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1 **Structural analysis for glycolipid recognition by the C-type lectins Mincle and**  
2 **MCL**

3 Atsushi Furukawa<sup>1, 2, †</sup>, Jun Kamishikiryo<sup>3, †</sup>, Daiki Mori<sup>4, †</sup>, Kenji Toyonaga<sup>4</sup>, Yuki  
4 Okabe<sup>1, ‡</sup>, Aya Toji<sup>1</sup>, Ryo Kanda<sup>1</sup>, Yasunobu Miyake<sup>4</sup>, Toyoyuki Ose<sup>1</sup>, Sho Yamasaki<sup>4, \*</sup>  
5 and Katsumi Maenaka<sup>1, 2, \*</sup>

6 <sup>1</sup>Laboratory of Biomolecular Science, Faculty of Pharmaceutical Sciences, Hokkaido  
7 University, Sapporo, Japan, <sup>2</sup>CREST, Japan Science and Technology Agency, Saitama,  
8 Japan, <sup>3</sup>Faculty of Pharmaceutical Sciences, Fukuyama University, Fukuyama, Japan,  
9 <sup>4</sup>Division of Molecular Immunology, Research Center for Infectious Diseases, Medical  
10 Institute of Bioregulation, Kyushu University, Fukuoka, Japan

11

12 \*Correspondence:

13 Katsumi Maenaka (maenaka@pharm.hokudai.ac.jp)

14 Laboratory of Biomolecular Science, Faculty of Pharmaceutical Sciences, Hokkaido  
15 University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan

16 Sho Yamasaki (yamasaki@bioreg.kyushu-u.ac.jp)

17 Division of Molecular Immunology, Research Center for Infectious Diseases, Medical  
18 Institute of Bioregulation, Kyushu University, Fukuoka, Japan

19 † These authors equally contributed to this work.

20 ‡ Present address: Department of Functional Biological Chemistry, Division of Science,  
21 Fukuoka University, Fukuoka, Japan

22

23 **Running title:** Structures and functions of C-type lectin receptors, Mincle and MCL.

24

25 **Key words:** C-type lectin receptor, cell surface receptors, X-ray crystallography,  
26 mycobacteria, innate immunity, glycolipid, adjuvant

27

28 **Abbreviations:** PRRs, Pattern recognition receptors, CLRs, C-type lectin receptors,  
29 TLRs, toll-like receptors, Mincle, Macrophage inducible C-type lectin, MCL,  
30 Macrophage C-type lectin, LPS, lipopolysaccharide, TDM, trehalose-6,6'-dimycolate,  
31 CARD9, caspase recruitment domain family member 9.

32

33

34 **Abstract**

35 Mincle (Macrophage inducible C-type lectin, CLEC4E) and MCL (Macrophage C-type  
36 lectin, CLEC4D) are receptors for cord factor, TDM (Trehalose-6,6'-dimycolate), a  
37 unique glycolipid of *Mycobacterium tuberculosis* cell surface components, and activate  
38 immune cells to confer adjuvant activity. Although the receptor-TDM interactions  
39 require both sugar and lipid moieties of TDM, the mechanisms of glycolipid recognition  
40 by Mincle and MCL remained unclear. We here report the crystal structures of Mincle,  
41 MCL and Mincle-citric-acid complex. The structures revealed that these receptors are  
42 capable of interacting with sugar in a Ca<sup>2+</sup>-dependent manner, as observed in other  
43 C-type lectins. However, Mincle and MCL uniquely possess shallow hydrophobic  
44 regions found adjacent to their putative sugar-binding sites, which reasonably locate for  
45 recognition to fatty acid moieties of glycolipids. Functional studies using mutant  
46 receptors as well as glycolipids ligands support this deduced binding mode. These  
47 results give insight on the molecular mechanism of glycolipid recognition through  
48 C-type lectin receptors, which may provide clues to rational design for effective  
49 adjuvants.

50

51

52 **Significance Statement**

53

54 Here we report the crystal structures of human C-type lectin receptors, Mincle  
55 (Macrophage inducible C-type lectin, CLEC4E) and MCL (Macrophage C-type lectin,  
56 CLEC4D), both of which are receptor for mycobacterial glycolipid adjuvant, cord factor  
57 (also called trehalose-6,6'-dimycolate; TDM). Our structural and functional studies  
58 clearly revealed the simultaneous recognition of sugar and lipid moieties by these  
59 C-type lectin receptors on myeloid cells, distinct from other C-type lectin receptors.  
60 Since better adjuvants are desired for enhancing vaccination effects on the medical  
61 treatments for infectious diseases, cancer, and etc, the structures provide a framework  
62 for rational design of more effective adjuvants than TDM.

63

64 /body **Introduction**

65

66 Pattern recognition receptors (PRRs) play important roles in innate immunity. PRRs  
67 recognize nucleotides, sugars, lipopolysaccharides (LPS), other pathogen components  
68 and self-ligands, and consequently trigger intracellular signaling cascades that initiate  
69 innate and adaptive immune responses (1). Among them, toll-like receptors (TLRs) are  
70 well-characterized receptors, in terms of their ligand specificities, ligand recognition  
71 mechanisms and signaling pathways (2-4). The C-type lectin receptors (CLRs) are also  
72 a large family of PRRs (5-7). The term 'C-type lectin' was introduced to distinguish a  
73 group of  $\text{Ca}^{2+}$ -dependent lectins from other lectins. In the CLRs, two amino acids  
74 harboring long carbonyl side chains separated by a proline in a *cis* conformation  
75 coordinate a  $\text{Ca}^{2+}$  ion, which forms hydrogen bonds with monosaccharides and  
76 determines the binding specificity. The CLRs have broad recognition abilities towards  
77 not only saccharides but also proteins (5, 7-9). For instance, human NKR-P1 interacts  
78 with Lectin-like transcript-1, and some members of the CD94/NKG2 family interact  
79 with HLA-E.

80 Macrophage inducible C-type lectin (Mincle, also called CLEC4E) is a type II  
81 transmembrane C-type lectin receptor that is expressed in macrophages, dendritic cells  
82 and monocytes upon stimulation (10). We have reported that Mincle is an  $\text{Fc}\gamma$ -coupled  
83 activating receptor that recognizes pathogenic fungus and mycobacteria (11-13).  
84 Detailed investigations of the ligands of Mincle revealed that Mincle binds glycolipids,  
85 such as trehalose-6,6'-dimycolate (TDM) from *M. tuberculosis*, and novel  
86 glyceroglycolipids from *Malassezia* fungus. The *Malassezia* and *M. tuberculosis* ligands  
87 are recognized through the carbohydrate recognition domain (CRD) in the extracellular

88 region of Mincle (11, 12). The binding of TDM to Mincle leads to the phosphorylation  
89 of the ITAM in the FcR $\gamma$  chain, which provides a binding site for the Syk tyrosine  
90 kinase. Syk activates the caspase recruitment domain family member 9  
91 (CARD9)-mediated NF- $\kappa$ B signaling pathway, to promote the expression of TNF and  
92 IL-6. A recent report revealed that Mincle plays a nonredundant role in T cell immune  
93 responses to infection by microbes and in the adjuvanticity of mycobacterial cord factor  
94 and its synthetic analog, trehalose-dibehenate (TDB) (14, 15).

