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Irreversible evolutionary loss of chitin-degrading ability in the chitinase-like protein Ym1 under positive selection in rodents

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Abstract

Ym1 (chitinase-like 3, Chil3) expressed in mice is a nonenzymatic chitinase-like protein, which shows 67% identity with mouse acidic chitinase (Chia). Similar to Chia, Ym1 is overexpressed in asthma and parasitic infections in mouse lungs. Due to the lack of chitin-degrading activity, the biomedical role of Ym1 under these pathophysiological conditions remains to be determined. In this study, we investigated what region and amino acid changes in Ym1 resulted in the loss of enzymatic activity. Replacing two amino acids at the catalytic motif to obtain a Chia-like sequence (N136D and Q140E; MT-Ym1) did not activate the protein. We conducted a comparative study of Ym1 and Chia. We found that three protein segments—(i) the catalytic motif residues, (ii) exons 6 and 7, and (iii) exon 10—are responsible for chitinase activity loss in Ym1. We show that replacing each of these three segments in Chia that are also involved in substrate recognition and binding by the Ym1 sequence can fully abolish the enzymatic activity. In addition, we show that there have been extensive gene duplication events at the *Ym1* locus specific to the rodent lineages. Consistent with this result, Ym1 orthologs from the rodent genome were under positive selection when analyzed through the CODEML program. These data suggest that numerous amino acid substitutions in the regions involved in the chitin recognition, binding, and degradation ability of the ancestor Ym1 molecule lead to the irreversible inactivation of the protein.

KEYWORDS

chitin, chitinase, chitinase-like protein Ym1, enzyme inactivation, mouse

1 | INTRODUCTION

Chitin is a polymer of *N*-acetyl-D-glucosamine linked by β-1,4-bonds. It is a structural component of crustaceans' and insects' exoskeleton, the microfilaria sheath of parasitic nematodes, and the fungi's cell wall (Bueter et al., 2013; Van Dyken & Locksley, 2018). Chitinase hydrolyzes the β-1,4-bonds of chitin polymers (Bueter et al., 2013; Van Dyken & Locksley, 2018; Lee et al., 2011).

Mammals do not synthesize chitin or its synthase, but mice and humans express two chitinases with chitin-

Kazuaki Okawa and Eri Tabata contributed equally to this article.

degrading activity, chitotriosidase (Chit1) and acidic chitinase (Chia) (Hollak et al., 1994; Renkema et al., 1995; Boot et al., 1995; Boot et al., 2001). Chia comprises the N-terminal catalytic domain (CatD) and the C-terminal chitin-binding domain (CBD). CatD of Chia has been shown to have chitinolytic and chitin-binding activities under physiological environments in mouse, chicken, and pig (Kashimura et al., 2015; Ohno et al., 2016; Tabata et al., 2018a; Tabata et al., 2019).

Mammalian chitinases belong to family 18 of glycosyl hydrolases (GH-18) (Henrissat, 1991; Cantarel et al., 2009; Bussink et al., 2007). The sequence of catalytic activity within family 18 chitinase is DXXDXDXE, where E is presumed to be the catalytic residue (Lee et al., 2011; Bussink et al., 2007; Watanabe et al., 1993). The GH-18 family consists of chitinase-like proteins (CLPs) that are structurally homologous to chitinase but incapable of degrading chitin (Lee et al., 2011; Bussink et al., 2007). CLPs are considered lectin proteins (Bussink et al., 2007; Kzhyshkowska et al., 2007; Schimpl et al., 2012; Ranok et al., 2015), and several have been identified in mice and humans. Mice express Ym1 (chitinase-like 3, also called ECF-L) encoded by *Chil3*, Ym2 coded by *Chil4*, and BRP-39 (also called Chi3l1) coded by *Chil1*. Humans express YKL-40 (human homolog of CHI3L1 or human cartilage glycoprotein 39) and YKL-39 (CHI3L2). Meanwhile, no human orthologs of Ym1 (Webb et al., 2001; Ward et al., 2001; Sun et al., 2001; Chang et al., 2001; Jin et al., 1998). Loss of chitinase activity in CLPs is generally believed to be due to evolutionary mutations of essential residues within the conserved catalytic sequence (Lee et al., 2011; Bussink et al., 2007; Watanabe et al., 1993; Schimpl et al., 2012).

Increased mRNA and/or protein levels of chitinase and CLP have been reported in many inflammatory diseases (Bueter et al., 2013; Lee et al., 2011). Chit1 levels are elevated in patients with Gaucher's disease, chronic obstructive pulmonary disease (COPD), atherosclerosis, diabetes mellitus, cystic fibrosis, Alzheimer's disease, and tobacco smokers (Hollak et al., 1994; Artieda et al., 2003; Seibold et al., 2008; Letuve et al., 2010; Sonmez et al., 2010; Watabe-Rudolph et al., 2012; Livnat et al., 2014). Chia expression and activity are enhanced by high molecular weight chitin administration during an allergic airway response in a mouse model of asthma (Zhu et al., 2004; Reese et al., 2007). In humans, elevated YKL-40 (CHI3L1) levels accompany asthma, COPD, cystic fibrosis, rheumatoid arthritis, inflammatory bowel disease, alcoholic cirrhosis, and various malignancies (Letuve et al., 2008; Hector et al., 2011; Johansen et al., 1999; Bernardi et al., 2003; Koutroubakis et al., 2003; Vind et al., 2003; Chupp et al., 2007; Vos et al., 2000; Johansen et al., 1997; Johansen et al., 1995; Cintin et al., 1999). Increased YKL-39 (CHI3L2) is observed in patients with osteoarthritis (Steck et al., 2002; Knorr et al., 2003). Thus, chitinases and CLPs play certain roles in many pathophysiological conditions (Bueter et al., 2013; Van Dyken & Locksley, 2018; Lee et al., 2011), although their exact contribution to these diseases remains unclear.

It is generally accepted that a protein-coding gene that has lost its function becomes a pseudogene. Despite CLPs losing chitin-degrading activity, their open reading frame is similar to active chitinases; it is highly expressed in normal tissues and overexpressed in inflammatory diseases (Ohno et al., 2014; Ohno et al., 2015).

Ym1 has been confirmed only in rodents (Sun et al., 2001; Chang et al., 2001). Despite the lost chitin-degrading activity, the mRNA and/or protein levels are increased during parasite infection (Reese et al., 2007; Hung et al., 2002) and allergic pulmonary inflammation (Webb et al., 2001) and are implicated in immunomodulation (Arora et al., 2006; Osborne et al., 2014; Sutherland et al., 2014). Thus, Ym1 plays an important role in the induction and progression of allergic pneumonitis (Draijer et al., 2018). Ym1 deficiency has been shown to alleviate pulmonary inflammation and promote the alternative activation of macrophages (Zhu et al., 2020). Ym1 and Ym2 have been reported to contribute to IL-17 production and be associated with neutrophil activity (Muallem & Hunter, 2014). In addition, Ym1 has been reported to promote the expression of genes and the activity of proteins related to neurogenesis and the maintenance and repair of lung tissue (Namiki et al., 2022; Sutherland et al., 2018). Thus, Ym1 plays an active role in many pathophysiological conditions (Bueter et al., 2013; Lee et al., 2011; Kang et al., 2022; Collmann et al., 2019). However, the contribution of Ym1 to the pathophysiology remains unclear.

Our present study shows that numerous amino acids are substituted in the regions involved in chitin recognition and binding and suggests that *Ym1* evolved from *Chia* while acquiring physiological functions unrelated to chitin binding and degradation. This study also shows that Ym1 is a molecule that actively lost its function as a chitinase while retaining the respective structural frame during its evolution.

