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Transglutaminase 2 regulates NF-κB activity through polyamination of COMMD1

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Transglutaminase 2 regulates NF-κB activity through polyamination of COMMD1

By

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Abstract

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Transglutaminase 2 (TG2) is a calcium-dependent enzyme that catalyzes polyamination of glutamine residues in protein substrates. COMMD (copper metabolism MURR1 domain-containing) family is associated with many biological processes. COMMD1 is the best studied isotype and is known to regulate NF-κB activity via promoting ubiquitin-dependent proteasomal degradation of p65. In numerous previous reports, TG2 has been reported to be involved in various pathophysiologies through NF-κB activation including tumorigenesis, inflammation, and fibrosis. However, the exact regulation mechanism of TG2 in NF-κB activation has not been fully elucidated. In this study, we found that all human COMMD paralogues, COMMD1 to COMMD10, except COMMD6 are polyaminated by TG2. Among them, we identified two modifiable glutamine (Q) residues in COMMD1 by TG2, using site-direct mutagenesis and mass spectrometry analysis, which were Q71 and Q112. We showed that 6xNF-κB-luc reporter activity was more down-

regulated by overexpression of polyamination-defective mutants of COMMD1 (Q71N and/or Q112N) and up-regulated by overexpression of its polyamination-mimic mutants (Q71R and/or Q112R) in 293 cells. In addition, overexpression of Q71N and/or Q112N COMMD1 in 293 cells and HeLa cells decreased the expression levels of CXCL1, CXCL5, CXCL8 and CXCL10 compared to overexpression of wild-type COMMD1. Interestingly, cells overexpressing Q71R and/or Q112R COMMD1, which are considered to mimic COMMD1 polyamination, showed higher mRNA expression levels of CXCL1, CXCL5, CXCL8 and CXCL10 than cells overexpressing wild-type COMMD1. Furthermore, ubiquitination assay revealed that Q71N and/or Q112N COMMD1 enhanced poly-ubiquitination of p65 in cells when compared to wild-type COMMD1. Therefore, these results indicate that polyamination of Q71 and Q112 of COMMD1 is responsible for TG2-mediated NF-κB activation.

Key words : Transglutaminase 2, NF-κB signaling, COMMD proteins, Polyamination

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INTRODUCTION

1. Transglutaminase

Transglutaminases (TGs) are members of calcium dependent enzyme and comprise a eight enzymes and one structural protein (Band 4.2) that lacks catalytic activity. TG incorporates primary amino groups of various compounds into glutaminyl side chains in proteins, catalyze post-translational modification of glutamine (Q) site of protein. There are three major function of TGs. It crosslinks between glutamine and lysine residue with isopeptide bond, catalyze polyamination and deamidation in glutamine residue (1)

TGs are involved in many biological processes such as blood clotting, skin barrier formation and extracellular matrix (ECM) assembly. TG participate in such activities by its protein post-translational modification potential. TGs can contribute to pathophysiological process such as inflammation, autoimmune and degenerative conditions. Some member of TG family have possibility that participate in biological process such as cell signal transduction like NF-κB signaling.

The human TG family consists of eight members. Eight of TGs (TG1 through TG7 and Factor XIIIa) are calcium dependent enzyme and have transamidation activity. One of it (Band 4.2) is cytoskeletal protein of erythrocyte, which has no enzymatic activity. Characteristic of TG family members are described in following *Table 1.*

TG1 (encoded by the gene *TGM1* on chromosome 14q11.2-13) is membrane associated transglutaminase that found in outermost layer of skin (epidermis). TG1 is involved in formation of the cornified cell envelope, which is structure that protective barrier between the body and environment. It also associated with adherent junction of epithelial cells in lung, kidney and liver. TG1 provides strength and stability to the tissue (2) TG1 synthesized as inactive zymogen and typically bound in membrane when cell proliferation or differentiation. When terminal differentiation of cell, TG1 undergo proteolysis and its activity is increase several fold. TG1 functions mainly in the formation of cornified cell envelope in differentiated keratinocytes. Mutations in the gene encoding TG1 are responsible for various types of autosomal recessive congenital ichthyosis (ARCI), such as lamellar ichthyosis (LI),

congenital ichthyosiform etythroderma (CIE) and some minor variants of ARCI (3). That finding indicates that TG1 not only has its transamidation activity, but also associated with lipid and protein barrier formation.

TG2 (encoded by the gene *TGM2* on human chromosome 20q 11-12) is a 74 kDa to 80kDa protein and found in both intracellular and extracellular spaces of various types of mammalian tissue (4). It found in many different organs including the heart, the liver, and the small intestine. Intracellular TG2 is abundant in the cytosol, nuclear and surface membrane, but it is not present in mitochondria (5). TG2 is a multifunctional enzyme that involved in several distinct biomedical functions at various cellular locations. It has transamidating, hydrolyzing and guanosine 5'-triphosphate (GTP) binding activities that mediates signal transduction (6). Transamidation activity can inhibited by GTP binding capacity. In addition to its intracellular activity, TG2 can externalized form cell (7) and mediates the interaction of integrin with fibronectin and crosslinks extracellular matrix (ECM)proteins to promotes cell-matrix interaction and matrix stabilization (8). Which is important in angiogenesis, wound healing and bone remodeling.

TG3 (encoded by *TGM3* on human chromosome 20q11-12) is widely expressed in epidermis and hair follicle (9). As it synthesized as inactive zymogen, requiring Cathepsin L's proteolysis for activation (10). When epidermal cell undergo terminal differentiation stage, TG3 establish outermost layer of the epidermis called the stratum corneum, certain of their proteins covalently cross linked. TG3 largely responsible for the resistance of skin to external environment. Despite these importance, a mouse model deficient in TG3 display no defect in skin development, no overt changes in barrier function or ability to heal wounds. In contrast in hair formation, TG3 deficient mouse has thinner and hair protein cross-linking is markedly decreased. While TG3 has unique functional importance in hair, in the epidermis TG3 can be compensated for by other TG family members (11).

TG4 (encoded by *TGM4* on human chromosome 3q21-22) distinct from other TG family members, TG4 is an abundantly expressed in prostate and prostatic fluid and its expression androgen-regulated (12). Not only fertility enhancing effect, TG4 has been implicated in prostate cancer progression. Overexpression of TG4 is reported in prostate cancer cell, it might be involved in prostate cancer cell

growth, migration, invasion, tumor-epithelial cell interaction and epithelial-mesenchymal transition (13). TG4 could function as a biomarker of aggressive cancer or potential therapeutic target (14).

TG5 (encoded by TGM5 on human chromosome 15q15.2) is similar to TG1 and TG3 in expression pattern and its role. It mainly expressed in epidermis and associated with tissue function (15). Even though the expression pattern of TG5 is not restricted to the epidermis, previous study suggest that TG5 probably plays a role in keratinocyte differentiation and cell envelope assembly. And transcription of TG5 can induced by keratinocyte differentiation agents (16). Like other TG members, TG5 involved in protein crosslinking and required for structural integrity of the outermost epithelial layers. Mutation in TGM5 can causes peeling skin syndrome (17).

TG6 (encoded by *TGM6* on human chromosome 20q11) is expressed in human central nervous system, human carcinoma cell line with neuronal characteristic and mouse brain. Due to alternative splicing, not only Full-sized proteins but also small sized proteins exist. In previous report, TG6 can allosterically regulated by Ca^{2+} and GTP. Molecular modelling suggest that TG6 has Ca^{2+} and GDP binding site related to those of TG2 and TG3. Temporal and spatial analysis in mouse indicates that TG6 is associate with neurogenesis (18).

TG7 (encoded by *TGM7* on chromosome 15q15.32) required for protein stabilization of protein assemblies. Little is known of the role of TG7, but its defects are highly related with TG5 mutant model.

Factor XIIIa (encoded by *F13A1* on human chromosome 6q24-25) also called fibrin stabilizing factor. It has two forms: a plasmatic form that flows freely in the blood plasma and a cellular from expressed in hematopoietic cells and placenta. Factor XIII is zymogen and activated by thrombin dependent proteolysis into Factor XIIIa requires Ca^{2+} as a cofactor. It required for blood coagulation system that crosslinking fibrin, defect of Factor XIIIa can affect clot stability (19).

Band 4.2 (encoded by *EPB42* on human chromosome 15q15.2) is TG-like protein that has strong sequence identity with other TG members. but Band 4.2 has no catalytic activity because of its active site cysteine (Cys) is substituted with alanine (Ala). It can found in erythrocyte membrane, as a cytoskeleton protein and it probably has a role in erythrocyte shape and mechanical property regulation.

Mutations in the Band 4.2 are associated with recessive spherocytic elliptocytosis and recessively transmitted hereditary hemolytic anemia (20).

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Table 1. Transglutaminase family

Gene	Protein	Chromosomal location	Molecular mass (kDa)	Main function	Tissue Distribution	Alternative Names
TGM1	**TG1**	14q11.2	90	Cell envelope formation during keratinocyte differentiation	Membrane-bound keratinocytes	TG_K, Keratinocyte TG, particulate TG
TGM2	**TG2**	20q11-12	80	Apotosis, cell adhesion, matrix stabilization and signal transduction	Many tissues: cytosol, nuclear, membrane and extracellular space	Tissue TG, TG_c, endothelial TG, Erythrocyte TG
TGM3	**TG3**	20q11-12	77	Cell envelope formation during keratinocyte differentiation	Hair follicle, epidermis and brain	TG_E, Callus TG, Hair follicle TG
TGM4	**TG4**	3q21-22	77	Reproduction, especially in rodents as a result of semen coagulation	Prostate	TG_P, androgen-regulated major secretory protein, vesciculase, Dorsal prostate protein 1
TGM5	**TG5**	15q15.2	81	Cell envelope formation in keratinocytes	Foreskin keratinocytes, epithelialbarrier and skeletal muscle	TG_X
TGM6	**TG6**	20q11	78	Unknown	Testis and lung	TG_y
TGM7	**TG7**	15q15.2	81	Unknown	Ubiquitous, predominantely in testis and lung	TG_z
F13A1	**FXIIIa**	6q24-25	83	Blood clotting, wound healing and bone formation	Platelets, placenta, synovial fluid, chondrocytes, astrocytes, macrophage, osteoclast and osteoblast	Fibrin-stabilizing factor, fibrinoligase, plasma TG, Laki-lorand factor
EPB4.2	**Band4.2**	15q15.2	72	Membrane integrity, cell attachment and signal transduction	Erythrocyte membrane, bone marrow, spleen	B4.2, ATP-binding erythrocyte membrane protein band 4.2

Modified from “Transglutaminase: crosslinking enzymes with pleiotropic functions.” (21)

1.1 Catalytic activity of Transglutaminases

Human genome encodes nine members of the Transglutaminase (TG) family and although one of them (Band4.2) lacks its enzymatic activity. The catalytic mechanism of TG members is highly conserved and related to cysteine protease. This process can be described in two step. First, when TG activates, cysteine residue (Cys277 in human TG2) in catalytic core domain attacks γ-glutaminyl residues of proteins or peptides (substrate A) and make thioester intermediate. Second, acylated TG react as an amine donor, it could be an ε-lysine residue from another protein, peptide or a small molecule (substrate B) amine. As a result of reaction, TG can make isopeptide bond between two substrate molecules and cross-linked product dissociated from enzyme. After all reaction, TG's active site is regenerated, allowing TG to participate another two steps enzymatic reaction. In the absence of suitable amine donor, the thioester intermediate can be hydrolyzed and make glutamic acid (E), which corresponds to 'deamidation' of substrate (22).

The enzymatic activity of TG can be devided into tree types: transamidation, esterification and hydrolysis. The direction of reaction is determined by its substrate (substrate B). In the case of substrate B has primary amine group, transamidation reaction occurs. Esterification and hydrolysis occurs substrate B contain an alcohol or water group respectively. Transamidation reaction can subclassified into tree: Crosslinking, amine incorporation and acylation. In addition, hydrolysis reaction subclassified into deamidation and isopeptide cleavage reactions. In TG-catalyzed deamidation reaction, a single amino acid change in substrate (neutral glutamine to negatively charged Glutamic acid) contributes only 2kcal/mol change in thermodynamic properties of the side chain, but it can cause a 100-fold shift in the substrate's equilibrium and affects protein conformation, stability, solubility and interaction with macromolecules. In this way, imposing positive charge on a neutral glutamine side chain with amine incorporation can influence the biochemical properties of the target protein (21). If covalent iso-peptide bond formed with crosslinking, the substrate can be resistant to proteolysis and increase its stability. As a result, the supramolecular structures may increase, which can provide structural resistance against proteolytic degradation.

These kinds of reaction is essential for physiological function such as blood coagulation, skin barrier formation and ECM stabilization. Reaction can be occurred by other TGs and its specific substrates. If same substrate encounters different TGs, different products may create with their own reactions. In addition to enzymatic activity of TGs, some of TG members have actions that are unrelated to transamidase activity. As an example of this, TG2, TG4 and TG5 can bind with GTP and have GTPase activity.

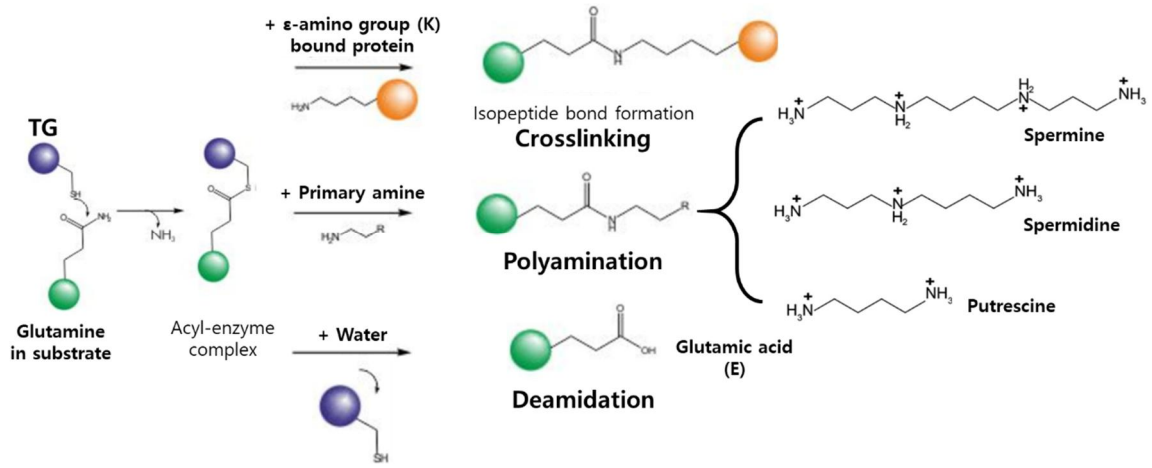


Figure 1. Catalytic activity of transglutaminase

Reaction pathway of transglutaminase. Transglutaminase catalyze isopeptide bond formation, polyamination and deamidation on specific glutamine site in its substrate.

1.2 Genomic organization and structure of Transglutaminases

Among transglutaminase members, Factor XIIIa (*F13A1*) and TG1 (*TGM1*) encoding genes are consist of 15 exons and 14 introns. Whereas other members from TG2 –TG7 (*TGM2-TGM7*) and Band 4.2 (*EPB4.2*) consist of 13 exons and 12 introns (Figure 2). *F13A1* and *TGM1* gene have non-coding exon 1 region and encode NH_2-termianl peptide from exon 2. After translation, these propeptide have to cleaved to get its TG activity. And one intron is present between exon 10 and 11 of *F13A1* and *TGM1* gene, but there are absent in *TGM2-TGM7* and *EPB4.2*.

All TG members have shares four distinct domain, which are structurally conserved N-terminal, β-sandwich, catalytic core subunit, and two β-barrel domain of C-terminal domain (in order of its sequence from N-terminal) (Figure 3A). In catalytic core domain, transition-state-stabilizing tryptophan (Trp), catalytic triad cysteine (Cys), histidine (His) and asparatic acid (Asp) are located in core protein. Among TG members, TG2 undergo a dramatic conformational change from closed to open form (Figure 3B). In order to TG2 have its activity, it is necessary to expose the active site with open conformation (23). With this distinct character, this paper will cover the TG family.

