A network of conserved proteases known as the intramitochondrial quality control (IMQC) system is central to mitochondrial protein homeostasis and cellular health. IMQC proteases also appear to participate in establishment of signaling cues for mitochondrial-to-nucleus communication. However, little is known about this process. Here, we show that in Saccharomyces cerevisiae, inactivation of the membrane-bound IMQC protease Oma1 interferes with oxidative-stress responses through enhanced production of reactive oxygen species (ROS) during logarithmic growth and reduced stress signaling via the TORC1-Rim15-Msn2/Msn4 axis. Pharmacological or genetic prevention of ROS accumulation in Oma1-deficient cells restores this defective TOR signaling. Additionally, inactivation of the Oma1 ortholog in the human fungal pathogen Candida albicans also alters TOR signaling and, unexpectedly, leads to increased resistance to neutrophil killing and virulence in the invertebrate animal model Galleria mellonella. Our findings reveal a novel and evolutionarily conserved link between IMQC and TOR-mediated signaling that regulates physiological plasticity and pancellular oxidative-stress responses.

Mounting evidence indicates that mitochondrial processes are tightly integrated with cellular signaling pathways (1–3). Such integration enables rapid modulation of mitochondrial functions upon homeostatic fluctuations in the intra- and extracellular environment and adaptation of cellular metabolism in response to changes within the mitochondrion. Studies in Saccharomyces cerevisiae have been instrumental for the elucidation of evolutionarily conserved aspects of mitochondrion-linked signaling. These analyses also provided insights into the mechanisms by which cells in general and mitochondria in particular are protected from oxidative insults (4).

Reactive oxygen species (ROS), a by-product of mitochondrial respiration, can damage a number of biological molecules, thereby affecting mitochondrial and cellular well-being (5, 6). Accumulating ROS-elicited oxidative stress and damage have long been known to be detrimental factors in many pathologies and aging (5, 7, 8). On the other hand, many ROS are now recognized as signaling molecules essential in multiple pathways, including mitochondrial-nucleus communication and longevity regulation (2, 6). The phenomenon, known as ROS adaptation, preconditioning, or hormesis, is believed to be one of the ways by which reduced signaling via the TOR pathway leads to stress-protective effects (2, 9). This effect partly stems from the derepression, through TOR inhibition, of the downstream signaling branch of the TOR pathway that includes the kinase Rim15 and a stress/nutrient signaling node containing the transcription factors Gis1 and the functionally redundant Msn2/Msn4 pair (4, 10). ROS adaptation requires respiration and is temporally restricted to logarithmic and diauxic growth phases (11).

A network of conserved proteases known as intramitochondrial quality control (IMQC) is central to normal mitochondrial activities (12). Studies in S. cerevisiae established that a specific IMQC protease, Oma1, is important in mitochondrial stress tolerance (13). This ATP-independent protease resides in the inner mitochondrial membrane (IM) as a high-mass complex and is dormant under physiological conditions (13–16). Conditions of mitochondrial stress, including acute treatment with H2O2 or respiratory uncoupling, lead to rapid activation of Oma1 through destabilization of the Oma1 oligomer (13, 16). Consistent with the protease’s stress response role, yeast cells lacking functional Oma1 are vulnerable to oxidative stress (13). However, in the context of cellular physiology, it remains unclear how Oma1 promotes resistance to oxidative insults.

Here, using the S. cerevisiae model, we demonstrate that loss of Oma1 leads to elevated ROS production during active growth. Genetic and biochemical analyses indicate that ROS emitted by Oma1-deficient mitochondria act as an adaptation signal that impinges on the conserved TORC1-Rim15-Msn2/Msn4 signaling pathway regulating stress survival responses. In addition, we show that inactivation of the Oma1 ortholog in the human fungal pathogen Candida albicans also modulates TOR signaling. This unexpectedly promotes survival of the pathogen in human neutrophils and enhances its virulence. Our findings reveal a novel link between IMQC and ROS-modulated TOR signaling in the
regulation of physiological plasticity and pancellular stress responses.

