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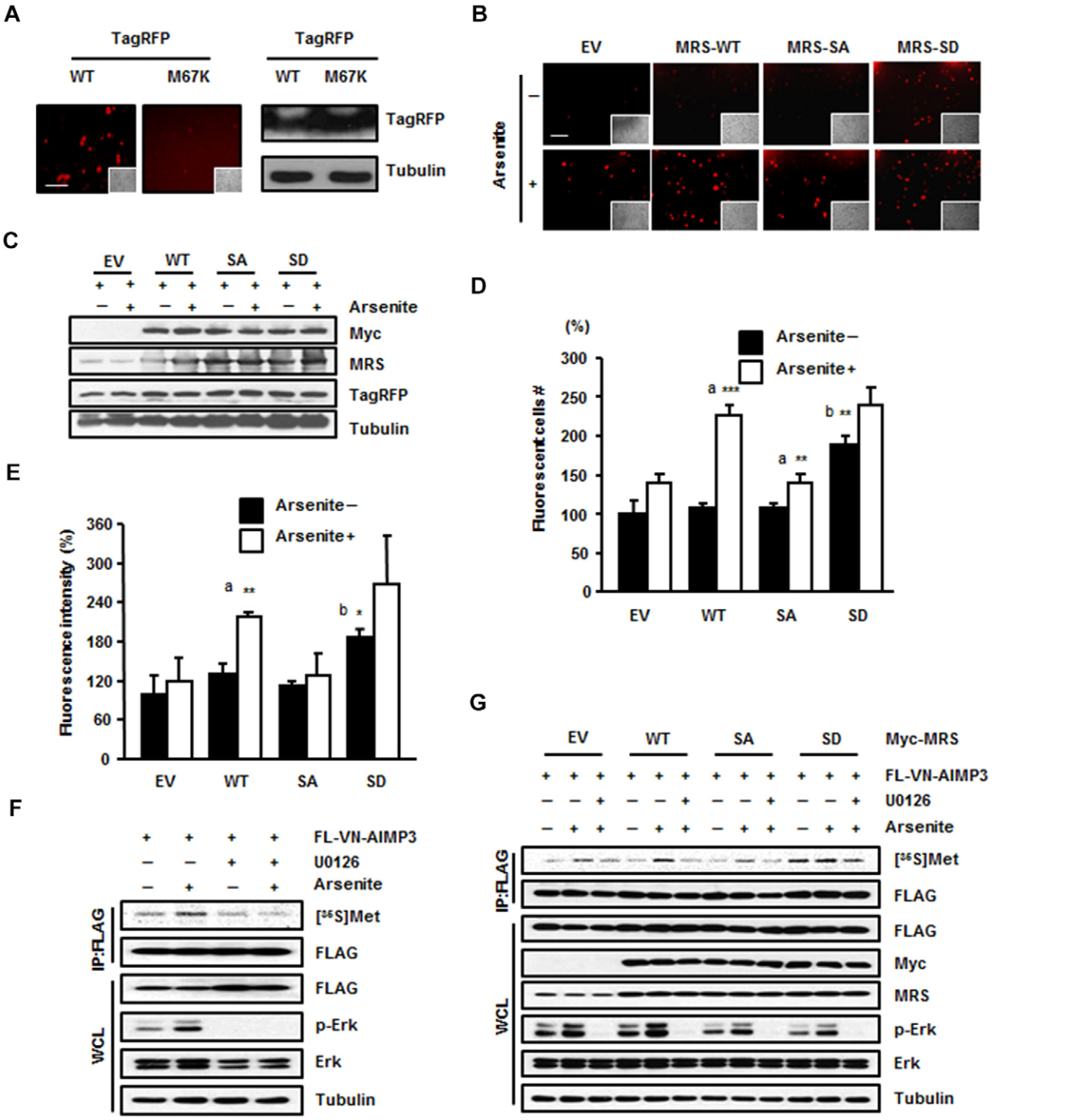