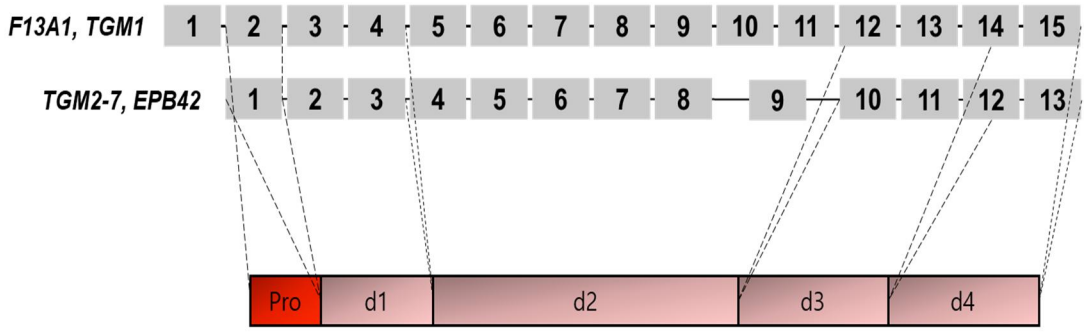
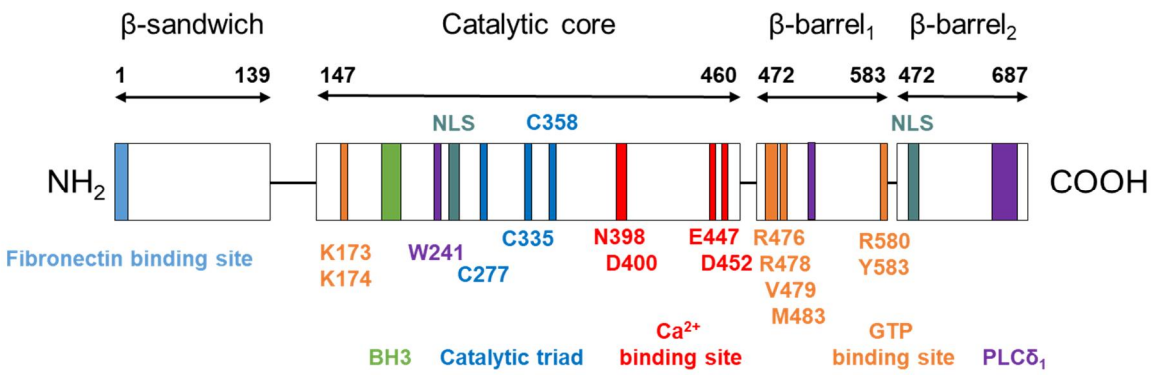


Figure 2. Genomic organization of Transglutaminases

Modified from 'Transglutaminases: lessons from genetically engineered mouse models and inherited disorders." (24)

(A)



(B)

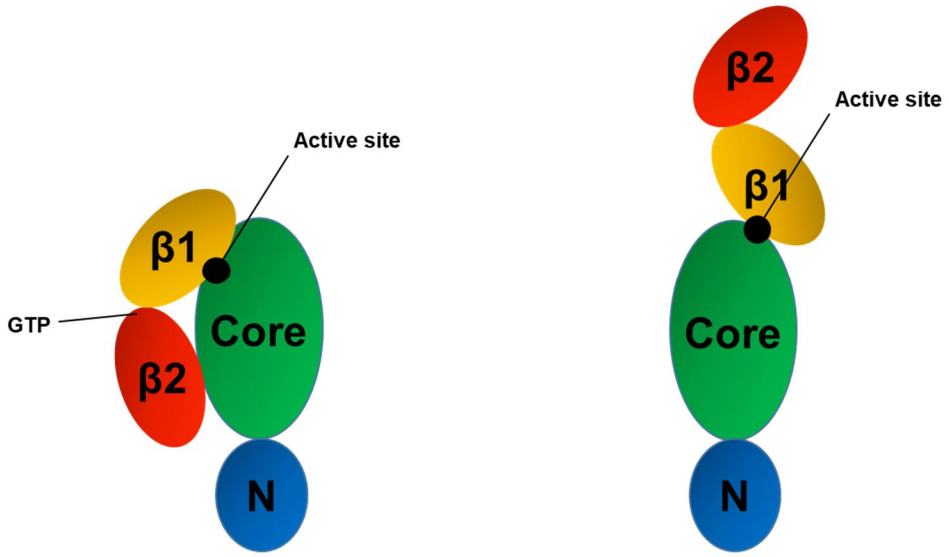


Figure 3. Transglutaminase protein domains, functional element and tertiary structure.

A) Domain of 'Transglutaminase. Modified from " Transglutaminase 2: an enigmatic enzyme with diverse functions." (25)

B) tertiary structure of transglutaminase. Modified from "Transglutaminase 2 Undergoes a Large Conformational Change upon Activation." (23)

2. Transglutaminase 2

Transglutaminase 2 is the first member of TG that discovered and researched intensively for many years however, its pathological role is not clear. TG2 is distinguished form other TG members with several unique characteristic. Ubiquitously expressed, bind and hydrolyze guanine nucleotides. TG2 involved in variety of cellular processes, including cell differentiation, migration, cell death, inflammation and wound healing.

2.1 Distribution and subcellular localization of Transglutaminase 2

TG2 expressed throughout all body due to its ubiquitous expression and widespread localization. Most of cells including endothelium, smooth muscle cells fibroblast and immune cell express TG2 and its expression level is different depends on its cell type. As an example of this, TG2 is constitutively expressed in mesangial cells, colonic pericryptal fibroblast, renomedullary epithelial cells and thymic subcapsular epithelial cells, while it is expressed only with stimulus in female breast epithelial cells (26). TG2 expressed both intracellular and extracellular location. In the cell, cytosolic TG2 is predominant type but it is present inner face of plasma, nuclear and mitochondrial membrane also. TG2 can bind to nuclear transporter importin-α3 and transported into the nucleus through this interaction (27). Notably, TG2 is not present inside of mitochondria (28). Extracellular TG2 is localized mainly in extracellular matrix or cell membrane associated with ECM (29). They involved in extracellular matrix stabilization, which are essential for wound healing, angiogenesis, and bone remodeling and reaction which requires TG2's transamidation activity.

2.2 Regulation of Transglutaminase 2 activity

Enzymatic activity of TG2 mainly regulated with Ca^{2+} and GTP in a reciprocal manner. When GTP binds, enzymatic activity of TG2 is inhibited and Ca2+ binding can prevent GTP mediate inhibition. When TG2 activated with Ca^{2+}, TG2 shows expended structure through its structural change, while bound with GTP shows compact form of TG2, which is enzymatic inactive state (4).

TG2 requires relatively high Ca^{2+} concentration to have its activity (21) and Ca^{2+} can be delivered from cytosol's intracellular stores or can be present extracellular environment. Latent TG activity can initiated or increased without newly synthesized TG2 protein.

2.3 Function of Transglutaminase 2

2.3.1 *Physiological function*

TG2 involved in many physiological process including cell adhesion, ECM formation and stabilization, cell cycle and apoptosis. Among these, TG2 play a key role in cell apoptosis. In various cell type, overexpression of TG2 leads to rapid cell death or become susceptible for apoptotic stimuli (30). As an example of this, expression of TG2 in cardiomyocytes is positively correlated with apoptotic signal under oxidative stress condition, whereas in TG2 knockdown model with small interfere RNA, shows decreased rate of cell apoptosis (31). In addition, TG2 is responsible for stabilizing apoptotic bodies with its crosslinking activity. Crosslinking of Nε (γ-glutamyl) lysine has been observed in apoptotic bodies. Taken together, Crosslinked protein polymers prevent intracellular contents leakage into surrounding tissue (32).

TG2 also involved in tissue repair by participating cell adhesion, ECM assembly, angiogenesis and basement membrane biogenesis. TG2 knockdown mouse model shows defective fibroblast adhesion in *in vitro* environment and impaired wound healing potential *in vivo*. Among ECM proteins, fibronectin, collagen type II, collagen type III, vitronectin, osteonectin and osteopontin are substrate of TG2. It is considered that TG2 secures ECM stability by making the matrix resistance for mechanical and biochemical stress.

2.3.2 *Pathological function*

Activity of latent TG2 is related to many pathologic processes including Celiac disease (Gluten sensitivity), cataract and inflammation. In celiac disease, anti-TG2 antibodies result in a form of gluten sensitivity in which a cellular response to *Triceae* glutens that are crosslinked to TG2 are able to

stimulate TG2 specific B-cell response that eventually result in the production of anti-TG2 antibodies IgA and IgG (33).

Age-related cataracts, can be caused by an aggregation of lens proteins (β-crystailin, αB crystalin and vimentin) *in situ* reaction with human lens epithelial cell, high level of oxidative stress can activate catalytic activity for TG2 and make protein aggregation via Transforming Growth Factor-β (TGF-β) mediate response (34).

It has also reported that TG2 can upregulate its activity with inflammation signal. As an example of this, pro inflammatory cytokine and growth factor such as tumor necrosis factor-α (TNFα) and TGF-β can enhance TG2 expression (35, 36)and increase its activity. This phenomenon leads to an increase polyamination in various substrate proteins (e.g., IκBα, PPAR-γ, Sp1), thereby promote an inflammatory response (37).

3. Regulation of NF-κB signal pathway

The nuclear factor-κB (NF-κB) family is the transcription factors that regulates various physiological process. It involved in wide arrange of gene expression, immunity and inflammation. In immune system, the central role of NF-κB signaling is activation of various immune cells by inducing pro-inflammatory mediator's transcription, such as cytokine, chemokine and cell adhesion molecules. This process consists defense system, and protect host from various external pathogens or injures. After immune system activation, NF-κB signaling has to be terminated in order to prevent chronic inflammation. Uncontrolled inflammation triggers chronic tissue damage (38), can caused of numerous disease such as atherosclerosis, rheumatoid arthritis, diabetes, and cancer (39). Therefore, NF-κB signaling and inflammatory response must be strictly controlled in terms of spatiotemporal.

3.1 NF-kB signal pathway

The Nuclear Factor κB (NF-κB) comprised of five subfamilies: RELA (p65), RELB, c-REL, p50/p105 (NF-κB) and p52/p100 (NF-κB2). NF-κB shares Rel Homology Domain (RHD domain) which is essential for various physiological events such as NF-κB dimerization, interaction with other proteins including IκB, nuclear translocation and DNA binding. Besides RHD domain, RELA, RELB and c-REL has Transcriptional activation domain (TAD domain) which is important for inducing NF-κB target genes transcription. This domain is absent in p50 and p52 therefore this two proteins function as NF-κB transcriptional repressor (40). In this signal pathway, whether NF-κB act as a transcriptional activator or repressor depends on the dimer combination of NF-κB members. When NF-κB dimer has at least one TAD domain, it can displace NF-κB repressor, such as histone deacetylase 1 or 2 (HDAC1 or 2) and recruit co-activator like histone diacetylase 3 (HDAC3) and CBP/p300 (41, 42). In contrast, p50 and p52 homodimers and its heterodimers have been shown transcriptional repressive function although both p50 and p52 bind to κB site. This function is elucidated with recruiting transcriptional repressor, HDAC1 (43, 44).

The activation of NF-κB can be classified into various signal pathway including Canonical (classical) and Non-Canonical (alternative) pathway. Which of the signaling event occurs depends on its stimuli type and combination of NF-κB in dimer complex. The NF-κB canonical pathway can activate by wide range of stimuli, such as pro-inflammatory cytokines, interleukin-1 (IL-1), tumor necrosis factor (TNF), lipopolysaccharide (LPS) and bacterial endotoxin. These stimuli induce formation of the RELA/p50 NF-κB dimer through various receptors: interleukin-1 receptor (IL-1R), TNF receptor (TNFR) and toll-like receptor 4 (TLR4) respectively. The non-canonical pathway can activated with lymphotoxin beta, CD40 ligand and B cell activating factor. This activation promote p100 processing, result in RELB/p52 complex formation and transcriptional activation (45, 46). Before canonical pathway activated, NF-κB dimer RELA/p50 dimer is kept in the cytosol binding with IκB proteins. This proteins retain RELA/p50 dimers in cytosol by masking their nuclear localization sequence (NLS) (Figure 4). This masking inhibits translocation of RELA/p50 dimers into nucleus. And it has been reported that IκB proteins, IκB

and IκBε can shuttle RELA/p50 dimers form nucleus to cytoplasm. This process is mediated by their nuclear exportation sequence (NES), which is absent in other IκB families.

The NF-κB signal transduction is start with IκB kinase (IKK) complex. Phosphorylation of the IκB proteins promote ubiquitination and proteasomal degradation of IκB, this result in nuclear translocation of RELA/p50 heterodimer. This complex bind to the target DNA sequence and regulate their gene transcription. The IKK complex consists of three subunit: two catalytic subunit with kinase activity (IKKα in IKK1 and IKKβ in IKK2) and regulatory subunit (IKKγ, also named NEMO) (47). IKK activation is mediated by various protein complexes underlying different receptor, which are responsible for NF-κB signal pathway activation (48).

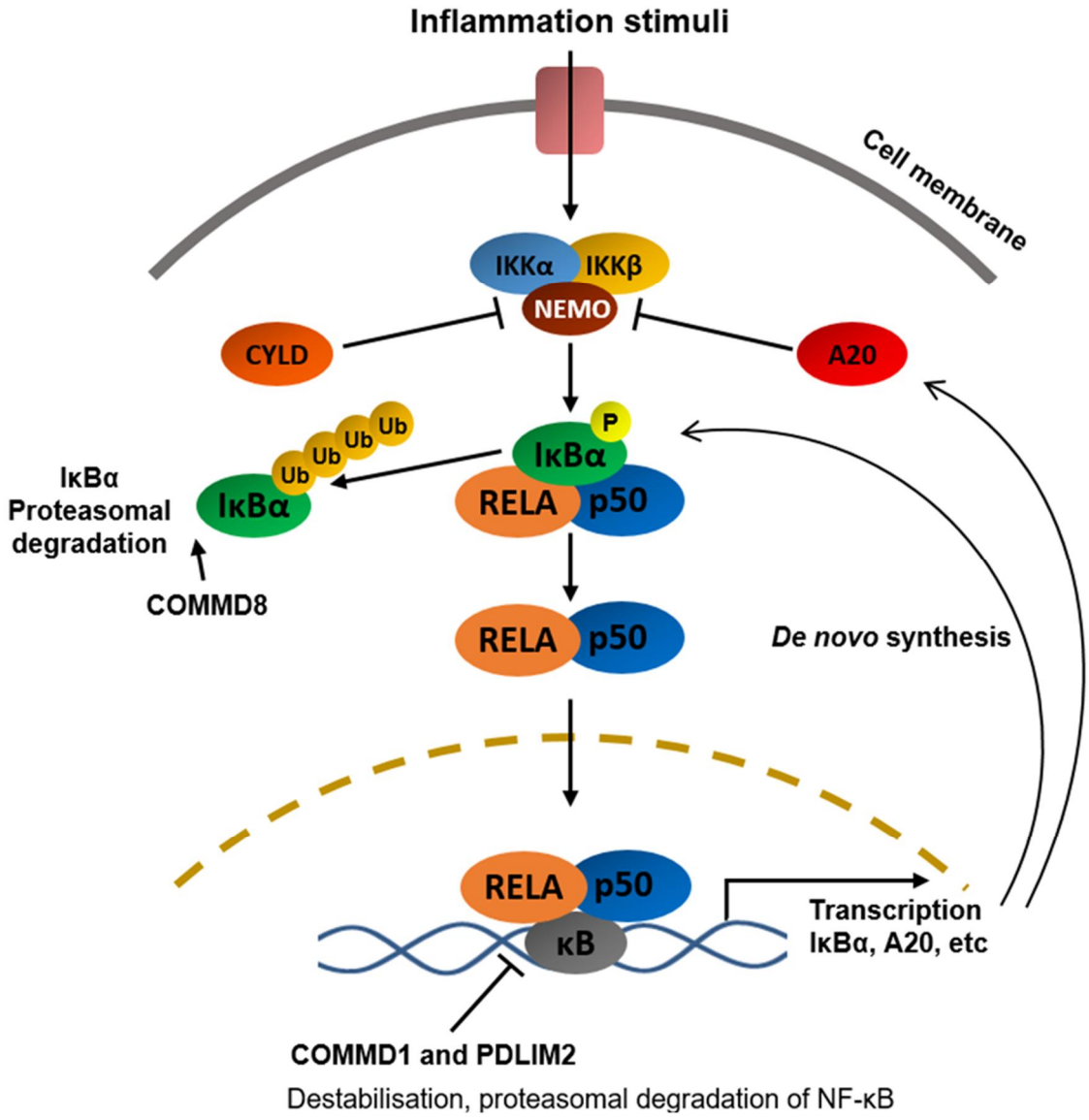


Figure 4. Simplified overview of NF-κB downregulation mechanism in cells.

Modified from "Tuning NF-κB activity: A touch of COMMD proteins." (49)

3.2 Downregulation of NF-κB signal pathway

On these days, only a few protein have been reported to negatively regulate NF-κB signaling (22, 23). Among them, IκB protein is most studied and well elucidated. When NF-κB activation, the expression of both IκBα and IκBε is increased as a negative feedback system. Although expression rate of IκBε is lower than IκBα, these two newly synthesized IκB proteins can sequestrate NF-κB complex into cytosol and inhibit induction of NF-κB signal pathway. On the other hands, phosphorylation of IκBβ can regulate NF-κB signaling either negatively and positively. Not only sequestrate NF-κB complex in cytosol, IκBβ can form DNA binding complex with RELA and prolong its target gene expression (50). In addition to IκB regulation, A20/TNFAIP3 also in negative feedback system in NF-κB signaling, A20 has both deubiquitinating, ubiquitinating activity. It removes remove K63-linked polyubiquitin chains in Receptor Interacting Protein 1 (RIP1) and promotes proteasomal degradation through formation of K48-linked polyubiquin chain. Not only RIP1, IKK regulators such as NEMO and TRAF8 can regulate by A20, these activity makes downregulation of canonical NF-κB signal pathway. In A20 conditional deficient mouse model, increased atherosclerotic lesion and hyper activation of immune cell such as Dendritic cells (DC), Macrophages, B cells and granulocytes are observed result in various inflammatory disease (51). Furthermore, A20 knockout mouse model shows uncontrolled inflammation and early death. Single nucleotide polymorphism (SNPs) in human A20 gene show several disease related with hyper inflammation such as rheumatoid arthritis (RA), psoriasis, celiac disease, Crohn's disease, systemic sclerosis and type 1 diabetes as its phenotype. In addition, defects of A20 gene has a important role in human B cell lymphoma (51).

Not only A20, culindromatosis (CYLD) also downregulates NF-κB signaling with deubiquitination activity. CYLD deubiquitinates several proteins including IKK regulation subunit NEMO and its upstream proteins such as RIP1, TAK1, TRAF2, 6 and 7. Like A20 protein, CYLD removes K-63 linked polyubiquitin chain and promotes proteasomal degradation of its target proteins. CYLD are essential for IKK activity downregulation, and variant CYLD deficient model in mouse shows uncontrolled inflammation and increased cancer generation (52). In human, CYLD mutations are result in development of cylindromas (53).

PDLIM2 also can directly inhibits NF-κB signaling. PDLIM is a nuclear protein that containing both postsymaptic density 65-discs large-zonula occludens 1 (PDZ) and abnormal cell linage 11-islet 1-mechanosensory abnormal (LIM) domains. It can subclassified in a large family of LIM proteins. PDLIM2 has E3 ligase activity in their LIM domain, act as a nuclear ubiquitin E3 ligase which targeting RELA. In addition, PDLIM2 targeted RELA sequestrate into intranuclear compartment promye-locytic leukemia protein (PML) and degraded by abundant proteasome inside. PDLIM2 downregulates NF-κB signaling with RELA ubiquitination and sequestration of nuclear RELA into PML nuclear bodies. PDLIM2 deficient mouse model shows uncontrolled NF-κB mediated immune response also. And it was reported that PDLIM2 is essential for the restrain of NF-κB activity which are inducted by TLR stimulation (54). Another factor for NF-κB inhibition, PIAS1 interferes NF-κB complex binding with κB binding site in target gene. Like PDLIM2 deficient mouse, PIAS1 null mice result in uncontrolled inflammation and hypersensitivity in endotoxin shock (55). In addition to these all protein, COMMD1 is a member of COMM protein family that identified as a NF-κB terminating agent in the level of DNA. Similar with PIAS1, COMMD1 inhibits transcription of NF-κB target genes.

