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Genome-wide toxicogenomic study of the lanthanides sheds light on the selective toxicity mechanisms associated with critical materials

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Lanthanides are a series of critical elements widely used in multiple industries, such as optoelectronics and healthcare. Although initially considered to be of low toxicity, concerns have emerged during the last few decades over their impact on human health. The toxicological profile of these metals, however, has been incompletely characterized, with most studies to date solely focusing on one or two elements within the group. In the current study, we assessed potential toxicity mechanisms in the lanthanide series using a functional toxicogenomics approach in baker's yeast, which shares many cellular pathways and functions with humans. We screened the homozygous deletion pool of 4,291 *Saccharomyces cerevisiae* strains with the lanthanides and identified both common and unique functional effects of these metals. Three very different trends were observed within the lanthanide series, where deletions of certain proteins on membranes and organelles had no effect on the cellular response to early lanthanides while inducing yeast sensitivity and resistance to middle and late lanthanides, respectively. Vesicle-mediated transport (primarily endocytosis) was highlighted by both gene ontology and pathway enrichment analyses as one of the main functions disturbed by the majority of the metals. Protein–protein network analysis indicated that yeast response to lanthanides relied on proteins that participate in regulatory paths used for calcium (and other biologically relevant cations), and lanthanide toxicity included disruption of biosynthetic pathways by enzyme inhibition. Last, multiple genes and proteins identified in the network analysis have human orthologs, suggesting that those may also be targeted by lanthanides in humans.

toxicogenomics | lanthanides | endocytosis | endosomes | *Saccharomyces cerevisiae*

Since their discovery, lanthanides have presented both difficulty and opportunity for researchers. As a series, these elements behave rather similarly: most of them form +3 ions in aqueous solution (1), prefer highly electronegative anionic ligands (2), and form insoluble hydroxide precipitates at neutral pH if not otherwise complexed (3). Although the chemical similarities between these elements made their initial isolation and characterization a significant challenge, they now have unique applications in industry and medicine. Several lanthanides have become critical materials for many clean and sustainable energy technologies that will drive the future of our societies and are used, for example, in the production of batteries, magnets, motors, and other electronic components (4); low-concentration mixtures of lanthanides are used in Chinese agriculture to increase body weight gain among livestock (5, 6); lanthanum carbonate (sold under the commercial name of Fosrenol) is a noncalcium phosphate binder used to control hyperphosphataemia (7); and gadolinium is employed in diagnostic medicine, as an essential component of MRI contrast agents (8, 9).

The growing use of lanthanides has increased the potential for human exposure to large concentrations of these metals, requiring more detailed investigations into their toxicological properties. For instance, administration of gadolinium-based contrast agents has been associated with the development of nephrogenic systemic fibrosis in patients with compromised renal function (10–12). Moreover, accumulation of gadolinium in the brains of patients who received repeated doses of gadolinium-based contrast agents has also been reported (13). Despite the current ubiquity of lanthanides, their toxicological profile has been incompletely characterized because until recently they were considered to be of low toxicological concern (14, 15). Previous toxicity studies primarily focused on lanthanum or cerium (and, to a lesser extent, neodymium and gadolinium), with the notion that these metals were representative of the series (14, 16–18). However, to our knowledge, no comprehensive mechanistic assay has been conducted to evaluate metal toxicity across the series, and little is known about what toxicological mechanisms may be shared by the different lanthanides.

Saccharomyces cerevisiae is one of the best-characterized model organisms (19, 20), and there are many tools available for analyzing its genomic data (21–23). For example, yeast functional toxicogenomic

Significance

The growing use of lanthanides in various industries has increased the potential for human exposure to large concentrations of these heavy metals throughout the life cycle of new technologies, requiring more detailed investigations into their toxicological properties. The eukaryote model organism *Saccharomyces cerevisiae* is ideal to apply functional toxicogenomics tools at the system level and probe fundamental cellular functions disrupted by rare-earth metals. A comprehensive mechanistic assay was conducted to evaluate toxicity across the entire lanthanide series and provide information about which toxicological mechanisms may be shared by the different metals. We identified distinct characteristic behaviors between early and middle/late lanthanides, highlighting the powerful discrimination capabilities of natural systems and pointing to detailed mechanistic pathways associated with lanthanide exposure.

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The authors declare no competing interest.

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screening is a powerful tool for investigating cellular mechanisms of cytotoxicity (24, 25). This method makes use of the yeast deletion libraries generated by the Yeast Deletion Project (26), a consortium of researchers across the United States and Canada, to establish relationships between genes and chemical exposures. Researchers used heterozygous and homozygous deletion pools of barcoded yeast strains to derive mechanistic toxicological information about a wide array of chemicals, pharmaceuticals, metals, and biological compounds (27, 28). As eukaryotes, yeast and humans share many cellular pathways and functions, and many components of cell biology identified in *S. cerevisiae* have homologs in human biology (29–31). Consequently, functional toxicogenomic screening offers unique opportunities to evaluate the mechanisms of cytotoxicity and general biological activity across the lanthanide series in yeast and explore potentially conserved mechanisms in humans.

