

# Inhibition of glutathione S-transferase omega 1-catalyzed protein deglutathionylation suppresses adipocyte differentiation

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**Glutathione S-transferase omega 1 (GstO1) is closely associated with various human diseases, including obesity and diabetes, but its functional mechanism is not fully understood. In the present study, we found that the GstO1-specific inhibitor C1-27 effectively suppressed the adipocyte differentiation of 3T3-L1 preadipocytes. GstO1 expression was immediately upregulated upon the induction of adipocyte differentiation, and barely altered by C1-27. However, C1-27 significantly decreased the stability of GstO1. Moreover, GstO1 catalyzed the deglutathionylation of cellular proteins during the early phase of adipocyte differentiation, and C1-27 inhibited this activity. These results demonstrate that GstO1 is involved in adipocyte differentiation by catalyzing the deglutathionylation of proteins critical for the early phase of adipocyte differentiation. [BMB Reports 2023; 56(8): 457-462]**

## INTRODUCTION

Glutathione S-transferase omega 1 (GstO1) belongs to the omega class of glutathione transferases, which has structural and functional properties distinctive from other glutathione transferase classes (1). Although the cellular function of GstO1 has not been fully elucidated, GstO1 is known to accept different types of substrate, including dehydroascorbate, monomethylarsonate (V), and phenylacetyl glutathione, and to catalyze the reductions of substrates (2-4). GstO1 also exhibits a thioltransferase activity, rather than the glutathione conjugation activity of other class enzymes, which more closely resembles the activity of glutaredoxin 1 (Grx1) (5). Furthermore, GstO1 catalyzes protein deglutathionylation, the removal of glutathione from glutathionylated proteins (6), and may be an important player in the protein glutathionylation cycle, which protects proteins against

irreversible oxidation and regulates protein functions.

GstO1 is closely associated with various human diseases such as cancers, neurological diseases, inflammation, and obesity (7). Hence, the enzyme has been extensively investigated as a target molecule for disease treatment. In particular, GstO1 is highly expressed in cancers and involved in the signaling for cancer development (8-10). In a previous study, a GstO1-specific inhibitor (chemical structure in Fig. 1B) was developed that covalently modified the catalytic cysteine residue of the enzyme (10). The inhibitor was highly cytotoxic to different types of cancer cells and efficiently suppressed the proliferation of colon cancer cells *in vitro* and *in vivo*. Another study showed that GstO1 inhibition efficiently suppressed LPS-induced inflammation and high-fat diet-induced obesity by reducing lipid accumulation in adipocytes (9).

In the present study, the GstO1-specific inhibitor C1-27 effectively suppressed the adipocyte differentiation of 3T3-L1 preadipocytes. GstO1 gene expression was immediately increased upon the induction of adipocyte differentiation, and GstO1 protein was accumulatively increased during the early phase of adipocyte differentiation. The glutathionylation of cellular proteins was inversely related to GstO1 levels, indicating GstO1-catalyzed protein deglutathionylation. Although C1-27 did not change GstO1 gene expression, it significantly reduced the stability of GstO1 protein *in vitro*, and decreased GstO1 levels in cells. Most importantly, GstO1 inhibition by C1-27 increased the levels of ~40 kDa glutathionylated proteins that are critically involved in the regulation of adipocyte differentiation. These results demonstrate that GstO1 plays an important role in the early phase of adipocyte differentiation by regulating the protein glutathionylation cycle.