4. COMMDs, a family of NF-κB regulators

With positional cloning strategy, COMMD1 identified in Bedlington terriers suffering from copper toxicosis (56). Copper toxicosis can developed by abnormal copper accumulation in the liver and its toxicity resulting in pathological changes. After discovery, COMMD1 belongs to a new family of proteins, called *CO*pper *M*etabolism gene *M*URR1 *D*omain-containing (COMMD) family (57). COMMD consists of 10 members of protein: from COMMD1 to COMMD10 and highly conserved in multicellular organism and in some protozoa. Structure of COMMD proteins are highly conserved and there is structurally conserved *CO*pper *M*etabolism gene *M*URR1 (COMM) domain in their carboxyl-terminal (57). COMMD domain is essential for protein-protein interaction between its specific interaction partners, and regulates many physiological changes as a platform for COMMD interaction.

In contrast, amino-terminal is unique in each of members and in COMMD6, there is no N-terminal region exceptionally. COMMD6 considered the primitive type of COMMD family (Figure 5). All COMMD members, including COMMD1 have no catalytic activity and they can interact with COMMD1 with their COMM domain (58). COMMD proteins are express ubiquitously, but its mRNA expression levels are varies between different tissues and there are no direct correlation between protein expression and its mRNA expression levels. All COMMD proteins can make direct interaction with their specific partners in NF-κB, and inhibit its activity. Only COMMD1 can interact with all of NF-κB subunits. Moreover, only and interaction between COMMD1 and IκB has been identified so far (59). This means each COMMD members can regulate NF-κB with distinct mechanism. In these days, more functions about COMMD1 has been elucidated, but its regulation, modification and about other COMMD members are not studied yet.

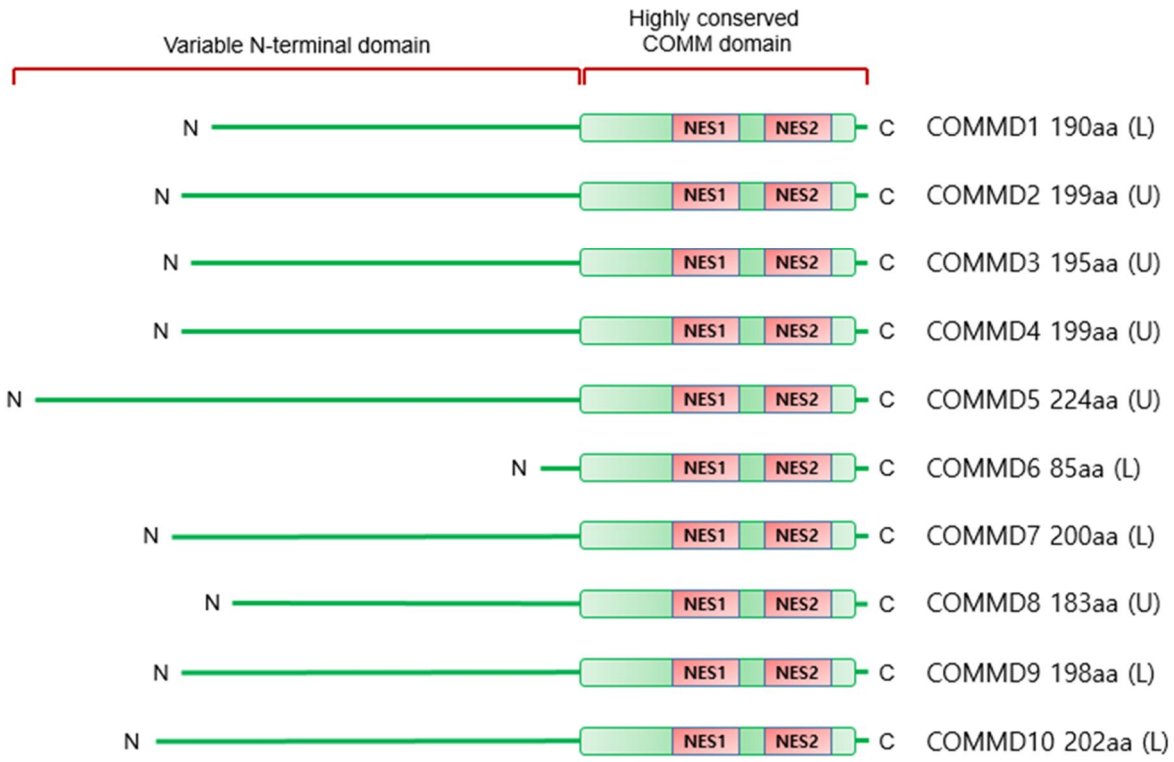


Figure 5. Structure of COMMD family proteins.

Size and simplified structure of COMMD family proteins. (L) means lethal and (U) means unknown in knockout phenotype.

Modified from "Tuning NF-κB activity: A touch of COMMD proteins." (49)

4.1 COMMD1 as a hub in NF-κB termination mechanism

Among COMMD protein members, detailed mechanism has only described for COMMD1. It promotes ubiquitin mediate proteasomal degradation of RELA in nuclear, which bound to DNA (57). In the situation where COMMD1 is depleted, result in prolonged NF-κB signal activation, increased nuclear levels of RELA and its target gene expression. COMMD1 also negatively regulate other NF-κB families such as RELB, p105 and p100. Like COMMD1 knockdown model, polyubiquitination level of RELA were decreased in COMMD6, 9 and 10 knockdown model (60). These data show that other COMMD members, including COMMD1 can participate in NF-κB signal termination by regulating the turnover of NF-κB families.

COMMD1 interacts with multimeric E3 ubiquitin ligase complex, called ECS^{SOCS1}. This complex consists of Elongin B, Elongin C, Cullin 2 and SOCS1 (Figure 6). COMMD1 facilitates interaction between SOCS1 in ECS^{SOCS1} complex and RELA, which result in increased RELA polyamination (60). In COMMD1 and RELA interaction, phosphorylation of RELA in serine 468 (ser468) is necessary and this modification is conducted by IKKα and IKKβ. In RELA S468A (substitution of Ser468 to Ala468) form, polyubiquitination and proteasomal degradation were completely prevented and NF-κB activation and its target gene expression were prolonged (61-63). Interestingly, even if RELA removed from its target promoter site, COMMD1 still exists in its target site (61). Through this result, COMMD1 occupies target promoter site after signal termination and it can inhibit NF-κB signal pathway with additional mechanism. Because COMMD1 has no DNA binding motif, it is assume that COMMD1 make complex with other proteins and occupies its target promoter site. However, composition of this complex has not elucidated.

4.2 Regulation of COMMD1

COMMD localized predominantly in cell cytosol, but in nuclear, they also detected in low levels (64). There is no nuclear localization signal (NLS) in COMMD1 and small size (21.7 kDa), they thought to be enter the nuclear with simple diffusion through the nuclear pores. However, COMMD1 has two

nuclear export signals (NESs) (Figure 5) and its exportation to cytosol is tightly regulated with CRM1 exportin in nuclear membrane (Figure 6). NES mutated COMMD1 shows increased NF-κB inhibition effect with abundant nuclear COMMD1.

Cytosolic COMMD1 can negatively regulated by X-linked inhibitor of apoptosis protein (XIAP) or redox sensor protein HSCARG (65). Both XIAP and HSCARG promote polyubiquination with K48-polyubiquitin chains and COMMD1 undergoes proteasomal degradation (Figure 6). With this mechanism, COMMD1 levels can controlled and thereby its function of NF-κB. With XIAP and HSCARG, tumor suppressor factor alternative reading frame (ARF) can regulate COMMD1 level with COMMD1 modification (66). ARF makes COMMD1 in stable by promoting K63-polyubiquitination and enhances its nuclear levels (Figure 6). In final, stress-induced small heat-shock chaperone secretory clusterin (sCLU) also mediates COMMD1's protein level (67). Normally, expression of sCLU protein is positively correlated with cellular survival signal in NF-κB, but it has negatively correlation with COMMD1 protein levels. In cancer cell, increased expression of sCLU enables survival of cancer cell against anti-cancer treatments. With this reslut, decreased COMMD1 level is observed in several kinds of cancers and the cancer which has lower COMMD1 expression shows increased invasion, metastasis and death rate of patients (68). Like this, expression of sCLU and COMMD1 are negatively correlated in various cancers, but its detail mechanism has not been elucidated.

As a result, function of COMMD1 can be modulated with various mechanism including protein, mRNA and nucleoplasmic transport regulation. And it can be activated with COMMD1 modification. So far, no other post-translational modification of COMMD1 have been reported except polyubiquitination.

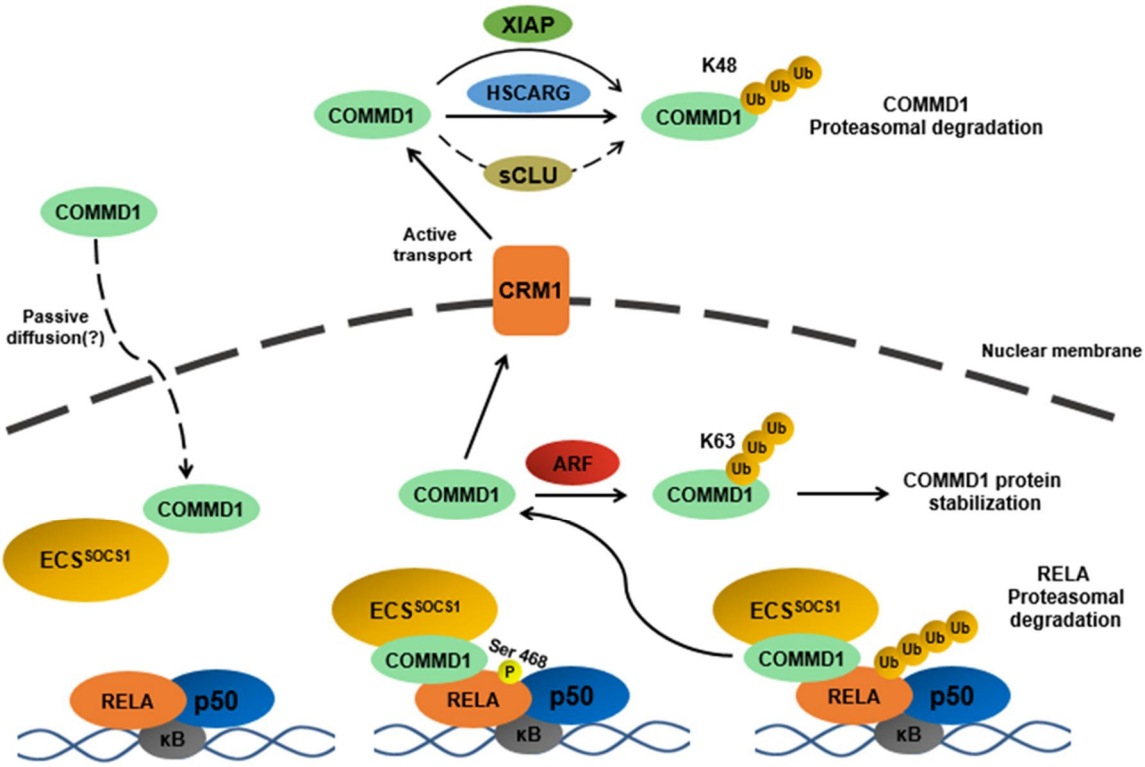


Figure 6. COMMD1 action in p65 degradation and its functional regulation.

Modified from "Tuning NF-κB activity: A touch of COMMD proteins." (49)

4.3 COMMD protein, as a binding partner of Cullins

All COMMD members can negatively regulate NF-κB signaling. And they can physically associate with Cullin as a binding partner (69). Cullin can act as scaffold protein of E3 ligase complex, and it forms Cullin-Ring-Ligases (CRLs) with RING box domain (Rbx1 or Rbx2). CRLs are largest family of ubiquitin ligases and they participate in ubiquitination of substrate proteins. CRLs are involved in wide range of physiological processes (70). Depending on the physiological conditions, Cullin can interact with their specific COMMD and plays a key role in several cellular processes including NF-κB signaling. Among COMMD members, COMMD8 can forms complex with coiled-coil domain-containing protein (CCDC22) and Cullin1 and promotes proteasomal degradation of IκBα. In depletion of COMMD8, degradation of IκBα is impaired and NF-κB activity is attenuated.

4.4 COMMD1 as a multifunctional protein

In addition to its NF-κB regulation ability, COMMD1 involved in other physiological process. Among them, role of COMMD1 in copper metabolism is best known (56, 67). COMMD1 participates in quality control of copper transporter ATP7A and ATP7B, and maintaining hepatic copper level by secreting copper ion to bile (71). Various cell lines with COMMD1 deficient show decreased copper transport capacity, but to date, no mutation in COMMD1 have been reported to be associated with copper accumulation disease in human.

COMMD1 also plays a key role in 1) sodium uptake in epithelial cell through regulation of epithelial sodium channel (ENaC), 2) negatively regulates HIF-1 signaling by interrupting dimerization of HIF1α and HIF1β, 3) Maturation of superoxide dismutase 1 (SOD1) and 4) Cystic fibrosis through interaction with cystic fibrosis transmembrane conductance regulator (CFTR) protein. Overall these physiological roles, COMMD1 normally involved in regulation of protein stability via ubiquitination of target proteins, also mediates intracellular target protein trafficking (72, 73).

PURPOSE

It has been reported that TG2 upregulates NF-κB signaling. However, its detail mechanism has not been elucidated yet. This study aims to investigate whether TG2-mediated COMMD1 transamidation is involved in the regulation of NF-κB signaling. To elucidate this, I will identify Q site(s) modified by TG2 in COMMD1 and confirm the role of TG2-mediated modification of COMMD1 in NF-κB signaling.

MATERIALS AND METHODS

Cell culture

HEK293FT cell (human embryonic kidney cell) and HeLa cell (human cervical cancer cell) were cultured in Dulbecco's modified Eagle's medium (WelGENE, #LM001-05) supplemented with 10% (v/v) fetal bovine serum (Hyclone, #SH30397), 1% (v/v) Penicillin-Streptomycin (GIBCO, #15140-122). Cells were maintained in a humidified atmosphere with 5% CO2 at 37℃.

Plasmid

The plasmids pEBB-COMMD1-Flag (Plasmid #74893), pEBB-COMMD4-Flag (Plasmid #74896), pEBB-COMMD5-Flag (Plasmid #74897) have received form Addgene. For bacterial expression, insert of COMMD1, COMMD4 and COMMD5 were transferred to pGEX4T-1 (67Q) plasmid. For purify COMMD1's N-term (amino acids 1-118) and COMM domain (amino acids 119-190), we cloned deletion mutant of COMMD1 in pGEX4T-1 (67Q) vector. Glutamine (Q) site mutant of COMMD1 protein (Q21N, Q38N, Q71N, Q79N, Q83N, Q90N, Q112N) were created through by site directed mutagenesis with Agilent primer design tool. To generate COMMD1 knockout HEK293FT cell line, pSpCas9 (BB)-2A-Puro (PX459) vector was used. pCDNA3-TG2 and pCMV GST-p65 were used for TG2 , p65 overexpression each. 6NF-κB luciferase reporter and pRL-TK plasmid were used for Dual-luciferase assay system.

Transient transfection

Cells were cultured in DMEM for 1day before transfection. Plasmid (COMMD members, construct for dual-luciferase assay system and pSpCas9 (BB)-2A-Puro) were incubated with Lipofectamine 3000 reagent (Invitrogen #18324-012) in Opti-MEM (GIBCO, 22600-043) over 5minutes. After incubation, add appropriate volume of mixture on complete cell culture media and incubated over 18hours. Detail

amount of plasmid and reagent are same as manufacturer's specifications.

Site-directed mutagenesis and deletion mutant cloning

Mutagenesis of glutamine to asparagine of COMMD1 was performed in pEBB-COMMD1-Flag, pGEX4T-1-COMMD1 plasmid as a template. The pfu-ultra polymerase (Stratagene, #600380) and following combination of primer were used : (a)Q21N (5'cccgtggaaagtgtcattggccagcgcattcag 3),(5'ctgaatgcgctggccaatgacactttCcacggg 3'), (b) Q38N(5' gcacctctggatatagattgctccgtagcagctcc 3'), (5' ggagctgctacggagcaatctatatccagaggtgc 3' (c) Q71N (5' agtcaagaatgcctccagattgttgaaatccatgtctgcag 3') , (5' ctgcagacatggatttcaacaatctggaggcattcttgact 3'), (d) Q79N(5' accttgctttttggtattagcagtcaagaatgcctccagc 3') (5' gctggaggcattcttgactgctaataccaaaaagcaaggt 3'), (e) Q83N(5' cagatgtgatcccaccattctttttggtttgagcagtcaa gaat 3'), (5' attcttgactgctcaaaccaaaaagaatggtgggatcacatctg 3'), (f) Q90N (5' atttggaaatgacagcagcattgtca gatgtgatcccacct 3'), (5' aggtgggatcacatctgacaatgctgctgtcatttccaaat 3'), (g) Q112N (5'gtgagagcctcatgaaca atagccgctggaatagcg 3'), (5' cgctattccagcggctattgttcatgaggctctcac 3'), (h) Q71K (5' agaatgcctccagcttgttg aaatccatgtctgca 3'), (5' tgcagacatggatttcaacaagctggaggcattct 3'), (I) Q112K (5' ctattccagcggctcttgttcatga ggctctcac 3'), (5' gtgagagcctcatgaacaagagccgctggaatag 3'), (J) Q71R (5' tcaagaatgcctccagccggttgaa atccatgtct 3'), (5' agacatggatttcaaccggctggaggcattcttga 3'), (K) Q112R (5' ctattccagcggctccggttcatgagg ctct 3'), (5' agagcctcatgaaccggagccgctggaatag 3'), (L)N terminal domain, ΔC mutant (5'cgggattcaccatg gcagccggcgagctt 3'), (5' ttttccttttgcggccgctcacccgctattccagcgg 3'), (M) C terminal domainΔN mutant (5' cgggatccaccatgcttcggggcctgagctgg 3'), (5' ttttccttttgcggccgctcagttaggctggctgatca 3')

Protein purification

pGEX4T-1 plasmid and BL21 (DE3) competent cell were used to COMMD1 protein purification. After transformation, cells were cultured in 1ml volume first, and transferred into 100ml of LB medium for amplify. When the absorbance reaches to 0.6 (measured in 600nm wavelength), proper concentration (500uM for COMMD1 and 100uM for COMMD4 and 5) of Isopropyl β-D-1-thiogalactopyranoside (IPTG) were treated for 18 hours in 18℃. Cells were collected and preserved in -20℃. Dissolve the pellet in 5ml of ice-cold cell lysis buffer which containing Triton-X100 and phenylmethane sulfonyl

fluoride (PMSF) and sonicated (2 sec pulse and 9.9 sec pause, total 5 minutes in 30% amplitude). Transfer the sample into pre-chilled 15ml tube and centrifuge in 12000G for 10 minutes. Discard the pellet and use lysate only for protein purification. 50 µl of Glutathione Sepharose^{TM} 4B beads (Sigma, 9012-36-6) were used for each reaction. Before reaction, wash the beads with ice-cold PBS five times (centrifuge in 500G, five minutes for each) and add PBS to make 50% slurry (v/v). Add beads mixture in cell lysate and binding was performed in room temperature for an hour. During reaction, tubes have to be rocked to prevent beads sedimentation. After reaction, elute beads bound protein with elution buffer (50mM Tris-Cl pH8.0 with10mM reduced-glutathione) and confirmed by western blotting method.

