TQKETWRTGLRRNFYGNCFVICGVLYAVDSYNQRNAN-----ISYAFTHHTNTQIVPRLLEFENEYSYTTQIDYNPKDRLLYAWDNGHQVTYHVI F-
OlfD HUMAN	1	LNDTTLQ--V--LNTWY-----
OlfA HUMAN	1	LDPVSLQ--T--LQTWNTSYPKRSAGEAFIICGTLVVTNGYSG-----GTKVHYAYQTNASTYEYIDIPFQNKYSHISMLDYNPKDRALYAWNNGHQILYNVTLF
OlfI WORM	1	V-DINNLTIIY--EAFNLTTVNHTDVANGIVVCGTIYLVESASEQSTY-----ISTAYDFYRNTYSQPLIKWINLYKKNANMISYNAYDKRIYIYDHGYMLSP-----
TIGR/MYOCILIN	1	LNPENLE--L--EQTWETNIRKQSVANAFIICGTLVTVSSYTSADAT-----VNFAYDTGTGISKTLTIPFKNRYKYSSMIDYNPLEKKLFAWDNLMVTVYDIKL-
PANCORTIN 3	1	LDPVSLQ--I--LQTWNTSYPKRSAGEAFIICGTLVVTNGYSG-----GTKVHYAYQTNASTYEYIDIPFQNKYSHISMLDYNPKDRALYAWNNGHQTLYNVTL-
OlfA MOUSE	1	LDPVSLQ--I--LQTWNTSYPKRSAGEAFIICGTLVVTNGYSG-----GTKVHYAYRTNASTYEYIDIPFQNKYSHISMLDYNPKDRALYAWNNGTQTLYNVTL-
OlfA RAT	1	LDPVSLQ--I--LQTWNTSYPKRSAGEAFIICGTLVVTNGYSG-----GTKVHYAYQTNASTYEYIDIPFQNKYSHISMLDYNPKDRALYAWNNGHQTLYNVTL-
OlfC HUMAN	1	LDPHTLE--V--MRSWDTGYPKRSAGEAFMVICGVLYYTNSHL-----AGAKVYFAYFTNTSSYEYTDVFPFHNYSHISMLDYNPRERALYTWNNGHQVLYNVTL-
Olf2 WORM	1	I-EPSLF--V--VNIWNVEVNTTEIADSFIMCGVWYGLKSANNLQTO-----ITHAYDLFRNDTIPGQVEWYNPYQGLTMLHYNPLDARLYFFDNSSLLSVNVR I-
Olf FROG	1	VNVATFT--V--DNIWITTONKSDASNAFMICGVLYVTRSLGPKMEE-----VFYMFDTKTGKEGHLSSIMMEKMAEKVHSLSYNSNDRKLYMFSEGYLLHYDIAL-
CL3 RAT	1	LNPYTLR--I--EGTWDTAYDKRSASNAFMICGILYVVKSVYEDDDNEATGNKIDYIYNTDQSKDSLVDVFPFNSYQYIAAVDYNPRDNLLYVWNNYHVVKYSLDF-
LATROPHILIN	1	LNPYTLR--F--EATWETVYDKRASNAFMICGVLYVRSVYQDNESETGKNSIDYIYNTRLNRGEYVDVFPFNPQYQYIAAVDYNPRDNQLYVWNNNFILRYSLEF-
CL2BA RAT	1	LNPYTLR--F--EATWETTYDKRASNAFMICGVLYVRSVYQDNESEAGKNVIDYIYNTRLSRGEHVVDVFPFNPQYQYIAAVDYNPRDNQLYVWNNNFILRYSLEF-
LATROPHILIN	1	LNPYTLR--I--EGTWDTAYDKRSASNAFMICGILYVVKSVYEDDDNEATGNKIDYIYNTDQSKDSLVDVFPFNSYQYIAAVDYNPRDNLLYVWNNYHVVKYSLDF-
LATROPHILIN	1	LNPYTLR--F--EGTWTGYDKRSASNAFMVCGVLYVLRVSVYVDDSEAAAGNRVDYAFENTNANREEPVSLAFPNPYQFVSSVDYNPRDNQLYVWNNYFVVRYSLEF-
LATROPHILIN	1	LNPYTLR--F--EGTWTGYDKRSASNAFMVCGVLYVLRVSVYVDDSEAAAGNRVDYAFENTNANREEPVSLTFFPNPYQFISVDYNPRDNQLYVWNNYFVVRYSLEF-
KIAA0821 H		LNPYTLR--F--EGTWTGYDKRSASNAFMVCGVLYVLRVSVYVDDSEAAAGNRVDYAFENTNANREEPVSLTFFPNPYQFISVDYNPRDNQLYVWNNYFVVRYSLEF-

