Chloramphenicol inhibits eukaryotic Ser/Thr phosphatase and infection-specific cell differentiation in the rice blast fungus

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Chloramphenicol (Cm) is a broad-spectrum classic antibiotic active against prokaryotic organisms. However, Cm has severe side effects in eukaryotes of which the cause remains unknown. The plant pathogenic fungus *Magnaporthe oryzae*, which causes rice blast, forms an appressorium to infect the host cell via single-cell differentiation. Chloramphenicol specifically inhibits appressorium formation, which indicates that Cm has a novel molecular target (or targets) in the rice blast fungus. Application of the T7 phage display method inferred that MoDullard, a Ser/Thr-protein phosphatase, may be a target of Cm. In animals Dullard functions in cell differentiation and protein synthesis, but in fungi its role is poorly understood. In vivo and in vitro analyses showed that MoDullard is required for appressorium formation, and that Cm can bind to and inhibit MoDullard function. Given that human phosphatase CTDSP1 complemented the MoDullard function during appressorium formation by *M. oryzae*, CTDSP1 may be a novel molecular target of Cm in eukaryotes.

Drugs frequently exhibit unexpected or unintended activities, as a result of unknown targets or interaction between drugs and other host molecules. A number of marketed drugs are assumed to have multiple targets. Although identification of new drug targets is vital for discovery and development of novel drugs, the procedure is challenging, labour intensive, and time-consuming for researchers and the pharmaceutical industry. By use of gene microarrays, candidate genes for intracellular drug targets affected by specific drug activities have been identified. However, this strategy generates a substantial amount of data and changes in gene expression may not correlate with drug action and hidden phenotypes. If target engagement is not validated, novel mechanistic targets are difficult to identify. Recent advances in validation analysis have enabled evaluation and estimation of novel targets of drugs. The use of fluorescent proteins in living cells has enabled the quantitation of spatial and temporal changes in drug-target engagement in protein complexes in response to drug treatment. Drug–target interactions are also predicted using machine learning-based and network-based methods. These powerful validation strategies accelerate identification of the drug-target engagement. In contrast, for development of new drugs together with target estimation, which integrates the relationship with known target factors, it is necessary to seek unknown target factors and to elucidate their functions.

The rice blast fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*) is one of the most destructive pathogenic filamentous fungi that infect rice. Rice blast decreases the total yield of crops by approximately 10–30%, therefore understanding the infection strategy of this pathogen and controlling damage to crops is critical for...
global food security. The infection cycle of this fungus is initiated by a three-celled conidium contacting the surface of a rice leaf. The conidium forms an elongating germ tube, and subsequently a hemispherical structure, termed the appressorium, develops at the tip of the germ tube. Considerable osmotic pressure attaining approximately 8 MPa is generated in the appressorium owing to accumulation of high concentrations of compatible solutes, such as glycerol. Using this turgor pressure, a penetration peg extends from the appressorium base and penetrates the plant cell wall to enter an epidermal cell. This infection process is necessary to invade a host plant and is dependent on mitotic division of the single-celled germ tube and cell differentiation. In addition, many molecules stored in the conidium are associated with appressorium formation, and expression of numerous appressorium-related genes increases dramatically during the cell differentiation process.

Given that innumerable cellular components are involved in cell differentiation, there are many potential molecular targets of a variety of drugs. Knowledge of drugs that inhibit cellular differentiation will contribute to understanding not only secondary targets of drugs but also the underlying cellular events. Ishii et al. focused on simple cell differentiation and unique genomic properties of M. oryzae, and reported that roxithromycin can inhibit appressorium formation via interaction with MoCDC27. These authors used a M. oryzae appressorium assay for drug screening and a genomic DNA library-based T7 phage display method for identification of the roxithromycin target. M. oryzae conidial germination and appressorium formation can be artificially induced on hydrophobic polyvinyl chloride as well as on plant surfaces. The genome size of M. oryzae is approximately 41.7 Mb and the proportion of non-coding DNA is about 60%, whereas the human genome is approximately 2.8 Gb and non-coding DNA comprises about 99% of the genome. Although a genomic library includes the peptides derived from non-coding regions, frame-shifting, and antisense sequences, M. oryzae genomic DNA library enables comprehensive analysis of the majority of proteins encoded by temporarily expressed genes. These genomic characteristics of M. oryzae are suitable for geneticists as its genome is approximately 41.7 Mb and the proportion of non-coding DNA is about 60%, whereas the human genome is approximately 2.8 Gb and non-coding DNA comprises about 99% of the genome.

