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Studies on the regulation mechanism of the NF-κB-like transcription factor Relish through the polyamine-modification catalyzed by transglutaminase

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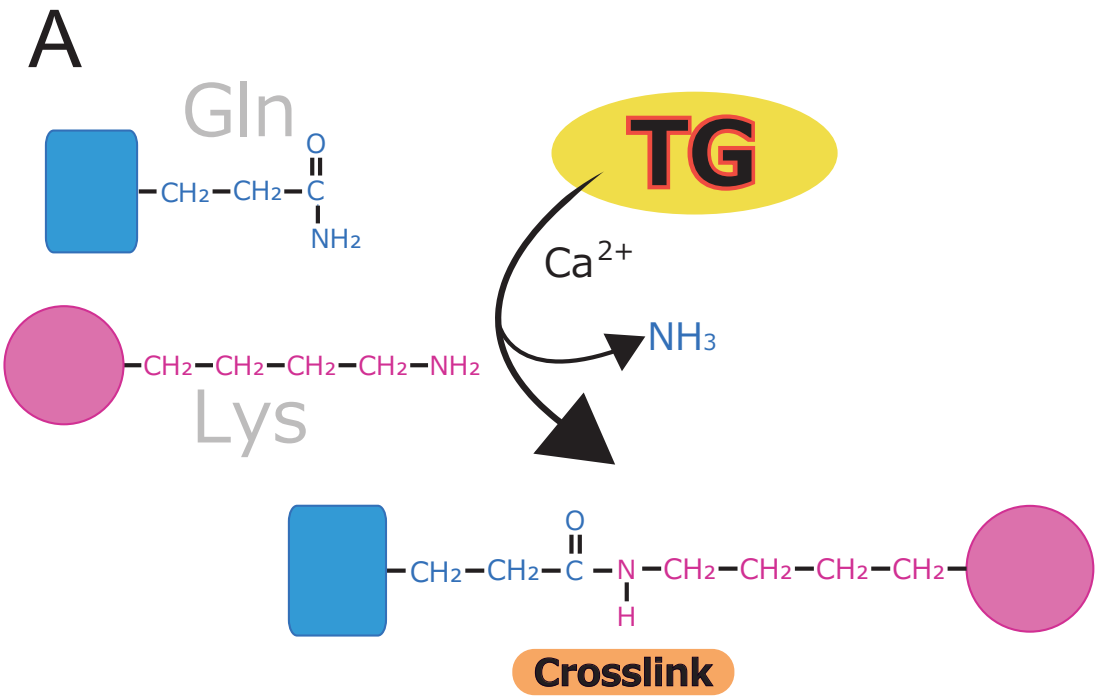
Contents

Preface

Transglutaminase (TG) is an enzyme that catalyzes protein-protein cross-linking between Lys and Gln residues to form ε(γ-glutamyl)lysine bonds involved in physiologic phenomena, such as blood coagulation, skin barrier formation, apoptosis, tissue repair and wound healing in mammals (Fig. A). TG is evolutionally conserved in all metazoans. The mammalian genome contains eight isoenzymes with each enzyme having diverse functions. On the other hand, genome analysis of the fruit fly *Drosophila melanogaster* identified a single TG gene encoding an 87-kDa protein that functions intracellularly and extracellularly. Previous studies performed in this laboratory showed that *Drosophila* TG suppresses the immune deficiency (IMD) pathway by polymerizing the nuclear factor-κB (NF-κB)-like transcription factor Relish to inhibit its nuclear translocation (Shibata *et al*. *Science Signaling*, 2013). TG also modifies Gln residue with primary amines, such as intracellular polyamines including putrescine, spermidine and spermine as shown in the following figure B. These polyamines are crucial for cell differentiations and growth, and are promising targets in anti-cancer drug development. The previous studies also demonstrated that orally ingested synthetic amines are TG-dependently incorporated into the transcription factor Relish. These results prompted me to investigate whether TG-catalyzed incorporation of these intracellular polyamines affects the transcriptional activity of Relish. The present study was undertaken firstly to determine amine-incorporation sites in Relish and secondly to examine the effects of the polyamine incorporation on the DNA-binding activity of Relish not only in *Drosophila* S2 cells but also *in vivo*.

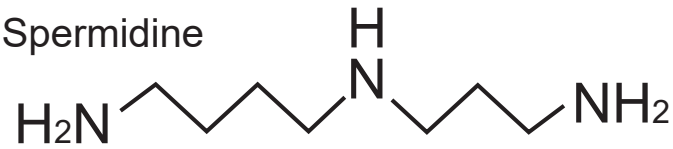
Figure



B

Putrescine

Spermidine



Spermine

Abbreviations

TG; transglutaminase

IMD; immune deficiency

NF-κB; nuclear factor–κB

DCA; monodansylcadaverine

CBB; Coomassie brilliant blue

I-κB; inhibitor of nuclear factor-κB

PGRP; peptidoglycan recognition protein

Abstract

In *Drosophila*, the final immune deficiency (IMD) pathway-dependent signal is transmitted through proteolytic conversion of the nuclear factor-κB (NF-κB)-like transcription factor Relish to the active N-terminal fragment Relish-N. Relish-N is then translocated from the cytosol into the nucleus for the expression of IMD-controlled genes. We previously demonstrated that transglutaminase (TG) suppresses the IMD pathway by polymerizing Relish-N to inhibit its nuclear translocation. On the other hand, we also demonstrated that orally ingested synthetic amines, such as monodansylcadaverine (DCA) and biotin-labeled pentylamine, are TG-dependently incorporated into Relish-N, causing the nuclear translocation of modified Relish-N in gut epithelial cells. It remains unclear, however, whether polyamine-containing Relish-N retains transcriptional activity. Here, we used mass spectrometry analysis of a recombinant Relish-N modified with DCA by TG activity after proteolytic digestion and show that the DCA-modified Gln residues are located in the DNA-binding region of Relish-N. TG-catalyzed DCA incorporation inhibited binding of Relish-N to the Rel-responsive element in the NF-κB-binding DNA sequence. Subcellular fractionation of TG-expressing *Drosophila* S2 cells indicated that TG was localized both in the cytosol and nucleus. Of note, natural polyamines, including spermidine and spermine, competitively inhibited TG-dependent DCA incorporation into Relish-N. Moreover, *in vivo* experiments demonstrated that Relish-N was modified by spermine and that this modification reduced transcription of IMD pathway-controlled *cecropin A1* and *diptericin* genes. These findings suggest that intracellular TG regulates Relish-N-mediated transcriptional activity by incorporating polyamines into Relish-N and via protein-protein crosslinking.

Introduction

Protein-protein crosslinking catalyzed by transglutaminase (TG) plays important and diverse roles in various physiologic phenomena, such as blood coagulation, skin barrier formation, and apoptosis (1). TG catalyzes the isopeptide formation of ε-(γ-glutamyl) lysine bonds in a Ca^{2+}-dependent manner (2). TGs function both intracellularly and extracellularly, and many TG substrates have been identified in metazoans (3–5). The mammalian genome contains eight encoded isoenzymes with each member exhibiting diverse functions: factor XIIIa is essential for blood coagulation and TG2 is involved in apoptosis, cell adhesion, and inflammatory responses (1). In invertebrates such as the horseshoe crab *Tachypleus tridentatus*, the crayfish *Pacifastacus leniusculus*, and the fruit fly *Drosophila melanogaster*, TGs also exhibit pleiotropic functions, including hemolymph coagulation, cuticle formation, and immobilization of invading pathogens, that depend on TG-mediated crosslinking of specific proteins (6-9). Genome analysis of *Drosophila* identified a single *TG* gene encoding an 87-kDa protein that functions in both intracellular and extracellular spaces, although there is no authentic secretion signal sequence in the N-terminus. *Drosophila* TG forms clots and traps invading pathogenic microbes (10, 11), and crosslinks drosocrystallin on the peritrophic matrix, a semi-permeable barrier structure in insects, to form a stabilized fiber structure against toxic proteases released by orally infected pathogenic bacteria (12). Moreover, RNAi directed against the *TG* gene in *Drosophila* leads to a pupal semi-lethal phenotype, abnormal morphology, and shorter lifespan compared with non-RNAi flies (8, 13).

Drosophila has two major immune signal pathways, the Toll and immune deficiency (IMD) pathways (14, 15). The Toll pathway is driven by yeast and Gram-positive bacteria, and the IMD pathway is driven by diaminopimelic acid-type peptidoglycans of Gram-negative bacteria or some strains of Gram-positive bacteria. The Toll and IMD pathways control the production of antimicrobial peptides by the activation

of nuclear factor–κB (NF-κB)–like nuclear factors, Dorsal/Dif and Relish (16–20), respectively. The transcriptional factor Relish, which is regulated by the IMD pathway, is endo-proteolytically activated by Dredd, a homolog of mammalian caspase-8 (21). Upon stimulation with diaminopimelic acid–type peptidoglycans, Relish is converted to the N-terminal fragment (Relish-N) containing the Rel homology domain with transcriptional activity and the C-terminal fragment (Relish-C) containing an ankyrin-repeat domain that inhibits the nuclear-translocation of Relish-N (Fig. 1*A*). We previously reported that the translocation of Relish-N from the cytosol into the nucleus is inhibited by TG-catalyzed protein-protein crosslinking in gut epithelial cells to promote immune tolerance against commensal bacteria, that full-length Relish must be proteolytically cleaved by Dredd before being crosslinked, and that the resulting Relish-N must be exposed by proteolytic conversion to become a suitable substrate for TG (13). On the other hand, synthetic amines, such as monodansylcadaverine (DCA) and biotin-labeled pentylamine, ingested by flies are TG-dependently incorporated into Relish-N, which inhibits protein-protein crosslinking of Relish-N and leads to the nuclear translocation of Relish-N modified with the amines as well as native Relish-N (13). It remains unclear, however, whether the amine-incorporated Relish-N retains transcriptional activity in the nucleus. Natural polyamines such as spermidine and spermine are ubiquitously present in organisms and are involved in critically important intracellular functions, such as gene transcription, cellular growth, and differentiation (22–24). In the present study, we examined the effects of these polyamines on TG-dependent regulation of the transcriptional activity of a recombinant Relish-N (rRelish-N) expressed in *Escherichia coli*. Moreover, TG-dependent polyamine incorporation into native Relish-N and the effects of the modification on the transcription of antimicrobial peptide genes controlled by the IMD pathway were also examined *in vivo*.

