Structural Consequences of Cysteine Substitutions C1977Y and C1977R in Calcium-binding Epidermal Growth Factor-like Domain 30 of Human Fibrillin-1*

Ji Young Suk‡, Sacha Jensen‡, Aileen McGetrick‡, Antony C. Willis, Pat Whiteman‡, Christina Redfield**, and Penny A. Handford***

From the *Division of Molecular & Cellular Biochemistry and the §Medical Research Council Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU and the **Department of Biochemistry and Oxford Centre for Molecular Sciences, Chemistry Research Laboratory, University of Oxford, Mansfield Road, Oxford OX1 3TA, United Kingdom

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

The largest group of disease-causing mutations affecting calcium-binding epidermal growth factor-like (cbEGF) domain function in a wide variety of extracellular and transmembrane proteins is that which results in cysteine substitutions. Although known to introduce proteolytic susceptibility, the detailed structural consequences of cysteine substitutions in cbEGF domains are unknown. Here, we studied pathogenic mutations C1977Y and C1977R, which affect cbEGF30 of human fibrillin-1, in a recombinant three cbEGF domain fragment (cbEGF29–31). Limited proteolysis, 1H NMR, and calcium chelation studies have been used to probe the effect of each substitution on cbEGF30 and its flanking domains. Analysis of the wild-type fragment identified two high affinity and one low affinity calcium-binding sites. Each substitution caused the loss of high affinity calcium binding to cbEGF30, consistent with intradomain misfolding, but the calcium binding properties of cbEGF29 and cbEGF31 were surprisingly unaffected. Further analysis of mutant fragments showed that domain packing of cbEGF29–30, but not cbEGF30–31, was disrupted. These data demonstrate that C1977Y and C1977R have localized structural effects, confined to the N-terminal end of the mutant domain, which disrupt domain packing. Cysteine substitutions affecting other cbEGF disulfide bonds are likely to have different effects. This proposed structural heterogeneity may underlie the observed differences in stability and cellular trafficking of proteins containing such changes.

The epidermal growth factor-like (EGF) domain is a widely distributed module found in transmembrane and extracellular proteins where it is often arranged as multiple tandem repeats. The EGF domain consists of ~40–50 amino acids that fold to produce major and minor β-hairpin structures. Six highly conserved cysteine residues pair up in a 1-3, 2-4, and 5-6 arrangement to stabilize the domain structure (1). A subset of EGF domains contain a consensus sequence for calcium binding, (D/N)(X)(D/N)(E/Q)Xm(D/N)n Xm(Y/F) (where m and n are variable and the asterisks indicate possible post-translational β-hydroxylation) (2–4). In tandem repeats of fibrillin-1 calcium-binding EGF (cbEGF) domains, the bound Ca2+ , together with a hydrophobic packing interaction, performs a key structural role restricting interdomain flexibility. This is thought to facilitate protein-protein interactions and also protect the modules against proteolytic cleavage in vitro (5–9). The biological importance of the cbEGF domain is highlighted by the number of inherited diseases caused by missense mutations in this domain type. These include Marfan syndrome (MFS), congenital contractual arachnodactyly, hemophilia B, familial hypercholesterolemia, and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy caused by mutations in genes encoding fibrillin-1, fibrillin-2, factor IX, low density lipoprotein receptor, and Notch, respectively (10–13).

A large subgroup of disease-causing mutations in cbEGF domains are those that result in cysteine substitutions. These are predicted to disrupt the fold of the domain by affecting native disulfide bond formation, but little is known of their long range structural effects. The functional consequences of these missense mutations include variable effects on protein trafficking and in vitro destabilization (14–20), suggesting heterogeneous effects on structure.