**MATERIALS AND METHODS**

Yeast strains, vectors, and media. The *S. cerevisiae* strains used in this study were isogenic to the W303 genetic background. A detailed description of the strains is provided in Table 1. Deletions of the indicated ORFs were performed with a URA3MX cassette as described previously [13] and followed by marker elimination through culturing on 5-fluoroorotic acid (5-FOA) plates. All deletions were confirmed by PCR. The pRS415-OMA1-6×His-Myc, pRS415-OMA1-6×His-Myc H203A [13], pADH1-Msn2-GFP [17], and pCUP1-6×Myc-Cki1 (2–200) [18] plasmids were used to transform the indicated strains.

Cells were cultured in standard yeast extract-peptone (YP) or synthetic complete (SC) medium supplemented with appropriate nutrients and either 2% glucose, 2% galactose, or 2% glycerol-lactate as sole carbon source.

**Construction of a *C. albicans* oma1ΔΔ mutant.** *C. albicans* strain SC5314 (a clinical isolate from the blood; clade 1) [19] was used to generate the oma1ΔΔ mutant by utilizing a NATT1-Clox deletion cassette.
developed by Shahana et al. (20). The mutant was obtained in two subsequent transformation rounds. First, an oma1::NAT1-Clox cassette was PCR amplified from the plasmid pLNMCL (20) with 92-bp flanking homology to the 5′ upstream region and 96-bp flanking homology to the 3′ downstream region of the OMA1 gene (primers OMA1_NAT1_F1, 5'-ATT TATCCTGGAAATTTGTCACCGCACCACCCCTTTAACATTGCTAC AGTGGCATAATACATTGTCGAGCGATACGTGAGAAAAT ATTATGCGCCGATGAAGTATTATAA-3′, and OMA1_NAT1_R1, 5′-TTGATGTCGTAATGTAATACATTACAAATTTGTTGGTC CATGATATGATTGATGATTCTTCCACAAATGGCACCACCA AACACCTTTAGACGAAATACCCCCACTAATA-3′). The transformants with one inactivated allele were selected on nourseothricin (Nou) containing rich medium with 2.5 mM methionine and 2.5 mM cysteine. The genotypes of heterozygous mutants were confirmed by diagnostic PCR. Single colonies were then restreaked on yeast extract–peptone–dextrose (YPD) medium without supplementation to reserve the oma1::NAT1-Clox cassette. The further selected Nou-susceptible (Nou') mutants were used for the next round of transformation with the PCR-amplified oma1::NAT1-Clox cassette (primers OMA1_NAT1_F2, 5′-TCT CGTGGGTTTCTATCAAACCTTTTTTCTTCTCCTGTGGTGTGGAC GATGGAAATGATTTGAGTTAGGAAAGAAGCCAAAGGATTAA CCAAAATCCGGCGTGAATTGTAATAA-3′, and OMA1_NAT1_R2, 5′-TGGATGACGACGAAAGGCTAAGAAAGAATATTACCTGAT ATTAACTGTTAAGCAATGTTAAAACTCTCAAGTCTGATCCA TGTAAACGCAATTACCCTCCTACTA-3′). The selected Nou-resistant (Nou') mutants were analyzed by PCR to confirm the disruption of both OMA1 alleles.

**Growth conditions.** Yeast cells were synchronized as described previously (21). Quantitative assessment of a strain’s survival under stress was done through determination of CFU as described previously (13). Cultures were grown to an A600 of 0.5 and treated with either 0.5 or 1 mM H2O2 for 1 h. The cells were then plated on YP-gluose plates to determine their ability to form viable colonies. Yeast cells for fluorescence microscopy were grown in synthetic selective medium and treated as described above.

**Fluorescence techniques.** For fluorescence microscopy, synchronized live cells were visualized using the Olympus IX81-FV5000 confocal laser scanning microscope at 488- or 543-nm laser lines with a 100× oil lens. Images were acquired and processed with Fluoview 500 software (Olympus America). Fluorescence-activated cell sorter (FACS) analyses were performed as described previously (15). The oxygen radicals generated by cells at various growth stages were quantitated using the fluorescent dyes dehydroethidium (DHE) (Life Sciences), MitoSox (Life Sciences), and dihydrorhodamine 123 (DHR123) (Cayman Chemical).