## RESULTS

### C1-27 suppresses adipocyte differentiation

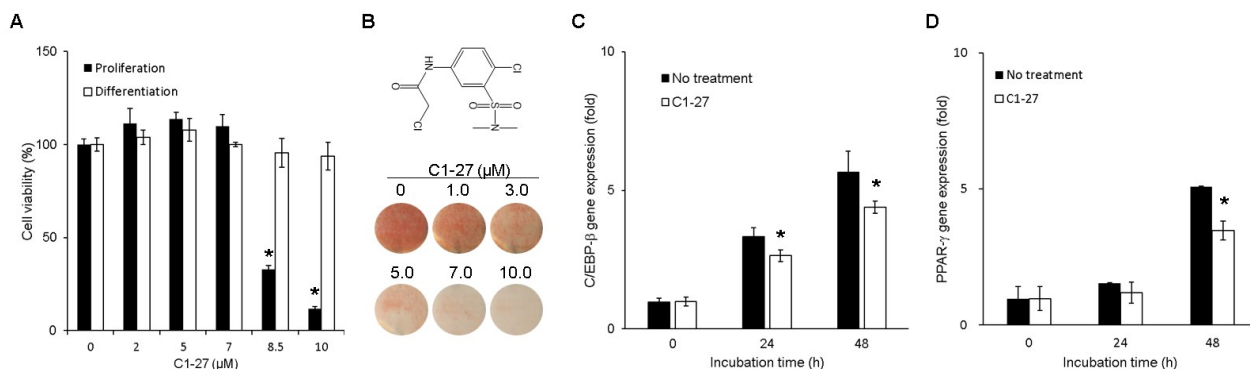
The GstO1-specific inhibitor C1-27 was cytotoxic to proliferating 3T3-L1 cells significantly at > 7.0  $\mu\text{M}$  (Fig. 1A). While, under conditions of adipocyte differentiation induction, C1-27 did not show significant cytotoxicity at  $\leq 10 \mu\text{M}$ . To examine the effect of C1-27 on adipocyte differentiation, the inhibitor was added into the medium of differentiation induction. Determination of adipocyte differentiation by measuring lipids in differentiated cells showed a dose-dependent suppression by

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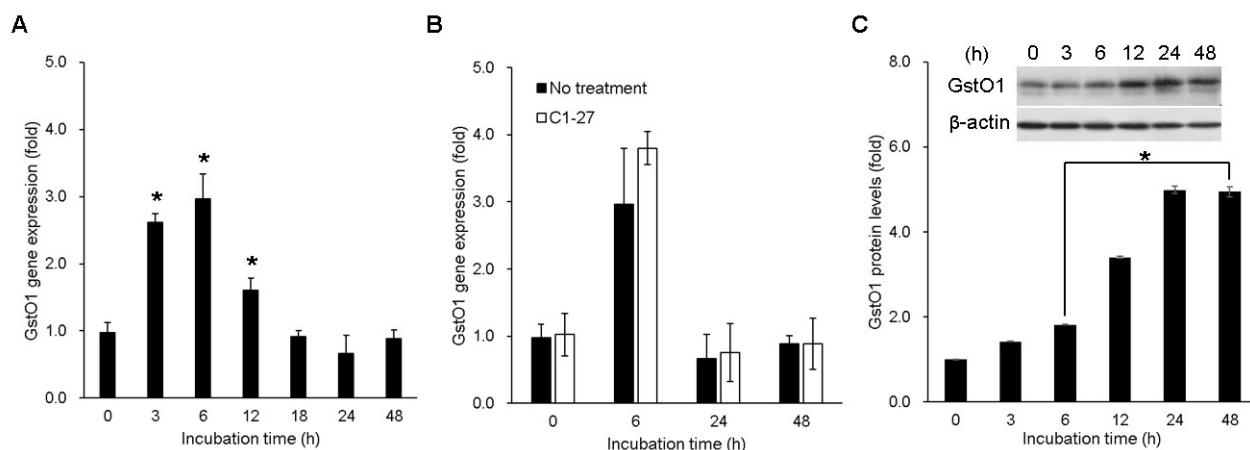
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**Fig. 1.** Suppression of adipocyte differentiation by C1-27. (A) The cytotoxic effect of C1-27 under cell proliferating (black bars) and differentiating conditions (white bars). (B) The chemical structure of C1-27 (upper), and its effect on adipocyte differentiation as determined by cellular lipid staining (lower). (C, D) Expressions of adipogenic marker genes at the indicated incubation times after induction and the effect of 7  $\mu$ M C1-27.



**Fig. 2.** GstO1 expression during the early phase of adipocyte differentiation. (A) GstO1 gene expressions at the indicated incubation times after differentiation induction. (B) The effect of C1-27 on the gene expression of GstO1. (C) Western blot (upper) of cellular GstO1 proteins at the indicated incubation times, and quantitative analysis for GstO1 protein levels normalized by  $\beta$ -actin (lower).

C1-27 at non-cytotoxic concentrations (Fig. 1B). The suppression of adipocyte differentiation by C1-27 was confirmed by determining the expressions of the adipogenic marker genes C/EBP- $\beta$  and PPAR- $\gamma$ , which were significantly reduced by C1-27 (Fig. 1C, D).