In this study, we show that simple screening and in vivo validation methods using M. oryzae as the study organism are valuable to discover unexpected drug targets. The appressorium formation assay revealed that a classic antibiotic, chloramphenicol (Cm), specifically inhibited appressorium formation in M. oryzae. It is well known that Cm binds to 50S ribosomal RNA and inhibits peptide synthesis in prokaryotes. Although a high concentration of Cm may cause mitochondrial malfunction in eukaryotic cells via the prokaryotic ribosome, the effect of Cm on appressorium formation in M. oryzae suggests the existence of novel secondary targets in fungi. The original genomic library-based T7 phage display method revealed that Cm can target the Ser/Thr phosphatase Dullard in M. oryzae and humans. Therefore, the Dullard protein may be a secondary target of Cm in humans, which has not been explored previously. This is the first report that Cm targets a eukaryotic molecule and inhibits cell differentiation. We demonstrated that fungal genomic library-mediated comprehensive screening and assay methods may contribute to identification of novel drug targets associated with cellular differentiation in eukaryotes.

**Results**

**Cm specifically inhibited appressorium formation of M. oryzae.** To test whether Cm affects infection-specific cell differentiation of M. oryzae, conidial suspensions supplemented with 3, 30, 300, or 3000 µM Cm were inoculated onto the surface of hydrophobic polyvinyl chloride. After 6 h post inoculation, the percentage of germinated conidia, germ-tube length, and percentage appressorium formation were calculated after microscopic observation. The germination percentage and germ-tube length were not affected by Cm, whereas the percentage of appressorium formation was significantly and specifically decreased in the presence of 30 and 300 µM Cm (Fig. 1). Addition of excessive Cm (3000 µM) resulted in inhibition of conidial germination and/or germ-tube elongation, which suggested that this Cm concentration inhibited the mitochondrial ribosome (Fig. S1). Interestingly, the Cm analogues thiamphenicol and florfenicol did not inhibit appressorium formation (Fig. S2). In these analogues the nitro group of Cm is modified, which suggests that the nitro group of Cm is involved in the inhibitory effect from binding to the target factor. These results indicated that Cm specifically inhibits appressorium formation of M. oryzae and has a novel target of cell differentiation in eukaryotic cells.

**Isolation of MoDullard as the Cm target using the T7 phage display method.** To screen the novel Cm target in M. oryzae, we used the M. oryzae genomic DNA library-based T7 phage display method. As the ligand for T7 phage display, we connected Cm to a biotinyl linker by organic synthesis (Fig. S3). Biotinylated Cm retained the ability to inhibit appressorium formation of M. oryzae (Fig. S4). Using this approach, we obtained 82 candidate peptide sequences, of which a BLASTP search revealed that 14 sequences showed homology to M. oryzae proteins. Among these sequences, two were coded in CDS regions (Table S2) and one of the candidate peptides showed high similarity (e-value: 1.6 × 10^-28) to the Dullard-like phosphatase domain in the Ser/Thr phosphatase Dullard (MGG_03646; MoDullard) (Fig. 2a). Dullard was identified in Xenopus laevis and Dullard-like phosphatase is highly conserved in eukaryotes. Orthologues in ascomycetes and yeast have been identified, and the phosphatase domain is also conserved between these organisms (Fig. S5). Dullard is involved in cell differentiation in higher organisms; for example, Dullard acts as a negative regulator of TGF-β signaling for endochondral ossification via phosphorylation of Smad2/3 in mice. M. oryzae harbored two paralogues of MoDULLARD, namely MoNEM1 (nuclear envelope morphology protein 1) (MGG_06001) and MoFCP1 (RNA polymerase II subunit A domain phosphatase 1) (MGG_03485). These genes also contained a putative Dullard-like phosphatase domain. Although pathogenicity was not affected in the deletion mutant monem1 in barley and rice, a functional analysis of MoDullard and MoFCP1 in M. oryzae has not been performed. Given
that MoDullard contained the completely identical sequence to the displayed peptide sequence, we performed a functional analysis of MoDullard.