Approximately 25% of all missense mutations associated with the connective tissue disorder MFS result in cysteine substitutions in cbEGF domains (Marfan data base, www.umd. necker.fr/). Here, we report the investigation of the structural effects of two MFS-causing substitutions, C1977Y and C1977R, which disrupt the 1-3 disulfide bond in cbEGF30 of fibrillin-1 (see Fig. 1). A well established in vitro protein production method (21–24) has been utilized to generate wild-type and mutant recombinant triple-domain constructs comprising cbEGF domains 29, 30, and 31 (cbEGF29–31) from fibrillin-1. We have applied a number of biochemical and biophysical methods to study these constructs (8, 21, 22, 24). Limited proteolysis of recombinant cbEGF29–31 constructs was used initially as a low resolution method to determine the range of structural effects conferred by the cysteine substitutions. Subsequent investigations using high resolution 1H NMR analysis and calcium chelation assays precisely delineated the extent of the effects of the two substitutions. Although both cbEGF30
domain structure and cbEGF29–30 domain-domain packing were significantly disrupted by the loss of the 1-3 disulfide bond, no effects on the calcium binding properties of N-terminal cbEGF domain 29 and the C-terminal cbEGF domain 31 were observed, suggesting that both of these cysteine substitutions have localized effects.

**EXPERIMENTAL PROCEDURES**

**Cloning of Wild-type and Mutant cbEGF Domain Constructs from Human Fibrillin-1—**DNA fragments (nucleotides 5918–6295 of human fibrillin-1 cDNA) encoding the wild-type cbEGF29–31 domains (residues 1929–2054) and fragments containing the C1977R and C1977Y mutant sequences were amplified by standard polymerase chain reaction techniques using *Pfu* polymerase (Stratagene). The DNA was amplified by the use of a forward primer that encodes a factor Xa cleavage site: 5′-TAATGGGATCCATAGAAGGACGATCAGCAATAGATGGTAGAAATGTC3′- and a reverse primer: 5′-TAATGAGGACGACCTCTTCTCAATCGGGAGGAC-3′.

The amplified DNA was cloned into pQE30 (Qiagen), which contains a sequence encoding a six-histidine affinity tag, and used to transform *Escherichia coli* NM554[pREP4]. The clones were sequenced to confirm the correct sequence had been amplified and that each mutation had been introduced into the inserted fragments with no other changes. Protein expression, refolding, and purification were carried out as described previously (21–24), and the identity of the purified proteins was confirmed by electrospray mass spectrometry.

**Limited Proteolysis of Wild-type and Mutant cbEGF Domain Constructs—**Proteolysis with trypsin (1:50, w/w) or endoproteinase Glu-C (1:125, w/w) was performed as described previously (24). N-terminal sequencing was used to characterize the proteolytic digestion products. Proteolysis performed in 50 mM EGTA or 50 mM CaCl2 was terminated after 60–120 min by acidification to pH 2. 50 mM CaCl2 was used to ensure saturation of the N-terminal cbEGF29 calcium-binding site, which was thought likely to have a weak affinity in the absence of cbEGF28. Samples were purified under nonreducing conditions by reverse phase HPLC. After lyophilization, aliquots of HPLC fractions (1:125, w/w) was performed as described previously (24). N-terminal proteolysis performed in 50 mM EGTA or 50 mM CaCl2 was terminated at 35 °C. The samples buffer to maintain approximate physiological ionic strength (I = 0.15) throughout the experiments. All of the experiments were conducted at 35 °C. The $K_c$ value of the low affinity site in cbEGF29 was calculated as described previously (21).
RESULTS

Purification of cbEGF29–31 Wild-type, C1977R, and C1977Y Triple-domain Constructs—The cbEGF29–31 wild-type and mutant triple-domain constructs of human fibrillin-1 (Fig. 1) were expressed and purified using a previously described method (21–24). After reduction and refolding in vitro, each construct was purified by reverse phase HPLC, and the major species was collected. The presence of Ca²⁺/H₁₁₀₀₁ in the refolding buffer was found to be essential for the production of a single peak in the HPLC chromatogram for all three constructs; refolding in the presence of EGTA resulted in multiple peaks, suggesting multiple conformations. Analysis of purified material on nonreducing and reducing SDS-PAGE gels (data not shown) confirmed the presence of a single, major protein species. The purified proteins were analyzed by electrospray mass spectrometry, and the experimental molecular masses were found to agree well with the predicted values (Table I). The analysis of the C1977Y and C1977R constructs by mass spectrometry showed an increased mass equivalent to a covalently bound cysteine residue. This is consistent with oxidation of the unpaired thiol in cbEGF29–31 by free cysteine present in the refolding reaction mixture.