Western blot analysis

Cells were washed with 4℃ cold phosphate-buffered saline (PBS, pH7.4) and lysed with Triton-X100 contained single detergent cell lysis buffer or Radio immunoprecipitation assay buffer (RIPA) with 1X protease inhibitor cocktail (Roche, #11 873 580 001). Collected cells were sonicated (2 sec pulse and 2 sec pause, total 10 seconds in 14% amplitude) in ice-cold condition. Cell lysates were centrifuged at 12000G for 10minites at 4℃. Sample were boiled in 100℃ for 10min after adding 5X sampling buffer and separated in acrylamide gel. Samples were transferred to Nitrocellulose membrane and the membrane was blocked with 5% skim milk or bovine serum albumin (BSA) in TBS-T (50mM Tris, 200mM NaCl, 0.05% tween 20 and pH 7.5) for 1hr. After 3 times washing in TBS-T (10minutes each), membrane was incubated with primary antibody of target (1:1000), in 5% BSA in TBS-T, containing NaN3 for overnight. Immuno-reactive antibodies were further incubated with secondary antibodies, conjugate to horse radish peroxidase (HRP) in 5% skim milk solution as previously mentioned. After TBS-T washing, antibodies were detected by Super-signal west Pico (Thermo #1862637, #1862638) with Las imaging system.

***In situ* TG2 substrate assay**

HEK293FT ells were incubated with 1mM biotinylated pentylamine (Pierce, #21345) in culture media

for an hour before harvest. After washed with ice-cold PBS, cell were scrapped and centrifuged in 12000G for 10minutes in 4℃ and subjected to SDS-PAGE in acrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with streptavidin-HRP (Zymed, #43-8323) and detected with SuperSignal West substrate.

***In vitro* TG2 substrate assay**

Target proteins were prepared with protein purification method as previously mentioned. *In vitro* TG2 reaction was proceeded with 1ug of sample proteins in proper condition (20nM purified TG2, 100uM Biotinylated Pentylamine, 150mM NaCl, 2mM CaCl2, 5mM DTT and 1% Triton-X100) for 45minutes. After reaction, 5mM of cystamine were added to stop the activity of TG2. After protein sampling, western bolt was performed and polyaminated proteins were detected with streptavidin-conjugated HRP.

***In situ* TG2 substrate assay**

HEK293FT cells were co-transfected with pEBB-COMMD1 (wild type or QN mutant)-Flag and pCDNA3-TG2. 18hrs after transfection, 10 μg/ml A23187 were treated for an hour to maximize TG2 activity. One hour prior to harvest, 1mM Biotinylated Pentylamine (Pierce, #21345) were added as a amine donor. Samples were washed with ice-cold phosphate buffered saline (PBS, pH7.4) twice and dissolved in a lysis buffer containing Triton-X100, PIC and PS. Sonicate the sample (2sec pulse and 2sec pause, 5times in 14% amplitude) in ice cold condition and centrifuge in 12000G for 20min in 4℃. Protein concentration was measured with BCA assay system (ThermoFisher, #23227). Load at least 500 μg protein per tube. 20 μl of Flag-M2 magnetic beads (Sigma, #8823) and Streptavidin magnetic beads (ThermoFisher, #10006D) was used for beads binding. To reduce non-specific binding, wash the beads 5 times with ice-cold RIPA buffer. Incubate lysate-beads mixture in 4℃ overnight. After binding reaction, centrifuge the sample at 1000rpm for 1min and discard supernatant. Wash the beads 5 times with 1ml of cold RIPA buffer, and get rid of residual buffer completely. Protein which bound on beads was eluted in 80 μl of 2X sampling buffer with 10 minutes boiling. Pull-downed protein was evaluated by western blot analysis.

p65 ubiquitin ligation test

HEK239 cell were co-transfected with pCMV-GST-p65 and pEBB-COMMD1 vectors. After 18 hours, 50 μg/ml of MG132 were treated for 6 hours to inhibit cell's proteasome activity. Wash the sample with 4°C cold phosphate-buffered saline (PBS, pH7.4) and lysed with single detergent cell lysis buffer which contains Triton-X100, PIC and PS. Sonicate the sample (2sec pulse and 2sec pause, 5times in 14% amplitude) in ice cold condition and centrifuge in 12000G for 20min in 4°C. Protein concentration was measured with BCA assay. Load 500 μg of protein per tube at minimum. 20 μl of Flag-M2 magnetic beads were used for each reaction. Before reaction, wash the beads 5 times with ice-cold PBS. Incubate lysate-beads mixture in 4°C overnight. After reaction, centrifuge the sample at 1000rpm for 1min and wash the beads 5 times with 1ml of cold PBS. Remove residual buffer completely and elude bound proteins with 80 μl of 2X sampling buffer. Immuno-precipated proteins were evalutated with anti-ubiquitin antibody in western blot analysis.

Nuclear fraction

Cells were collected in prechilled microcentrifuge tube and dissolved in 350μl of 1X hypotonic buffer (10mM NaCl, 3mM $MgCl_2$ in 20mM Tris-Cl pH7.5 solution) with pipetting. Incubate lysate on ice for 15 minutes. Add 15μl of 10% NP40 solution as detergent and vortex for 10 seconds. Centrifuge lysate for 10 minutes at 3000rpm at 4℃ to isolate nuclear fraction. Save the pellet (nuclear fraction) and supernatant (Cytoplasmic fraction). Wash the nuclear fraction with 350μl of hypotonic buffer and resuspend it in 200ul of Triton-X100 contained cell lysis buffer. Sonicate (2 sec pulse and 2 sec pause, total 10 seconds in 14% amplitude) it twice and centrifuge the sample for 30minutes at 14000G in 4℃.

Stable COMMD1 knockout cell line

HEK293FT cells were cultured at 100mm dish in DMEM. In 60% confluence cells were transfected with pSpCas9 (BB)-2A-Puro (PX459) DNA which designed to target COMMD1 gene with sgRNA insert (5' cccgaggagttc 3'). 24hours after transfection, cells were treated with 1 μg Puromycin (Invivogen, #58-58-2) for selection. Cells were maintained over 2 weeks and puro-resistance cell clones

derived from single cells were isolated to new culture dishes. Selected cell line were tested expression of COMMD1 with western blot, quantitative PCR and dual-luciferase assay.

Dual-luciferase assay

To measure NF-κB activity, Dual-luciferase assay system was used. Cells were plated in 48well scale, in triplicate for each group. 18hr after transfection, Luciferase activity was quantified using the Luciferase Assay System (Promega, #E1910)

Real-Time qPCR

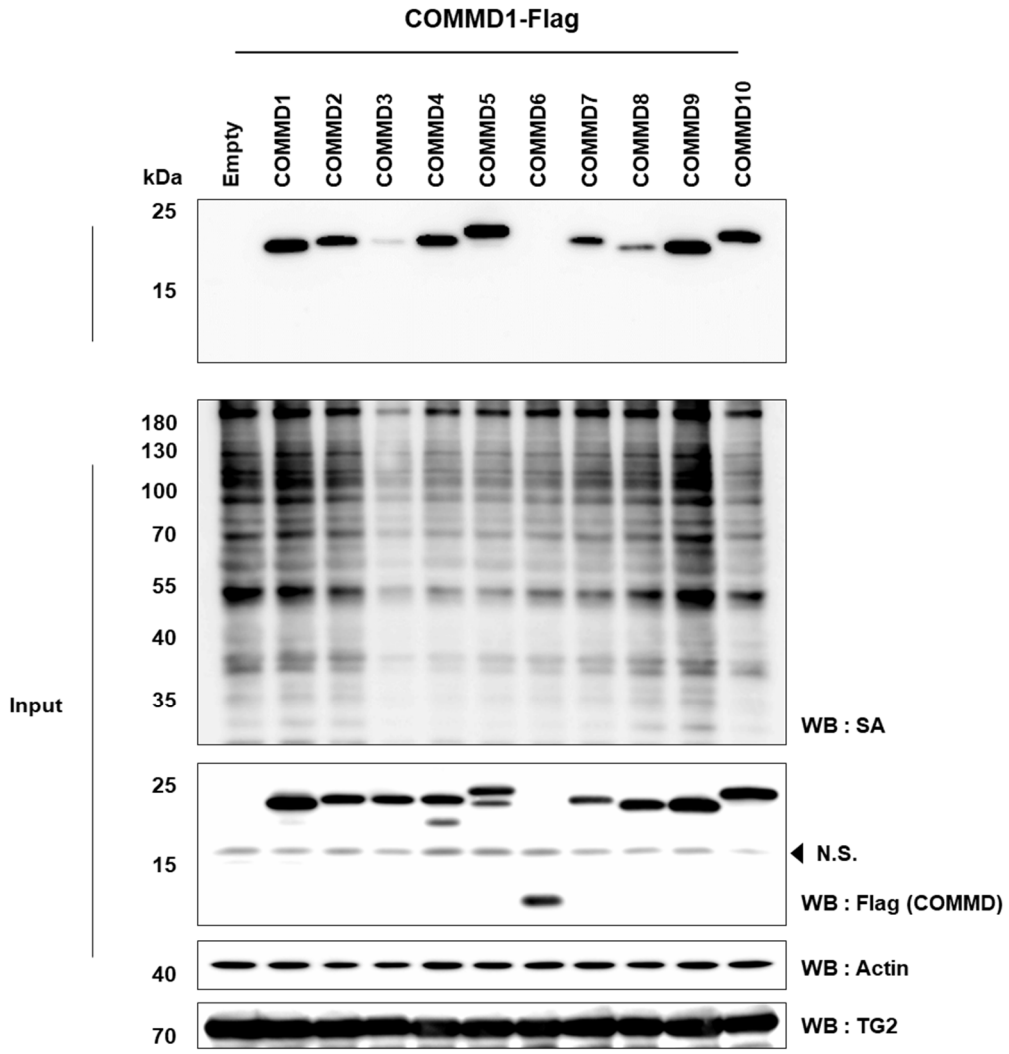
mRNA expression level of COMMD members and various pro-inflammatory cytokines were determined by real-time RT-PCR procedures. Isolation of RNA and reverse transcription were performed following procedures. Total RNA was isolated with RNA-spinTM kit (iNtRON, #17211) and its instruction. RNA was diluted in sterie diethyl pyrocarbonate (DEPC) treated water and normalized. Obtained RNA was reverse transcribed using Superscript II (Invitrogen, #18064014) to cDNA with oligo dT priming. With cDNA, mRNA expression level were measured with KAPA SYBR FAST (Biosystem, #4602) and quantative-PCR, normalized with GAPDH expression rate.

RESULTS

COMMD families, except COMMD6 are substrate of TG2 in HEK293FT cells.

So far, there are no post-translational modification of COMMD1 have been reported except polyubiquitination. We explored the possibility that TG2 might be participates in post-translational modification of COMMD members including COMMD1. To investigate this, *In situ* TG2 substrate assay was performed. HEK293FT cells were co-transfected with pEBB-COMMD (1-10)-Flag and pCDNA3-TG2 plasmids to express both proteins. 18hrs after transfection 10uM of calcium ionopore A23187 were added for an hour prior to harvest and 1mM of BP were added as a amine donor. In cell lysate, pull down experiment was performed with Streptavidin. BP incorporated proteins were pull-down with Streptavidin beads and COMMDs were detected with anti-Flag antibodies (Figure 7B). With this data, we can make sure that all COMMDs except COMMD6 are modified by TG2, and its reactivity is different depends on its type. Densitrometery result shows that every COMMD has different reactivity depends on its type as a substrate of TG2. COMMD5, COMMD7 have relatively high reactivity against TG2 and COMMD3 and COMMD8 have lower reactivity than other COMMD families (Figure 7C). All this result indicate that COMMDs, except COMMD6 can be modified by TG2 and because of COMMD6 is the only COMMD without N-terminal domain of its protein, we hypothesized that substrate glutamine sites of TG2 are present in N-terminal domain of COMMD. Interestingly, COMMD3 transfected group shows relatively lower viability and TG activity than other COMMD groups. It is possible that COMMD3 is involved in unknown mechanism that can effect cell death and TG's enzymatic activity.

A)



B)

Subtype	M.W (kDa)
COMMD 1	21.7
COMMD 2	23
COMMD 3	22
COMMD 4	22
COMMD 5	27
COMMD 6	8
COMMD 7	23
COMMD 8	21
COMMD 9	22
COMMD 10	23

C)

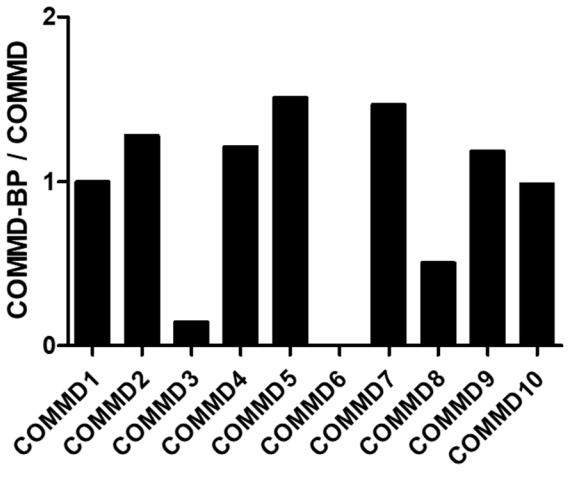


Figure 7. COMMD families, except COMMD6 are substrates of TG2 in cells.

In HEK293FT cells, all COMMD members (COMMD1-COMMD10) and TG2 were expressed with pEBB-COMMD (1-10)- Flag and pCDNA3-TG2 expression system. TG2 was activated 10uM of A23187, 1mM BP were added as a amine donor. After reaction, In situ substrate assay was performed. In A), BP incorporated proteins in cell lysate were pull-downed with Streptavidin and COMMD were detected with anti-Flag antibodies. BP were incorporated in all COMMD families except COMMD6 and pull-downed. But its degree of polyamination is different depend on its type, COMMD5 and COMMD7 showed relatively high reactivity and COMMD3 and COMMD8 showed lower than other COMMD families. Relative polyamination level (COMMD-BP / COMMD) was quantified with densitrometry in C) and COMMDs protein expression level, TG activity was confirmed in A). (N.S. in A) means non-specific detection)

The polyamination site of COMMD1 is conserved

Among COMMD members, we focused on COMMD1 because its physiological role and its mechanism is relatively clear. COMMD1 has highly conserved sequence and structure between species. COMMD1 has two distinct domains: C-terminal domain, also called 'COMMD domain' (119~190 amino acid) and N terminal domain (1~118 amino acid) in human COMMD1 protein. In full length sequence, it has 11 preserved glutamine site (Q21, Q38, Q71, Q79, Q83, Q90, Q112, Q131, Q137, Q154 and Q170) that can be candidates for substrate of TG2 (Figure 8).

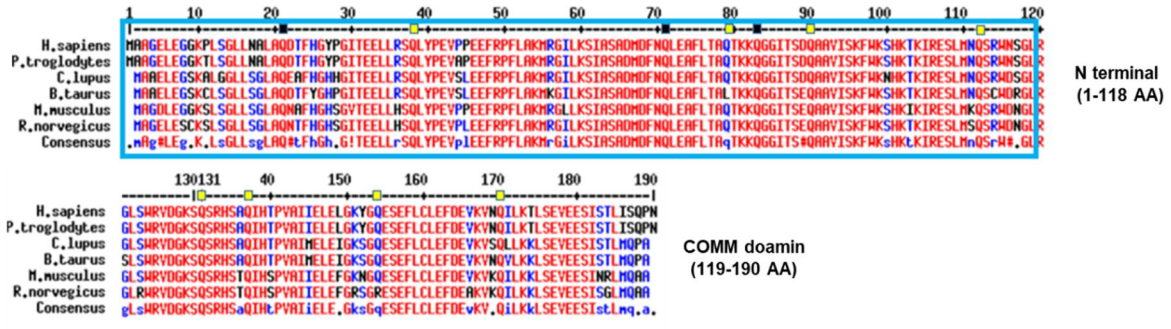


Figure 8. COMMD1, sequence homology in species.

COMMD1 has highly conserved sequence between species. In human COMMD1, there are eleven preserved glutamine site (marked on upper side of sequence) and protein can be divided into two distinct domains: COMM domain in 119~190 amino acid and N terminal domain in 1~118 amino acid except COMM domain.

The glutamine site, which can be a substrate for TG2, is located in the N-terminal domain of COMMD1.

