The Glaucoma-associated Olfactomedin Domain of Myocilin Is a Novel Calcium Binding Protein

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Background: Myocilin is an extracellular protein linked to glaucoma but is of unknown structure and function. Myocilin is a unique component of the trabecular meshwork linked to glaucoma. Myocilin is a protein found in the trabecular meshwork extracellular matrix tissue of the eye that plays a role in regulating intraocular pressure. Both wild-type and certain myocilin variants containing mutations in the olfactomedin (OLF) domain are linked to the optic neuropathy glaucoma. Because calcium ions are important biological cofactors that play numerous roles in extracellular matrix proteins, we examined the calcium binding properties of the myocilin OLF domain (myoc-OLF). Our study reveals an unprecedented high affinity calcium binding site within myoc-OLF. The calcium ion remains bound to wild-type OLF at neutral and acidic pH. A glaucoma-causing OLF variant, myoc-OLF(D380A), is calcium-depleted. Key differences in secondary and tertiary structure between myoc-OLF(D380A) and wild-type myoc-OLF, as well as limited access to chelators, indicate that the calcium binding site is largely buried in the interior of the protein. Analysis of six conserved aspartate or glutamate residues and an additional 18 disease-causing variants revealed two other candidate residues that may be involved in calcium coordination. Our finding expands our knowledge of calcium binding in extracellular matrix proteins; provides new clues into domain structure, function, and pathogenesis for myocilin; and offers insights into highly conserved, biomedically relevant OLF domains.

Results: The myoc-OLF domain contains a buried calcium ion ligated by Asp-380. The myocilin olfactomedin domain binds calcium with an unprecedented ligand arrangement. The presence of calcium within the OLF domain provides new clues into normal myocilin function, myocilin glaucoma pathogenesis, and biomedically important olfactomedin domains.

Conclusion: The myocilin olfactomedin domain binds calcium with an unprecedented ligand arrangement. Conclusion: The myocilin olfactomedin domain binds calcium with an unprecedented ligand arrangement. Conclusion: The myocilin olfactomedin domain binds calcium with an unprecedented ligand arrangement.
and molecular characterization of other OLF-containing proteins, such as amassin-1, a sea urchin protein involved in cell-cell adhesion of coelomocytes (17), gliomedin involved in nerve conduction within myelinating fibers (18), and olfactomedin-4, which has recently emerged as a factor in a variety of human disorders, including some cancers (19–21) and irritable bowel syndrome (22).