### GstO1 expression during the early phase of adipocyte differentiation

The GstO1 gene expression was increased immediately upon inducing adipocyte differentiation (Fig. 2A). The gene expression peaked at 6 h post-induction, and decreased to the basal level after 18 h. C1-27 did not significantly change the GstO1 gene expression with a slight increase (Fig. 2B). Western blot analysis also showed increases in GstO1 protein levels upon inducing adipocyte differentiation (Fig. 2C). However, GstO1

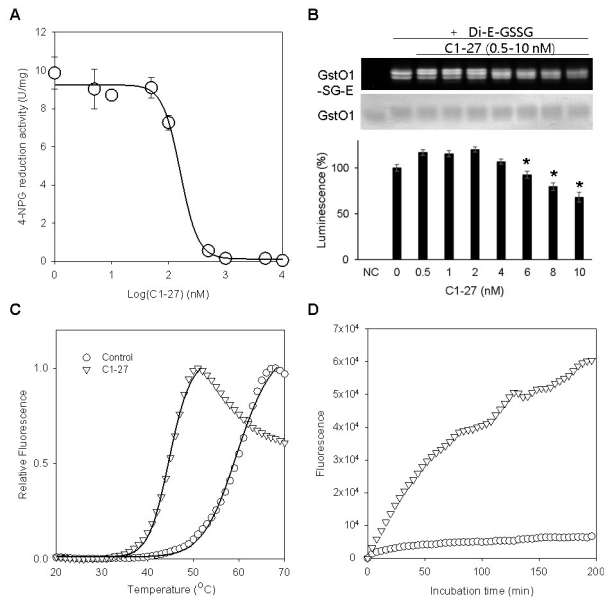
protein reached a maximum level at 24 h post-induction, and the changes in the protein levels were different from the changes in the gene expression. In addition, C1-27 significantly reduced GstO1 proteins during the early phase of adipocyte differentiation (Fig. 4D).

### C1-27 inhibits GstO1 activity by covalently modifying a cysteine residue

Recombinant mouse GstO1 (mGstO1) was prepared homogeneously, as described in supplementary information. Size-exclusion chromatography revealed that mGstO1 is a homodimer (MW =  $\sim$ 60 kDa, calculated MW = 28.5 kDa) (Supplementary Fig. 1). mGstO1 exhibited a thiol transferase activity of 0.3 U/mg and a 4-NPG reduction activity of 10 U/mg. While glutathione transferase activity was not detected. C1-27

effectively inhibited the 4-NPG reduction activity of mGstO1 with an IC<sub>50</sub> = ~220 nM (Fig. 3A). Covalent binding of C1-27 to mGstO1 was examined using di-eosin-glutathione (E-GSSG), which provides luminescent labeling of cysteine residues by forming mixed disulfide bonds (Cys-SSG-E) through thiol-disul-

fide exchange (11) (Fig. 3B). Luminescent labeling of GstO1 was reduced by ~25%, when the protein was incubated with an equimolar amount of C1-27, indicating that C1-27 covalently modified one of four cysteine residues, likely the conserved catalytic cysteine C32, of mGstO1, as was previously reported for human GstO1 (5, 10).



**Fig. 3.** GStO1 inhibition and reduced stability by C1-27. (A) 4-NPG reduction activity of GStO1 after incubation with the indicated concentrations of C1-27. The solid line fitting the data determined the IC<sub>50</sub> = 220 ± 16 nM for C1-27. (B) E-GSH labeling of mGstO1 after incubation of 10 nM mGstO1 with the indicated increasing concentrations of C1-27 in the graph. mGstO1-SG-E was detected by 12% SDS-PAGE (lower) under UV-light (upper), and quantitatively analyzed by densitometry normalizing amount of loaded proteins (lower). (C, D) The thermal stability of GStO1 was determined by DSF and ITD at 37°C, respectively.