2 | RESULTS

2.1 | The introduction of Chia catalytic residues does not activate Ym1

Ym1 and Chia share 67% sequence identity. In addition to having similar amino acid sequences, Ym1 and Chia CatD have been shown to form comparable three-dimensional structures. The lack of chitinase activity in CLPs has been believed to be caused by mutations of crucial residues

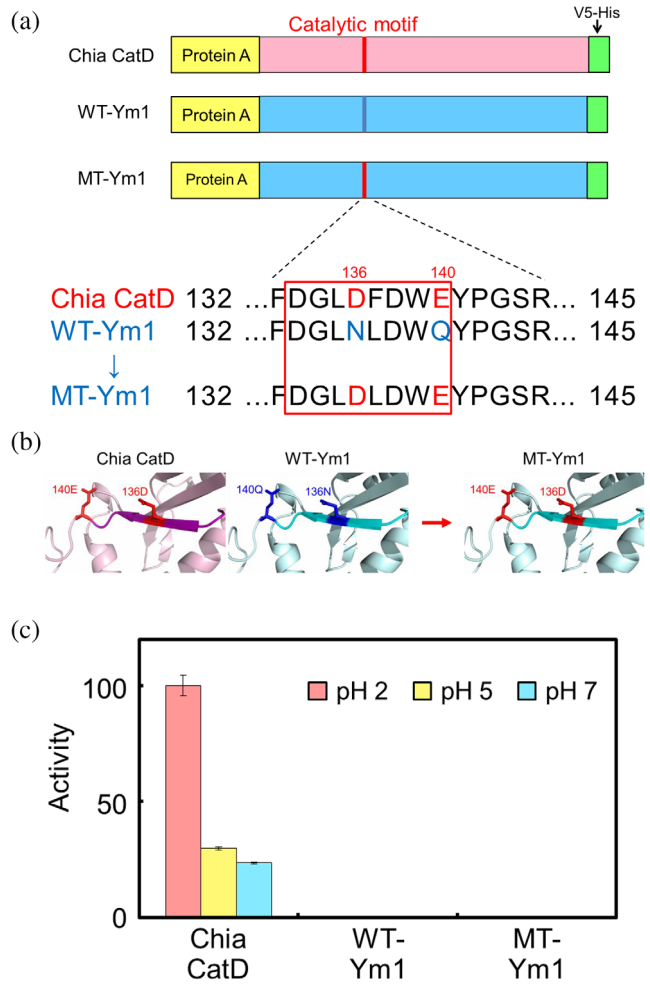

FIGURE 1 The introduction of N136D and Q140E into WT-Ym1 did not activate MT-Ym1. (a) Schematic representation of *E. coli*-expressed Chia CatD, WT-Ym1 and MT-Ym1 fusion proteins. MT-Ym1 was constructed by substituting N136D and Q140E with WT-Ym1. The amino acid sequences are color-coded: pink, Chia CatD; blue, Ym1. (b) Comparison of catalytic residues of Chia and MT-Ym1 using PyMOL (http://www.pymol.org). The simulation was done using AlphaFold2 (Jumper et al., 2021). The catalytic residues are color-coded: red, Chia CatD; blue, WT-Ym1. (c) Comparison of chitinolytic activities of the recombinant proteins using the fluorogenic substrate. Values are represented as relative activity. Error bars represent the mean ± standard deviation from a single experiment conducted in triplicate.

within the conserved catalytic sequence (Lee et al., 2011; Bussink et al., 2007; Watanabe et al., 1993). To test this hypothesis in Ym1, we introduced amino acid substitutions N136D and Q140E in the catalytic motif of the protein, obtaining "mutant" Ym1 (MT-Ym1) in the catalytic motif (Figure 1a). In the simulation using AlphaFold2 (Jumper et al., 2021), the three-dimensional structures of the catalytic residues of Chia CatD, WT-Ym1, and MT-Ym1 are similar (Figure 1b), suggesting that MT-Ym1 was expected to have chitin-degrading activity.

We expressed Chia CatD, WT-Ym1, and MT-Ym1 in *Escherichia coli* as fusion proteins containing *Staphylococcus aureus* Protein A and V5-His tag (Okawa et al., 2016) as described in Section 4 (Figures 1a; S1, S2, S3, and Table S1). To compare the chitinolytic activity of these recombinant proteins, we used a fluorogenic substrate 4-methylumbelliferyl (4-MU) *N,N′*-diacetyl-β-D-chitobioside [4-MU-$(GlcNAc)_2$].

As shown in Figure 1C and in agreement with previous reports (Lee et al., 2011; Bussink et al., 2007; Watanabe et al., 1993), the only protein to degrade the substrate was Chia CatD, with the highest activity at pH 2.0 and decreased with higher pH (pH 5.0 and 7.0), whereas WT-Ym1 nor MT-Ym1 showed no chitinolytic activity at any pH condition (Figure 1C). These results indicate that the lack of chitinase activity in Ym1 is not caused by the amino acid substitutions within the conserved catalytic motif (Figure 1C).

2.2 | Activation of MT-Ym1 using chimeric proteins

Attempting to activate MT-Ym1, we constructed and expressed chimeric proteins using MT-Ym1 and Chia CatD (Figures 2a, S2, S3, and Table S1). Chimeras, where the C-terminal region of MT-Ym1 containing the catalytic residue was replaced with Chia CatD, were designated C1 and C2 (Figure 2a). Conversely, chimeras in which the C-terminal region of Chia CatD was replaced with MT-Ym1 were named C3 and C4.

Chimera C1 (MT-Ym1 containing Chia CatD exons 8–11) remained inactive (Figure 2b). However, in chimera C2, which included two more Chia CatD exons, enormous chitinolytic activity was detected exceeding those of Chia CatD probably due to the lack of CatD/CBD linker (see in the following text). In contrast, C3 had no chitin-degrading activity even though the amino acid sequence of exons 6 and 7 was that of Chia CatD. These results suggest that the replacement of exons 6–11 with Chia CatD is necessary to express the chitinolytic activity of MT-Ym1. In other words, the cause of evolutionary Ym1 inactivation is placed within exons 6–11 (Figure 2C). Thus, our results in Figures 1 and 2 suggest multiple inactivation causes in Ym1 (Figure 2C).

2.3 | Exons 6 and 7 and exons 10 and 11 of Ym1 suppress the chitinolytic activity of Chia CatD

To narrow down the regions involved in the inactivation of Ym1, we examined whether it is inactivated by substituting Ym1 exons 6–11 with Chia CatD sequences.

