The protein transportation pathway from Golgi to vacuoles via endosomes plays a role in enhancement of methylmercury toxicity

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Methylmercury causes serious damage to the central nervous system, but the molecular mechanisms of methylmercury toxicity are only marginally understood. In this study, we used a gene-deletion mutant library of budding yeast to conduct genome-wide screening for gene knockouts affecting the sensitivity of methylmercury toxicity. We successfully identified 31 genes whose deletions confer resistance to methylmercury in yeast, and 18 genes whose deletions confer hypersensitivity to methylmercury. Yeast genes whose deletions conferred resistance to methylmercury included many gene encoding factors involved in protein transport to vacuoles. Detailed examination of the relationship between the factors involved in this transport system and methylmercury toxicity revealed that mutants with loss of the factors involved in the transportation pathway from the trans-Golgi network (TGN) to the endosome, protein uptake into the endosome, and endosome-vacuole fusion showed higher methylmercury resistance than did wild-type yeast. The results of our genetic engineering study suggest that this vesicle transport system (proteins moving from the TGN to vacuole via endosome) is responsible for enhancing methylmercury toxicity due to the interrelationship between the pathways. There is a possibility that there may be proteins in the cell that enhance methylmercury toxicity through the protein transport system.

Methylmercury is a major environmental pollutant. Because it is highly lipophilic and easily penetrates the blood-brain barrier, its excessive consumption causes serious central nervous system (CNS) damage, including sensory paralysis, speech disorders, motor ataxia, restricted vision, and impaired hearing. Neurobehavioral consequences have recently been found in children born to mothers whose diet during pregnancy included large amounts of fish known to accumulate high levels of methylmercury; the severity of neurobehavioral symptoms is reported to be highly correlated with the level of methylmercury intake during pregnancy. Because brain development in human embryos is highly sensitive to methylmercury toxicity, it is believed that the function of the embryonic brain is affected by smaller quantities of methylmercury compared to the adult brain. Therefore, foetal consequences of methylmercury are a serious global problem. However, little is known about the molecular mechanisms of methylmercury toxicity and cellular protective mechanisms against such toxicity.

To clarify the molecular mechanisms of methylmercury toxicity, we screened for intracellular proteins affecting the sensitivity of methylmercury toxicity in budding yeast (Saccharomyces cerevisiae). Budding yeast is an ideal model because it is a eukaryotic unicellular organism and maintains many gene functions in common with humans and other mammals. We successfully identified several genes that confer resistance to methylmercury when overexpressed in yeast in previous work. However, it is expected that yeast cells have many more proteins that may affect the manifestation of methylmercury toxicity. In this study, we aim to clarify the molecular mechanisms of methylmercury toxicity as well as biological defence mechanisms against such toxicity. We conducted a genome-wide screening for genes whose deletions affect the sensitivity of budding yeast to methylmercury using a gene-deletion mutant library of the S. cerevisiae strain BY4742.
Table 1 | Genes whose deletion cause resistance to methylmercury

| Functional group               | Gene name | ORF     | Description of gene product | IC50 of deletion cells/IC50 of control cells |
|-------------------------------|-----------|---------|-----------------------------|--------------------------------------------|
| Metabolic pathway             | ACO2      | YJR200C | Aconitase                   | 1.29                                       |
|                               | ALD6      | YPL061W | Aldehyde dehydrogenase      | 1.31                                       |
|                               | MET25     | YLR303W | O-acetyl homoserine-O-acetyl serine sulfhydrolase | 1.64                                       |
| Protein degradation           | BAR1      | YIL015W | Aspartyl protease           | 1.29                                       |
|                               | BRE5      | YNR051C | Ubiquitin protease cofactor  | 1.18                                       |
|                               | ELA1      | YNL230C | F-box protein               | 1.24                                       |
|                               | SKP2      | YNL311C | F-box protein               | 1.39                                       |
|                               | RAV2      | YDR202C | Regulator of (H\(^{-}\))ATPase | 1.31                                       |
|                               | UBPI6     | YPL072W | Ubiquitin-specific protease  | 1.31                                       |
|                               | VPS28     | YIL017C | Vacular degradation         | 1.23                                       |
| Protein phosphorylation       | RH2R      | YLP053W | DGlycerol-3-phosphatase     | 1.34                                       |
| Transcription                 | ACE2      | YLR131C | Transcription factor        | 1.44                                       |
|                               | MET31     | YPL038W | Sulphur amino acid metabolism | 1.18                                       |
|                               | NAM2      | YLR382C | Leucyl-RNA synthetase       | 1.42                                       |
|                               | RPD3      | YNL330C | Histone deacetylase         | 1.14                                       |
|                               | RRP8      | YDR083W | rRNA processing             | 1.17                                       |
|                               | TRM1      | YDR120C | mRNA methyltransferase      | 1.16                                       |
| Vacuoles and vesicular transport | SFP8     | YPL002C | Component of the ESCRT-I complex | 1.31                                       |
|                               | VPS4      | YPR173C | AAA-type ATPase             | 1.10                                       |
|                               | VPS28     | YPL065W | Component of the ESCRT-I complex | 1.14                                       |
|                               | VPS45     | YGL095C | Vacular protein sorting     | 1.79                                       |
|                               | VPS63     | YLR261C | Vacular protein sorting     | 1.76                                       |
|                               | VPS68     | YOL129W | Vacular protein sorting     | 1.25                                       |
|                               | VTA1      | YLR181C | Endosomal protein sorting   | 1.29                                       |
| Unknown function or poorly characterised | BUD22 | YMR014W | Bud-site selection         | 1.57                                       |
|                               | IRS4      | YKR019C | Silencing of ribosomal DNA  | 1.27                                       |
|                               | NBF2      | YGR089W | Unknown function            | 1.23                                       |
|                               | RNY1      | YPL123C | Ribonuclease                | 1.94                                       |
|                               | RPS0B     | YLR048W | Component of the small (40S) ribosomal subunit | 1.18                                       |
|                               | YKE4      | YLR023C | Unknown function            | 1.40                                       |