95 MCL (also called Clec4D) is another C-type lectin receptor expressed in myeloid cells  
96 (16, 17). Recently, we found that MCL is also an FcR $\gamma$ -coupled activating receptor that  
97 binds to TDM (15). MCL is distinct from Mincle, in the following manners: 1) The  
98 expression of Mincle is inducible, whereas MCL is constitutively expressed in myeloid  
99 cells. 2) MCL shows weaker binding affinity to TDM than that of Mincle. 3) EPN motif,  
100 a typical glucose/mannose binding motif, is conserved in Mincle but not in MCL.

101 We now report the crystal structures of Mincle and MCL, as well as Mincle complexed  
102 with citric acid. They have similar overall structures to other typical CLRs, but exhibit  
103 characteristic conformations in the vicinity of the Ca<sup>2+</sup> binding motif. A patch of  
104 hydrophobic amino acids located adjacent to the carbohydrate binding site may likely  
105 contribute to the recognition of the fatty acid chain of TDM. The mutational analysis  
106 essentially supports this TDM binding model, and may also explain the different  
107 affinities of MCL and Mincle.

108

109

## 110 **Results**

### 111 **Preparation, crystallization and structural determination of MCL**

112 The extracellular domain of human MCL (residues 61-215, Fig. 1) was expressed in *E.*  
113 *coli* as inclusion bodies, and was refolded *in vitro* by a dilution method.  $\text{Ca}^{2+}$  ions were  
114 required in the refolding procedure, and the crude, refolded MCL was purified by  
115 sequential gel filtration chromatography steps (Fig. S1A and C). The purified MCL was  
116 crystallized by the hanging drop method with 0.1 M Bis-Tris propane, pH 6.5, 0.2 M  
117 potassium thiocyanate and 20% (w/w) PEG 3350 (PACT, 64). Crystals of the MCL  
118 protein (the space group was I centered orthorhombic (*I*222), and the unit cell  
119 parameters were  $a = 85.19 \text{ \AA}$ ,  $b = 96.06 \text{ \AA}$ ,  $c = 104.53 \text{ \AA}$ ) were obtained, and the data  
120 set was collected to the resolution limit of  $2.2 \text{ \AA}$  at the BL32XU beam line at SPring-8  
121 (Table S1). The crystal structure of MCL has two  $\alpha$ -helices ( $\alpha 1$  and  $\alpha 2$ ) and eleven  $\beta$   
122 -strands ( $\beta 1$  to  $\beta 11$ ) (Fig. 2A), which is typical structural organization of CLRs and  
123 partly similar to the solution structure of MCL registered in the Protein Databank (PDB:  
124 2LS8) (Fig. S1E). Two MCL molecules exist in the asymmetric unit. The gel filtration  
125 analysis showed the mixture of the peaks (Fig. S1A), suggesting that MCL may have  
126 some conformational variation.

127

### 128 **Preparation, crystallization and structural determination of Mincle**

129 Using a similar refolding method to that for MCL, we also prepared the extracellular  
130 domain of Mincle (residues 74-219) (Fig. 1). The expression, refolding and purification  
131 were successful. However, the crystallization was not successful, because the refolded  
132 Mincle was not sufficiently soluble at high concentrations. To improve the protein  
133 solubility, we performed site-directed mutagenesis, and changed the hydrophobic amino

134 acids presumably located on the surface of the Mincle protein to hydrophilic amino  
135 acids, as found in the corresponding residues of MCL. Among them, the mutant with  
136 the substitution of isoleucine to lysine at residue 99 (I99K mutant) formed good crystals  
137 by the hanging drop method with two conditions. One is 1 M lithium chloride, 0.1 M  
138 citric acid (pH 4), and 20% (w/v) PEG6000, and the other is 0.2M NH<sub>4</sub>SO<sub>4</sub>, 0.1M  
139 Bis-Tris (pH 5.5), 25% (w/v) PEG3350. These diffraction data were collected to the  
140 resolution limit of 1.3 Å and 1.35 Å at the BL5A and BL17A beam lines at KEK  
141 (Tsukuba, Japan), respectively. Both crystals have the same space group, primitive  
142 trigonal (*P*3<sub>1</sub>), and the unit similar cell parameters (Table S1). Mincle exhibits typical  
143 CLR fold, as shown in Fig. 2B. The asymmetric unit contained one molecule of Mincle  
144 and no physiologically important packing was detected. This is consistent with the gel  
145 filtration analysis showing that Mincle behaves as a monomer, although the eluted time  
146 is later than the expected one likely due to affinity of Mincle to the glucose-based  
147 dextran resin of Supderdex column (Fig. S1B and D).

148

#### 149 **Structural comparison between Mincle, MCL and other C-type lectins**

150 MCL and Mincle superimposed well on each other (root mean square deviation (r. m. s.  
151 d.) 1.5 Å for 124 C $\alpha$  atoms) (Fig. 2A and B, and Fig. S2B). However, Mincle has two  
152 calcium ions, while MCL has only one. A DALI analysis indicated that MCL and  
153 Mincle share high homology with mouse collectin (2OX9) (r. m. s. d. of 1.3 Å for 120  
154 C $\alpha$  atoms, 28% identity (MCL), and 1.07 Å for 118 C $\alpha$  atoms, 35% identity (Mincle))  
155 (18) and DC-SIGNR (1K9J) (r. m. s. d. of 1.7 Å for 114 C $\alpha$  atoms, 37% identity (MCL)  
156 and 1.3 Å for 121 C $\alpha$  atoms, 45% identity (Mincle)), which has been extensively  
157 studied as an entry receptor of HIV (19, 20). Since collectin recognizes fucose-based

158 oligosaccharides, rather than glucose- or mannose-based ones, we chose to compare the  
159 structural features of MCL, Mincle, and DC-SIGNR (Fig. 2C and D). The entire  
160 structures and the positions of the amino acid residues in the putative CRD are similar.  
161 Specifically, the positions of the  $\text{Ca}^{2+}$  ions (site 1) are the same among the three proteins.  
162 The glutamic-acid-proline-asparagine (EPN) motif (residues 169–171 in Mincle) is  
163 often observed in C-type lectins, and contributes to carbohydrate recognition via a  $\text{Ca}^{2+}$   
164 ion-mediated binding network (Fig. 3A and B). In contrast, the EPD motif of MCL  
165 (residues 173–175) is an unusual sequence among the C-type lectins (6) (Fig. 1).  
166 However, the  $\text{Ca}^{2+}$  ion and other amino acids involved in carbohydrate recognition are  
167 located in this region, as in other C-type lectins (Fig. 2 and 3A-C). These results  
168 indicated that Mincle and MCL recognize carbohydrates through these motifs in slightly  
169 different, but similar manners.