Here, we identify fundamental cellular functions disrupted by lanthanides using functional toxicogenomics in *S. cerevisiae*. The metals studied had distinct behaviors: early lanthanides showed limited unique functional effects, while middle and late lanthanides had prominent and distinct ones. Although the functional effects of each lanthanide were different and suggested some

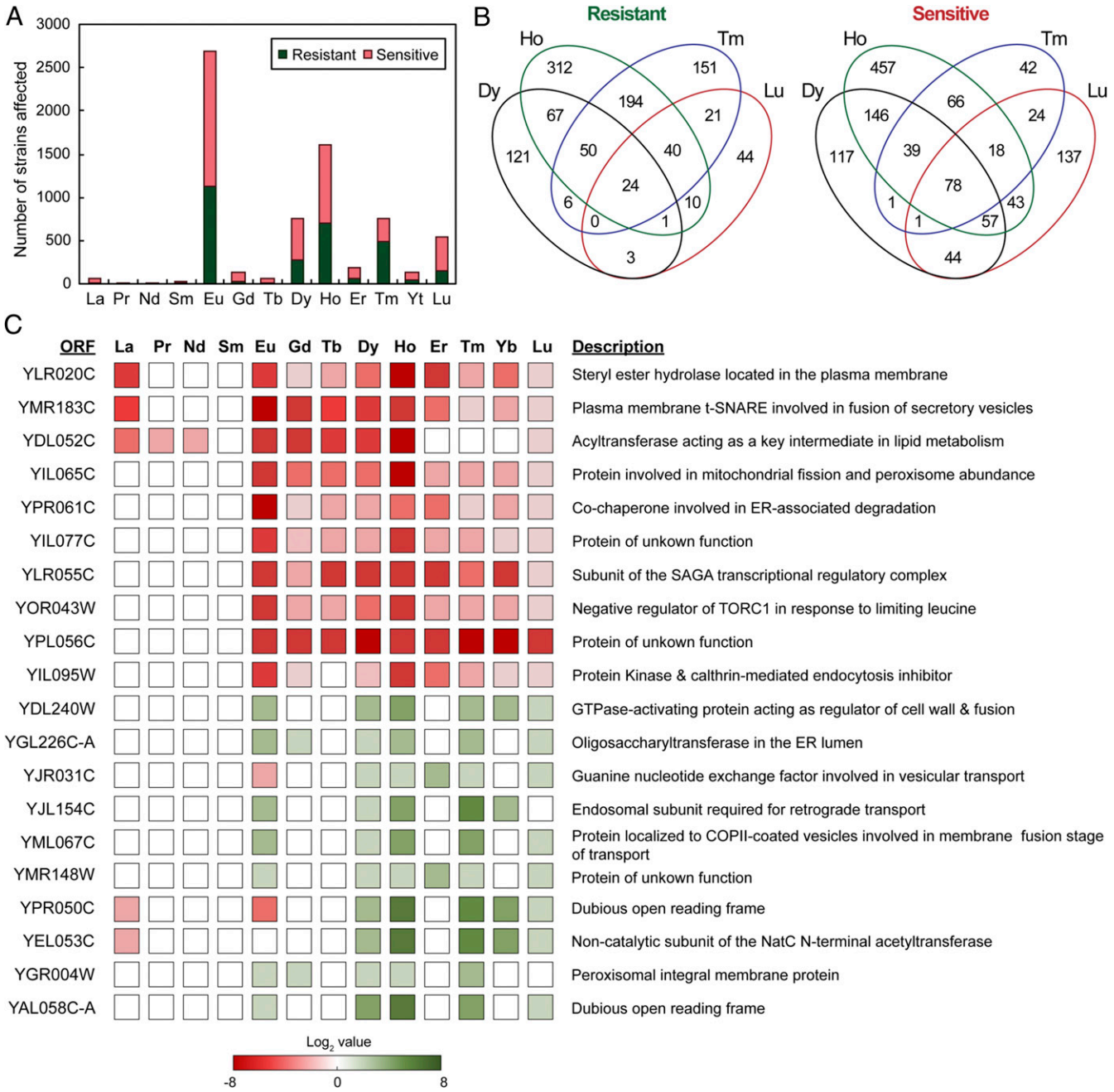

Fig. 1. Sensitive and resistant deletion strains identified by DSSA. (*A*) Total number of strains identified after being exposed to concentrations of lanthanides equivalent to IC20 for 15 generations. (*B*) Venn diagrams of lanthanides with the most sensitive and resistant strains. (*C*) Genes most affected by exposure to lanthanides and corresponding growth variation in $\log_2$ scale. These genes include the top 10 whose deletion induced sensitive strains and the top 10 whose deletion promoted resistance to lanthanides at IC20.

very efficient element discrimination by endogenous molecules, we observed a few common trends. In particular, vesicle-mediated transport (primarily endocytosis) was perturbed by the majority of the lanthanides tested. Moreover, protein–protein network analysis suggested that lanthanides mimic calcium ions, interacting with calcium-binding proteins and disrupting processes regulated by this cation. Finally, several of the highly interconnected proteins targeted by multiple lanthanides in the network analysis are conserved in humans, suggesting their roles in the origin of the human health issues associated with lanthanide exposure and opening many directions for the determination of mechanisms associated with toxicity.

