Promiscuous Methionyl-tRNA Synthetase Mediates Adaptive Mistranslation against Oxidative Stresses

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JCS Advance Online Article. Posted on 5 August 2014

ABSTRACT

Aminoacyl-tRNA Synthetases (ARSs) acylate tRNAs with amino acids. Charging tRNAs with the right amino acids is the first step in translation; therefore, the accurate and error-free functioning of ARSs is an essential prerequisite for translational fidelity. A recent study found that methionine (Met) can be incorporated into non-Met residues of proteins through methionylation to non-cognate tRNAs under oxidative stress. However, it was not understood how this mis-methionylation is achieved. Here, we report that methionyl-tRNA synthetase (MRS) is phosphorylated at Ser209 and Ser825 by extracellular signal-related kinase (ERK) upon reactive oxygen species (ROS) stress, and that this phosphorylated MRS showed increased affinity to non-cognate tRNAs with lower affinity to $tRNA^{Met}$, leading to an increase in Met residues in cellular proteins. The expression of a mutant MRS containing the substitutions S209D and S825D, mimicking dual phosphorylation, reduced ROS levels and cell death. This controlled inaccuracy of MRS seems to serve as a defense mechanism against ROS-mediated damage at the cost of translational fidelity.

INTRODUCTION

Reactive oxygen species (ROS), which are generally unstable and highly reactive, are continuously produced because of normal physiological events such as aerobic metabolism as well as extracellular stress including radiation or chemicals. Low levels of ROS promote cellular proliferation and differentiation, but long-term or high-level exposure to ROS induces oxidative damage, causing cell death. Maintaining redox homeostasis is therefore essential for normal cell growth and survival (Trachootham et al., 2009). Conversely, an ROS imbalance is related to several pathophysiological conditions, including cancer, diabetes, chronic inflammation, atherosclerosis, ischemia-reperfusion injury, and neurodegenerative disorders (Jones et al., 2012; Waris and Ahsan, 2006).

To protect against oxidative damage, cells have recruited various endogenous enzymatic and non-enzymatic antioxidants, including peroxidase, superoxide dismutase, glutathione, NADPH, ubiquinone, vitamin, and carotenoids (Birben et al., 2012). Apart from these antioxidant molecules, a distinct antioxidant mechanism uses methionine (Met) as a ROS scavenger to protect proteins (Luo and Levine, 2009). Met residues on the surface of proteins intercept ROS such as hydrogen peroxides and they are converted to methionine sulfoxides. The oxidized Met is then reduced to Met by methionine sulfoxide reductases (Levine et al., 1996; Levine et al., 2000; Luo and Levine, 2009; Stadtman et al., 2002; Stadtman et al., 2003).

It was recently shown that methionylation to non-cognate tRNAs, termed ‘Met-misacylation’, increased up to 10% in mammalian cells under oxidative stress (Netzer et al., 2009; Wiltrout et al., 2012). This Met-misacylation predominantly occurs in tRNA families that originally carry charged or polar amino acids (Netzer et al., 2009), implying that methionyl-tRNA synthetase (MRS) might be involved in Met-misacylation to non-cognate tRNAs such as $tRNA^{Lys}$, $tRNA^{Gly}$ and $tRNA^{Leu}$ (Netzer et al., 2009). However, the molecular mechanism responsible for Met-misacylation under oxidative stress remains unidentified.

MRS is an enzyme responsible for the ligation of Met to the cognate initiator or elongator $tRNA^{Met}$. In the mammalian system, MRS is normally bound to the multi-tRNA synthetase complex (MSC), making specific interaction with aminoacyl-tRNA synthetase (ARS)-interacting multifunctional protein 3 (AIMP3/p18) (Park et al., 2005).

Our recent study suggests that affinity to the cognate tRNA is reduced by MRS phosphorylation, resulting in suppression of global translation (Kwon et al., 2011). This study suggested that the tRNA binding affinity of MRS can be modulated by post-translational modification. Further expanding this discovery, we hypothesized that certain modifications of MRS could alter its tRNA specificity and induce Met-misacylation to non-cognate tRNAs, which would serve as a protective mechanism against oxidative stress. In this study, we investigated the functional significance of MRS in protection against ROS damage and in the molecular mechanism that controls the specificity of tRNA recognition.

RESULTS

MRS is phosphorylated by extracellular signal-related kinase (ERK) under oxidative stress

Based on previous studies showing the possibility of MRS-mediated Met-misacylation (Jones et al., 2011; Netzer et al., 2009; Wiltrout et al., 2012) and modulation of MRS catalytic activity by phosphorylation (Kwon et al., 2011; Pendergast and Traugh, 1985), we first examined MRS modification under ROS stress. Protein extracts from HeLa cells treated with arsenite, a ROS-inducing agent, were separated by 2 dimensional-polyacrylamide gel electrophoresis (2D-PAGE) and then immunoblotted using the anti-MRS antibody. Additional spots of MRS were generated on the acidic side by treatment with arsenite; these spots disappeared after alkaline phosphatase treatment, indicating that MRS was phosphorylated under ROS stress (Fig. 1A). To identify which amino acid residues were involved in ROS-mediated phosphorylation, proteins from control and ROS-induced HeLa cells were immunoprecipitated with anti-MRS antibody and immunoblotted with phospho-specific antibodies. Phosphorylation of MRS at serine residues was observed in MRS extracted from arsenite-treated cells (Fig. 1B), whereas phosphorylation at threonine or tyrosine residues was not detected. Serine-specific phosphorylation was also confirmed in H_2O_2-treated HeLa cells (Fig. S1A). Removal of ROS by treatment with diphenyleneiodonium (DPI), a broad inhibitor of oxidases, apparently reduced the arsenite-dependent phosphorylation of MRS (Fig. 1C), indicating that kinases activated under ROS signaling would be involved in MRS phosphorylation. Since mitogen-activated protein kinases (MAPKs) are well known for

their various functions upon stimulation by ROS (Lau et al., 2004; Pan et al., 2009; Son et al., 2011), we treated HeLa cells with MAPK-specific inhibitors and monitored MRS phosphorylation. Serine-specific phosphorylation of MRS induced by arsenite was dramatically suppressed in cells treated with the ERK inhibitor PD98059, whereas p38 MAPK or c-Jun N-terminal kinase (JNK) inhibitors did not influence MRS phosphorylation (Fig. 1D). Next, we performed an *in vitro* kinase assay by incubating glutathione sulfotransferase (GST) or GST-MRS with purified active ERK and [γ-^{32}P]ATP to confirm whether MRS is a real substrate for ERK. GST-MRS, but not GST, showed obvious phosphorylation signal by ERK (Fig. 1E); therefore, we concluded that MRS was phosphorylated at serine residues by ERK under ROS stress.