Fig. 3. Dually phosphorylated MRS induces mismethionylation under ROS stress. (A) HEK293T cells were transfected with wild-type (WT) TagRFP or the TagRFP M67K mutant and the fluorescence of each protein was compared (left). Insets show the same field as in the phase-contrast image. Scale bar: 80 mm. The expression level of wild-type TagRFP and the TagRFP M67K mutant were determined by immunoblotting (right). (B E) MRS-dependent Met-misincorporation was monitored using the TagRFP M67K mutant, the fluorescence of which disappeared due to the M to K substitution. HEK293T cells co-transfected with Tag-RFP M67K and empty vector (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 (6200). Insets show the same field as in the phase-contrast images. Scale bar: 80 mm (B). Expression levels of total MRS, Myc MRS (wild type, SA or SD mutant) and TagRFP M67K mutant were analyzed by immunoblotting (C). The relative number (D) and the relative fluorescence intensity (E) of red fluorescent cells are presented as bar graphs. Data are represented as the mean±s.d. (n=3); *P<0.05; **P<0.01; ***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-untreated empty vector and SD groups. (F) HEK293T cells transfected with FLAG-VN AIMP3 were incubated with arsenite, [35S]Met and with or without the ERK inhibitor U0126. The radioactive signal from the immunoprecipitated (IP) FLAG-VN AIMP3 was detected by autoradiography. WCL, whole-cell lysate. (G) HEK 293T cells co-transfected with FLAG-VN AIMP3 and empty vector or each type of Myc MRS (wild type, SA or SD mutant) were incubated with [35S]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. [35S]Met signals from the FLAG-VN AIMP3 were monitored by autoradiography.

Fig. 5. Dually phosphorylated MRS reduces intracellular ROS levels and promotes cell survival under ROS stress. (A) HEK293T cells were transfected with empty vector (EV) or Myc-tagged wild-type (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 mm. (B) Bax and Bcl-2 levels were detected with their specific antibodies under the same conditions as shown in A. Exogenous (Exo) and endogenous (Endo) MRS were separated and detected using the anti-MRS antibody to show the expression level. (C) MRS level in HEK293T cells was reduced by treatment with MRS-specific siRNA (si-MRS) for 72 h. The effect of MRS expression on cell viability under ROS stress was determined by the MTT assay. (D,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). In C E: 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 stably transfected HeLa cells were monitored in the presence and absence of ROS stress. (G) ROS-dependent apoptosis was determined in MRS-expressing stably transfected 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 mm (upper panel). The number of TUNEL-positive cells was normalized to that of DAPI-positive cells, and the quantitative analysis is shown (lower panel). (H) HeLa cells expressing wild-type MRS were subjected to TUNEL assay as described for G with or without U0126 pre-treatment, and the images (upper panel) and the quantitative analysis (lower panel) for positive cells are shown. Scale bar: 200 mm. All quantitative data show the mean±s.d. (n=3);*P<0.05; **P<0.01; ***P<0.001.

little difference between the overexpression of wild-type MRS and the MRS-SD mutant in their effect on global translation (data not shown).

Although MRS-SD revealed evident $tRNA^{Lys}$-charging activity, MRS might have to compete with lysyl-tRNA synthetase (KRS) for capturing $tRNA^{Lys}$ during ROS stress unless there is spare $tRNA^{Lys}$. We analyzed free $tRNA^{Lys}$ as well as charged $tRNA^{Lys}$ using acidic urea PAGE gels and observed that some portion of free $tRNA^{Lys}$ seems to be available even when KRS is fully active (data not shown). It also implies that other spare non-cognate tRNAs are available for misacylation. Met-misincorporation does not require high amounts of other tRNAs. It is known that the total increase in Met-misincorporation is about 10% under conditions of ROS stress (Netzer et al., 2009). Other possible mechanisms of increasing the availability of tRNAs during oxidative stress, such as changes in the accessibility of 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 benefits to cells, such as the localization of Met residues to the protein surface and an increased number of Met residues. According to Levine et al., not all of the Met residues in the original positions are used as ROS scavengers (Levine et al., 1996). Approximately 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 idea (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 that were basally exchanged with Met did not perfectly match those detected following 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 probably 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 for a broad range of tRNAs, not all tRNAs were charged by MRS under ROS stress (Netzer et al., 2009). Generally, tRNAs 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 a strategy involving MRS-mediated mistranslation to survive under ROS stress by tolerating a reduced fidelity of translation is unique. The sacrifice of translational fidelity does not seem to cause severe

