Regulation of MntH by a Dual Mn(II)- and Fe(II)-Dependent Transcriptional Repressor (DR2539) in Deinococcus radiodurans

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Abstract

The high intracellular Mn/Fe ratio observed within the bacteria Deinococcus radiodurans may contribute to its remarkable resistance to environmental stresses. We isolated DR2539, a novel regulator of intracellular Mn/Fe homeostasis in D. radiodurans. Electrophoretic gel mobility shift assays (EMSA) revealed that DR2539 binds specifically to the promoter of the manganese acquisition transporter (MntH) gene, and that DR0865, the only Fur homologue in D. radiodurans, cannot bind to the promoter of mntH, but it can bind to the promoter of another manganese acquisition transporter, MntABC. β-galactosidase expression analysis indicated that DR2539 acts as a manganese- and iron-dependent transcriptional repressor. Further sequence alignment analysis revealed that DR2539 has evolved some special characteristics. Site-directed mutagenesis suggested that His98 plays an important role in the activities of DR2539, and further protein-DNA binding activity assays showed that the activity of H98Y mutants decreased dramatically relative to wild type DR2539. Our study suggests that D. radiodurans has evolved a very efficient manganese regulation mechanism that involves its high intracellular Mn/Fe ratio and permits resistance to extreme conditions.

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Introduction

Deinococcus radiodurans belongs to the Deinococcaceae family of bacteria. These bacteria are characterized by extreme resistance to ionizing radiation (IR), ultraviolet (UV) radiation, oxidative stress, and desiccation [1–3]. To evaluate its robust viability in extreme environments, extensive studies on its ability to repair its DNA have been carried out [4,5]. However, more and more evidence supports the view that proteins, especially those involved in DNA repair and replication, must be protected from damage by radiation toxicity before DNA repair can begin. The antioxidants present in D. radiodurans seem to play critical roles in cell survival during irradiation. Recent studies have demonstrated that independent antioxidant mechanism can protect proteins from reactive oxygen species (ROS)-induced damage in many species, including D. radiodurans [6–8]. D. radiodurans contains approximately 100 times more Mn(II) than Escherichia coli when grown in a defined minimal medium. This suggests that the regulation of Mn(II) homeostasis in D. radiodurans may be more complicated than in E. coli [9].

So far, two types of bacterial high-affinity manganese acquisition systems have been identified, the natural resistance-associated macrophage protein (Nramp) transporter and the ATP-binding cassette (ABC) transporters [10,11]. Both Nramp and ABC-type acquisition system homolog have been found in D. radiodurans [12,13]. Nramp is a ubiquitous proton-coupled divalent metal ion transporter, and bacterial homologues have been reported in Mycobacterium tuberculosis, Bacillus subtilis, Escherichia coli, and Salmonella enteric serovor Typhimurium [10,14–16]. The primary role of bacterial Nramp homologues is to transport Mn(II), so it was renamed MntH (manganese transport, H-). MntH is a ubiquitous proton-coupled divalent metal ion transporter, and bacterial homologues have been reported in Mycobacterium tuberculosis, Bacillus subtilis, Escherichia coli, and Salmonella enteric serovor Typhimurium [10,14–16]. The primary role of bacterial Nramp homologues is to transport Mn(II), so it was renamed MntH (manganese transport, H-dependent) [10,11,14]. Manganese is an essential enzyme cofactor. It protects cells from oxidative damage. However, it can be toxic at high concentrations, and so the expression of MntH must be strictly regulated [6,17]. The regulation of mntH genes in response to Mn(II) availability is mediated by the manganese transport regulator (MntR), among other regulators, including Fe(II)-sensing ferric uptake repressor (Fur) and the peroxidase-sensing OxyR factor [10,16,18,19]. MntR is a diphtheria toxin repressor (DtxR) homologue. It represses the transcription of genes encoding manganese acquisition systems, such as mntH and mntABCD in B. subtilis. Unlike other DtxR family members, which can sense both Mn(II) and Fe(II), the MntRs in B. subtilis, E. coli, and S. aureus
exhibit pronounced selectivity for Mn(II) due to amino acid substitutions at the metal-binding site [20–23].