**Killing of C. albicans strains by neutrophils.** Polymorphonuclear neutrophils (PMNs) were isolated from peripheral blood of healthy donors by gradient density centrifugation in Histopaque 1077 and Histopaque 1119 (Sigma) as described previously (22). The isolated PMNs were resuspended in RPMI 1640 medium with fetal bovine serum (FBS) (Life Technologies) at a concentration of 10⁵ cells/ml. Neutrophil killing was assayed as described previously (23), with minor modifications. Briefly, the C. albicans cells were collected in mid-logarithmic phase by centrifugation at 4,000 × g for 5 min, counted, and resuspended in sterile phosphate-buffered saline (PBS) at a concentration of 10⁶ cells per ml. C. albicans cells (200 μl) were incubated with 200 μl of PMNs at a final ratio of 1:10 and 200 μl of RPMI medium. The cultures were incubated for 2 h at 37°C under 5% CO₂. The PMNs were lysed by addition of 100 μl of 0.25% SDS and a short incubation at 30°C for 5 to 10 min. Ice-cold water (900 μl) was added and vigorously mixed, and the mixture was incubated with 50 U of DNease I in 100 μl 10× PBS for 15 min at 30°C; 100 μl of the mixture was plated on YPD agar, and CFU were counted after 48 h at 30°C.

**G. mellonella killing assay.** Virulence assays were performed in the Galleria mellonella larva infection model. Animals were injected with 6 × 10⁶ C. albicans cells in 10 μl of PBS or with PBS alone in the last proleg. The larvae were maintained at 37°C, and viability was assessed twice daily.

**Quantitative real-time PCR.** Total RNA was prepared with an RNA purification kit (Epicentre) by following the manufacturer’s instructions. cDNA was prepared using a cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed on a Bio-Rad iCycler by using Sybr green master mix (Bio-Rad). All samples were examined in triplicate, and the values were normalized to the expression of β-actin. The values were calculated using the ΔΔCₚ method. Statistical significance was determined using the threshold cycle (Cₚ) value.

**Miscellaneous biochemical assays.** Catalase enzymatic activity was determined as described previously (24). The activity was normalized to total protein in the sample and is presented as micromoles of decomposed hydrogen peroxide per minute and milligram of protein.

Whole-cell trichloroacetic acid (TCA) extracts were generated as previously described (15). The extracts were separated by SDS-PAGE and transferred onto nitrocellulose. All the gels were run under the same experimental conditions. Immobilized polypeptides were incubated with the indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies and visualized with chemiluminescent substrates (Thermo Scientific). Antiporin, antithemagglutinin (anti-HA), anti-Kar2, anti-green fluorescent protein (anti-GFP), and anti-Pgk1 antibodies were obtained from Life Technologies, Santa Cruz Biotechnology, Abcam, and Sigma. Images were scanned and cropped to prepare the figures, with cropping lines indicated. The intensities of the bands were quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

**Statistical analyses.** Statistical analyses were conducted using Microsoft Excel. At least three independent replicates were obtained for each experiment presented. Unless otherwise specified, the results were analyzed using one-way analysis of variance (ANOVA) or an unpaired t test. P values of <0.05 were considered statistically significant.

**RESULTS**

Oma1-deficient cells are resistant to rapamycin and exhibit reduced TOR signaling. Oma1 activation in the mitochondria occurs under various stress conditions, but beyond this, much remains to be learned regarding its role in stress tolerance. To better understand the role of Oma1 in mitochondrial stress tolerance, we conducted a miniscreen aiming to test the fitness responses of the Oma1-deficient mutant to 25 varied chemical compounds known to impact the growth of yeast cells (Table 2). Our screen revealed that, unlike wild-type (WT) cells, oma1Δ cells showed enhanced resistance to compounds linked to TOR signaling, including caffeine and vanillin (25, 26) (Fig. 1A) and, most notably, the TOR pathway-inactivating antibiotic rapamycin (Fig. 1A and 2A). This effect is specific to Oma1 deficit, as loss of the other IM-bound proteases, Pcp1 and Yml1 (1-AAA), or m-AAA (via deletion of its Yta10 subunit) did not produce increased rapamycin tolerance (Fig. 2A and data not shown). Moreover, the oma1-null allele is dominant in the yml1Δ background but not in the yta10Δ background, as an oma1Δ yml1Δ double-deletion strain was also mildly resistant to rapamycin. Importantly, lack of resistance to the antibiotic oligomycin (Fig. 1B) and unaltered expression of the key multidrug resistance proteins Pdr5 and Pdr15 (Fig. 1C) indicate that the enhanced rapamycin tolerance of the oma1Δ strain is not due to the activated multidrug resistance pathway. Collectively, these observations suggest that loss of Oma1 likely exerts a negative effect on TOR signaling (27, 28).

