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Posted Date: 14 January 2025

doi: 10.20944/preprints202501.1062.v1

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GEF1 Is a Novel Factor Regulating Nickel Ion Metabolism in *Saccharomyces cerevisiae*

Qianqian Li [1], Lixuan Zong [1,*], Joseph Brake [2], Rongqiu Huang [1], Chaoyang Luo [1], Jiang Bian [1,3,*] and Xiaobin Wu [1,*]

[1] Development Center of Plant Germplasm Resources, College of Life Sciences, Shanghai Normal University, Shanghai, China 200234

[2] Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-0600

[3] Department of Obstetrics and Gynecology, Shanghai Everjoy Medical Polyclinic, 675 Minbei Road, Shanghai, China, 201107

* Correspondence: 13287323629@163.com; bianjiang07110924@163.com; xwu6@shnu.edu.cn; Tel.: +86-21-64322762

Abstract: Nickel is a trace element essential for human life. Currently, studies on the metabolism and function of nickel ions mainly focus on prokaryotes, while the role of nickel ions in eukaryotes is still being explored. In this study, we found for the first time that *GEF1* which encodes Gef1p, a chloride transport protein located in the Golgi membrane, is involved in nickel ion metabolism in yeast. The *GEF1* knockout strain (*gef1Δ*) showed strong resistance to excess nickel ions, and the content of nickel ions in *gef1Δ* cells was significantly elevated. The results of transcriptomics analysis showed significant upregulation of *MMT2* and *CUP1* in *gef1Δ* cells supplemented with nickel. Both *MMT2* and *CUP1* overexpressed in the *gef1Δ* strain showed a growth advantage on nickel media. Nickel ion content in the mitochondria of cells overexpressing *MMT2* was significantly elevated, and the levels of reactive oxygen species were significantly decreased in strains overexpressing either the *MMT2* or *CUP1* genes. This study reveals that the *GEF1* gene plays an important role in nickel homeostasis, and that upregulation of *MMT2* and *CUP1* is critical for *gef1Δ* strains to counteract ROS formation and growth defect by nickel ions.

Keywords: nickel; *GEF1*; *MMT2*; *CUP1*; yeast

1. Introduction

Nickel is a necessary metal for plants, animals and microorganisms to participate in various physiological activities, and is also a necessary micronutrient for the human body[1]. Adults consume approximately 79 to 105 μg/day of nickel from diet and supplements[2]. After being ingested into human body, nickel resides in various organs such as the brain[3], liver and heart[4], where it is believed to play a role in the normal physiological activities of the human body. Nickel is redox active metal which is normally found in the +2 oxidation state. Excess nickel has detrimental effects through its action as a ROS generator. For example, nickel inhibits differentiation of neuronal cells[5], causes premature ovarian failure by inhibiting cell proliferation[6], and causes oxidative damage to the testis which inhibits sperm formation[7]. Nickel was shown to inhibit the proliferation of cultured immune cells[8], and reversal of this inhibition is of great interest for the prevention and treatment of nickel-induced cancer. On the other hand, many microbial enzymes depend on the presence of nickel for their functions, such as urease, methyl coenzyme M reductase, CO-dehydrogenase, nickel superoxide dismutase, glyoxalase, acireductase dioxygenase, lactate racemase, prolyl cis-trans isomerase and [NiFe] hydrogenase[9,10]. Therefore, organisms must maintain nickel homeostasis to prevent toxicity but allow for its essential functions.

At present, two metabolic pathways of nickel have been found in microorganisms. One of the ways for microorganisms to absorb nickel is the nickel-specific ABC transporter found in *Escherichia coli*, which is composed of five proteins NikA, B, C, D and E and relies on ATP hydrolysis to provide

 doi:10.20944/preprints202501.1062.v1

energy. Other microorganisms have similar nickel transporters such as NixA, a high-affinity nickel transporter responsible Ni^{2+} uptake in Helicobacter pylori[11]. The second way for microorganisms to absorb nickel is by facilitated diffusion through nickel-specific permeases. There are few studies on nickel metabolism in higher eukaryotes such as humans or animals and no specific nickel-containing enzymes have been identified, despite nickel being recognized as an essential trace mineral. Therefore, it is particularly necessary to study the role of nickel in eukaryotes. Many genes are involved in the metabolism of nickel ions, and more related genes need to be discovered. Our preliminary data found that *GEF1* showed significant resistance to nickel ions.

The *GEF1* gene belongs to the Guanine nuclear exchange factor (*GEF*) family and encodes Gef1p, a multifunctional membrane protein located on the Golgi membrane of *Saccharomyces cerevisiae* cells. Previous studies in unicellular eukaryotes have shown that *GEF1* is located on the cilia or matrix and may activate G proteins and find nuclear targeting sequences[12,13]. Greene JR et al. found that the 13 transmembrane domains encoded by *GEF1* have high homology with chloride ion channel proteins of humans and mammals, suggest that *GEF1* may encode a protein of intracellular iron metabolism[14]. Flis K et al. also found that Gef1p has high amino acid homology with the CLC chloride ion channel family[15]. Gaxiola et al. found that the deletion of *GEF1* leads to increased iron demand and sensitivity to cations in cells[16]. López-Rodríguez A et al. found that the expression of *GEF1* in HEK293 cells keeps chloride ion channels open, and are essential to the protein's function[17]. Sasvari et al. found that Gef1p played a key role in the RNA replication of TBSV (Tomato bushy stunt virus), and verified in Saccharomyces cerevisiae that Gef1p knockout inhibited RNA replication of the virus by regulating intracellular copper[18]. At present, many functions of *GEF1* have not been explored, and the metabolic regulation of nickel ions by this gene has not been reported in the literature. Therefore, it is of great significance to investigate the regulatory mechanism between *GEF1* and nickel ions. This work found for the first time that *GEF1* is related to nickel ion metabolism, which provides a new idea for the study of nickel metabolism in eukaryotes.

To better understand the function and metabolism of nickel in eukaryotes, we used a yeast knockout library to screen nickel-related genes in *Saccharomyces cerevisiae* with excessive nickel supplementation. In this paper, we found that *GEF1* participated in the metabolic balance of nickel ions, a *GEF1* knockout (*gef1*Δ) strain showed resistance to nickel ions, and *gef1*Δ in the presence of nickel caused changes to the expression of several genes related to metal homeostasis. We identified *MMT2* and *CUP1* as the key factors for *gef1*Δ-mediated resistance to nickel ions. *MMT2* is a gene located in mitochondrial membrane whose function is related to export of iron ions out of the mitochondria[19], while *CUP1* is a metallothionein, which is a metal-binding gene[20]. We showed that these genes reduce the content of bioactive nickel ions in the cytoplasm resulting in lower ROS and recovered cell growth, which provides a new perspective for elucidating the metabolism of nickel ions in eukaryotes.