In *D. radiodurans*, *dr1709* encodes the manganese transporter, which is believed to be responsible for its high Mn(II) accumulation in *D. radiodurans* [7–9,13]. Unfortunately, *dr1709* could not be disrupted and its repression is still unknown [12]. *dr2539* encodes a DtxR homologue, and previous studies have implied that it may regulate the intracellular Mn/Fe ratio [24]. However, which genes *DR2539* regulates and the mechanism by which it regulates them remains to be investigated. In this study, we successfully expressed the *DR2539 in vivo* and analyzed its biochemical characteristics both in *vivo* and in *vivo*. We found that the transcription of *dr1709* was strictly repressed by *DR2539*. Unexpectedly, the Fe(II)-induced repression of *dr1709* was also found to be mediated by *DR2539* but not by the Fur homologue *DR0865*. We demonstrated that *DR2539* can be activated by both Fe(II) and Mn(II) in *vivo* and in *vivo*. Site-directed mutagenesis analysis showed that His98 plays an important role in the DNA binding activity of *DR2539*. Our study suggests that the metal binding mode of *DR2539* may be different from what has been previously reported for the MntH repressor. It may represent a new subset of DtxR family proteins.

**Results**

**Binding of DR2539 to the mntH (dr1709) promoter**

The MntH is a major component of the MntII uptake system. It is involved in oxidative stress resistance in many bacteria [10]. A *dr1709* null mutant of *D. radiodurans* showed increased intracellular manganese concentration and enhanced oxidative resistance, suggesting that *DR2539* may be involved in the regulation of MntH [24]. To determine whether *DR2539* binds to the *dr1709* promoter, we performed electrophoretic gel mobility shift assays (EMSA) in the presence of MnII. The *dr1709* promoter was amplified by PCR from the genome of *D. radiodurans* strain R1 (Fig. 1A). MntRs in *E. coli* and *S. enterica* were found to regulate the mntH by binding to the reverted repeat region of mntH promoters. In *D. radiodurans*, a similar reverted repeat region was found in the *dr1709* promoter. In order to confirm whether *DR2539* binds to this region, we designed two pairs of primers: one pair was capable of generating *dr1709* promoter DNA fragments containing reverted repeat regions (*dr1709b*), and the other pair of primers was capable of generating *dr1709* promoter DNA fragments lacking the reverted repeat region (*dr1709a*) (Fig. 1A). As indicated by the gel shift, the *DR2539* protein bound to the *dr1709b* promoter, and binding increased with protein concentration (Fig. 1B). In contrast, the *DR2539* protein cannot bind to *dr1709* promoters that lack inverted repeat regions (Fig. 2A). This suggests that the binding between *DR2539* and *dr1709* promoters depends on the presence of the inverted repeat region. The presence of EDTA in the EMSA reaction abolished binding to the *dr1709* promoter, suggesting that MnII is necessary for *DR2539*–DNA binding (Fig. 2B). These results indicate that *DR2539* specifically binds to the mntH promoter of *D. radiodurans*.

**Effects of Mn(II) and Fe(II) on DR2539 in vitro**

Generally, the regulation of MntH genes involves iron-sensing by Fur regulatory proteins [18,23]. In *D. radiodurans*, *dr0865* encodes the only Fur homologue. To investigate whether *DR0865* might be involved in the regulation of the mntH gene, we expressed the *DR0865* protein in *vivo* and performed DNA binding experiments. Unexpectedly, the EMSA results demonstrated that *DR0865* cannot bind to the promoter of *dr1709* (p1709b is the full length version of *dr1709* promoter) (Fig. 2C). Current evidence supports the idea that MntR is a strictly Mn(II)-specific metalloregulatory protein, and that the repression of MntH by Fe(II) is always mediated by Fur [20,21,25]. We therefore wondered whether *DR2539* can respond to both MnII and FeII in *vivo*. We studied the binding efficiency of *DR2539* to the *dr1709b* promoter using EMSA in the presence of varying concentrations of MnII and FeII. As shown in Fig. 1C, the binding activity of *DR2539* increased as the concentration of MnII rose from 25 μM to 150 μM. The same response was observed when MnII was replaced by FeII (Fig. 1D), indicating that the activity of *DR2539* can be regulated by both MnII and FeII in a concentration-dependent manner.