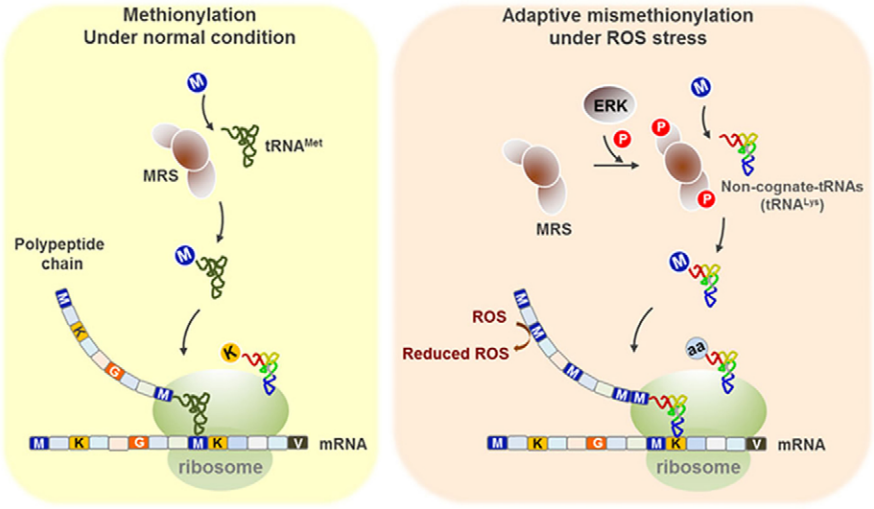


Fig. 6. Schematic model for the protective role of MRS under ROS stress. Upon ROS stress, ERK is activated and phosphorylates MRS at the Ser209 and Ser825 residues. Phosphorylated MRS enhances the mischarging of Met on non-methionyl tRNAs, such as $tRNA^{Lys}$. Met carried by non-cognate tRNAs is incorporated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

side effects in 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, might 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 might 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 function of dually phosphorylated MRS during ROS stimulation is advantageous, at least for a short period, because it can induce Met-misacylation to remove ROS and to protect against 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 under 5% CO_2. To establish stably transfected HeLa cell lines, cells were transfected with the pcDNA3-Myc empty vector, wild-type pcDNA3-Myc-MRS, pcDNA3-Myc-MRS-SA or pcDNA3-Myc-MRS-SD plasmid using FuGENE HD (Roche). Stable cells were selected and maintained under antibiotic pressure (800 mg/ml of geneticin; Duchefa Biochemie).

ROS induction and inhibitor treatment

Cells were cultured until they reached 80% confluence. Cells were treated with 4 mM sodium arsenite (Sigma) or 200 mM 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 mM. All inhibitors were purchased from Calbiochem (Billerica). For the DPI chase (Enzo), cells were pre-treated with 50 mM DPI 30 min before ROS induction.

DCFH-DA assay

HEK293T cells transfected with pcDNA3-Myc empty vector, wild-type pcDNA3-Myc-MRS, pcDNA3-Myc-MRS-SA, or pcDNA3-Myc-MRS-SD plasmids were exposed to sodium arsenite (4 mM) for 24 h. After treatment with 20 mM 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 in lysis buffer (50 mM Tris-HCl pH 7.4, 0.5% Triton X-100, 5 mM EDTA, 10% glycerol and 150 mM NaCl) containing phosphatase inhibitor and protease inhibitor (Calbiochem) for 30 min at 4 C. After centrifugation, supernatants were collected and the protein amounts were quantified by using the Bradford assay (BioRad). Proteins extracts from the cells were separated by SDS-PAGE, transferred to PVDF membrane and incubated with specific primary antibodies. Antibodies against phosphorylated (p)-Ser (Abcam), p-Thr (Cell Signaling Technology), p-Tyr (Cell Signaling Technology), 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 a concentration of 0.2 0.4 mg/ml (Abcam, Sigma and Santa Cruz) or with a 1:1000 dilution (Cell Signaling Technology).

Immunoprecipitation

Protein extracts were incubated with primary antibody (2 mg) for 4 h at 4 C with agitation, and then incubated for a further 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-mg protein extract was rehydrated in resolubilization buffer [7 M urea, 2 M thiourea, 2% ASB-14, 0.5% Triton X-100, 1% (v/v) ampholyte, 1% (v/v) tributylphosphine and 0.1% Bromophenol Blue]. Samples were loaded onto the immobilized pH gradient (IPG) strip gels (linear pH gradient 4 7, 7 cm; 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 Rosetta 2. Protein expression was induced by treatment with 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG), followed by culturing cells at 18 C overnight. Harvested cells were lysed by sonication, and lysates were incubated with glutathione Sepharose 4B (GE Healthcare) in 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 mM ATP for 10 min at room temperature, and the kinase reactions were performed at 30 C for 30 min by adding ERK (Cell Science), [c-^{32}P]ATP (Izotop, SBP301, 1000 mCi) 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 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 the 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 allowed to react with the GFP ERK at 30 C for 30 min. Each sample was filtered through a streptavidin-coated matrix biotin-capture membrane (Promega) using a 96-well Minifold filtration apparatus. The membrane was washed as described previously (Schaefer and Guimond, 1998) and exposed for autoradiography.