170 The regions surrounding the  $\text{Ca}^{2+}$ -bound sites in MCL and Mincle are distinctly  
171 different from that in DC-SIGNR. In DC-SIGNR, two additional bound  $\text{Ca}^{2+}$  ions are  
172 observed close to the site (red, **2** and **3**) (Fig. 2C), and they stabilize the typical protein  
173 conformation of the C-type lectins (6). The Ca (**2** and **3**) ions push the loop (residues  
174 312-317) close to the Ca (**1**) ion (Fig. 2D, red dotted circle). In contrast, the  
175 corresponding loops in MCL and Mincle are located far from the Ca (**1**) ion. The  
176 asparagine/aspartate residues just after the EPD/EPN sequences are conserved (Fig. 1).  
177 The directions of the asparagines in MCL (residue 176) and Mincle (residue 172) are  
178 different from those in other CLRs, such as DC-SINGR (Fig. 3A-C). The asparagine in  
179 DC-SIGNR is used to bind the Ca (**2**) ion, and therefore the side chain faced to the  
180 opposite direction of the Ca (**1**) ion. In contrast, neither Mincle nor MCL coordinates Ca  
181 (**2** and **3**) ions, and their asparagine side chains extend in different directions, as

182 compared to other C-type lectins.

183

#### 184 **Calcium binding and ligand recognition**

185 In the crystals of Mincle grown in 1 M lithium chloride, 0.1 M citric acid (pH 4), and  
186 20% (w/v) PEG6000, a strong electron density in addition to that of the Mincle protein  
187 was observed close to the Ca(1) ion and matched a citric acid molecule (Fig. 3D). The  
188 superimposition of the amino acids in the Ca<sup>2+</sup> ion binding regions of the ligand  
189 complex structures of Mincle and DC-SIGNR revealed the well conserved locations of  
190 the oxygen atoms of the ligands, citrate and mannose (equatorial 3- and 4-OH groups),  
191 respectively (Fig. 3E). Because the chemical property of the sugar moiety is different  
192 from the citric acid, we cannot simply compare the recognition modes but these data  
193 may support the idea that Mincle can utilize this Ca ion to bind nucleophiles for the  
194 sugar moieties of TDM and Malassezia ligands, in essentially the same manner as  
195 generally observed in CLRs including DC-SIGNR.

196 The calcium binding site in the human and mouse Mincles includes the EPN motif, a  
197 well conserved in the mannose-recognizing C-type lectins, as described above. We  
198 examined whether the EPN motif in Mincle is involved in direct TDM recognition using  
199 soluble Mincle protein (Mincle-Ig). Mincle-Ig (Mincle<sup>WT</sup>), but not control Ig,  
200 selectively bound to plate-coated TDM, as previously reported (11, 12). This  
201 recognition was shown to require the EPN motif, as the binding was eliminated by  
202 introducing a mutation of EPN into QPD, a putative galactose-recognition sequence  
203 (Mincle<sup>QPD</sup>) (21). Substitution of EPN motif into MCL-type EPD (Mincle<sup>EPD</sup>) also  
204 impaired the binding capacity, although their reactivities to anti-hIgG were comparable  
205 (Fig. 3F and Fig. S3A). This data suggested that EPN in human Mincle is indispensable

206 for TDM recognition, as previously shown in mouse Mincle (11, 12). In contrast, the  
207 direct binding of MCL to TDM was much weaker than that of Mincle (Fig. 3G),  
208 consistent with the previous report that MCL recognizes TDM with less affinity than  
209 Mincle (15). Mutation of EPD sequence of into QPD (MCL<sup>QPD</sup>) did not have large  
210 impact on the TDM binding in higher concentrations. Unexpectedly, however, the EPD  
211 to EPN mutation in MCL, which was expected to coordinate the Ca ion location well  
212 and facilitate carbohydrate binding, did not improve the affinity for TDM (Fig. 3G).  
213 These results suggested that the TDM binding site of MCL might be distinct from that  
214 used by Mincle. Furthermore, the side chain of Arg183 in Mincle is in a suitable  
215 position to interact with the hydroxyl groups of TDM, based on the crystal structure of  
216 Mincle (22) (Fig. 4A). This arginine residue of Mincle is well conserved from fishes to  
217 mammals. In contrast, the valine (Val186) at the corresponding position of human MCL  
218 is conserved among placentalia, however, its side chain cannot reach the putative  
219 carbohydrate recognition site (Fig. 4B). To verify the role of Arg183 in TDM  
220 recognition, we introduced the R183V mutation in Mincle and tested its function in an  
221 NFAT-GFP reporter assay. This mutation reduced the NFAT-GFP activity in the  
222 reporter cell assay, suggesting that Arg183 of Mincle is crucially involved in the ligand  
223 recognition (Fig. 4C).

224 Taken together, these results strongly suggested that the binding mode of the two OH  
225 groups of citrate acid to Ca<sup>2+</sup> reflects the equatorial 3- and 4-OH groups of mannose and  
226 glucose of Mincle/MCL ligands, in a similar, but slightly different manner than the  
227 CLRs (6).