Limited Proteolysis of cbEGF29–31 Mutant Proteins Indicates Disruption of cbEGF30 but Not cbEGF31—In the presence of Ca²⁺, fibrillin cbEGF domains are resistant to proteolysis (6, 8, 9, 19, 20, 24, 29). Digestion of cbEGF constructs in the presence and absence of Ca²⁺ and subsequent identification of cleaved N termini can therefore be used to identify the structural effects of amino acid substitutions on each domain. Comparative digestion by trypsin in the presence of EGTA (50 mM) or CaCl₂ (50 mM) followed by SDS-PAGE analysis revealed significant protection by Ca²⁺ against proteolysis in the wild-type construct but increased susceptibility to proteolysis in the two mutant constructs (Fig. 2A). Similar SDS-PAGE analysis of endoproteinase Glu-C, elastase, and chymotrypsin digests also demonstrated loss of protection by Ca²⁺ in the mutant constructs, whereas the wild-type constructs were protected by Ca²⁺ against proteolysis (data not shown).

N-terminal sequence analysis was performed on the HPLC-purified trypsin digestion products obtained in the presence of EGTA or Ca²⁺ as described previously (24). The proteins were purified under nonreducing conditions, and quantitative N-terminal sequencing was used to determine the amount of each cleavage product (Table II). Comparative analysis of these data are shown in Fig. 2B. Upon trypsin digestion, four cleavage sites were mapped in the protein constructs. In the wild-type digests, the cleavage sites 1983KCAPG and 1998CICPP in cbEGF30 and 2038CLCPE in cbEGF31 were only detected in the absence of CaCl₂. This indicated that the cbEGF30 and cbEGF31 domains of the wild-type construct were protected by Ca²⁺ against proteolysis, consistent with a native calcium-binding fold for each of these domains. The positions of these sites are indicated on a schematic structure and a three-dimensional model of the triple construct in Fig. 3. The fourth cleavage site, 2052RCQ, was not protected against proteolysis by Ca²⁺ in any of the proteins. This sequence is located close to the C terminus of cbEGF31 and most likely reflects the flexibility commonly observed at the termini of recombinant domain constructs (30).

In the trypsin digests of the C1977Y and C1977R mutants, the same four cleavage sites were observed in the absence of Ca²⁺ as were seen in the wild-type protein. One of these sites,
2038CLCPE located in cbEGF31, was significantly protected against proteolysis in Ca\(^{2+}\) and suggested that the C-terminal cbEGF31 domain of the mutant proteins contained a native-like calcium-binding fold. However, in contrast to the wild-type protein, the cleavage sites 2038KCAGP and 1999CICPP in cbEGF30 were detected in significant amounts in Ca\(^{2+}\) (Table II and Fig. 2B). This demonstrated new proteolytic susceptibility in cbEGF30 and suggested a loss of Ca\(^{2+}\) binding to this domain. No cleavage sites in the cbEGF29 domain were identified with trypsin or with the subsequent use of endoprotease Glu C, chymotrypsin, or elastase (data not shown), and it was therefore not possible to determine whether or not the

![Table II](image)

Table II

| Fragment | Cleavage site in CaCl\(_2\) | Amount (pmol (%)) | Cleavage site in EGTA | Amount (pmol (%)) |
|----------|-----------------------------|------------------|-----------------------|------------------|
| Wild type | SAIDV | 489.1 (100) | SAIDV | 416.7 (100) |
| C1977Y | 2053RCQ | 165.0 (33.7) | 2053RCQ | 55.4 (13.2) |
| C1977R | 2052RCQ | 113.1 (29.7) | 2052RCQ | 33.2 (11.4) |

Changes in the aromatic region of the NMR spectrum (6–8 ppm), specifically shifts of resonances assigned to the calcium-binding consensus aromatic residue at the N terminus of the cbEGF domain, have been used previously to characterize Ca\(^{2+}\) binding (21, 23, 31, 33, 34). In this study, equivalent residues within cbEGF 29–31 are Phe\(^{1954}\), Tyr\(^{1996}\), and Phe\(^{2036}\) (Fig. 3, asterisks). Initially, in the absence of specific assignments for these residues, the aromatic region of one-dimensional spectra of the wild-type and mutant proteins in selected Ca\(^{2+}\) concentrations was examined (Fig. 4). In the wild-type protein, spectral changes were observed in both low (200 \(\mu\)M) and high (50 \(\mu\)M) concentrations of Ca\(^{2+}\), indicative of both high and low affinity calcium-binding sites. The spectra of the mutant proteins also showed characteristics of both high and low affinity calcium-binding sites. However, some of the spectral changes observed for the wild-type protein were not observed for the
mutants, indicating a change in calcium binding properties.