Circular dichroism spectrum analysis

Wild-type MBP MRS 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 circular dichroism spectrum was measured using a Jasco J-815 circular dichroism spectrometer at 25 C and 70 C in the far-UV range from 190 to 250 nm. Samples were loaded into a 1-mm path-length absorption microcell. The results are shown as an average of three repeated scans after subtraction of buffer background.

Aminoacylation assay

His-tagged MRS (wild type, SA and SD) expressed in E. coli RIL strain was purified using ProBond Resin (Invitrogen) and washed with lysis buffer (pH 7.8, 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 by in vitro transcription. The MRS aminoacylation reaction 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 mM Met, 500 mg/ml of each tRNA, 400 nM purified MRS and 25 mCi [^{35}S]Met (Izotop, 1000 Ci/mmol). For kinetics analysis, each tRNA (tRNAe^{Met}, tRNAi^{Met} and tRNA^{Lys}) was used at a concentration of 0.5 mM to 80 mM. Aminoacylation reaction samples were spotted on 3-mm filter paper pre-wetted with 5% trichloroacetic acid (TCA) containing 1 mM Met.

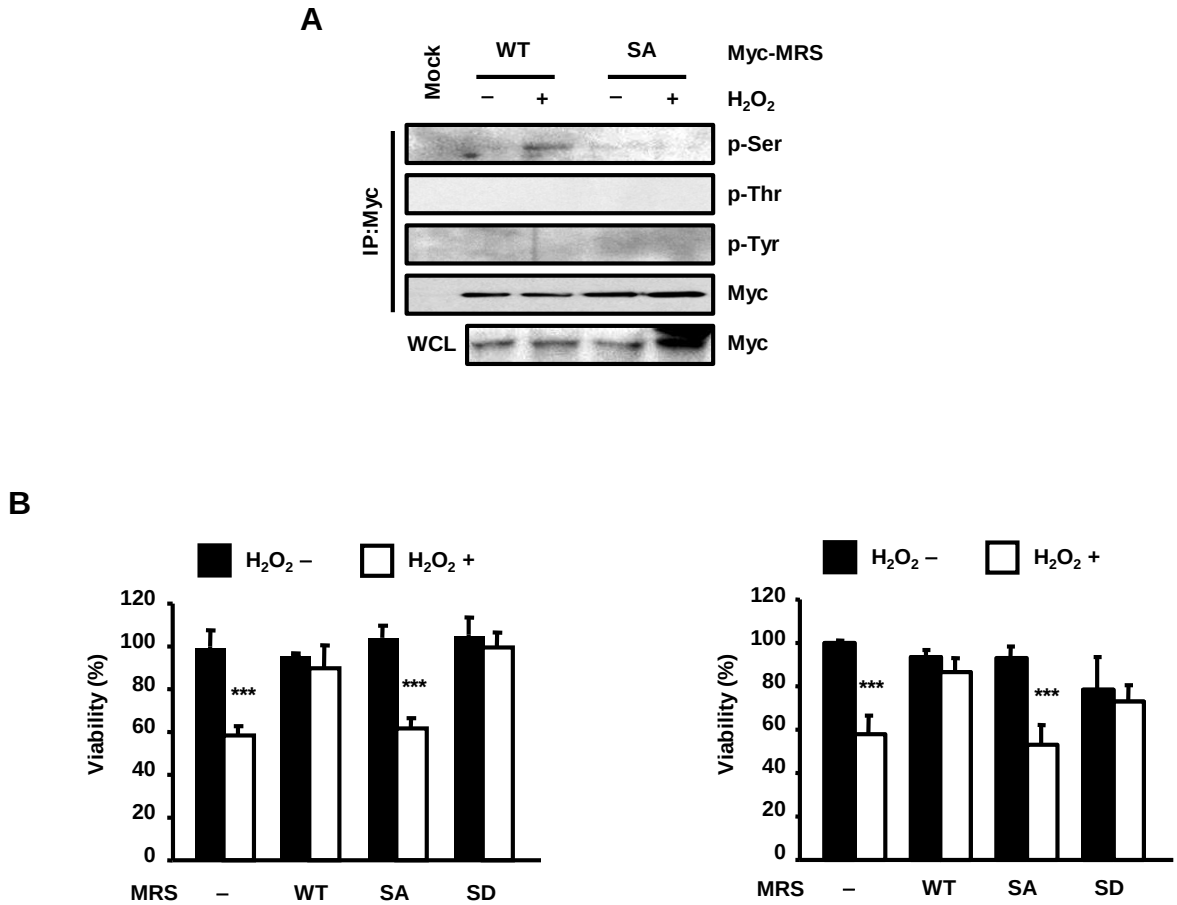
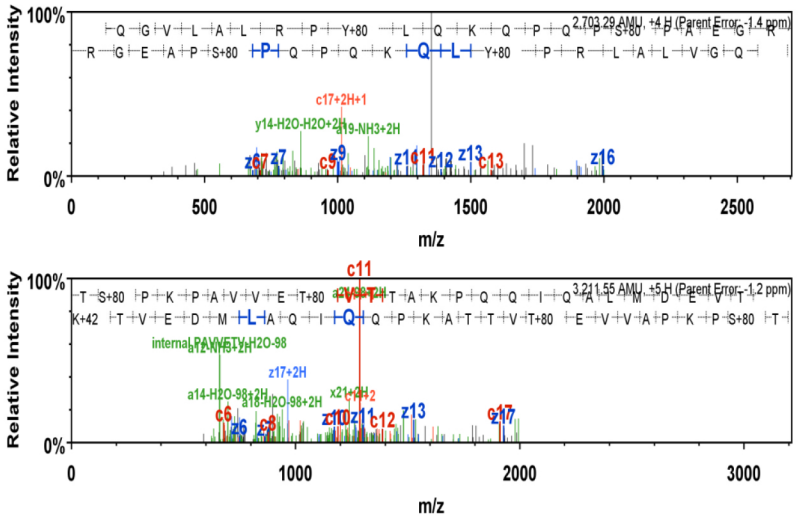