Fig. 2. Amino acid sequence alignments of the olfactomedin homology domains of 18 olfactomedin-related proteins. GenBank accession numbers for these sequences are indicated in the legend to Fig. 3. For proteins with splice variants the longest sequences were chosen for analysis. The Dialign2 program was used to construct the alignment (Morgenstern, 1999). Residues indicated in bold print are conserved among at least 50% of the sequences compared.

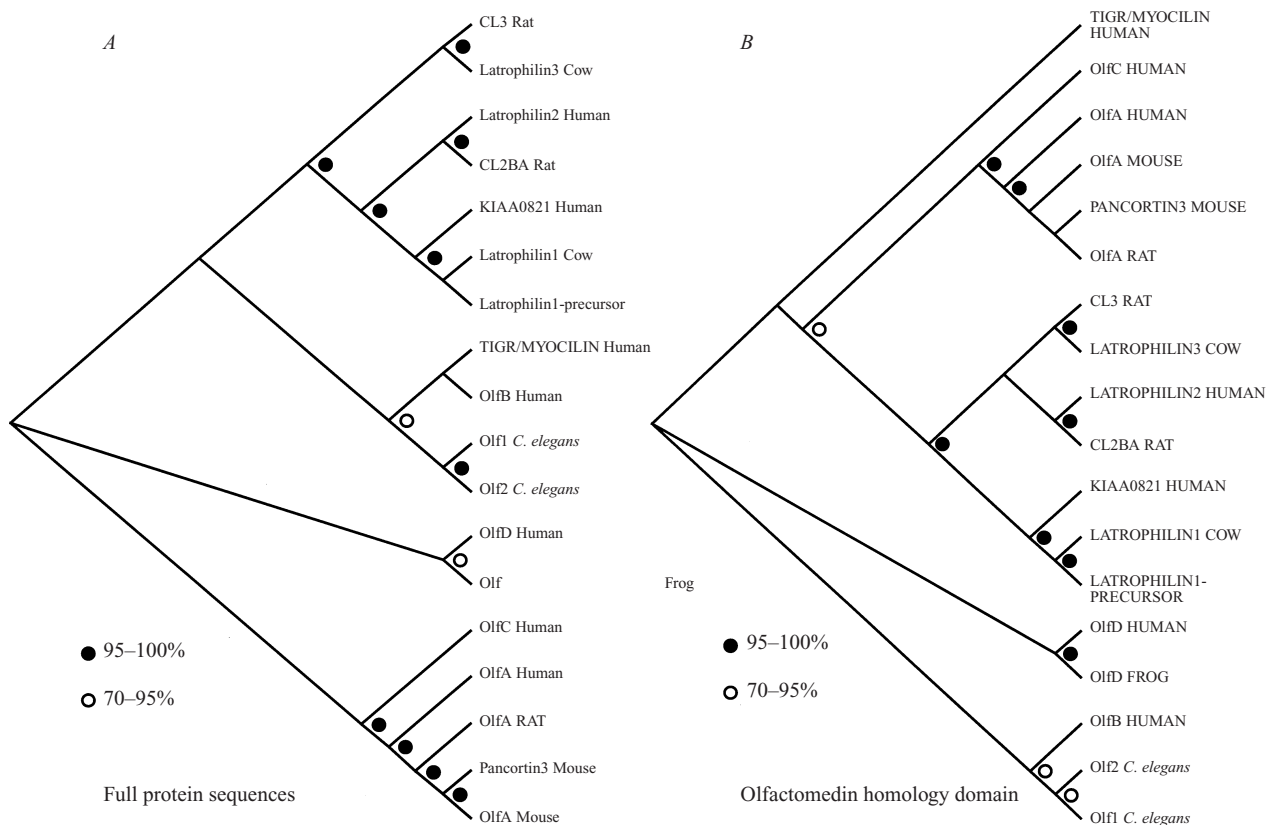


Fig. 3. Unrooted neighbour-joining trees of 18 olfactomedin-related proteins (A) and their olfactomedin homology domains (B). The bootstrap values are coded such that a filled circle refers to a value of 95–100% and an open circle refers to values between 70 and 94%. GenBank accession numbers for these protein sequences are as follows: CL2BA\_Rat, gi|3695129|gb|AAC62657-1; CL3\_Rat, gi|3695143|gb|AAC62664-1; KIAA0821\_Human, gi|4240128|dbj|BAA74844-1; Latrophilin1\_Cow, gi|4185802|gb|AAD09191-1; Latrophilin1\_precursor, gi|2213659|gb|AAC98700-1; Latrophilin2\_Human, gi|4034486|emb|CAA10458-1; Latrophilin3\_Cow, gi|4164059|gb|AAD05324-1; Olf\_Frog, gi|585611|sp|Q07081; Olf1\_Worm, gi|1947128|gb|AAB52933-1; Olf2\_Worm, gi|3875750|emb|CAB04088-1; OlfA\_Human, gi|3024228|sp|Q99784; OlfA\_Mouse, gi|2599125|gb|AAB84058-1; OlfA\_Rat, gi|3024210|sp|Q62609; OlfB\_Human, gi|2159929|gb|AA447264-1; OlfC\_Human, gi|4406679|gb|AAD20056-1; OlfD\_Human, gi|1349928|gb|W53028-1; TIGR/myocilin\_Human, gi|3024209|sp|Q99972; Pancortin3\_Mouse, gi|3218528|dbj|BAA28767-1.

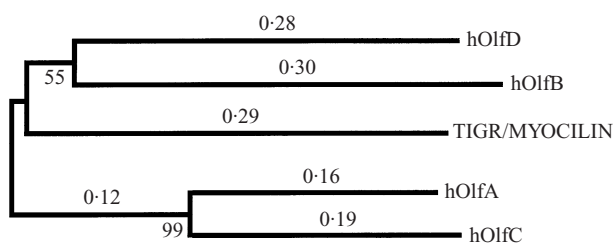