### C1-27 reduces the stability of GStO1

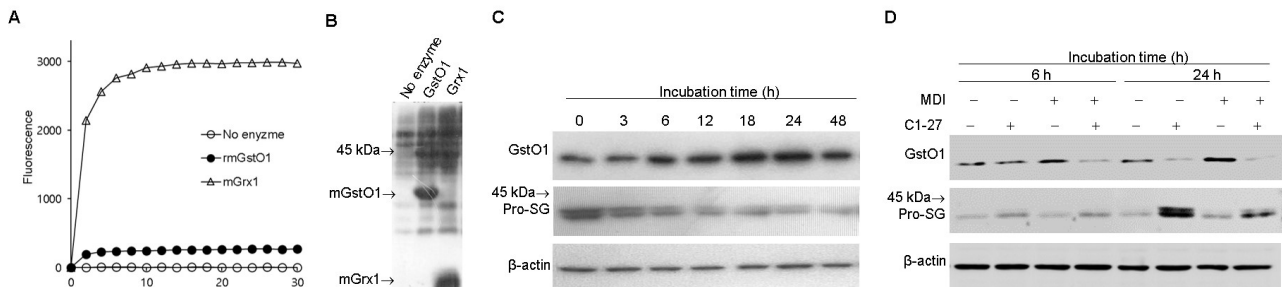
The effect of C1-27 on mGstO1 stability was examined by differential scanning fluorimetry (DSF) (Fig. 3C). The melting temperature of mGstO1 was determined to be  $T_m = 60 \pm 0.5^\circ\text{C}$ . In the presence of C1-27, the thermal stability of mGstO1 was dramatically reduced, and its  $T_m$  fell to  $45 \pm 0.7^\circ\text{C}$ . Another experiment, isothermal denaturation (ITD) at 37°C showed the insignificant denaturation of mGstO1 within 200 min (Fig. 3D). While, in the presence of C1-27, mGstO1 was rapidly denatured showing increases in reporter fluorescence with a half-denaturation time of 1.5 h.

### GstO1 catalyzes protein deglutathionylation *in vitro*

Protein deglutathionylation was examined using glutathionylated proteins with luminescent E-GSH (Pro-SG-E). The control enzyme glutaredoxin 1 (mGrx1) catalyzed protein deglutathionylation as determined by increased luminescence of free E-GSH removed from Pro-SG-E (Fig. 4A). mGstO1 also showed increased free E-GSH levels, indicating that mGstO1 catalyzed deglutathionylation. Protein thiols generated by enzyme-catalyzed deglutathionylation were labeled by alkylation using biotin-NEM. Western blot analysis confirmed the mGstO1 catalyzed deglutathionylation, showing deglutathionylated proteins labeled with biotin-NEM preferentially around the molecular weight of ~40 kDa (Fig. 4B).

### C1-27 inhibits GStO1 with deglutathionylation of cellular proteins

Glutathionylated proteins (Pro-SG) in 3T3-L1 cells during the early phase of adipocyte differentiation were examined by we-



**Fig. 4.** Protein deglutathionylation by GStO1. (A) Fluorescence of free E-GSH released from E-GSH-labeled proteins following incubation with the indicated enzymes. (B) Western blot analysis for Pro-SG in reactions of (A) after labeling with biotin-NEM. (C) Western blot analysis for GStO1 and Pro-SG at the indicated incubation times after differentiation induction. (D) Western blot analysis for GStO1 and Pro-SG at 6 h and 24 h post-induction with 7 μM C1-27.

stern blot analysis using anti-Pro-SG antibody. Cellular Pro-SG was remarkably reduced following differentiation initiation and was inversely related to GstO1 protein levels (Fig. 4C). Most prevalent Pro-SG was detected at ~40 kDa, consistent with *in vitro* mGstO1-catalyzed deglutathionylation of proteins (Fig. 4B). Cellular GstO1 proteins were dramatically decreased by treatment of C1-27 for the differentiation induction at 6 h and 24 h post-induction (Fig. 4D), which was consistent with the reduced mGstO1 stability by C1-27 observed *in vitro* (Fig. 3C, D). In addition, C1-27 significantly increased cellular Pro-SG levels with decreased GstO1 proteins, indicating that GstO1 is responsible for the deglutathionylation of proteins in cells (Fig. 4D).

## DISCUSSION

GstO1 has been suggested to play a critical role in different human diseases, including cancers, neurodegenerative diseases, inflammation, and obesity. The involvement of GstO1 in obesity was previously reported in GstO1 knock-out mice, which were less susceptible to high-fat diet-induced obesity (9). This study also showed that GstO1 might be involved in insulin resistance, although a molecular mechanism was not elucidated. In the present study, we found that the GstO1-specific inhibitor C1-27 suppresses adipocyte differentiation. C1-27 inhibits GstO1 by covalent modification of a catalytic cysteine and reducing the protein stability of the enzyme. In addition, we present evidences indicating that GstO1 is responsible for the deglutathionylation of cellular ~40 kDa proteins that play important roles in the early phase of adipocyte differentiation.