2. Materials and Methods

2.1. Yeast Strains, Culture Media, and Growth Assays

A haploid control yeast S. cerevisiae strain, BY4741, and other knock out mutants (gef1Δ, mtm2Δ)*[21]*were purchased from the Open Biosystems. Yeast strains were grown in synthetic complete media lacking uracil (SC-ura) for plasmid selection as indicated in the figure legends. 1.5% agar was supplemented into the liquid media for solid medium plates. Yeast strains were cultured at 30 °C.

For yeast cell growth assays*[22]*, wild-type (WT) or knock out cells transformed with either empty vector, MMT2 or CUP1 plasmids were grown overnight in SC-ura media, re-inoculated (OD_{600}= 0.2) into fresh media, and grown to the mid-log phase (OD_{600}= 0.8–1.0). After dilution to OD_{600}= 0.1 and 3× serial dilutions in sterilized water, ~5 µl of cells were spotted on SC plates supplemented with metal at specified concentrations. Cells were incubated at 30 °C for 2-3 days prior to photography. Each assay was repeated at least three times using three different colonies to confirm results.

2.2. Plasmid Manipulation

GEF1 gene cloned by PCR was inserted into Bam HI and XhoI sites in the p416-TEF vector for TEF2 gene promoter-mediated constitutive expression in yeast*[23]*. The GFP epitope-tag was inserted at the C-

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terminus of Gef1p at a NotI restriction enzyme site which was generated in the PCR primer before the stop codon. Common molecular biology techniques, including plasmid amplification, purification using Escherichia coli and expression in S. cerevisiae, followed previously established methods*[24]*. All plasmids were sequenced to confirm inserted gene sequences. Yeast transformations were performed via the lithium acetate method*[25]*.

2.3. Western Blot Nalysis

WT and *gef1Δ* cells expressing empty vector (p416-TEF) or *GEF1* tagged with GFP were cultured in SC-ura media beginning at OD_{600} 0.2 for 4 h. Total protein was prepared via glass bead disruption in phosphate-buffered saline (PBS) containing 1% Triton X-100 and protease inhibitor complex (Roche Applied Science). The BCA kit (Pierce) was used for protein concentration measurements following the manufacturer's instructions. Total protein was denatured in SDS buffer containing 100 mM DTT (dithiothreitol) for 15 min at 60 °C and subjected to SDS-PAGE. Green fluorescent protein (GFP) epitope-tagged Gef1p was detected by anti-GFP antibody (Rockland) and the secondary antibody was anti-rabbit IgG antibody (Santa Cruz). The stained gel was used as a loading control.

2.4. Metal Measurement

Yeast cells were cultured to mid-log phase in SC media. 5 $OD._{600}$ cells ($OD._{600}$ = 5 cell amount) were collected and washed four times in PBS buffer with 10 mM EDTA to remove cell surface Ni. The washed cell pellets were dissolved in 70% nitric acid at 70 °C for 3 h. After overnight at room temperature, these samples were diluted in 10% nitric acid. ICP-MS (inductively coupled plasma mass spectrometry, Agilent Model 7500cs, Santa Clara, CA) was used to measure metal ion levels. Metal ion contents were normalized to protein concentrations[26].

2.5. Quantitative PCR

WT and *gef1Δ* cells were cultured in the SC media overnight. Cells were then re-inoculated (OD.600 0.2) into fresh media for 4h culture containing $NiSO_4$ (0, 0.5, 1 mM). Total RNA was subjected to cDNA synthesis followed by quantitative PCR (qTOWER, 3.0 G) using primer sets that are specific for *MMT2* (F:5′-AGC AGCGGGTTCTATCTTGG; R:5′-ACCAACGCTACCAACGATGT), *CUP1* (F:5′-GCCAATGTGGTAGCTGCAAA; R:5′-TCAGACTTGTTACCGCAGGG) and ß-actin (F:5′-AAATGCAAACCGCTGCTCA-3′; R:5′-TACCGGCAGATTCCAA ACCC -3′).

2.6. Measurement of Intracellular ROS Level

Intracellular ROS levels were determined by nitroblue tetrazolium (NBT) assay with some modifications[27]. Briefly, WT and *gef1Δ* cells were cultured to mid-log phase with indicated $NiSO_4$ treatments. Cells were incubated for 90 minutes in PBS containing 0.2% NBT before being collected. ROS reduced NBT to a dark blue insoluble form of NBT named formazan. The mixtures were centrifuged at 5,000 rpm for 2 min at 4°C. The supernatant was removed and the formazan was dissolved in 300 µl 50% acetic acid by sonication (three pulses of 6 s). The samples were shortly spun down and the absorbance of the supernatant was determined at 560 nm by a spectrometer.

2.7. Fluorescence Microscopy

Mid-log phase cells (p416TEF-GEF1-GFP) were cultured with and without $NiSO_4$ in SC-ura media. Cells were collected by centrifugation and washed once with fresh media and then were washed once with phosphate-buffered saline by centrifugation. GFP-fused Gef1p signals were photographed on a confocal microscope (Olympus FV500). Differential interference contrast (DIC) images were also captured to present cell morphology. GFP signals and cell images were overlaid to determine GEF1-GFP subcellular distribution*[26]*.

2.8. Statistical Analysis

The image quantification was determined by ImageJ software (http://rsbweb.nih.gov/ij/). Descriptive analyses were presented as the means ±S.D. and statistical comparisons of control and experimental groups were performed using Student's t-test. $p<0.05$ was considered to be significant.