**MoDullard is associated with appressorium formation.** To examine whether Dullard phosphatase is associated with appressorium formation by *M. oryzae*, we analysed the expression patterns of MoDullard by semi-quantitative RT-PCR (semi-qPCR). MoDULLARD was expressed during vegetative growth and appressorium formation but a higher expression level was observed in the appressorium formation phase (Fig. S6a). To investigate the effect of MoDullard on appressorium formation, we generated the Δmodullard mutant by *Agrobacterium tumefaciens*-mediated transformation (AtMT)27. The mutant often produced aberrant conidia that were narrow and/or lacking one of the two septa (Fig. S6b), and showed a slightly reduced growth rate on oatmeal medium, YG medium, and YPD medium (data not shown). The conidia from the Δmodullard mutant and the wild-type P2 strain showed similar germination frequencies (Fig. 2b), whereas the percentage appressorium formation of the Δmodullard mutant was severely decreased compared with that of the wild type (Fig. 2c). The MoDULLARD-complemented strain showed restoration of appressorium formation and susceptibility to Cm. In addition, the MoDULLARD overexpression strain showed tolerance to Cm (Fig. 2d). These results indicated that MoDullard plays an important role in appressorium formation and that Cm may directly bind to MoDullard. To analyse the interaction between MoDullard and Cm, the GST-tag and 6xHis-tag were fused to the N-terminus and C-terminus of MoDullard, respectively. The recombinant MoDullard and control proteins were injected into avidin-beads to immobilise Bio-Cm or Bio-Ctrl. The bound proteins were eluted and detected by western blotting. The tag-fused recombinant MoDullard protein bound weakly to Bio-Cm (Fig. S7). From these inhibition analyses, it was inferred that Cm inhibits appressorium formation by means of the loss or decline in Ser/Thr phosphatase function.

**MoDullard function is similar to that of human CTDSP1.** Chloramphenicol causes side effects in humans but its target molecule has not been elucidated in eukaryotes. Given that fungal Dullard was targeted by Cm, we investigated whether human orthologues complement the MoDullard function in *M. oryzae*. Five MoDullard orthologues, namely CTDSP1 (CCDS56166.1), CTDSP2 (CCDS41801.1), CTDNBP (CCDS11093.1), CTDSP1 (CCDS33734.1), and CTDSP1 (CCDS10110.1), were identified from the human genome (Fig. 3a). Each orthologue also contained a phosphatase domain in the C-terminal region with high similarity (Fig. S8), but these orthologues have different molecular functions in human cells28–32. We cloned cDNA for each orthologue from human U937 cell mRNA and expressed the cDNA under the constitutive promoter in Δmodullard cells. The appressorium formation assay surprisingly showed that only CTDSP1 expression complemented the MoDullard function (Figs 3b, S9) and Cm inhibited CTDSP1-complemented appressorium formation (Fig. 3c). To confirm the importance of phosphatase activity for appressorium formation, we obtained the complementary strains expressing the active-site-mutated MoDullard and CTDSP1 in the Δmodullard mutant, and the appressorium formation ability of these mutants was analysed. All complemented strains containing the mutated phosphatases
could not restore appressorium formation (Fig. 3d). These results suggested that MoDullard affected phosphorylation of the carboxy-terminal domain (CTD) in RNA polymerase II (RNAPII) as well as CTDSP1.

Discussion

Organisms are composed of many thousands of compounds, thus many drugs may have multiple molecular targets and cause side effects. Analysis of secondary targets of drugs will lead to drug repositioning or analysis of the mechanism of the side effects1. Chloramphenicol was isolated from Streptomyces venezuelae as a broad-spectrum antibiotic for prokaryotes but often causes side effects in humans; therefore, use of Cm has been avoided as a first-line treatment33. In the present study, Cm showed specific inhibition of appressorium formation in M. oryzae, although conidial germination and germ-tube elongation were not affected. This effect cannot be explained by the well-known action of Cm binding to the mitochondrial ribosome. These results suggested that Cm may target a novel eukaryotic molecular factor (or factors) and inhibit cell division and differentiation factors in the rice blast fungus.