Determination of ERK-induced phosphorylation sites in MRS

Human MRS consists of three functional domains, the GST-like (MD1, residues 1–266), catalytic (MD2, residues 267–597), and tRNA-binding (MD3, residues 598–900) domains (Fig. 1F). Using these domain fragments, we conducted an *in vitro* kinase assay to narrow down the ERK-mediated phosphorylation domain of MRS. Since a strong phosphorylation signal was observed in MD1 and MD3, but not in MD2 (Fig. 1G), we analyzed phosphorylation sites in MD1 and MD3 after the *in vitro* kinase assay by mass spectrometry to determine the ERK-dependent phosphorylation site in MRS. Among the phosphorylation sites of MRS detected (Fig. S2A), we selected the serine residues Ser209 and Ser825, because ROS-induced MRS phosphorylation is serine-specific (Fig. 1B). We synthesized biotinylated MRS peptides containing Ser209 and Ser825, respectively, as well as the same peptides with serine to alanine substitution. The peptide kinase assay revealed the apparent phosphorylation of both Ser209- and Ser825-containing peptides by ERK, whereas little signal was observed in alanine-substituted mutant peptides (Fig. 1H, left) or ERK inhibitor-treated conditions (Fig. 1H, right). The same results were obtained when the *in vitro* kinase assay was performed with wild-type (WT) and mutant GST-MRS proteins. The GST-MRS S209A/S825A (SA) mutant, in which both serine residues were substituted with alanines, showed minimal phosphorylation upon incubation with ERK, compared with WT MRS (Fig. 1I). We also transfected HEK293T cells with WT Myc-MRS or the Myc-MRS SA mutant and analyzed serine-specific phosphorylation by immunoblotting. The phosphorylation

signal was increasingly detected in WT MRS by arsenite treatment, but was not detected in the dual alanine-substituted SA mutant (Fig. 1J). Moreover, H_2O_2 treatment did not induce phosphorylation in the MRS SA mutant (Fig. S1A). We also checked the phosphorylation state in single alanine-substituted mutants. Although the serine-specific phosphorylation signal was slightly lower in the S209A and S825A single mutants compared to WT MRS, these single alanine-substituted mutants did not show a dramatic decrease as seen with the MRS SA mutant, suggesting that the Ser209 and Ser825 residues are dually phosphorylated by ERK under ROS stress (Fig. S2B).

ERK is activated upon various stimuli including UV, therefore we wondered whether Ser209 and Ser825 phosphorylation is specific to ROS. We transfected Myc-MRS S662A into HEK293T cells along with Myc-MRS WT and investigated MRS phosphorylation. Ser662 residue of MRS is known to be phosphorylated by general control nonderepressible 2 (GCN2) upon UV irradiation (Kwon et al., 2011). If Ser209 or Ser825 can be phosphorylated by UV-activated ERK, phosphosylation signal will be detected in MRS S662A by UV. Phosphorylation of MRS S662A, however, was only detected under ROS stress but not by UV suggesting that Ser209 and Ser825 phosphorylation is specific to ROS stress (Fig. S2C).

Phosphorylation of MRS at Ser209 and Ser825 induces Met-misacylation under ROS stress

Because MRS was modified by phosphorylation under ROS stress, we investigated the correlation between Met-misacylation and the dual phosphorylation of MRS under ROS stress conditions. We first analyzed circular dichroism (CD) spectra of WT MRS, the MRS SA mutant and the S209D/S825D (SD) mutant and observed a temperature-dependent structural change of MRS SD mutant in the far-UV spectra (Fig. 2A). To evaluate the Met-misacylation ability of the MRS SD mutant, we performed *in vitro* tRNA binding and aminoacylation activity assays. Based on a previous report suggesting that ROS-dependent Met-misacylation predominantly occurs in $tRNA^{Lys}$(CUU) (Netzer et al., 2009), we tested $tRNA^{Lys}$(CUU) first. We examined the interaction between the MRS SD mutant and $tRNA^{Lys}$(CUU) via electrophoretic mobility shift assay (EMSA). The MRS SD mutant showed clearly increased association with radioactively labeled $tRNA^{Lys}$(CUU) in a dose-dependent manner,

whereas WT MRS did not (Fig. 2B, top). Both WT MRS and the SD mutant interacted with elongator $tRNA^{Met}$ ($tRNAe^{Met}$)(CAU) to a similar extent (Fig. 2B, bottom).

In the aminoacylation activity assay, the K_M value of the MRS SD mutant for $tRNA^{Lys}$(CUU) was about six-fold higher than that of WT MRS for $tRNAe^{Met}$(CAU) (Table 1). It is notable that the MRS SD mutant showed relatively higher affinity to $tRNA^{Lys}$(CUU) rather than to $tRNAe^{Met}$(CAU), whereas the K_M of WT MRS for $tRNA^{Lys}$(CUU) was not measurable. Although MRS SA mutant showed significant decrease in its catalytic activity to $tRNA^{Met}$, it retained its specificity to the cognate tRNA. We further confirmed the Met-misacylation ability of the MRS SD mutant to other types of tRNA such as $tRNA^{Ala}$(AGC), $tRNA^{Gly}$(GCC), $tRNA^{His}$(GUG), and $tRNA^{Leu}$(CAG). The MRS SD mutant generally showed considerable binding affinities to these non-cognate tRNAs compared with WT MRS (Fig. S3A). Consistently, the MRS SD mutant charged non-cognate tRNAs with Met at levels similar to those seen with $tRNAe^{Met}$(CAU), with the highest activity for $tRNA^{Lys}$(CUU) among the tRNAs (Fig. S3B). These results suggested that dual phosphorylation of MRS at residues Ser209 and Ser825 can endow MRS with the ability to recognize non-cognate tRNAs and misacylate them with Met.

Next, we investigated whether Met-misacylated tRNA families can be used for translation. Since MRS incorporation into the MSC may affect translational efficiency, we firstly performed immunoprecipitation assay using anti-EPRS (glutamyl-prolyl tRNA synthetase) and anti-Myc antibodies to see the existence of the Myc-MRS SD mutant in the MSC. EPRS, the representative subunit of the MSC, and the Myc-MRS SD mutant were co-immunoprecipitated together, therefore we concluded that exogenous MRS SD mutant can be localized in the MSC like endogenous MRS (data not shown). We set up a fluorescence system using TagRFP to monitor Met-misacylation. TagRFP is a red fluorescent protein whose Met67 residue is critical for its fluorescence. We mutated the ATG codon for Met67 to AAG for Lys to turn off the fluorescence and observed that the red fluorescence signal from the TagRFP M67K mutant was undetectable (Fig. 3A). If MRS mismethionylated $tRNA^{Lys}$(CUU), then Met-$tRNA^{Lys}$(CUU) could be incorporated into the mutated codon of TagRFP M67K to restore the Lys residue to original Met, thereby restoring red fluorescence. We transfected the TagRFP M67K mutant together with empty vector (EV), WT Myc-MRS,

the Myc-MRS SA mutant, or the Myc-MRS SD mutant into HEK293T cells. EV-, WT MRS-, and MRS SA-expressing cells exhibited increased red fluorescence signal upon arsenite treatment, with the most enhanced fluorescence in WT MRS-expressing cells (Fig. 3B). The MRS SD mutant showed increased fluorescence signal regardless of arsenite treatment (Fig. 3B). Based on the results of immunoblotting, this difference in fluorescence among the MRS proteins was not due to the expression level of TagRFP (Fig. 3C). We analyzed the images based on the fluorescence-positive cell number (Fig. 3D) as well as fluorescence intensity (Fig. 3E). Both of these results suggested three notable points: 1) ROS-responsive Met-misincorporation by endogenous and exogenous WT MRS; 2) similar increase in Met-misincorporation under ROS condition in the cells expressing EV or the MRS SA mutant; and 3) basally enhanced Met-misincorporation by the MRS SD mutant. Similar patterns were also observed with the TagRFP M67H and M67G mutants, proving the MRS-mediated production of Met-tRNA^{His}(GUG) and Met-tRNA^{Gly}(UCC) and their incorporation into the TagRFP mutant protein, respectively (Fig. S3C).