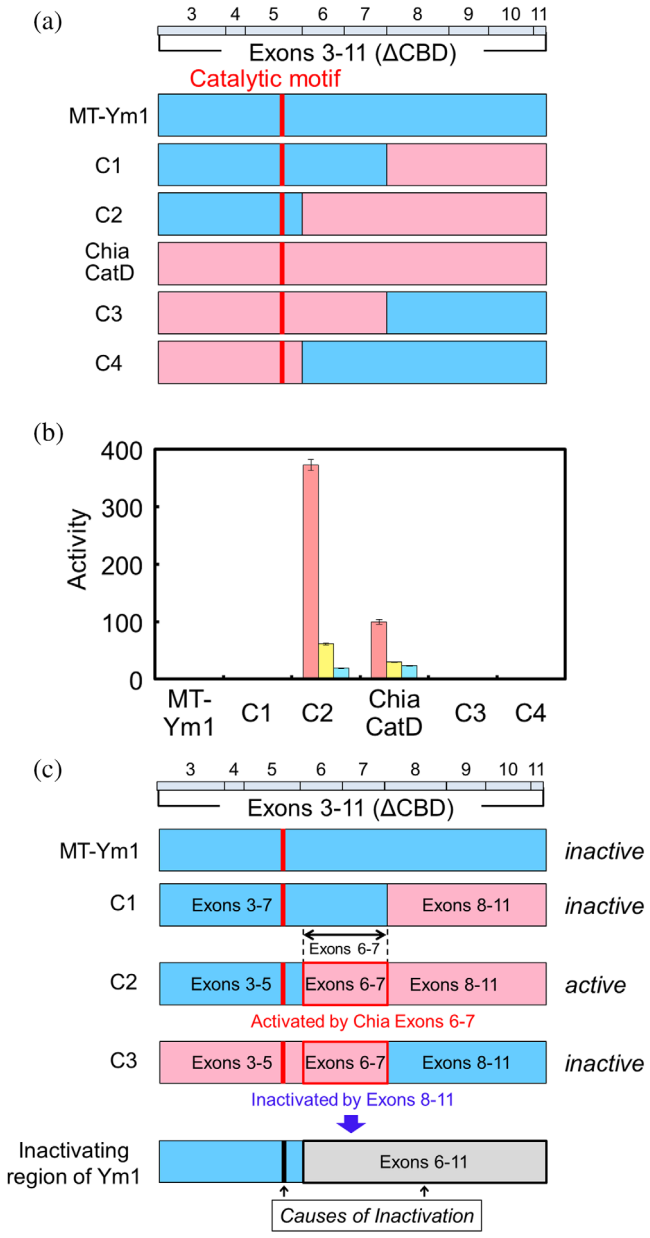

FIGURE 2 Activation of MT-Ym1 using chimeric proteins. (a) Schematic representation of MT-Ym1 and WT-Chia CatD chimeric proteins (C1–C4). The coding region of Chia CatD and MT-Ym1 consists of exons 3–11. The schematic exon structures of the chimera are shown above the Figures. The amino acid sequences are color-coded: pink, Chia CatD; blue, Ym1. (b) The chitinolytic activities of MT-Ym1-Chia CatD chimeric proteins (C1-C4), MT-Ym1 and Chia CatD. (c) Causes of inactivation in the C-terminal region of Ym1. Exons 6 and 7 of Chia (shown in the red frame) play an important role in activating MT-Ym1. In contrast, exons 6–11 of Ym1 inactivated Chia CatD. These data suggest that the multiple arrows show the causative regions for Ym1 inactivation (indicated by the black). Schematic exon structure is shown above the schemes in panels A and C.

We constructed chimeras C5-C10 where Ym1 exons 6–11 were replaced with Chia CatD's corresponding exons (Figures 3a, S2, S3, and Table S1). The chitinase activity of chimeras C5 and C8 was higher than those of Chia (Figures 3b, S3 and Table S1). In contrast, chimeras C6 and C7 were lower than those of Chia (Figure 3b). In addition, C9 and C10, in which Chia CatD's exons 10–11 and 6–7, respectively, were replaced with Ym1, showed no chitinase activity (Figure 3b). These results show that some single Chia region substitutions increase its chitinolytic activity (exons 6 and 9) while others have an opposite effect (exons 7 and 8). On the other hand, substitutions of exons 6 and 7, 10 or 11 completely inactivated Chia. Thus, exons 6–7 and exon 10 of Ym1 suppress the chitinolytic activity.

Next, we prepared chimeras C11–C13 by substituting Chia CatD exons 8–11 of C2 with exons of Ym1 to clarify the effect of these regions on the chitinolytic activity (Figures 3c, S2, S3, and Table S1). The activity of C11 (C2 with Ym1 exon 8) and C12 (C2 with Ym1 exons 8 and 9) was lower than that of C2 but similar to Chia CatD (Figure 3D). In contrast, in C13 (C2 with Ym1 exons 8–11) no chitinolytic activity was detected (Figure 3D). These results indicate that activation of Ym1 requires the substitution of exons 6, 7, 10, and 11 with Chia CatD.

To further determine the inactivating region in Ym1, we expressed Ym1-Chia CatD chimeras where parts of exons 6 and 7 of C12 were gradually replaced by the Ym1 sequence from the N-terminal side (Figure 3e, C14–C16; Figures S2, S3, and Table S1) and chimeras where exons 10 and 11 of C12 were gradually replaced from the C-terminal side (Figure 3e, C17 and C18). Chimeras C14, C15, and C16 displayed massively reduced chitinolytic activity directly proportional to the extent of the replaced sequence (Figure 3f). Meanwhile, chimera C17 showed increased activity. In contrast, the activity of C18 was decreased (Figure 3f). The reason for the activity of C17 being significantly higher than that of C12 appears to be the missing Chia linker between CatD and CBD in exon 11 that may regulate the chitinase activity of Chia.

Thus, our results revealed that exons 6, 7, and 10 are involved in the inactivation of the chitin-degrading activity in Ym1 (Figure 3g). Based on our results, we revealed that there are three regions causing inactivation in Ym1; (i) catalytic residues, (ii) exons 6 and 7, and (iii) exons 10 (Figure 3g, upper). Comparing the amino acid sequences of Chia and Ym1, 53 residues differed in these three regions.

2.4 | Numerous amino acid substitutions are involved in Ym1 inactivation

Exons 7 and 10 of Chia CatD contain important amino acid residues in cognitive binding to the substrate

(Figure S4), and there is an enzymatic subsite for recognizing substrate in exon 7 (218 W) (Olland et al., 2009). The subsite and surrounding area sequence differ in multiple amino acids between Ym1 and Chia CatD. Similarly, there is a conserved catalytic residue (360 W) in exon 10 (Olland et al., 2009), and similarly to exon