Domain-specific Assignments of Aromatic Resonances in cbEGF29–31 Identification of a Low Affinity Calcium-binding Site in cbEGF29 of Wild-type and Mutant Proteins—More detailed, domain-specific information about the calcium binding affinities of cbEGF29–31 was obtained using two-dimensional correlated spectroscopy, total correlation spectroscopy, and NOESY experiments and the aromatic residues within cbEGF29–31. In addition to the calcium-binding consensus aromatic residue, which can be used to measure intradomain calcium binding, another consensus aromatic residue closer to the C terminus of each domain (Tyr1962 in cbEGF29 and Tyr2004 in cbEGF30; Fig. 3) is predicted by homology to be involved in an interdomain packing interaction with the following domain in the calcium-bound protein (5, 7). The chemical shift of this residue is therefore sensitive to calcium binding to the following domain. The spin systems of all three tyrosines and two of the three phenylalanine residues in cbEGF29–31 were identified using correlated and total correlation spectra collected in 10 mM EDTA and in 50 mM CaCl₂; integration of one-dimensional spectra indicated that the peaks of the third phenylalanine residue overlap at ~6.7 ppm and do not give resolved cross-peaks in two-dimensional spectra. Residue-specific assignments for the tyrosine and phenylalanine spin systems (see supplemental data) were made by analysis of their behavior in calcium titrations, comparison of the wild-type and mutant spectra and comparison with spectra obtained for a related triple-domain construct cbEGF28–30.²

One of the peaks assigned to Phe₁⁹⁵⁴ in cbEGF29 is observed to shift in the one-dimensional NMR spectra shown in Fig. 4 at calcium concentrations up to 50 mM; this is indicative of a low affinity calcium-binding site. The Phe₁⁹⁵⁴ peak shows similar behavior in the C1977Y and C1977R mutant spectra (Fig. 4), indicating that low affinity calcium binding is maintained in the cbEGF29 domain. The chemical shift changes of the Phe₁⁹⁵⁴ peak from its starting position in 10 mM EDTA were plotted against the free calcium concentrations in the protein

![Graph](https://via.placeholder.com/150)

**Fig. 5.** The change in chemical shift (H) of the H⁴ resonance of Phe₁⁹⁵⁴ in cbEGF29–31 wild-type (□, dots and dashes), C1977R (C, unbroken line), and C1977Y (A, dotted line) plotted against free calcium concentration. The method used to calculate free calcium concentration and derive the values for dissociation constants has been previously described (18). Calculated Kᵣ values were ~1.5 mM for the wild-type construct, ~1.8 mM for the C1977R construct, and ~2 mM for the C1977Y construct.

² J. Y. Suk, S. Jensen, A. McGettrick, P. Whiteman, C. Redfield, and P. A. Handford, unpublished data.
samples to obtain $K_d$ values (21). The estimated $K_d$ values were within the 1.5–2 mM range in all three constructs (Fig. 5), as expected for an N-terminal cbEGF domain. It is likely in the native protein, however, that covalent linkage of cbEGF28 will enhance the affinity of cbEGF29 for calcium.

Two-dimensional NMR Identifies High Affinity Calcium Binding in cbEGF31 of Wild-type and Mutant Proteins—

NOESY spectra collected at varying concentrations of CaCl$_2$. Wild-type spectra collected with 0 (A), 200 (B), or 400 μM CaCl$_2$ (C) and C1977R spectra collected with 0 (D), 100 (E), and 200 μM CaCl$_2$ (F) are presented as an example. Minor differences in the cross-peaks in 0 CaCl$_2$ spectra compared with the 10 mM EDTA spectra were observed, which indicated a small amount of residual CaCl$_2$ present in the samples. The cross-peaks for Phe$^{2036}$ in the Ca$^{2+}$-free and Ca$^{2+}$-bound forms are colored in red and violet, respectively. The cross-peaks for Tyr$^{1996}$ in the Ca$^{2+}$-free and Ca$^{2+}$-bound forms are colored in gray and orange, respectively. The cross-peaks for Tyr$^{1996}$ in the Ca$^{2+}$-free form are colored dark blue; the Ca$^{2+}$-bound peak is located close to the diagonal and is indicated by an arrow in B and C. The exchange peaks, which link the free and bound states of the respective aromatic residue, are observed. The exchange peaks for Tyr$^{1996}$ and Phe$^{2036}$ are colored cyan and green, respectively. The cross-peaks for Phe$^{2036}$, which shows low affinity behavior, are labeled. The mutant spectra are similar to the wild-type spectra with the exception of the region (indicated with a bracket) where multiple peaks are observed for Tyr$^{1996}$, indicating the possibility of multiple species for the cbEGF30 domain.