To further investigate the possible residue positions of a protein at which Met can be misincorporated, we first transfected HEK293T cells with the pBiFC-VN173-AIMP3 vector (Kwon et al., 2011) to use the Flag-VN (N-terminal fragment of Venus)-AIMP3 protein as a reporter for monitoring Met-misincorporation. We chose Flag-VN-AIMP3 because this protein is small (347 net amino acids without linker) and contains a relatively small number of Met residues (4 amino acids). Flag-VN-AIMP3 was immunoprecipitated after the addition of [^{35}S]Met to the cells, and the autoradioactivity emanating from the Flag-VN-AIMP3 was detected (Fig. 3F,G). The radioactive signal from Flag-VN-AIMP3 was increased upon arsenite treatment, whereas the enhanced signal disappeared upon treatment with ERK inhibitor (Fig. 3F). This signal was not observed by serum stimulation either, which is another activation signal for ERK, suggesting that Met-misincorporation is a phenomenon specifically occurs upon ROS stress (data not shown). We also confirmed MRS-dependent [^{35}S]Met incorporation into Flag-VN-AIMP3 in HEK293T cells expressing EV, WT MRS, the SA or SD mutant. Autoradioactivity was detected in the protein isolated from arsenite-treated HEK293T cells with different signal intensity according to the expressional and mutational status of MRS. MRS WT-transfected cells showed the topmost signal intensity of ROS-

dependent Met-misincorporation among the EV, WT, and the SA mutant expressing cells (Fig. 3G). The MRS SD mutant induced the production of radioactive Flag-VN-AIMP3 regardless of arsenite or ERK inhibitor treatment (Fig. 3G). This Met-misincorporation under ROS stress was MRS specific because leucine-misincorporation into Flag-VN-AIMP3 was not observed by overexpression of leucyl-tRNA synthetase (LRS) (Fig. S3D). To further validate the MRS-mediated Met-misincorporation, we immunoprecipitated Flag-VN-AIMP3 from HEK293T cell lysates and analyzed the Met-misincorporated residues using mass spectrometry. We identified several peptides containing Met at non-Met residue positions in the MRS SD-expressing cells, although there was basal Met-misacylation in WT HEK293T cells (Table 2; Fig. 4; Fig. S4). Interestingly, residues swapped with Met were detected on the surface of AIMP3 as well as Venus (Fig. 4A,B). In addition, the swapped residues of AIMP3 do not participate in the MRS interaction, and they are expected to be exposed outside of the MSC (Fig. 4A, bottom). This supports the idea that Met-misincorporation is not an accidental event, but it results in relocating Met on the surface of proteins as a ROS scavenger.

MRS-mediated Met-misacylation reduces intracellular ROS levels and protects cells from oxidative damage

Since residue switching with Met was detected on the protein surface where ROS attacks vulnerable amino acids, we examined whether MRS-mediated Met-misacylation can actually reduce intracellular ROS levels by using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. Whereas EV-expressing control cells or MRS SA mutant-expressing cells showed a little bit increased ROS levels upon arsenite treatment, WT MRS- and SD mutant-expressing cells did not (Fig. 5A). It is known that ROS increases pro-apoptotic Bax while reducing the expression level of Bcl-2, the Bax inhibitor (Chen et al., 1998; Fleury et al., 2002; Hossain et al., 2000). Thus, we checked the expression levels of these markers and found that cells expressing WT MRS and the SD mutant were more resistant to apoptotic cell death upon arsenite-induced ROS generation than EV- or MRS SA mutant-expressing cells (Fig. 5B). Together, these results suggest that MRS phosphorylation at Ser209 and Ser825 can reduce intracellular ROS level resulting in cell protection from ROS-mediated apoptosis.

To investigate the effect of MRS-mediated Met-misacylation on cell survival, we performed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. First, we reduced the expression level of MRS using siRNA treatment. MRS knockdown significantly reduced the cell viability under ROS stress suggesting the critical role of MRS in cell protection against ROS stress (Fig. 5C). Overexpression of WT MRS or the MRS SD mutant under ROS stress, whether transient (Fig. 5D) or stable (Fig. 5E), maintained cell survival at levels similar to that of normal growth in the MTT assay. In contrast, the MRS SA mutant could not protect cells from oxidative damage as much as MRS WT or the SD mutant did, resulting in significant reduction in cell viability as shown in ROS-treated control cells. Growth curve analysis also showed the same results as in the MTT assay. ROS retarded the growth of stable HeLa cells expressing EV and the MRS SA mutant by up to 23.14% and 23.62%, respectively, but it did not cause any significant effects on the growth of stable HeLa WT and SD mutant cells (Fig. 5F). A similar pattern of cell viability under ROS stress was also observed with H_2O_2 treatment (Fig. S1B). Moreover, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay indicated that cells expressing MRS WT or SD mutant were more resistant to apoptosis upon ROS stimuli than EV or MRS SA-expressing cells (Fig. 5G). The cell protective effect of MRS WT under ROS stress was significantly reduced by U0126 suggesting that ERK activation is required for the cell protective function of MRS (Fig. 5H; Fig. S3E). In addition, unlike MRS, LRS overexpression did not compensate for the reduced cell viability caused by ROS (Fig. S3F). These results indicate that dual phosphorylation of MRS is critical for cell protection against oxidative damage.

In summary, under oxidative stress conditions, MRS is phosphorylated at Ser209 and Ser825 by activated ERK. Dually phosphorylated MRS induces Met-misacylation of noncognate tRNAs. Increased numbers of Met residues via misincorporation during translation can serve as ROS scavengers and protect cells from ROS-induced damage and apoptosis (Fig. 6).

DISCUSSION

Previous studies reported the importance of the Met residue as an antioxidant agent (Levine et al., 1996; Luo and Levine, 2009; Stadtman et al., 2002; Stadtman et al.,

2003) and an increase in Met-misacylation and Met-misincorporation under ROS stress (Netzer et al., 2009). Although MRS mutation- or MRS condition-dependent Met-misacylation was identified in *Escherichia coli* and *Saccharomyces cerevisiae* (Jones et al., 2011; Wiltrout et al., 2012), the exact mechanism and meaning of this phenomenon in human cells is not yet understood. In this study, we identified human MRS as a new substrate for ERK under ROS stress and demonstrated a novel function of phosphorylated MRS, changing tRNA specificity to increase the rate of codon-independent Met-mistranslation during oxidative damage.