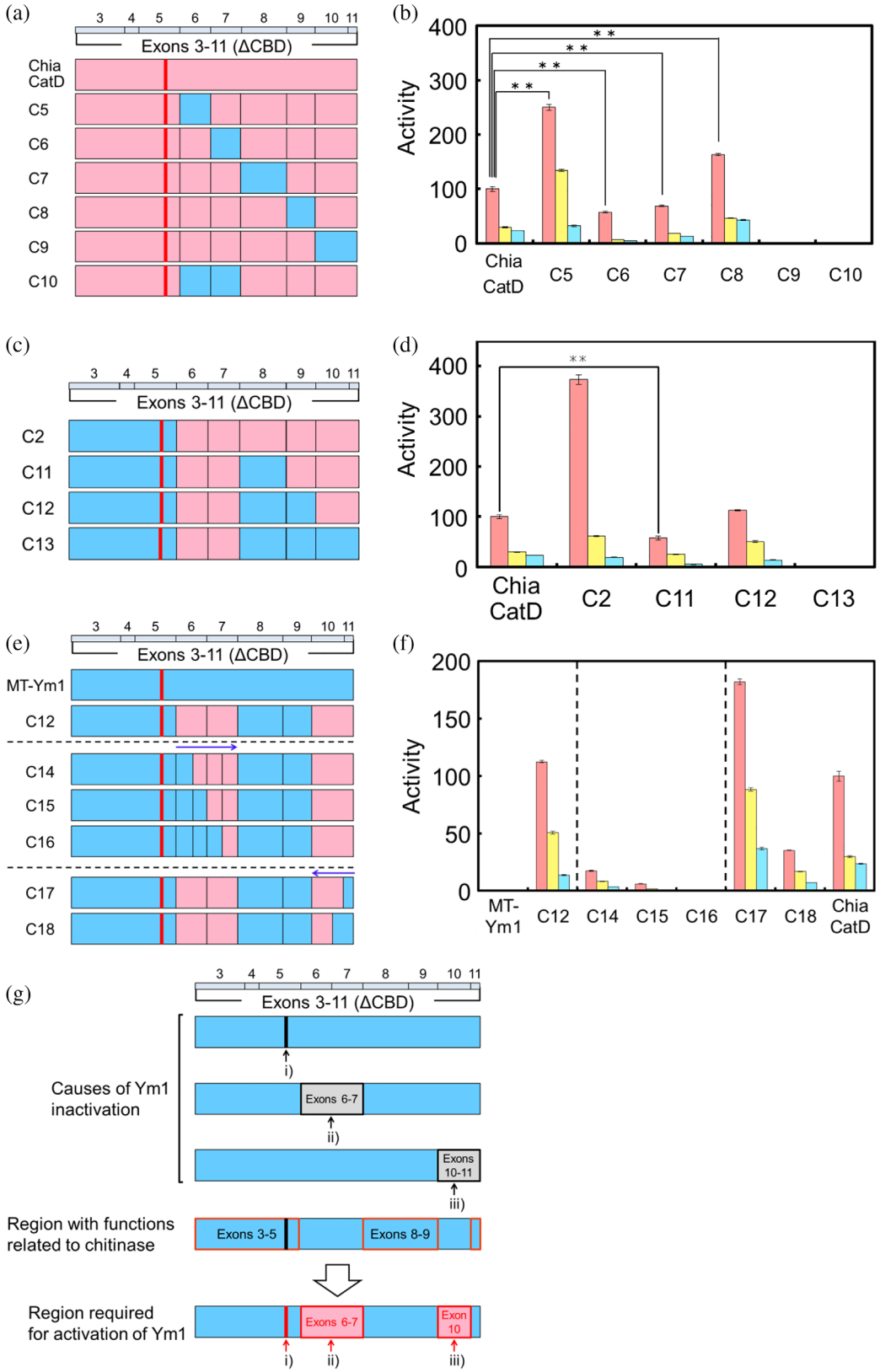

FIGURE 3 Legend on next page.

7, numerous differences between the two proteins are present in the adjacent sequence. Therefore, we assumed that sequences around these two residues could contain causative amino acids of Ym1 inactivation. In an attempt to activate Ym1, exon 6 of MT-Ym1 was replaced with Chia CatD and amino acid substitutions Q208H, D216G, P217S, and K218W in exon 7 and L354F, V358M, V359I, and P361A in exon 10 were introduced (chimera C19) (Figures 4a, S2, S3, and Table S1). In addition, we created chimeras based on C19, C20, C21, and C22 by dividing exon 6 in C19 into exon 6A, 6B, and 6C. We detected chitin-degrading activity in C19, but it was very low compared to Chia (Figure 4b). The activity of chimeras C20, C21, and C22 was further reduced with C21, particularly lacking activity completely. Thus, the evaluated amino acids are involved in Ym1 inactivation, but it is clear that the responsible sequence change is much more complex at exons 7 and 10. In addition, the complete loss of chitin-degrading activity in C21 suggests that the sequence at part B of exon 6 (Figure 4a) significantly impacts the protein activity. These results indicate that a sequence modification in exons 6, 7, and 10 leads to Ym1 inactivation.

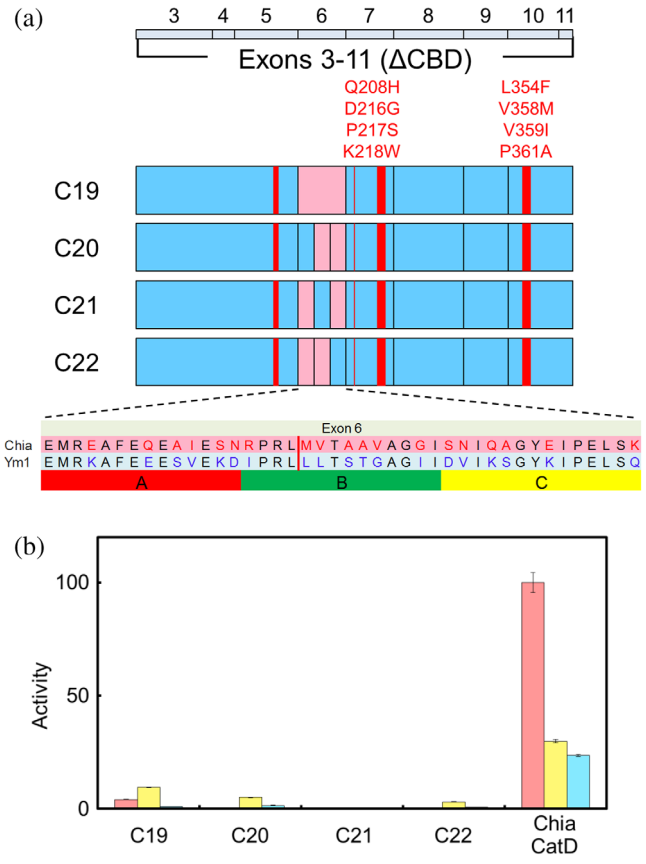

FIGURE 4 Multiple amino acids are involved in the Ym1 inactivation. (a) Schematic representation of Ym1-Chia chimeras (C19–C22). The divided parts of exon 6 of C19 are 6A, 6B, and 6C. (b) Comparison of the chitin-degrading activity of Chia CatD and C19–C22.

2.5 | Ym1 lacks chitin recognition/binding and degradation

Next, we used chitin beads to compare the chitin-binding ability of Chia CatD, WT-Ym1, MT-Ym1, and chimera C19.

Active Chia CatD was confirmed to have chitin-binding activity (Figure 5a) (Kashimura et al., 2015; Tabata et al., 2018a). In contrast, chitin-binding activity was negligible in WT-Ym1 (Figure 5b). Binding of MT-Ym1 to chitin was weaker than Chia CatD but higher than WT-Ym1 (Figure 5C), suggesting that the presence of active catalytic residues slightly improved the reduced recognition and binding function to the substrate.

Interestingly, C19, with multiple amino acid substitutions into MT-Ym1, had chitin-binding activity comparable to Chia CatD (Figure 5D), indicating that Chia CatD exon 6 and substituting at least several amino acids substitutions into exons 6, 7, and 10 of MT-Ym1 led to the reactivation of chitin recognition/binding in Ym1. These results suggest that WT-Ym1 almost completely lost the chitin recognition/binding function.

Based on our results in Figures 1-5, we concluded that there are three causes of inactivation in Ym1 (Figure 5e); (i) catalytic residues primarily causing loss of the

FIGURE 3 Identification of the inactive region other than the catalytic residue of Ym1. (a) Schematic representation of the Chia CatD and Ym1 chimeras and their chimeric proteins (C5–C10) in which Ym1 was replaced for each exon. (b) Comparison of the chitin-degrading activity of Chia CatD and Ym1-Chia chimeric proteins (C5–C10). Each experiment was performed in triplicate. $^{**}p < 0.01$. p-Values were determined using Student's t-test for each pH. (c) Schematic representation of Ym1-Chia chimeras (C11–C13) in which C2 was replaced with MT-Ym1. (d) Comparison of the chitin-degrading activity of Chia CatD and Ym1-Chia chimeric proteins (C2, C11–C13). (e) Schematic representation of Ym1-Chia chimeras (C14–C18) in which C6 was replaced with MT-Ym1. (f) Comparison of the chitin-degrading activity of Chia CatD and Ym1-Chia chimeric proteins (C6, C14–C18). (g) Location of three inactivating regions in Ym1. Exons 6, 7, and 10 of Ym1 play an important role in activating MT-Ym1. The black arrows show the causative regions for Ym1 inactivation. The red arrows indicate the region required for Ym1 activation. Exons 3, 4, 5 (excluding catalytic residues), 8, and 9 of Ym1 had the same function as Chia (shown in orange frames).