This type of slow exchange behavior, characteristic of high affinity calcium binding in the micromolar range, can be seen clearly for Tyr$^{1996}$ of cbEGF30 and for Phe$^{2036}$ of cbEGF31 in wild-type spectra. The exchange peak for Phe$^{2036}$ (shown in green in Fig. 6A) is visible at a lower Ca$^{2+}$ concentration than the exchange peak for Tyr$^{1996}$ (shown in cyan in Fig. 6B). These residues are markers for Ca$^{2+}$ binding in their respective domains. Thus, the cbEGF31 domain appears to have a higher Ca$^{2+}$ affinity than the cbEGF30 domain within the cbEGF29–31 wild-type construct. In the C1977R (Fig. 6) and C1977Y (data not shown) mutant spectra, the exchange peak and high affinity behavior is observed for Phe$^{2036}$, indicating that high affinity binding is maintained in cbEGF31 in these mutant proteins.

The behavior of the peaks corresponding to Tyr$^{1996}$ in the mutants, however, differs from that observed in the wild-type protein. No exchange peak or calcium-dependent chemical shift changes are observed for Tyr$^{1996}$ in the spectra of the mutants. This indicates that calcium binding within the cbEGF30 domain is lost as a result of the cysteine substitutions. Multiple cross-peaks, corresponding to Tyr$^{1996}$, are observed in the spectra of the mutants, which may suggest a mixture of conforma-
Structural Effects of Cysteine Substitutions in cbEGF Domains

Cysteine substitutions in cbEGF domains are a significant cause of disease in a number of different extracellular and transmembrane proteins. Although predicted to cause domain misfolding, there have been few studies of their structural consequences. Here we have used low and high resolution techniques to examine the effects of two pathogenic amino acid changes, C1977R and C1977T, in human fibrillin-1. Each substitution was expressed in a triple-domain construct, cbEGF29–31, where the mutant cbEGF30 domain is placed in a native-like context. Initial comparative analysis by limited proteolysis identified structural changes within cbEGF30, consistent with a degree of misfolding in the mutant domain. Further analysis by NMR revealed that despite the disruption of the 1-3 disulfide bond in cbEGF30 domain 30, relatively localized structural effects of each Cys substitution were observed.

By observing spectral changes of assigned aromatic resonances within cbEGF29–31 on the addition of Ca$^{2+}$, it was possible to identify a normal interdomain packing interaction of cbEGF30–31. However, the absence of spectral changes for Tyr$^{1962}$, located in the minor $\beta$-sheet of cbEGF29, indicated that the interdomain packing interaction between cbEGF29 and cbEGF30 was missing in the mutant proteins. Despite this disruption, calcium binding to cbEGF29 in the mutant proteins was indistinguishable from wild-type protein, suggesting that each cysteine substitution did not affect the N-terminal region of this domain. In addition, a high affinity calcium-binding site was observed in cbEGF31 of both mutant and wild-type proteins, which gave rise to slow exchange peaks in NMR spectra. Measurement of the $K_d$ value of this site in each protein using chromophoric chelation gave similar values of 0.3–0.8 $\mu$M. The retention of a high affinity calcium-binding site in cbEGF31 of the mutant proteins, together with the observed hydrophobic packing interaction between cbEGF30–31 characteristic of tandem cbEGF pairs, confirmed that the cysteine substitutions did not result in disruption of the C-terminal region of the cbEGF29–31 protein.