As previously mentioned, there are 11 preserved glutamine site (Q21, Q38, Q71, Q79, Q83, Q90, Q112, Q131, Q137, Q154 and Q170) and it can be divided into two distinct domain: COMM domain in C-terminal (119~190 amino acid) and N terminal domain (1~118 amino acid) in human COMMD1 protein (Figure 8). To identify which of the glutamine site is the substrate of TG2, we cloned two deletion mutants of COMMD1: ΔC mutant (38.19 kDa, consists of only N-terminal domain of COMMD1), ΔN mutant (33.01 kDa, consists of only C-terminal domain of COMMD1) and Wild type (48kDa, Full length of COMMD1) in pGEX4T-1 bacterial expression system with BL21(DE3) cells. After purify with Glutatione-4B beads, *in vitro* TG2 reaction performed for an hour. Streptavidin-HRP was used to detect substrate domain of COMMD1. In result, polyamination reaction was occurred only in full length (wild type) and N terminal domain (ΔC), whereas there were no reaction in GST and C-terminal domain (ΔN) of COMMD1 (Figure 9). This data indicates, there is (are) a substrate Q site of TG2, among the N-terminal domain of COMMD1 (Q21, Q38, Q71, Q79, Q83, Q90, Q112).

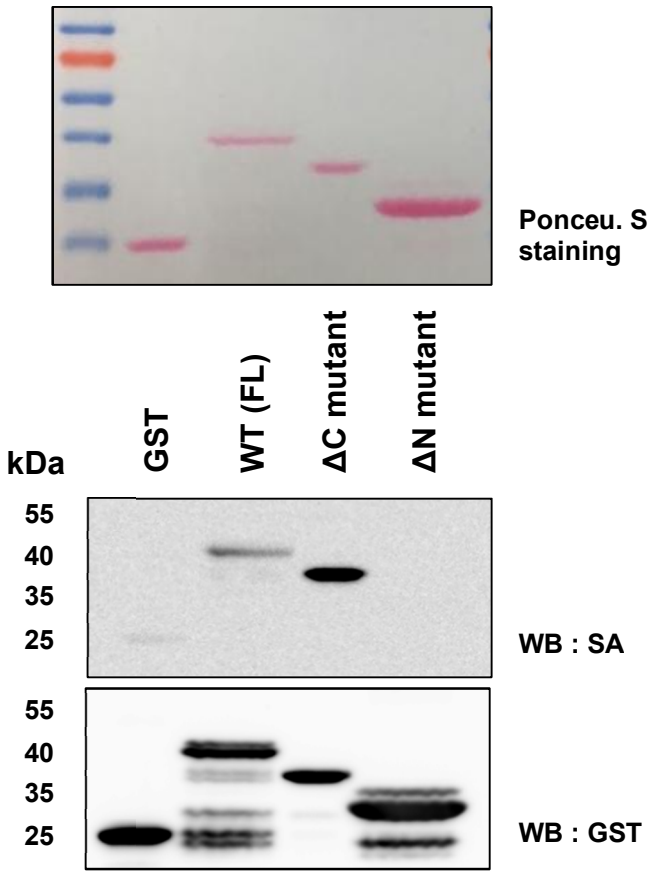


Figure 9. N-terminal domain of COMMD1 is substrate of TG2

In vitro TG2 reaction were performed with Wild type (48kDa, Full length)ΔC mutant (38.19 kDa, N-terminal domain) and ΔN mutant (33.01 kDa, C-terminal domain) of COMMD1. Proteins were expressed in BL21 cell with pGEX4T-1 bacterial expression system, purified with Glutatione 4B beads. In reaction, proteins were incubated for an hour with 20nM of TG2 and polyaminated proteins were detected with SA-HRP in western blot. Result shows that only full length (wild type) and N terminal domain (ΔC) of COMMD1 were polyaminated, and these two type of protein can be substrate of TG2.

Three-dimensional structure of COMMD1 N-terminal domain.

Residue 1-118 in amino acid is defined as the N-terminal domain of COMMD1. About this region, there are nothing known in its physiological role and modification. In COMMD1's N-terminal domain, there are seven glutamine site that can be substrate of TG2 (in figure, only six out of seven glutamine are displayed). And among the glutamine site, three glutamine site: Q21, Q71 and Q83 are exposed to the outside as a loop structure (Figure 10). Therefore, we assumed that, these three glutamine sites are likely to be modified by TG2.

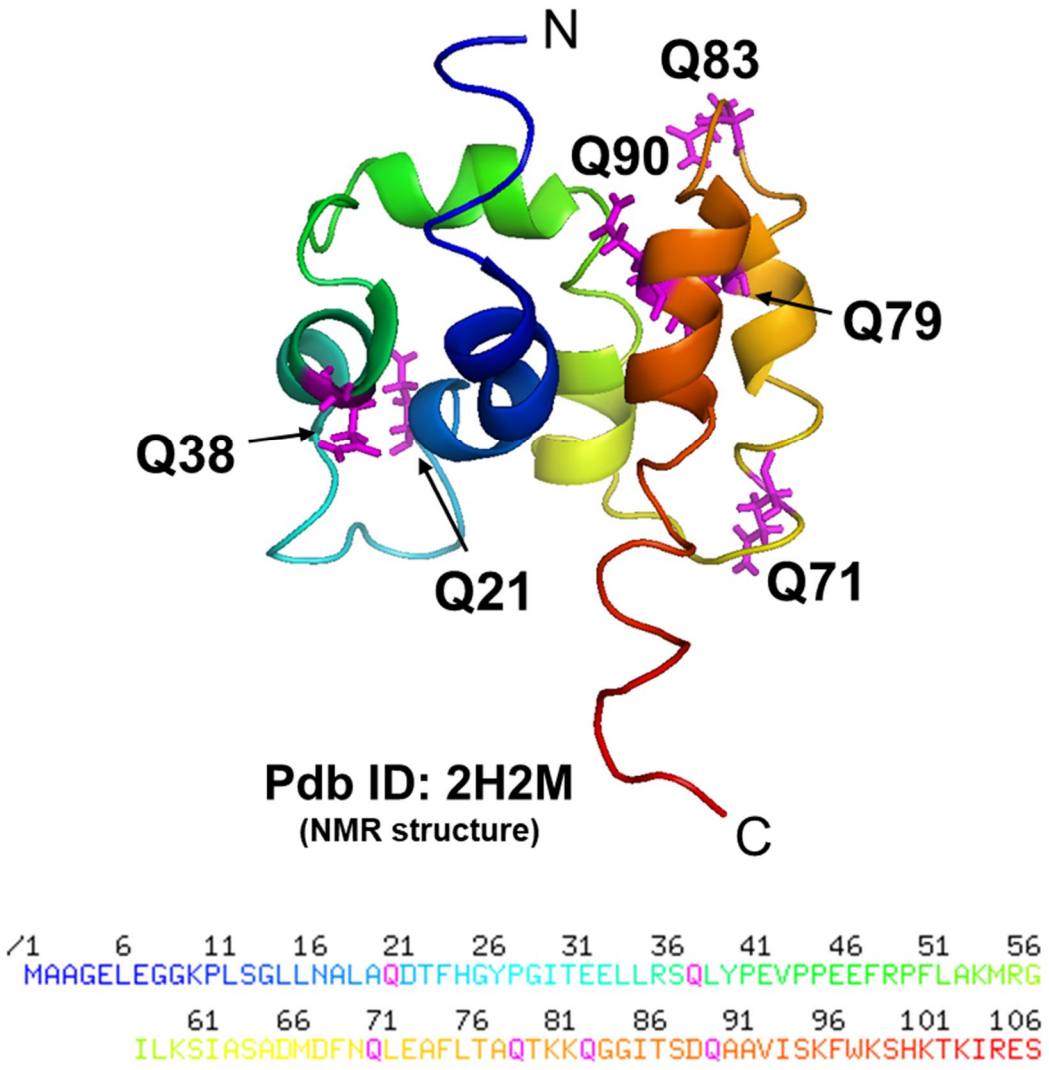


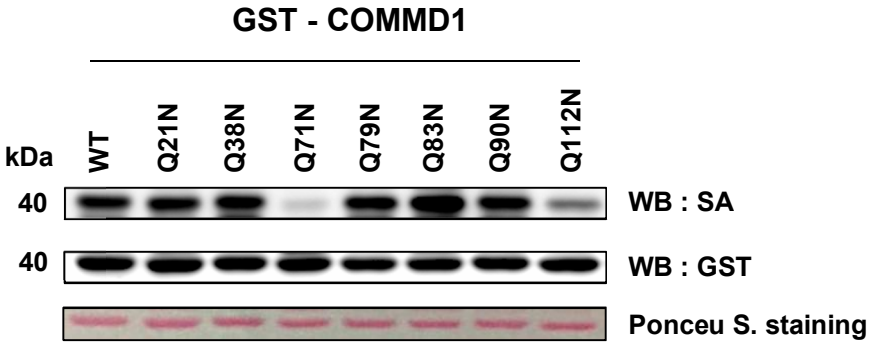
Figure 10. 3D structure of COMMD1 N-terminal domain.

In N-terminal domain, there are seven glutamine site that can be substrate of TG2 (in figure, only six out of seven glutamine are displayed). Three of glutamine sites are exposed to the outside as flexible loop structure (Q21, Q71 and Q83).

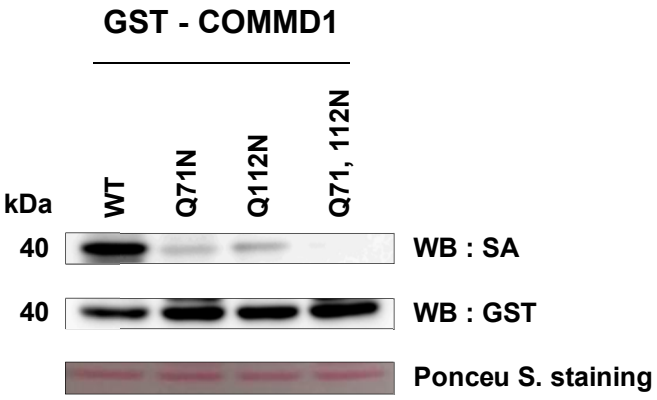
Glutamine 71 and 112 in COMMD1 are modified by TG2 *in vitro*

Seven preserved glutamine site in N-termianl domain (Q21, Q38, Q71, Q79, Q83, Q90 and Q112) were changed to asparagine (N). To identify which of the glutamine is can be modified by TG2, we cloned set of mutants in pGEX4T-1 bacterial expression system with BL21(DE3) cells. Eluted purified proteins were incubated with 20nM of TG2 for 30minutes to *in vitro* TG2 reaction. As previously mentioned, Streptavidin-HRP was used for detection. A) *In vitro* TG2 reaction is performed with wild type and single QN mutant of COMMD1. Data shows that compared with the wild type, there were significant decrease of polyamination level in Q71N and Q112N single mutant of COMMD1. Furthermore of these two, Q71N decreased more than Q112N (Figure 11A). Result indicates that glutamine 71 and 112 are major modification target of TG2 and this modification occurs more strongly at Q71 than Q112. Total protein amount was measured by GST antibody and Ponceu S. staining method. B) With result A), we can assume that glutamine 71 and 112 can be modified by TG2 and it occurs strongly in Q71 site, better than Q112. To confirm this again, we made Q71, 112N double mutant of COMMD1 with mutagenesis. Protein purification and *in vitro* reaction were conducted same with A). As we observed earlier, compared with the wild type, there was significant decrease of polyamination level in single mutant of COMMD1 (Q71N and Q112N) and Q71N decreased more than Q112. Q71, 112N double mutant COMMD1 shows much lower level of polyamination compared to wild type and two single mutants with almost disappeared level (Figure 11B). This data indicates that glutamine 71 and 112 are major modification site of TG2 in COMMD1, and in absence of these two glutamine sites, COMMD1 is rarely polyaminated by TG2. Same with A), total protein amount was measured by GST antibody and Ponceu S. In C), polyamination site of COMMD1 was validated with Mass-spectrometry analysis method. Purified wild type COMMD1 protein was prepared with pGEX4T-1 bacterial expression system with BL21(DE3) cells. After purify with GST-purification method, *in vitro* TG2 reaction was performed with 2 μg of wild type COMMD1 protein for an hour. In result, there was a polyamination in glutamine 71 of COMMD1 but not in glutamine 112(Figure 11C). We assumed that the reason for this result is due to deamidation activity of TG2. Taken together, all these data indicates that glutamine 71 and 112 are major modification target of TG2 and this modification occurs more strongly at Q71 than Q112.

A)

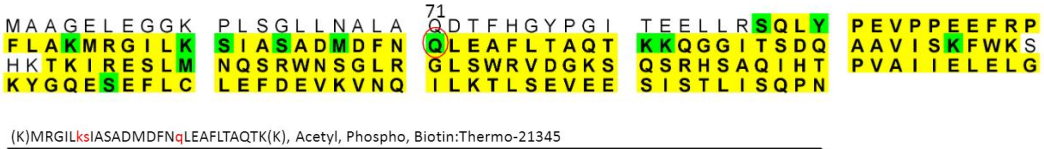


B)



C)

COMD1_HUMAN (100%), 21,178.9 Da
COMM domain-containing protein 1 OS=Homo sapiens GN=COMMD1 PE=1 SV=1
21 exclusive unique peptides, 57 exclusive unique spectra, 1819 total spectra, 151/190 amino acids (79% coverage)



(K)MRGILksIASADMDFNqLEAFLTAQTK(K), Acetyl, Phospho, Biotin:Thermo-21345

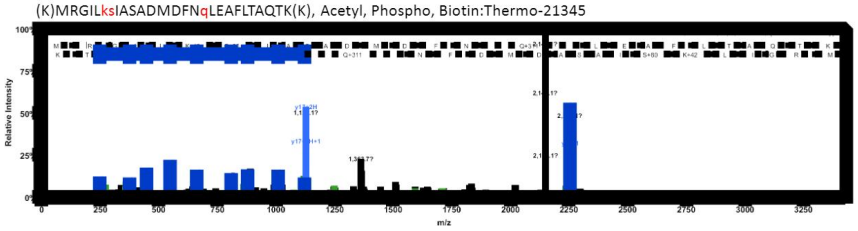


Figure 11. Glutamine 71 and 112 in COMMD1 are modified by TG2 *in vitro*.

A) *In vitro* TG2 reaction was performed with Wild type and QN single mutant of COMMD1 (all of COMMD1 are 48kDa). Proteins were expressed in BL21 cell with pGEX4T-1 bacterial expression system and purified with GST purification system. For reaction, proteins were incubated for 30minutes with 20nM of TG2 and polyaminated proteins were detected with SA-HRP in western blot as previously mentioned. Result shows that compared with the wild type COMMD1, there were significant decrease of polyamination level in Q71N and Q112N single mutant COMMD1. Furthermore of these two, Q71N decreased more than Q112N. in B), *In vitro* TG2 reaction was performed with QN single mutant and double mutant of Q71 and Q112. As we observed in A), compared with the wild type, there was significant decrease of polyamination level in single mutant of COMMD1 (Q71N and Q112N) and Q71N decreased more than Q112. Double mutant of COMMD1 (Q71, 112N) shows much lower level of polyamination compared to wild type and two single mutant form. C) Polyamination site of COMMD1 was identified with Mass-spectrometry analysis method. *In vitro* TG2 reaction was performed with 2 μg of wild type COMMD1 for an hour. In result, there was a polyamination in glutamine 71 site, but not in 112 site. The reason for this result is probably due to deamidation activity of TG2.

Glutamine 71 and 112 in COMMD1 are modified by TG2 in cells

Next, in order to confirm whether glutamines 71 and 112 of COMMD1 are modified by TG2 in cells, *In situ* TG2 substrate assay was performed. HEK293FT cells were co-transfected with pEBB-COMMD1 (wild type, QN single and double mutant)-Flag and pCDNA3-TG2 plasmids to express both proteins. 18hrs after transfection, 10uM A23187 were added 1 hour prior to harvest and 1mM of BP were added as a amine donor. In cell lysate, two pull down experiment was performed simultaneously: First, Immuno-precipitate (IP) Flag tagged COMMD1 with Flag-M2 antibodies and detect Biotinylated pentylamine (BP) incorporated COMMD1 with HRP conjugated streptavidin (SA-HRP). Second, BP incorporated proteins were pull-down with Streptavidin beads and among these proteins, COMMD1 was detected with anti-Flag antibodies (Figure 12B). In both pull down experiment, compared with the wild type, there were significant decrease of polyamination level in Q71N and Q112N single mutant of COMMD1 and much more decrease occurred in Q71, 112N double mutant of COMMD1. Between samples, same amount of COMMD1 expression and same degree of TG2 activity can confirmed in input blot (Figure 12A). This result indicates that both glutamine 71 and 112 are major modification target of TG2 not only *In vitro* but also *In vivo,* but TG2's reactivity to glutamine 71 and Q112 can be different from *in vitro* system.