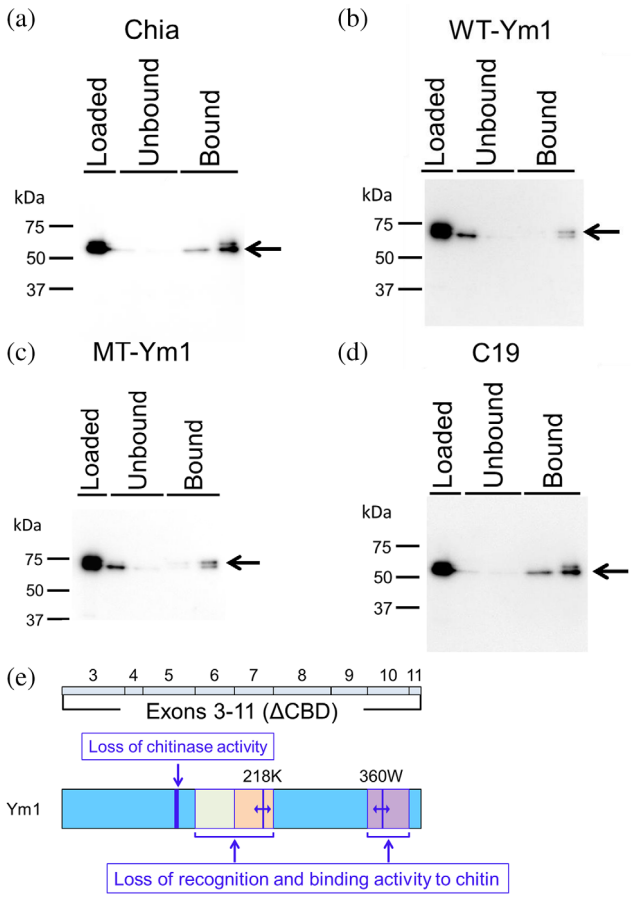

FIGURE 5 Loss of function involved in recognition, binding and degradation toward chitin substrates through Ym1. The affinity of recombinant proteins Chia CatD (a), WT-Ym1 (b), MT-Ym1 (c), and C19 (d) to chitin beads. Unbound and bound fractions were collected and analyzed by Western blot using an anti-V5-HRP antibody. (e) Schematic diagram of the inactivation mechanisms in three Ym1 regions.

chitinolytic activity and (ii) exons 6 and 7, and (iii) exon 10 primarily leading to the loss of substrate recognition/binding.

2.6 | *Ym1* has evolved under positive selection within an independent lineage of rodents

Ym1 gene in mice is generally thought to be a CLP evolved from *Chia* and is located on the same chromosome (Bussink et al., 2007). In addition, both genes are present between *Dennd2d* and *Pifo* (Figure 6). We used the NCBI database to elucidate the *Chia* and CLP genes present around *Dennd2d* and *Pifo* in rodents and other mammals for evolutionary analysis of *Chia* and *Ym1*.

In mammals other than rodents, the positions of *Chia*, *Chia-like* genes, and *Chi3l2* were confirmed to be between *Dennd2d* and *Pifo* (Figure 6, upper). In other words, *Chi3l2* is the only CLP gene located near *Chia*, except for rodents. In contrast, many CLP genes, *Ym1* (*Chil3, Chi3l3*), *Ym2* (*Chil4, Chi3l4*), *Chil5*, and *Chil6*, have been found in rodents at this location (Figure 6, middle and lower). The distance between *Dennd2d* and *Pifo* in nonrodent mammals was 100–200 kbp, while in rodents, the shortest distance was more than 220 kbp (in *Jaculus jaculus*). These data indicate that many gene duplications generated CLP genes present between the *Dennd2d* and *Pifo* genes in rodents during evolution. Therefore, these, including *Ym1*, have undergone unique evolution in rodents and are functionally more diverse than Chia.

To better understand the evolution of *Ym1*, the CLP and *Chia* gene sequences were collected from 30 rodents using the NCBI database. We added human, monkey, and cattle *Chi*a and *Chi3l2* as an outgroup. We used the retrieved collection of protein sequences to build a bootstrapped neighbor-joining phylogenetic reconstruction. Phylogenetic trees of rodent CLP gene sequences showed respective monophyletic clades within multiple species (Figure 7). Since mouse *Ym1* and *Ym2* are highly identical (91%), these genes are thought to be paralogs that arose following a recent (5–7 MYA) segmental duplication. These results showed that duplication of CLP genes frequently occurred in rodents.

Within the CODEML program in the PAML package (Yang, 2007), we used three pairs of site models to test whether any of the *Chia* or *Ym1* has been subjected to positive selection. Model M0, which assumes a single ω for all codons in the sequence, was used to estimate a general ω value for each data set. The ω value of the rodent *Chi*a family was smaller than that of the *Ym1*/*Ym2* family ($\omega = 0.249$ for *Chia* genes and $\omega = 0.556$ for *Ym1*/*Ym2* genes), suggesting that the evolution of *Chia* is dominated by purifying selection operating on the majority of sites. Among the models implemented, M2a and M8 can account for positive selection by adding a class of sites where $\omega > 1$. Results of the analysis for the rodent *Chi*a family indicated that a small number of sites (1.0%–1.5%) might be subject to positive selection. However, most residues in *Chia* are under purifying selection (76% of sites with $\omega < 0.034$) (Table 1). Comparisons of model M1a with M2a ($\chi^2 = 0.279$, $p = .870$) and model M7 with M8 ($\chi^2 = 0.729$, $p = .695$) failed to support any hypothesis of positive selection.

For the *Ym1*/*Ym2* genes, both comparisons (M1a and M2a, $\chi^2 = 18.20$, $p < .001$; M7 and M8, $\chi^2 = 22.94$, $p < .001$) favored the hypothesis that the sites in *Ym1*/*Ym2* family have been under significant positive selection over the null hypothesis. Models M2a and M8 assigned 2.9% and 4.9% of codons to the class of positively selected