Accurate translation is an important issue for cells to maintain their normal cellular integrity (Yadavalli and Ibba, 2012). To maintain translational fidelity, ARSs possess an editing function: they remove misactivated aminoacyl-adenylate or mischarged tRNAs via several mechanisms. While other class Ia ARSs have at least one connecting peptide 1 (CP1) with an editing site, and perform pre-transfer and post-transfer editing, MRS has a truncated CP1 domain and performs pre-transfer editing in a catalytic site-dependent manner (Englisch et al., 1986; Fersht and Dingwall, 1979; Jakubowski, 1991; Yadavalli and Ibba, 2012). Although the manner in which human MRS carries out its editing function and recognizes its cognate tRNA is structurally unclear, it is known that bacterial MRS recognizes the anticodon region and acceptor A73 in $tRNA^{Met}$ (Senger et al., 1992). In eukaryotes, MRS is expected to have a certain additional mode for the recognition and aminoacylation of tRNA. First, the C-terminal WHEP (824–900 amino acids) domain strengthens the association of $tRNA^{Met}$ to MRS under suboptimal tRNA concentrations (Kaminska et al., 2001). Second, the N-terminal 215–267 amino acids on the GST-like domain are critical for MRS catalytic activity (He et al., 2009). Our results are in line with these studies in that phosphorylation in these appended domains, but not in the core domain, can modulate MRS enzymatic activity. The structural change in the MRS SD mutant also supports the possibility of functional modulation by MRS modification (Fig. 2A). Although further investigation is required to understand the interaction of phosphorylated MRS with a non-cognate tRNA, it does not seem to recognize either the anticodon region or the discriminator base in the non-cognate tRNAs. The common bases between Met-misacylated tRNAs and $tRNA^{Met}$ are mainly located in the D and TψC arms, implying that phosphorylated MRS may recognize tRNAs in a different way.

WT MRS and the SD mutant showed similar levels of $tRNAe^{Met}$ binding in EMSA, whereas their kinetic analyses suggested that MRS SD mutant charges $tRNAe^{Met}$ less efficiently than WT MRS does (Table 1; Fig. 2B). The MRS SD mutant, on the other hand, showed more effective aminoacylation activity to $tRNA^{Lys}$. Considering only the Met-charging efficacy, the reduced susceptibility of phosphorylated MRS to $tRNAe^{Met}$ is unnecessary. The reduced affinity to the cognate $tRNAe^{Met}$ may be an inadvertent result of the increased selectivity to other tRNAs. The activity of MRS SD mutant to the $tRNAi^{Met}$, however, was similar to that of MRS WT indicating that MRS phosphorylation under ROS stress may not cause adverse effect on translation initiation. Indeed, there was little difference between the overexpression of MRS WT and the SD mutant in their effect on global translation (data not shown).

Although MRS SD mutant revealed evident charging activity to $tRNA^{Lys}$, MRS may have to compete with lysyl-tRNA synthetase (KRS) for capturing $tRNA^{Lys}$ under ROS stress unless there is spare $tRNA^{Lys}$. We analyzed free $tRNA^{Lys}$ as well as charged $tRNA^{Lys}$ using acidic urea PAGE gel and observed that some portion of free $tRNA^{Lys}$ seems available even when KRS is fully active (data not shown). It also implies the availability of other spare non-cognate tRNAs for misacylation. Met-misincorporation does not require high amounts of other tRNAs. It is known that total increase of Met-misincorporation is about 10% under ROS stress (Netzer et al., 2009). Also other possibilities to increase the tRNAs availability under oxidative stress, such as the accessibility changes of the tRNAs to their cognate ARSs, cannot be excluded.

Considering that a series of tRNAs that mainly carry charged or polar amino acids are mismethionylated under ROS stress (Netzer et al., 2009), the shift in substrate preference can confer apparent merits to cells, such as relocation of Met residues on the surface and an increased number of Met residues. According to Levine *et al*., all the Met residues in the original positions are not used as ROS scavenger (Levine et al., 1996). About 50% of the original Met residues in glutamine synthetase were oxidized by ROS, with the intact Met residues being buried within the core of protein. When repositioned in exposed spots with increased occupancy by misacylation, Met has a greater chance of reacting with ROS. In fact, the Met-misincorporated residues in Flag-VN-AIMP3 were all detected on the surface of protein, supporting this point of view (Fig. 4). Met-misincorporation increased the number of Met residues from the original 3 to 11 in VN

(173 amino acids) and from 1 to 8 in AIMP3 (174 amino acids) (Table 2; Fig. 4).

Cells under normal conditions also had a basal level of Met-misincorporation on the protein surface (Fig. 3B; Fig. 4). This is probably due to endogenous ROS levels. Interestingly, the residues basally exchanged with Met were not perfectly matched up to those detected under MRS SD expression. This suggests that Met-misacylation by MRS can be arbitrary in some respects but can provide equal results under independent circumstances. This is probable due to the characteristics of non-cognate tRNAs, which can be used as substrates for phosphorylated MRS. Although the MRS SD mutant showed extended affinity to a broad range of tRNAs, all tRNAs were not charged by MRS under ROS stress (Netzer et al., 2009). Generally, tRNAs in charge of carrying hydrophilic amino acids were preferentially used for Met-misacylation. Therefore, the selection of non-cognate tRNAs for Met-misacylation and coupled Met-incorporation seems to be regulated in a flexible way but within a limited range to cope with different environments.

Our finding that cells adopt MRS-mediated mistranslation to survive under ROS stress via tolerating reduced translational fidelity is unique. The sacrifice of translational fidelity does not seem to cause severe side effects during the short term, because cells transiently transfected with the MRS SD mutant did not show any signs of apoptosis (Fig. 5B,D). Long-lasting misacylation, however, may cause adverse effects on cells due to the accumulation of misfolded or inactive proteins. Consistent with this expectation, stable cells expressing the MRS SD mutant showed slightly reduced viability in the MTT assay (Fig. 5E). There may be a correlation between long-lasting misacylation and human diseases such as cancer or degenerative diseases, and this should be further studied to uncover their relationship. Nevertheless, the role of dually phosphorylated MRS upon ROS stimulation is advantageous at least for a short period because it can induce Met-misacylation to remove ROS and to protect protein damage while maintaining cell viability.

MATERIALS AND METHODS

Cell culture

HeLa and HEK293T cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1%

penicillin/streptomycin at 37°C in a 5% CO_2 incubator. To establish stable HeLa cell lines, cells were transfected with the pcDNA3-Myc EV, pcDNA3-Myc-MRS WT, pcDNA3-Myc-MRS SA or pcDNA3-Myc-MRS SD plasmid using FuGENE HD (Roche) in HeLa cells. Stable cells were selected and maintained under antibiotic pressure (800 μg/ml of geneticin; Duchefa Biochemie).