Results

TG-dependent DCA incorporation into rRelish-N

We previously demonstrated that *Drosophila* TG catalyzes the crosslinking of Relish-N to suppress antimicrobial production (13). To confirm whether rRelish-N, as shown in Fig. 1*A*, functions as a substrate for TG, rRelish-N was incubated with a recombinant TG (rTG) in the presence of DCA and the fluorescence of the DCA-incorporated rRelish was detected by UV illumination. DCA was incorporated into rRelish-N in a time-dependent manner (Fig. 1, *B* and *C*). In Coomassie Brilliant Blue (CBB)-stained gels, the amount of the protein of rRelish-N decreased depending on the incubation time, because a part of rRelish-N was transformed into high-molecular weight polymers through TG-dependent protein-protein crosslinking, as shown in the following experiments. In addition, the incorporation of DCA into rRelish-N was equivalent to that into *N,N'*-dimethylcasein, which is a standard TG substrate, indicating that rRelish-N is a proper substrate for TG and equivalent to *N,N'*-dimethylcasein (Fig. 1*D*). In the present study, rRelish-N was tagged with GST derived from *Schistosoma japonicum* at its N-terminus, because human GST is a known substrate of TG2 (25). To confirm that the GST tag in rRelish-N is not a target of *Drosophila* TG, rRelish-N was cleaved by PreScission Protease (GE Healthcare, Pittsburgh, PA), and the resulting proteins were incubated with rTG in the presence of DCA. As a result, DCA was only incorporated into rRelish-N, clearly indicating that rRelish-N, but not the GST-tag, is a substrate for TG (Fig. 2, *A* and *B*).

We previously reported that rRelish-N expressed in *Drosophila* S2 cells is crosslinked by TG to form a high-molecular weight polymer (13). To examine whether rRelish-N can form a homopolymer *in vitro*, rRelish-N and rTG were incubated and subjected to SDS-PAGE. The high-molecular weight bands were clearly detected by anti-His tag antibody depending on the incubation time (Fig. 3*A*, upper panel), but not

by anti-TG antibody (Fig. 3*A*, lower panel), indicating that rRelish-N forms TG-dependent covalent homopolymers. We also previously reported that the full-length Relish must be proteolytically cleaved by Dredd to release Relish-N, leading to the exposure of Gln or Lys residues on Relish-N for TG-dependent crosslinking (13). On the other hand, the C-terminal portion of Relish, Relish-C, contains an Ankyrin-repeat domain that inhibits the dimerization of Relish-N (16–20). To examine the inhibitory effects of Relish-C on the protein-protein crosslinking of Relish-N, rRelish-N was incubated with rTG in the presence of rRelish-C. In Western blotting, the intensity of the monomer band of rRelish-N in the absence of rRelish-C was increased approximately 65% by the addition of an equimolar of rRelish-C (Fig. 3, *B* and *C*), and increased dramatically depending on the dose increase of rRelish-C (Fig. 3*D*), indicating that rRelish-C interacts with rRelish-N through masking TG-dependent crosslinking sites on rRelish-N. To determine whether rRelish-C itself has an inhibitory effect on the crosslinking activity of rTG, TG-dependent DCA-incorporation into dimethylcasein was measured in the presence of rRelish-C (Fig. 3*E*). The band intensity of DCA-incorporated dimethylcasein on SDS-PAGE was not affected by increasing concentrations of rRelish-C, indicating that the blocking mechanism of TG-dependent rRelish-N polymerization is due to the presence of rRelish-C at the rRelish-N crosslinking sites.

Determination of amine-incorporation sites in Relish-N

In mammals, I-κB (inhibitor of NF-κB), a mammalian homolog of Relish-C, is a substrate of TG2, and the TG2-catalyzed crosslinking sites have been identified by mass spectrometry (26). To identify the amine-incorporation sites of Relish-N, rRelish-N was incubated with rTG in the presence of DCA, and DCA-incorporated Gln residues were

determined by mass spectrometry after proteolytic digestion (Tables 1–3). In total, 58 peptides were identified by LC-MS/MS analysis of the tryptic or chymotryptic digestion of DCA-modified rRelish-N, covering 90% of the whole sequence of Relish-N (Tables 1–3). Six Gln residues in rRelish-N were identified as DCA incorporation sites, including Gln^{157}, Gln^{219}, Gln^{275}, Gln^{285}, Gln^{287}, and Gln^{304} (Table 3). Interestingly, the modified Gln residues were all located in the Rel homology domain of Relish-N, functioning as the binding site for the κB site of target genes (Fig. 4*A*).

To assess the mechanism underlying the specificity of the identified Gln residues for TG-dependent crosslinking of rRelish-N, all six Gln residues were mutated to Asn residues (rRelish-N^{6QN}). The expression of rRelish-N^{6QN}, however, was too low to be used for the experiments. Fortunately, rRelish-N^{5QN} with five Gln/Asn replacements at Gln^{157}, Gln^{275}, Gln^{285}, Gln^{287}, and Gln^{304} was successfully prepared. rRelish-N^{5QN} was incubated with rTG in the presence of DCA and subjected to SDS-PAGE. The DCA incorporation into rRelish-N^{5QN} was strongly inhibited by the mutations and the fluorescence intensity of the band of DCA-incorporated rRelish-N^{5QN} was approximately 30% that of wild-type rRelish-N (Fig. 4, *B* and *C*). Moreover, the amount of homopolymers of rRelish-N^{5QN} crosslinked by rTG was reduced to an undetectable level (Fig. 4*D*) and the ratio of Relish-N monomers to Relish-N polymers in Western blotting was significantly higher in rRelish-N^{5QN} than in rRelish-N (Fig. 4*E*), indicating a loss of the reactivity of rRelish-N^{5QN} against the TG-dependent protein-protein crosslinking. These results demonstrated that the Gln residues identified by LC-MS/MS analysis are fairly specific for TG-dependent crosslinking in rRelish-N.

Polyamine-incorporation of rRelish-N inhibits its ability to bind to the κB site

Mammalian TG2 functions not only in the cytosol but also in the nucleus (27-31). To confirm the subcellular localization of TG in *Drosophila*, rTG was expressed in S2 cells

with no detectable endogenous TG antigen, and cytoplasmic and nuclear fractions were prepared. Analysis of the relative intensity of the rTG band by Western blotting indicated that the amount of rTG in the nucleus was approximately half that in the cytoplasm (Fig. 5, *A* and *B*), suggesting that *Drosophila* TG works not only in the cytosol but also in the nucleus.

DCA or biotin-pentylamine-incorporated Relish-N is translocated into the nucleus in gut epithelial cells, but whether the amine-incorporated Relish-N retains transcriptional activity in the nucleus is unknown (13). To evaluate the DNA-binding ability of rRelish-N crosslinked with DCA, rRelish-N treated with 5 mM DCA was incubated with the biotinylated-κB oligonucleotide and the protein-oligonucleotide complex was collected using Streptavidin-immobilized agarose. The amount of κB-bound rRelish-N was significantly decreased only when incubated with rTG and DCA (Fig. 5, *C* and *D*). To confirm that the decrease in the binding activity of DCA-treated rRelish-N to the κB oligonucleotide was not caused by TG-dependent protein-protein crosslinking of rRelish-N, rRelish-N was incubated with rTG in different concentrations of DCA and subjected to SDS-PAGE. The polymer band of rRelish-N on SDS-PAGE disappeared at 5 mM DCA (Fig. 5*E*), indicating that the protein-protein crosslinking of rRelish-N by rTG was almost completely inhibited under these conditions.

Polyamines such as putrescine, spermidine, and spermine are natural substrates for TG (32), and intracellular polyamines are present in millimolar concentrations (33, 34). To confirm whether these polyamines are TG-dependently incorporated into rRelish-N, DCA was incubated with rRelish-N in the presence of different concentrations of spermidine or spermine. The incorporation of DCA into rRelish-N was inhibited in the presence of the polyamines in a concentration-dependent manner, suggesting that these

polyamines function as substrates for TG in the cytosol or nucleus (Fig. 6, *A* and *B*). The incorporation of spermine into rRelish-N was also confirmed by Western blotting using anti-spermine antibody in the presence of DCA (Fig. 6, *C* and *D*). DCA incorporation into Relish-N was strongly inhibited by increasing concentrations of spermine, consistent with the incorporation of spermine in a concentration-dependent manner, indicating that spermine and DCA were competitively crosslinked with Relish-N by rTG (Fig. 6, *E* and *F*).

To examine polyamine modification of Relish-N *in vivo*, intestinal proteins extracted from flies fed a spermine-containing diet were analyzed by Western blotting using anti-spermine and anti-Relish-N antibodies. Protein bands of ~68 kDa corresponding to native Relish-N in wild-type flies not fed a spermine-containing diet were detected by anti-spermine antibody (Fig. 7*A*), and endogenous spermine was incorporated into Relish-N in the absence of exogenous spermine (Fig, 7A, left panel). The relative intensity of the ~68-kDa bands increased 2-fold depending on the amount of spermine ingested (Fig. 7, *A* and *B*). In contrast, the incorporation of spermine into the ~68-kDa bands was not detectable in *Relish*-null mutant flies (Fig. 7, *A* and *B*). These findings indicate that polyamines are incorporated into Relish-N *in vivo*. To investigate the effect of polyamine incorporation on the transcriptional activity of Relish-N, mRNA expression of the genes encoding the antimicrobial peptides cecropin A1 and diptericin, which are controlled by the IMD pathway, was measured by quantitative PCR. S2 cells with no TG activity exhibited the same expression level of *cecropin A1* both in the presence and absence of spermine (Fig. 7*C*). On the other hand, in cells expressing TG, the transcription of *cecropin A1* was decreased significantly in the presence of spermine (Fig. 7*C*). Moreover, the transcription of *diptericin* was significantly reduced in wild-type flies fed a spermine-containing diet (Fig. 7*D*). In

TG-RNAi flies, the transcriptional level of *diptericin* remained constant regardless of the presence of spermine. These findings suggest that the transcriptional activity of Relish-N is controlled by polyamines in a TG-dependent manner. Figure 7*D* shows that *diptericin* expression was not significantly enhanced in *TG*-RNAi flies, because 3-day-old flies were used for the experiments. We recently found that the overexpression of antimicrobial peptide is not induced in young *TG*-RNAi flies (35).