**Effects of Mn(II) and Fe(II) on DR2539 in vivo**

In order to confirm our results in *vivo*, the transcriptional regulation of the mntH promoter by different ions were tested by linking the *dr1709* promoter to the β-galactosidase gene *lacZ*. As shown in Fig. 2A, the activity of β-galactosidase was repressed dramatically when MnII or FeII was included in the growth medium, but not when NiII, CuII, or ZnII was added in the absence of MnII and FeII. This strongly suggests that the expression of *D. radiodurans* MntH is regulated by both MnII and FeII in *vivo*. A *dr2539* null mutant was constructed to investigate whether the MntH repression induced by FeII was actually mediated by *DR2539*. The expression of *lacZ* was repressed when wild-type bacteria were treated with different concentrations of MnII or FeII (Fig. 2B). On the other hand, MnII- and FeII-dependent repression of β-galactosidase was abolished in the *dr2539* null mutant, indicating that MnII/FeII-dependent repression of MntH (*dr1709*) was mediated by *DR2539* (Fig. 2C). Using Real-time PCR analysis, we compared the expression levels of *dr1709* in the *dr2539* mutant and wild-type strains. As shown in Fig. 2D, the mRNA of the wild-type strain decreased dramatically when the concentration of MnII increased relative to that cultured in media without additional MnII (indicating control). No difference was observed in *dr2539* mutants, indicating that *dr2539* encodes the *dr1709* repressor.

**Binding of DR0865 to mntABC promoters**

Because *DR0865* cannot regulate MntH transcription, we turned our attention to *dr2283*, *dr2284*, and *dr2284*, which encode the other putative MnII acquisition transporters. The EMSA results indicate that *DR0865* binds to the promoters of *dr2283*, *dr2284*, and *dr2284* (*dr2283* and *dr2284* share one promoter), which encode the MntABC subunits in a manganese-dependent manner (Fig. 3A/3B). The manganese resistance assay showed that the *dr0865* null mutant is comparable to the wild strain while the manganese resistance of the *dr2539* null mutant decreased significantly (Fig. 3C/3D). These results imply that the MntABC in *D. radiodurans* may not be involved in manganese transport. Therefore, *DR2539* may be the most important regulator of intracellular manganese homeostasis in *D. radiodurans*.

**Effects of H98Y mutation on the metal-sensing function of DR2539**

The protein sequences of various members of the bacterial MntRs/DtxR family were aligned to define those structure features of *DR2539* that could confer dual MnII/FeII-dependent functional regulation. Sequence alignment analysis indicated that *DR2539* possesses a conserved His98 that is also present in MnII/FeII-dependent DtxR but absent from the
MntR regulator (MntR). ScaR represents a new group of Mn(II)-responsive transcription factors, and its metal binding residues are conserved in DR2539 except Asp126 (Fig. 4). Site mutants H98Y and D126A were constructed to evaluate the metal binding mode of DR2539. These dr2539 mutants were reverted to dr2539 null mutants. The manganese resistance of the dr2539 mutant complementation strain was examined in TGY plates supplemented with 6 mM manganese. As shown in Fig. 5A, the manganese resistance of the D126A complementation strain is comparable to that of the wild-type dr2539 complementation strain. In contrast, the manganese resistance of H98Y complementation strain decreased dramatically. To investigate the DNA binding activity of the H98Y mutant, the H98Y mutant protein was expressed in vitro, and the DNA binding activity was examined. The DNA binding activity of the H98Y mutant was found to be much weaker than that of wild type DR2539 at the same manganese concentration (Fig. 5B/C). This shows that His98 may play a very important role in the function of DR2539 and that D126 may not be involved in the metal binding activity of DR2539.

**Discussion**

*D. radiodurans* contains more intracellular Mn(II) than other bacteria [9]. It has been suggested that the high Mn/Fe ratio in *D. radiodurans* facilitates the pronounced resistance to extreme oxidative stress observed in this species [8,13]. Intracellular Mn(II) is known to confer protection against oxidative stress and other forms of injury, including ionizing radiation [13]. We analyzed the genetic regulation of the MntH gene dr1709 to study manganese homeostasis in *D. radiodurans*.