Results
Screening for genes whose deletion affects the sensitivity of yeast to methylmercury. The S. cerevisiae genome contains approximately 6000 genes, and yeast is able to survive single-gene knockouts in the case of 4850 of these genes. We used the 4850 single-gene mutants and conducted genome-wide screening for genes whose deletions affected the sensitivity of the yeast to methylmercury. In this screen, each gene deletion mutant was cultured for 24 or 48 hrs in a liquid medium containing 60 nM of methylmercuric chloride. When wild-type yeast was cultured in the presence of 60 nM of methylmercuric chloride, minimal growth was observed after 24 hrs due to delayed growth in the presence of methylmercury. However, wild-type cultures were approximately fully grown 48 hrs later. In this study, we designated the gene deletion mutants showing growth after 24 hrs of culture in the medium containing methylmercuric chloride as putative methylmercury-resistant strains and those not showing full growth even after 48 hrs of culture as putative methylmercury hypersensitive strains. Yeast cells of each putative strain were cultured in liquid media containing 60 nM of methylmercuric chloride, minimal growth was observed after 48 hrs of culture due to delayed growth in the presence of methylmercury. In this study, our results showed that deletion of RNY1, VPS45, or VPS63 conferred strong resistance to methylmercury on yeast (Table 1) but that deletion of YHR029C, YJR120W, or FPS1 markedly enhanced yeast sensitivity to methylmercury (Table 2). Figure 1 shows the sensitivity of each deletion mutant of the six genes for methylmercury toxicity.

All of the proteins identified in this study were found to be involved in methylmercury toxicity. The functions of these proteins are classified into many types, suggesting that the sensitivity of yeast cells to methylmercury is determined using the combinatorial contributions of many protein functions.

Of particular note, during our analysis of methylmercury toxicity, we identified many genes encoding factors involved in protein transportation to vacuoles via endosomes. Therefore, we performed further analysis of the relationship between methylmercury toxicity and the protein transportation system.

Relationship between methylmercury toxicity and protein transportation systems to vacuoles via endosomes. There are two types of proteins transported to vacuoles via endosomes; one becomes functional in the vacuole, and the other is degraded in the vacuole. Membrane proteins such as signalling receptors and transporters are incorporated into the cell by endocytosis and then transported to the vacuole via the endosome and degraded, resulting in regulation of their cell surface abundance. Newly synthesised membrane proteins in the cell are transported to the trans-Golgi