The TOR complex 1 (TORC1) effector kinase Npr1, which exists in both hyperphosphorylated and dephosphorylated forms, exhibits a specific separation pattern on SDS-PAGE, with the former form migrating slower than the latter form (29). Published data indicate that the hyperphosphorylated state is significantly
TABLE 2 Sensitivity of oma1Δ cells to various stressors

<table>
<thead>
<tr>
<th>Compound or chemical</th>
<th>Concentration</th>
<th>Response (relative to WT control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>1 M</td>
<td>No difference</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0.8 M</td>
<td>No difference</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>100 mM</td>
<td>No difference</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>2 mM</td>
<td>No difference</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>2 mM</td>
<td>No difference</td>
</tr>
<tr>
<td>NaAsO₃</td>
<td>1 mM</td>
<td>No difference</td>
</tr>
<tr>
<td>Buthionine sulfoximine (BSO)</td>
<td>5 mM</td>
<td>No difference</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>4 mM</td>
<td>No difference</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>1 μg/ml</td>
<td>No difference</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>1 μg/ml</td>
<td>No difference</td>
</tr>
<tr>
<td>Canavanine</td>
<td>10 μg/ml</td>
<td>No difference</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
<td>4 mM</td>
<td>No difference</td>
</tr>
<tr>
<td>2-Deoxyglucose (2-DG)</td>
<td>0.1%</td>
<td>No difference</td>
</tr>
<tr>
<td>Dibutyryl cyclic AMP (dbcAMP)</td>
<td>1 mM</td>
<td>No difference</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>30 ng/ml</td>
<td>Resistant</td>
</tr>
<tr>
<td>Myriocin</td>
<td>25 μg/ml</td>
<td>No difference</td>
</tr>
<tr>
<td>Caffeine</td>
<td>15 mM</td>
<td>Resistant</td>
</tr>
<tr>
<td>Vanilllin</td>
<td>10 mM</td>
<td>Resistant</td>
</tr>
<tr>
<td>Concanamycin A</td>
<td>5 μM</td>
<td>No difference</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>0.1 μg/ml</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Idoacetamide</td>
<td>30 μM</td>
<td>No difference</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>5 μM</td>
<td>No difference</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM</td>
<td>No difference</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>10 μg/ml</td>
<td>No difference</td>
</tr>
</tbody>
</table>

decreased in tor1Δ cells (29, 30). Therefore, to further corroborate reduced TOR signaling in the oma1Δ mutant, we chromatically tagged Npr1 with a 3×HA epitope tag in WT, oma1Δ, and tor1Δ cells and monitored its electrophoretic behavior by immunoblotting. We reproducibly observed a significant decrease in the abundance of hyperphosphorylated Npr1 in tor1Δ cells, as well as in WT cells treated with rapamycin (Fig. 2B and C). Importantly, the basal levels of hyperphosphorylated Npr1 were significantly lower in oma1Δ cells than in the WT (Fig. 2B and C), reflecting decreased basal TORC1 activity. The expression levels of Tor1 and Tor2 (components of the TORC1 and TORC2 complexes, respectively) were comparable between the oma1Δ strain and the wild-type control (Fig. 2D). Altogether, our data suggest that certain aspects of TORC1-related signaling are impaired in oma1Δ cells.