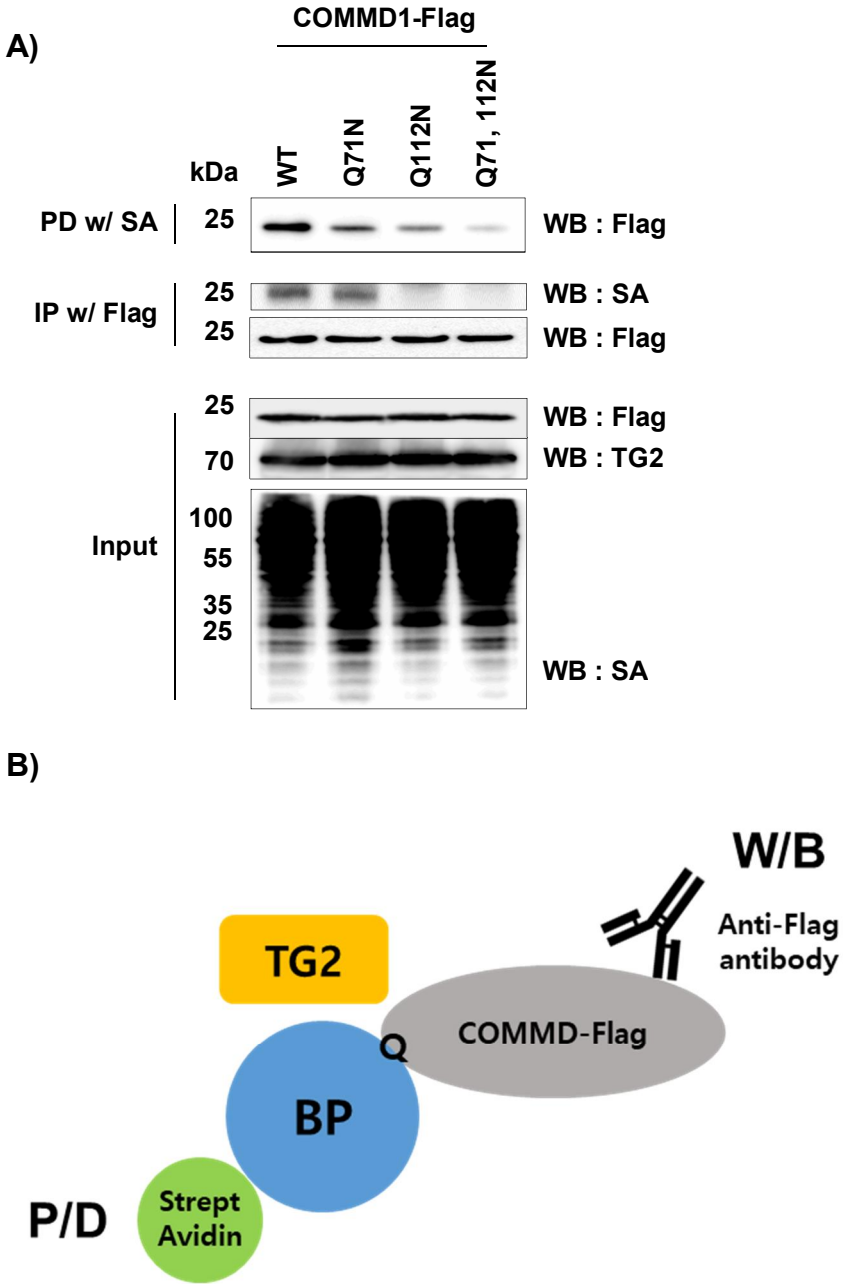


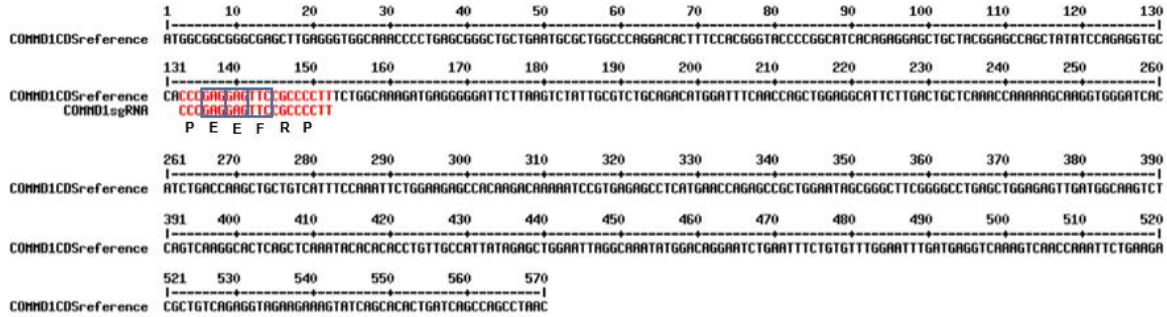
Figure 12. Glutamine 71 and 112 in COMMD1 are modified by TG2 in HEK293FT cells.

A) In both pull down experiment, there were significant decrease of polyamination level in Q71N and Q112N single mutant than that of wild type COMMD1. In addition, much more decrease occurred in Q71, 112N double mutant of COMMD1. Activity of TG2 and COMMD1 expression are same between samples. B) Two methods of immunoprecipitation were performed simultaneously.

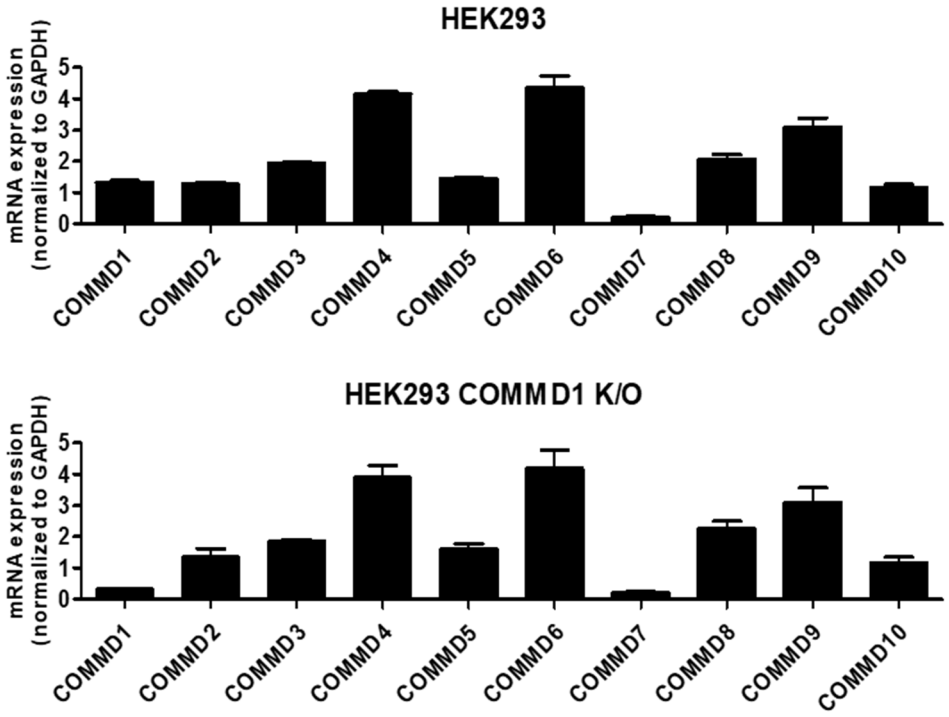
COMMD1 knockout HEK293 cell line were generated and showed decreased NF-κB activity.

To observe functional difference between wild type of COMMD1 and its QN mutant type in cells, COMMD1 knockout HEK293FT cell line were generated. Cells were transfected with pSpCas9(BB)-2A-Puro (PX459) DNA which designed to target COMMD1 gene with sgRNA insert (Figure 13A) and selected with puromycin selection method. mRNA expression level of all COMMDs was measured in wild type and COMMD1 knockout cells. There was significant decrease in COMMD1 mRNA level in knockout cells, but the expression of rest of COMMDs has no difference. Other COMMDs do not seem to compensate for the loss of COMMD1 (Figure 13B). The amount of COMMD1 protein expression was almost disappeared in knockout cells (Figure 13C) and since its main function is to inhibiting NF-κB activity, we assume that NF-κB activity would be enhanced when COMMD1 is deficient. As expected, dual-luciferase assay result shows enhanced NF-κB activity in COMMD1 knockout cells than wild type (Figure 13D).

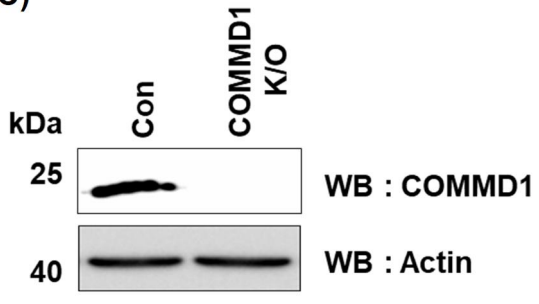
A)


COMMD1CDSreference
COMMD1sgRNA
P E E F R P

B)


HEK293
mRNA expression (normalized to GAPDH)
COMMD1
COMMD2
COMMD3
COMMD4
COMMD5
COMMD6
COMMD7
COMMD8
COMMD9
COMMD10
HEK293 COMMD1 K/O

C)


kDa
Con
COMMD1 K/O
25
40
WB : COMMD1
WB : Actin

D)

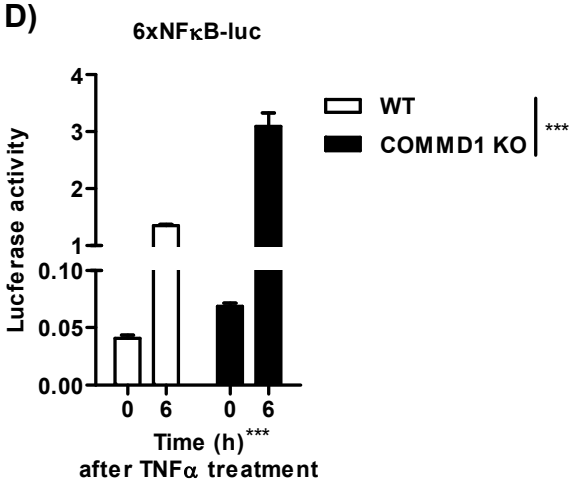

6xNFκB-luc
Lucferase activity
WT
COMMD1 KO
***
Time (h)***
after TNFα treatment

Figure 13. COMMD1 knockout 293 cell line were generated and showed decreased NF-κB activity. A) COMMD1 knockout HEK293FT cells were generated with pSpCas9 (BB)-2A-Puro (PX459) system. B) mRNA expression level of all COMMDs, including COMMD1 was measured by qRT-PCR. COMMD1 knockout cell line showed significant decrease in mRNA expression and C) protein expression of COMMD1. D) Furthermore, its NF-κB activity was enhanced in knockout cells than wild type cells.

Codon-optimization to overexpress COMMD1s in COMMD1 knockout cells.

To substitute endogeous COMMD1 to QN mutant of COMMD1, codon-optimization system was established. Codon-optimization system is a strategy that can avoid CRISPR/Cas9 system by switching its codon sequence, but maintain its protein functions with same amino acid sequence. In this system, cccgaggagttccgccctt sequence targeted by CRISPR/Cas9 system was substituted in cccggaggatttcgccctt (Figure 14A). Based on this sequence, wild type and QN mutants of COMMD1 were made. The amount of protein expression between these wild type and mutants were constant in COMMD1 knockout cell line (Figure 14B).

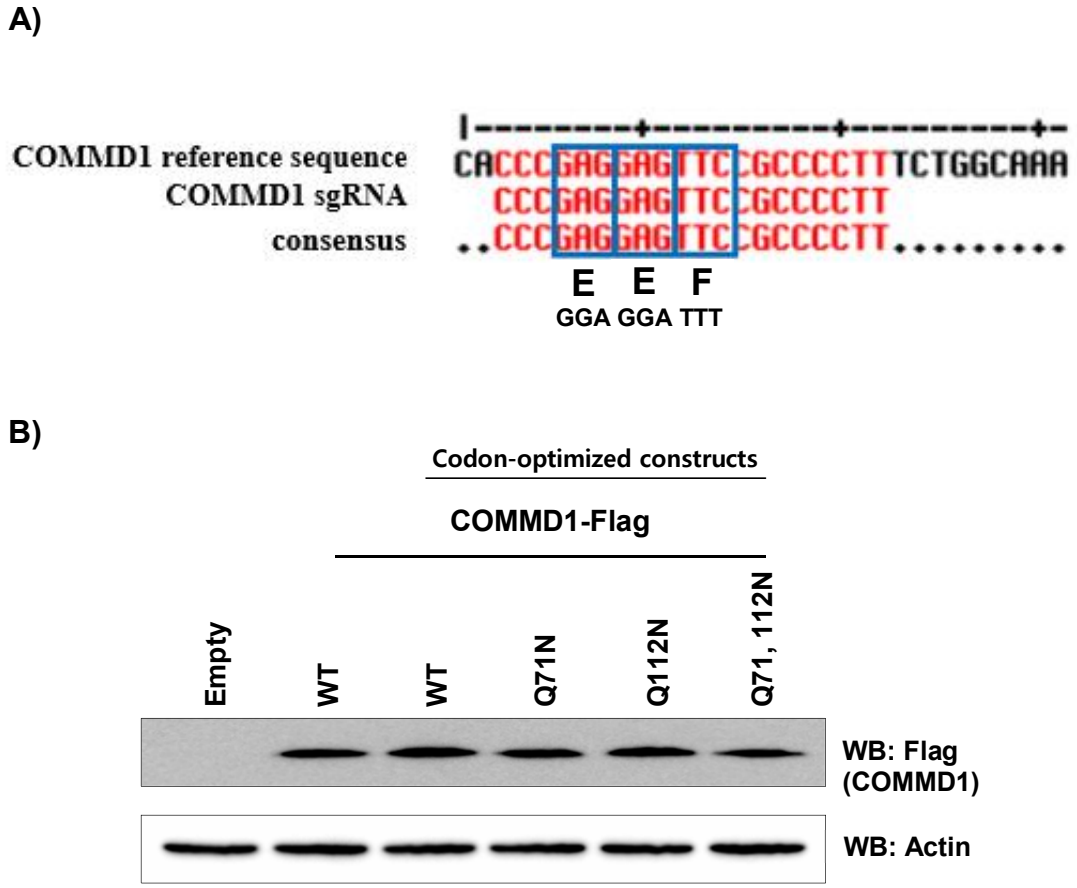


Figure 14. COMMD1 Codon-optimization to overexpress in COMMD1 knockout cells

A) To substitute endogenous COMMD1 to QN mutant of COMMD1, codon-optimization system was applied. The COMMD1 sequence, which are newly transfected into COMMD1 knockout cell was replaced with cccgaggagttccgccctt to cccggaggatttcgccctt. Whereas its amino acid sequence was maintained. Protein expression level of codon-optimized COMMD1 was confirmed in B). All constructs show same amount of protein expression in COMMD1 knockout cell line.

Q71N and Q112N mutants of COMMD1 show greater inhibitory effects on NF-κB activity than wild type COMMD1.

To verify functional difference between wild type and QN mutant of COMMD1, a set of codon-optimized constructs (wild type, Q71N, Q112N and Q71, 112N) were delivered into COMMD1 knockout HEK293FT cell line. With this system, almost of endogenous COMMD1 in the cell were replaced with newly expressed codon optimized COMMD1. Among the various physiological function of COMMD1, inhibitory effect of NF-κB were observed. In dual-luciferase assay, comparing with Empty group, there was an inhibitory effect on NF-κB signaling when transfected with wild type COMMD. Furthermore, comparing wild type and other mutants, QN mutants show greater inhibitory effect than wild type COMMD1. Of these mutants, Q112N has the strongest NF-κB inhibitory effect, followed by Q71, 112N (Double Mutant, written in DM) and Q71N. This phenomenon was common in the wild type and COMMD1 knockout HEK293FT cells (Figure 15A). These results suggest that TG2 enhances NF-κB signaling by the modification of COMMD protein. And with QN mutant model, (which cannot be modified by TG2) its NF-κB signaling is inhibited compared to the wild type. We assumed that the NF-κB inhibitory effect of QN mutant can cause of different expression amount of pro-inflammatory cytokine mRNA level. In B) mRNA expression levels of various pro-inflammatory cytokines were measured. In CXCL1, CXCL5, CXCL8, CXCL10 and TNFα, a suppression pattern was similar to luciferase activity previously observed. On the other hand IL-6, IL-1α and IL-1β showed no such inhibitory effect (Figure 15B). This result indicates that NF-κB modulation by TG2 is mediated by COMMD1 and its modification is essential for upregulation. Also it has target specific effect in downstream of NF-κB signaling.

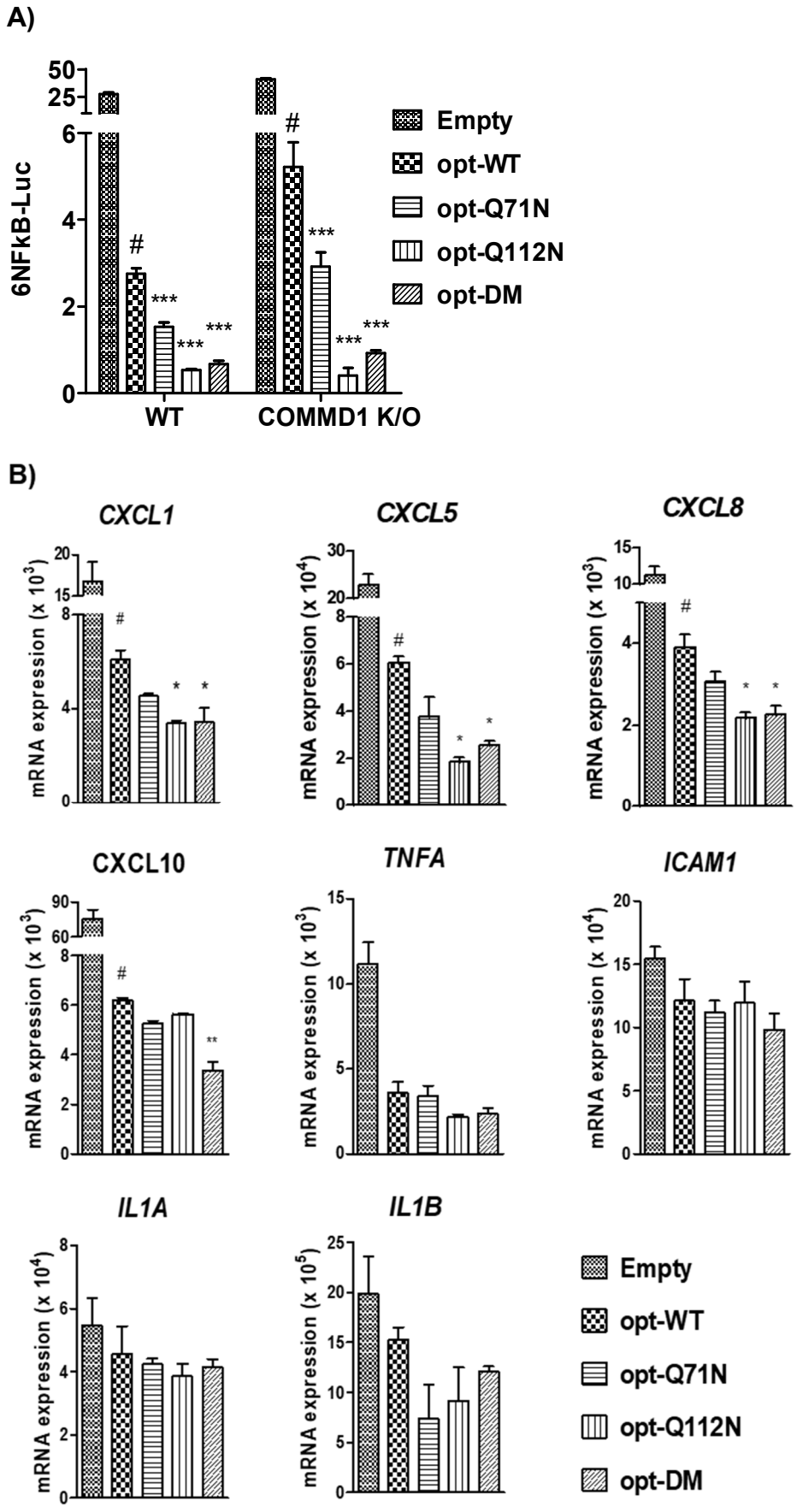

A)
6NFkB-Luc
50
25
6
4
2
0
#
***
***
***
#
***
***
***
WT
COMMD1 K/O
Empty
opt-WT
opt-Q71N
opt-Q112N
opt-DM
B)
CXCL1
CXCL5
CXCL8
CXCL10
TNFA
ICAM1
IL1A
IL1B
mRNA expression (x 10^3)
mRNA expression (x 10^4)
mRNA expression (x 10^5)
Empty
opt-WT
opt-Q71N
opt-Q112N
opt-DM

Figure 15. Q71N and Q112N mutants of COMMD1 show greater inhibitory effects on NF-κB activity than wild type.