Fig. 4. Neighbour-joining tree showing evolutionary relationships among the olfactomedin homology domains of the five human olfactomedin-related proteins. Bootstrap values over 500 iterations are indicated at the nodes. Branch lengths, given in italics, reflect the proportion of amino acids that differ (*p*-distance).

information is available about the possible functions of olfactomedin-related proteins. As a first approach to gain insights into the functions of these proteins, we examined by Northern blotting the expression of each of the five olfactomedin-related proteins in a panel of 16 human tissues (Fig. 5). The expression patterns we observed are highly selective, suggesting that each

member of this family of proteins serves a distinct, specialized function.

As expected from its strong homology with the rat AMZ protein (Danielson *et al.*, 1994), hOlfA is expressed uniquely in brain, where Northern blot analysis reveals an ~ 3 kb message. To gain further insights into the distribution of this message in the brain, we performed dot blot hybridizations with the same antisense oligonucleotide probe on an RNA Masterblot from ClonTech Laboratories, which contains RNA samples from 50 different human tissues (Table 1). Hybridization signals were observed only in samples obtained from brain regions and from pituitary and adrenal gland, but not in spinal cord and samples from 26 other tissues. The expression pattern of hOlfA closely resembles that of the rat AMZ protein (Danielson *et al.*, 1994). It should be noted that our panel of tissues does not contain olfactory tissue, known to express the mouse AMZ protein (Karavanich & Anholt, 1998). Additionally, possible truncated forms of hOlfA, similar to truncated forms