Discussion

Many factors are involved in regulating the IMD pathway in *Drosophila* (36, 37): peptidoglycan-recognition protein (PGRP)-LB and PGRP-SC (amidases against peptidoglycans) digest peptidoglycans to non-stimulatory fragments (38–40), Casper represses the expression of IMD-controlled antimicrobial peptide genes (41), PIMS/Rudra/Pirk interact with the complex of PGRP-LC (a membrane-bound receptor for peptidoglycans) and IMD to reduce IMD immune signaling (42–44), the Pvr receptor tyrosine kinase attenuates the JNK and NF-κB arms of the IMD pathway (45), PGRP-LF forms a non-signaling complex with PGRP-LC (46), *Drosophila* Ring and YY1 binding protein promote the proteasomal degradation of Relish (47), and defense repressor 1 regulates the activation of the IMD pathway in the brain (48, 49). Chronic activation of the IMD pathway leads to inflammatory gut diseases and reduces lifespan (50, 51), clearly indicating that distinct proteins and enzymes have evolved into essential regulatory factors to evade uncontrollable conditions for the IMD pathway.

We previously reported that TG-catalyzed Relish-N crosslinking suppresses the IMD pathway to maintain the threshold required for immune tolerance against microbes in the gut, and that the regulated amounts of antimicrobial peptides may function to maintain gut microbiota (13). In mammals, specific transcription factors involved in intracellular signaling pathways are regulated through TG2-dependent protein-protein crosslinking in the cytosol or the nucleus. Park *et al*. reported an I-κB kinase-independent activation mechanism of NF-κB in a lipopolysaccharide-treated microglial cell line: the TG2-dependent polymer formation of I-κB in the cytosol results in the translocation of NF-κB into the nucleus and the subsequent upregulation of inflammatory genes (26). On the other hand, Tatsukawa *et al*. reported hepatocyte apoptosis by TG2-dependent crosslinking of the transcription factor Sp1 in the nucleus: the crosslinking of Sp1 downregulates expression of the *c-Met* gene, which encodes a receptor for hepatocyte

growth factor required for hepatocyte viability (30). Therefore, the regulation of signaling pathways through TG-dependent protein-protein crosslinking of specific transcription factors or regulatory components appears to be highly conserved between mammals and insects.

On the other hand, our previous study revealed that synthetic amines, such as DCA and biotin-labeled pentylamine, ingested by flies are incorporated into Relish-N by TG activity, leading to nuclear translocation of the modified Relish-N into gut epithelial cells (13). Here we found that the incorporation sites for DCA identified in rRelish-N are all localized in the DNA-binding domain of Relish-N and that the DCA modification of rRelish-N reduces the binding of the κB oligonucleotide (Fig. 5, *C* and *D*). We previously reported that ingested or injected DCA triggers the activation of the IMD pathway in the fly gut, although the expression of *diptericin* in DCA-treated flies was not as high as that in *TG*-RNAi flies (13). The present study did not investigate the transcriptional activity of rRelish-N partially modified with DCA except that DCA incorporation of rRelish-N^{5QN} was reduced to approximately 30% that in wild-type rRelish-N (Fig. 4*C*). Some Relish-N partially modified with DCA that is translocated into the nucleus may retain a lower level of transcriptional activity. Natural polyamines, including spermidine and spermine, are also incorporated into Relish-N (Fig. 6). These findings suggest that the polyamine-incorporated Relish-N in the nucleus has reduced transcriptional activity in the nucleus. These polyamines are ubiquitously located in the cytosol and the nucleus, and play critical roles in gene regulation, cell growth, and wound healing after injury (22–24). Their intracellular concentrations are strictly regulated through metabolism, and cellular import and export. We found that TG localizes not only in the cytosol but also in the nucleus in *Drosophila* S2 cells (Fig. 5, *A* and *B*). Therefore, these polyamines may be TG-dependently incorporated into Relish-N to reduce its transcriptional activity under

physiologic or pathologic conditions.

TG requires Ca^{2+} for its activity (1). In addition to the production of antimicrobial peptides by the IMD pathway, reactive oxygen species-dependent innate immunity mediated by the dual oxidase pathway in the gut is very important for host survival (52). Lee *et al*. reported that uracil secreted from non-resident microbes is recognized by a G protein–couple receptor(s), triggering the activation of phospholipase Cβ to increase intracellular Ca^{2+}, sufficient for spontaneous activation of the dual oxidase pathway (53). We speculate that this basal concentration of Ca^{2+} in gut epithelial cells is both required and sufficient to maintain TG activity for the crosslinking of Relish-N to suppress its nuclear translocation or for polyamine-incorporation into Relish to reduce its transcriptional activity (Fig. 8). We conclude that TG regulates the transcriptional activity of Relish-N through the incorporation of polyamines into Relish-N as well as through the protein-protein crosslinking of Relish-N in *Drosophila*.

Experimental procedures

Materials

All chemicals used in this study were of analytical grade unless otherwise stated. DCA, *N,N'*-dimethylcasein, and streptavidin-immobilized on agarose CL-4B were purchased from Sigma-Aldrich (Tokyo, Japan). *Drosophila* S2 cells were purchased from Thermo Fisher Scientific (Waltham, MA). The biotinylated κB oligonucleotide was obtained from Hokkaido System Science (Sapporo, Japan).

Fly stocks

Da-Gal4 and w^{1118} were obtained from Bloomington Stock Center; UAS-*TG*-RNAi was obtained from National Institute of Genetics; Rel^{E20} was obtained from Prof. D. Hultmark, Umeå University. All flies were maintained in standard medium at 25°C.

Preparation of primers for PCR

The sequence of *Relish* (CG11992) or the *TG* (CG7356) gene was amplified by PCR. For the genetic template, the complimentary DNA library of *Drosophila* was used. A mutant of Relish-N was generated using a site-directed mutagenesis method. The primers used in this study were as follows:

Relish-N (residues No. 1–545), 5'-CGGGATCCGATGAACATGAATCAGTACTACGAC-3' (forward) and 5'-TAGTTTAGCGGCCGCATCGTGCTGCAACTC-3' (reverse);

Relish-C (residues No. 546–971), 5'-CGCGGATCCGGGTCATAACCGGGCGGAA-3' (forward) and 5'-ATAGTTTAGCGGCCGCAGTTGGGTTAACCAGTAG-3' (reverse);

TG, 5'-GGCGGATCCATGAGTTATTGGTATCGG-3' (forward) and 5'-CCGCCCGGGAGCTATTACATCGGTGCG-3' (reverse);

Relish-N (Q157N), 5'-AACCCGGTGGAGAAGTTCCGC-3' (forward) and 5'-CTCAACGATCCGCAGCTG-3' (reverse);

Relish-N (Q275N), 5’-GTCTTTAACATGAACCGCCGCGAG-3’ (forward) and 5’-CAGGCGATCCTGCTTCTTC-3’ (reverse);

Relish-N (Q285, 287N), 5’-CTAAACGAACTGCATCAGGAGACAGAG-3’ (forward) and 5’-GTTTTTGTGGGACAACTCGCGGC-3’ (reverse);

Relish-N (Q304N), 5’-AACGTGCGGCTCTGCTTTGAGGCC-3’ (forward) and 5’-GTTCAAGTTCATGTCCTTGGC-3’ (reverse).

The constructed plasmids were confirmed by DNA sequencing.

Western blotting

Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl). The blocked membrane was reacted to each antibody at room temperature for 1 h, including anti-His-tag mAb-HRP-DirecT (Catalog No. D291-7, MBL, Nagoya, Japan), anti-TG polyclonal antibody (8), anti-Relish-N polyclonal antibody (13), anti-V5-tag HRP-DirecT (Catalog No. PM003-7, MBL), anti-α-tubulin HRP-DirecT (Catalog No. PM054-7, MBL), anti-spermine antibody (Catalog No. ab26975, Abcam, Cambridge, UK), or anti-histone H1 antibody (Catalog No. 39575, Active Motif, Carlsbad, CA). Protein bands were visualized by WesternBright Quantum (Advansta, Menlo Park, CA). Chemiluminescence was detected using an Omega Lum G fluorescence imager (Aplegen, Pleasanton, CA).

Preparation of recombinant proteins

The nucleotide sequences of Relish-N and Relish-C were inserted into the pET-22b vector (Novagen, Madison, WI) containing GST and 6×histidine tags (Fig. 1*A*). For wild-type TG (rTG), the entire TG sequence was inserted into the pGEX-4T-2 vector (GE Healthcare). Transformed colonies were picked up and grown in Luria-Bertani

medium to reach an optical density at 600-nm wavelength of ~0.6. At this stage, to induce protein expression, 0.05 mM isopropyl β-D-1-thiogalactopyranoside was added and incubated at 18°C for 24 h with shaking at 180 rpm. After induction, the collected bacteria were sonicated in lysis buffer (50 mM Tris-HCl, pH 8.0, containing 200 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM EDTA, and 1 mM PMSF) by a Branson Sonifier 250 for 1 min, output control 2.5, and duty cycle 50%. The supernatant containing soluble recombinant proteins was separated by centrifugation (13000×g, 4°C, 20 min) and filtered. GST-tagged recombinants were loaded on a glutathione Sepharose 4B column (GE Healthcare). The column was washed with washing buffer (50 mM Tris-HCl, pH 8.0, containing 200 mM NaCl) and eluted with elution buffer (50 mM Tris-HCl, pH 8.0, containing 200 mM NaCl and 20 mM reduced-glutathione).

TG-dependent DCA incorporation into rRelish-N

rRelish-N was incubated with rTG in the TG buffer (50 mM Tris-HCl, pH 8.5, containing 10 mM $CaCl_2$, 10 mM DTT, and 1 mM DCA) at 37°C. The resulting rRelish-N modified with DCA was precipitated with TCA and separated by SDS-PAGE. The fluorescence of DCA was detected by UV illumination (wavelength = 365 nm) and loaded proteins were also stained by CBB.