Based on the high calcium levels measured in ocular fluid (23, 24), similar to other ECM environments (25), we set out to investigate whether the OLF domain of myocilin harbors a calcium binding site. The presence of numerous highly conserved aspartates among OLF domains (Fig. 1) and interaction of myocilin with negatively charged glycosaminoglycans (14) further suggest a need for such charge stabilization. Although sequence gazing and bioinformatics approaches failed to identify any canonical calcium binding motifs, we experimentally identified and characterized an unprecedented, single, high affinity, calcium binding site within the OLF domain of myocilin. This site is likely prevalent among OLF domains. Our results suggest new roles for myocilin in the TM and possible contribution to the pathogenesis of glaucoma.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification**—myo-OLF and variants were expressed using a modified pMAL-c4x plasmid encoding an N-terminal maltose binding protein (MBP) fusion (New England Biolabs) in Rosetta Gami 2 (DE3)pLysS (Novagen) cells, as described previously (26). Cells were grown at 37 °C in Superior Broth (US Biological) to an optical density at 600 nm of 0.6–0.8, cooled to 18 °C, induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside and allowed to grow overnight (14–16 h). Cells were flash frozen with liquid nitrogen and stored at −80 °C. Cell pellets were lysed via French Press after suspension in amylase wash buffer (10 mM KH2PO4, 10 mM Na2HPO4, 200 mM NaCl, and 1 mM EDTA) containing Roche Complete EDTA-free Protease Inhibitor Mixture. Cellular debris was removed via ultracentrifugation (162,000 g for 45 min at 4 °C), and the supernatant was loaded onto a 20-ml column containing high flow amylose resin (New England Biolabs) equilibrated with amylose wash buffer. The MBP-OLF fusion protein was eluted using amylose wash buffer supplemented with 10 mM maltose. Elution fractions were concentrated using Amicon Ultra-15 centrifugal filtration devices and loaded onto a Superdex 75 prep grade column (GE Healthcare) equilibrated with gel filtration buffer (10 mM KH2PO4, 10 mM Na2HPO4, and 200 mM NaCl, pH 6.8). Fractions of MBP-OLF monomer were identified by SDS-PAGE analysis, pooled, and concentrated for further use or for protease cleavage. Cleavage of MBP-OLF was accomplished using Factor Xa (New England Biolabs or Roche Applied Science) incubated for 16–18 h in 50 mM Tris, pH 8, 100 mM NaCl, and 5 mM CaCl2 at 37 °C (wild-type) or room temperature (variants). Cleaved protein was loaded onto the amylose resin column to remove MBP and uncleaved fusion protein. Flow-through fractions containing cleaved myo-OLF and Factor Xa were concentrated and subjected to Superdex 75 prep grade column chromatography. Fractions containing cleaved, pure, myo-OLF were identified by SDS-PAGE, pooled, and concentrated for further use.

**Generation of myo-OLF Variants**—Site-directed mutagenesis was accomplished using the QuickChange II® site-directed mutagenesis kit (Stratagene). Primers were designed using PrimerX and synthesized by MWG Operon (sequences not published previously (9, 26) appear in supplemental Table S1).
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All mutated plasmid sequences were confirmed by DNA sequencing (MWG Operon). Protein expression and purification proceeded as above. The structural core of myoc-OLF (core-OLF), which lacks Asp-490, was generated by limited proteolysis as described previously (27).

**Thermal Stability Assay**—Changes in thermal stability were assessed by differential scanning fluorimetry (28), as modified by us previously for MBP-OLF (26, 27). Briefly, 30-μl reactions containing final concentrations of 1–3 μM myoc-OLF or MBP-OLF variants were diluted into buffer containing 10 mM Hepes, pH 7.5, 200 mM NaCl, and 5× Sypro Orange dye (Invitrogen). For MBP-OLF variants, 50 mM maltose was added to stabilize OLF variants were diluted into buffer containing 10 mM Hepes, pH 7.5, 150 mM NaCl, MgCl\(_2\), or Mg(OAc)\(_2\) at final concentrations of 1–3 μM, myoc-OLF(D380A), and MBP were purified as reported except EDTA was omitted from amylose wash buffer, and gel filtration buffer was chelated with Chelex (Sigma) resin. Duplicate independent reactions containing 8 μM wasthen measured under native and denaturing conditions. For MBP-OLF variants, 50 mM maltose was added to stabilize myoc-OLF(D380A) in gel filtration buffer, pH 7.2. For myoc-OLF at pH 4.6, purified protein was subjected to 3× concentration and dilution into 10 mM sodium acetate, 200 mM NaCl, pH 4.6, using an Amicon Ultra 15 centrifugal device. For thermal melts, protein samples were first diluted into 10 mM MES, pH 6.0, buffer and supplemented with 0 or 1 mM CaCl\(_2\) to a final concentration of 10–12 μM. No differences in secondary structure were observed between samples prepared in pH 6.0 or 7.2 (data not shown). Far-UV spectra were acquired at 4 or 20 °C with 30 averaged scans from 300 to 200 nm at a 500 nm min\(^{-1}\) scan rate, using a 0.1-cm cuvette. Far-UV melts were performed in duplicate utilizing a 1 °C min\(^{-1}\) increase in temperature from 5 to 95 °C. Ten scans from 300 to 200 nm at a 500-nm min\(^{-1}\) scan rate were averaged for each temperature. Data were blank-subtracted and converted to mean residue ellipticity \(\Theta = M_{res} \times \Theta_{obs}/10 \times d \times c\), where \(M_{res}=112.9\) is the mean residue mass calculated from the protein sequence; \(\Theta_{obs}\) is the observed ellipticity (degrees) at wavelength λ; \(d\) is the path length (cm); and \(c\) is the protein concentration (g/ml). The \(T_{m}\) was determined using mean residue ellipticity values recorded at 215 nm via Boltzmann Sigmoid analysis using Igor Pro.