Loss of Oma1 impinges on the TORC1-Rim15-Msn2/Msn4 signaling axis. Loss of Oma1 may lead to elevated mitochondrial ROS production during active growth, which acts as an adaptation signal that impinges on the conserved TORC1 pathway (11). Several signaling cascades intersect with TORC1 or converge on its downstream targets (4, 31). To determine which facet of TOR signaling is impeded by Oma1 loss-associated ROS emission, we examined genetic interactions of OMA1 and genes encoding key components of TORC1-related pathways (Fig. 3A). Deletion of OMA1 in Sch9 kinase-deficient cells improved the growth of the mutant on rapamycin-containing plates (Fig. 3B, row 4), indicating the dominant nature of the oma1-null allele in the sch9Δ background. Deletions of OMA1 in combination with genes encoding retrograde pathway components (the transcription factor Rtg1 or its sensor, Rtg2) did not significantly influence the rapamycin phenotype of the cells in question (Fig. 3B, row 6, and data not shown), probably because the rtg1Δ mutant alone also exhibited resistance to the drug (Fig. 3B, line 5). Similarly, reduction of cyclic AMP (cAMP)-protein kinase A (PKA) signaling through deletion of the adenylate cyclase activator Ras2 (Fig. 3B, row 12) or enhancement of PKA activity via disruption of the CAMP phosphodiesterase Pde2 (Fig. 3B, row 14) did not influence the rapamycin resistance of oma1Δ cells. In contrast, the rapamycin resistance of oma1Δ cells was abolished by deleting either Tor1 or its downstream target, the PAS kinase Rim15 (Fig. 3B, rows 8 and 10), mimicking single deletions of these genes (32). Inactivation of Avo2, a nonessential subunit of the rapamycin-insensitive complex TORC2 (33), did not affect the rapamycin resistance of the oma1Δ strain (Fig. 3C), indicating that loss of Oma1 primarily reduces signaling via the Tor1-containing complex. In line with these observations, TORC1 appears to be a limiting factor in the oma1Δ-mediated epistatic effects, as loss of TOR1 ablated the rapamycin-resistant phenotype of the sch9Δ oma1Δ cells (Fig. 3D).

We thus conclude that modules of the TORC1-Rim15 signaling axis are located downstream of OMA1.

Because the TORC1-Rim15 axis is known to control certain aspects of pancellular stress responses (4), we examined the oxidative-stress resistance of the above-mentioned strains by testing their ability to form viable colonies following treatment with 1 mM H₂O₂ in log phase. As reported previously (13), oma1Δ cells were sensitive to H₂O₂. Consistent with the dominant nature of the oma1-null allele in the sch9Δ background, loss of SCH9 did not significantly improve the sensitivity of oma1Δ cells to H₂O₂. On the other hand, deletion of TOR1 or RIM15 in the oma1Δ background conferred H₂O₂ resistance (Fig. 3E), which was markedly increased for tor1Δ oma1Δ cells relative to the WT control. Loss of TOR1 even enhanced the H₂O₂ resistance of the sch9Δ oma1Δ strain (data not shown), further indicating that sensitization of oma1Δ cells to oxidants occurs via TORC1 signaling. Interestingly, tor1Δ oma1Δ cells also exhibited a slight, but statistically significant, increase in H₂O₂ survival relative to the tor1Δ mutant, suggesting that the relationship between Oma1 and TORC1 may be more complex and involve additional factors.

In S. cerevisiae, the TORC1-Rim15 axis orchestrates several transcription factors, including Gis1 and Msn2/Msn4 (4, 10). While the above-described results suggest that the Msn2/Msn4-mediated oxidative-stress response is altered in oma1Δ cells, it is possible that Gis1 is also involved. To distinguish between these scenarios, we analyzed the sensitivity of oma1Δ gis1Δ and oma1Δ msn2Δ msn4Δ cells to rapamycin. The loss of Gis1 (Fig. 3F, rows 9 and 10), Msn2 (Fig. 3F, rows 3 and 4), or Msn4 (Fig. 3F, rows 5 and 6) in the oma1Δ background had no appreciable effect on drug resistance. Conversely, the disruption of Msn2 and Msn4 in oma1Δ cells abolished rapamycin resistance (Fig. 3F, row 8). Moreover, the oma1Δ msn2Δ msn4Δ cells showed improved H₂O₂ survival relative to the oma1Δ mutant alone (Fig. 3G). These data indicate that deregulation of TORC1-Rim15-Msn2/Msn4 signaling is a likely contributor to several phenotypes seen in the oma1Δ strain, including rapamycin resistance and oxidative-stress sensitivity.