Mass spectrometry

DCA-incorporated rRelish-N separated by SDS-PAGE was detected by silver staining or CBB staining, and the protein band was excised, digested with trypsin or chymotrypsin, and subjected to LC-MS/MS analysis. Peak lists obtained from the mass spectra were used to identify fragments using the Mascot search engine (Matrixscience, Boston, MA). The analysis was carried out at the Proteomics center, Laboratory for Technical Support, Medical Institute for Bioregulation, Kyushu University.

Pull-down assay

rRelish-N was reacted with rTG in the presence of DCA with the TG buffer at 37°C for 1 h. The resulting DCA-incorporated rRelish-N was incubated with 50 nM the biotinylated κB oligo (biotin-5'-CATCGGGGATTCCTTTT-3') in the κB-binding buffer (10 mM Tris-HCl, pH 7.5, containing 50 mM KCl, 1 mM DTT, and 1 mg/ml BSA) in the presence of 2.5% glycerol, 0.02% Nonidet P-40, and 5 mM $MgCl_2$ at 4°C overnight. Streptavidin-immobilized agarose was added to the sample and the resulting biotin-κB–bound rRelish-N was obtained by centrifugation. The proteins were separated by SDS-PAGE and detected by Western blotting.

Subcellular fractionation of the nucleus and cytosol

Drosophila S2 cells expressing rTG were prepared as described previously (13). The NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Fisher Scientific) was used to prepare the nuclear and cytoplasmic fractions according to the manufacturer's instructions.

Total RNA extraction and reverse transcription

Drosophila S2 cells were maintained at 27°C in Insect-Xpress protein-free medium (Lonza Japan, Tokyo, Japan) with 1% penicillin-streptomycin, 50 μg/ml gentamicin. The cells were transfected with FuGENE HD (Promega, Madison, WI). A pIB vector (Thermo Fisher Scientific) was used for expression of the C-terminal FLAG-tagged TG. Transfected cells were incubated for 18 h with 1 μM 20-hydroxyecdysone (Sigma). After the incubation, the cells were treated with or without 0.5 mM spermine for 1 h, and then stimulated with 1 μg/ml peptidoglycan from *Escherichia coli* O111:B4 (InvivoGen, San Diego, CA) for 8 h. The cells were washed with PBS and total RNA was extracted with RNAiso Plus (Takara, Shiga, Japan), and treated with deoxyribonuclease I. Total RNA (500 ng) was used for reverse transcription with PrimeScript RT Master Mix (Perfect Real Time, Takara)

according to the manufacturer's instructions. Three-day-old adult female flies were fed 5 mM spermine for 48 h. Spermine was dissolved in 5% sucrose. The reagent was added to a vial containing standard medium covered with glass microfiber filters GF/A (diameter 21 mm; GE Healthcare). After feeding, flies' midguts (three replicates of three intestines) were dissected in PBS. Total RNA (300 ng) extracted from midguts was used for reverse transcription.

Quantitative PCR

Quantitative PCR was performed using FastStart Essential DNA Green Master (Roche, Basel, Switzerland) on a LightCycler Nano (Roche). Copy number was determined by relative standard curve method. Target gene inserted into pMD19 (Takara) was used as the standard. *Ribosomal protein 49* (*rp49*) was used as a housekeeping gene. The following primers were used:

rp49, 5'-AGATCGTGAAGAAGCGCACCAAG-3' (forward) and 5'-CACCAGGAACTTCTTGAATCCGG-3' (reverse);

diptericin, 5'-GGCTTATCCGATGCCCGACG-3' (forward) and 5'-TCTGTAGGTGTAGGTGCTTCCC-3' (reverse);

cecropin A1, 5'-CATCTTCGTTTTCGTCGCTC-3' (forward) and 5'-CGACATTGGCGGCTTGTTGA-3' (reverse).

Spermine incorporation into rRelish-N

rRelish-N was reacted with rTG in the presence of DCA (1 mM) and different concentrations of the polyamines. Reacted proteins were separated by SDS-PAGE and detected by UV illumination and Western blotting with anti-spermine antibody. To detect spermine-incorporated into Relish, flies were fed 5 mM spermine for 48 h. Spermine-fed flies were dissected, and midguts proteins were extracted by lysis buffer. The proteins were separated by SDS-PAGE and confirmed by Western blotting.

Statistical analysis

Student's t test was used to calculate *P*-values. A *P*-value of less than 0.05 was considered significant.

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FIGURE LEGENDS

FIGURE 1. TG-dependent incorporation of DCA into rRelish-N.

A, Schematic domain structures of full-length Relish, rRelish-N, and rRelish-C. *B*, rRelish-N (50 nM) was reacted with rTG in the presence of DCA (1 mM) for various incubation times. Reacted proteins were separated by SDS-PAGE and detected by UV illumination and CBB staining. *C*, density of bands stained with CBB or modified with DCA fluorescence was quantitated using ImageJ software (54) and the quantity of DCA incorporation was calculated based on CBB staining. Relative intensity of DCA incorporation at 180 min was defined as 1.0. *D*, rTG was incubated with *N,N'*-dimethylcasein or rRelish-N in the presence of DCA (1 mM) for various incubation times. Reacted proteins were separated by SDS-PAGE and detected by UV illumination and CBB staining. Relative intensity of DCA incorporation was quantified as described in the legend for Fig. 1*C*. Data for *B*, *C*, and *D* are representative of three independent experiments.

FIGURE 2. TG-dependent incorporation of DCA into rRelish-N without the GST-tag.

A, rRelish-N digested with PreScission Protease (2 units) was reacted with rTG in the presence of DCA (1 mM) for different incubation times. Reacted proteins were separated by SDS-PAGE and detected by UV illumination (left) and CBB staining (right). *B*, bands of the CBB and the DCA fluorescence were quantitated using ImageJ software. Data for *A* and *B* are representative of three independent experiments.

FIGURE 3. Polymerization of rRelish-N by TG.

A, rRelish-N was reacted with rTG. Reacted proteins were separated by SDS-PAGE and detected by Western blotting. *B*, rRelish-N (5 nM) was reacted with rTG in the presence of rRelish-C (5 nM). Reacted proteins were separated by SDS-PAGE and detected by Western blotting. *C*, the intensity of rRelish-N (monomer) was analyzed by UltraQuant ID Gel Analysis Software (Aplegen). Error bars represent standard errors of mean values (n = 3). *, $P < 0.05$. *D*, rRelish-N (5 nM) was reacted with rTG in the presence of different

concentrations of rRelish-C (0, 5, 50, and 500 nM). The intensity of the rRelish-N (monomer) band was analyzed by ImageJ software. *E*, dimethylcasein (5 nM) was reacted with rTG in the presence of different concentrations of rRelish-C (0, 5, 50, and 500 nM). The intensity of the DCA-incorporated dimethylcasein band was analyzed by ImageJ software. Data for *A*, *B*, *D*, and *E* are representative of three independent experiments.

FIGURE 4. TG-dependent DCA-incorporation into rRelish-N and rRelish-N^{5QN}.

A, Amino acids detected by mass spectrometry are shown in bold letters. Asterisks indicate the DCA-incorporated Gln residues. A box indicates the Rel homology domain. *B*, rRelish-N or rRelish-N^{5QN} was reacted with rTG in the presence of DCA. The reacted proteins were separated by SDS-PAGE, and detected by UV illumination and CBB staining. *C*, the bands were analyzed by ImageJ software. *D*, rRelish-N or rRelish-N^{5QN} was reacted with rTG. The reacted proteins were separated by SDS-PAGE, and determined by Western blotting. *E*, the intensity of the rRelish-N monomer band was analyzed by ImageJ software. Data for *B* and *D* are representative of three independent experiments. Error bars for *C* and *E* represent standard errors of mean values (n = 3). *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$.

FIGURE 5. TG localization in S2 cells and the inhibition of the κB-binding ability of rRelish-N by TG-dependent DCA incorporation.

A, TG was expressed in S2 cells and the cytoplasmic (C) and nuclear (N) fractions were prepared; V5-tagged rTG in the subcellular fractions was detected by anti-V5 antibody, anti-α-Tubulin, and anti-histone H1 antibodies were used to detect the reference proteins of the cytoplasmic and nuclear fractions. *B*, the intensity of the rTG band was analyzed by ImageJ software. *C*, the DNA binding ability of rRelish-N was analyzed by pull-down assay. rRelish-N was reacted with rTG in the presence of DCA (5 mM) with the TG buffer at 37°C for 1 h. The resulting DCA-incorporated rRelish-N was incubated with 50 nM the biotinylated κB oligo at 4°C overnight. Streptavidin-immobilized agarose was added to the sample and the resulting biotin-κB–bound rRelish-N was obtained by centrifugation.

Proteins were separated by SDS-PAGE and detected by Western blotting using anti-histidine tag antibody. *D*, the intensity of the Relish-N monomer band was analyzed by ImageJ software. *E*, rRelish-N was reacted with rTG in different concentrations of DCA (0, 0.05, 0.5, and 5 mM). Reacted proteins were separated by SDS-PAGE and analyzed by UV illumination. Data for *A*, *C*, and *E* are representative of three independent experiments. Error bars for *B* and *D* represent standard errors of mean values (n = 3). *, $P < 0.05$.

FIGURE 6. Polyamines and DCA were competitively incorporated into rRelish-N.

A, rRelish-N was reacted with rTG in the presence of DCA (1 mM) and different concentrations of spermidine or spermine. Reacted proteins were separated by SDS-PAGE and detected by UV illumination and CBB staining. *B*, the density of bands stained with CBB or modified with DCA fluorescence was quantitated using ImageJ software and the quantity of DCA incorporation was calculated based on CBB staining. Relative intensity of DCA incorporation at 0 mM spermidine or spermine was defined as 1.0. *C*, rRelish-N was reacted with rTG in the presence of DCA (1 mM) and different concentrations of the spermine. Reacted proteins were separated by SDS-PAGE and detected by Western blotting using anti-spermine antibody. *D*, the bands of spermine-incorporated rRelish-N were quantified by ImageJ software. Relative intensity of spermine-incorporated rRelish-N at 100 mM spermine was defined as 1.0. *E*, rRelish-N was reacted with rTG in the presence of DCA (1 mM) and different concentrations of spermine. Reacted proteins were separated by SDS-PAGE and detected by UV illumination. *F*, the quantity of DCA incorporation was measured by ImageJ software. Relative intensity of DCA incorporation at 0 mM spermine was defined as 1.0. Data for *A*-*F* are representative of three independent experiments.