Near-UV CD experiments were conducted with myoc-OLF at pH 7.2, at pH 4.6, and myoc-OLF(D380A) at pH 7.2 (40–50 μM protein concentration) prepared as described above. Scans were measured from 250 nm to 320 nm at a rate of 50 nm/min and a data pitch of 1 nm using a 0.1-cm cuvette. Each measurement was an average of 10 scans, converted to mean residue ellipticity.

**Estimate of Ca\(^{2+}\) Dissociation Constant \(K_d\)**—The \(K_d\) of Ca\(^{2+}\) for myoc-OLF was estimated with the binding constant macro in Origin (version 7) using data from differential scanning calorimetry (MicroCal VP-Capillary DSC) conducted at 15.4 μM protein concentration in gel filtration buffer. The unfolding transitions for both myoc-OLF and myoc-OLF(D380A) are not reversible but can be fit well to a non-two-state model (data not shown). Data from myoc-OLF(D380A) were used as an approximation for apo myoc-OLF (see “Results”). Relevant parameters: For myoc-OLF \(T_{m}=56.3^\circ C\),

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**Tables**

**Table 1**

Analysis of stabilization of myoc-OLF by divalent metal ions

| Sample                  | \(T_m\) \(^{\circ C}\) | \(\Delta T_m\) \(^{\circ C}\) |
|-------------------------|------------------------|-----------------------------|
| Myoc-OLF                | 53.0 ± 0.5             |                            |
| Myoc-OLF + 10 mM CaCl\(_2\) | 59.6 ± 0.2             | 6.6                         |
| Myoc-OLF + 10 mM Ca(OAc)\(_2\) | 60.0 ± 0.1             | 7.0                         |
| Myoc-OLF + 10 mM MgCl\(_2\) | 52.8 ± 0.2             | −0.2                        |
| Myoc-OLF + 10 mM Mg(OAc)\(_2\) | 53.6 ± 0.5             | 0.6                         |
| Myoc-OLF, pH 4.6        | 48.9 ± 0.1             |                             |
| Myoc-OLF, pH 4.6 + 10 mM CaCl\(_2\) | 53.6 ± 0.1             |                             |

**Table 2**

Elemental analysis for Ca\(^{2+}\) by ICP-OES

| Sample                  | Calcium:protein (per mol) |
|-------------------------|---------------------------|
| MBP-OLF as-isolated     | 0.81                      |
| EDTA-free MBP-OLF       | 0.96                      |
| MBP-OLF(D380A)          | 0.10                      |
| MBP                      | 0.01                      |

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RESULTS

Initial Identification of Ca$^{2+}$ in myoc-OLF—We previously examined the effects of metal ions on the stability of myoc-OLF, but aspects of experimental design excluded calcium ions (27). Reassessment of myoc-OLF stability with calcium ions in a compatible buffer revealed a clear, anion-independent increase in thermal stability, as measured by differential scanning fluorimetry (Table 1; see “Experimental Procedures”), a technique that reports ligand binding as an increase in thermal stability (28). This phenomenon is observed even in the case of a fully bound protein when the ligand only binds the folded state of the protein (31).