In *E. coli*, mntH transcription can be repressed either by the Mn(II)-dependent MntR or by the Fe(II)-dependent transcriptional regulator Fur [18]. The MntH of *S. enterica* has also been reported to be responsive to Fe(II) and the repression induced by Fe(II) was mainly mediated by Fur [23]. In contrast to previous studies, the gene dr0865 was here found to encode the only Fur homologue in *D. radiodurans*, but it cannot bind to dr1709 promoter. This indicates that Fe(II)-dependent regulation of MntH must be mediated by another Fe(II)-responsive transcriptional regulator. *D. radiodurans* DR2539 exhibited both Mn(II)-dependent and Fe(II)-dependent repression of dr1709. This explains the Fe(II)-dependence of MntH regulation in the absence of a functional Fur homologue.

The activation of metalloregulators is closely correlated with their structures. We compared the protein sequences of DR2539 to DtxR homologues and found substantial structural and functional differences. The Glu11 that provides two metal binding ligands in the MntR binuclear metal binding site is substituted by a basic amino acid (Lys) in DR2539. The presence of a positive charge at this position suggests that DR2539 cannot form a regular MntR-type metal binding site (Fig. 4). In addition, the His98 and SH3-like domains that are conserved in DtxR homologues (but missing in other MntRs) are present in DR2539.

In this paper, we observed that DR2539 responded to both Mn(II) and Fe(II), and the impact of H98Y and D126A on activity was assessed. The results indicate that the Mn(II) resistance of the C- H98Y complementation strain decreased significantly after exposure to Mn(II) and Fe(II), while the C- D126A strain was relatively resistant to Mn(II). A DNA binding assay implied that His98 is crucial to protein activity. This is different from previously
reported MntR, because His98 is not involved in the metal binding of these proteins. This shows that DR2539 may represent a new subset of the DtxR family.

The disruption of the MntABC transporter could induce the accumulation of iron but could not change the manganese level in *D. radiodurans* [26]. Our results indicate that the DR0865 (Fur homologue) participates in the transcriptional regulation of the MntABC. The disruption of *dr0865* did not affect the manganese resistance of *D. radiodurans*. Therefore, we speculate that MntABC in *D. radiodurans* may be responsible for the intracellular iron efflux. However, there is still some controversy as to whether such an iron efflux mechanism exists in bacteria [27]. The function of DR0865 still needs to be elucidated.

Many studies have attempted to determine why MntR can selectively respond to Mn(II) while DtxR prefers to bind Fe(II). Here, we described a novel MntH repressor (DR2539) from *D. radiodurans*, and found it capable of responding to both Mn(II) and Fe(II). Moreover, the His98 in DR2539 is crucial to its activity. This is quite different from with results of previous reports on MntH repressors. However, crystallography analysis is still needed to confirm this and clarify the structure-function relationship.

**Materials and Methods**

**Strains and media**

All strains and plasmids used in this study are listed in Table S1. *D. radiodurans* strains were cultured at 30°C on TGY medium (0.5% Bacto tryptone, 0.1% glucose, 0.3% Bacto yeast extract) with aeration or on TGY plates supplemented with 1.2% Bacto agar. *E. coli* strain DH5α was used for propagation of plasmids and grown at 37°C on LB medium with appropriate antibiotics.

**Construction of pET-29b-IF-dr2539 fusion expression vector**

The *IF* and *dr2539* transcripts were cloned from *E. coli* using primers IFF/IFR and RFF/RFR (listed in Table S2). Then the PCR products were ligated separately into the pMD18-T simple vector (TaKaRa Biotechnology (Dalian) CO., Ltd, China) for
sequencing (Invitrogen Corporation). The pMD18-T-IF plasmid was digested with NdeI and EcoRI and ligated to pET-29b digested with the same enzymes to form pETIF. The pMD18-T-dr2539 vector was digested with EcoRI and HindIII and ligated to pETIF (digested with the same enzymes) to yield pET-29b-IF-dr2539 (pIFMR), as shown in Fig. S1.

Overexpression and purification of DR2539

Wild-type DR2539 was overexpressed in E. coli strain BL21 (DE3) (pLysS) transformed with pIFMR. Typically, 500 ml of LB medium was inoculated with 5 ml of a clone expressing DR2539 and cultured until OD600 was <0.6. Then 200 μM IPTG was added and the cultures were incubated for another 4 h. Cells were harvested by centrifugation and resuspended in 20 ml of lysis buffer.