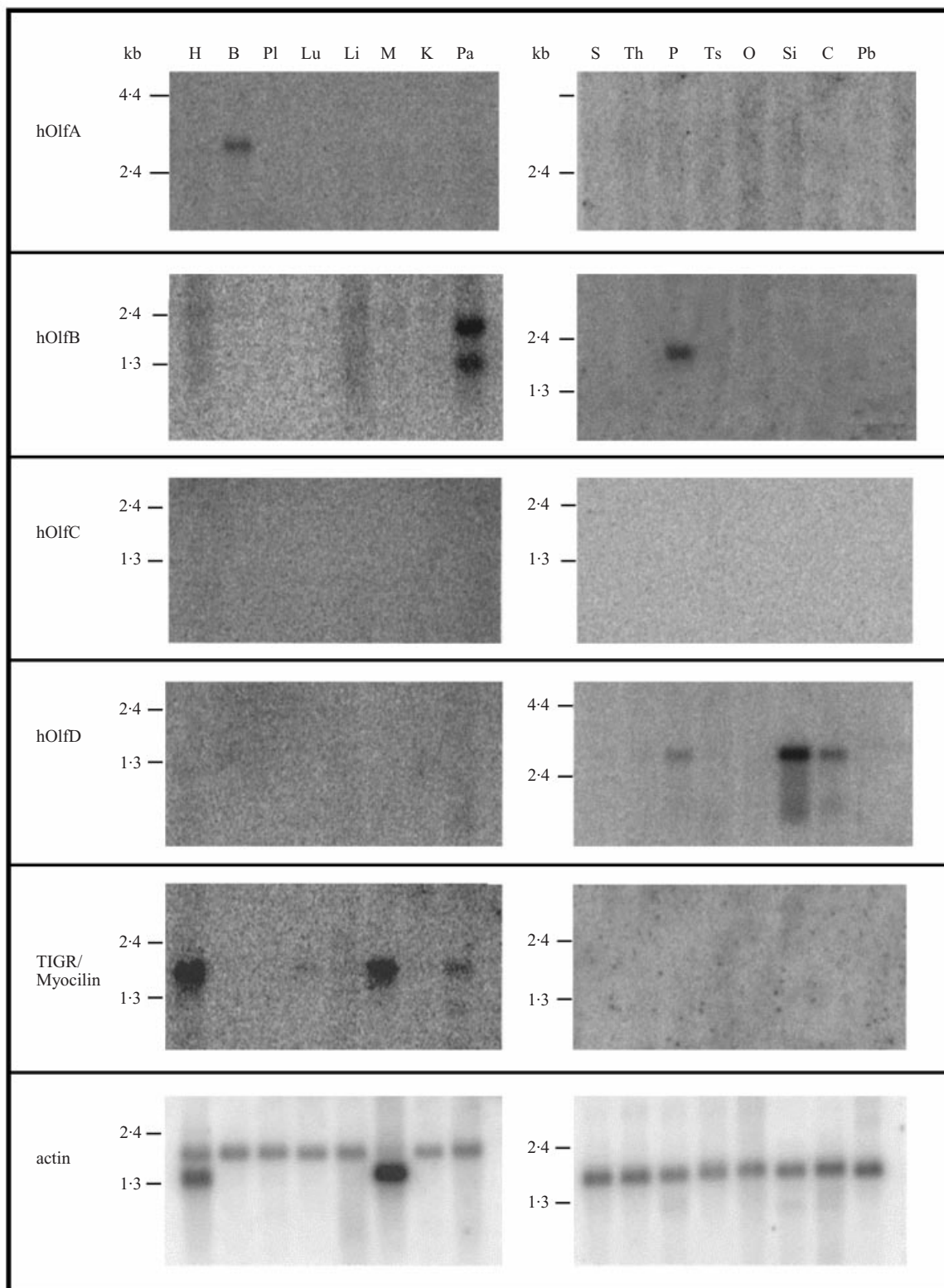


Fig. 5. Tissue-specific expression of olfactomedin-related gene products. Non-crosshybridizing radiolabelled oligonucleotide probes were hybridized to a Northern blot containing samples of human heart (H), brain (B), placenta (Pl), lung (Lu), liver (Li), skeletal muscle (M), kidney (K), pancreas (Pa), spleen (S), thyroid (Th), prostate gland (P), testis (Ts), ovary (O), small intestine (Si), colon (C) and peripheral blood leucocyte (Pb). Two blots were hybridized sequentially to different probes and complete removal of the previous probe was verified between hybridizations. A faint band of hybridization is detected in pancreas with the TIGR/myocilin probe. This band is not an artifact due to incomplete erasure of the hOlfB probe, since hybridization with TIGR/myocilin was preceded by hybridization with hOlfA to this blot.

Table 1. Expression of hOlfA in different regions of the central nervous system<sup>a</sup>

Brain region	Hybridization with hOlfA probe
Cerebral cortex	+
Frontal lobe	+
Temporal lobe	+
Occipital lobe	+
Hippocampus	+
Amygdala	+
Thalamus	+
Cerebellum	+
Putamen	+
Caudate nucleus	±
Substantia nigra	±
Subthalamic nucleus	±
Medulla oblongata	±
Spinal cord	–

<sup>a</sup> The radiolabelled hOlfA-specific oligonucleotide probe was hybridized to a human RNA Multiple Tissue Masterblot from ClonTech according to the manufacturer's instructions. The dot blot contains 89–514 ng polyA<sup>+</sup> RNA per sample standardized to ubiquitin message. Signals from hybridizing dots were scored as strong (+), weak, but unambiguously present (±), or absent (–). In addition to regions of the central nervous system, hybridization was observed only with pituitary (+) and adrenal gland (±), consistent with the data presented in Fig. 5 and in agreement with the previously observed distribution of the rat neuronal AMZ protein (Danielson *et al.*, 1994).