Oxidative-stress signaling is compromised in oma1Δ cells. To better understand the reduced tolerance of Oma1-deficient cells for oxidative stress, we monitored the expression of the two Msn2/Msn4-controlled oxidative-stress-responsive genes: catalase (CTT1) and manganese superoxide dismutase (SOD2). Whereas expression of these genes was greatly upregulated in WT cells treated with 0.5 mM H₂O₂, as reported previously (34), their expression was largely unchanged in peroxide-treated oma1Δ cells.
Furthermore, attenuated CTT1 transcript levels in the oma1Δ/H9004 mutant were reflected by reduced catalase activity following H2O2 exposure (Fig. 4B). No significant difference in basal catalase levels between WT and oma1Δ/H9004 cells was observed.

Given that Msn2/Msn4 accumulate in the nucleus upon H2O2 treatment (35), we wanted to test if localization is altered in oma1Δ cells upon cellular stress. Using confocal microscopy (CFM), we examined the nuclear accumulation of GFP-tagged Msn2 in glucose-grown cells exposed to 1 mM H2O2. Compared with the WT control, oma1Δ cells accumulated significantly more

FIG 1 Tolerance of the oma1Δ mutant for selected chemical compounds. (A) Growth of WT and oma1Δ cells on plates with or without 10 mM vanillin, 30 ng/ml rapamycin, 15 mM caffeine, and 0.1 μg/ml caspofungin. Synchronized cells were grown to mid-logarithmic stage (8 h of growth; A600 = 0.8), serially diluted, and spotted onto the respective plates. Photographs were taken after 2 (No treatment) or 4 (plus compound) days at 28°C. (B) Growth of WT and oma1Δ strains on plates containing increasing concentrations of oligomycin. The cells were handled as in panel A and spotted onto plates containing 10 μg/ml or 250 μg/ml oligomycin. Photographs were taken after 2 days of incubation at 28°C. (C) Relative expression of PDR5 and PDR15 in the indicated strains. The error bars indicate standard deviations (SD) (n = 4 biological replicates); n.s., nonsignificant by Student’s t test. AU, arbitrary units.

(4A). Furthermore, attenuated CTT1 transcript levels in the oma1Δ mutant were reflected by reduced catalase activity following H2O2 exposure (Fig. 4B). No significant difference in basal catalase levels between WT and oma1Δ cells was observed.

Given that Msn2/Msn4 accumulate in the nucleus upon H2O2 treatment (35), we wanted to test if localization is altered in oma1Δ cells upon cellular stress. Using confocal microscopy (CFM), we examined the nuclear accumulation of GFP-tagged Msn2 in glucose-grown cells exposed to 1 mM H2O2. Compared with the WT control, oma1Δ cells accumulated significantly more

FIG 2 Oma1-deficient cells are resistant to rapamycin and exhibit attenuated TOR activity. (A) Growth of the indicated strains on YPD plates with (+Rapa) or without (−Rapa) 30 ng/ml rapamycin. Synchronized cells were grown to mid-log stage (8 h of growth; A600 = 0.8). Photographs were taken after 2 (−Rapa), 4 (+Rapa), or 6 (+Rapa, Prolonged incubation) days at 28°C. (B) WT, oma1Δ, and tor1Δ strains expressing genomically tagged Npr1-HA were cultured in the presence or absence of rapamycin. Log-phase cells were harvested, lysed, and analyzed by immunoblotting with antibodies against HA epitope and Kar2 (loading control). Npr1HA and P-Npr1HA, dephosphorylated and hyperphosphorylated forms of the protein, respectively. The asterisk indicates an HA tag-cross-reacting band. (C) Quantitation of the P-Npr1-HA form in WT, oma1Δ, and tor1Δ cells. The error bars indicate standard errors of the mean (SEM) (n = 3 biological replicates). (D) Relative expression of TOR1 and TOR2 in the indicated strains. The error bars indicate SD (n = 4 biological replicates). P values were determined as for Fig. 1 (n.s., nonsignificant; *, P < 0.05 by Student’s t test). Ø, no detectable signal.
Msn2-GFP in the nucleus upon peroxide treatment (Fig. 4C and D). Importantly, Msn2-GFP steady-state levels were not altered by the oma1Δ mutation (Fig. 4E). Similar results were obtained when 0.1 mM or 0.5 mM H2O2 was used (Fig. 4F and G). To test the specificity of this response, we examined Msn2-GFP localization in response to a hyperosmotic insult. Unlike with H2O2 stress, wild-type and oma1Δ/H9004 cells showed similar levels of nuclear Msn2-GFP accumulation upon exposure to 1 M NaCl (Fig. 4H and I). This is consistent with the finding that Oma1-deficient cells show no sensitivity to NaCl. The more robust nuclear localization of Msn2-GFP in response to NaCl treatment (relative to H2O2 treatment) is likely due to the fact that Msn2/Msn4-mediated core (environmental) stress responses are better “tuned” toward more natural environmental stressors, such as osmotic shock. Taken together, these data led us to conclude that Msn2/Msn4-mediated oxidative-stress signaling is specifically compromised by Oma1 inactivation.