Results and Discussion

Identification of Genes Required for Sensitivity and Tolerance to Lanthanides by Functional Profiling. The concentration at which wild-type yeast growth is inhibited by 20% (IC20) was initially determined for each lanthanide (*SI Appendix*, Fig. S1). IC20 is frequently used in functional toxicogenomics because it allows the identification of subtle, compound-specific biological effects rather than general, nonspecific cell-death pathways (27, 28). All IC20 values were found between 70 and 170 μM, which corresponds to the same concentration range as that of lanthanides in other biologically relevant systems, such as gadolinium in the blood of patients with kidney disfunction after the administration of gadolinium-based contrast agents (32). All lanthanides were screened in our study except for cerium, which is the only lanthanide with a +4 stable oxidation state under physiological conditions, and promethium, whose isotopes are all radioactive. Pools of yeast homozygous diploid deletion mutants (n = 4,291) were grown with IC20 concentrations of lanthanides (one treatment per lanthanide, 13 different treatments in total) for 15 generations. Differential strain sensitivity analysis (DSSA) (33) was used to identify the strains whose growth was inhibited (sensitive strains) or increased (resistant strains) in the presence of metal. Three clear trends (Fig. 1*A*) were observed when classifying the number of strains affected by each lanthanide: 1) Eu affected the growth profiles of 2,686 out of 4,291 strains (more than 60% of all strains); 2) Dy, Ho, Tm, and Lu showed moderate effects, affecting between 545 and 1,602 mutants (~13 to 37% of all strains); and 3) the rest of the metals had relatively lower biological impact, since each of them altered growth in fewer than 200 strains (<5% of the pool). We considered both sensitive and resistant strains in our analysis. Although the growth of many strains was affected by at least one metal, only a few were altered by multiple lanthanides as observed in the Venn diagrams of the lanthanides with the most sensitive and resistant strains (Fig. 1*B*). *SI Appendix*, Dataset S1 lists all the pooled strains and their $\log_2$-fold growth change in the presence of the individual lanthanide metals compared to controls. Fig. 1*C* shows the top 20 deletion mutants that displayed sensitivity (negative $\log_2$ value) or resistance (positive $\log_2$ value) to the greatest number of lanthanides. Although these genes regulate a wide range of processes, 5 (of the 20) were involved in vesicle-mediated transport, and 7 were related to enzymes.

Gene-Ontology Enrichment Analysis Highlights Biological Attributes Required for Lanthanide Resistance and Sensitivity. The strains highlighted by DSSA were analyzed using gene-ontology (GO) enrichment analysis, which discriminates overrepresented gene groups, identified by GO terms, based on their functional characteristics (34). Based on the number of significantly overrepresented (P value < 0.05) GO terms for each metal, the lanthanide series can be separated into three major groups (Fig. 2): early lanthanides (from La to Eu) that show a low number of enriched GO terms, middle lanthanides (from Gd to Dy) that have a high number of sensitive GO attributes, and late lanthanides (from Ho to Lu) that have a high number of resistant GO groups. Within the late lanthanides, however, Er and Yb show a limited number of overrepresented GO terms. Notably, Eu affects many strains (Fig. 1*A*), but these deletion mutants include only a small number of overrepresented GO terms, suggesting that at IC20 concentration, this metal affects many aspects of cell biology in a nonspecific manner. Eu is the lanthanide with the most accessible divalent chemistry (higher standard reduction potential) (35), which may contribute to its greater involvement in chemical bonding with biological receptors. Although the exact reasons for the three different trends within the lanthanide series are not fully understood, distinct behaviors between lighter and heavier lanthanides had been previously observed in other biological systems, including bacteria (36, 37). The metal selectivity of endogenous receptors in bacteria has been explained based on the balance between affinity (Lewis acidity of lanthanides increases with atomic number) and coordination chemistry (early lanthanides can accommodate higher coordination numbers) (37); both properties are also likely participating in the trends observed in our study. Furthermore, the transition from low to high numbers of enriched GO terms occurred at Gd, which was consistent with the "gadolinium break," a discontinuity in the lanthanide series observed for multiple properties, including ionic radii, stability constants, and solvent extraction equilibria (38, 39). Thus, even though lanthanides have similar properties, their different