of the AMZ protein (AMY and BMY; Danielson *et al.*, 1994), would escape detection with the hOlfA antisense oligonucleotide probe used in the experiment shown in Fig. 5. None of the other 15 tissues examined in Fig. 5 show message corresponding to hOlfA.

hOlfB is prominently expressed in the pancreas and the prostate gland. In pancreas two splice variants of ~1.4 kb and ~2 kb are evident (Fig. 5). In prostate gland, only the larger splice variant is detected, indicating differential processing of the message in the two tissues. No message for hOlfB is detected in any of the other 14 tissues examined.

The only member of this family of proteins without detectable message in any of the 16 tissues examined is hOlfC. The original EST was identified from cDNA derived from human cerebellum. Failure to detect message for hOlfC in human brain mRNA on our ClonTech multiple tissue Northern blot may be due to the possibility that this mRNA does not represent cerebellum. Apparent absence of hOlfC in any of the 16 tissues examined indicates highly localized expression.

The fourth member of this family of proteins, hOlfD, is predominantly found in the small intestine with lower levels of message detectable in colon and prostate gland. In each case an ~3 kb message is evident (Fig. 5). The prostate gland is the only organ

that expresses two members of the olfactomedin-related protein family, predominantly hOlfB. Since no hybridization is evident with the hOlfD antisense probe and pancreas, hybridization to mRNA from the prostate gland is not due to cross-hybridization of the probe with hOlfB.

Finally, an ~2 kb message is detected for TIGR/myocilin in heart and skeletal muscle, as reported previously (Ortego *et al.*, 1997) and to a lesser extent in pancreas (Fig. 5). Expression of this protein is induced in trabecular meshwork cells in the eye in response to glucocorticoids and stress (Polansky *et al.*, 1997; Adam *et al.*, 1997; Sarfarazi, 1997; Polansky & Nguyen, 1998; Nguyen *et al.*, 1998). We did not examine mRNA from trabecular meshwork cells in the present study. No expression of TIGR/myocilin is observed in any of the 13 other tissues examined.

#### 4. Discussion

We analysed the family of human olfactomedin-related proteins. Thus far, we have studied in detail only five olfactomedin-related gene products. Since less than 10% of the human genome has been sequenced to date, it is likely that the olfactomedin-related gene family is considerably larger and that other olfactomedin-related proteins, yet to be discovered, are expressed in other tissues. Recent entries in the GenBank data base, in fact, indicate a human EST expressed in testis (accession number AA609525), which may be an additional member of the olfactomedin-related protein family and the GW112 protein (accession number AF097021), which is expressed in haematopoietic cells and shows extensive homology with *Rana catesbeiana* olfactomedin and similarities with hOlfD.

hOlfB and hOlfD have been identified on the basis of partial EST sequences. Previous studies on the molecular evolution of olfactomedin showed that the N-terminal region underwent large insertions and deletions during the course of evolution, whereas evolution of the C-terminal half, containing the characteristic olfactomedin homology domain, was more constrained and proceeded primarily through single base substitutions (Karavanich & Anholt, 1998). This gave rise to the notion that the N-terminal and C-terminal halves of the protein fulfil distinct functions and made it tempting to speculate that the olfactomedin homology domain may mediate protein–protein interactions. Thus, although it would be desirable to determine the complete sequences of these olfactomedin-related proteins, the currently available sequences are likely to contain all the information necessary to deduce phylogenetic interrelationships and sufficient sequence specificity to determine in which tissues each gene product is expressed.



Of the five olfactomedin-related proteins analysed two, hOlfA and hOlfC, are expressed in the central nervous system. Both the sequence and expression pattern of hOlfA closely resemble that of its homologue, the rodent AMZ protein (Danielson *et al.*, 1994; Karavanich & Anholt, 1998). It is of interest to note that hOlfA is most closely related to its cerebellar counterpart, hOlfC (Figs. 3, 4). We cannot exclude expression of any of these olfactomedin-related proteins at levels below the detection limit of our Northern hybridizations. It is clear, however, that above our detection limit the expression patterns of the five olfactomedin-related proteins show remarkable tissue specificity. Future studies will be needed to identify the exact cellular locations within the tissues in which these proteins are expressed.