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network (TGN) from the endoplasmic reticulum via the Golgi body. They are subsequently sorted in the TGN. Some proteins are transported to the cell membrane to become functional there, and the rest are transported to the vacuole via the endosome and degraded. Six pathways are involved in protein transportation to vacuoles: 1) a transportation pathway from the endoplasmic reticulum to the TGN, 2) a direct transportation pathway from the TGN to vacuoles (the alkaline phosphatase (ALP) pathway), 3) a reticulum to the TGN, 4) the TGN to vacuoles (the alkaline phosphatase (ALP) pathway), and 5) endocytosis, and 6) endosome-vacuole fusion.12,13 (Figure 2a). Our study showed that proteins involved in the transportation pathway from the TGN to endosomes, 4) the TGN to vacuoles (the alkaline phosphatase (ALP) pathway), 3) a reticulum to the TGN, 2) a direct transportation pathway from the TGN to vacuoles without passing through endosomes (the second pathway, as previously listed), and genes encoding End3, Ent1, and EndoA play a role in the enhancement of methylmercury toxicity. However, because this study used a simple screening method conducted in the presence of a fixed concentration of methylmercury (60 nM), some factors involved in the protein transportation pathway from the TGN to vacuoles may not be detected despite potentially impacting methylmercury toxicity. To elucidate the relationship between methylmercury toxicity and proteins involved in the protein transportation pathway from the TGN to vacuoles and to identify the pathway playing a role in the enhancement of methylmercury toxicity among those transportation pathways, we investigated the sensitivity of each gene deletion mutant to proteins known to be involved in those pathways. Several single-gene deletion mutants have a similar level of sensitivity to methylmercury as do wild-type yeast. These include, the genes encoding Apl5 and Apl6, both of which are involved in the pathway for direct transportation of substrate proteins from the TGN to vacuoles without passing through endosomes (the second pathway, as previously listed) and the genes encoding End3, Ent1, Ent2, and EndoA, all of which are involved in endocytosis (the fifth pathway, as previously listed; data not shown). Based on these results, it appears that neither of these pathways is involved in methylmercury toxicity enhancement. A subset of single-gene deletion mutants showed higher resistance to methylmercury compared to wild-type yeast. These included genes encoding Vps45 and Pep12, both of which are involved in transportation from the TGN to endosomes (the third pathway, as previously listed), genes encoding Vps27, Hse1, Stp22, Snr2, Vps28, Vps36, Vps25, Snf8, Did4, Vps20, Vps24, Snf7, and Doa4, all of which are involved in the MVV sorting pathway (the fourth pathway, as previously listed), and genes encoding Vam3 and Vam7, both of which are involved in endosome-vacuole fusion (the sixth pathway previously listed; Figure 2b). We also examined the methylmercury sensitivity of yeast cells with genetic deletion of Ypt7 and Mon1, which are involved in the same pathway as Vam3 and Vam7. However, these deletion mutants did not affect the yeast’s sensitivity to methylmercury. It has been reported that the knockout of Ypt7 or Mon1 inhibited approximately 50% of vacuole transport activity and that the knockout of Vam3 or Vam7 inhibited a majority of vacuole transport activity. Therefore, Ypt7 and Mon1 may not be essential for vacuole transport pathway via Vam3 and Vam7. From these results, it appears that the protein transportation system from the TGN to vacuoles via endosomes is involved in the enhancement of methylmercury toxicity. However, the three pathways identified in the enhancement of methylmercury toxicity (pathways three, four, and six) may enhance toxicity independently via different mechanisms. Therefore, we prepared double deletion mutants with deletions of Vps45 (involved in transportation from the TGN to endosomes) or Vam7 (involved in endosome-vacuole fusion) in addition to the deletion of the Vps27 gene involved in the MVV sorting pathway (vps45Δ vam7Δ and vps27Δ vam7Δ) and studied their sensitivity to methylmercury. In this experiment, we selected Vps27 as the key factor of the MVV sorting pathway because Vps27 is known to function upstream in the MVV sorting pathway. Single-gene deletion mutants with VPS27, VPS45, or VAM7 showed strong resistance to methylmercury. However, an additive increase in resistance level was not observed during double deletion of VPS45 and VPS27 (Figure 3a) or VPS27 and VAM7 (Figure 3b). These results suggest that transportation from the TGN to endosomes (pathway three), the MVV sorting pathway (pathway four), and endosome-vacuole fusion (pathway six), are jointly involved in the enhancement of methylmercury toxicity. Therefore there may be proteins in the cell that enhance methylmercury toxicity via transportation from the TGN to vacuoles via endosomes.