Cells were transfected with Codon-optimized constructs of COMMD1 (WT, Q71N, Q112N and Q71, 112N). In luciferase assay result A), comparing with 'Empty', there was an inhibitory effect on NF-κB signaling when transfected with 'opt-WT' COMMD. Furthermore, comparing 'opt-WT' with other mutants, QN mutants show greater inhibitory effect than wild type COMMD1. Of these mutants, Q112N has the strongest NF-κB inhibitory effect, followed by DM and Q71N. In B) mRNA expression levels of pro-inflammatory cytokines were measured. CXCL1, CXCL5, CXCL8, CXCL10 and TNFα show a suppression pattern similar to luciferase activity in A). In contrast, ICAM1, IL-6, IL-1α and IL-1β show no such inhibitory effect.

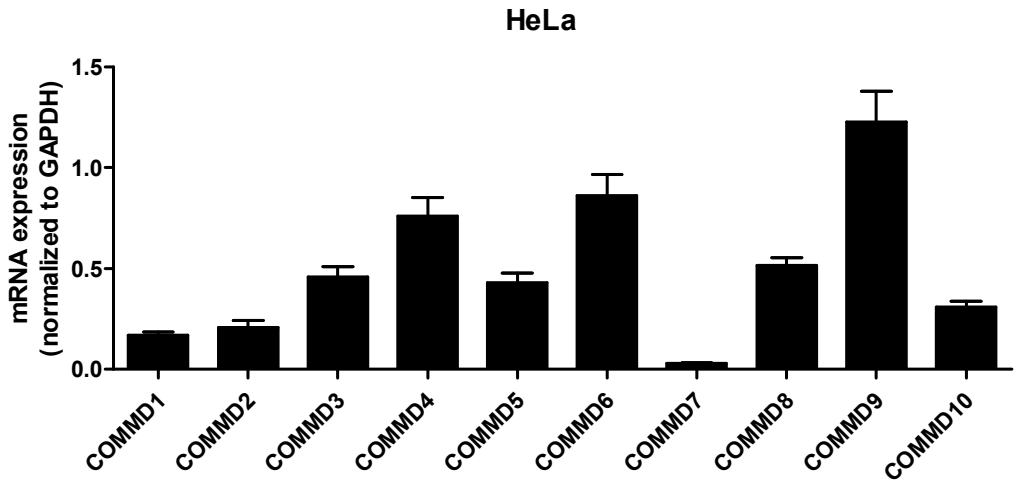
Glutamine 71 and 112 sites in COMMD1 are responsible for TG2- mediated NF-κB activation.

In HeLa cells, mRNA expression of all COMMDs was measured with qRT-PCR method. COMMD4, COMMD6 and COMMD9 showed relatively high expression, but COMMD7 was scarcely expressed in cells (Figure 16A).

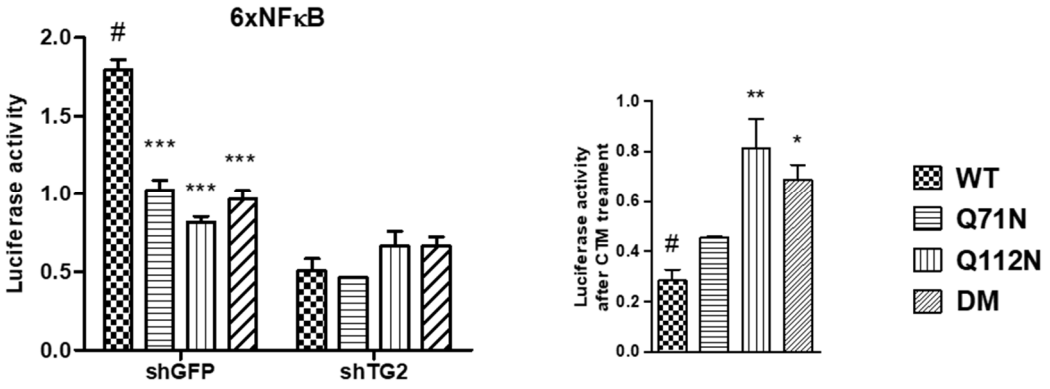
In order to confirm whether the NF-κB inhibitory effect, shown by QN mutant is due to TG2, experiments with inhibitor was performed. Cells were transfected with set of codon-optimized COMMDs (WT, Q71N, Q112N and Q71, 112N) and 1mM of cystamine (TG2 inhibitor) were treated 6 hours after transfection. After 12 hours incubation, NF-κB activity was measured with dual-luciferase assay system. Compare to the absence and presence of cystamine, COMMD1's NF-κB inhibitory effect was attenuated in the presence of cystamine. The ratio after cystamine treat also shows that NF-κB inhibitory effect of COMMD1s, including wild type and QN mutants, was disappeared in the presence of cystamine (Figure 16C). This result indicates that the difference between wild type and QN mutant of COMMD1 is due to TG2 and suggesting that glutamine 71 and 112 are essential for this mechanism.

To clarify this result, TG2 knock down cells were used. shGFP and shTG2 HeLa cells were transfected with codon-optimized COMMD1s (WT, Q71N, Q112N and Q71, 112N). 18 hours after transfection, NF-κB activity was measured with dual-luciferase assay system. Same as previous result comparing with control cells (shGFP), COMMD1's NF-κB inhibitory effect was attenuated in TG2 knockdown cells (shTG2). Furthermore, difference of NF-κB inhibitory effect between wild type and QN mutant of COMMD was disappeared in TG2 knockdown cell line (Figure 16B).

A)


HeLa
mRNA expression (normalized to GAPDH)
1.5
1.0
0.5
0.0
COMMD1
COMMD2
COMMD3
COMMD4
COMMD5
COMMD6
COMMD7
COMMD8
COMMD9
COMMD10

B)


6xNFκB
Luciferase activity
2.0
1.5
1.0
0.5
0.0
#
***
***
***
shGFP
shTG2
Luciferase activity after CTM treament
1.0
0.8
0.6
0.4
0.2
0.0
#
**
*
WT
Q71N
Q112N
DM

C)

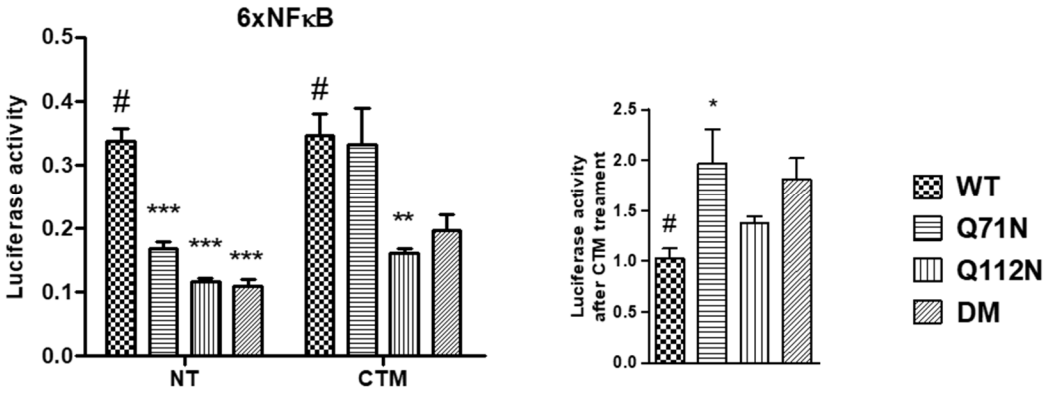
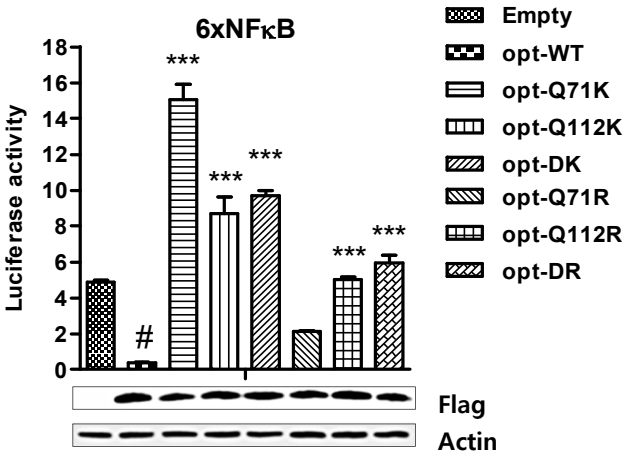

6xNFκB
Luciferase activity
0.5
0.4
0.3
0.2
0.1
0.0
#
***
***
***
#
**
NT
CTM
Luciferase activity after CTM treament
2.5
2.0
1.5
1.0
0.5
0.0
#
*
WT
Q71N
Q112N
DM

Figure 16. Q71 and Q112 site in COMMD1 are responsible for TG2- mediated NF-κB activation. A) mRNA expression of all COMMDs was measured in wild type HeLa cells. COMMD4, COMMD6 and COMMD9 showed relatively high expression level, and COMMD7 was scarcely expressed. B) Cells were transfected with codon-optimized COMMDs (optimized WT, Q71N, Q112N and DM) and 1mM of cystamine (TG2 inhibitor) were treated 6 hours after transfection. COMMD1's NF-κB inhibitory effect was attenuated in cystamine treated group. Ratio also shows that with cystamine, NF-κB inhibitory effect in wild type and QN mutant of COMMD1 was disappeared. B) shGFP and TG2 shTG2 HeLa cells were transfected with codon-optimized COMMDs and measured its NF-κB activity. Comparing with control (shGFP), NF-κB inhibitory effect of COMMD1 was weakened in TG2 knockdown cell line (shTG2) and its difference between wild type and QN mutants also disappeared in TG2 knockdown cell line

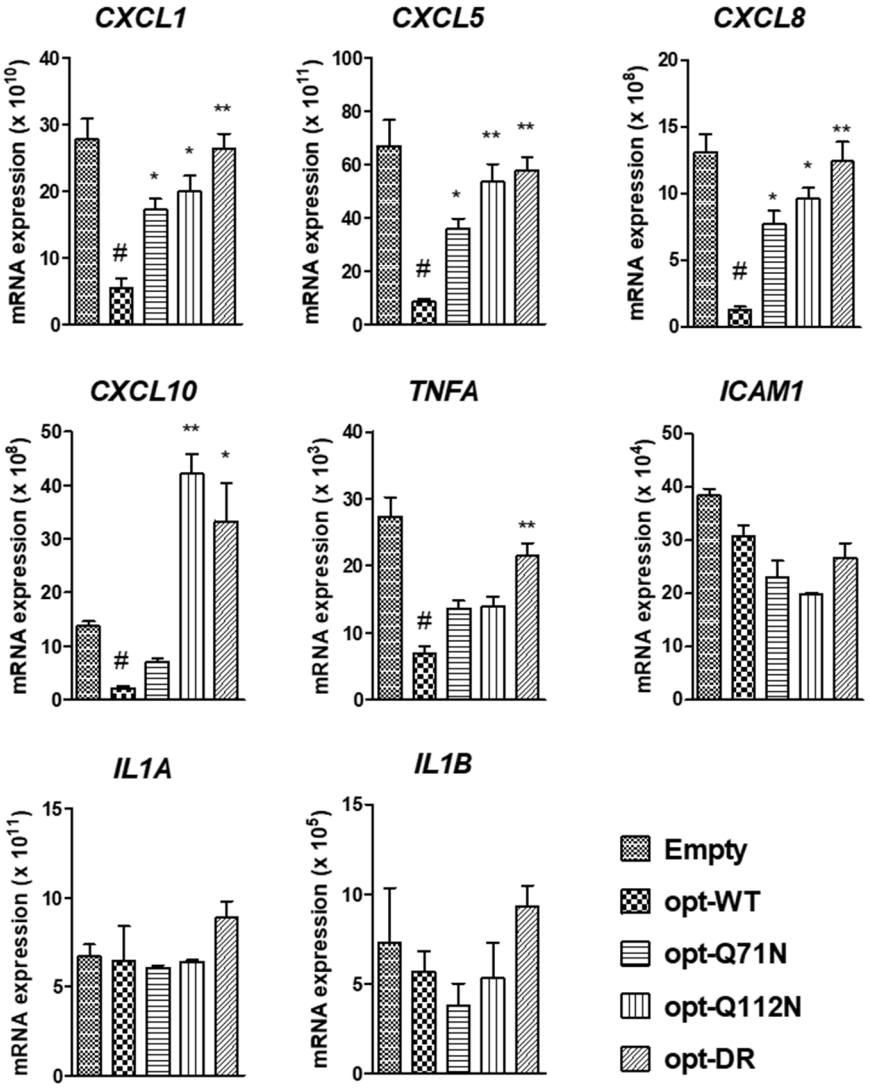
Polyamination-mimic form of glutamine 71 and 112 in COMMD1 shows enhanced NF-κB activity than wild type.

In order to confirm the effect of polyamination of COMMD1 on NF-κB signaling, both of the 71 and 112 glutamine (Q) residues in COMMD1 are substituted with Arginine (R) and Lysine (K). Because of Arginine and Lysine are positively charged amino acid, these amino acid can mimic polyaminated glutamine and QR or QK mutants of COMMD1 can act as polyaminated COMMD1. QR mutants (Q71R, Q112R and Q71, 112R) and QK mutants (Q71K, Q112K and Q71, 112K) were obtained through single-direct mutagenesis of codon-optimized pEBB-COMMD1 plasmid. QR and QK mutants were delivered into COMMD1 knockout HEK293FT cells, and its NF-κB activity was measured with dual-luciferase assay system (Figure 17A). Both of polyamination-mimic mutant shows enhanced NF-κB activity than wild type COMMD1. QK mutants have greater NF-κB enhancing effect than QR mutants, but QR mutants show exactly opposite pattern with QN mutants. mRNA expression levels of various pro-inflammatory cytokines were measured in COMMD1 QR and QK mutants transfected cells (Figure 17B, C). CXCL1, CXCL5, CXCL8, CXCL10 and TNF shows significant increase in both of QK, QR mutants than wild type COMMD1. But ICAM1, IL-1α and IL-1β are not affected. Among these two groups, mRNA expression level of COMMD1 QR mutants shows exactly opposite pattern from the data in QN mutants. This result indicates that NF-κB upregulation by TG2 is mediated by COMMD1 modification (polyamination) and it has target specific effect in downstream of NF-κB signaling.