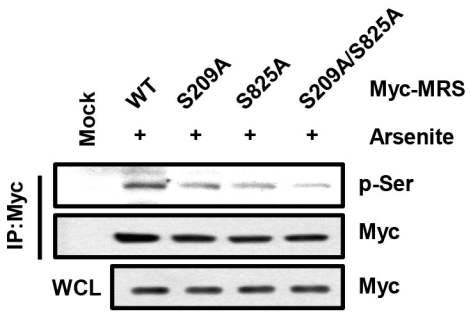
Fig. S1. MRS was phosphorylated and induced cell protection upon H_2O_2 stress. (A) HEK293T cells transfected with WT Myc-MRS or the Myc-MRS SA mutant were incubated with 200 μM H_2O_2 and the lysates were immunoprecipitated with anti-Myc antibody. H_2O_2-dependent phosphorylation of MRS was detected by p-Ser, p-Thr, and p-Tyr antibody. (B) The effect of WT MRS, the MRS SA or SD mutant on the cell viability under H_2O_2-induced ROS stress was determined by MTT assay. HEK293T cells transiently expressing each MRS protein (left) or HeLa cells stably expressing each MRS (right) were used for the viability analysis. The values of relative cell viability are represented as mean ± s.d. (n = 3). ***, P<0.001.

A



Domain	Sequence	Site
MD1	QGVLALRPyLQKQPQPsPAEGR	Y201, S209
MD3	TsPKPAVVEtVTTAKPQQIQALMDEVTK	S825, T833