228

229 **Putative lipid recognition sites**

230 To determine whether Mincle and MCL utilize unique amino acids for their  
231 interactions with the lipid regions of glycolipids, we verified the characteristics of the  
232 surfaces surrounding the putative sites for the Ca<sup>2+</sup>-mediated sugar binding. A series of  
233 hydrophobic regions were specifically found in Mincle and MCL, but not in other  
234 C-type lectins in the vicinity of the putative sugar-binding sites (dotted circles in Fig.  
235 4A and B, yellow surfaces in Fig. 4D-F, and yellow-shaded amino acid residues in the  
236 box enclosed with a blue line in Fig. 1). The regions are composed of Val195, Thr196,  
237 Phe198, Leu199, Tyr 201 and Phe 202 in Mincle, and Val197, Pro198 and Phe201 in  
238 MCL. The Mincle has larger hydrophobic areas than MCL, while DC-SGMR has only  
239 much smaller one than both Mincle and MCL (Fig. 4D-F). If the trehalose part of TDM  
240 is placed on the sugar binding site of Mincle, as in the binding mode of mannose to  
241 DC-SGMR, then the mycolic acid attached to the 6-*O* of the glucose of TDM (Fig. 4A  
242 and B, red arrow) is oriented toward the hydrophobic regions of Mincle and MCL, as  
243 described above. To investigate whether the hydrophobic region of Mincle contributes  
244 to the recognition of TDM, the Ala substitutions of both Phe198 and Leu199 in this  
245 region was introduced in reporter cells expressing Mincle (Fig. 4C). The cells  
246 expressing the Mincle<sup>F198A/L199A</sup> mutant exhibited reduced NFAT activity in response to  
247 TDM. Moreover, we replaced the hydrophobic region of Mincle (residues 195-202)  
248 with the corresponding region of another CLR, Dectin-2 (residues 192-199), which  
249 lacks the hydrophobic residues (23, 24). The reporter cells expressing this  
250 Mincle-Dectin-2 chimeric molecule (Mincle<sup>MD chimera</sup>) still retained the activity against  
251 anti-Mincle mAb, 13D10-H11 (Fig. S3C), which recognizes the conformational epitope  
252 on Mincle (Fig. S3D), indicating that the mutation as well as other mutations of this  
253 study did not make remarkable effect on the overall protein folding and stability on the

254 cell surface. However, the TDM recognition of Mincle<sup>MD chimera</sup> was severely  
255 compromised (Fig. 4C). As described above, the O $\delta$  atom of the corresponding residue  
256 Asn172 just after the EPN motif in Mincle does not face toward the Ca ion, which is an  
257 unusual type of Ca coordination among the C-type lectins (Fig. 3A and B). Instead, the  
258 N $\delta$  atom of Asn172 forms a hydrogen bond with the O $\delta$  atom of Thr196 of the  
259 hydrophobic patch (Fig. 4A). The reporter cells expressing the mutant Mincle  
260 (Mincle<sup>N172Q</sup>), which has only one additional methylene group, showed reduced  
261 NFAT-GFP activity (Fig. 4C). This result may suggest that the N172Q mutation  
262 indirectly influences the hydrophobicity of the putative lipid binding patch via the side  
263 chain of Thr196.

264 In order to further examine the effect of a set of acyl chains, we performed surface  
265 plasmon resonance binding assays using a set of trehalose-based glycolipids, which  
266 have a single acyl chain with different carbon lengths (C8, C10, C12). These glycolipids  
267 have a single and short tail and thus are expected to be water-soluble while retaining the  
268 ligand activity. The single acyl chains with trehalose (C10 and C12) bound to Mincle  
269 (Figs. 4G and S5). The affinity of C8 to Mincle is much lower than those of C10 and  
270 C12 (Fig. 4G). The crystal structure clearly indicated that the 10-carbon acyl chain with  
271 trehalose is reasonably accommodated within the hydrophobic portion in Mincle (Fig.  
272 S6).

273

## 274 **Discussion**

275 We have determined the crystal structures of the ectodomains of Mincle and MCL,  
276 which confirmed that the overall structures of Mincle and MCL are similar to those of  
277 other CLRs. Furthermore, we have also solved the crystal structure of Mincle

278 complexed with citric acid, which revealed that the binding mode to citric acid  
279 essentially resembles that of glucose/mannose recognition by typical CLRs. We further  
280 performed the competition binding of glycolipids with citric acid as Fig. S7, clearly  
281 showing that the citric acid inhibits the glycolipid binding to Mincle, while the acetic  
282 acid does not. Notably, other mannose-binding c-type lectin, codakine, bound the  
283 similar positions of oxygens of glycerol and glycan in  $\text{Ca}^{2+}$  ion-mediated manner (25).  
284 The citric acid is likely accommodated at this position to block the ligands and hydroxyl  
285 groups are likely utilized following the coordination of  $\text{Ca}^{2+}$  ions generally observed in  
286 CLRs.

287 Glycolipids play pivotal roles in innate immunity, as exemplified by the functions of  
288 CD1-mediated natural killer T cells (NKT) (26, 27). The CD1 family molecules display  
289 a variety of glycolipids toward semi-invariant NKT cell receptors to activate NKT cells.  
290 The structural analyses of CD1 family proteins have revealed that the lipid parts of the  
291 glycolipids are deeply accommodated inside the hydrophobic cores of the proteins (28,  
292 29), and thus only the sugar moieties are exposed, for recognition by NKT cell receptors  
293 (Fig. S6). On the other hand, our present study showed that the putative TDM binding  
294 sites of Mincle and MCL include hydrophobic loops uniquely found in Mincle and  
295 MCL, which distinguish them from the other C-type lectins (Fig. 4A, B and D-F). These  
296 loops form shallow hydrophobic patches extending from the corresponding position of  
297 the 6-OH of glucose on the structure of the mannose complex of DC-SIGNR, which is  
298 attached to mycolic acid, in the case of TDM (Fig. S6). The mutational study suggested  
299 that these CLRs directly recognize the acyl groups of the glycolipid TDM using this  
300 shallow hydrophobic region, which is close to the  $\text{Ca}^{2+}$  binding site (Fig. 4D and E).  
301 Notably, the SPR binding study using a set of glycolipids clearly showed that the single

302 acyl chain is sufficient for Mincle binding. In addition, importantly, at least a C10  
303 length of the lipid moiety is required (Fig. 4G). These observations might suggest that  
304 Mincle recognizes only the sugar-proximal part of the acyl chain of glycolipids. The  
305 hydrophobic patch branches out from the potential sugar binding site (downward and to  
306 right in Fig. 4D and Fig. S6). These might confer the sites accommodating the branched,  
307 acyl chains in mycolic acids, such as TDM and trehalose mono-mycolate (TMM) (11).  
308 The recently discovered ligands of Mincle (44-1 and 44-2), which also have branched  
309 acyl chains, may interact similarly with TMM and TDM (13). Therefore, the recognition  
310 of glycolipids by Mincle and presumably MCL seems to be significantly distinct from  
311 those of lipid recognition proteins, such as CD1 and Toll-like receptor 4/MD2 complex,  
312 which have deep hydrophobic grooves to accommodate the acyl moieties of glycolipids  
313 (Fig. S6). Thus, a minimum acyl chain length is required for glycolipid recognition by  
314 CLRs. The unique modes of CLR-glycolipid recognition would be advantageous for  
315 host defense responses, because they may allow receptors to recognize these bipolar  
316 ligands even within a microbial cell wall or in the micellar form in aqueous solution.  
317 Future study for the co-crystallization with glycolipids harboring short branched acyl  
318 chains, which might have increased binding affinity, would elucidate the lipid binding  
319 modes.