Olfactomedin from the olfactory neuroepithelium of *Rana catesbeiana*, the archetype of this family of proteins, is expressed by Bowman's glands and sustentacular cells in the olfactory neuroepithelium (Snyder *et al.*, 1991; Bal & Anholt, 1993). It represents about 5% of the total tissue protein (Yokoe & Anholt, 1993) and is secreted into the mucus layer on top of the chemosensory surface of the olfactory epithelium in close contact with dendritic cilia of olfactory neurons (Anholt *et al.*, 1990; Snyder *et al.*, 1991; Bal & Anholt, 1993; Yokoe & Anholt, 1993). This protein is highly glycosylated (Snyder *et al.*, 1991; Bal & Anholt, 1993) and forms polymers through intermolecular disulphide bonds (Snyder *et al.*, 1991; Yokoe & Anholt, 1993). It represents the basic structure of the viscous lower mucus layer of this extracellular matrix (Yokoe & Anholt, 1993).

Similarly, the TIGR/myocilin protein is produced by cells of the trabecular meshwork under conditions of stress in response to ocular hypertension or glucocorticoids (Polansky *et al.*, 1997; Adam *et al.*, 1997; Sarfarazi, 1997; Polansky & Nguyen, 1998; Nguyen *et al.*, 1998). Substantial evidence suggests that this protein is also secreted into the canal of Schlemm, where it may contribute to modifying outflow characteristics of fluid from the eye (reviewed by Polansky *et al.*, 1997; Sarfarazi, 1997; Polansky & Nguyen, 1998). Furthermore, genetic and biochemical studies indicate that this protein may form dimers (Morissette *et al.*, 1998) and even higher molecular weight polymers (Nguyen *et al.*, 1998) through leucine zipper domains and intermolecular disulphide bonds. In addition, the AMZ protein and its human homologue, hOlfA, contain a C-terminal endoplasmic reticulum targeting signal and are thought to be secreted by neurons (Danielson *et al.*, 1994; Fig. 1).

These observations and our finding that hOlfB is expressed in glands, namely pancreas and prostate and that hOlfD is prominent in the intestine, which is another mucus-producing organ, suggest that olfactomedin-related proteins constitute a new family

of extracellular matrix components. Based on current knowledge of regulation of expression of the TIGR/myocilin protein, it is possible that these proteins may allow communication between the extracellular matrix and the cells responsible for its production. It is especially intriguing that both the archetypical olfactomedin from *Rana catesbeiana* and TIGR/myocilin are associated with sensory ciliary components of olfactory and visual receptor cells, respectively, suggesting that they may play a vital protective role in maintaining the integrity of these structures (Anholt *et al.*, 1990; Snyder *et al.*, 1991; Bal & Anholt, 1993; Kubota *et al.*, 1997). Both the localization of olfactomedin in olfactory tissue to the viscous lower mucus layer (Snyder *et al.*, 1991) and the relationship between ocular hypertension and overproduction of TIGR/myocilin (Sarfarazi, 1997; Stoilova *et al.*, 1997; Stone *et al.*, 1997; Adam *et al.*, 1997; Polansky & Nguyen, 1998; Morissette *et al.*, 1998; Alward *et al.*, 1998; Richards *et al.*, 1998) together with the expression of several of these proteins in mucus-lined tissues suggest that these proteins may play a role in regulating rheostatic properties of the extracellular environment. The functions of the neuronal olfactomedins and latrophilin are even less clear. Evolutionary conservation of the olfactomedin homology domain raises the speculation that this domain may mediate protein-protein interactions. Thus, homophilic interactions between neuronal olfactomedin-related proteins and the extracellular olfactomedin homology domain of latrophilin could conceivably contribute to the regulation of neurotransmitter release. However, no evidence is currently available to support this hypothesis. Although the precise functions of the olfactomedin-related proteins remain to be elucidated, their importance for human health is unambiguously evident from the close association between mutations in the TIGR/myocilin protein and the risk for development of glaucoma (Sarfarazi, 1997; Stoilova *et al.*, 1997; Stone *et al.*, 1997; Adam *et al.*, 1997; Polansky & Nguyen, 1998; Morissette *et al.*, 1998; Alward *et al.*, 1998; Richards *et al.*, 1998). The well-established link between TIGR/myocilin and the pathogenesis of glaucoma suggests that it is not unlikely that other members of this new family of proteins may in future studies become implicated in a variety of human diseases.

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