FIGURE 7. Polyamine incorporation into native Relish *in vivo*.

A, Flies were fed 5 mM spermine for 48 h and then dissected, and midguts of the flies were examined by Western blotting. Rel^{E20} indicates *Relish*-null mutant flies. *B*, the density of the bands of spermine-incorporated Relish-N was quantitated using ImageJ software. *C*, TG was

expressed in S2 cells with medium containing 5 mM spermine. The expression level of *cecropin A1* was analyzed by quantitative PCR. *D*, *TG*-RNAi flies were fed 5 mM spermine for 48 h. The expression level of *diptericin* was analyzed by quantitative PCR. Data for *A* and *B* are representative of three independent experiments. Error bars for *C* and *D* represent standard errors of mean values (n = 3). *, $P < 0.05$, ***, $P < 0.001$; n.s, not significant.

FIGURE 8. TG functions as a suppressor of Relish.

Intracellular TG catalyzes the polymerization of Relish-N to inhibit its translocation into the nucleus. On the other hand, polyamines are incorporated into Relish-N to reduce the DNA-binding or the transcriptional ability of Relish-N.

Table 1. Mass spectrometry of trypsin-digested peptides.

Mass		Δmass	Amino acid number	Expected Peptide
Measured	Calculated			
2641.098	2641.102	-0.004	14–38	K.NVMFMNDASSTSGYSSSTSPNSTNR.S
956.471	956.472	-0.001	39–47	R.SFSPAHSPK.T
748.434	748.434	0.000	148–153	K.HVPQLR.I
1243.692	1243.693	-0.001	154–163	R.IVEQPVEKFR.F
1916.878	1916.880	-0.002	166–183	R.YKSEMHGTHGSLNGANSK.R
1950.948	1950.951	-0.003	188–204	K.TFPEVTLCNYDGPAVIR.C
2237.097	2237.101	-0.004	205–223	R.CSLFQTNLDSPHSHQLVVR.K
1806.794	1806.796	-0.002	225–239	K.DDRDVCDPHDLHVSK.E
1761.921	1761.924	-0.003	242–257	R.GYVAQFINMGIIHTAK.K
1228.616	1228.616	-0.001	258–266	K.KYIFEELCK.K
1305.659	1305.661	-0.002	269–278	K.QDRLVFQMNR.R
1409.688	1409.690	-0.002	285–295	K.QLQELHQETER.E
988.476	988.476	0.000	299–306	K.DMNLNQVR.L
913.437	913.437	0.000	307–313	R.LCFEAFK.I
2537.302	2537.303	-0.001	314–336	K.IEDNGAWVPLAPPVYSNAINNRK.S
1228.688	1228.689	-0.001	337–347	K.SAQTGELRIVR.L
2112.147	2112.150	-0.003	348–367	R.LSKPTGGVMGNDELILLVEK.V
2319.028	2319.033	-0.005	375–393	K.VRFFEEDEDGETVWEAYAK.F
2171.009	2171.011	-0.002	396–413	R.ESDVHHQYAIVCQTPPYK.D
1945.973	1945.975	-0.002	420–435	R.EVNVYIELIRPSDDER.S
933.507	933.507	0.000	436–443	R.SFPALPFR.Y
659.397	659.397	0.000	448–453	R.SVIVSR.K
2225.986	2225.988	-0.003	458–480	R.TGSSANSSSSGTESSNNSLDLPK.T
1733.835	1733.837	-0.002	508–521	R.EKHLNEFIASEDFR.K
1411.729	1411.731	-0.002	522–533	R.KLIEHNSSDLEK.I

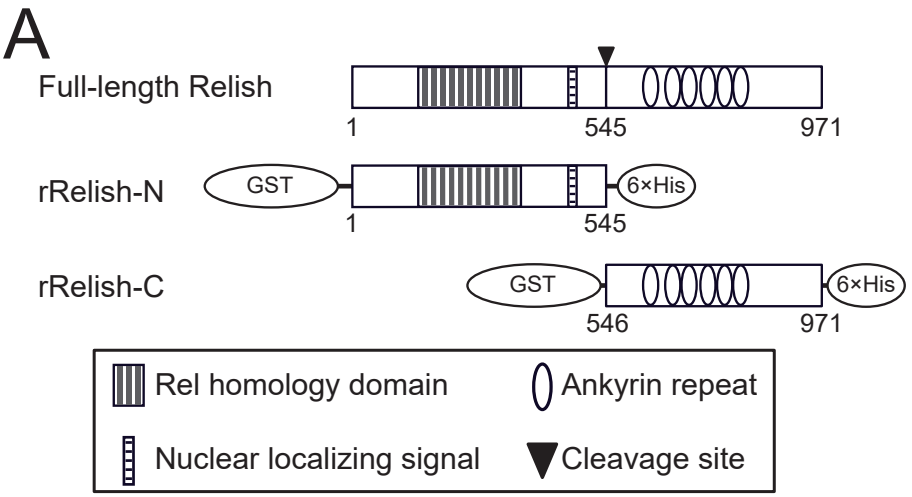
Table 2. Mass spectrometry of chymotrypsin-digested peptides.

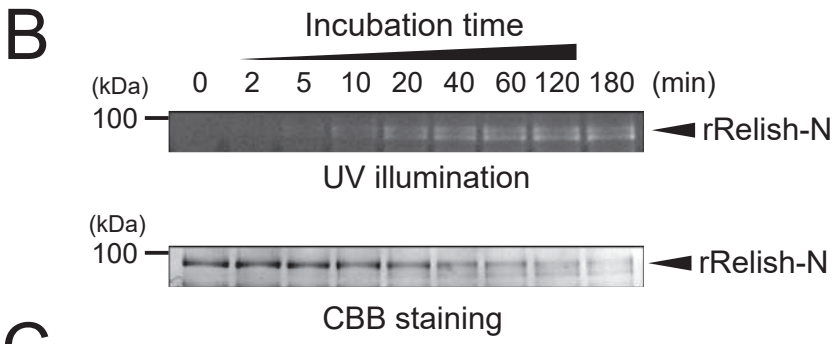
Mass		Δmass	Amino acid number	Expected Peptide
Measured	Calculated			
1222.474	1222.463	0.011	1–9	-.MNMNQYYDL.D + 2 Oxidation (M)
1151.527	1151.528	-0.001	8–17	Y.DLDNGKNVMF.M
1031.387	1031.387	0.000	18–27	F.MNDASSTSGY.S
1370.606	1370.606	0.000	28–40	Y.SSSTSPNSTNRSF.S
1687.788	1687.788	0.000	41–55	F.SPAHSPKTMELQTDF.A
2175.012	2175.017	-0.005	56–76	F.ANLNLPGGNSPHQPPMANSPY.Q
1470.727	1470.725	0.002	77–89	Y.QNQLLNNGGICQL.G
1279.638	1279.641	-0.002	90–102	L.GATNLINSTGVSF.G
1258.564	1258.565	-0.001	103–114	F.GVANVTSFGNMY.M
1157.427	1157.427	0.000	111–119	F.GNMYMDHQY.F
1470.750	1470.751	-0.001	120–133	Y.FVPAPATVPPSQNF.G
787.361	787.361	0.000	134–140	F.GYHQNGL.A
1182.545	1182.545	-0.001	167–177	Y.KSEMHGTHGSL.N
1670.753	1670.755	-0.002	195–208	L.CNYDGPAVIRCSLF.Q
1837.871	1837.874	-0.003	221–235	L.VVRKDDRDVCDPHDL.H
974.493	974.493	0.000	236–243	L.HVSKERGY.V
1387.763	1387.765	-0.001	248–259	F.INMGIIHTAKKY.I
981.523	981.524	-0.001	282–289	L.SHKQLQEL.H
628.366	628.366	0.000	303–307	L.NQVRL.C
1767.918	1767.920	-0.002	313–328	F.KIEDNGAWVPLAPPVY.S
1601.812	1601.812	0.000	329–343	Y.SNAINNRKSAQTGEL.R
1658.854	1658.855	-0.001	349–364	L.SKPTGGVMGNDELILL.V + Oxidation (M)
1515.722	1515.722	0.000	392–403	Y.AKFRESDVHHQY.A
1047.505	1047.506	-0.001	404–412	Y.AIVCQTPPY.K
1478.736	1478.737	0.000	413–424	Y.KDKDVDREVNVY.I
685.401	685.401	0.000	477–482	L.DLPKTL.G
1189.645	1189.646	0.000	483–494	L.GLAQPPNGLPNL.S
1077.581	1077.582	-0.001	515–523	F.IASEDFRKL.I

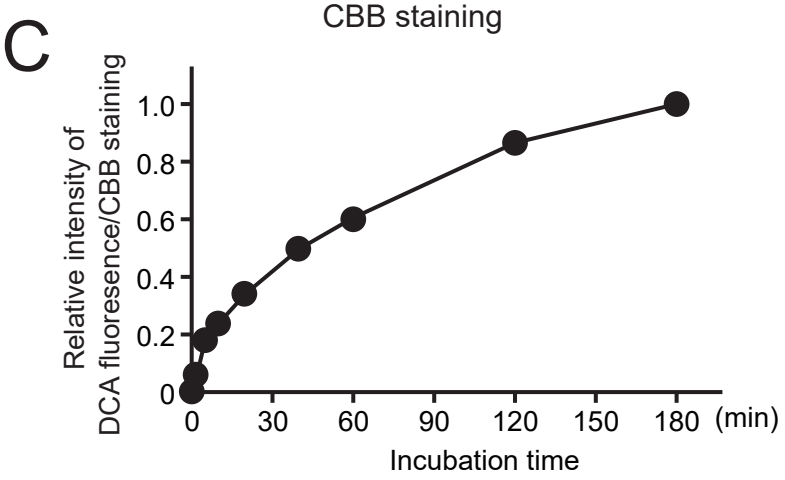
Table 3. Dansylated peptides.