Given the fact that the typical purification procedure involves numerous hours of contact with buffers containing 1 mM EDTA ($K_M = 3.2 \times 10^{-8}$ M for Ca$^{2+}$ (32)), we expected the as-isolated MBP-OLF fusion protein to lack Ca$^{2+}$. However, elemental analysis by ICP-OES (Table 2) revealed significant levels of Ca$^{2+}$. Omission of EDTA from the purification procedure yielded nearly stoichiometric values consistent with a singly bound Ca$^{2+}$ ion to the monomeric MBP-OLF; Ca$^{2+}$ does not copurify with MBP (Table 2). When incubated with Quin-2, a fluorescent EGTA analog with $K_J = 2.9 \times 10^{-9}$ M (29), high fluorescence values indicative of Ca$^{2+}$ release from myoc-OLF were only detected under denaturing conditions (Fig. 2). To date, we have not been able to prepare a native form of apo myoc-OLF or fully reload myoc-OLF. Isothermal titration calorimetry using myoc-OLF reveals only nonspecific binding; no additional binding sites are apparent (supplemental Fig. S1).

Mutational Analysis of Carboxylic Acid-containing Residues as Ligands for Ca$^{2+}$ Reveals Asp-273 and Glaucoma-associated Asp-380—To deduce the metal binding residues in myoc-OLF, the tertiary structure observed in the aromatic region of myoc-OLF(D380A) is somewhat different from wild-type at pH 7.2. However, the spectrum overlays with wild-type myoc-OLF at pH 4.6 (Fig. 3B). Because myoc-OLF at pH 4.6 is a well folded (27), Ca$^{2+}$-stabilized protein (Table 1), the structural changes in myoc-OLF(D380A) that lead to ablation of Ca$^{2+}$ binding are due to the loss of coordination of Asp-380 to Ca$^{2+}$ and not a change in the structure of myoc-OLF or the Ca$^{2+}$ binding pocket.

Investigation of Ca$^{2+}$ Stabilization of 18 Other Disease-causing myoc-OLF Variants Reveals No Other Impaired Variants—Due to the documented participation of other polar residues or main chain-derived carbonyls in Ca$^{2+}$ binding (33), combined with the glucoma relevance of myoc-OLF(D380A), we looked at the extent of stabilization by Ca$^{2+}$ for 18 disease-causing OLF mutants (Table 3). With the exception of D380A, all of the disease-causing variants were stabilized by $+5.7\text{ to }-9.1$ °C in the presence of 10 mM Ca$^{2+}$; wild-type myoc-OLF is stabilized by 6.5 °C. Although the extent of stabilization varies somewhat, lack of calcium binding is not a general feature of disease-causing variants, and we were not able to statistically correlate initial $T_m$ or position in the amino acid sequence with extent of stabilization. Thus, the remaining cryptic Ca$^{2+}$ coordination sphere likely involves some combination of other side chains not yet identified, main chain carbonyls, or water molecules.
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**a**

**b**

**c**

FIGURE 3. Structural and stability comparison of myoc-OLF and myoc-OLF(D380A). Shown is a comparison of secondary structure from far-UV spectra (a) and tertiary structure from near-UV spectra (b) among wild-type myoc-OLF at pH 7.2, wild-type myoc-OLF at pH 4.6, and myoc-OLF(D380A) at pH 7.2. c, CD thermal melts for myoc-OLF and myoc-OLF(D380A) in MES pH 6.0, monitored at 215 nm in the presence and absence of exogenous Ca\(^{2+}\).