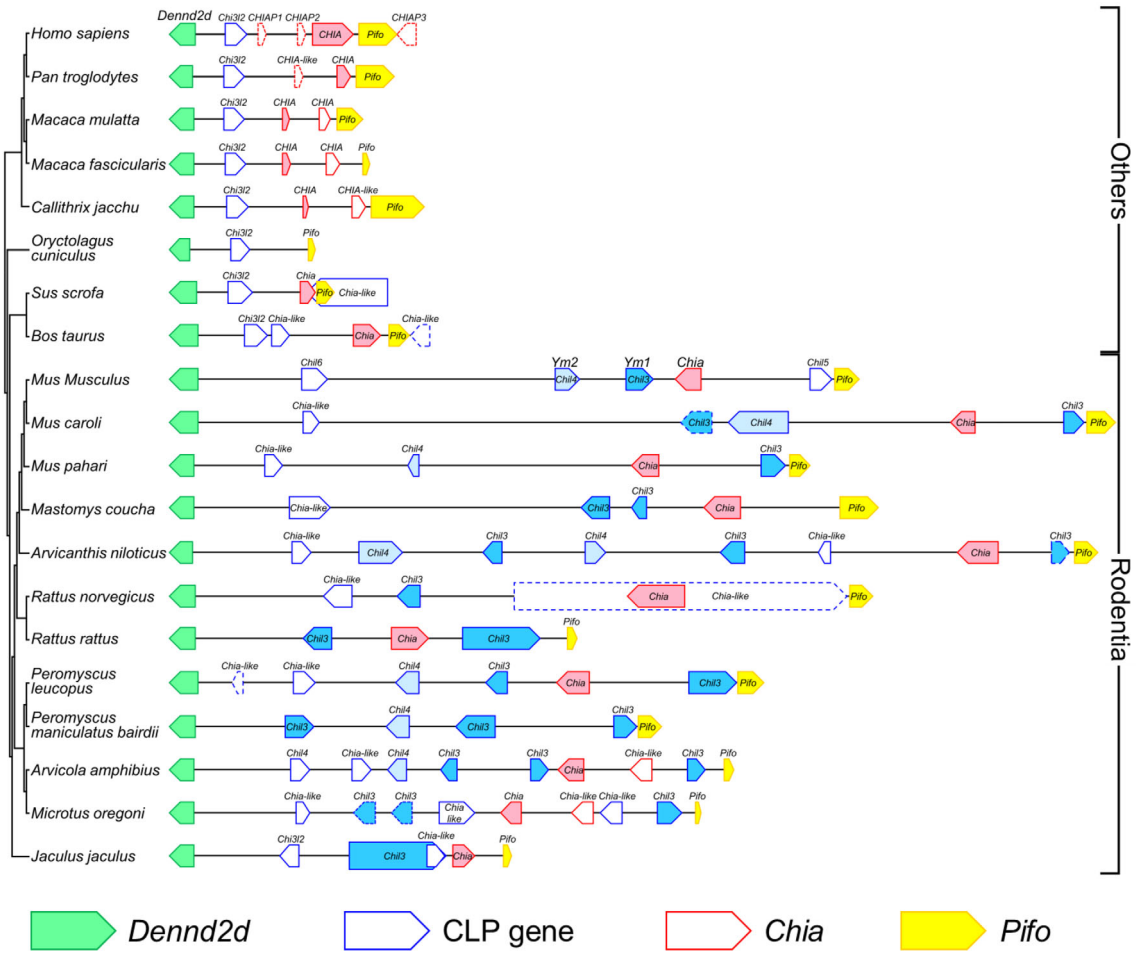

FIGURE 6 Rodent CLP genes, including Ym1, are uniquely evolved molecules through multiple gene duplications. Schematic representation of the chitinase-related genes existing between the *Dennd2d* and the *Pifo* genes. Each frame is categorized: solid lines, coding genes; dotted lines, pseudogenes.

sites, respectively ($\omega = 3.570$ and 2.910, respectively) (Table 1). Bayes Empirical Bayes (BEB) analysis also indicated several sites in the *Ym1*/*Ym2* family with a significant probability of being under positive selection. Two sites (171 V and 302S) were inferred to have $\omega > 1$ for those with high posterior probabilities (>0.95) under both models of M2a and M8 (Table 1).

3 | DISCUSSION

Gene duplication plays an important role in developing genes with novel functions. However, studies investigating how the function of the duplicated CLPs changes during their evolution are scarce. Our study focuses on *Ym1*, a gene in the rodent lineage that emerged through the duplication of a chitinase gene. This study provides important findings considering the abundant expression of this family of proteins during pathological environment. It presents several new concepts in the chitin-degrading activity of CLPs, whose specific functions still need to be revealed. We also show evidence arguing against the hypothesis of the catalytic activity of the CLPs being inhibited due to two amino acid changes.

Chil3 encodes mouse-specific protein Ym1 and is thought to be a gene duplication of *Chia* (Bussink et al., 2007). However, the inactivity of MT-Ym1 with two amino acid substitutions (N136D and Q140E) in the catalytic motif of WT-Ym1 shows that Ym1 inactivation was not caused merely by this change (Figure 1). We identified exons 6, 7, and 10 of Ym1 as the cause of the protein inactivation using Ym1-Chia chimeras (Figures 2 and 3). The Ym1 inactivation involves many amino acids, and loss of chitinolytic activity leads to substrate recognition/binding depletion (Figures 4 and 5). CLP genes in rodents frequently occur due to gene duplication (Figure 6). We have also shown that the *Ym1*/*Ym2* family accumulates many nonsynonymous single nucleotide polymorphisms (nsSNPs) under positive selection, despite being similar to *Chia* (Figure 7

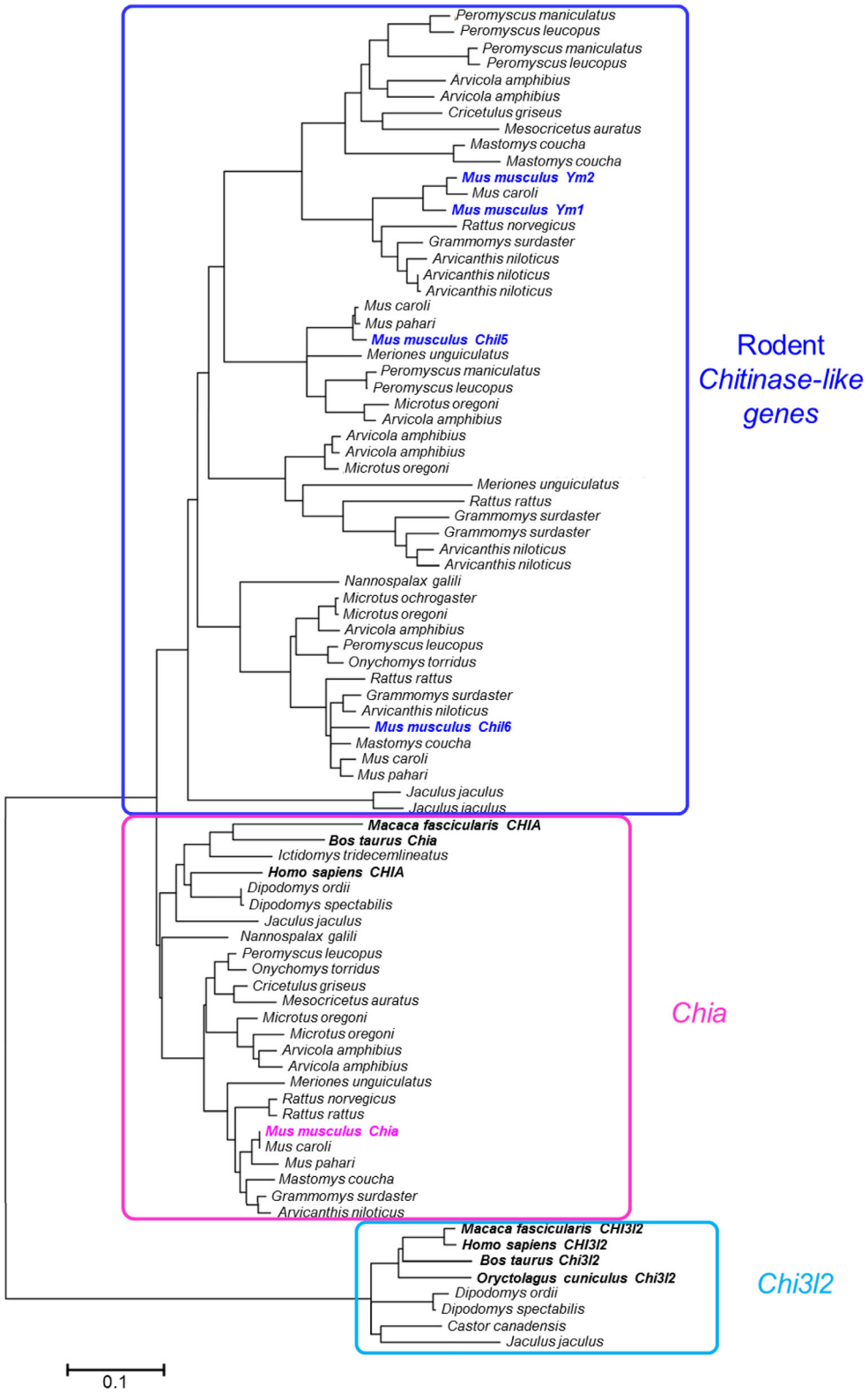

FIGURE 7 Phylogenetic relationship of CLP gene homologs. Phylogenetic reconstruction of rodent CLP gene and mammals *Chia* and *Chi3l2* using the neighbor-joining algorithm. Scores for 1000 bootstrap iterations over 60 are indicated.