### Table 2 | Genes whose deletion causes hypersensitivity to methylmercury

| Functional group | Gene name | ORF | Description of gene product | IC50 of deletion cells/IC50 of control cells |
|------------------|-----------|-----|------------------------------|------------------------------------------|
| Metabolic pathway | PSD2 | YGR170W | Phosphatidylycerine decarboxylase | 0.78 |
|                  | RPE1 | YJR121C | Dribulos-5-phosphate 3-epimerase | 0.67 |
|                  | ETR1 | YBR026C | 2-enoyl thioester reductase | 0.68 |
| Protein phosphorylation | CKB2 | YOR039W | Subunit of the casein kinase 2 | 0.65 |
| Transporter | PK1 | YGR240C | Subunit of the phosphofructokinase | 0.74 |
|                  | BAB2 | YBR068C | Branched-chain Amino acid Permease | 0.75 |
| Transcription | CDC39 | YCR093W | Transcription factor | 0.72 |
|                  | BRE2 | YLR015W | Subunit of the COMPASS (Set1C) complex | 0.76 |
| Unknown function or poorly characterised | GVP1 | YOR070C | GTPase-activating protein | 0.68 |
|                  | VMA1 | YDL185W | Vacular ATPase V1 domain subunit A | 0.78 |
|                  | YDJ1 | YNL064C | Protein chaperone | 0.74 |
|                  | YDL173W | YDL173W | Unknown function | 0.89 |
|                  | YHR029C | YHR029C | Unknown function | 0.33 |
|                  | YIL120W | YIL120W | Unknown function | 0.52 |
|                  | YLL014W | YLL014W | Unknown function | 0.71 |
|                  | YLR111W | YLR111W | Unknown function | 0.78 |
|                  | YOL087C | YOL087C | Unknown function | 0.55 |

Role of substrate recognition proteins in the MVV sorting pathway. As shown in Figure 2b, deletion of any single protein involved in the MVV sorting pathway conferred resistance to methylmercury in yeast. Vps27-Hse1 (a complex existing on the endosome membrane) and its downstream factor ESCRT (Endosomal Sorting Complex Required for Transport, consisting of Stp22, Snr2, Vps28, Vps36, Vps25, Snf8, Did4, Vps20, Vps24, and Snf7 shown in Figure 2b) are both involved in the MVV sorting pathway.15,16 Substrate proteins of the MVV sorting
pathway first undergo monoubiquitination in the cell, which serves as the recognition signal for the Vps27-Hse1 complex. After recognition, the protein is transferred to ESCRT. The HSE1 deletion mutant showed weaker methylmercury resistance than the VPS27 deletion mutant or deletion mutants of genes encoding other proteins within the MVB sorting pathway (Figure 2b).

Next, we prepared a double-deletion mutant for Vps27 and Hse1 (vps27Δ hse1Δ), which showed a similar level of methylmercury resistance as the VPS27 single-deletion mutant (Figure 3c). This indicates that the presence of Vps27 is necessary for acquired methylmercury resistance following HSE1 deletion. Hse1 binds directly to Vps27 at amino acids 416–418. Therefore, Hse1 may not be essential for substrate protein recognition in the MVB sorting pathway. However, Hse1 may play a role in enhancing the activity of Vps27 by binding to Vps27.

Vps27 contains a VHS (for Vps27, Hrs and STAM; 1–145 amino acids) domain involved in substrate recognition, the FYVE (for Fab1, YGL023, Vps27, and EEA1; 168–231 amino acids) domain, which is
necessary for binding to phosphatidylinositol 3-phosphate on endosome membranes, and two UIM (Ubiquitin-Interacting Motifs; 257–274 amino acids and 300–317 amino acids) domains that bind to ubiquitinated proteins (Figure 4a) 18. To study the relationship between these domains and the methylmercury toxicity enhancement shown by Vps27, we investigated methylmercury sensitivity in mutants with single-domain deletions of Vps27 fused to an HA tag (Figure 4c). The methylmercury resistance shown by the VPS27 deletion mutant disappeared in yeast expressing wild-type HA-Vps27. However, the expression of mutant Vps27 protein with the FYVE domain deleted (HA-Vps27 FYVE D) only minimally affected methylmercury resistance in the VPS27 deletion mutant. This result indicates that the FYVE domain is essential for the enhancement of methylmercury toxicity shown by Vps27. When the VHS domain deleted Vps27 (HA-Vps27 VHS D) or double UIM domain deleted Vps27 (HA-Vps27 UIM1 D UIM2 D) were expressed in the VPS27 deletion mutant, a significant reduction in resistance level was observed. However, the decrease in resistance level in both mutants was smaller than that in the mutant expressing wild-type HA-Vps27 (Figure 4c). Based on these results, the function of Vps27 on endosome membranes is essential for Vps27 to enhance methylmercury toxicity and the Vps27 substrate recognition function (VHS domain) and binding ability to ubiquitinated proteins (UIM domain) play a lesser role in enhancing methylmercury toxicity. Vps27 proteins lacking the VHS domain may be able to bind to substrate proteins with reduced efficiency via the two UIM domains. Hrs, a mammalian homologue of Vps27, is able to bind to ubiquitin via the VHS domain 19, and the Hse1-Vps27 complex also contains the UIM domain in its structure 20. Therefore, when both UIM domains are deleted, the VHS domain in Vps27 or the UIM domain in Hse1 may take on their role.