| Variant | Rationale | \(T_m\) | \(T_m + 10\) \(\text{mM CaCl}_2\) | \(\Delta T_m\) |
|---------|-----------|--------|-----------------|------------|
| myoc-OLF | Wild-type | 53.0 ± 0.5 | 59.6 ± 0.2 | 6.6 |
| myoc-OLF core | Identified structural core\(^a\) | 49.7 ± 0.3 | 57.7 ± 0.1 | 8.1 |
| MBP-OLF(G246R) | Disease-causing variant | 42.5 ± 0.2 | 50.6 ± 0.0 | 8.1 |
| MBP-OLF(G252R) | Disease-causing variant | 43.0 ± 0.2 | 51.5 ± 0.2 | 8.5 |
| MBP-OLF(R272G) | Disease-causing variant | 41.0 ± 0.3 | 48.6 ± 0.1 | 7.6 |
| MBP-OLF(D273A) | Carboxylate side chain? | 21.7 ± 0.8 | 21.1 ± 0.7 | -0.6 |
| MBP-OLF(E323K) | Disease-causing variant | 44.0 ± 0.5 | 50.3 ± 0.2 | 6.2 |
| MBP-OLF(G364V) | Disease-causing variant | 45.0 ± 0.4 | 51.9 ± 0.1 | 6.9 |
| MBP-OLF(G367R) | Disease-causing variant | 42.7 ± 0.1 | 50.9 ± 0.4 | 8.1 |
| MBP-OLF(T377M) | Disease-causing variant | 44.3 ± 0.3 | 50.6 ± 0.6 | 6.3 |
| MBP-OLF(D378A) | Carboxylate side chain? | N/A | N/A | N/A\(^a\) |
| MBP-OLF(D380A) | Carboxylate side chain ligand? and disease causing variant | 46.6 ± 0.3 | 45.1 ± 0.5 | -1.5 |
| MBP-OLF(D384A) | Carboxylate side chain ligand? | 42.5 ± 0.7 | 53.6 ± 0.5 | 11.1 |
| MBP-OLF(E385A) | Carboxylate side chain ligand? | 39.7 ± 0.4 | 49.7 ± 0.3 | 10 |
| MBP-OLF(A427E) | Disease-causing variant | 34.2 ± 0.4 | 43.2 ± 0.1 | 9.0 |
| MBP-OLF(A427T) | Disease-causing variant | 41.5 ± 0.1 | 49.9 ± 0.1 | 8.4 |
| MBP-OLF(A433R) | Disease-causing variant | 48.3 ± 0.3 | 55.2 ± 0.4 | 6.9 |
| MBP-OLF(Y437H) | Disease-causing variant | 40.4 ± 0.4 | 49.4 ± 0.5 | 9.0 |
| MBP-OLF(L477N) | Disease-causing variant | 37.7 ± 0.8 | 46.8 ± 0.2 | 9.1 |
| MBP-OLF(S480K) | Disease-causing variant | 39.7 ± 0.2 | 48.2 ± 0.5 | 8.5 |
| MBP-OLF(P481L) | Disease-causing variant | 42.4 ± 0.2 | 48.1 ± 0.1 | 5.9 |
| MBP-OLF(A499F) | Disease-causing variant | 45.5 ± 0.4 | 51.2 ± 0.3 | 5.7 |
| MBP-OLF(E902K) | Disease-causing variant | 42.8 ± 0.1 | 50.4 ± 0.4 | 7.6 |

\(^{a}\) From Ref. 27.

\(^{b}\) Variant could not be purified in sufficient quantities in folded state for measurement.

Estimation of Ca**\(^{2+}\)** Dissociation Constant—Because a strictly apo wild-type myoc-OLF protein could not be prepared for direct calorimetric measurement of calcium binding, we estimated the dissociation constant from experimental \(T_m\) values obtained by differential scanning calorimetry, which are corroborated by CD (Fig. 3C) and differential scanning fluorimetry (Table 3), as well as experimental values for the enthalpy of Ca**\(^{2+}\)** binding at specific pH conditions (38), obtained by differential scanning calorimetry, which are corroborated by CD (Fig. 3).