320 The production of NO and IL6 by Bone Marrow derived Macrophages (BMM $\phi$ ),  
321 which express Mincle and MCL, was reportedly changed by stimulation with several  
322 lengths of acyl chains, revealing the importance of the acyl chain length (30). The  
323 fungal glycolipids, recently identified as Mincle ligands, have more complicated and  
324 branched lipid moieties. The structural and functional data presented here showed that  
325 Mincle and MCL probably require an acyl chain longer than 10 carbons for glycolipid

326 recognition, thus clearly providing important clues for the design of better adjuvants  
327 than TDM.

328 The present study indicated that Mincle has a higher affinity for TDM than MCL,  
329 which is consistent with our *in vitro* binding study (Fig. 3F and G). The crystal  
330 structures of MCL and Mincle clearly revealed that MCL has a smaller hydrophobic  
331 patch next to the putative Ca<sup>2+</sup>-mediated sugar binding site, as compared to that in  
332 Mincle. The different sizes of these hydrophobic sites might explain the affinity  
333 differences of two CLRs observed in the binding data.

334 The typical CLRs that simply recognize sugars, such as DC-SIGNR and CEL-IV,  
335 exhibit remarkably low affinities (K<sub>d</sub> ~mM) (31, 32). They require multiple valencies of  
336 sugar ligands to mediate signaling. However, the SPR analysis revealed that Mincle  
337 seemed to show higher affinity, suggesting that it can detect small numbers of  
338 glycolipids on fungal surfaces. On the other hand, MCL showed much lower affinity  
339 than Mincle, but essentially the same ligand specificity. It is plausible that  
340 MCL-mediated signaling requires multiple valencies of glycolipid ligands. Therefore,  
341 Mincle and MCL may play distinct roles in physiological events.

342 CLRs often form homodimers or heterodimers on the cell surface. As described above,  
343 the multivalent ligands on the bacterial surface likely induce the multimerization of the  
344 CLRs (either monomeric or dimeric structures), which may mediate efficient signaling.  
345 The recent report by Lobato-Pascual et al. demonstrated that Mincle and MCL form the  
346 disulfide-linked heterodimer associated with the FcεRIγ chain (33). The heterodimeric  
347 complex formation between Mincle and MCL through the N-terminal β strand and/or  
348 stalk regions, as previously reported for maltose-binding protein (34), for efficient  
349 recognition/signaling would be an intriguing issue to be addressed.

350

351

## 352 **Materials and Methods**

### 353 **Plasmid construction**

354 The *E. coli* expression plasmids encoding the partial extracellular domain of human  
355 Mincle (residues 74-219), pET22-Mincle, and the extracellular domain (residues 61 -  
356 215) of human MCL, pET22-MCL, were constructed (the detail in Supplemental  
357 information) (35).

358 In order to improve the solubility and crystallization of Mincle, we synthesized,  
359 purified and crystallized several mutated Mincle proteins. Among them, the I99K  
360 mutant was produced with a high yield and generated good crystals.

361

### 362 **Preparation of recombinant proteins**

363 The pET22-Mincle, pET22-Mincle I99K and pET22-MCL plasmids were transformed  
364 into *Escherichia coli* strain BL21(DE3) plysS, and the protein was obtained as inclusion  
365 bodies. The protein was solubilized in a buffer containing 6 M guanidine-HCl, 50 mM  
366 MES, pH 6.5, 100 mM NaCl, and 10 mM EDTA for 12 hours at 4°C. 1h after the  
367 addition of dithiothreitol (10mM), the solubilized proteins were slowly diluted into 1  
368 liter of buffer, containing 0.1 M Tris-HCl, pH 8.5, 1 M l-arginine, 2 mM EDTA, 6.3 mM  
369 cystamine, 3.7 mM cysteamine, and 0.1 mM phenylmethylsulfonyl fluoride. The  
370 refolding mixture was purified by gel filtration chromatography. The buffer was finally  
371 exchanged to 20 mM Tris-HCl, pH 8.0, with 5 mM CaCl<sub>2</sub> for crystallization.

372

### 373 **Crystallization and Structure Determination**

374 Crystals of purified Mincle I99K and MCL were grown at 20°C (reservoir solutions: 1  
375 M lithium chloride, 0.1 M citric acid (pH 4), 20% (w/v) PEG6000 and 0.1 M Bis-Tris  
376 propane, pH 6.5, 0.2 M potassium thiocyanate, 20% (w/w) PEG 3350, respectively) by  
377 the hanging drop vapor-diffusion method. Crystals were equilibrated in a  
378 cryo-protectant consisting of reservoir solution supplemented with 16% (v/v) glycerol.  
379 X-ray data were collected on beamlines, BL32XU in SPring-8 and BL5C in KEK. The  
380 data were processed with HKL2000 (36) or XDS (37). The structure was solved by  
381 molecular replacement with PHASER (38), using CD69 as the search model (PDB:  
382 1FM5). Several rounds of model building in COOT (39) and refinement in PHENIX  
383 (40) were performed. The final refinement statistics are provided in Table S1. The  
384 coordinates for the refined Mincle, Mincle-citrate complex, and MCL structures have  
385 been deposited in the Protein Data Bank (accession codes 3WH3, 3WH2 and 3WHD,  
386 respectively).

387

### 388 **Binding assay using Ig-fusion proteins**

389 The MCL-Ig and Mincle-Ig fusion proteins were prepared as described previously.  
390 Briefly, the C terminus of the extracellular domain of human MCL (residues 42-215),  
391 human Mincle (residues 46-219), or their mutants was fused to the N terminus of the  
392 hIgG1 Fc region. The Ig-fusion proteins were incubated with 0.2 µg/well of  
393 plate-coated TDM or plate-coated anti-human IgG, and the bound proteins were  
394 detected by using HRP-labeled anti-human IgG.

395

### 396 **Reporter Assay**

397 Reporter cells were prepared as described previously (11, 35). Briefly, 2B4-NFAT-GFP  
398 reporter cells were transfected with FcR $\gamma$ , together with Mincle and mutants. The  
399 reporter cells were stimulated with various concentrations of plate-coated TDM or  
400 anti-human Mincle antibody (13D10H11). The activation of NFAT-GFP was monitored  
401 by flow cytometry.

402

### 403 **SPR analysis**

404 The SPR analysis was performed similarly as described previously for other cell  
405 surface receptors (41). Briefly, Mincle and MCL were each dissolved in 10 mM sodium  
406 acetate (pH 4), containing 5 mM CaCl<sub>2</sub> with or without 5% dimethyl sulfoxide (DMSO).  
407 SPR experiments were performed with a BIAcore T3000 (GE Healthcare). All of the  
408 proteins were covalently immobilized on the CM5 sensor chip by amine-coupling (GE  
409 Healthcare).  $\beta$ 2 microglobuline was used as a negative control protein. All glycolipids  
410 (C12, C10 and C8), and trehalose as a negative control, were injected in 10 mM HEPES,  
411 pH 7.4, containing 150 mM NaCl and 5 mM CaCl<sub>2</sub>. The data were analyzed using the  
412 BIAevaluation software, version 4.1 (GE Healthcare).