3. Results

 doi:10.20944/preprints202501.1062.v1

3.1. Gef1p Is a New Molecular Factor Involved in Nickel Ion Metabolism in Saccharomyces cerevisiae

In order to study the metabolic mechanism of nickel ion in *S. cerevisiae* and screen genes related to nickel ion metabolism, we prepared SC solid media containing 0 mM, 0.5 mM and 1 mM $NiSO_4$ respectively, then observed the growth of gene-deficient strains on the media over 3 days. *GEF1* gene knockout cells (*gef1Δ*) grew better than WT cells in SC solid medium containing 0.5 mM and 1 mM nickel ions, which demonstrates *gef1Δ* cells had resistance to nickel ion toxicity (Figure 1A). Next, we confirmed the specificity of the phenotype by repeating the experiment using a dose curve of several other heavy metal ions. *Gef1Δ* cells were sensitive to both copper ions and cadmium ions, and their growth trend was noticeably worse than that of WT cells when 0.5 mM copper (Figure 1B) or 5 μM cadmium (Figure 1C) was added. However, iron ions (Figure 1D) and zinc ions (Figure 1E) had little effect on *gef1Δ* growth. Therefore, we showed the ion resistance phenotype of the *gef1Δ* strain was specific to nickel ions. To further verify the experimental results, we prepared SC liquid culture medium with nickel ion concentrations of 0 mM, 0.5 mM and 1 mM respectively, then observed the growth of WT and *gef1Δ* cells. In SC liquid medium without nickel ions, the growth of WT and *gef1Δ* cells were almost the same (Figure 1F). After adding 0.5 mM nickel ion, the growth of *gef1Δ* cells in SC liquid medium was much higher than that of WT cells (Figure 1G). The growth trend of *gef1Δ* cells was still better than that of WT cells in SC liquid medium containing 1 mM nickel ions (Figure 1H). Adding excessive nickel ions promoted the growth of *gef1Δ* cells, which was consistent with the results of the solid growth experiment and further confirmed that *gef1Δ* cells are resistant to nickel ions. Next, we cloned the *GEF1* gene, transformed it into WT and *gef1Δ* cells, and observed their growth trends under the condition of excessive nickel. *Gef1Δ* cells still showed resistance to nickel ions after being transformed with the empty plasmid. However, after expressing the *GEF1* gene in *gef1Δ* cells, the cells became sensitive to nickel (Figure 1I). These results collectively indicate that there is a regulatory mechanism between the *GEF1* gene and nickel ion homeostasis.

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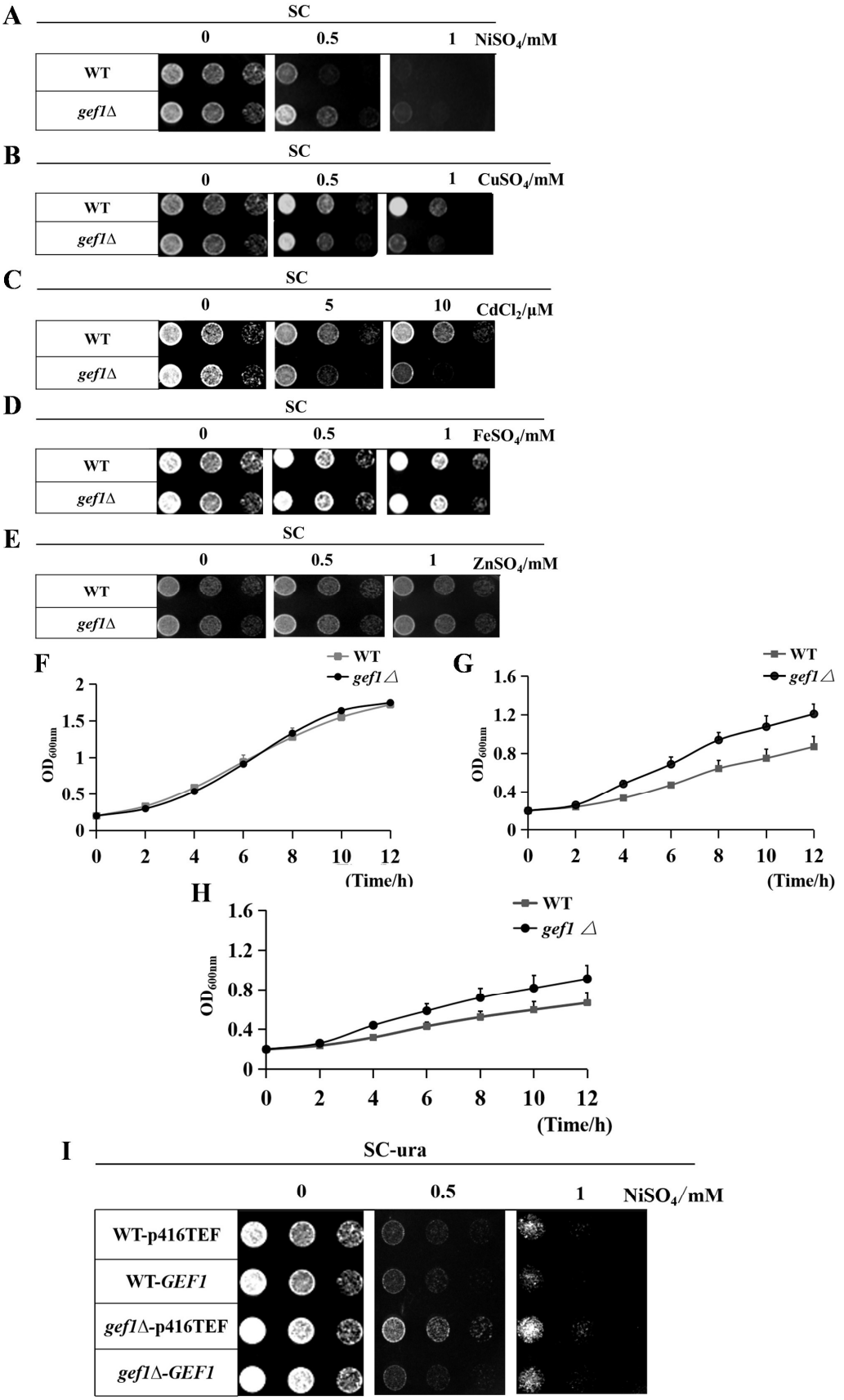

Figure 1. Gef1p is a new molecular factor involved in nickel ion metabolism in *Saccharomyces cerevisiae*. WT control and *gef1Δ* strains were cultured with $NiSO_4$ (A) or $CuSO_4$ (B) or $CdCl_2$ (C) or $FeSO_4$ (D) or $ZnSO_4$ (E) on SC plates containing glucose to assess growth. WT control and *gef1Δ* strains in were cultured in SC liquid medium with 0 mM (F), 0.5 mM (G) and 1 mM (H) $NiSO_4$ and growth curves are shown. (I) WT or *gef1Δ* cells

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were transformed with empty vector (p416TEF) or *GEF1* and grown on SC-Ura medium with or without nickel supplementation. All growth tests were carried out with at least four different clones.

3.2. GEF1 Deletion Significantly Increases the Nickel Content in Cells Under Condition of Nickel Excess

We suspected that the reason for the resistance of *gef1Δ* strain was that nickel ions in the culture medium would supplement the deficiency of nickel ions in *gef1Δ* cells. Therefore, we used ICP-MS to detect the changes in nickel and other divalent metal ions in cells cultured with and without supplemental nickel. The content of nickel in *gef1Δ* cells supplemented with nickel increased relative to WT controls (Figure 2A). However, the contents of iron (Figure 2B) and zinc (Figure 2C) decreased due to competitive absorption with nickel. Taken together, these data indicate that *GEF1* has a regulatory effect on nickel ions in yeast cells.