The TORC1 effector PP2A phosphatase contributes to abnormal Msn2 activity and impaired H2O2 tolerance in the oma1Δ mutant. Despite the enhanced oxidative-stress-induced nuclear localization of Msn2, its function appears to be impeded in the oma1Δ mutant. A recent report implicated protein phos-
phatase 2A (PP2A) in facilitating Msn2/Msn4 association with chromatin via modulation of nuclear sequestration mechanisms in response to signals from TORC1 (17). Moreover, alterations in TORC1 cues can attenuate the transcriptional activity of Msn2/Msn4 via several factors, including PP2A phosphatase (36). That is, response to H$_2$O$_2$ treatment requires not only nuclear translo-

**FIG 4** Oxidative- but not osmotic-stress response mediated via the Msn2/Msn4 signaling node is altered in oma1Δ cells. (A) Transcription changes in synchronized WT and oma1Δ cells in response to acute treatment with 0.5 mM H$_2$O$_2$. Relative transcript levels of OMA1, cytosolic catalase (CTT1), and manganese superoxide dismutase (SOD2) were assessed by quantitative PCR (qPCR) ($n = 5$ independent experiments). (B) Catalase activity in whole-cell extracts derived from the indicated cells ($n = 3$ biological replicates). The bars show mean values and SD. (C and D) Nuclear accumulation of Msn2-GFP in 1 mM H$_2$O$_2$-exposed WT and oma1Δ cells. (C) Representative images showing Msn2-GFP localization at 15 min posttreatment. (D) Quantitation of time-dependent nuclear accumulation of Msn2-GFP. The error bars indicate SD ($n = 3$, with 800 cells per biological replicate). (E) Steady-state levels of Msn2-GFP in WT and oma1Δ cell lysates. Proteins were visualized with antibodies against the GFP moiety. (F and G) Nuclear accumulation of Msn2-GFP in 0.1 or 0.5 mM H$_2$O$_2$-exposed WT and oma1Δ cells. (F) Representative images showing Msn2-GFP localization at 15 min posttreatment. (G) Quantitation of nucleus-localized Msn2-GFP. The error bars indicate SD ($n = 3$, with 800 cells per biological replicate). (H and I) Nuclear accumulation of Msn2-GFP in response to 1 M NaCl was analyzed as for panels C, F, and G. n.s., nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Student’s $t$ test.
was very sensitive to this TORC1 inhibitor (Fig. 5B, row 8), reflecting the importance of PP2A in the rapamycin resistance of Oma1-deficient cells. Moreover, combined loss of PPH21 and PPH22 in oma1Δ/H9004 cells restored their tolerance for acute H2O2 stress (Fig. 5C).

Deletion of the PP2A subunits in the oma1Δ strain resulted in even higher nuclear accumulation of Msn2-GFP reporter than in the control strains (Fig. 5D and E). These observations are consistent with the idea that PP2A functions downstream of the events leading to nuclear accumulation of Msn2/Msn4. These data indicate that the PP2A phosphatase modulates Msn2/Msn4 activity and oxidative-stress sensitivity in the oma1Δ strain.