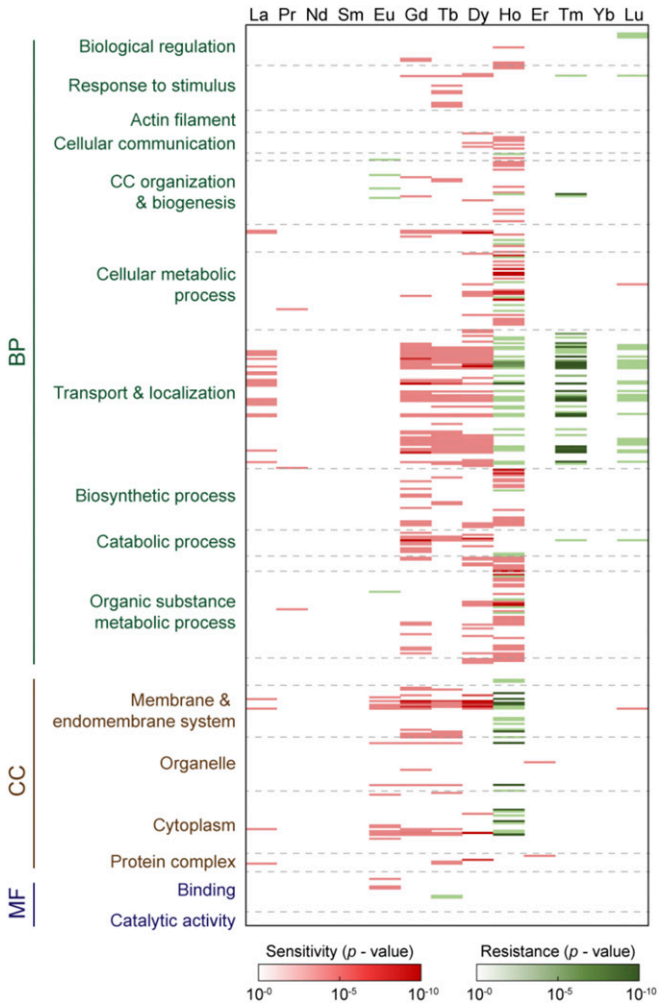

Fig. 2. GO enrichment analysis of strains identified by DSSA. Heat map of different overrepresented GO terms based on their adjusted P value. BP, CC, and MF refer to the three GO domains: biological process, cellular component, and molecular function.

chemistries and ionic radii (40) seem to affect their interaction with yeast.

In total, 269 different overrepresented GO terms were identified (*SI Appendix*, Datasets S2 and S3), of which 92 were affected by more than one lanthanide. Of those 92 terms, 41 (44.6%) were transport or localization GO terms, and 11 (12.0%) were membrane or endomembrane GO terms. Analysis of the overlapping groups associated with yeast resistance to Ho, Tm, and Lu (the lanthanides with the most resistant GO terms) confirmed the predominance of transport and localization categories (Fig. 3*A*). That same category (Fig. 3*B*) was also linked to yeast sensitivity to Gd, Dy, and Tb (three of the four lanthanides with the most sensitive GO terms). The behavior of Ho deviated from the other late lanthanides as its sensitive GO terms were spread across multiple categories. The three different trends for early, middle, and late lanthanides shown in Fig. 2 were also observed among the 20 most-overrepresented GO terms (Fig. 3*C*), which were within transport and localization, membrane and endomembrane, and cytoplasm categories. Eight of these GO terms were directly involved in vesicles and vesicle/endosome-mediated transport.

Pathway Enrichment Analysis Identifies Endocytosis as Primary Path Affected by Lanthanides. To further explore the biological interactions between lanthanides and *S. cerevisiae*, we performed pathway enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (41). Unlike GO, which examines genes independently, pathway enrichment analysis considers how genes interact with one another to form pathways, providing additional mechanistic insights (42). Eight KEGG pathways were significantly overrepresented (adjusted P value < 0.05, Fig. 4) among the genes identified by DSSA. Endocytosis was enriched for seven lanthanides (i.e., Eu, Gd, Tb, Dy, Ho, Tm, and Lu), which was consistent with our GO analysis that highlighted multiple overrepresented groups related to vesicle/endosome-mediated transport. Moreover, the different behaviors of early, middle, and late lanthanides were also observed in KEGG endocytosis pathway. These results were consistent with a previous study that reported endocytosis being disrupted by lanthanides in plants (43). Other pathways, such as ribosomal translation, mitogen-activated protein kinase (MAPK) signaling, and n-glycan biosynthesis, were also enriched for multiple lanthanides.