B



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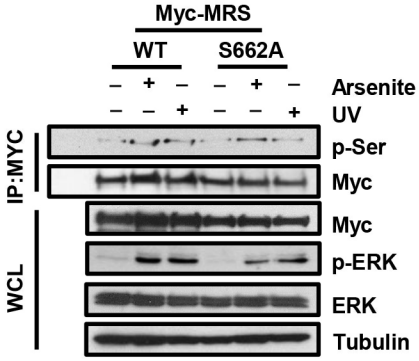
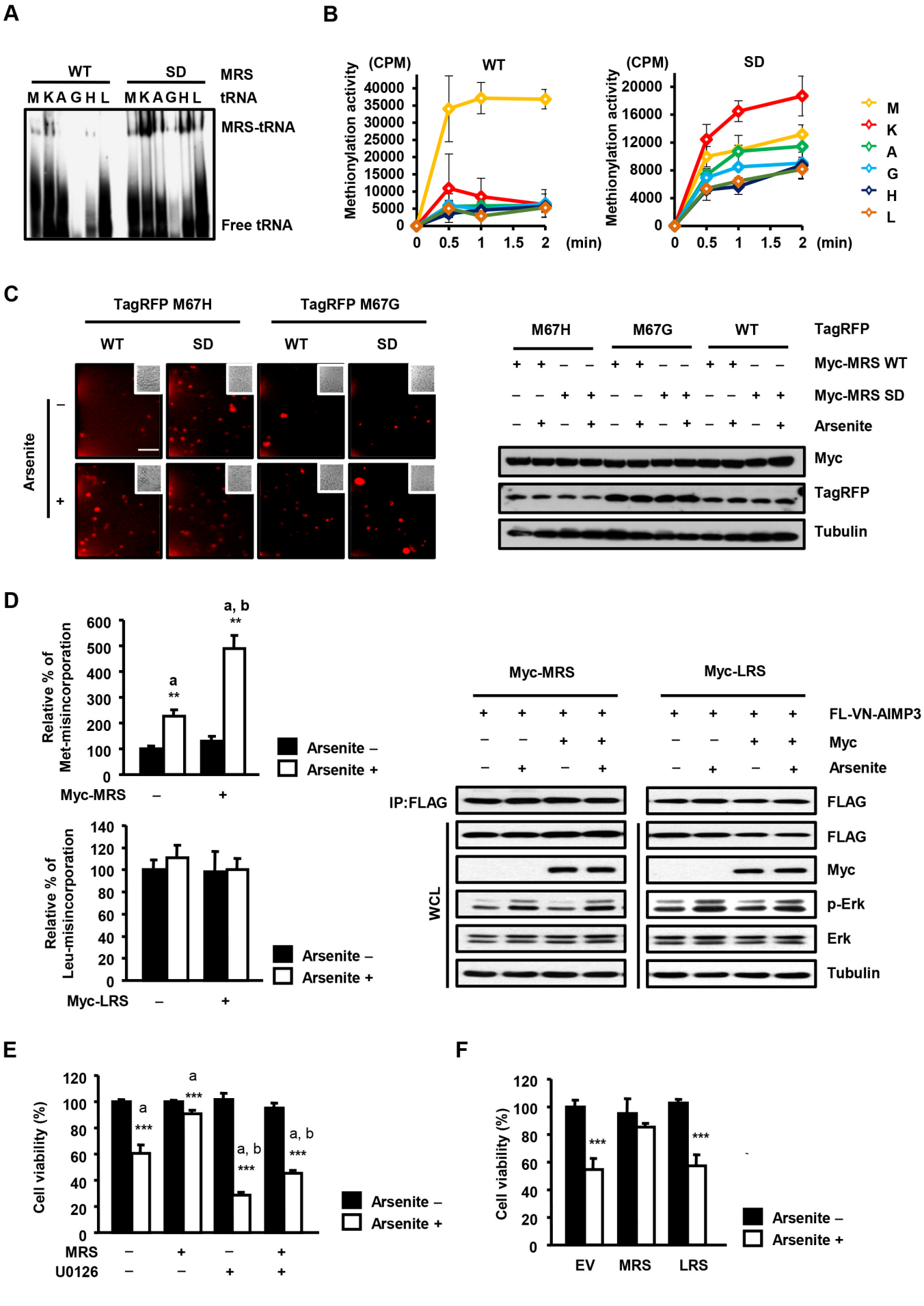


Fig. S2. Ser209 and Ser825 residues are the ERK-dependent phosphorylation sites in MRS under ROS stress. (A) GST-fused domains of MRS (MD1 and MD3), which were subjected to *in vitro* kinase reaction with purified active ERK, were processed for nano LC-MS/MS analysis as described in Materials and Methods. Peaks of peptides containing Tyr201 and Ser209 residues in MD1 (top) and Ser825 and Thr833 residues in MD3 (middle) are shown. The peptide sequences containing ERK-mediated phosphorylation were presented (bottom). (B) HEK293T cells transfected with WT Myc-MRS, Myc-MRS S209A, Myc-MRS S825A or Myc-MRS S209A/S825A mutant were treated with sodium arsenite. Cell lysates were immunoprecipitated with anti-Myc antibody and then immunoblotted with p-Ser antibody. (C) HEK293T cells were transfected with Myc-MRS WT and Myc-MRS S662A (GCN2 kinase phosphorylates S662 residue upon UV irradiation). Transfected cells were supposed to arsenite or UV (50 J/m^2) stress for 4 h or 30 min, respectively. Myc-MRSs were immunoprecipitated and then phosphorylation state was detected using p-Ser antibody.