ROS induction and inhibitor treatment

Cells were cultured until they reached 80% confluence. Cells were treated with 4 μM sodium arsenite (Sigma) or 200 μM H_2O_2 for 4 h in DMEM with FBS (2% for short-term and 5% for long-term treatment) and 1% penicillin and streptomycin. Cells were pre-treated with the MAPK inhibitors SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), and PD98059 or U0126 (ERK inhibitor) 1 h before ROS induction at a concentration of 20 μM. All inhibitors were purchased from Calbiochem (Billerica). For the DPI chase (Enzo), cells were pre-treated with 50 μM DPI 30 min before ROS induction.

DCFH-DA assay

HEK293T cells transfected with pcDNA3-Myc EV, pcDNA3-Myc-MRS WT, pcDNA3-Myc-MRS SA, or pcDNA3-Myc-MRS SD plasmids were exposed to sodium arsenite (4 μM) for 24 h. After treatment with 20 μM DCFH-DA (Invitrogen) (Ruiz-Ramos et al., 2009) for 15 min, cells were washed twice with PBS. The DCF signals were detected using a fluorescence microscope equipped with a green fluorescence filter (470 nm excitation, 525 nm emission) (Nikon) to monitor intracellular ROS levels.

Immunoblotting

Cells were lysed by lysis buffer (50 mM Tris-HCl pH 7.4, 0.5% Triton X-100, 5 mM EDTA, 10% glycerol and 150 mM NaCl) with phosphatase inhibitor and protease inhibitor Calbiochem) for 30 min at 4°C. After centrifugation, supernatants were collected and the protein amounts were quantified by Bradford assay (BioRad). Proteins extracts from the cells were separated by SDS-PAGE, transferred to PVDF membrane and incubated with specific primary antibodies. Antibodies for p-Ser (Abcam), p-Thr

(Cell Signaling), p-Tyr (Cell Signaling), Myc (Santa Cruz), Flag (Sigma), MRS (Abcam), tubulin (Sigma), DsRed (Clontech), Bax (Santa Cruz) and Bcl-2 (Santa Cruz) were used in this study. Primary antibodies were used at the concentration of 0.2-0.4 μg/ml (Abcam, Sigma and Santa Cruz) or with a 1:1000 dilution (Cell Signaling).

Immunoprecipitation

Protein extracts were incubated with primary antibody (2 μg) 15 for 4 h at 4°C with agitation, and then incubated further for 4 h at 4°C with protein A agarose (Invitrogen). Beads were washed three times with cold lysis buffer and supernatants were removed. Samples were dissolved in the SDS sample buffer and separated by SDS-PAGE.

2D-PAGE

Protein extracts from HeLa cells were incubated with alkaline phosphatase (Roche) for 2 h. Each 500 μg protein extract was rehydrated in resolubilization buffer (7 M urea, 2 M thiourea, 2% ASB-14, 0.5% Triton X-100, 1% (vol/vol) ampholyte, 1% (vol/vol) tributylphosphine and 0.1% bromophenol blue). Samples were loaded onto the immobilized pH gradient (IPG) strip gels (linear pH gradient 4-7, 7cm, Bio-Rad) and subjected to isoelectric focusing (Bio-Rad).

***In vitro* kinase assay and filter binding assay**

GST-fusion MRS proteins were purified from *Escherichia coli* Rossetta 2. Proteins were induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cultured at 18°C for overnight. Harvested cells were lysed by sonication, and lysates were incubated with glutathione Sepharose 4B (GE Healthcare) in the lysis buffer (PBS containing 0.5% Triton X-100 and protease inhibitor) at 4°C for 6 h. Before the kinase reaction, the GST-fusion MRS proteins were pre-incubated with 500 μM ATP for 10 min at room temperature and the kinase reactions were performed at 30°C for 30 min by adding ERK (Cell science), [γ-^{32}P]ATP (IZOTOP, SBP301-1000 μCi), and kinase buffer (100 mM Tris-HCl pH 7.4, 75 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, phosphatase inhibitor and protease inhibitor). Reactions were stopped by adding the SDS sample buffer and the samples were separated by SDS-PAGE gel and detected by autoradiography.

For the peptide kinase assay (Kwon et al., 2011), GFP-ERK expressed in HEK293T cells with or without U0126 pre-treatment was immunoprecipitated with anti-GFP antibody. N-terminal biotinylated peptides were chemically synthesized (GL Biochem). Each peptide [3 mM MRS Ser209 (QKQPFQPSPAEGR), MRS S209A (QKQPQPAPAEGR), MRS Ser825 (GGQAKTSPKPA), MRS S825A (GGQAKTAPKPA), positive control (APRTPGGRR), and negative control (APRAPGGRR)] was reacted with the GFP-ERK at 30°C for 30 min. Soup from each sample was filtered through a streptavidin-coated matrix biotin-capture membrane (Promega) using a 96-well Minifold filtration apparatus. The membrane was washed according to the previous report (Schaefer and Guimond, 1998) and exposed for autoradiography.

CD Spectrum analysis

MBP-MRS WT and MBP-MRS SA and SD mutants were purified and eluted with a buffer containing 50 mM maltose at 4°C for 24 h and then dialyzed with 10 mM potassium phosphate buffer (pH 7.4). The CD spectrum was measured using a Jasco J-815 CD spectrometer at 25°C and 70°C in the far-UV range from 190 to 250 nm. Samples were loaded into a 1mm path-length absorption micro cell. The results are shown as an average of three repeated scans after subtraction of buffer background.

Aminoacylation assay

His-tagged MRS (WT, SA and SD) expressed in *E. coli* RIL was purified using ProBond Resin (Invitrogen) and washed with lysis buffer (pH7.8, 20 mM KH_2PO_4, 500 mM NaCl, ,10% glycerol and 2 mM 2-mecaptoethanol) changing the buffer pH from 7.8, to 6 to 5.2, and back to 6, with 24 mM imidazole at the final step. His-MRS was eluted in the presence of 300 mM imidazole (pH 6.0) and dialyzed with PBS containing 20% glycerol. $tRNAe^{Met}$(CAU), $tRNAi^{Met}$(CAU), $tRNA^{Lys}$(CUU), $tRNA^{Ala}$(AGC), $tRNA^{Gly}$(GCC), $tRNA^{His}$(GUG) and $tRNA^{Leu}$(CAG) were synthesized via *in vitro* transcription. MRS aminoacylation activity was performed at 37°C in reaction buffer (30 mM HEPES, pH 7.4, 100 mM potassium acetate, 10 mM magnesium acetate, 4 mM ATP, 20 µM Met, 500 µg/mL each tRNA, 400 nM purified MRS and 25 µCi [^{35}S]Met (IZOTOP, 1000 Ci/mmol). For kinetics analysis, each tRNA ($tRNAe^{Met}$, $tRNAi^{Met}$ and

tRNA^{Lys}) was used at the concentration from 0.5 μM to 80 μM. Aminoacylation reaction samples were spotted on 3 MM filter paper pre-wetted with 5% trichloroacetic acid (TCA) containing 1 mM Met. Washing three times with 5% TCA and dried, radioactivity was detected by liquid scintillation counter (Wallac 1409).