Protein–Protein Interaction Network Analysis Highlights Endosomal Sorting Complexes Required for Transport in Yeast Response to Lanthanides. Both GO and pathway enrichment analyses distinguished vesicle-mediated transport, particularly endocytosis, as one of the main targets of lanthanides. These analyses, however, did not identify the mechanism by which endocytosis and other pathways were disrupted. Hence, we performed protein–protein interaction network analysis to observe whether the effects of lanthanides could be associated to specific protein interactions. We mapped the products of knocked-out genes from the strains identified by DSSA to the STRING database (44) of *S. cerevisiae* protein–protein interactions and identified several protein clusters. Because we wanted to study general trends across the lanthanide series rather than element-specific mechanisms, we initially considered both sensitive and resistant strains that were affected by five or more lanthanides ($n = 200$). The resulting protein–protein interaction network presented one primary subnetwork made of highly interconnected proteins with 8 to 15 degrees each (Fig. 5*A*).

The whole network was assessed for significantly overrepresented KEGG pathways, and endocytosis was the most significant (P value of $1.6 \cdot 10^{-6}$), corroborating our previous GO and pathway enrichment analyses. Furthermore, 12 out of 15 proteins associated with endocytosis were in the primary subnetwork (Fig. 5*B*), highlighting their importance in the yeast response to lanthanides. Of these 12 proteins, 10 (i.e., VPS25, VPS36, VPS28, SNF7, SNF8, VPS24, DID4, DID2, VTA1, and VPS60) are part of the endosomal

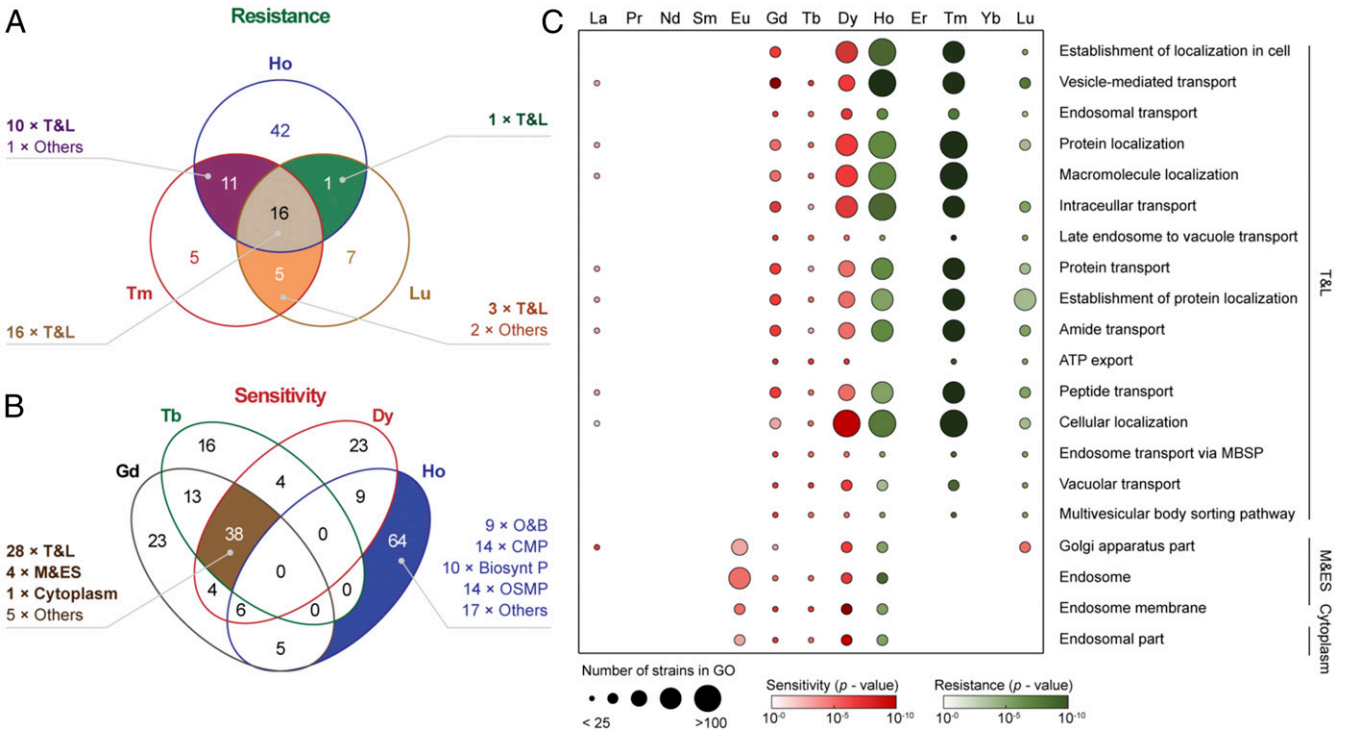

Fig. 3. Overlapping GO terms between different metal treatments. Venn diagrams for (*A*) resistance and (*B*) sensitive GO terms. The majority of overlapping terms are within transport and localization (T&L), membrane and endomembrane system (M&ES), or cytoplasm GO groups. (*C*) Top 20 overrepresented GO terms across the lanthanide series. The following abbreviations were used in the figure: O&B (cellular organization and biogenesis), CMP (cellular metabolic process), Biosynt P (biosynthetic process), OSMP (organic substance metabolic process), and MBSP (multivesicular body sorting pathway).