413

414 **Note Added in Proof.** While this paper was under revision, Feinberg *et al.* (42) reported  
415 the crystal structure of bovine Mincle complexed with trehalose, whose binding mode  
416 is similar to that of human Mincle for glycolipids we proposed here.

417

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424

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533

### 534 **Figure legends**

535 **Fig. 1.** Structure-based sequence alignment of Mincle, MCL, DC-SIGNR and Dectin-2.

536 The sequence alignment of the ecto-domains of Mincle, MCL, DC-SIGNR and Dectin-2  
537 (h and m indicate that human and mouse, respectively) is shown, as depicted with  
538 ESPript (43). Identical residues are highlighted in red, and similar residues are framed  
539 in blue. The secondary structure elements ( $\alpha$ :  $\alpha$ -helix,  $\beta$ :  $\beta$ -strand, T: turn) of Mincle  
540 and MCL are shown above the sequences. The box enclosed by the thick black line  
541 indicates the EPN motifs, which are usually involved in carbohydrate recognition by  
542 C-type lectins. The box enclosed by the blue line indicates the hydrophobic amino acids  
543 loops and yellow-shaded amino acids residues are hydrophobic residues within Mincle  
544 and MCL. The asterisks below the sequences indicate the residues involved in calcium  
545 binding in Mincle and MCL. The red filled circles below the sequences indicate the  
546 residues changed to other amino acid residues in the mutational studies. The numbers  
547 under the cysteine residues indicate disulfide bond formation with the cysteine residue  
548 with the same number.

549

550 **Fig. 2.** Structures of MCL and Mincle, and structural comparison with DC-SIGNR.

551 (A and B) Cartoon models of overall structures of MCL (A) and Mincle (B). The  
552 secondary structure elements are shown. Gradient rainbow color from blue to red  
553 indicates N- to C-terminal. The yellow and cyan spheres are Ca<sup>2+</sup> ions in MCL and  
554 Mincle, respectively. (C and D) Overall structures (C) and putative ligand binding sites  
555 (D), close-up view of black box of C, of MCL (yellow), Mincle (cyan) and DC-SIGNR  
556 (pink) are shown. Yellow, blue and pink spheres are Ca<sup>2+</sup> ions in Mincle, MCL and  
557 DC-SIGNR, respectively. Red dotted circle indicates the large structural difference of  
558 loops among these CLRs (see Text).

559

560 **Fig. 3.** Structural comparison of the putative ligand binding sites in MCL, Mincle and  
561 DC-SIGNR, and *in vitro* binding assays of Mincle and MCL mutants.

562 (A-C) Close-up views of the putative ligand binding sites of MCL (yellow) (A), Mincle  
563 (cyan) (B), and DC-SIGNR (pink) (C) are shown. The amino acid residues involved in  
564 and close to Ca<sup>2+</sup> ion binding are shown as stick models. Interactions with Ca<sup>2+</sup> ions are  
565 shown in black dotted lines. (D) Composite OMIT map (2Fo-Fc) for citric acid in  
566 Mincle. The electron-density map is contoured at 1.0σ, and the resolution is 1.3 Å. The  
567 citric acid is shown with the O atoms colored red and the C atoms in green. Putative  
568 amino acids involved in Ca<sup>2+</sup> binding are depicted by sticks. (E) The superimposed  
569 structures of Mincle (cyan, the same as (D)) and DC-SIGNR (pink) are shown. The  
570 stick model indicates the mannose (the O atoms colored red and the C atoms in pink) in  
571 the DC-SIGNR complex. (F and G) Mincle-Ig, mutated Mincle-Igs or hIgG (F) and  
572 MCL-Ig, mutated MCL-Igs or hIgG (G) were incubated with plate-coated TDM. Bound  
573 proteins were detected by anti-hIgG-HRP.

574

575 **Fig. 4.** Unique amino acid residues in MCL and Mincle, reporter assays of mutant  
576 Mincles, and SPR analysis.

577 **(A-B)** The superimposed structures of Mincle (cyan) and DC-SIGNR **(A)**, and MCL  
578 (yellow) and DC-SIGNR (pink) **(B)** are shown. Arrowheads indicate the oxygen atom  
579 connected with mycolic acid in TDM (mannose binding to DC-SIGNR is shown in the  
580 figure). Dotted circles indicate the hydrophobic loops found in Mincle and MCL. A  
581 sequence comparison between human Mincle and Dectin-2 is shown. **(C)** Analyses of  
582 Mincle and its mutants were performed. Reporter cells expressing human Mincle or its  
583 mutants were stimulated with TDM for 18 h. **(D-F)** Electrostatic potentials of Mincle  
584 **(D)**, MCL **(E)** and DC-SGINR **(F)** are shown. Electrostatic surface potentials were  
585 calculated using the program APBS (44) and represented by PyMOL, with the color of  
586 the surface potentials in the scale ranging from negatively charged (-4.0 kbT/ec, red) to  
587 positively charged amino acids (4.0 knT/ec blue). Black spheres are Ca<sup>2+</sup> ions. The  
588 yellow surface indicated the hydrophobic site. **(G)** SPR analysis of Mincle and several  
589 lengths of acyl chains with trehalose were performed. The C12, C10 and C8 glycolipids  
590 used in this experiment are shown.