Model for Ca**\(^{2+}\)** Binding Motif in myoc-OLF—On the basis of bioinformatics approaches, neither the popular D(D/N)DG sequence found among integrins, EF-hands, and \(\beta\)-blades (36, 37) nor the EGF-like motif DXD(Q/E)X\(_{14}\)(D/N) (38), is present in myoc-OLF. Thus, to gain additional structural insight, we probed the region of the myoc-OLF sequence containing Asp-380 (378–393) using HH-PRED (39) and Robetta (40), revealing as expected from CD, a high \(\beta\)-strand propensity. Although no structurally similar Ca**\(^{2+}\)** binding proteins were identified by HH-PRED, the Mg**\(^{2+}\)**-dependent \(\delta\)-alanine \(\delta\)-alanine ligase (Protein Data Bank code 1IOW) and a Zn**\(^{2+}\)**-dependent Haemophilus influenzae enzyme (Protein Data Bank code 1NO5) use a \(\delta\)-strand-embedded aspartate at a position equivalent to Asp-380 for metal ion binding. Thus, even though calcium binding sites are generally found within a loop region, the site in myoc-OLF may instead resemble one of these other metalloproteins. Notably, the sequence Gly-387–Tyr-392, which forms a predicted \(\beta\)-strand separate from that containing Asp-380, bears resemblance to the C-terminal region of \(\gamma\)-S-crystallin (Protein Data Bank code 1HA4), the cataract-associated lens protein. Although an equivalent aspartate...
to Asp-380 is not present in γS-crystallin, the βγ-crystallin superfamily does bind calcium (41) using consensus sequence (N/D)(N/D)X₆(S/T/S)₃ (42), which is also absent in myoc-OLF.

**Prediction of Ca²⁺ Binding Motifs in the OLF Domain Family**—Finally, we broadened our scope beyond myocilin to include other OLF domain containing proteins to gain insight into whether calcium binding may be an inherent characteristic of such domains. Among myocilin orthologs, Asp-380 is located in a well conserved region of the OLF domain peppered with acidic residues that were subjected to mutagenesis in our study (see above). Expansion of sequence analysis to include 45 OLF homologs available in ProSite (43), combined with an evolutionary trace (44), reveals that all but one distant branch harbors an aspartate or glutamate at the equivalent position of 380 in myocilin (supplemental Fig. S2). Instead of aspartate, these distant relatives, the gliomedins, harbor asparagine, which is unlikely to be a ligand for calcium. Asp-273 is also highly conserved among the expanded list of OLF domains, with the only outliers in the same branch lacking a Asp-380. Asp-378 is far less conserved, being replaced with tyrosine, leucine, and phenylalanine. Thus, although many variants appear to have a well positioned aspartate for calcium binding, additional characterization of other OLF domains will be required to assess further generality.

**DISCUSSION**

We have identified a novel, high affinity Ca²⁺ site within the OLF domain of myocilin. The myoc-OLF Ca²⁺ binding site contains an unprecedented motif that includes Asp-380, also the site of a glaucoma-causing lesion. Of the 23 total myoc-OLF variants we investigated, including mutants of conserved aspartate/glutamate residues, as well as disease-causing mutants, only two additional candidate ligands emerged, namely, Asp-273 and Asp-378. However, neither position could be confirmed unambiguously due to the severely impaired biophysical properties of the resulting recombinant protein. The combination of low thermal stability of myoc-OLF(D273A) and its high level of conservation among orthologs at this position underscores the importance of this residue to the integrity of the OLF domain. This stability reduction is highly residue-specific; myoc-OLF(R272G), the adjacent disease variant, is a moderately stable protein that is stabilized by calcium. By comparison, although Asp-378 could hypothetically form part of the prevalent DXD Ca²⁺ binding loop found in EF-hands and β-blades, the remaining motif is absent. Asp-378 is not well conserved among OLF domains, and Asp-380 is predicted to be located within a β-strand, not a loop. The varied nature of calcium binding sites in proteins, which include not only oxygen-containing amino acid side chains but also main chain carbonyls, hydroxyl moieties, and water molecules for a total coordination number of 6–8 (45), may render the remaining Ca²⁺ coordination environment in myoc-OLF inaccessible by site-directed mutagenesis. At present, however, there is no OLF structure or high-confidence homology model for further insight.