and Table 1). Based on these results, we constructed a conceptual diagram of the Chia, and Ym1 evolutionary process (Figure 8). The evolution of the rodent *Chia* was under purifying selection and was not inactivated by nsSNPs (Figure 8, left). In contrast, *Ym1* evolved under positive selection and accumulated nsSNPs resulting in the dysfunction of the catalytic motif, exons 6 and 7, exon 10, and the introduction of a stop codon into exon 11 (Figure 8, right). Thus, Ym1 exons 6–10 have each evolved to affect chitinolytic activity.

TABLE 1 Results of evolutionary analysis of *Chia* and *Ym1* using PAML CODEML site model.

Gene family	Model	lnL	np	TL	*k*	Parameter estimates	Test	LR	*p*-Value	Positively selected sites
Chia	M0	−4242.53	19	1.01	3.34	$\omega = 0.249$				
	M1a	−4192.52	20	1.03	3.45	$\omega_0 = 0.0321$ (75.6%)				
						$\omega_1 = 1.00$ (24.5%)				
	M2a	−4192.38	22	1.03	3.47	$\omega_0 = 0.0342$ (75.8%)	M1a vs. M2a	0.279	0.870	Not allowed
						$\omega_1 = 1.00$ (23.7%)				
						$\omega_2 = 2.95$ (0.0442%)				
	M7	−4192.65	20	1.03	3.43	$p = 0.0569$				
						$q = 0.159$				
	M8	−4192.29	22	1.03	3.46	$p_0 = 0.991$	M7 vs. M8	0.729	0.695	230 K (0.955*)
						$p = 0.0715$				
						$q = 0.213$				
						$\omega = 2.58$ (0.946%)				
Ym1/ Ym2	M0	−6141.83	25	2.57	1.91	ω =0.556				
	M1a	−6049.96	26	2.67	1.89	$\omega_0 = 0.106$ (53.0%)				
						$\omega_1 = 1.00$ (47.0%)				
	M2a	−6040.86	28	2.72	1.95	$\omega_0 = 0.108$ (51.2%)	M1a vs. M2a	18.2	$p < 0.001$	171 V (0.994**), 302S (0.958*)
						$\omega_1 = 1.00$ (45.9%)				
						$\omega_2 = 3.57$ (2.92%)				
	M7	−6054.11	26	2.67	1.87	$p = 0.234$				
						$q = 0.219$				
	M8	−6042.64	28	2.71	1.95	$p_0 = 0.951$	M7 vs. M8	22.9	$p < 0.001$	171 V (0.996**), 302S (0.972*)
						$p = 0.282$				
						$q = 0.287$				
						$\omega = 2.91$(4.88%)				

Note: Positively selected sites (*: $p > 95\%$; **: $p > 99\%$).

The single exon substitution of Chia CatD by Ym1 sequences has revealed that Ym1 exons 6 and 9 increase, exons 7 and 8 decrease, and exon 10 eliminates the chitinase activity. Thus, we revealed that the causes of Ym1 inactivation are amino acid substitutions at (i) catalytic residues, (ii) exons 6 and 7, and (iii) exon 10, suggesting that reactivation of Ym1 requires amino acid substitutions of at least 29 residues.

The three-dimensional structures of Ym1 and Chia are very similar (Figure 1b). Examining the coding region from exons 6 to 10 by crystallographic analysis of Ym1, exons 6, 7, and 10 forms a substrate-binding groove (Figures S5A,B,D). In contrast, regions encoded by exons 8 and 9 are not involved in chitin-binding groove formation (Figure S5C). Chimeras in which Chia's exons 6 and 7 (C10) or exon 10 (C9) were replaced with Ym1 were not active (Figure 3a,b), suggesting that the conformation of the substrate-binding groove was affected by the amino acid substitution, resulting in the loss of chitinase activity. In contrast, chimeras C17 and C19 showed chitinase activity (Figures 3e and 4a,b). C17 had the exons 6, 7, and 10 of MT-Ym1 replaced with Chia sequences (Figure 3e). C19 was the MT-Ym1 with Chia exon 6 and 4 amino acid substitutions in both exon 7 (Q208H, D216G, P217S, and K218W) and exon 10 (L354F, V358M, V359I, and P361A) (Figure 4a). AlphaFold2 simulations showed that Chia and Ym1 had a slightly different structure of the substrate-binding groove and varied even upon single amino acid substitutions (Figure S6A,B). However, simulations in C17 and C19 showed substrate-binding groove

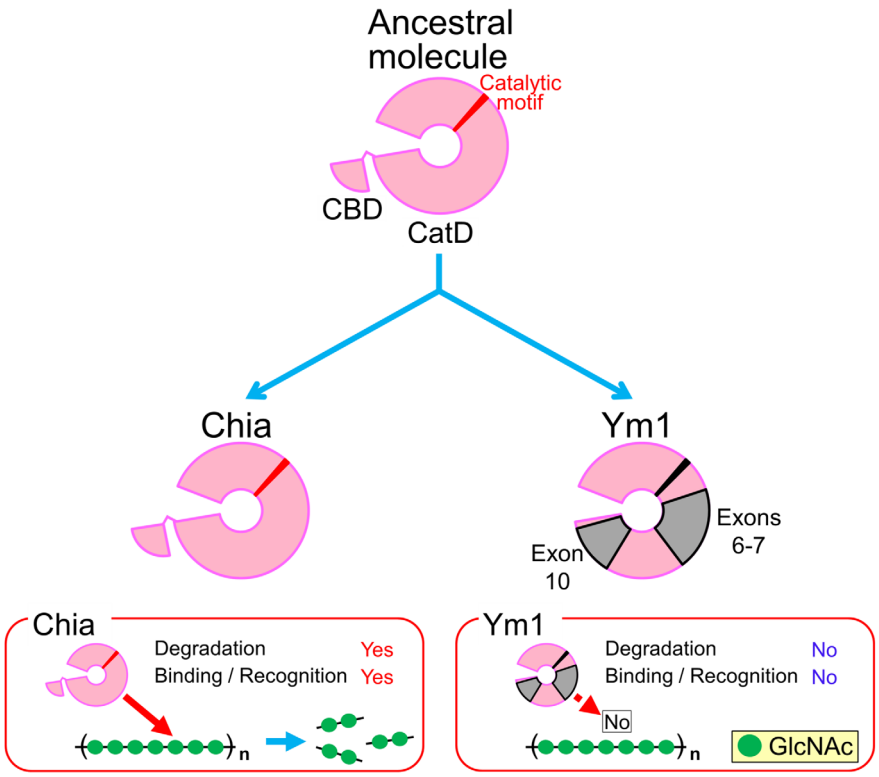

FIGURE 8 The process of evolutionary inactivation of Ym1 in mice. Chia has evolved from its ancestral molecule to retain its function as chitinase. In contrast, Ym1 is functionally modified by introducing amino acid substitutions into the sites involved in recognizing, binding and degrading chitin substrates in CatD. Ym1 also lost CBD by introducing a stop codon. The black region in each schematic representation indicates a loss of function.

structures similar to those of Chia. Thus, multiple amino acid substitutions into exons 6, 7, and 10 of MT-Ym1 "opened" the chitin-binding groove, allowing recognition, binding, and degradation of chitin substrates.