591



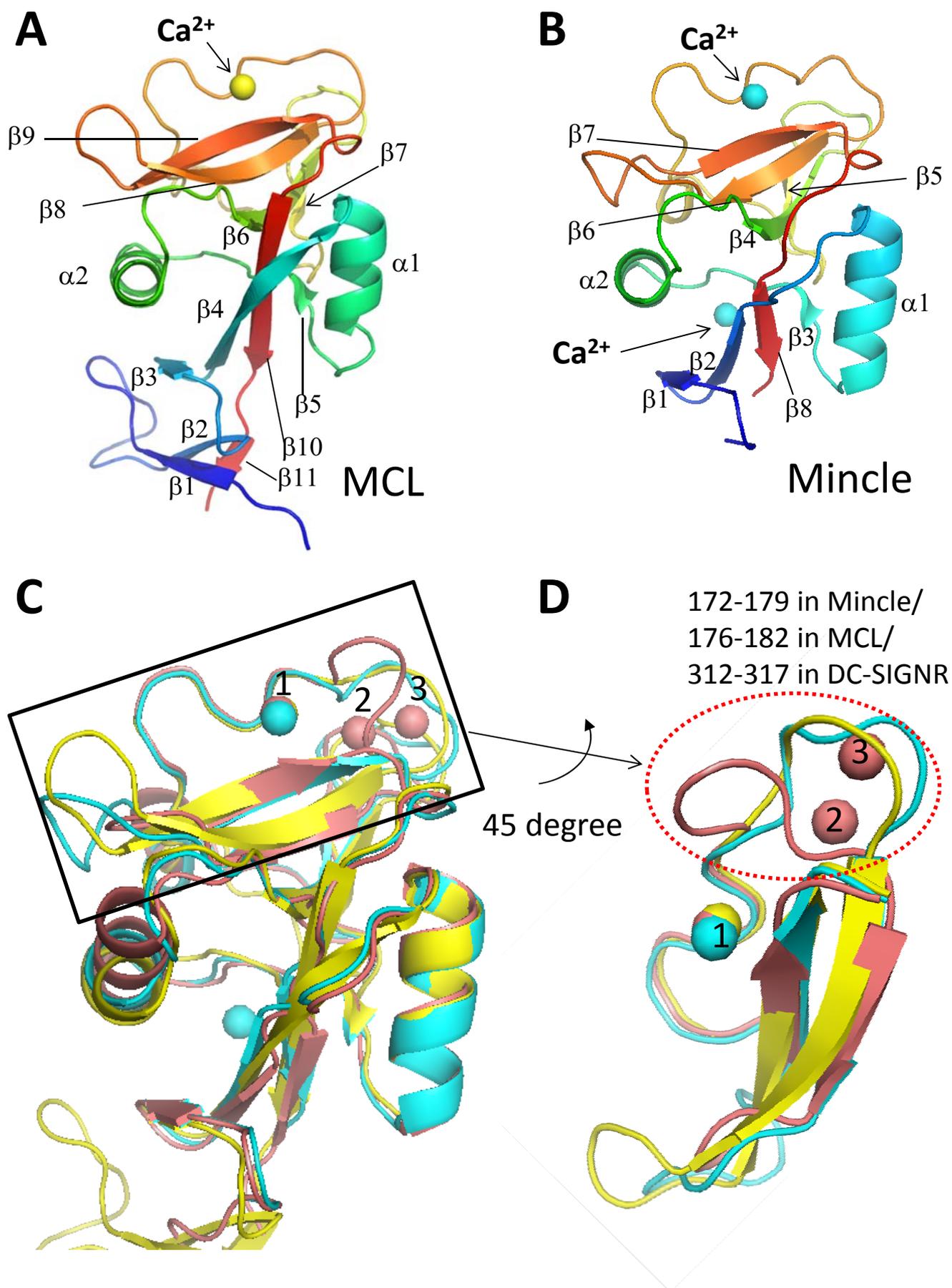


Fig.2

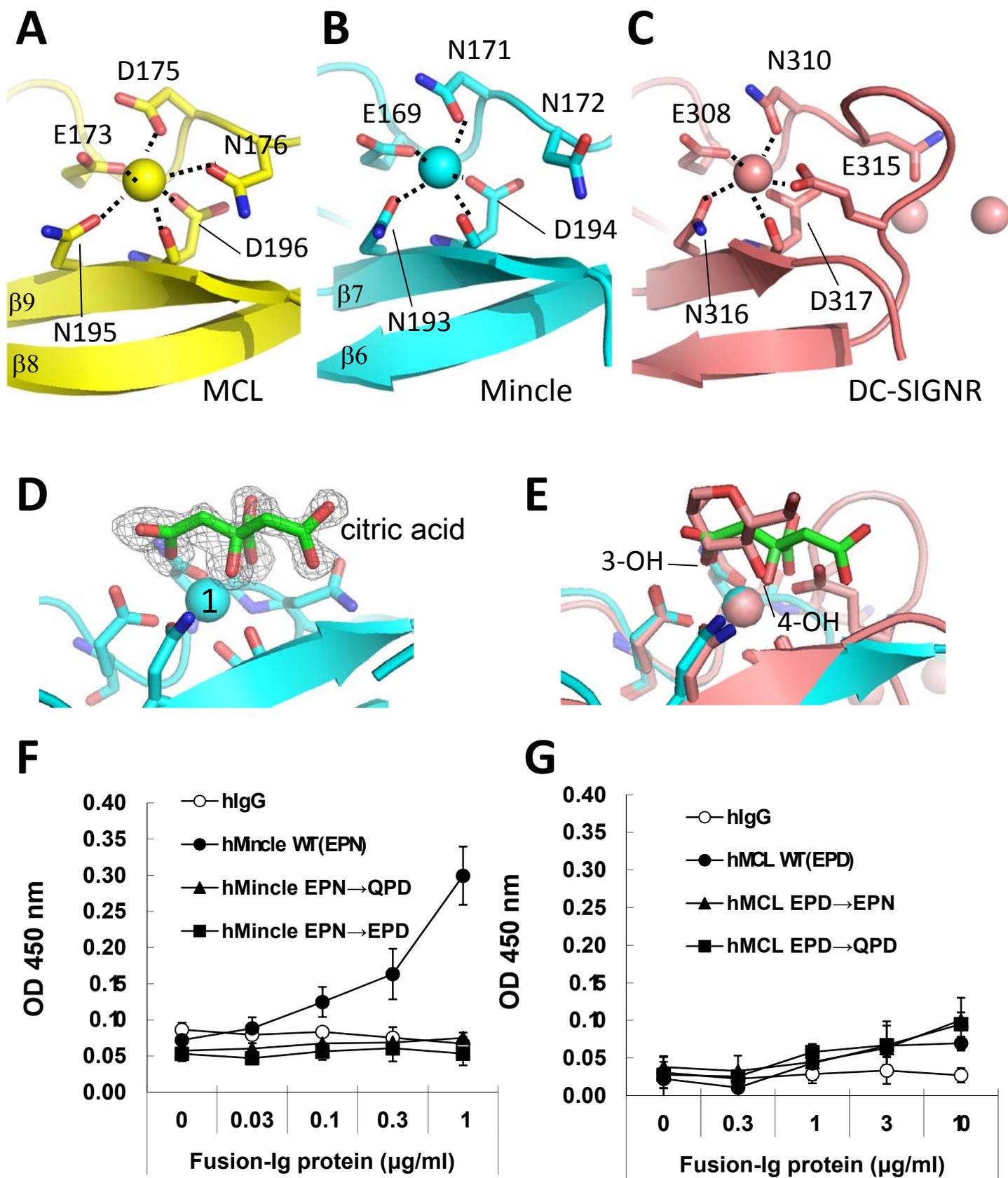


Fig.3

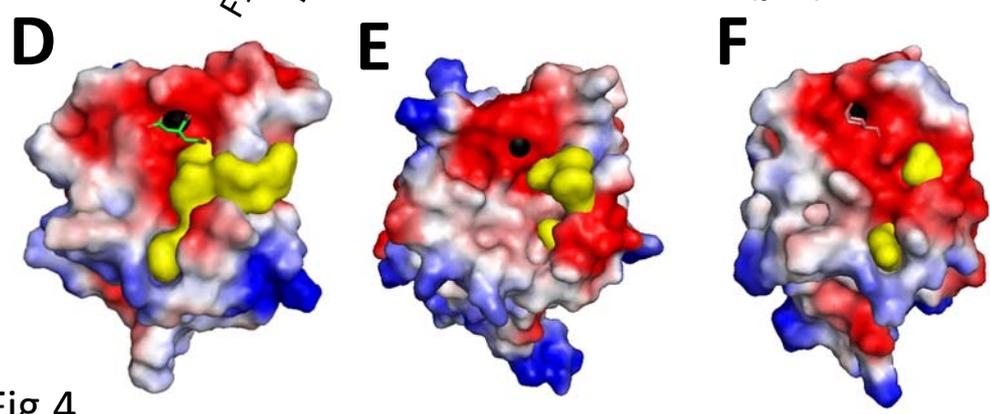