Mass		Δmass	Amino acid number	Expected Peptide
Measured	**Calculated**			
1989.088	1989.087	0.001	148–161	K.HVPQLRIVEQPVEK.F + DCA (Q)
1693.785	1693.788	-0.003	209–220	F.QTNLDSPHSHQL.V + DCA (Q)
1240.607	1240.610	-0.003	272–278	R.LVFQMNR.R + DCA (Q); Oxidation (M)
2045.978	2045.970	0.008	285–295	K.QLQELHQETER.E + 2 DCA (Q)
1306.615	1306.616	-0.002	299–306	K.DMNLNQVR.L + DCA (Q)

Figure 1


A
Full-length Relish
1
545
971
rRelish-N
GST
6×His
1
545
rRelish-C
GST
6×His
546
971
Rel homology domain
Ankyrin repeat
Nuclear localizing signal
Cleavage site


B
Incubation time
(kDa)
0 2 5 10 20 40 60 120 180 (min)
100
rRelish-N
UV illumination
(kDa)
100
rRelish-N
CBB staining


C
Relative intensity of DCA fluoresence/CBB staining
1.0
0.8
0.6
0.4
0.2
0
0 30 60 90 120 150 180 (min)
Incubation time

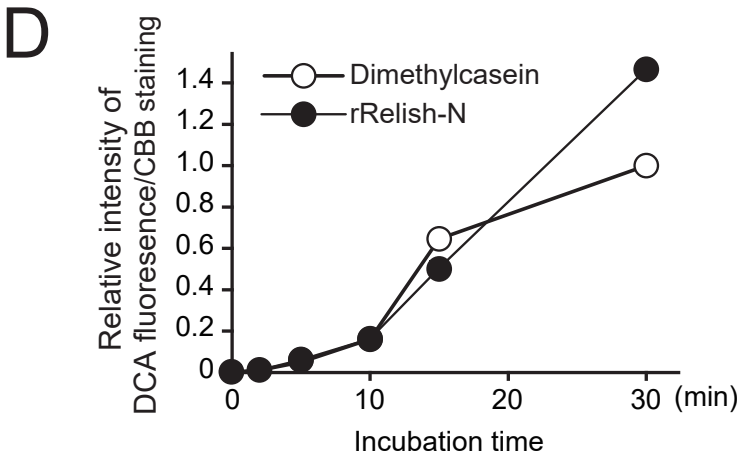
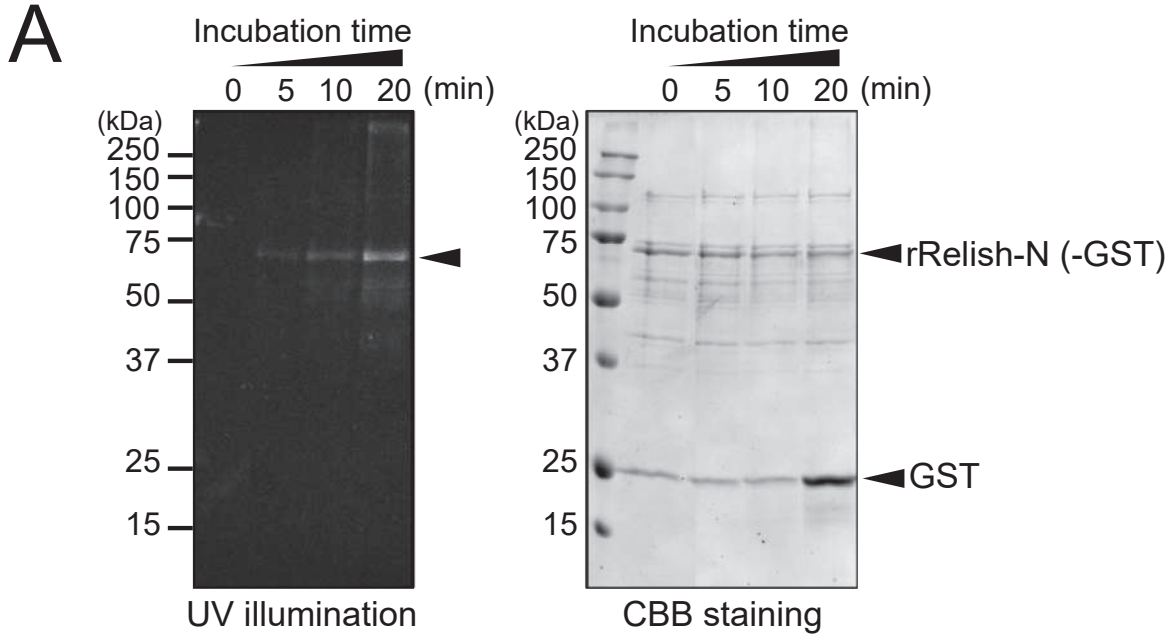

D
Relative intensity of DCA fluoresence/CBB staining
Dimethylcasein
rRelish-N
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0 10 20 30 (min)
Incubation time

Figure 2


A
Incubation time
0 5 10 20 (min)
(kDa)
250
150
100
75
50
37
25
15
UV illumination
CBB staining
rRelish-N (-GST)
GST

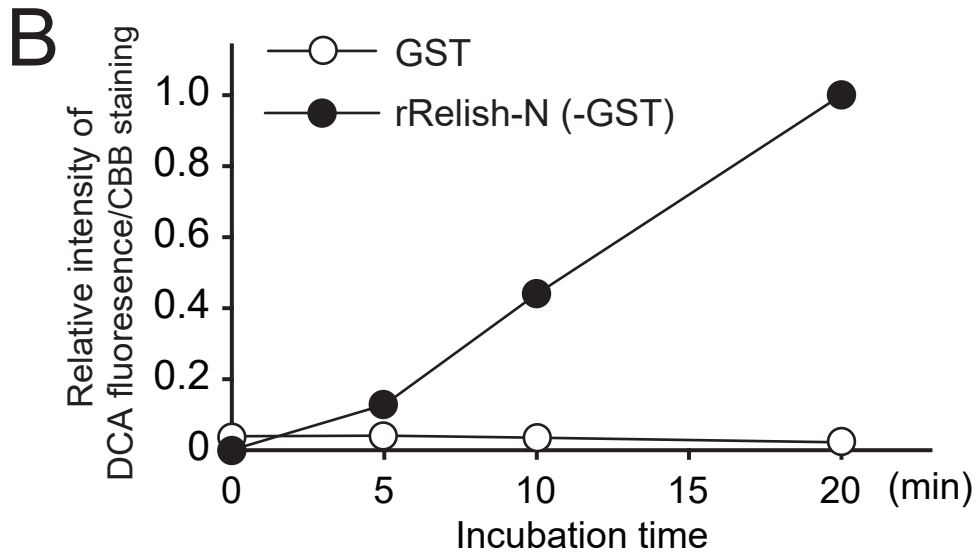

B
GST
rRelish-N (-GST)
Relative intensity of
DCA fluoresence/CBB staining
1.0
0.8
0.6
0.4
0.2
0
0 5 10 15 20 (min)
Incubation time

Figure 3

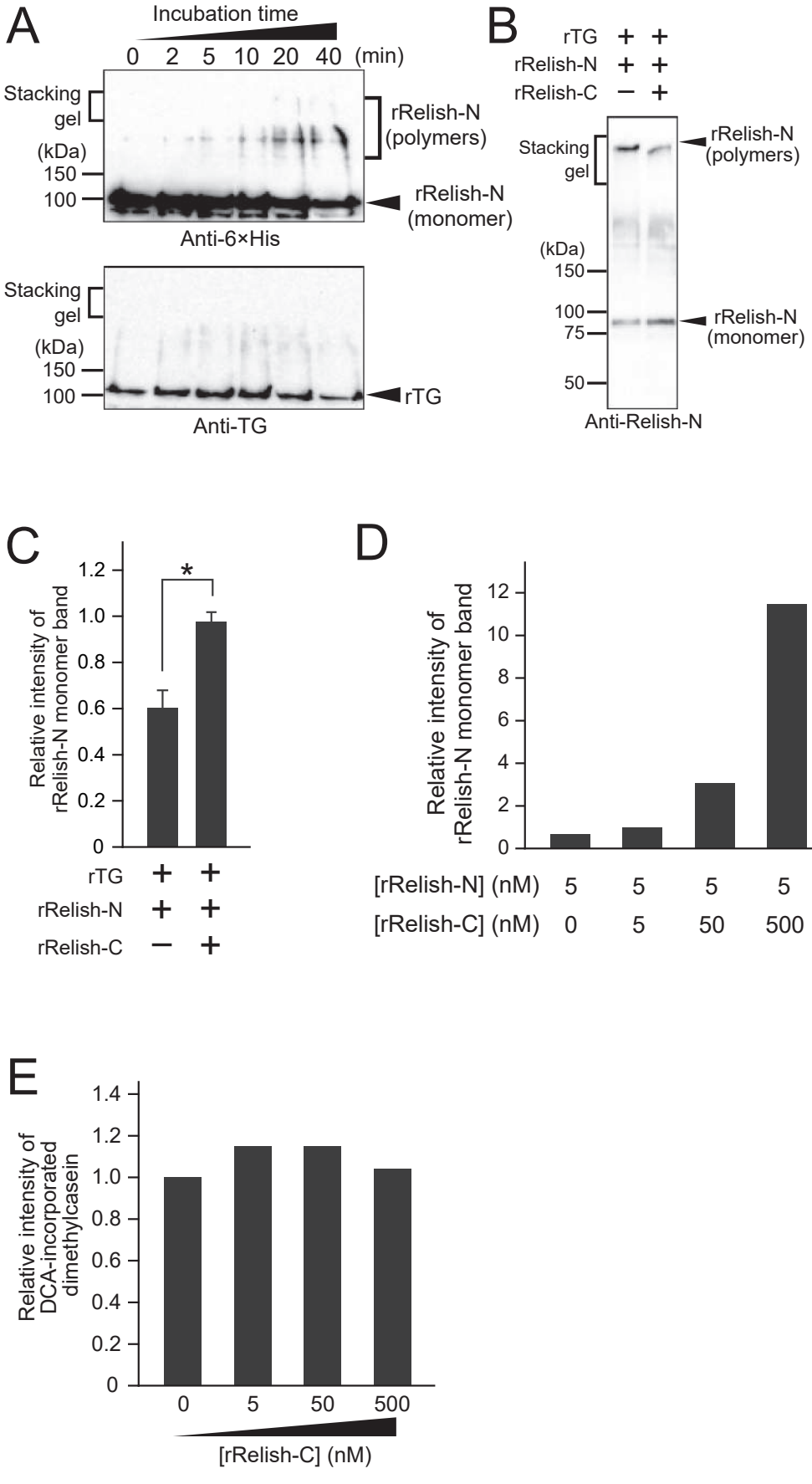

A
Incubation time
0 2 5 10 20 40 (min)
Stacking gel
(kDa)
150
100
rRelish-N (polymers)
rRelish-N (monomer)
Anti-6×His
Stacking gel
(kDa)
150
100
rTG
Anti-TG
B
rTG + +
rRelish-N + +
rRelish-C − +
Stacking gel
rRelish-N (polymers)
(kDa)
150
100
75
50
rRelish-N (monomer)
Anti-Relish-N
C
Relish-N monomer band
1.2
1.0
0.8
0.6
0.4
0.2
0
*
rTG + +
rRelish-N + +
rRelish-C − +
D
Relish-N monomer band
12
10
8
6
4
2
0
[rRelish-N] (nM) 5 5 5 5
[rRelish-C] (nM) 0 5 50 500
E
DCA-incorporated dimethylcasein
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0 5 50 500
[rRelish-C] (nM)