**Discussion**

In this study, we conducted a genome-wide screen using a gene-deletion mutant library and successfully identified many genes affecting methylmercury sensitivity in yeast. In the future, it is expected that a detailed examination of the role of each of these proteins in methylmercury toxicity may provide a useful clue to elucidate the molecular mechanisms of methylmercury toxicity.

The genes whose deletions confer methylmercury resistance in yeast include four genes encoding proteins involved in the ubiquitin-proteasome system, representing a selective proteolytic pathway (BRE5, ELA1, SIP2, UBP16; Table 1). In a previous study, we found that yeast gains resistance to methylmercury by enhancing proteolysis in this ubiquitin-proteasome system 21. Among the genes identified in this study, BRE5 20 and UBP16 22 encode enzymes involved in deubiquitination of proteins. In a cell, the dissociation of ubiquitin from proteins occurs via deubiquitinating enzymes, resulting in regulation of protein levels. When deubiquitinating enzymes such as BRE5, ELA1, SIP2, and UBP16 are deleted, proteolysis via ubiquitination at the proteasome is promoted. It is possible that through this process, yeast may show resistance to methylmercury.

In this study, genes whose deletion increased methylmercury sensitivity were also successfully identified. These include genes encoded transporters of branched-chain amino acids (Bap2) and glycerol (Fpsl). Because Fpsl is involved in the exocytosis of metal ions, including As 3+ and Sb 5+ 23, these proteins may enhance elimination of methylmercury from the cell. We also measured the accumulation of mercury in the cell when FPS1 knockouts or BAP2 knockouts were treated with methylmercury. Because we did not observe a significant difference in accumulation between the two mutants and the wild-type yeast (data not shown), the hypersensitivity of yeast to methyl-
Our study revealed that deletion of any single protein involved in the protein transportation pathway from the TGN to vacuoles via endosomes (Figure 2a; pathways three, four, and six as previously stated) makes yeast highly resistant to methylmercury (Figure 2b). This shows that the protein transport pathways are deeply involved in the enhanced toxicity of methylmercury (Figure 3). There are transport pathways from the endoplasmic reticulum to the TGN at the starting point of this transport system (pathway one, as previously stated; Figure 2a). Because many proteins involved in this pathway (pathway one) are essential for the survival of yeast and thus difficult to delete, a detailed examination of the relationship between this pathway and methylmercury toxicity was not carried out in this study. However, there are a few proteins involved in the transport pathway from the endoplasmic reticulum to the TGN, for example, Emp24 and Emp47, whose deletions do not affect the survival of yeast. We investigated the impact of EMP24 or EMP47 deletions on the sensitivity of yeast to methylmercury, but minimal impact was observed (data not shown). Although some abnormalities in protein transportation are observed in EMP24 deletion yeast, ALP and carboxypeptidase Y (CPY) are transported normally in EMP47 deletion yeast. More detailed examination is required to determine the relationship between methylmercury toxicity and the transportation pathway from the endoplasmic reticulum to the TGN.

It has been reported that methylmercury damages lysosomes (corresponding to vacuoles in yeast) in human astrocytoma cells and causes cell death\(^2\). Because protein transportation pathways from the TGN to vacuoles (lysosomes) via endosomes are conserved in a wide variety of organisms ranging from yeast to humans, methylmercury may impact this transportation pathway by causing damage to the function of vacuoles, resulting in cell death. However, there have been no reports that protein transportation systems to vacuoles are involved in methylmercury toxicity; the present study provides telling clues for the elucidation of the molecular mechanisms of methylmercury toxicity.

The MVB sorting pathway for endosomes is involved in making cellular decisions regarding whether to reuse substrate proteins or send them to vacuoles for degradation and processing. In this MVB sorting pathway, Vps27 plays a crucial role in recognising substrate proteins. Our data suggest that the binding of Vps27 to the endosome membrane is essential for Vps27-enhanced methylmercury toxicity and that the substrate recognition function of Vps27 and its ability to bind ubiquitinated proteins also play crucial roles (Figure 4). It is thought that cells have proteins that enhance methylmercury toxicity via transportation from the TGN to vacuoles via endosomes and that during this transportation, the proteins may be recognised as substrates of the MVB sorting pathway that exists on the endosome. Although the results of the present study suggest that the proteins involved in the protein transportation system via the MVB sorting pathway are likely to be associated with methylmercury toxicity in yeast, the exact mechanism remains to be explained.