EMSA

tRNAe^{Met}(CAU), tRNA^{Lys}(CUU), tRNA^{Ala}(AGC), tRNA^{Gly}(GCC), tRNA^{His}(GUG) and tRNA^{Leu}(CAG) were synthesized via *in vitro* transcription with [α-^{32}P] UTP (IZOTOP, 3000 Ci/mmol). Purified His-tagged MRS proteins (WT and SD; 0, 1, 2 and 2 μM) were mixed with each tRNA probe in the binding buffer (20 mM Tris-HCl pH 7.4, 75 mM KCl, 10 mM $MgCl_2$, and 5% glycerol) and incubated at 30°C for 30 min. Samples were mixed with same volume of sample buffer and separated by 6.5% non-denaturing polyacrylamide gel. Radioactivity was detected by autoradiography.

Mass spectrometry analysis

GST-fusion MRS domains (MD1 and MD3) were incubated with ERK and ATP, and were subjected to SDS-PAGE. MRS bands were cut from the SDS-PAGE gel and in gel-digested with trypsin (Promega). tryptic digests of MRS are subsequently separated by online reversed-phase chromatography for each run using a Thermo Scientific Eazynano LC II autosampler with a reversed-phase peptide trap EASY-Column (100 μm inner diameter, 2 cm length) and a reversed-phase analytical EASY-Column (75 μm inner diameter, 10 cm length, 3 μm particle size, both Thermo Scientific), and electrospray ionization was subsequently performed using a 30 μm (i.d.) nano-bore stainless steel online emitter (Thermo Scientific) and a voltage set at 2.6 V, at a flow rate of 300 nl/min. The chromatography system was coupled on-line with an LTQ VelosOrbitrap mass spectrometer equipped with an ETD source. To improve peptide fragmentation of phosphopeptides, we applied a data dependent neutral loss MS3 ETD mode or a data dependent decision tree (DDDT) to select for collision induced dissociation (CID) or electron transfer dissociation (ETD) fragmentation depending from the charge sates, respectively. Protein identification was accomplished utilizing the Proteome Discoverer v1.3 database search engine (Thermo scientific) and searches were performed against IPI.human.v3.2 FASTA database or human MRS FASTA database. A fragment mass

tolerance of 1.2 Da, peptide mass tolerance of 15 ppm, and maximum missed cleavage of 2 was set. Result filters was performed with peptide rank (Maximum rank: 1), peptides number per protein (Minimal number of peptides: 2, Count only rank 1 peptides: True, Count peptide only in top scored proteins: True) and Charge State versus Score (Score to which the filter is applied: Sequest Node (XCorr), Minimal Score for charge state = +1: 1.7, + 2: 2.5, +3: 3.2, > +4: 3.5). The Carbamidomethylation (+57.021 Da) of cystein (C) is set as a Static Modification, and the following variable modification were allowed: GlyGly / +114.043 Da (K), Acetyl / +42.011 Da (K), HexNAc / +203.079 Da (N, S, T), Phospho / +79.966 Da (S, T, Y), Oxidation / +15.995 Da (M), deamidated / +0.984 Da (N, Q). Each processed data was subsequently transformed to .sf file with Scaffold 3 program and finally all MRS PTMs identified from control or stimulated sample, respectively were scored and compared with Scaffold PTM software.

For Met-misincorporation data analysis, protein identification was accomplished utilizing the Proteome Discoverer v1.3 database search engine (Thermo scientific) and searches were performed against Flag-VN-AIMP3 FASTA database. The carbamidomethylation (+57.021 Da) of cystein (C) or deamidated (+0.984 Da) of asparagine or glutamine (N, Q) is set as a static modification or as a variable modification, respectively. To identify the exchange of specific amino acid to Met, the following variable modification was additionally allowed; K→M/ +2.946 Da (K), D→M / +16.014 Da (D), V→M / +31.972 Da (V), G→M / +74.019 Da (G), H→M / –6.018 Da (H), L→M /+17.956 Da (L), A→M / +60.003 Da (A) and Oxidation / +15.995 Da (M). Finally all Flag-VN-AIMP3 PTMs identified from both conditions were manually inspected.

Met-misincorporation detection by radioisotope

HEK293T cells were transfected with pBiFC-VN173-AIMP3 and pcDNA3-Myc EV or pcDNA3-Myc-MRS (WT, SA or SD) plasmids using Fugene HD transfection reagent (Roche). After 24 h, the cells were treated with 50 μM ALLN in DMEM methionine free media (Invitrogen) for 30 min. To confirm the effect of ERK on MRS-mediated mismethionylation, ERK inhibitor was co-treated with ALLN. Then 1 mCi [^{35}S]Met was added to the cells and incubated for 2 h. Cells were further incubated with 4 μM

sodium arsenite for 4 h, washed twice with PBS and then subjected to immunoprecipitation. After SDS PAGE, the [^{35}S]Met incorporation into the Flag-AIMP3 protein was detected by autoradiography.

TagRFP mutant generation and Met-misincorporation detection by fluorescence

PCR-based site-directed mutagenesis was performed to change Met67 in TagRFP (GenBank: BAI43881.1) to Lys (M67K), His (M67H) and Gly (M67G). Briefly, Hind III fragment in pHAG016 in which TagRFP was cloned after SRα promoter was swapped with Hind III digests of PCR fragments containing mutations in the 3' PCR primer region.

Each TagRFP plasmid was co-transfected with pcDNA3-Myc EV or pcDNA3-Myc-MRS (WT, SA and SD) into the HEK293 cells. After 24 h, the cells were treated with 4μM arsenite-treated and incubated for 4 h. Images were analyzed for snap pictures using MetaMorph software. The red fluorescent cells were counted using 3 randomly selected scopes in high-power fields (x20). Because of differences in the light absorption rate, light path, and material properties of cell culturing gels, the brightness is not uniform over the microscopic image. Non-uniform illumination correction is required as pre-processing before image analysis.

To analysis cell images based on fluorescence intensity, image contrast was adjusted using a localized version of the modification framework histogram smoothing (MF-HS) algorithm (Arici et al., 2009) to enhance the low-dynamic input image without producing over-enhancing artifacts in the resulting image. Then, the advanced simple linear iterative clustering (SLIC) superpixels method as used for cell segmentation. To identify the approximate boundary of cell, the degree of initial size and compactness parameters were used. Interested cell region was manually selected after SLIC superpixel method. The feature vectors were extracted from the selected cell object and used to analyze the differences in cell size and intensity.

Cell growth and viability assay

Stable HeLa cells expressing pcDNA3-Myc EV or pcDNA-Myc-MRS (WT, SA and SD) were treated with 4 μM sodium arsenite and the cell growth curve was monitored at 37°C for 48 h using IncuCyte Kinetic Live Cell Imaging System (Essen BioScience).