Figure 4

A

```
  1 MNMNQYYDLD NGKNVMFMND ASSTSGYSSS TSPNSTNRSF SPAHSPKTME
 51 LQTDFANLNL PGGNSPHQPP MANSPYQNQL LNNGGICQLG ATNLINSTGV
101 SFGVANVTSF GNMYMDHQYF VPAPATVPPS QNFGYHQNGL ASDGDIKHVP
151 QLRIVEQPVE KFRFRYKSEM HGTHGSLNGA NSKRTPKTFP EVTLCNYDGP
201 AVIRCSLFQT NLDSPHSHQL VVRKDDRDVC DPHDLHVSKE RGYVAQFINM
251 GIIHTAKKYI FEELCKKKQD RLVFQMNRRE LSHKQLQELH QETEREAKDM
301 NLNQVRLCFE AFKIEDNGAW VPLAPPVYSN AINNRKSAQT GELRIVRLSK
351 PTGGVMGNDE LILLVEKVSK KNIKVRFFEE DEDGETVWEA YAKFRESDVH
401 HQYAIVCQTP PYKDKDVDRE VNVYIELIRP SDDERSFPAL PFRYKPRSVI
451 VSRKRRRTGS SANSSSSGTE SSNNSLDLPK TLGLAQPPNG LPNLSQHDQT
501 ISEEFGREKH LNEFIASEDF RKLIEHNSSD LEKICQLDMG ELQHD
```

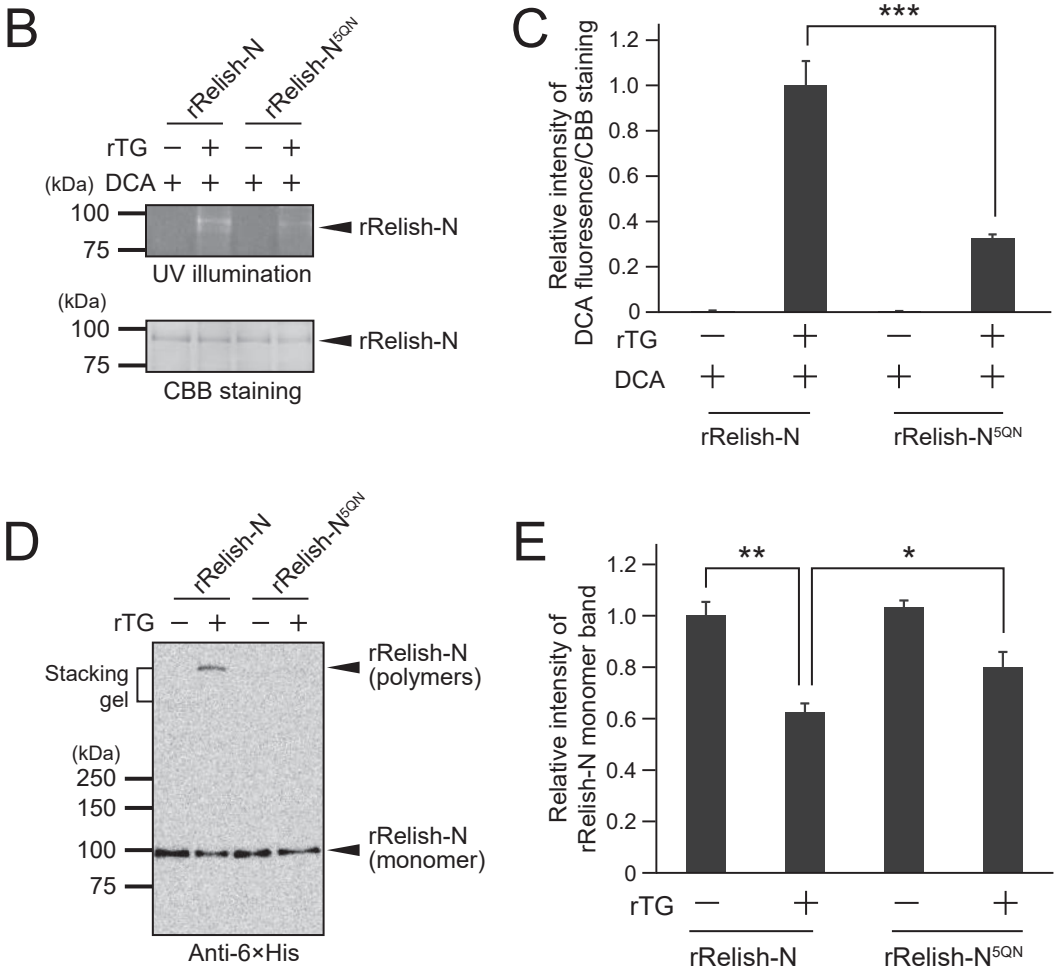


Figure 5

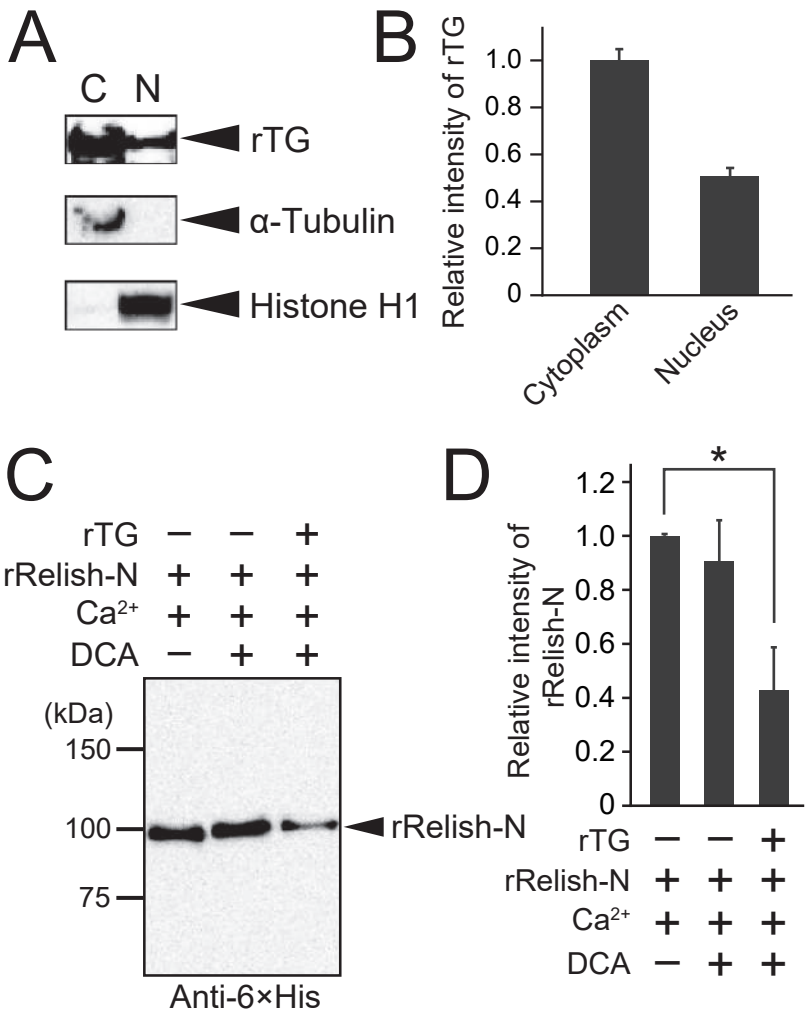

A
C N
rTG
α-Tubulin
Histone H1
B
Relative intensity of rTG
1.0
0.8
0.6
0.4
0.2
0
Cytoplasm
Nucleus
C
rTG − − +
rRelish-N + + +
Ca2+ + + +
DCA − + +
(kDa)
150
100
75
rRelish-N
Anti-6×His
D
*
Relative intensity of rRelish-N
1.2
1.0
0.8
0.6
0.4
0.2
0
rTG − − +
rRelish-N + + +
Ca2+ + + +
DCA − + +
E
DCA
0 0.05 0.5 5 (mM)
Stacking gel
Crosslinked rRelish-N
(kDa)
150
100
75
rRelish-N
UV illumination