Complementation experiments indicated that MoDullard and the human orthologue CTDSP1 fully complemented the function of MoDullard in M. oryzae. This is the first report that MoDullard and human CTDSP1 play an important role in cell differentiation in filamentous fungi and are targeted by Cm in eukaryotic cells. MoDullard contains the highly conserved Dullard phosphatase domain and is a member of the halo acid dehalogenase (HAD) superfamily, which is classified in the RNA polymerase CTD phosphatase family34. In human cells, CTDSP1 functions as a Ser/Thr phosphatase, especially in the dephosphorylation of the CTD of the RNAPII largest subunit (Rbp1)35. The human RNAPII CTD contains highly conserved heptad repeats Tyr-Ser-Pro-Thr-Ser-Pro-Ser (YSPTSPS)36. After RNAPII-mediated transcription, dephosphorylation of the CTD heptad repeats of RNAPII released from the DNA template is required to form the complex for the next transcription cycle37. Therefore, the phosphorylation status of the CTD repeat regulates RNAPII-mediated transcription, and dephosphorylation of repeats is needed to recycle RNAPII. A mutation in the phosphatase domain causes loss of the dephosphorylation activity of CTDSP138. Human CTDSP1 complemented the MoDullard function in M. oryzae cells, and the function was inhibited by treatment with Cm during appressorium formation. In
addition, mutation of the phosphatase domain in this protein led to a severe decrease in frequency of appressorium formation. These results suggested that MoDullard also plays a role as a Ser/Thr phosphatase for dephosphorylation of the CTD of the RNAPII largest subunit Rbp1. The abundance of total RNAPII cannot be changed but raising the rate of RNAPII recycling improves the transcription efficiency. RNAPII is phosphorylated stepwise during initiation, accumulation of RNA, and release of the expression products. The stepwise phosphorylation is the key to start each step and at the termination RNAPII is hyperphosphorylated; RNAPII is dephosphorylated to enter the next transcriptional stage. Consequently, the phosphorylated RNAPII must be dephosphorylated to restart the transcription cycle. During infection of a rice plant by *M. oryzae*, energy resources are limited to those stored in the conidium. Thus, energy management is important for successful infection under the energy-limited condition. Conidial autophagic cell death occurs and subsequently the substrates and energy are recycled for appressorium formation. Excluding the treatment with a high concentration of Cm (3000 µM), Cm did not inhibit conidial germination and germ-tube elongation (Fig. S1). If MoDullard functions similar to CTDSPI in the dephosphorylation of RNAPII, Cm may affect the recycling of RNAPII via phosphatase activity during appressorium formation. Previous studies suggest that dormant conidia of ascomycetes store a pre-existing pool of mRNAs and ribosomes for immediate use in conidial germination and germ-tube elongation. In the *M. oryzae*
MoDullard, co-functions with Whi2p and is involved in the sodium stress response 50,51. The proteins Psr1 and
Colletotrichum orbiculare library was used, which was constructed as previously reported15. As bait, Bio-Cm was immobilised on 5
genomic DNA WI, USA) following the manufacturer’s protocol. For the T7 phage display method, a
was dissolved in ethanol (Nacalai Tesque) or dimethyl sulfoxide (DMSO; Wako) and a stock solution (10 mg ml
broadly conserved among eukaryotes and regulates cell growth and proliferation in response to nutrients53. The
conidial germling stage was performed following a previously described method54.

Extraction of DNA and RNA from

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Materials and Methods

Fungal strains and growth conditions. M. oryzae P2 strain, a Japanese rice blast pathogenic isolate, was
used as the wild-type strain in this study. For preservation, P2 and derived strains were cultured on oatmeal agar
medium containing 5.0% oatmeal (Quaker Oats Company, Chicago, IL, USA), sucrose (Nacalai Tesque, Kyoto,
Japan), and 1.5% agar (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at 28 °C. For induction of
conidiation, aerial hyphae were removed with a sterilised brush and stationary-cultured under blacklight blue
lamps (FL20S, 20 W; Toshiba Co. Ltd, Tokyo, Japan) at 28 °C for 2–3 days. Conidia were brushed off into sterile
water and used in subsequent experiments.

Appressorium formation assay. The percentages of germinated conidia and appressorium formation were
determined by means of an appressorium formation assay as described previously49. Chloramphenicol (Wako)
was dissolved in ethanol (Nacalai Tesque) or dimethyl sulfoxide (DMSO; Wako) and a stock solution (10 mg ml
−1) was prepared. The stock solutions were diluted to the appropriate concentrations with each solvent. Before plac-
ing drops of conidial suspension on the surface of hydrophobic polyvinyl chloride (Thermo Fisher Scientific, Inc.,
Waltham, MA, USA), diluted Cm solution was mixed with conidial suspensions at various concentrations in 0.1%
etanol or 0.5% DMSO.