A)


6xNFκB
Luciferase activity
18
16
14
12
10
8
6
4
2
0
***
***
***
***
***
#
Empty
opt-WT
opt-Q71K
opt-Q112K
opt-DK
opt-Q71R
opt-Q112R
opt-DR
Flag
Actin

B)


CXCL1
CXCL5
CXCL8
CXCL10
TNFA
ICAM1
IL1A
IL1B
mRNA expression (x 10^10)
mRNA expression (x 10^11)
mRNA expression (x 10^8)
mRNA expression (x 10^8)
mRNA expression (x 10^3)
mRNA expression (x 10^4)
mRNA expression (x 10^11)
mRNA expression (x 10^5)
#
*
**
Empty
opt-WT
opt-Q71N
opt-Q112N
opt-DR

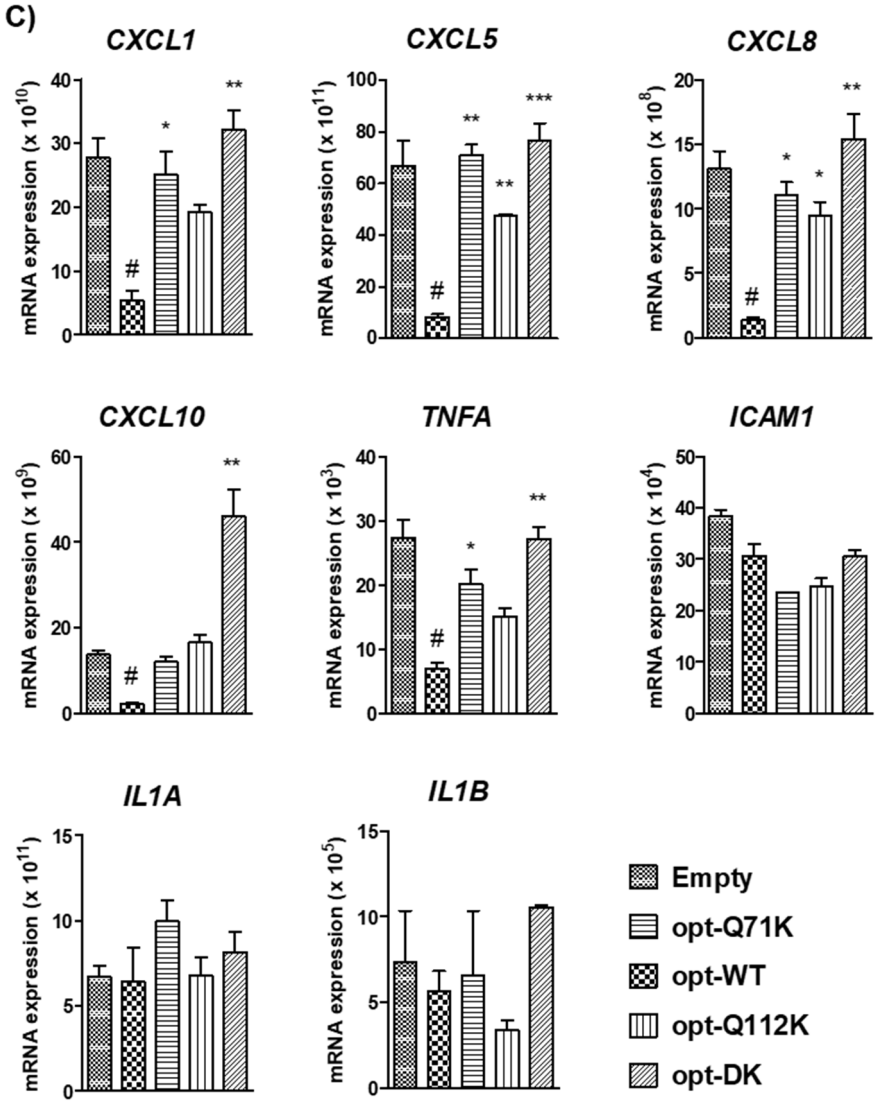


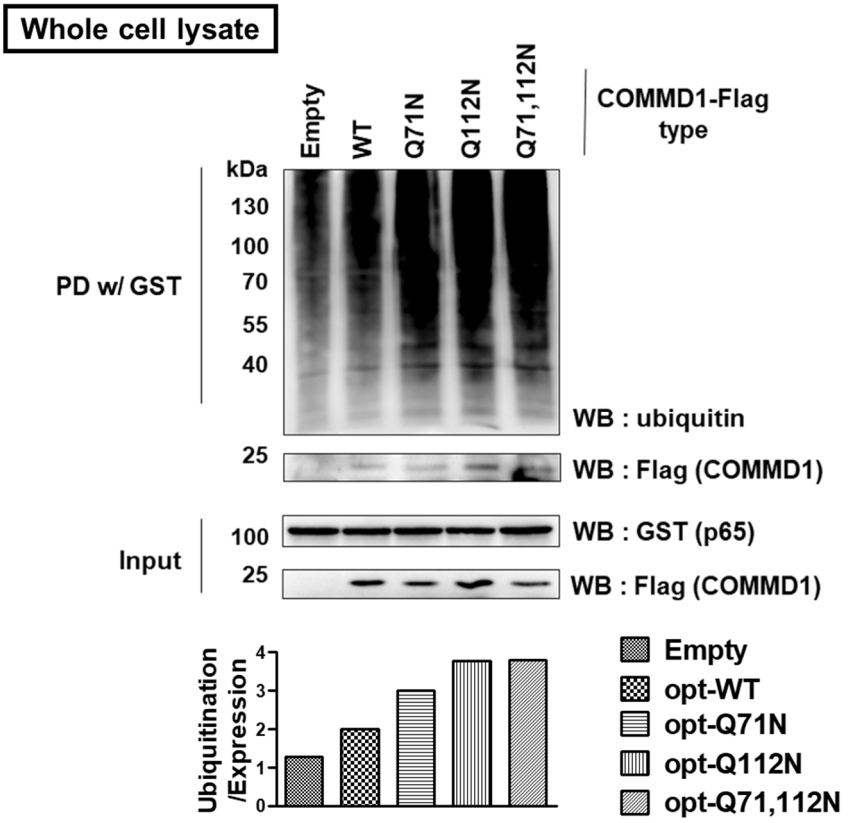
Figure 17. Polyamination-mimic form of glutamine 71 and 112 in COMMD1 shows enhanced NF-κB activity than wild type.

Both of the 71 and 112 glutamine residues in COMMD1 are substituted to Arginine (R) and Lysine (K) with direct-mutagenesis method. Sets of QR, QK constructs are delivered into HEK293FE cells and its NF-κB activity was measured with dual-luciferase assay system (A). Both QR and QK mutants show enhanced NF-κB activity than wild type COMMD1. Protein expression level of COMMD1 mutants were confirmed with western blot assay. (C) and (D) show mRNA expression levels of pro-inflammatory cytokines what measured by real-time qPCR method. Both of QR (C) and QK (D) mutants transfected cells show significantly increased mRNA expression level in CXCL1, CXCL5, CXCL8, CXCL10 and TNF. But not in ICAM1, IL-1α and IL-1β.

QN mutants show enhanced polyubiquitination of p65 than wild type COMMD1

To elucidate how the glutamine 71 and 112 in COMMD1 regulates NF-κB signaling, ubiquitin ligation on p65 was measured. COMMD1 knockout HEK293FT cells were co-transfected with pEBB-COMMD1 (wild type, QN single and double mutant)-Flag and pCMV-GST-p65 plasmids to express both proteins. 18hrs after transfection, 50 μg/ml of MG132 were treated for 6 hours to inhibit proteasomal degradation of polyubiquitinated proteins including p65. In whole cell lysate, GST-tagged p65 was pull downed with GSH-sepharose beads and ubiquitin ligated p65 was detected with HRP conjugated anti-ubiquitin antibody. Densitometry result shows significant increase of polyubiquitinated p65 in all QN mutants (Q71N, Q112N and Q71, 112N double mutant) compare to wild type COMMD1 transfected cells. In order, Q112N was the most increased, followed by double mutant (Q71, 112N) and Q71N (Figure 18A). To confirm whether this regulation occurs in nucleus, the same experiment was performed with nuclear fraction of cells. Lamin B and COX1 blot show successful separation of nuclear fraction and cytoplasmic fraction. Consistent with previous data, all of QN mutants show enhanced polyubiquitination of p65. Among them, the highest increase was observed in Q112N mutant, followed by double mutant (Q71, 112N) and Q71N (Figure 18B). All this data shows same pattern with NF-κB activity which shown in dual-luciferase assay with 6NF-κB. Taken together, this indicates that TG2 upregulates NF-κB signaling by modifying COMMD1 and this phenomenon caused by blocking of poly-ubiquitination and proteasomal degradation of p65.

A)


Whole cell lysate
Empty
WT
Q71N
Q112N
Q71,112N
COMMD1-Flag type
kDa
130
100
70
55
40
PD w/ GST
WB : ubiquitin
25
WB : Flag (COMMD1)
Input
100
WB : GST (p65)
25
WB : Flag (COMMD1)
Ubiquitination /Expression
0
1
2
3
4
Empty
opt-WT
opt-Q71N
opt-Q112N
opt-Q71,112N

B)

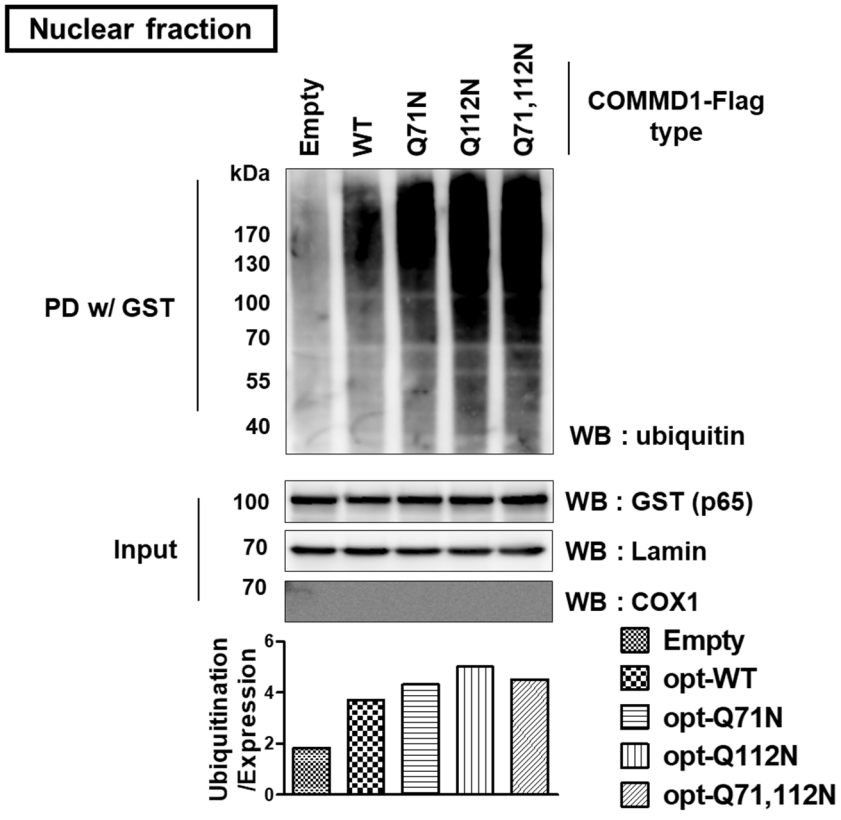

Nuclear fraction
Empty
WT
Q71N
Q112N
Q71,112N
COMMD1-Flag type
kDa
170
130
100
70
55
40
PD w/ GST
WB : ubiquitin
Input
100
WB : GST (p65)
70
WB : Lamin
70
WB : COX1
Ubiquitination /Expression
0
2
4
6
Empty
opt-WT
opt-Q71N
opt-Q112N
opt-Q71,112N

Figure 18. QN mutants show enhanced polyubiquitination of p65 than wild type COMMD1

A) COMMD1 knockout HEK293FT cells were co-transfected with set of pEBB-COMMD1-Flag (wild type and QN mutants) and pCMV-GST-p65 plasmids and 50 μg/ml of MG132 were treated for 6 hours. Poly-ubiquitination of p65 was measured with whole cell lysate. Compare to wild type COMMD1, QN mutants showed increased p65 poly-ubiquitination. In order, Q112N was the most increased, followed by double mutant (Q71, 112N) and Q71N. Expression of COMMD1 and p65 were confirmed with anti-Flag antibody and anti-GST antibody each. B) Same experiment was performed with nuclear fraction of cells. Lamin B and COX1 blot show successful separation of nuclear fraction and cytoplasmic fraction. Consistent with A), all of QN mutants show enhanced polyubiquitination levels of p65. Q112N mutant shows highest increase, followed by double mutant (Q71, 112N) and Q71N.

DISCUSSION

Transglutaminase 2 (TG2)is a enzyme that catalyzes Post-translational modification (PTM) of many proteins and its function and physiological role have been researched. Among them, many studies found NF-κB upregulation effects of TG2, but its detail mechanism has not been elucidated.

In this paper, we suggest that the NF-κB upregulation effect of TG2 is caused by modification of COMMD proteins. COMMD protein family was was discovered recently and little is known about its physiological roles and functions. In current, its ability to control copper homeostasis and anti-cancer effect studied intensively. Also, COMMD's are known as hub of NF-κB regulation. It can downregulates NF-κB signal by promoting ubiquitination and proteasomal degradation of intracellular NF-κB dimers. However, nothing has known about COMMD's post-translational modification and its functional changes. In this study, we suggest that such NF-κB upregulation effect can caused of COMMD1 modification by TG2. To prove this hypothesis, we checked whether COMMDs (COMMD1 to COMMD10) could be a substrate for TG2. Flag tagged COMMDs were expressed in HEK293 cells with pEBB expression system, and *In-situ* substrate assay was performed. With *In-situ* experiment, we can confirm that all COMMDs except COMMD6, are substrate of TG2 and its degree of polyamination is different depends on type of COMMDs. COMMD5 and COMMD7 showed relatively high reactivity and COMMD3, COMMD8 showed lower than other COMMD families (Figure 7). After that, we focused of COMMD1, which its role and mechanism in NF-κB downregulation are relatively well known. To identify Q sites, which can modified by TG2 in COMMD1, we used domain deletion mutant, direct mutagenesis that changes glutamine to asparagine (Figure 8 and 9). With these experiments, we elucidated N-terminal domain of COMMD1 can modified by TG2 and especially, glutamine 71 and 112 are target of TG2. In mass spectrometry, result, shows glutamine 71 was modified by TG2, but not in glutamine 112. The difference between this two results is presumably due to deamidation activity of TG2. To determine whether this modification is reproducible *In vitro* as well as in cell, *in situ* substrate assay was performed (Figure 12). HEK293FT cells were co-transfected with TG2 and COMMD1s (wild

type, Q71N, Q112N and Q71, 112N). TG2 was activated by TNFα induction and its activity was maximized with calcium ionopore. Two kinds of pull-down results indicate that this modifications of COMMD1 occurs not only in vitro environment, but also in cells. Based on this fact, to observe physiological role of COMMD1 modification by TG2, we generated COMMD1 knockout HEK293FT cell line with CRISPR/Cas9 system (Figure 13). And codon-optimized COMMD1 constructs (Figure 14). As predicted, COMMD1 knockout cell shows reduced endogenous COMMD1 level and upregulated NF-κB activity. But when transfected with codon-optimized COMMD1, its NF-κB activity was suppressed and its inhibition capacity was different depends on its mutant type. Normally, polyamination-defect QN mutants showed greater inhibitory effect than wild type and Q112N has strongest effect among them. This effect was verified with luciferase assay and mRNA quantification of pro-inflammatory cytokines (Figure 15). In order to confirm whether the NF-κB inhibitory effect, which shown in QN mutants is due to TG2, cystamine (inhibitor of TG2) and TG2 knock down HeLa cells were used. In both experiment, when TG2 lost its activity, NF-κB inhibitory effect of COMMD1 was attenuated and its difference between wild type and QN mutant of COMMD1 was disappeared (Figure 16). Taken together, all of results indicate that the difference between wild type and QN mutant of COMMD1 is due to TG2 and glutamine 71 and 112 in COMMD1 are essential for NF-κB regulation by TG2. To verify the effect of COMMD1 polyamination of in NF-κB signaling, both of the 71 and 112 glutamine (Q) residues in COMMD1 are substituted with Arginine (R) and Lysine (K). Because of these amino acids are positive charged, it can mimic polyaminated glutamine and QR or QK mutants of COMMD1 can act as polyaminated COMMD1. Both of polyamination-mimic mutant showed enhanced NF-κB activity than wild type COMMD1 in luciferase assay and mRNA quantification of pro-inflammatory cytokines. QK mutants has greater NF-κB enhancing effect than QR mutants, but QR mutants show exactly opposite pattern with QN mutants (Figure 17) This result indicates that NF-κB upregulation by TG2 is mediated by COMMD1 modification and it has target specific effect in downstream of NF-κB signaling. In final, to elucidate how the glutamine 71 and 112 in COMMD1 regulates NF-κB signaling, ubiquitin ligation on p65 was measured. There was significant increase of p65 ubiquitination in COMMD1 QN mutants than wild type COMMD1 this phenomenon was occurred

in both whole cell lysate and nuclear fraction. Taken together, this indicates that TG2 upregulates NF-κB signaling by modifying COMMD1 and this phenomenon caused by blocking of poly-ubiquitination and proteasomal degradation of p65.With this study, we found new NF-κB modulation pathway that mediates TG2 and COMMD1. Not only COMMD1, all COMMD members except COMMD6 are modified by TG2. COMMD-TG2 mediated NF-κB regulation needs to be studied more. Furthermore, not only NF-κB activity regulation, TG2 mediated COMMD modification has a possibility that changes physiological function of COMMD1 such as copper homeostasis, ROS scavenging and anti-tumoric effects.

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국문 초록

트랜스글루타미네이즈 2 (TG2) 는 칼슘 의존성 효소로 단백질 및 펩타이드 기질에 존재하는 글루타민 잔기에 이소펩티드 교결합 형성, 폴리아민화 반응과 탈아민화 반응을 촉매한다. COMMD(copper metabolism MURR1 domain-containing) 족은 세포내 다양한 생리활성 조절에 관여하는 단백질이며, 특히 COMMD1의 p65의 유비퀴틴-프로테아좀 분해를 통한 NF-κB 신호전달계의 조절이 잘 연구되어 있다. 다수의 선행 연구를 통해 TG2가 NF-κB 신호전달계를 활성화 시키며, 이는 여러 질환의 발생에 관여함이 보고되었지만, 정확한 기전은 밝혀지지 않았다. 본 연구의 목적은 NF-κB 신호전달계에 미치는 TG2의 영향을 COMMD1을 통해 밝히고자 함이다. 기질 분석법을 통해 COMMD6을 제외한 모든 COMMD (1-10) 가 TG2에 의해 폴리아민화 됨을 확인하였고 글루타민 잔기를 아스파라긴으로 치환한, 폴리아민화 될 수 없는 QN 돌연변이를 통해 COMMD1의 71번과 112번 글루타민이 TG2에 의해 폴리아민화 되는 것을 확인하였다. 또한 이중 루시퍼라아제 분석을 통해 COMMD1 QN 돌연변이가 야생형 COMMD1에 비해 뛰어난 NF-κB 신호전달 억제효과를 보이고, 폴리아민화 모방 돌연변이 COMMD1 QR, QK 돌연변이는 NF-κB 신호전달을 활성화시킴을 확인했다. NF-κB 신호전달계 조절 효과는, 293세포와 HeLa 세포에서의 CXCL1, CXCL5, CXCL8, CXCL10 mRNA 발현량을 통해 확인되었다. 더하여, p65의 폴리 유비퀴틴화 실험을 통해 야생형 COMMD1에 비해 QN 돌연변이 COMMD1에서 핵 내 p65의 폴리 유비퀴틴화 반응이 증가한 것을 확인하였다. 이 결과 TG2에 의한 NF-κB 신호전달계의 활성은, COMMD1의 71번과 112번 글루타민의 폴리아민화로 인한 핵 내 p65의 유비퀴틴-프로테아좀 분해 억제로 일어나는 현상임을 확인하였다..

주요어 : TGase 2, NF-κB 신호전달계, COMMD, 단백질 번역 후 변형

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