Figure 3. DR0865 binds to the promoter of MntABC in an ion-dependent manner. (A) and (B) DR0865 binding to p2523 and p2284 as the concentration of DR0865 increased. (C) Wild-type R1, dr2539 null mutant (Δdr2539), and dr0865 null mutant (Δdr0865) were cultured on TGY plates overlaid with filter discs saturated with 1 M solution MnCl2. (D) The zone of inhibition was measured from edge of disc after three days. *, P<0.05. Data represent the means± deviations of three independent experiments.

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Figure 4. Sequence alignment of the metal binding sites of DR2539 with other DxtR/MntR family members. ScaR (Streptococcus gordonii), SloR (Streptococcus suis), LCAS (Lactobacillus casei), SirR (Corynebacterium glutamicum), TroR (Treponema pallidum), TroR (Treponema denticola), IdeR (Mycobacterium tuberculosis), DtxR (Corynebacterium diptheriae), DR2539 (Deinococcus radiodurans), MntR (Staphylococcus aureus), MntR (Escherichia coli), and MntR (Bacillus subtilis). The sequences were aligned using the CLUSTAL W software. Residues shaded with black represent metal-binding sites that have been studied while residues shaded with grey represent predicted metal binding sites.

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buffer (50 mM Tris-HCl (pH 8.0), 0.22 mg/ml lysozyme, 100 μM PMSF, and 10 mM DTT) and sonicated on ice. The lysate was centrifuged at 39,000 × g for 30 min at 4°C. The clear supernatant containing the soluble protein was mixed with Ni-NTA slurry (QIAGEN) and rocked for 60 min at 4°C. The mixture was poured through a Ni-NTA column and washed with 50 ml of phosphate wash buffer (20 mM imidazole, 1 M NaCl, 10% glycerol, 50 mM Tris-HCl, pH 8.0). Purified His-IF-DR2539 protein was eluted with elution buffer (500 mM imidazole, 1 M NaCl, 10% glycerol, 50 mM Tris-HCl, pH 8.0). The imidazole and excess NaCl were removed by dialysis in 50 mM Tris-HCl, plus 300 mM NaCl and 10% glycerol. Then, the TEV protease, 5 mM EDTA and 0.1% DTT were added to the purified protein solution and digested overnight at 4°C. The DRA0865 was purified using the same method. The purity of the proteins was analyzed with the SDS-PAGE (12% acrylamide) (Fig. S1).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were performed as previously described with some modifications [28]. Briefly, FITC-labeled promoters were amplified from the genome of D. radiodurans strain R1 with primer pairs MhF/MhRa, MhF/MhRb, and Maf/Mar (listed in Table S2). The (NH₄)₂Fe(SO₄) solution used for the DNA binding assay was prepared immediately before use to limit oxidation. The binding buffer contained 30 mM Tris-HCl (pH 6.8), 50 mM NaCl, and 5% glycerol. The DR2539 protein was incubated for 30 min at 4°C in EMSA binding buffer supplemented with 50 μg/mL calf thymus DNA, 50 μg/mL bovine serum albumin, Mn²⁺ or Fe²⁺ (at various concentrations), and 10 nM FITC-labeled promoter DNA fragments containing the promoter. After incubation, the reaction mixtures were analyzed on 5% non-denaturing polyacrylamide gels. The labeled DNA was detected using a LAS-3000 cooled CCD camera system (Fujifilm).

Construction of promoter-lacZ transcriptional fusions and β-galactosidase assays

D. radiodurans dr1709 gene promoter was amplified by PCR using the primer pair HPF/HPR (Table S2). The PCR products were ligated to pMD18-T simple vector for sequencing (Sango Biotech (Shanghai) Co., Ltd). Then, the dr1709 gene promoter was digested with BglII and SpeI and further ligated into pRADZ vector yielding pRAZH [29]. The pRAZH vector was transformed into the dr2539 null mutant as described previously [4,24].

For β-galactosidase activity assays, cells were grown to OD₆₀₀≈1.0 and permeabilized with Triton X100 as described previously [30]. β-galactosidase activity was assayed using standard methods. Activity was expressed in Miller units, defined as 1,000 times the scattering-corrected OD₄₂₀ per OD₆₀₀ of cells per minute.