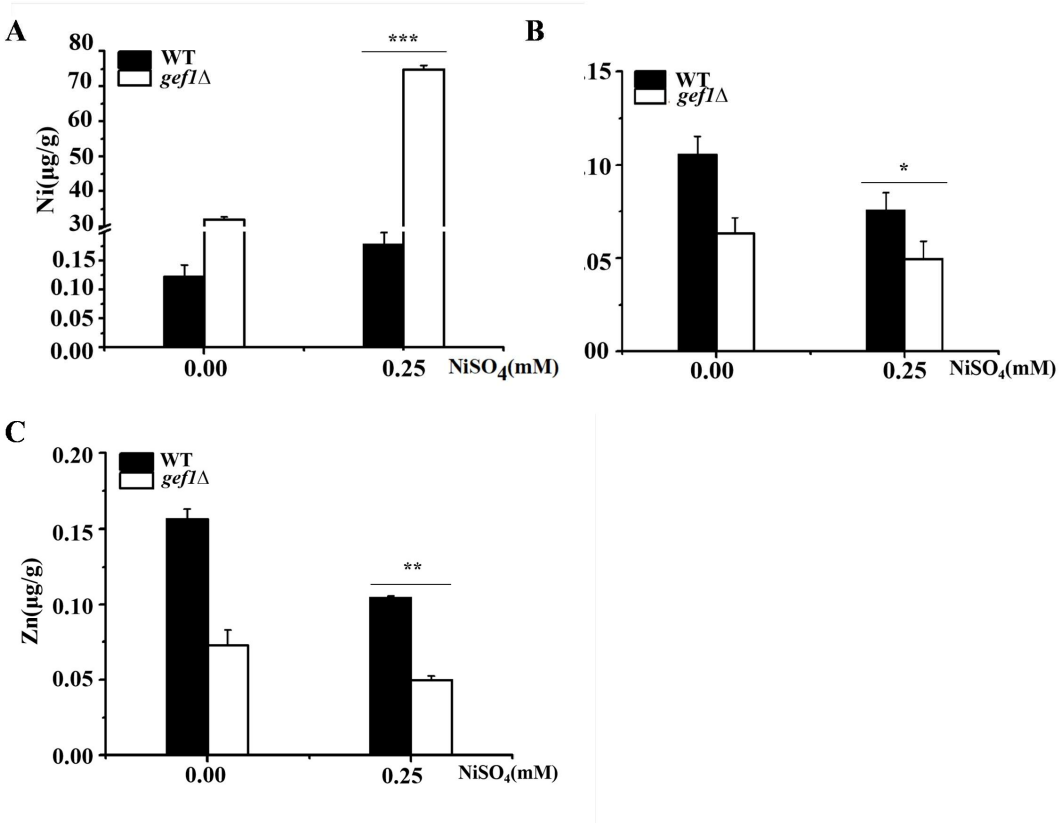

Figure 2. Metal content in cells of WT control and gef1Δ strains. Nickel (A), iron (B) and zinc (C) in cells were determined by ICP-MS from cells cultured in SC medium with or without $NiSO_4$, and then normalized to protein concentration. All metal level analyses were performed with at least four different clones. Average and standard deviation (n=4) are indicated. The asterisk (*) indicates that there are significant differences among different strains with the same treatment (*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$).

3.3. MMT2 Transports Excess Nickel to Mitochondria and Changes the Distribution of Nickel Ions

Given *GEF1* is not known to directly affect nickel, we supposed that changes to gene expression in *gef1Δ* cells exposed to nickel was responsible for mediating nickel resistance. Therefore, we performed transcriptome sequencing to determine changes in gene expression resulting from supplemented nickel in *gef1Δ* cells. We assessed the related functions of differentially expressed genes and selected nine Ni-related genes which are all related to metal ion metabolism to test mRNA expression levels in WT and *gef1Δ* cells under supplemental nickel ions. Compared with the WT cells, the expression levels of *FRE2*, *FIT2*, *ARN2*, *FET3*, *FRE1*, *MMT2*, *ARN1*, *FTR1* and *CUP1* were all significantly increased (Figure 3). Of these 9, *MMT2* and *CUP1* had exceptionally large increase in expression relative to WT controls and therefore were prioritized for further study. The *MMT2* gene is located on the mitochondrial membrane where it functions to export iron ions from the mitochondria into the cytosol[28]. According to previous research in our laboratory, most of the genes

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that transport iron ions can also transport nickel ions[29], so we speculated that *MMT2* can transport nickel ions from the outside of mitochondria to the inside. To verify this conjecture, we used ICP-MS to detect the content of nickel ions in mitochondria when *MMT2* gene was overexpressed in WT and *gef1Δ* cells, respectively. Compared with the overexpression of empty plasmid in *gef1Δ* cells treated with nickel ion, the content of nickel ion in the mitochondria of the *gef1Δ* cells with *MMT2* gene overexpressed increased significantly (Figure 4A). Moreover, the contents of iron, zinc and copper also increased (Figure 4B,C,D). It can be concluded that Mmt2p can transport nickel ions from cytoplasm to mitochondria, thus reducing the toxic effect of nickel ions on cells, which is one of the reasons *gef1Δ* cells are resistant to nickel ions.