Recent evidence suggests that the ubiquitin modification of proteins functions as an important signal not only in proteolysis at the proteasome but also for intracellular membrane trafficking and the proteolysis process in lysosomes\(^22,23\). In the MVB sorting pathway, which was found to be involved in methylmercury toxicity enhancement during this study, ubiquitin modification is known to play a vital role as a recognition signal for substrate proteins\(^25\). It has also been reported that proteasome activity is necessary for protein uptake into endosomes by the MVB sorting pathway\(^25\). Therefore, it is difficult to deny the possibility that the function of the MVB sorting pathway is regulated by the ubiquitin-proteasome system. We have identified Cdc34\(^6\) and Rad23\(^9\) as ubiquitin-proteasome system-related factors involved in the reduction of methylmercury toxicity. Methylmercury resistance was observed during the overexpression of Cdc34 or Rad23 in wild-type yeast and was similarly observed when Cdc34 or Rad23 was highly expressed in VPS27 defective yeast (data not shown). From these findings, it is thought that the MVB sorting pathway is not involved in the resistance acquired by overexpression of Cdc34 or Rad23.

We have also reported that overexpression of Bop3 (a protein of unknown function)\(^7\) or L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT)\(^10\) confers resistance to methylmercury in yeast. Yeast cells overexpressing Bop3 or GFAT showed methylmercury resistance even when Vps27 was disrupted (data not shown). Therefore, the MVB sorting pathway might not participate in the acquired resistance by overexpression of Bop3 or GFAT.

The present study suggests that there may be proteins in the cell that enhance methylmercury toxicity through transportation from the TGN to vacuoles via the MVB sorting pathway. It has been reported that yeast strains lacking the genes involved in the MVB sorting pathway have a higher sensitivity to hygromycin B\(^11\), bleomycin\(^12\), fluorozamide\(^13\), tunicamycin\(^13\), metansulfonate\(^14\), and tert-butyl hydroperoxide\(^15\) than do wild-type yeast, suggesting that the MVB sorting pathway may play a role as a defence mechanism against various toxins. However, our study revealed that methylmercury toxicity is enhanced by the protein transport system from TGN to vacuoles via endosomes (including the MVB sorting pathway). We know of no other toxic substances whose toxicity is enhanced by the protein transport system. The protein transport system is believed to show specific毒性-enhancing action for methylmercury.
Figure 4 | Effect of Vps27 functional domains on the sensitivity of yeast cells to methylmercury. (a) Structural domains of Vps27 and construction of mutant proteins. (b) A yeast strain expressing Vps27 fused to the HA tag (HA-Vps27) was transformed with pRS316, pRS316-HA-VPS27 FYVE, pRS316-HA-VPS27 FYVE + UIM2, or pRS316-HA-VPS27 FYVE + UIM2. Cell extracts were prepared from the various strains and analysed via immunoblotting with the monoclonal HA-specific antibody. (c) Yeast vps27Δ cells (10⁶ cells/ml) that harboured pRS316 (control), pRS316-HA-VPS27, pRS316-HA-VPS27 FYVE, pRS316-HA-VPS27 FYVE + UIM2, or pRS316-HA-VPS27 FYVE + UIM2 were grown in SD liquid media with or without methylmercuric chloride at the indicated concentrations. After incubation for 3 hrs at 30°C, cells from each strain were diluted in sterilised water to 10⁷ cells/ml. Five microlitres of each of the resulting cell suspensions were spotted on agar-solidified SD media. For further details, refer the Figure 4c legend.

Methods
Yeast strains and media. Saccharomyces cerevisiae BY4742 (MATa; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) and the complete collection of haploid deletion strains (MATα) constructed by inserting KanMX4 cassettes (G418 resistance) as selective markers into the genome of BY4742 cells were obtained from Euroscarf (Frankfurt, Germany). Yeast cells were grown at 30°C in a yeast extract-peptone-dextrose (YPD) media or synthetic dextrose (SD) media. Deletion mutant strains were maintained on YPD or synthetic dextrose (SD) media. Deletion mutant strains were inoculated into 200 μl SD liquid media and then cultured to reach a stationary phase (approximately 10⁸ cells/ml) for 48 hrs at 30°C. Portions (5 μl) of the culture were transferred into 195 μl SD liquid media and then cultured to reach a stationary phase (approximately 10⁸ cells/ml) for 48 hrs at 30°C. To screen for genes whose deletions confer sensitivity to methylmercury, dilutions of yeast cells (approximately 10⁹ cells/5 μl) were plated on YPD or synthetic dextrose (SD) media. Deletion mutant strains were maintained on YPD or synthetic dextrose (SD) media. Plasmid DNA was introduced into BY4742 cells using the high-efficiency lithium acetate transformation method.