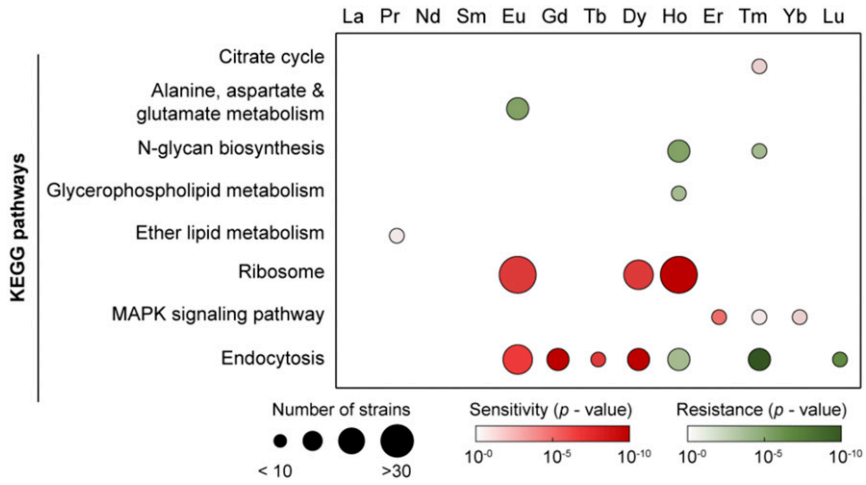

Fig. 4. Pathway enrichment analysis of strains identified by DSSA. Endocytosis was the KEGG pathway significantly affected by more lanthanides.

sorting complexes required for transport (ESCRT) machinery, which is involved in the formation of multivesicular bodies (a class of endosomes), and the sorting of certain proteins (45). The ESCRT system is also responsible for activating multiple yeast responses to high intracellular concentrations of calcium (46) and copper (47). Previous studies have shown that yeast with deletion mutations in some of the genes that code ESCRT proteins are highly sensitive to calcium exposure (48). Part of ESCRT response to calcium involves RIM101 activation (46), which was also a highly interconnected node (14 connections) in our network analysis. Besides endocytosis, the other overrepresented KEGG pathway in the network was mitophagy (P value of $4.2 \cdot 10^{-5}$), a process that involves the selective degradation of mitochondria, which reduces the amount of reactive oxygen species produced by the yeast (49, 50). One of the main causes of heavy-metal toxicity is oxidative stress, which originates from accumulation of high levels of oxidizing species that damage intracellular components (51). Thus, mitophagy is the yeast attempt to decrease endogenous oxidizing species to compensate the metal-induced oxidative stress (52). The proteins related to mitophagy, however, were scattered rather than clustered throughout the network, suggesting lower importance compared to endocytosis in the yeast response to lanthanides. The other nodes with the most connections outside the endocytosis subnetwork included YPT6 (10 degrees), a GTPase enzyme involved in endosome-to-Golgi, intra-Golgi, and retrograde Golgi-to-endoplasmic reticulum transport (53), and HOG1 (9 degrees), a protein within the osmoregulation-related MAPK pathway that binds to the calcium-binding protein calmodulin (54). These results were consistent with previous in vitro studies that showed similarities between the biological coordination chemistry of calcium and lanthanides (37), with f-block elements competing for calcium binding sites in transport proteins (55–57) and ion channels (58).

Next, we assessed why some mutations promoted yeast sensitivity to lanthanides (highlighting genes involved in detoxification pathways), while others induced tolerance (highlighting genes being targeted by lanthanides that caused toxicity). We performed protein–protein interaction network analysis of the top gene deletions that promoted sensitivity and resistance separately. The network associated with sensitive mutations contained proteins related to three different detoxification responses: vesicle-mediated transport, cation homeostasis, and mitophagy (Fig. 5*C*). Vesicle-mediated transport likely participated in the lanthanide discharge path and included the following proteins: 1) ESCRT proteins (SNF7, SNF8, VPS25, VPS28, and VPS36), which mediate in vesicle formation and calcium homeostasis (46); 2) GARP proteins (VPS51 and VPS52) that participate in vesicle fusion to the Golgi apparatus and vesicle formation in the cytoplasm to the vacuole targeting pathway; and 3) COG proteins (COG5, COG6, and COG8), which mediate in vesicle fusion to the Golgi apparatus and intra-Golgi trafficking (59). Taken together, the network analysis seemed to indicate that vesicle-mediated response to lanthanides used the Golgi apparatus (and perhaps the vacuole) as transient storage during metal discharge. It is worth noting these two organelles serve as storage to other biologically relevant metals, including calcium, manganese, and iron (60, 61). Regarding the proteins involved in preserving cation homeostasis, these included the following: 1) calcium-binding proteins, such as calcium channels (CCH1 and MID1) and calcium signaling pathways (CNB1 and CRZ1); 2) Nramp proteins (SMF1 and SMF2), which transport divalent and trivalent cations (62, 63); and 3) MAPK pathway-related proteins (HOG1, SSK1, SSK2, and PBS2) that participate in yeast osmoregulation (64). These results were consistent with previous studies that showed yeast repurposing osmoregulatory paths (used to control the osmotic pressure of biologically relevant cations) to respond to heavy-metal exposures (51). The third response highlighted in the network was mitophagy, which decreases the oxidative stress induced by the metal (52). Thus, protein–protein network analysis indicated that yeast response to lanthanides included proteins from three different paths, two responsible for controlling lanthanide levels in yeast (vesicle-mediated transport and metal homeostasis) and one involved in decreasing metal-induced damage (mitophagy).