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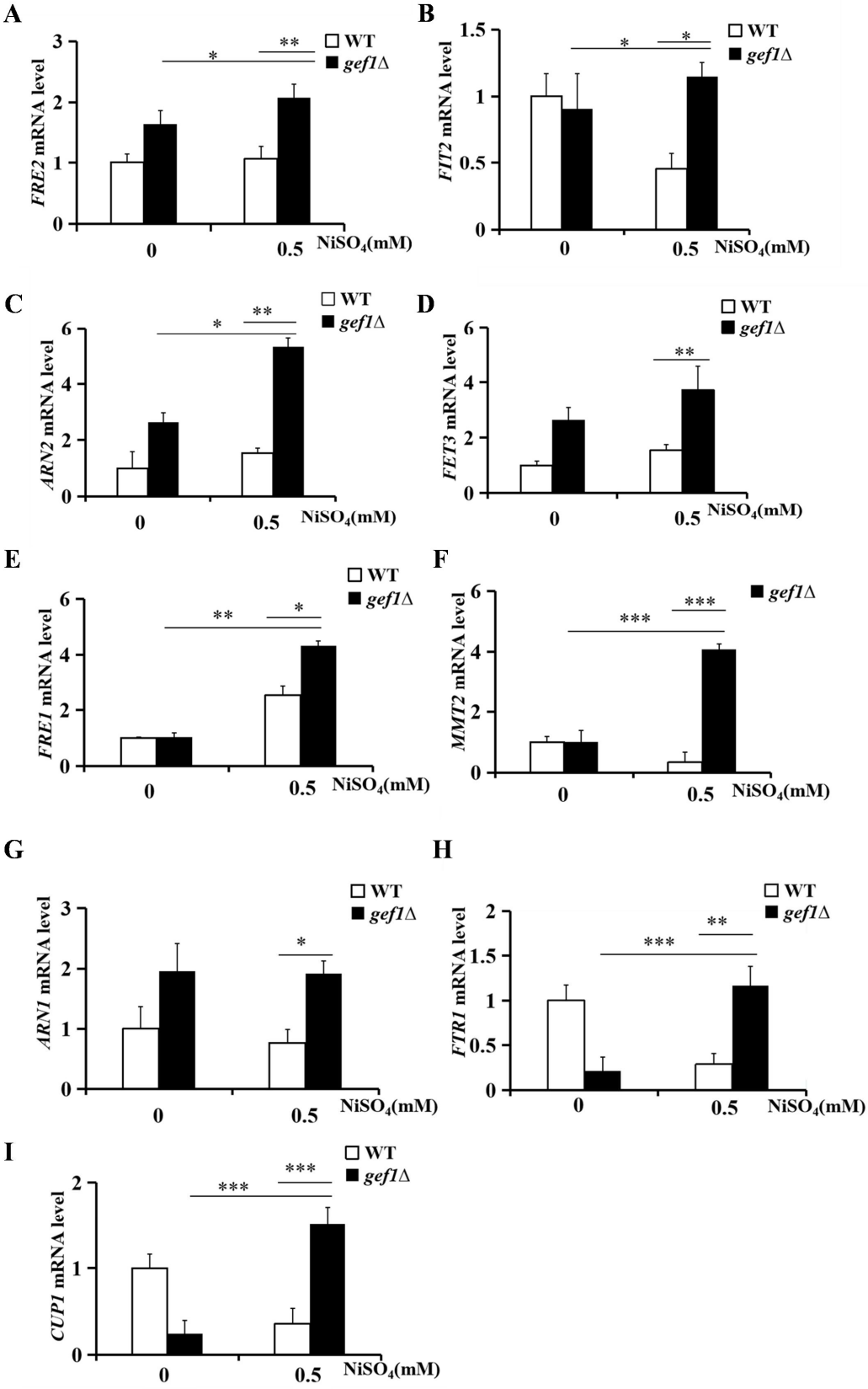

Figure 3. The mRNA expression levels of different genes in WT control and *gef1Δ* strains treated with or without $NiSO_4$. The mRNA expression levels of *FRE2* (A), *FIT2* (B), *ARN2* (C), *FET3* (D), *FRE1* (E), *MMT2* (F), *ARN* (G), *FTR1* (H) and *CUP1* (I). All treatments were carried out on at least four different clones. The mean and standard deviation is given. Significant differences are indicated by asterisks (*),.(* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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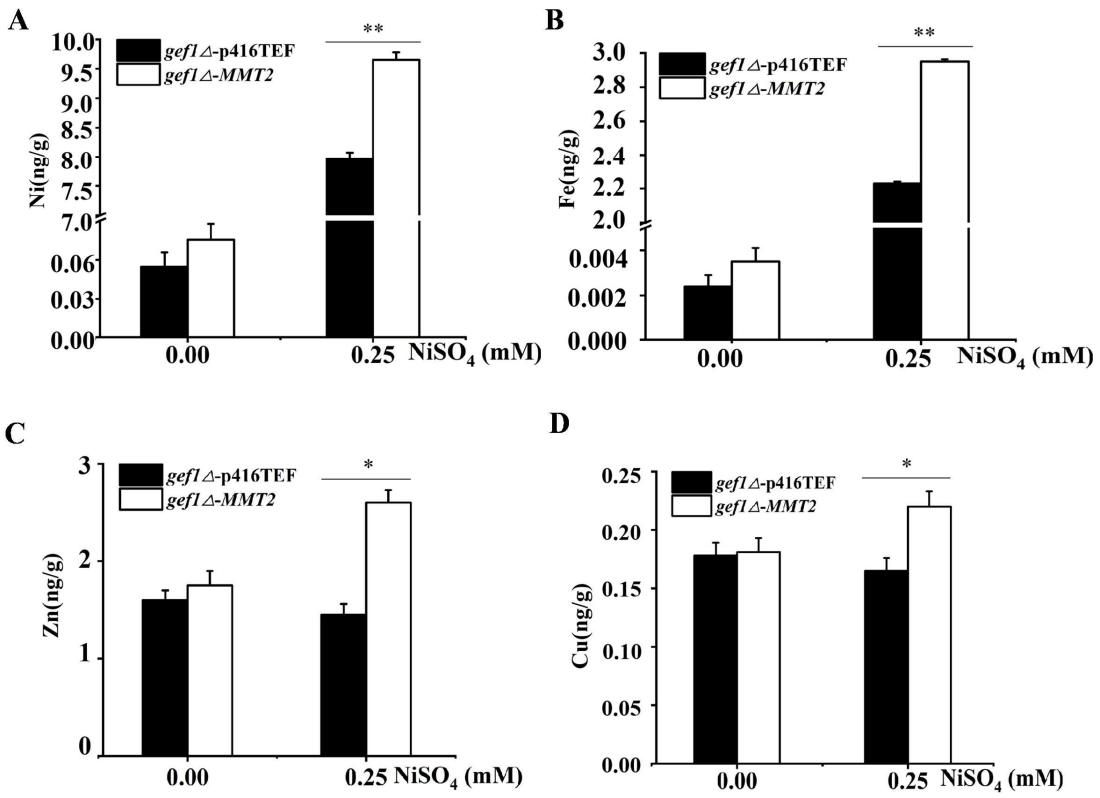

Figure 4. Heavy metal content in mitochondria during *MMT2* overexpression in *gef1Δ* strain. Nickel (A), iron (B), zinc (C), and copper (D) levels in mitochondria during *MMT2* overexpression were determined by ICP-MS and normalized to protein concentrations. All metal level analyses were performed with at least four different clones. Mean ± standard deviation (n=4) is indicated. Asterisks (*) indicate significant differences between lines with the same treatment.(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.4. Over-Expression of MMT2/CUP1 Reduces Intracellular ROS Level