To assess the cell viability, MTT (USB) stock solution (5 mg/ml) was added to a final concentration of 0.5 mg/ml in each well containing 200 μl medium and incubated for 30 min. The precipitated crystal was dissolved in 100 μl of DMSO (Sigma). Absorbance was measured at 450 nm using microplate reader (Sunrise, TECAN).

TUNEL assay

Stable HeLa cells expressing pcDNA3-Myc EV or pcDNA-Myc-MRS (WT, SA and SD) were treated with 4 μM sodium arsenite for 72 h and apoptosis was measured using Dead End Fluorometric TUNEL assay system (Promega) according to the manufacturer's manual. ERK inhibitor was pre-treated 1 h before arsenite treatment when required.

Statistical analysis

Statistical significance of data was determined by applying Student's *t* test or analysis. Significance of analysis of variance is indicated in the figures as *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$. Statistics were calculated with the Prism 5 software (GraphPad Software).

Acknowledgments

We thank to Dr. T. Pan and Dr. T. Jones (University of Chicago) for the discussion.

Competing interests

The authors declare that they have no competing interests.

Author contributions

N.H.K. and S.K. conceived the project, and designed the experiments. J.Y.L., N.H.K. and S.K. drafted the manuscript. J.Y.L., D.G.K., B.G.K., W.S.Y., J.H., T.K., Y.S.O., K.R.K., B.W.H., B.J.H., M.S.K., M.H.K. and N.H.K. performed the experiments and analyzed and interpreted the data. All authors contributed to discussion.

Funding

This work was supported by the Global Frontier Project grants (NRF-M3A6A4-2010-

0029785 [S.K.], NRF-2013M3A6A4045452 [B.J.H.], NRF-2013-045813 [B.S.K]) of National Research Foundation funded by the Ministry of Science, ICT & Future Planning (MSIP) of Korea, and by the Korea Science & Engineering Foundation through the Acceleration Research Program (NRF-2008-0060617 [M.H.K]). This study was also funded by a grant from Gyeonggi Research Development Program [S.K.]. MRS purification was supported by the National R&D Program for Cancer Control (Grant number 1120170) from National Cancer Center of Korea.

Supplementary material

Supplementary material is available online.

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Table 1. Comparison of catalytic activities between MRS WT, SA and SD.

Enzyme	Constants	Met-tRNAe^{Met}	Met-tRNAi^{Met}	Met-tRNA^{Lys}
WT	K_M (μM)	3.03 ± 0.063	5.515 ± 0.0768	N.D
	k_{cat} (s^{-1})	0.157 ± 0.002	0.0176 ± 0.00009	N.D
	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	0.051 ± 0.042	0.00319 ± 0.00004	N.D
SD	K_M (μM)	41.83 ± 14.19	7.616 ± 0.195	25.56 ± 4.94
	k_{cat} (s^{-1})	0.127 ± 0.052	0.0155 ± 0.000111	0.089 ± 0.0045
	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	0.0031 ± 0.0002	0.002 ± 0.00003	0.0035 ± 0.0004
SA	K_M (μM)	7.96 ± 2.84	8.3003 ± 0.4264	N.D
	k_{cat} (s^{-1})	0.011 ± 0.0005	0.0143 ± 0.0002	N.D
	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	0.0015 ± 0.0005	0.00172 ± 0.00006	N.D

Catalytic activities of WT MRS and the SD and SA mutants were measured using tRNAe^{Met}(CAU), tRNAi^{Met}(CAU) and tRNA^{Lys}(CUU) to obtain kinetic parameters (K_M, k_{cat} and k_{cat}/K_M values). tRNAs were used in the concentration range of 0.5–80 μM. Data are represented as mean ± s.d. (n = 3). N.D., not detected.

Table 2. Met-misincorporated residues in Flag-VN-AIMP3.

Target	Sequence	Site
FLAG-Venus N-term	GEELFTGVVPILVELDGDVNGHK	D20, D22
	GEELFTGVVPILVELDGDVNGHK	V23
	FSVSGEGEGDATYGK	D37
	LPVPWPTLVTTLGYGLQCFAR	V56
	FEGDTLVNRIELK	G117
	LEYNYNSHNVYITADKQK	V151
	EDGNILGHKLEYNYNSHNVYITADKQK	K157
AIMP3	ANFKIRHNIEMAAAAELSLLEK	A2, A4, A5
	HNIEMAAAAELSLLEKSLGLSK	L7, L9
	SLGLSKGNKYSAQGER	K21
	QANKEYLLGSTAEEKAIVQQWLEYR	K57

Peptide sequences detected by mass spectrometry analysis. Red color indicates the residues identified only from the HEK293T cells expressing the MRS SD mutant. Orange color indicates the residues detected from HEK293T cells expressing WT MRS under normal condition as well as cells expressing the MRS SD mutant.

FIGURE LEGENDS

Fig. 1. Determination of ERK-mediated phosphorylation sites in MRS under ROS stress. (A) Lysates from untreated and sodium arsenite-treated HeLa cells were subjected to 2D-PAGE. The gel was immunoblotted with anti-MRS antibody. To check ROS-dependent phosphorylation of MRS, lysates from sodium arsenite-treated cells were incubated with alkaline phosphatase (AP). (B) Lysates prepared as above were immunoprecipitated with anti-MRS antibody and immunoblotted with phosphoserine (p-Ser), phosphothreonine (p-Thr), and phosphotyrosine (p-Tyr) specific antibodies. (C) Lysates from HeLa cells treated with sodium arsenite with or without DPI (ROS inhibitor) were immunoprecipitated using anti-MRS antibody. The gel was immunoblotted with p-Ser antibody. (D) HeLa cells pre-treated with each MAPK inhibitor, SB203580 (p38 MAPK), SP600125 (JNK) and PD98059 (ERK), were incubated in sodium arsenite-containing media. The lysates were immunoprecipitated with anti-MRS antibody and immunoblotted with p-Ser antibody. (E) Purified GST (EV, empty vector) and GST-MRS was subjected to an *in vitro* kinase reaction by incubating with ERK and [γ-^{32}P]ATP. After staining with coomassie brilliant blue, radioactivity was detected by autoradiography. (F) Schematic representation of functional domains in human MRS. The domains of MRS can be divided into MD1 (GST-like, residues 1–266), MD2 (Catalytic, residues 267-597) and MD3 (tRNA-binding, residues 598–900) fragments. (G) Individual GST-fused domains of MRS were subjected to *in vitro* kinase assay with ERK in the presence of [γ-^{32}P]ATP. Phosphorylation signal was detected by autoradiography. (H) HEK293T cells transfected with GFP-ERK were immunoprecipitated with anti-GFP antibody. The immunoprecipitated GFP-ERK was mixed with each biotinylated synthetic peptide (WT, wild type; SA, phosphorylation-inactive form) and [γ-^{32}P]ATP. A consensus sequence phosphorylated by ERK was used as a control (P, positive control; N, phosphorylation-inactive form of positive control) (left). Positive control peptide and peptides containing each S209 and S825 were incubated with [γ-^{32}P]ATP and immunoprecipitated ERK from U0126 pre-treated cells. Autoradioactivity from the peptides was detected after dot blotting (right). (I) To confirm the ERK-dependent phosphorylation sites in MRS, *in vitro* kinase assay was performed with WT GST-MRS and the GST-MRS S209A/S825A (SA) mutant as described above. The phosphorylation signal was detected by autoradiography. The

relative quantification obtained by densitometer analysis for the phosphorylation signal of WT MRS and SA mutant was 1 and 0.11, respectively. (J) HEK293T cells transfected with Myc-MRS WT or Myc-MRS SA mutant were treated with sodium arsenite and immunoprecipitated using anti-Myc antibody. Arsenite-dependent phosphorylation of MRS was detected using the p-Ser antibody.