Our recent studies activated human CHIA (AMCase) and dog Chia by 1 or 2 amino acid substitutions, respectively (Okawa et al., 2016; Tabata et al., 2022). In contrast, Ym1 requires multiple sequence modifications indicating that Ym1 has evolved to lose the chitin-degrading activity actively. Therefore, it is highly unlikely that Ym1 could be reactivated during a short-term evolution.

Chia is a crucial digestive enzyme in the stomach of omnivorous animals, including mice, while in carnivorous and herbivorous mammals with reduced insect consumption, the functional constraints of the Chia paralog were relaxed and pseudogenized (Tabata et al., 2022; Tabata et al., 2018b; Emerling et al., 2018). Rodent Ym1, on the other hand, is functionally inactivated under positive selection. They, however, maintain their chitinase frame, and their expression levels increase under pathological conditions. In addition, the Ym1 expression level in mouse stomachs is less than 1/10,000 of that of the *Chia* gene (Ohno et al., 2014), suggesting that Ym1 does not play a relevant role in food digestion in mice. Thus, Ym1 is thought to have evolved to actively lose its chitinolytic activity and acquire distinct roles in pathologies such as inflammatory conditions and immune responses in mouse lung tissues.

Mouse Ym1 and Ym2 are paralogs that emerged from recent gene duplication. These CLPs are very similar, and the amino acid sequences of the three regions involved in Ym1 inactivation are highly conserved. However, Ym1 and Ym2 are expressed in different lung cells in allergic mice and may have different roles in related inflammation (Parkinson et al., 2021). Therefore, the different physiological roles of Ym1 and Ym2 in mouse lungs may be attributed to their slightly different amino acid sequences. This dissimilarity could be a source of another inactivating mechanism in Ym2. The same hypothesis could be applied to other mouse CLP genes.

Ym1 is an interesting and potentially important protein, and more research is needed to understand its biological functions and potential therapeutic applications fully. Mammals possess various CLP genes, which are overexpressed under different pathological conditions. Our results may open new avenues toward understanding the pathophysiological function of CLPs and their utilization for medical purposes (e.g., disease markers).

4 | MATERIALS AND METHODS

4.1 | Construction of Ym1 vector for *E. coli* expression

Mature Ym1 was amplified from mouse lung tissue cDNA by PCR using KOD Plus DNA polymerase (Toyobo, Kyoto,

Japan) and oligonucleotide primers (Eurofins Genomics, Tokyo, Japan) anchored with the restriction sites for EcoRI and XhoI as described previously (Table S2) (Kashimura et al., 2013). We digested the amplified DNA fragments with EcoRI and XhoI and cloned them into the same sites of the pEZZ18/pre-Protein A-AMCase-V5-His (Kashimura et al., 2013). We then confirmed the entire nucleotide sequence of the resulting plasmid pEZZ18/Ym1/V5-His by sequencing (Eurofins Genomics). We constructed MT-Ym1 and Ym1-Chia chimeras by site-directed mutagenesis by primer extension (Okawa et al., 2016).

4.2 | Preparation of recombinant mouse Chia CatD and Ym1 proteins expressed in *E. coli*

Preparation of Protein A-Chia CatD-V5-His (Kashimura et al., 2015) and Protein A-Ym1-V5-His from the *E. coli* was performed and purified by IgG Sepharose described previously (Kashimura et al., 2013).

4.3 | SDS-polyacrylamide gel electrophoresis and Western blot

The protein fractions were analyzed using standard SDS-polyacrylamide gel electrophoresis (PAGE), followed by Western blot using an anti-V5-HRP monoclonal antibody (Invitrogen, Carlsbad, CA, USA). According to the manufacturer's instructions, the immunoblots were analyzed and quantified using a Luminescent Image Analyzer (Amersham ImageQuant 800, GE Healthcare) (Figure S3 and Table S1).

4.4 | Chitinase activity

We determined the chitinase activity using the fluorogenic substrate of 4-MU-$(GlcNAc)_2$ (Sigma-Aldrich, St. Louis, MO, USA) in McIlvaine's buffer (0.1 M citric acid and 0.2 M Na_2HPO_4; pH 2.0, 5.0, and 7.0) at 37°C for 30 min as described previously (Okawa et al., 2016). The fluorescence of released 4-MU was measured using a Glo-Max Discover Multimode Microplate Reader (Promega, Madison, WI, USA) with excitation at 365 nm and emission at 445 nm. The product (4-MU) was estimated using a standard curve based on 4-MU (Sigma-Aldrich).

4.5 | Chitin binding assay

Chitin binding assays are performed as described previously (Kashimura et al., 2015; Tabata et al., 2019). Briefly, column equilibration was performed using McIlvaine buffer (pH 7.0) and 0.5 M NaCl. Bound protein was eluted with 8 M urea in McIlvaine buffer (pH 7.0), followed by SDS-sample buffer (Laemmli, 1970).

4.6 | Phylogenetic analysis

Mammalian chromosomal information and cDNA sequences were retrieved from the NCBI GenBank (Tables S3–S5). A phylogenetic tree was constructed by MEGA X (http://www.megasoftware.net) (Kumar et al., 2018). The phylogenetic tree was constructed by the unweighted pair group method with arithmetic mean (UPGMA). Rate ratios of nonsynonymous-to-synonymous substitutions d*N*/d*S* were calculated using the CODEML program of PAML to compare the rodent Ym1 and Chia as described (Tabata et al., 2022). We used a site model, which allows the ω ratio to vary among sites to detect signatures of positive selection at each codon. Likelihood ratio tests (LRTs) using a χ^2 distribution with two degrees of freedom were conducted to test for positive selection between each pair of models. To test for site-specific positive selection among all gene sets, we fit two sets of nested models: (1) discrete ω ratio models M1a vs. M2a, the null model M1a allows two categories of codon sites in p_0 and p_1 proportions, with $\omega_0 < 1$ and $\omega_1 = 1$, whereas the alternative model M2a allows an additional category of codons (p_2) with $\omega_2 > 1$, indicating positive selection; and (2) continuous ω ratio models M7 versus M8, which M7 specifies a null model with a beta distribution, beta (p, q), of ω values between 0 and 1, and M8 specifies an alternative model with an additional category for sites that have $\omega > 1$, indicating positive selection.

4.7 | Statistical analysis

The Student's *t*-test compared biochemical data at each pH. We carried out experiments in triplicate for statistical analysis.

AUTHOR CONTRIBUTIONS

Kazuaki Okawa: Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); writing – original draft (equal); writing – review and editing (equal). **Eri Tabata:** Formal analysis (equal); investigation (equal); methodology (equal); validation (equal); writing – review and editing (equal). **Yuta Kida:** Investigation (equal); methodology (equal); validation (equal). **Kyohei Uno:** Investigation (equal); methodology (equal); validation

(equal). **Hidetoshi Suzuki:** Investigation (equal); methodology (equal); validation (equal). **Minori Kamaya:** Investigation (equal); methodology (equal); validation (equal). **Peter O. Bauer:** Conceptualization (equal); methodology (equal); validation (equal); writing – review and editing (equal). **Fumitaka Oyama:** Conceptualization (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); supervision (equal); validation (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest with the contents of this article.

DATA AVAILABILITY STATEMENT

Data supporting the reported results will be available from the corresponding author (Fumitaka Oyama).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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