CUP1 encodes a metallothionein which binds copper and other intracellular metals. We suspected that the upregulation of *GEF1* gene expression in *gef1Δ* cells led to an increase in bound nickel ions, thus reducing the content of ROS-producing free nickel ions. Furthermore, by transporting nickel ions into the mitochondria, MMT2 was predicted to mitigate ROS production, potentially through increase in nickel-requiring Sod2p antioxidant enzyme activity [26]. Therefore, we *overexpressed MMT2* and *CUP1* in *gef1Δ* cells treated with excessive nickel ions and analyzed intracellular ROS levels. The ROS levels in both knockouts decreased (Figure 5A,B) compared to WT controls which demonstrated the ability of genes upregulated by *gef1Δ*+nickel to protect cells from nickel-mediated ROS. As further evidence, we overexpressed MMT2 and CUP1 in *gef1Δ* grown on nickel-supplemented plates to assess cell growth. The MMT2 overexpressing *gef1Δ* cells grew better than control strains (Figure 5C). The growth of *CUP1* overexpressing *gef1Δ* cells was also better than that of control cells (Figure 5D). The superior growth of both overexpression cells supports the finding of decreased ROS relative to controls.

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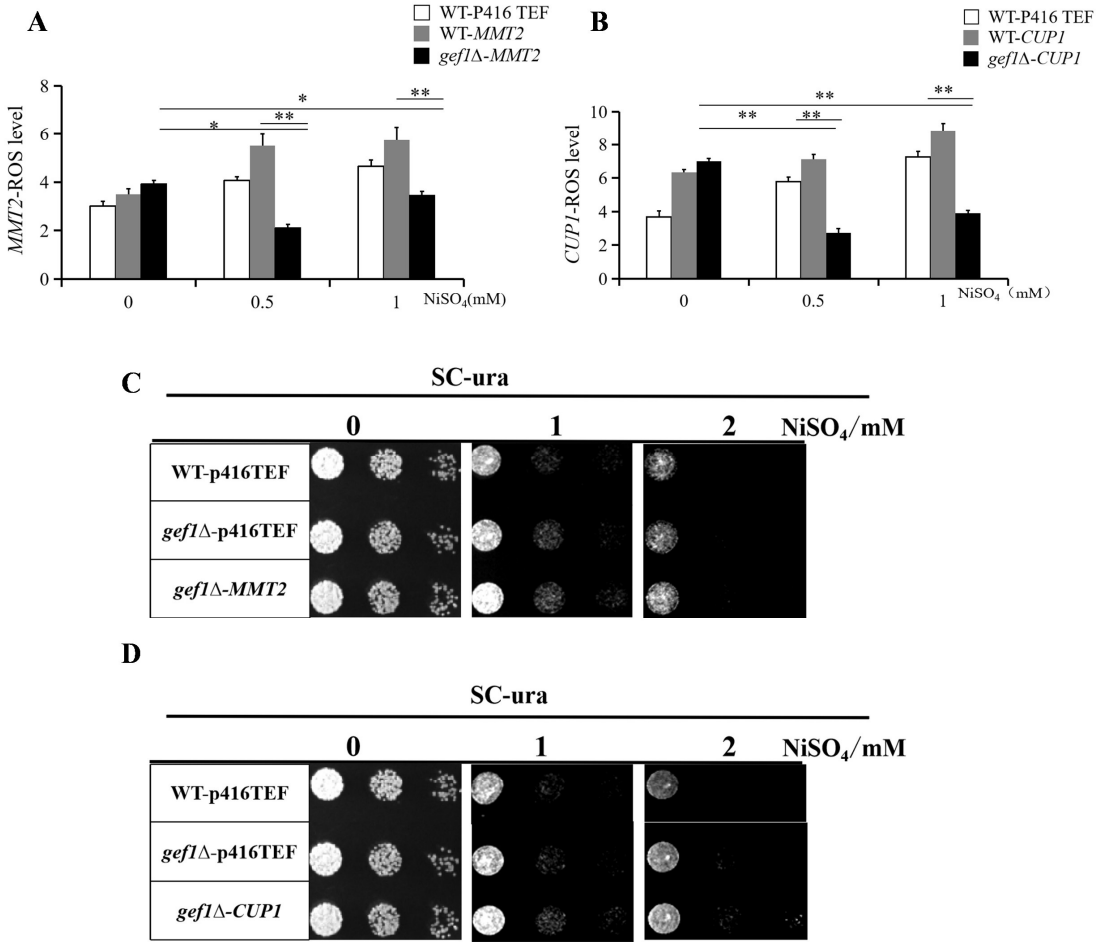

Figure 5. *MMT2*/*CUP1* overexpression reduces intracellular ROS levels. (A) Levels of reactive oxygen species produced by *MMT2* when overexpressed in WT and *gef1Δ* strains. (B) Levels of reactive oxygen species produced by *CUP1* when overexpressed in WT and *gef1Δ* strains. (C) Spot assay of *MMT2* overexpressed in WT and *gef1Δ* strains. (D) Spot assay of *CUP1* overexpressed in WT and *gef1Δ* strains. All treatments were performed on at least four different cell lines. Means ± standard deviation are given. Significant differences are indicated by an asterisk (*). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.5. Nickel Ions Play an Important Role in Regulating mRNA and Protein Expression of GEF1

GEF1 regulates the expression of *MMT2* and *CUP1* and reduces the intracellular ROS level, yet it is not clear whether the increase of nickel ion content in the *gef1Δ* cells affects the expression of *GEF1*. We cultured WT cells with three densities of nickel ions (0 mM, 0.5 mM and 1 mM) in the medium, then extracted the RNA of the cells and detected the mRNA expression levels. The mRNA expression level of *GEF1* decreased by more than ten times under the interference of nickel ions (Figure 6A). Next, we added the three densities of nickel ions to SC-Ura medium and using WT-p416TEF as a blank control, assessed the protein stability of WT-*GEF1* and *gef1Δ*-*GEF1* by western blot. Both *GEF1*-overexpressing cell lines showed increased Gef1p expression proportional to the concentration of nickel in the medium (Figure 6B). Overall, these results revealed that the addition of exogenous nickel ions to the culture medium enhances the protein stability of gef1p, which indirectly indicates that not only can *GEF1* regulate nickel ions, but also nickel ions can regulate *GEF1*.