It is interesting to speculate how the native-like properties of the C-terminal region are retained in the mutant proteins. Our data suggest that, although the mutant domain was misfolded as a consequence of the 1-3 disulfide disruption, the 5-6 disulfide bond of cbEGF30 may have been formed correctly. This would then allow the packing interaction of cbEGF30 and cbEGF31, which is required for high affinity calcium binding to cbEGF31, to form (5, 7). Thus, it can be speculated that if cysteine substitutions disrupting the 5-6 disulfide bond of cbEGF30 were to be introduced, different structural effects, including destabilization of the interdomain interface and a decrease in calcium affinity, might be observed for the C-terminal domain. In support of this, a recent study by Vollbrandt et al. (20) demonstrated that a C750G substitution that affects the C-5 residue of cbEGF7 (and therefore disrupts the 5-6 disulfide bond) caused increased proteolytic susceptibility of cbEGF8. Although calcium binding to this fragment was not measured, it is likely that the increased proteolysis observed occurs as a result of the disruption of domain packing between cbEGF7 and 8 and the decrease in calcium binding affinity in cbEGF8. As observed previously for mutations that specifically affect calcium-binding residues within fibrillin-1 cbEGF domains, cysteine substitutions also have the potential to cause considerable structural heterogeneity. This may result in a variety of pathogenic mechanisms, despite the apparent similarity of the mutation.
Protease digestion studies and NMR analyses have previously been used to probe the structural and calcium binding properties of wild-type and mutant fibrillin-1 cbEGF-containing fragments. It has been shown that the effect of the G1127S substitution in cbEGF13 on protease susceptibility is confined to the mutant domain, and this domain retains a native-like fold (22). In contrast, a calcium-binding mutation E1073K in cbEGF12, which causes severe neonatal MFS, demonstrated a longer range structural effect where a new protease-sensitive site in the N terminus of the adjacent domain was revealed (6).

The effects of the cysteine substitutions studied here appear to be heterogeneous and lead to more complex pathogenic mechanisms than previously thought. Our data, and that of others (20), indicating the structural and calcium binding properties of the mutant domain and the 29–30 domain interface are disrupted, but effects on the N-terminal cbEGF29 calcium-binding site are not observed.

In addition to identifying structural consequences of specific cysteine substitutions, this study has provided further insight into the calcium binding properties of fibrillin-1. Previous studies have indicated high affinity calcium-binding sites in cbEGF13 and 14 (<30 and <100 μM) (24) of the “neonatal region” and a moderate affinity site in cbEGF33 (~350 μM) (21). The analyses performed on the wild-type fragment in this study demonstrate that other cbEGF domains such as cbEGF30 and 31, located away from the neonatal region, have a high affinity for calcium. Thus, although the high affinity observed in the neonatal region may be important for function, it is not a unique property of this region.

Pulse-chase studies on MFS patient fibroblasts containing cysteine substitutions in fibrillin-1 cbEGF domains have commonly reported normal synthesis and a delay in secretion of fibrillin-1 that leads to severe reduction of matrix deposition (14–18). It was suggested that the delay in secretion might be due to targeted recognition of misfolded protein. In contrast, a smaller number of cases (24% of those studied) have been found in MFS patients with cysteine substitutions where normal secretion of fibrillin-1 has been observed (14, 18). The C1977R substitution is one example where pulse-chase analysis of patient fibroblasts harboring this mutation showed normal synthesis, secretion, and deposition of fibrillin-1 compared with normal fibroblasts (Ref. 37 and data not shown). One may speculate that this is due to the relatively localized effects of the mutation.

Our data, and that of others (20), indicating the structural heterogeneity of this group of mutations suggests that both the disulfide bond affected and the degree of structural destabilization are factors that determine the pathogenic role of the mutant protein. The misfolded domain of a mutant protein may be recognized and targeted to degradation pathways by intracellular mechanisms or may be retained within the cell. In contrast, partially misfolded fibrillin-1 may escape quality control surveillance in the cell. On encountering the extracellular space, mutant proteins may be rapidly degraded by proteases in the surrounding environment or may subsequently disrupt a specific protein-protein interaction required for the assembly of fibrillin-1 or interactions of microfibrils with other cell-matrix components. The structural effects of misfolding mutations in other proteins containing tandem repeats of EGF domains may also be heterogeneous and lead to more complex pathogenic mechanisms than previously thought.