Phage display method. For screening we used the T7Select® Phage Display System (Novagen, Madison,
WI, USA) following the manufacturer’s protocol. For the T7 phage display method, a M. oryzae genomic DNA
library was used, which was constructed as previously reported16. As bait, Bio-Cm was immobilised on 5 μg
NeutrAvidin™ Protein (Pierce, Rockford, IL, USA) placed on the sensor chip of an AFFINIX Q QCM appa-
ratus (Initium, Kanagawa, Japan). Non-immobilised avidin was removed using an air duster (Sanwa Supply,
Tokyo, Japan). Bio-Cm dissolved in 20 μl QCM buffer (10 mM Tris-Cl, 200 mM NaCl; Nacalai Tesque) with 10% DMSO
was placed on the avidin-immobilised chip and left at room temperature for 80 min. Non-immobilised Bio-Cm was
removed using an air duster. The chip was set up for the QCM apparatus with the cuvette containing
8 μl QCM buffer. An aliquot (80 μl) of the T7 phage display library was injected into the cuvette. The frequency
changes, which were caused by binding between phages and Bio-Cm on the sensor chip, were monitored for
10 min. The chip was detached from the apparatus and air-dried, then bound phages were recovered by applying
a 10 μl drop of host Escherichia coli (BLT5615). The isolated phage DNA sequence was amplified by PCR and
sequenced.

Extraction of DNA and RNA from M. oryzae. For induction of hyphal growth to extract genomic DNA, M.
oryzae strains were cultured in 20 ml YG liquid medium containing 0.5% yeast extract and 2% glucose (Nacalai
Tesque) and incubated at 28 °C on a rotary shaker at 150 rpm for 2 days. The fungal mycelium samples were inoc-
ulated into 100 ml YG liquid medium and incubated at 28 °C on a rotary shaker at 150 rpm for 1 day. The fungal
fluid was concentrated by centrifugation at 2000 × g at room temperature for 10 min. After freeze-fracturing with
liquid nitrogen, genomic DNA was extracted using a previously described method17. The extraction of RNA at
the conidial germling stage was performed following a previously described method14.
Construction of MoDullard deletion mutant. To establish a Δmodullard strain knockout vector using the AtMT method, we constructed pNR011 as the AtMT knockout vector. Restriction enzymes and calf intestinal alkaline phosphatase (New England Biolabs Inc., Ipswich, MA, USA), Ligation Convergence Kit (Wako), and QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany) were used for vector construction. First, the vectors pBI121 and pRI910 were digested using PcmI and EcoRI. The extracted pRI replicator region (9265 bp) from pRI910 and multiple cloning site from pBI121 were ligated into the vector pNR01. The AtMT knockout vector, pNR011, was constructed by cloning TrpP-C-HPH from pCSN43 into pNR01 using SmaI. The upstream and downstream regions of Modullard were amplified from genomic DNA using the primers Modul-Up-Fwd-KpnI, Modul-Up-Rvs-KpnI, Modul-Down-Fwd-XbaI, and Modul-Down-Rvs-XbaI, and each region was cloned into pNR01 digested with KpnI or XbaI. The cloned vectors were digested with RsrII, and several fragments containing the upstream or downstream region were ligated to construct the modullard knockout vector pANKO03.

To obtain Δmodullard mutants, the AtMT method was performed using a previously reported procedure27. After co-culture fungal and bacterial cells on paper filters were plated on YG agar medium supplemented with hygromycin (500 µg ml−1; Nacalai Tesque) and Meropenem (25 µg ml−1; Nacalai Tesque) at 28 °C for 4 days. Individual transformants that emerged at the edge of the paper filters were transferred onto oatmeal medium and incubated for 4 days. The deletion of modullard in the transformants was checked by PCR and Southern blot analysis.

Cloning and construction of plasmid vectors for transformation of M. oryzae. To express each gene in Δmodullard, we constructed the pBFT vector by cloning the promoter of the translation elongation factor gene (Ptef) fragment from pMK41256, amplified using the primers pTEF-Fwd-PmeI and pTEF-Rvs-BamHI, and digested with PcmI and BamHI. The pBF101 vector7 was also digested by PcmI and Smal, and ligated with the digested PCR fragment to generate pBFT. To construct each expression vector, corresponding genes were amplified with the primers ModUL-D-Fwd, ModUL-Rvs CTDS-P1-Fwd, CTDS-P1-Rvs, CTDS-P2-Fwd, CTDS-P2-Rvs, CTDTNEP-Rvs, CTDTEP-Fwd, CTDTEP-Rvs, and CTDTEP2-Rvs from M. oryzae cDNA (extracted from germling-stage conidia) or human cDNA (extracted from U937 cultured cells using RNAzol; Cosmo Bio Co., Ltd., Tokyo, Japan) following the manufacturer’s protocol. The amplified DNA fragments were digested by SpeI or BamHI, then cloned into pBFT to construct each expression vector. The sequence in each vector was checked by sequencing.