Identification of gene deletion yielding sensitivity to methylmercury. Deletion mutant strains were inoculated into 200 μl SD (+G418) liquid media in 96-well plates using a 96-pin replicator (Funakoshi, Tokyo, Japan) and then cultured for 48 hrs at 30°C (preculture). Portions (5 μl) of the preculture were transferred into 195 μl SD liquid media and then cultured to reach a stationary phase (approximately 10⁸ cells/ml) for 48 hrs at 30°C. To screen for genes whose deletions confer sensitivity to methylmercury, dilutions of yeast cells (approximately 10⁹ cells/5 μl) were plated into 195 μl SD liquid media that contained methylmercuric chloride (60 nM) in a 96-well microtitre plate. The optical density of the culture after 24 hrs and 48 hrs was measured at 600 nm using a microtitre plate reader (Benchmark plus; Bio-Rad, Hercules, CA, USA). Putative methylmercury-resistant or -hypersensitivity strains were further analysed by the rate of cell proliferation in the SD liquid media. The IC50 was calculated using Probit analysis with a 95% detection limit.

Quantitation of the methylmercury toxicity in yeast cells. Yeast cells (10⁶ cells/200 μl) were cultured in SD liquid media containing methylmercuric chloride at various concentrations. Following incubation for 48 hrs, we measured the absorbance of each culture at 600 nm to quantify cell growth. For the colony-formation assay, we cultured yeast cells (10⁶ cells/ml) in SD liquid media that contained methylmercuric chloride at various concentrations. After incubation for 3 hrs at 30°C, the cells were washed and pelleted via centrifugation and each pellet was suspended and diluted in 0.1 ml sterilised water to yield 10⁷ cells/ml. Five microlitres of each yeast cell suspension were spotted on agar-solidified SD media, and colony formation was examined after 24 hrs of culture at 30°C.

Construction of plasmids. The hemaglutinin (HA) - VPS27 gene was amplified from a yeast genomic DNA template via polymerase chain reaction (PCR) using the following oligonucleotides as primers: VPS27-HA-F, 5'-GTCAGCTAGCTTAAAGCTCTATTAGCAGTTC-3' and VPS27-HA-R, 5'-GTCAGTCGACTTAAAGCTCTATTAGCAGTTC-3' for deletion of the VPS27 gene. The PCR-generated HA-VPS27 plasmid was digested with the restriction endonucleases Smal and Sall (New England Biolabs, Beverly, MA, USA). The resulting fragment was ligated into the Smal and Sall cloning site of the pRS316 yeast expression vector. Sequences of constructs were verified with an automated DNA sequencer. The resulting plasmid was designated pRS316-HA-VPS27.

Site-directed mutagenesis of the gene for HA-VPS27 was performed as previously described. We constructed the HA-VPS27 FYVE, HA-VPS27 UIM1, and HA-VPS27 UIM2 genes by creating pairs of NheI sites in the open reading frame (ORF) of the HA-VPS27 gene and excising the fragments between the respective pairs of NheI sites. We amplified the fragments via PCR using the pRS316-HA-VPS27 plasmid as a template and the following oligonucleotides as primers: FYVE-F, 5'-GTCAGCTAGCAGACATGACGACAGTAAAAAAA-3' and FYVE-R, 5'-GTCAGCTAGCAGACATGACGACAGTAAAAAAA-3' for deletion of the VPS27 gene; VHS-F, 5'-GTCAGCTAGGAACATGACGACAGTAAAAAAA-3' and VHS-R, 5'-GTCAGCTAGGAACATGACGACAGTAAAAAAA-3' for deletion of the VPS27 gene; VHS-F, 5'-GTCAGCTAGGAACATGACGACAGTAAAAAAA-3' and VHS-R, 5'-GTCAGCTAGGAACATGACGACAGTAAAAAAA-3' for deletion of the VPS27 gene; and VHS-F, 5'-GTCAGCTAGGAACATGACGACAGTAAAAAAA-3' and VHS-R, 5'-GTCAGCTAGGAACATGACGACAGTAAAAAAA-3' for deletion of the VPS27 gene.

Gene disruption. For construction of the vps27::HIS5 vector, the HIS5 gene, which is derived from Saccharomyces pombe and complements the Saccharomyces cerevisiae HIS5 gene, was amplified via PCR from plasmid pUG27 with primers VPS27-HIS5-F (5'-GGTATTAATAGGGGGGCGATCCTG-3') and VPS27-HIS5-R (5'-ATGGGGCGGTATGTTAGAATCTGA-3'). The HIS5 promoter and terminator of pUG27 plasmid are homologous to the KanMX4 marker containing the G418.
resistance gene. Consequently, the PCR product was introduced into the
vps27::KanMX strain from the EUROSCARF deletion strain collection to construct the
vps27::HisS disruptant. Replacement of the gene was verified via PCR analysis
with primers VPS27-F2 (5′-CTGCTAATGTCATCGACTT-3′) and VPS27-R1 (5′-TATGCAGGAAAGCTTCC-3′). Subsequently, using genomic DNA of
vps27::HisS strain as the template, the PCR product was used to transform
vps27::KanMX, vam3::KanMX, and his3::KanMX strains from the
EUROSCARF deletion strain collection to construct the vps27::HisS vps45::KanMX, vps27::HisS vam3::KanMX, and vps27::HisS
his3::KanMX double disruptants, respectively. Disruption of the gene was verified via
PCR using primers VPS27-F2 (5′-TGGTCGTTACAACTCAGGA-3′) and
VPS27-R2 (5′-AAAGAACGTCGCAAGGAAAGCAG-3′).