Regarding the network of genes whose absence promoted tolerance, it had two significantly overrepresented KEGG pathways (Fig. 5*D*): *N*-glycan biosynthesis (P value of $3.9 \cdot 10^{-4}$), which was consistent with our previous KEGG pathway analysis (Fig. 4), and endocytosis (P value of $1.2 \cdot 10^{-3}$), which included VPS35 and PEP8 proteins, whose absence had also been reported to improve tolerance to other transition metals, such as nickel cations (65, 66). Hence, although endocytosis was associated with both lanthanide resistance and sensitivity, the specific proteins that participated in each event were different. As to the proteins related to *N*-glycan biosynthesis, they included transferases (ALG5, ALG6, ALG8, and OST5) and a glucosidase (ROT2). Lanthanides targeting enzymes and disrupting their activities was consistent with previous studies that reported heavy metals directly binding to enzymes (primarily through cysteines), causing function inhibition, which led to yeast toxicity (51).

Lastly, 11 genes coding highly interconnected proteins (with 10 or more degrees) in the network map are conserved in humans (Table 1). The majority of genes are endosome and endocytosis

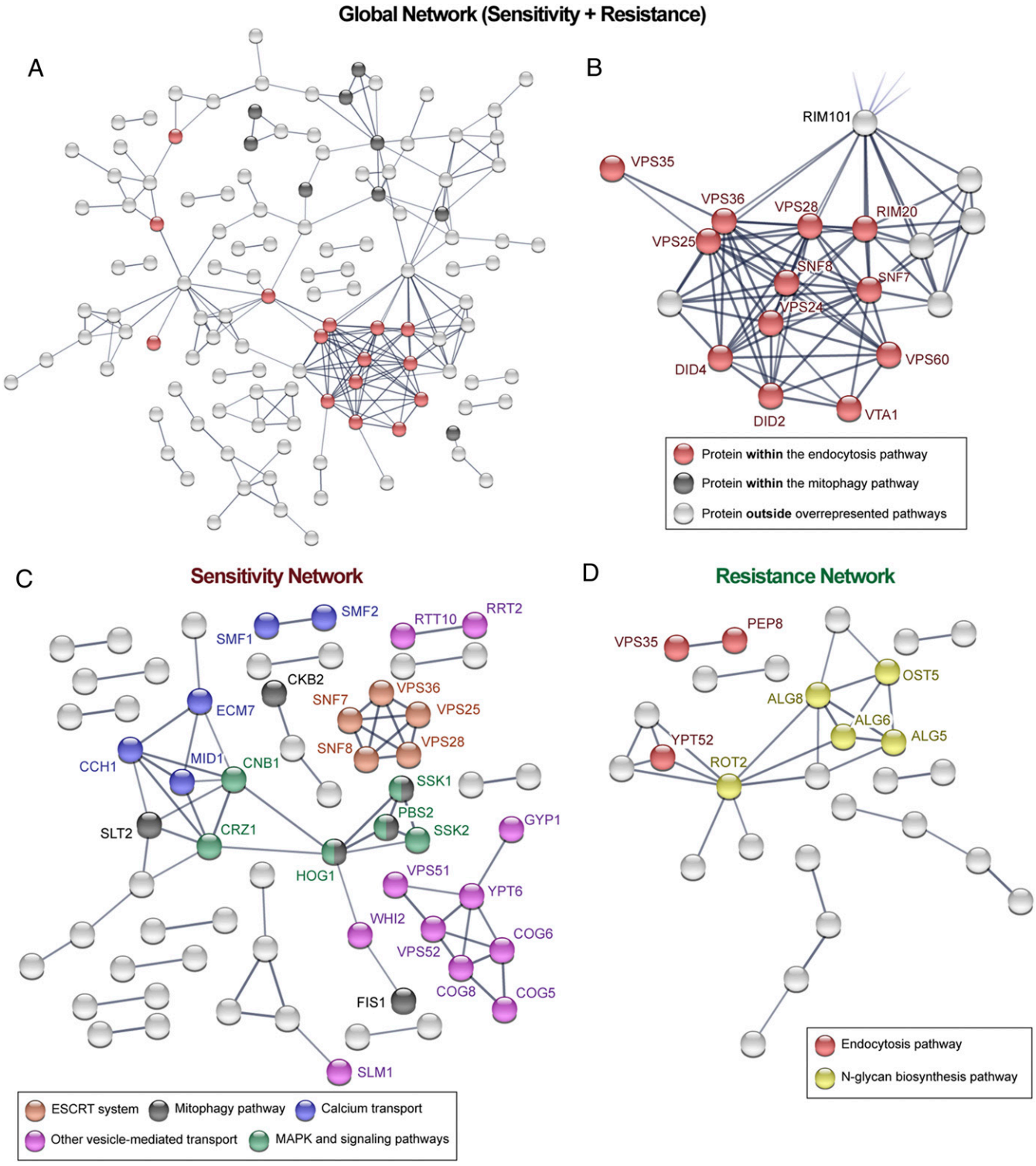

Fig. 5. Protein–protein interaction network analysis identifies mechanisms of lanthanide interaction with yeast. (*A*) Global network of proteins coded by the top 200 genes identified by DSSA. Proteins within the KEGG endocytosis pathway are highlighted in red. (*B*) Main subnetwork, which contains the majority of endocytosis pathway proteins. (*C*) Network of proteins coded by genes whose deletion promoted sensitivity to lanthanides. All genes affected by at least five lanthanides were considered ($n = 134$). (*D*) Network of proteins coded by genes whose deletion promoted resistance to lanthanides. All genes affected by at least four lanthanides were considered ($n = 71$). Some protein names, including those of proteins without connections, are removed for clarity (refer to *SI Appendix*, Figs. S2–S4 for fully labeled networks). The network analysis was performed with STRING and a cutoff for confidence interactions of 0.70 (high confidence).

related, including nine encoding ESCRT components. Therefore, it is possible that those gene products play a role in lanthanide toxicity in humans.

Conclusions

In summary, we employed functional toxicogenomics to identify the biological functions and mechanisms that are disrupted by

Table 1. Human orthologs of genes targeted by lanthanides in yeast

	Yeast gene	Human gene ortholog	Human protein
ESCRT system	SNF7	CHM4A	Charged multivesicular body protein 4a
	VPS36	VPS36	Vacuolar protein-sorting–associated protein 36