A
WT
SD
MRS
M K A G H L
M K A G H L
tRNA
MRS-tRNA
Free tRNA
B
(CPM)
WT
Methionylation activity
40000
35000
30000
25000
20000
15000
10000
5000
0
0 0.5 1 1.5 2 (min)
(CPM)
SD
Methionylation activity
20000
16000
12000
8000
4000
0
0 0.5 1 1.5 2 (min)
M
K
A
G
H
L
C
TagRFP M67H
TagRFP M67G
WT
SD
WT
SD
Arsenite
–
+
M67H
M67G
WT
TagRFP
Myc-MRS WT
Myc-MRS SD
Arsenite
Myc
TagRFP
Tubulin
D
Relative % of Met-misincorporation
600
500
400
300
200
100
0
a
**
a, b
**
Arsenite –
Arsenite +
Myc-MRS – +
Relative % of Leu-misincorporation
140
120
100
80
60
40
20
0
Arsenite –
Arsenite +
Myc-LRS – +
Myc-MRS
Myc-LRS
FL-VN-AIMP3
Myc
Arsenite
IP:FLAG
FLAG
WCL
FLAG
Myc
p-Erk
Erk
Tubulin
E
Cell viability (%)
120
100
80
60
40
20
0
a
***
a
***
a, b
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a, b
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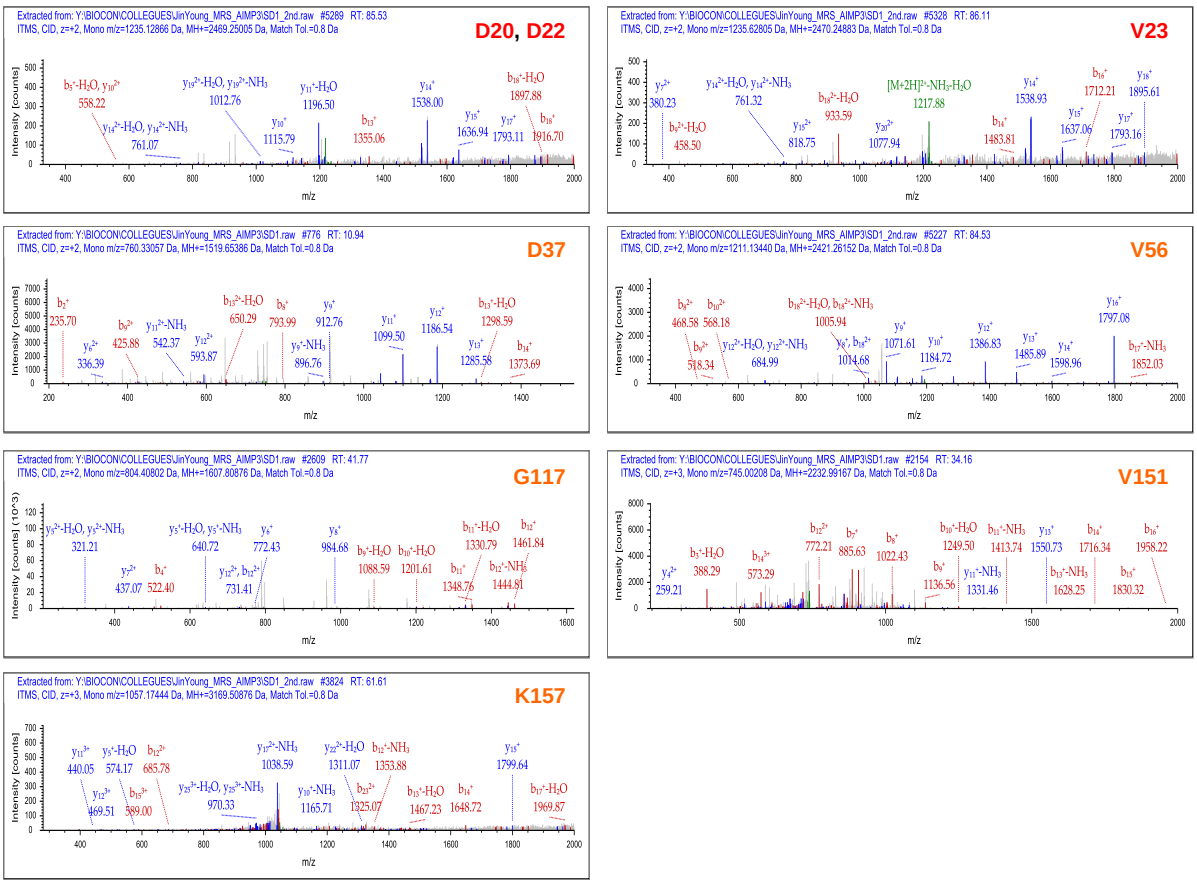
Arsenite –
Arsenite +
MRS – + – +
U0126 – – + +
F
Cell viability (%)
120
100
80
60
40
20
0
***
***
Arsenite –
Arsenite +
EV MRS LRS