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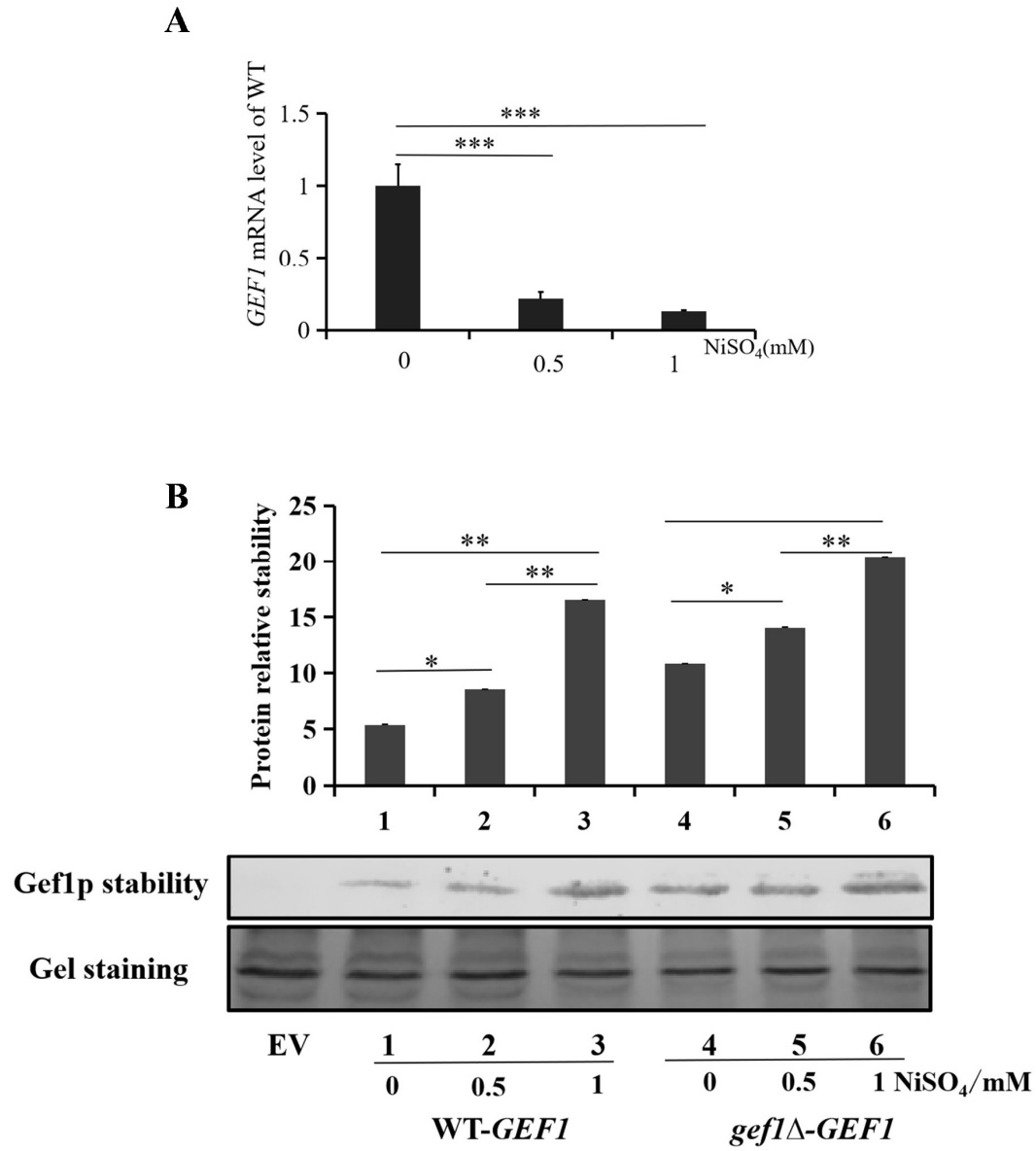

Figure 6. Nickel ions regulate the mRNA expression and protein expression level of *GEF1*. (A) The mRNA expression levels of *GEF1* in WT strains treated with different concentrations of nickel ions. (B) Protein expression levels of *GEF1*-GFP in WT cells and *gef1Δ* cells. EV represents empty vector. All treatments were performed on at least four different cell lines. Mean values ± standard deviation are given. Significant differences are indicated by an asterisk (*).(*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

4. Discussion

In this study, we studied the function of *GEF1* in nickel metabolism of *S. cerevisiae*. Previously, we found that the *GEF1*-deficient strain showed resistance to toxicity from medium containing 0.5 mM and 1 mM nickel ions. From there we assessed the regulatory mechanism of *GEF1* on nickel ions, thus discovering new functions of *GEF1* and laying a foundation for studying diseases related to nickel ion metabolism.

We proposed a model for the regulation of Ni distribution by Mmt2p and reduction of ROS levels by *MMT2*/*CUP1* overexpression (Figure 7). According to transcriptomics, *GEF1* can maintain intracellular nickel ion levels in a stable range by regulating gene expression. We verified the regulation of nine genes by qPCR and focused on two highly upregulated genes with potential roles in nickel metabolism, *MMT2* and *CUP1*. *MMT2* is located on the mitochondrial membrane and functions to transport iron from the mitochondria to the cytosol. According to previous results in our laboratory, we judged that most of the genes that transport iron ions can also transport nickel ions,

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so we speculated that *MMT2* gene could transport nickel ions from the cytosol to the mitochondria. *CUP1* is a metallothionein, which is a metal-binding gene whose expression is upregulated in *gef1Δ* cells. We speculated this could lead to an increase in bound nickel ions, allowing for the increase in total nickel ions which we observed in the *gef1Δ* strain. We used inductively coupled plasma mass spectrometry (ICP-MS) and solid-state growth experiments in cells overexpressing *MMT2* and *CUP1* to show that *MMT2* transported nickel from cytosol to mitochondria, and both *MMT2* and *CUP1* restored growth in nickel-supplemented media. Furthermore, given that nickel ions can induce the production of ROS, we assessed redox status of MMT2 and CUP1-overexpressing cells in the presence of excess nickel in the media. For both cell lines ROS were greatly decreased by over 50% compared to controls. These data suggest the *gef1Δ* strain was resistant to nickel ions through upregulation of *MMT2* and *CUP1* which act to reduce intracellular redox active nickel ions via their sequestration in the mitochondria and cytosol, respectively.

Finally, we identified a reciprocal relationship between *GEF1* and nickel whereby just as *GEF1* can regulate the metabolism of nickel ions, the nickel ions can also affect the expression of *GEF1*. Real-time fluorescence quantitative PCR experiments showed that nickel ions reduced the mRNA expression level of *GEF1*. The results of western blot showed that nickel ions could stabilize Gef1p. A possible explanation for this regulation is that excess nickel in the media impairs the uptake of iron and other metals through competitive inhibition (supported by Figure 2). Therefore, stabilization of gef1p, a chloride channel implicated in iron and copper homeostasis [30], may be a compensatory mechanism to maintain proper micronutrient balance. To summarize, this work demonstrated new roles for *GEF1* in the absorption, utilization and storage of nickel ions in cells, which provides new insight for studying diseases related to nickel ion metabolism.