To obtain complementary strains harboring the inactivated DxDxT/V motif, the mutation induction vectors were constructed using improved methods for site-directed mutagenesis using the Gibson Assembly® Master Mix (New England Biolabs) following the manufacturer’s protocol. Using the primers mutDxDxT-Fwd and mutDxDxT-Rvs, the sequence of the DxDxT/V motif in the expression vectors was changed from 5′-GATCTCGACGAGACG-3′ to 5′-aATCTCaACGAGACG-3′ to mutate DxDXT to NxxXT (lower-case letters indicate the nucleotides changed from the original sequence). The sequences of the constructed vectors were checked by sequencing.

Expression and purification of proteins. To obtain purified proteins for the binding assay, we used pGEX-6p-1 (GE Healthcare UK Ltd, Amersham, UK) as an expression vector. The cDNA of interest was amplified from complementary vectors by MoD-EX-Fwd-Xhol and MoD-EX-Rvs-BamHI. Amplified fragments were digested using Xhol or BamHI and were cloned into pGEX-6p-1. The sequence of the vectors and that each cDNA was in-frame was checked by sequencing. The obtained vector was introduced to Rosetta™ (DE3) Competent Cells (Merck KGaA, Darmstadt, Germany) and used as an expression host. For the expression experiment Auto Induction medium was used, which contains autoclaved A solution (final concentration 2.4% yeast extract and 1.2% Trypton; Nacalai Tesque), autoclaved B solution (1.1% KH2PO4, Nacalai Tesque; and 4.7% K2HPO4, Kanto Chemical Co., Tokyo, Japan), and filtered sugar solution (0.6% glycerol, 0.5% glucose, and 0.08% lactose; Nacalai Tesque) following the manufacturer’s protocol. The amplified DNA fragments containing the upstream or downstream region were ligated to construct the modullard knockout vector pANKO03.

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The purification of sonicated soluble fractions was performed using Glutathione Sepharose® 4B (GE Healthcare) as a resin following the manufacturer’s protocol. Resin (100 µl) and 1 ml soluble fractions were mixed in a 2 ml tube and incubated at 4 °C for 16 h at 25 rpm with a RT-30mini rotator (TAITEC, Saitama, Japan). The resin was centrifuged at 500 × g for 5 min and the supernatant was discarded. The resin was washed five times with 1 ml TBS buffer. To the bound proteins 100 µl elution buffer (50 mM Tris-Cl, 10 mM reduced glutathione, pH 8.0; Nacalai Tesque) were added and the solution was incubated at 4 °C for 20 min at 25 rpm. After incubation, the resins were centrifuged at 500 × g for 5 min and the eluted fraction was collected. The elution step was repeated three times. The resulting solution was frozen with liquid nitrogen and stored at −80 °C prior to the pull-down assay.
Pull-down assay. The pull-down assay was performed as previously described. An aliquot (40 µl) of the elution fractions of GST-fused proteins purified with GST-Sepharose was used for the pull-down assay with Streptavidin Sepharose High Performance beads (GE Healthcare) to immobilize 40 nmol Bio-Cm or Bio-Ctrl. After mixing overnight, the resins were washed three times with 200 µl TBS buffer, mixed with SDS loading buffer containing 0.002% bromophenol blue, 5% glycerol, and 0.1% sodium dodecyl sulfate (Nacalai Tesque), and then incubated at 95 °C for 10 min. After centrifugation at 17,900 × g for 1 min, the supernatants were subjected to SDS-PAGE using 10% polyacrylamide gel. The separated proteins were blotted onto a PVDF membrane and detected by western blot analysis using Anti-Glutathione S-transferase antibody (Wako) as a primary antibody and goat anti-IgG AP (Santa Cruz Biotechnology Inc., Dallas, TX, USA) as a secondary antibody. The colorimetric detection of alkaline phosphatase activity was performed using CDP-Star (Thermo Fisher Scientific) with the ChemiDoc™ Imaging System (BIO-RAD Laboratories, Tokyo, Japan).

Homology search and alignment of amino acid sequences. Protein sequences were downloaded from the National Center for Biotechnology Information database (http://www.ncbi.nlm.gov). We used the BLAST tool to search for homologous sequences and CLUSTALW to align and compare amino acid sequences.

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Author Contributions
A.N. and T.A. wrote the manuscript. A.N., A.N., Y.N., S.E., M.K., M.N., S.K., T.A., H.T., F.S. and M.N. acquired the data. T.K. designed and conducted this study.

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