Real-time quantitative PCR

Real-time quantitative PCR was used to determine whether DR2539 modulates the transcription of dr1709. In short, cells were grown to OD₆₀₀≈0.2 and then 10 mM, 20 mM, or 30 mM MnCl₂ was added. Cells were harvested by centrifugation at 4000 rpm at 4°C after the OD₆₀₀ was 0.4–0.45. Total RNA was extracted from 200 ml of cell cultures using TRIZOL Reagent (Invitrogen Corp., Carlsbad, CA, U.S.) after liquid nitrogen grinding and then treated with 10 units of RNase free DNase I (Promega, Mannheim, Germany). First-strand cDNA synthesis was carried out in 20 μl reaction mixtures containing 1 μg of each

Figure 5. His98 plays an important role in DNA binding activity of DR2539. (A) 10 μl cell dilution was dripped on the TGY plate to which 6 mM of Mn(II) had been added. The cells were cultured for 3 days. (B) H98Y mutant and wild-type DR2539 proteins were incubated with p1709b at different concentrations of Mn(II). (C) Quantification of the fluorescence intensity of binding bands was performed using ImageJ. *, P<0.05. doi:10.1371/journal.pone.0035057.g005
DNase I-treated and purified total RNA sample, combined with 3 μg of random hexamers. The Real-time PCR amplification used SYBR Premix Ex Taq™ (TaqKaRa Biotechnology [Dalian] Co, Ltd, China) following the manufacturer’s instructions. All assays were performed using the STRATAGENE Mx3005p™ Real-time detection.

Site-directed mutagenesis of dr2539 and complementation assays of the dr2539 null mutant

Site-directed mutagenesis of dr2539 was performed as described previously [31]. Briefly, dr2539 was amplified by PCR using the primer pair MRF/MRR (Table S2) and cloned into pMD-18 simple vector. It was amplified using M126F/M126R (Table S2) as a primer and pMD-18-dr2539 as a template. Amplified vector was treated with the enzyme DpnI, which cleaves only when its recognition site is methylated. Following digestion, the DNA fragments were cloned into the pMD-18 simple vector. The D126A site mutation was confirmed by DNA sequencing. The confirmed pMD19-D126A was digested with NdeI and BsmII and cloned into pRADK [32]. It was treated with the same enzymes to yield the plasmid vector pD126A. Identical methods were used to construct pH98Y using primers M98F/M98R (Table S2).

Manganese-sensitive assay

Complementation strains were cultured in TGY until OD_{600}^\text{max} = 1.0. Cells were collected and diluted with PBS. Cell suspensions (10 μL) were dripped on plates with different concentrations of Mn(II). (A plate without Mn(II) was used as a control.) Plates were cultured for 3 days at 30°C. The results were captured using a scanner. For plate counting, 10^4 and 10^5 cells were inoculated on plates containing different concentrations of Mn(II) (Plates without Mn(II) were used as controls). The number of clones was determined after 3 days at 30°C.

Supporting Information

Figure S1 Overexpression and purification of the DR2539, H98Y mutant and DR0865 in E. coli. (A) Schematic of the construction of the HP-dr2539 fusion expression vector. (B) DR2539 was expressed as a fusion protein with solubility partner IF, and purified following digestion by TEV protease (1%) and Ni-NTA chromatography. Samples were analyzed by SDS-PAGE (12% acrylamide). Lane 1, Fusion expressed DR2539. Lane 2, Fusion expressed DR2539 treated with TEV. Lane 3, Purified DR2539 protein. Lane 4, Purified IF protein which was expressed by pETIF. MW, molecular size markers. (C) and (D) H98Y mutant of DR2539 and DR0865 proteins analyzed by SDS-PAGE (12% acrylamide).

Table S1 Strains and plasmids used in this study.

Table S2 Primers used in this study.

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Author Contributions

Conceived and designed the experiments: YHJ LYW HXS MFL. Performed the experiments: HXS MFL. Analyzed the data: HXS MFL. GZX. Contributed reagents/materials/analysis tools: LYW HC JDJ BT. Wrote the paper: HXS MFL. Purified the proteins needed in this work and did the site mutants: HXS GZX.

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