Immunoblotting. Immunoblotting was performed as previously described. Yeast
cells (107 cells/ml) were grown in 10 ml of SD liquid media for 3 h and harvested.
Approximately 50 µl of cell pellet was suspended in 0.2 to 0.3 ml of Buffer C (20 mM of
Tris-HCl (pH 7.5), 1 mM of EDTA, 5 mM of MgCl2, 50 mM of KCl, 5% glycerol,
3 mM of DTT, 1 mM of phenylmethylsulfonyl fluoride, and 1 µg/ml of pepstatin A)
and lysed with glass beads (425–600 µm; Sigma, St. Louis, MO, USA) in a cell
disruptor (Micro Smash® MS-100R; Tomy, Tokyo, Japan). Cell extracts were
adjusted to equal the volumes and concentrations of the protein and analysed using
immunoblotting. HA-Vps27 was visualised with HA-specific monoclonal primary antibodies
(Roche, Basel, Switzerland) and peroxidase-conjugated goat secondary antibodies
against rat immunoglobulins (Dako A/S, Glostrup, Denmark).

1. Yuan, Y. Methylmercury: a potential environmental risk factor contributing to
epileptogenesis. Neurotoxicology 33, 119–126 (2012).
2. Grandjean, P. et al. Cognitive deficit in 7-year-old children with prenatal exposure
to methylmercury. Neurotoxicol. Teratol. 19, 417–428 (1997).
3. Murata, K., Weihe, P., Araki, S., Budtz-Jorgensen, E. & Grandjean, P. Evoked potentials
in Faroese children prenatally exposed to methylmercury. Neurotoxicol. Teratol. 21, 471–472 (1999).
4. Mozaffarian, D. & Rimm, E. B. Fish intake, contaminants, and human health:
evaluating the risks and the benefits. JAMA 296, 1885–1890 (2006).
5. Schrope, M. US to take temperature of mercury threat. Nature 409, 124 (2001).
6. Furuchi, T., Hwang, G. W. & Naganuma, A. Overexpression of the ubiquitin-
conjugating enzyme Cdc34 confers resistance to methylmercury in
Saccharomyces cerevisiae. Mol. Pharmacol. 61, 738–741 (2002).
7. Hwang, G. W., Furuya, Y., Hiroshima, A., Furuchi, T. & Naganuma, A. Overexpression of
Bop3 confers resistance to methylmercury in Saccharomyces cerevisiae through interaction with other proteins such as
Fkh1, Rts1, and Msn2. Biochem. Biophys. Res. Commun. 330, 378–385 (2005).
8. Hwang, G. W., Ishida, Y. & Naganuma, A. Identification of F-box proteins that are
involved in resistance to methylmercury in Saccharomyces cerevisiae. FEBS Lett. 580, 6813–6818 (2006).
9. Hwang, G. W., Sasaki, D. & Naganuma, A. Overexpression of Rad23 confers resistance to methylmercury in
Saccharomyces cerevisiae via inhibition of the degradation of ubiquitinated proteins. Mol. Pharmacol. 68, 1074–1078 (2005).
10. Miura, N. et al. Overexpression of L-glutamine:D-fructose-6-phosphate
amidotransferase provides resistance to methylmercury in
Saccharomyces cerevisiae. FEBS Lett. 458, 215–218 (1999).
11. Gaever, G. et al. Functional profiling of the Saccharomyces cerevisiae genome. Nature 418, 387–391 (2002).
12. Kucharczyk, R. & Rytkö, J. Saccharomyces cerevisiae—a model organism for the
studies on vascular transport. Acta. Biochim. Pol. 48, 1025–1042 (2001).
13. Rabiger, C., Rusten, T. E. & Stenmark, H. Protein sorting into multivesicular
endosomes. Curr. Opin. Cell Biol. 15, 446–455 (2003).
14. Wang, C. W., Stromhaug, P. E., Shima, J. & Klionsky, D. J. The Ccz1-Mon1 protein
ubiquitin-interacting motif. J. Cell Biol. 163, 237–243 (2003).