Glutathionylation is an important post-translational modification, and several proteins appear to be glutathionylated during adipocyte differentiation (12). The GstO1 gene expression was immediately upregulated upon the induction of adipocyte differentiation, indicating a critical role of the enzyme (Fig. 2A). The GstO1 gene expression was returned to a basal level after 18 h post-induction, however, interestingly, the GstO1 protein was increased with a delay and reached a peak at 24 h (Fig. 2C). These results indicated a potential post-translational regulation of GstO1 in cells. It was shown that GstO1 is sensitive to oxidative degradation, and the enzyme is protected by reduced glutathione (10). We also observed the similar effect of reduced glutathione, which increased the thermal stability of mGstO1 in the presence of hydrogen peroxide (data not shown). It is well known that intracellular oxidative stress is significantly increased during the early phase of adipocyte differentiation. In this sense, GstO1 should be quantitatively regulated, not only on gene expression level, but also on a post-translational level. Therefore, we speculate that the delayed increase of the GstO1 protein is due to a post-translational regulation of the enzyme stability, likely by glutathione, and its accumulation in cells, which needs to be confirmed by further studies.

During the early phase of adipocyte differentiation, actin depolymerization and cytoskeletal rearrangements dramatically

change cell morphology (13). Actin depolymerization has been shown to enhance adipocyte differentiation (14) and to be negatively regulated by glutathionylation in different cells (15, 16). We showed, in this study, that C1-27 significantly reduced the *in vitro* stability of GstO1 (Fig. 3C, D). This effect of C1-27 was consistently obtained in cells, showing decreased cellular GstO1 proteins by treatment of C1-27 in the differentiation induction (Fig. 4D). Moreover, C1-27 significantly increased cellular Pro-SG levels, indicating that GstO1 catalyzes protein deglutathionylation during the early phase of adipocyte differentiation. Interestingly, GstO1 substrate proteins were detected to be ~40 kDa, which might include actin (42 kDa). In fact, actin has been identified as a GstO1 substrate for deglutathionylation in several types of cells (6). Therefore, these results suggest that actin is a probable GstO1 substrate during the early phase of adipocyte differentiation, which requires further studies to confirm.

Adipogenic transcription factor C/EBP- $\beta$  (38 kDa) is another potential GstO1 substrate for deglutathionylation. C/EBP- $\beta$  expression is rapidly induced within an hour after differentiation induction, and that C/EBP- $\beta$  played a crucial role during the early phase of adipocyte differentiation (17). Glutathionylation of C/EBP- $\beta$  increases its stability and facilitates its nuclear translocation, and Grx1 has been reported to catalyze the deglutathionylation of C/EBP- $\beta$  (18, 19). However, Grx1 was shown to be upregulated in relatively later phase of adipocyte differentiation (18). We also observed significant upregulation of Grx1 at 4 days post-induction, but only a basal level of Grx1 expression was detected during 2 days after differentiation induction. We speculate that the glutathionylation of C/EBP- $\beta$  would be regulated by different enzymes at different times during adipocyte differentiation.

In summary, we found that the GstO1-specific inhibitor C1-27 effectively suppressed adipocyte differentiation. GstO1 inhibition by C1-27 was mediated by covalent modification of a catalytic cysteine residue, and reduced the protein stability of GstO1 during the early phase of adipocyte differentiation. Notably, the present study demonstrates that GstO1 plays a key role during the early phase of adipocyte differentiation by catalyzing the deglutathionylation of proteins, especially ~40 kDa proteins that critically regulate adipocyte differentiation.

## MATERIALS AND METHODS

### Cell culture

Mouse-derived 3T3-L1 preadipocytes were maintained in the media DMEM/10% calf serum (CS, Hyclone), as described previously (11). For adipocyte differentiation, 3T3-L1 cells were grown in the media DMEM/10% CS to confluency, and another 2 days to reach growth arrest. Adipocyte differentiation was induced by changing the media to DMEM/10% fetal bovine serum (FBS, Hyclone) containing an induction cocktail (MDI, 0.5 mM 3-isobutyl-1-methylxanthine, 1  $\mu$ M dexamethasone, and 5  $\mu$ g/ml of insulin) with supplementing the indicated

concentrations of C1-27. After incubation for 2 days, cells were grown for another 6 days in DMEM/10% FBS containing 5 µg/ml insulin with media changes on alternate. Adipocyte differentiation was estimated by Oil Red-O staining of accumulated lipids in cells (11).