Fig. S3. The MRS SD mutant charges Met to non-cognate tRNAs and Met-misincorporation is specific to MRS and ERK. (A) Binding affinity of WT MRS and the MRS SD mutant to several radioactively labeled tRNAs such as $tRNA_e^{Met}$(CAU), $tRNA^{Lys}$(CUU), $tRNA^{Ala}$(AGC), $tRNA^{Gly}$(GCC), $tRNA^{His}$(GUG) and $tRNA^{Leu}$(CAG) was determined by EMSA. Each tRNA probe was represented as M, K, A, G, H and L, respectively. (B) Methionylation activities of WT MRS (left) and the MRS SD mutant (right) to the several tRNAs as described above were compared by aminoacylation assay. The CPM (count per minute) values of methionylation to these tRNAs are represented as mean ± s.d. (n = 3). (C) HEK293T cells co-transfected with TagRFP M67H mutant (Met to His substitution) and Myc-MRS (WT or SD mutant) were treated with sodium arsenite. The red fluorescence increased by Met-misincorporation to the H67 residue was observed by fluorescence microscopy (×200). Met-misincorporation to G67 was also monitored in the TagRFP M67G mutant (Met to Gly substitution)-transfected cells. Insets show the same field as in the phase-contrast image. Scale bar = 80 μm (left). The expression level of Myc-tagged MRS proteins and TagRFP mutants were analyzed by immunoblotting (right). (D) HEK293T cells co-transfected with Flag-VN-AIMP3 and MRS were incubated with [^{35}S]Met in the presence with arsenite (left, top). For the cells transfected with Flag-VN-AIMP3 and leucyl–tRNA synthetase (LRS) together, [^{3}H]Leu was added instead of [^{35}S]Met (left, bottom). The cell extracts were immunoprecipitated with anti-Flag antibody. [^{35}S]Met and [^{3}H]Leu signals from the Flag-VN-AIMP3 were detected using liquid scintillation counter. The amounts of immunoprecipitated Flag-VN-AIMP3 and transfected Myc-MRS or Myc-LRS were analyzed using immunoblotting (right). **, 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-treated EV and MRS groups. (E) HEK293T cells transfected with EV or Myc-MRS were treated with arsenite. To inhibit ERK activation, cells were pretreated with U0126 1 h before being exposed to arsenite. After 72 h incubation, cell viability was analyzed using MTT assay. ***, 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 U0126 – and U0126 + groups. (F) HEK293T cells were transfected with EV, Myc-MRS and Myc-LRS, and the overexpression effect of these ARSs on cell viability under ROS stress was analyzed after 72 h incubation. ***, P<0.001.

FLAG-Venus N-term



AIMP3

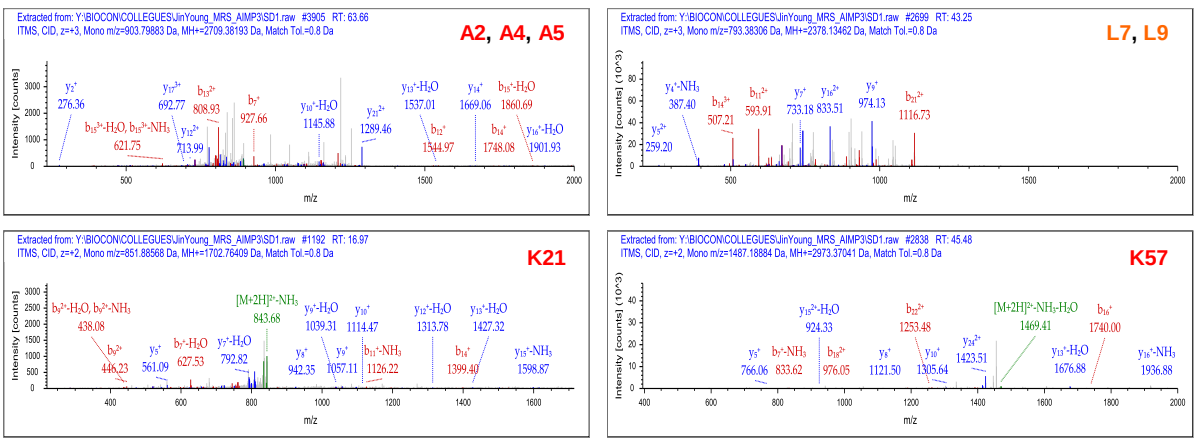


Fig. S4. Met-misincorporated residues in Flag-VN-AIMP3. Peptides from Flag-VN-AIMP3, which contain Met-exchanged residues were identified via mass spectrometry analysis. Peaks of peptides containing Met in the non-Met residues are shown.