To further explore the relationship between *GEF1* and nickel ions, it is necessary to identify the mechanism of how nickel ions reduce the mRNA level of *GEF1* and enhance stability of the gef1p protein. A possibility is that nickel ions may directly interact with transcription factors to affect GEF1 expression in a manner similar to NikR in microbes [31]. Nickel ions may also directly interact with gef1p protein to affect its stability. For example, nickel binding to HpNikR was shown to affect its conformation and stability [32] and nickel dramatically increased the thermostability of UreE [33]. Additionally, it is necessary to explore how *GEF1* regulates the transcription level of *MMT2* and *CUP1* including the extent to which nickel and other metal ions are implicated. Furthermore, it is unknown whether higher eukaryotes or humans have processes analogous to the relationship between *GEF1* and nickel shown in *S. cerevisiae.* Our work hopes to provide a basis for further study of nickel metabolism in eukaryotes which is greatly lacking despite being considered an essential trace mineral. It is hoped that industrial yeast strains with high nickel tolerance can be constructed by using the function of *GEF1* gene in the future, thus making certain contributions to industrial development.

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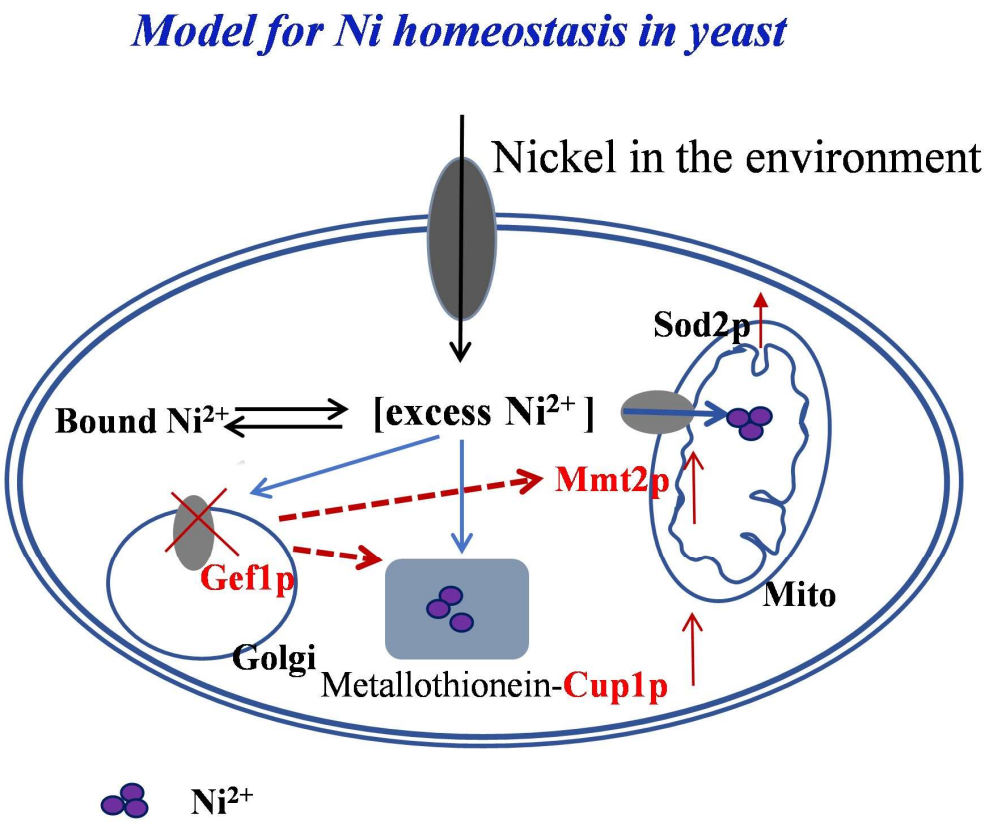

Figure 7. Proposed model for the regulation of Ni distribution by Mmt2p and reduction of ROS levels by MMT2/CUP1 overexpression.

Finally, we identified a reciprocal relationship between *GEF1* and nickel whereby just as *GEF1* can regulate the metabolism of nickel ions, the nickel ions can also affect the expression of *GEF1*. Real-time fluorescence quantitative PCR experiments showed that nickel ions reduced the mRNA expression level of *GEF1*. The results of western blot showed that nickel ions could stabilize Gef1p. A possible explanation for this regulation is that excess nickel in the media impairs the uptake of iron and other metals through competitive inhibition (supported by Figure 2). Therefore, stabilization of gef1p, a chloride channel implicated in iron and copper homeostasis [32], may be a compensatory mechanism to maintain proper micronutrient balance. To summarize, this work demonstrated new roles for *GEF1* in the absorption, utilization and storage of nickel ions in cells, which provides new insight for studying diseases related to nickel ion metabolism.

To further explore the relationship between *GEF1* and nickel ions, it is necessary to identify the mechanism of how nickel ions reduce the mRNA level of *GEF1* and enhance stability of the gef1p protein. A possibility is that nickel ions may directly interact with transcription factors to affect GEF1 expression in a manner similar to NikR in microbes [33]. Nickel ions may also directly interact with gef1p protein to affect its stability. For example, nickel binding to HpNikR was shown to affect its conformation and stability [34] and nickel dramatically increased the thermostability of UreE [35]. Additionally, it is necessary to explore how *GEF1* regulates the transcription level of *MMT2* and *CUP1* including the extent to which nickel and other metal ions are implicated. Furthermore, it is unknown whether higher eukaryotes or humans have processes analogous to the relationship between *GEF1* and nickel shown in *S. cerevisiae*. Our work hopes to provide a basis for further study of nickel metabolism in eukaryotes which is greatly lacking despite being considered an essential trace mineral. It is hoped that industrial yeast strains with high nickel tolerance can be constructed by using the function of *GEF1* gene in the future, thus making certain contributions to industrial development.

 doi:10.20944/preprints202501.1062.v1

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: Formal analysis, L.Z., Q. L.; Investigation, Q.L., L.Z., R.H., C.L., J.B. and X.W.; Writing—review & editing, J.B., J.B. and X.W.; Project administration, X.W.; Funding acquisition, X.W. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Shanghai Agriculture Applied Technology Development Program, China (No. 2021-02-08- 00-12-F00779), National Key R&D Program of China (No.2018YFC1604403) and the Fund of Shanghai Engineering Research Center of Plant Germplasm Resources (Grant No. 17DZ2252700).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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