Two of the major proposed functions of Ca²⁺ binding sites in ECM proteins are the enhancement of thermal stability and protection against proteolysis (25). In support of these roles, we previously observed resistance of myoc-OLF to protease treatment (27) and core-OLF is still stabilized by calcium. Unlike other known calcium-containing ECM proteins like osteonectin, in which the binding of calcium induces a large conformational change (46), wild-type myoc-OLF at pH 7.2, myoc-OLF at pH 4.6, and myoc-OLF(D380A) are stable proteins with highly similar structural features. Thus, although myoc-OLF at pH 7.2 is more stable than the D380A mutant to thermal denaturation, calcium is not absolutely required for OLF folding.

The estimated binding affinity of the myoc-OLF calcium site based on available thermodynamic parameters is also in line with Ca²⁺ equilibrium dissociation constants of other ECM proteins, which are usually in the micromolar range (2). However, myocilin is atypical in that the site is largely inaccessible to the strong chelators EDTA and Quin-2. This indicates that metallation likely occurs upon folding in the calcium-rich endoplasmic reticulum (47). Based on experimental measurements of millimolar levels of calcium ions in aqueous humor (23, 24), the myocilin OLF domain should be continually saturated with Ca²⁺ once trafficked to the TM. In addition to conferring stability, Ca²⁺ sites in ECM proteins play regulatory or signaling roles, for example, in response to local calcium ion gradients (2). The emerging picture appears true in proteins with a stabilizing, high affinity Ca²⁺ site, regardless of whether Ca²⁺ is bound within a single protein domain or at the interface between domains. Calcium ions may directly facilitate ligand interaction or membrane association, or stabilize a particular protein conformation so that it is primed for ligand binding or activated for catalysis (1). Full-length myocilin is a modular protein like other ECM proteins, but myocilin is unusual in that it has a coiled-coil for oligomerization instead of a repeated domain structure within a single polypeptide chain. The OLF domain behaves as a monomer in vitro (26, 27), and it has been suggested that the myocilin domain structure brings OLF domains in close proximity, albeit in an unknown configuration and for an unclear purpose. Even though all but one (48) of the interacting partners for myocilin identified to date appear to not require the OLF domain, our results hint at the possibility that these and other interactions may be calcium-dependent or require a transient calcium gradient. In support of this hypothesis, in the case of amassin, cell-cell interactions were found to be contingent upon the presence of calcium ions (49).

Although currently there is no experimental evidence for calcium involvement in biomechanical stress response in the TM, it is well known that calcium is associated with muscle contraction, cell shape, and adhesion, by altering myosin interaction with actin (50). Relevant to the myocilin system, mechanical stress of TM cells leads to rearrangements of actin filaments (51) and elevated levels of myocilin mRNA (52). Myocilin has also been proposed to interact directly with actin via its coiled-coil (15). This syllogism suggests that myocilin could be sensitive to shear and/or other biomechanical stress via a calcium-dependent mechanism. Alternatively, myocilin may play a part in the regulation of TM calcification. Genes associated with calcification are abundantly expressed in the TM. Although the details of calcification and/or prevention in the TM and their physiological or pathological role(s) are still unknown (53),
myocilin mutants can alter the expression of calcification genes (54), suggesting interplay is possible.

In sum, the new knowledge of a calcium site in myoc-OLF opens a completely new context in which to probe the biological and pathogenic roles of myocilin. Additional characterization of the OLF domain in the context of full-length myocilin, calcium fluxes, and mechanical stress in the TM should both yield new functional insights for myocilin and contribute to our still poor comprehension of the role of Ca\textsuperscript{2+} in the anterior segment of the eye.

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