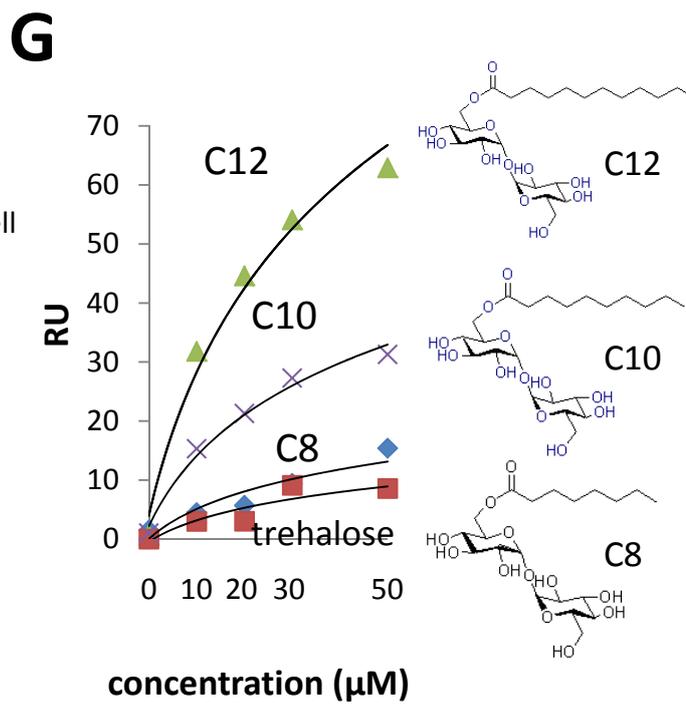
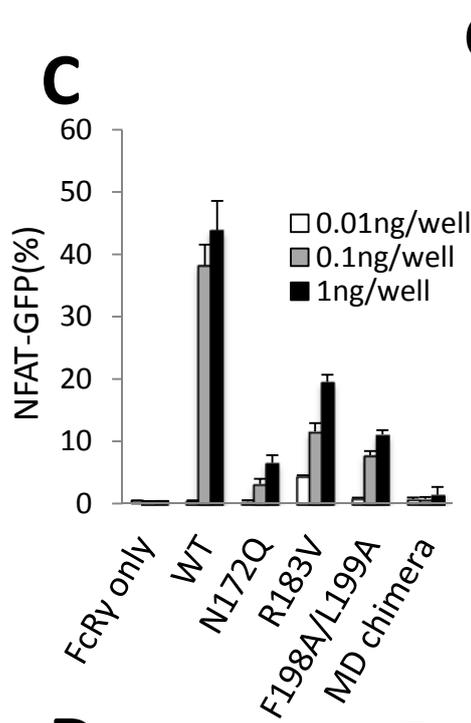
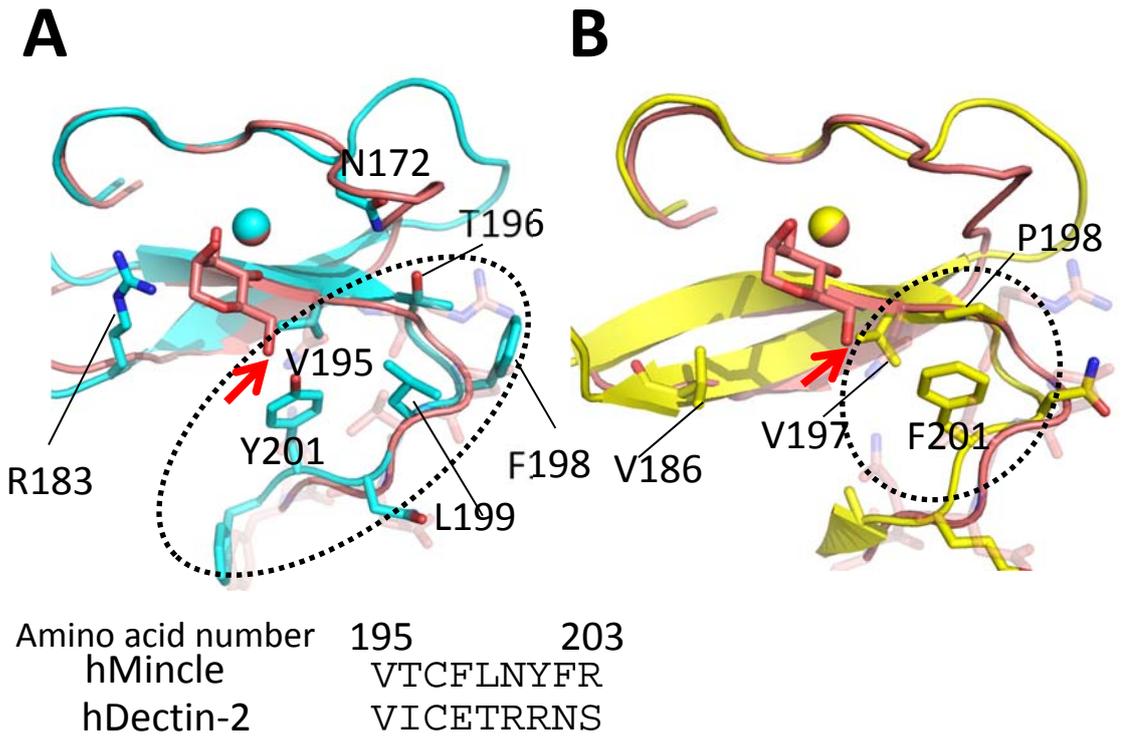


Fig.4