### Cytotoxicity assay

For the assay of proliferating cells, 3T3-L1 cells (3,000-5,000/well) were seeded in a 96-well culture plate containing DMEM/10% CS. After 24 h incubation, the indicated concentrations of C1-27 were added to culture media, and cells were grown for 48 h. Viable cells were determined using a D-Plus CCK kit (Dongin Biotech). For the assay under differentiation conditions, of proliferating cells, 3T3-L1 cells were grown in DMEM/10% CS till growth arrest. Cell culture media were changed to DMEM/10% FBS containing MDI and the indicated concentrations of C1-27. After incubation for 48 h, viable cell numbers were determined.

### Quantitative real-time PCR

Total RNAs were isolated from 3T3-L1 cells using TRIzol reagent, and cDNA was synthesized from 1 µg of RNA using a cDNA reverse transcription kit (Invitrogen). PCR amplification was conducted using an ABI 7500 Real-Time PCR system (Applied Biosystems) and SYBR green (Biorad). Gene expressions were normalized versus β-actin and appropriate negative controls. The PCR primers used are summarized in Supplementary Table 1.

### Western blot analysis

Cell proteins were separated by 12% SDS-PAGE under reducing conditions for GStO1 and β-actin, or non-reducing conditions for glutathionylated proteins (Pro-SG). Separated proteins were transferred to PVDF membrane. GStO1 and β-actin were probed using an anti-GstO1 antibody (Santa Cruz) and an anti-β-actin antibody (Santa Cruz), respectively. Pro-SG was probed using an anti-Pro-SG antibody (Merck). Quantitative analyses were conducted using Image J software by normalizing with β-actin.

### Enzyme activity assays

The reduction of 4-nitrophenacyl glutathione (4-NPG) to 4-nitroacetophenone was determined in 50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1.5 mM EDTA, and 10 mM β-mercaptoethanol. The reaction was started by adding 0.5 mM 4-NPG, and absorbance decreases at 305 nm ( $\epsilon_{305 \text{ nm}} = 1.1 \text{ mM}^{-1} \text{ cm}^{-1}$  (20)) were monitored at room temperature (RT) using a Cary 100 UV-Vis spectrophotometer (Varian). To investigate the inhibition of 4-NPG reduction activity by C1-27, mGstO1 (12 µg/ml) was incubated in the assay buffer for 1 h at RT at the indicated inhibitor concentrations. Thiol transferase and glutathione transferase activities were measured as previously described (6).

### Determination of mGstO1-catalyzed deglutathionylation

mGstO1-catalyzed deglutathionylation was performed in 0.2 ml of 50 mM Tris/HCl (pH 8.0) containing 150 mM NaCl and 15 µg of 3T3-L1 cell proteins labeled with eosin-glutathione (E-GSH), as previously described (11). Deglutathionylation was initiated by adding 100 µg mGstO1 and measuring the fluorescence of released E-GSH at 540 nm (excitation at 490 nm). mGstO1-catalyzed protein deglutathionylation was also determined by western blot analysis after alkylating protein thiols with 0.5 mM biotin-NEM (*N*-ethylmaleimide). Proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes. Deglutathionylated proteins labeled with biotin-NEM were detected by western blot analysis using avidin-HRP.

### Determination of thermal stability

The thermal stability of GStO1 was determined by differential scanning fluorimetry (DSF) and isothermal denaturation (ITD), as described in (21). Briefly, mGstO1 (0.1 mg/ml) was incubated with the fluorescent reporter Sypro orange (Invitrogen) from 20 to 80°C by increasing the temperature at 1°C/min or at 37°C for 3 h. Protein unfolding was assessed by following fluorescence increases using an ABI 7500 Real-Time PCR system (Applied Biosystems).

### Statistical analysis

The significance of differences between gene expression levels was determined using the Student t-test for unpaired samples and one-way ANOVA. Results are presented as means ± standard errors, and a P-value < 0.01(\*) was considered statistically significant.

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### CONFLICTS OF INTEREST

The authors have no conflicting interests.

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