Figure 6

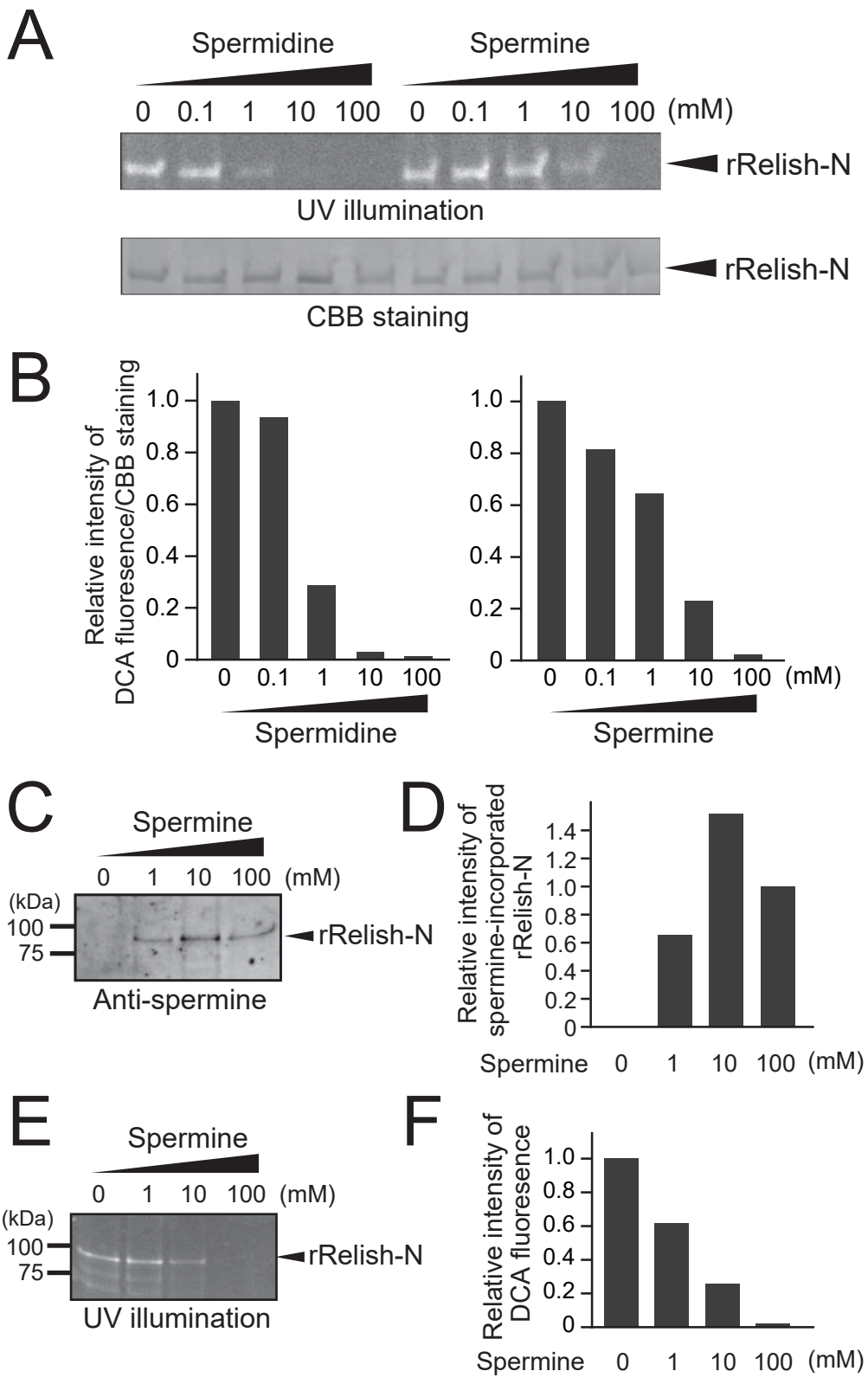

A
Spermidine
Spermine
0 0.1 1 10 100 0 0.1 1 10 100 (mM)
rRelish-N
UV illumination
rRelish-N
CBB staining
B
Relative intensity of DCA fluoresence/CBB staining
1.0
0.8
0.6
0.4
0.2
0
0 0.1 1 10 100
Spermidine
0 0.1 1 10 100 (mM)
Spermine
C
Spermine
0 1 10 100 (mM)
(kDa)
100
75
rRelish-N
Anti-spermine
D
Relative intensity of spermine-incorporated rRelish-N
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Spermine 0 1 10 100 (mM)
E
Spermine
0 1 10 100 (mM)
(kDa)
100
75
rRelish-N
UV illumination
F
Relative intensity of DCA fluoresence
1.0
0.8
0.6
0.4
0.2
0
Spermine 0 1 10 100 (mM)

Figure 7

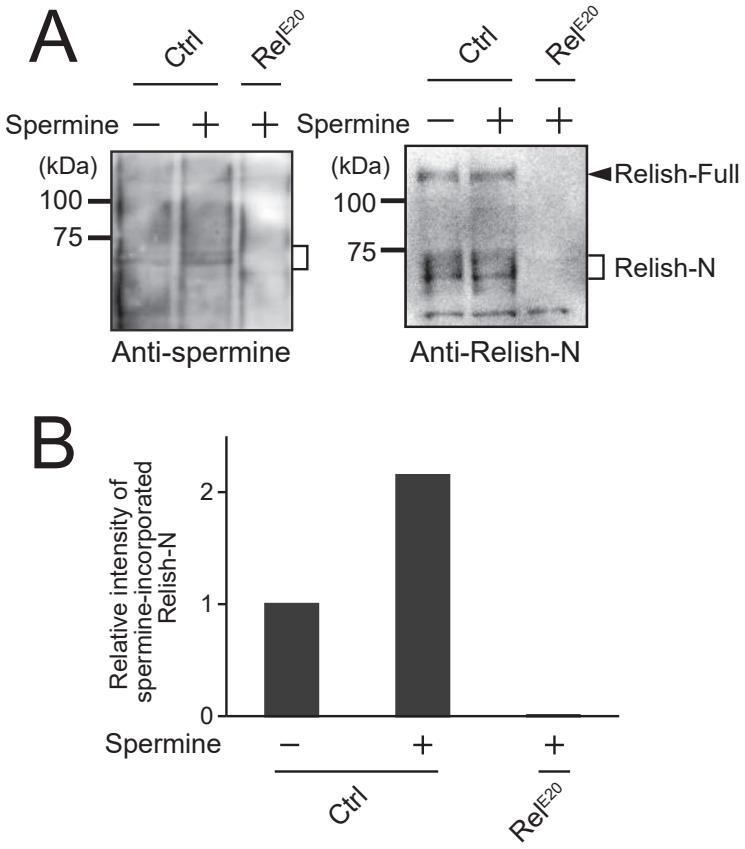
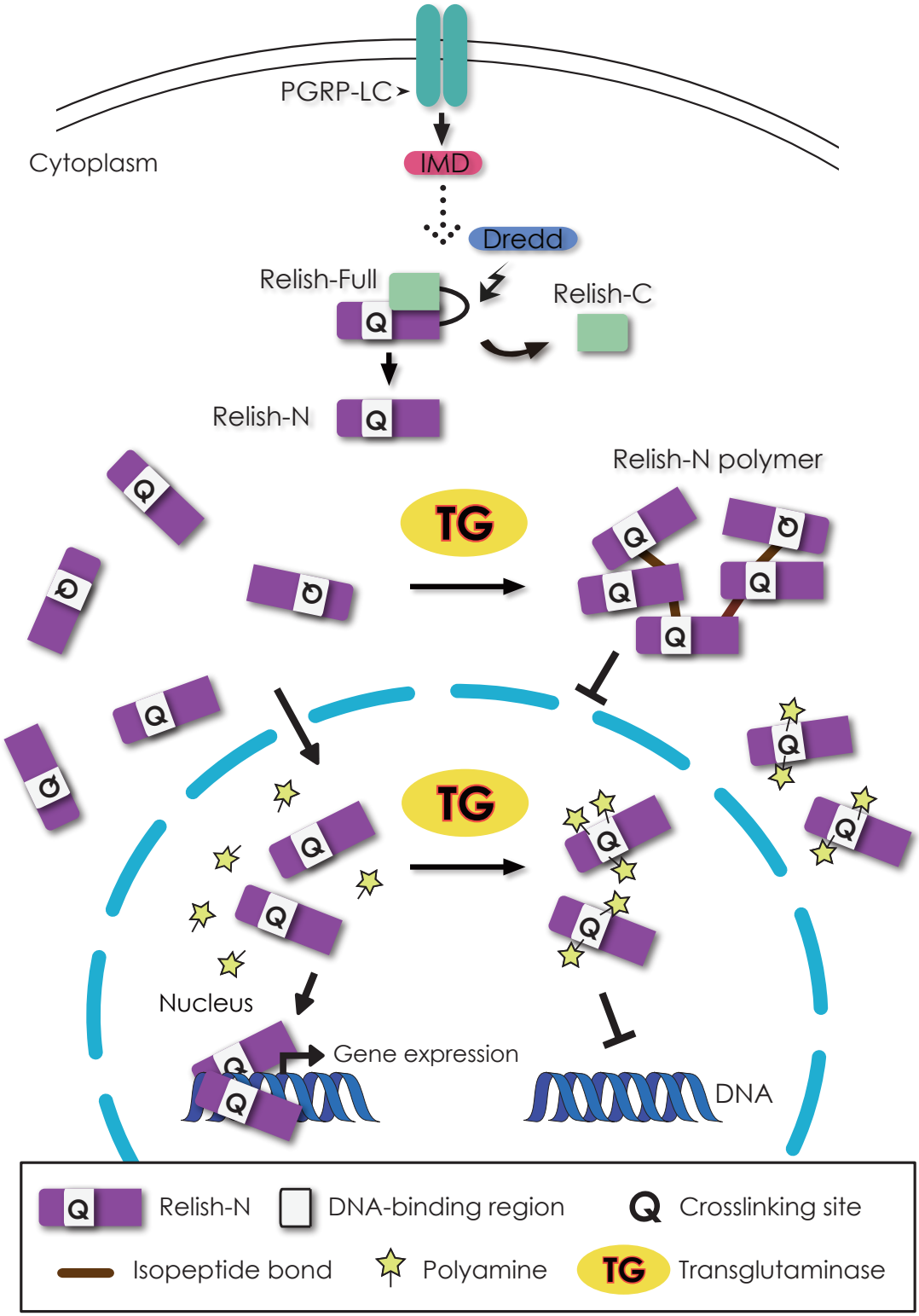

A
Ctrl
Rel^E20
Spermine
(kDa)
100
75
Anti-spermine
Relish-Full
Relish-N
Anti-Relish-N
B
Relative intensity of spermine-incorporated Relish-N
2
1
0
Spermine
Ctrl
Rel^E20
C
Cecropin A1/rp49
0.01
0
n.s.
***
*
Spermine
TG
D
Diptericin/rp49
0.008
0.006
0.004
0.002
0
n.s.
*
Spermine
TG-RNAi

Figure 8


PGRP-LC
Cytoplasm
IMD
Dredd
Relish-Full
Relish-C
Relish-N
Relish-N polymer
TG
TG
Nucleus
Gene expression
DNA
Relish-N
DNA-binding region
Crosslinking site
Isopeptide bond
Polyamine
TG
Transglutaminase