	VPS28	VPS28	Vacuolar protein-sorting–associated protein 28 homolog
	VPS24	CHMP3	Charged multivesicular body protein 3
	DID4	CHMP2A	Charged multivesicular body protein 2a
	VPS25	VPS25	Vacuolar protein-sorting–associated protein 25
	SNF8	SNF8	Vacuolar-sorting protein SNF8
	DID2	CHMP1A	Charged multivesicular body protein 1a
	VPS60	CHMP5	Charged multivesicular body protein 5
Other endosome-related proteins	RIM20	PDCD6IP	Programmed cell death 6-interacting protein
Others	YPT6	RAB6B	Ras-related protein Rab-6B

lanthanides in *S. cerevisiae*. Both GO and pathway enrichment analyses distinguished vesicle-mediated transport, particularly endocytosis, as one of the main pathways affected by lanthanides. Moreover, three different trends were observed within the series: early lanthanides showed a small number of significant GO terms, middle lanthanides were highly enriched in sensitive GO categories, and later lanthanides were predominantly associated with resistant GO terms. Protein–protein interaction network analysis indicated that multiple proteins involved in biological response to calcium participate in yeast response to lanthanides, with ESCRT machinery in the endosomes being one of the most significant. The data suggest that although lanthanides can, as a group, mimic calcium and disrupt calcium-regulated processes, element-specific effects among the series were observed, likely as a consequence of specific metal discrimination at the molecular level. Lastly, several of the genes and proteins highlighted in the network analysis are conserved in humans, suggesting that these may also be targeted by lanthanides in humans.

Materials and Methods

Diploid yeast deletion strains (BY4743 background) used for functional profiling were cultured for 15 generations in yeast extract–peptone-dextrose media at different lanthanide concentrations in an in-house–built automated dispensing system robot. Detailed materials and methods are provided in *SI Appendix*, including materials and reagents, yeast strains and culture protocols, and procedures for functional screening of the yeast genome, including DNA extraction, amplification, and sequencing, as well as methodologies for DSSA.

Data Availability. All study data are provided in the article or as datasets. Dataset S1 includes a list of all pooled strains and their log2-fold growth change in the presence of the individual lanthanide metals compared to controls. Dataset S2 includes a list of different overrepresented sensitive GO terms across the lanthanide series and their adjusted *P* values. Dataset S3 includes a list of different overrepresented tolerant GO terms across the lanthanide series and their adjusted *P* values.

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