Fig. 2. Dually phosphorylated MRS undergoes a conformational change and binds to tRNA^{Lys}(CUU). (A) Maltose-binding protein (MBP) tagged WT MRS and the MBP-MRS SA and SD mutants were purified and the CD spectra of these proteins were obtained in the far-UV at two different temperatures (left). The purified proteins were run using SDS-PAGE and stained with coomassie brilliant blue (right). (B) Binding affinities of WT MRS and the S209D/S825D (SD) mutant to tRNA^{Lys}(CUU) (top) and tRNAe^{Met}(CAU) (bottom) were determined by the electrophoretic mobility shift assay. Each tRNA probe was incubated with WT His-MRS and His-MRS SD mutant proteins and separated by non-denaturing PAGE.

Fig. 3. Dually phosphorylated MRS induces mismethionylation under ROS stress. (A) HEK293T cells were transfected with WT TagRFP or the TagRFP M67K mutant and the fluorescence of each protein was compared. Insets show the same field as in the phase-contrast image. Scale bar = 80 μm (left). The expression level of WT TagRFP and the TagRFP M67K mutant were determined by immunoblotting (right). (B-E) MRS-dependent Met-misincorporation was monitored using the TagRFP M67K mutant whose fluorescence disappeared due to the M to K substitution. HEK293T cells co-transfected with Tag-RFP M67K and EV or each type of Myc-MRS (WT, SA or SD mutant) were treated with sodium arsenite. Revival of fluorescence due to Met-misincorporation at the M67K residue position was observed by fluorescence microscopy (×200). Insets show the same field as in the phase-contrast images. Scale bar = 80 μm (B). Expression levels of total MRS, Myc-MRS (WT, SA or SD mutant) and TagRFP M67K mutant were analyzed via immunoblotting (C). The relative number (D) and the relative fluorescence intensity (E) of red fluorescent cells are presented as a bar graph. Data are represented as mean ± s.d. (n = 3). ***, P<0.001; **, P<0.01. a, P value indicates a significant difference between the arsenite-untreated and -treated groups; b, P value

indicates a significant difference between arsenite-untreated EV and SD groups. (F) HEK293T cells transfected with Flag-VN-AIMP3 were incubated with arsenite, [^{35}S]Met and with or without ERK inhibitor, U0126. The radioactive signal from the immunoprecipitated Flag-VN-AIMP3 was detected by autoradiography. (G) HEK 293T cells co-transfected with Flag-VN-AIMP3 and EV or each type of Myc-MRS (WT, SA or SD mutant) were incubated with [^{35}S]Met in the presence with arsenite. To see the effect of ERK inhibitor, cells were pre-treated with U0126 1 h before the arsenite treatment. The cell extracts were immunoprecipitated with anti-Flag antibody. [^{35}S]Met signals from the Flag-VN-AIMP3 were monitored by autoradiography.

Fig. 4. Met-misincorporated residues in Flag-VN-AIMP3 and their location in the AIMP3 and Venus structures. (A,B) The Met-exchanged residues identified by mass spectrometry analysis were depicted in the AIMP3 (pdb2uz8) (A) and the full Venus (pdb3t6h) (B) structures, respectively. Residues only identified from HEK293T cells expressing WT MRS (A,B top) and MRS SD (A,B middle) are depicted in cyan and red, respectively. Common residues identified from both cells are represented in orange. The original Met residue in AIMP3 is depicted in green. Mismethionylated residues on AIMP3 in SD-overexpressing conditions are also represented on the MRS-AIMP3 complex structure (pdb4bl7) (A, bottom). The GST-domain of MRS is shown in light green. Each VN and VC (deleted C-terminus of Venus) in the full Venus protein structure is shown in green and red, respectively, for convenience (B, bottom).

Fig. 5. Dually phosphorylated MRS reduces intracellular ROS levels and promotes cell survival under ROS stress. (A) HEK293T cells were transfected with EV or Myc-tagged WT MRS, or the SA or SD mutants, and incubated with arsenite. ROS levels were detected by the DCFH-DA assay. Insets show the same field as in the phase-contrast images. Scale bar = 200 μm. (B) Bax and Bcl-2 levels were detected with their specific antibodies under the same conditions as shown in (A). Exogenous and endogenous MRS were separated and detected using MRS antibody to show the expression level. (C) MRS level in HEK293T cells was reduced by si-MRS treatment for 72 h. The effect of MRS expression on cell viability under ROS stress was determined by the MTT assay. (D and E) The effect of MRS proteins on cell viability

under ROS stress was determined by the MTT assay. MRS proteins were transiently expressed in HEK293T cells (D) and stably expressed in HeLa cells (E). The values of relative cell viability in (C) (D) and (E) are represented as mean ± s.d. (n = 3). *, P<0.05; ***, P<0.001. a, P value indicates a significant difference between the arsenite-untreated and -treated groups; b, P value indicates a significant difference between arsenite-treated si-control and si-MRS groups. (F) The growth curves of MRS-expressing stable HeLa cells were monitored in the presence and absence of ROS stress. The values of relative cell growth are represented as mean ± s.d. (n = 3). (G) ROS-dependent apoptosis was determined in MRS-expressing stable HeLa cells using the TUNEL assay. Cells incubated with or without arsenite for 72 h were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI; blue) and fluorescein-labeled dUTP. Green fluorescence indicates TUNEL-positive cells. Scale bar = 200 μm (top). The number of TUNEL-positive cells was normalized to that of DAPI-positive cells, and the quantitative analysis is represented as mean ± s.d. (n = 3) (bottom). **, P<0.01; ***, P<0.001. (H) HeLa cells expressing WT MRS were supposed to TUNEL assay as described in (G) with or without U0126 pre-treatment, and the images (top) and the quantitative analysis (bottom) for positive cells are present. *, P<0.05; **, P<0.01.

Fig. 6. Schematic model for the protective role of MRS under ROS stress. Upon ROS stress, ERK is activated and it phosphorylates MRS at the Ser209 and Ser825 residues. Phosphorylated MRS enhances the mischarging of Met on non-methionyl tRNAs, such as $tRNA^{Lys}$. Met carried by non-cognate tRNAs is incorporated into growing polypeptides during translation and used as a ROS scavenger, protecting cells from oxidative damage and apoptosis.