**Transiently elevated ROS production contributes to reduced TOR signaling in Oma1-deficient cells.** Mitochondria generate cues that regulate a number of cellular processes, including oxidative-stress tolerance (11, 37–40). Mitochondrion-emitted ROS appear to be central to many of these events (6). We recently demonstrated that loss of Oma1 leads to unbalanced respiration and enhanced ROS production (41). To further explore this observation, we stained synchronized WT and oma1Δ cells at various growth stages with the superoxide (O2·−)-sensitive dye DHE and monitored their fluorescence by flow cytometry. Consistent with previous reports (11), we observed low basal levels of DHE fluorescence in exponentially growing WT cells. We also confirmed the increase in endogenous O2·− production in stationary-phase cells. However, we found that during log phase, oma1Δ cells reproducibly accumulate relatively high levels of O2·− (Fig. 6A). The superoxide levels were comparable in diauxic oma1Δ and WT cells (data not shown), indicating that ROS levels in WT cells eventually catch up to those in the oma1Δ mutant. We therefore tested ROS levels in stationary phase and found that the DHE staining in the oma1Δ mutant was comparable to that in the WT (Fig. 6A).

(continued)
partially phenocopy mutants with reduced TOR signaling (11) (Fig. 6A and B), we hypothesized that reduced TORC1 activity in oma1Δ cells may be a consequence of transiently elevated intracellular ROS.

To examine the link between elevated radicals in log-phase oma1Δ cells and deregulated TOR signaling, we tested whether preventing ROS accumulation in these cells ameliorates the rapamycin resistance of the strain. Electron transport chain (ETC) complexes are one of the primary sources of ROS in a cell (6). Thus, we compared the rapamycin growth phenotypes of oma1Δ strains with ([rho]+) and without ([rho]) mtDNA. (D to G) Growth of antioxidant-pretreated WT and oma1Δ strains. Synchronized exponential-phase cells (8 h of growth; A600 = 0.8) cultured with or without 5 mM sodium ascorbate (D) or 20 mM the ROS scavenger Tiron (F) were analyzed as described above. (E and G) Ascorbate pretreatment (E) and Tiron pretreatment (G) showing growth of the same strains analyzed in stationary phase (24 h of growth; A600 = 8.0). n.s., nonsignificant; **, P < 0.01; ***, P < 0.001 by Student’s t test.

FIG 6 Transiently elevated ROS contribute to rapamycin resistance of the oma1Δ mutant. (A and B) ROS production determined by FACS analysis of synchronized DHE-stained (A) or DHR123-stained (B) WT and oma1Δ cells at log (Log.) (A600 = 0.5 6 h postinoculation) and stationary (Stat.) (A600 = 3.0 12 h postinoculation) stages. The data show mean values and SD (n = 3 to 5 biological repeats). (C) Growth of the indicated strains with ([rho]+) or without ([rho]) mtDNA. (D to G) Growth of antioxidant-pretreated WT and oma1Δ strains. Synchronized exponential-phase cells (8 h of growth; A600 = 0.8) cultured with or without 5 mM sodium ascorbate (D) or 20 mM the ROS scavenger Tiron (F) were analyzed as described above. (E and G) Ascorbate pretreatment (E) and Tiron pretreatment (G) showing growth of the same strains analyzed in stationary phase (24 h of growth; A600 = 8.0). n.s., nonsignificant; **, P < 0.01; *** P < 0.001 by Student’s t test.
Loss of mitochondrial DNA (mtDNA) (encoding several key ETC subunits) completely abrogated the rapamycin resistance of the oma1Δ mutant (Fig. 6C). Moreover, pretreatment of log-phase oma1Δ cells (A600 = 0.5) with the ROS scavenger ascorbate (Fig. 6D) or the antioxidant Tiron (Fig. 6F) negated their ability to grow on rapamycin-containing plates. In line with the idea of hormetic-like ROS adaptation in exponential-phase cells (11), postdiauxic oma1Δ cells were unable to survive on rapamycin plates (Fig. 6E and G). This is likely due to the fact that stationary-phase oma1Δ cells do not exhibit elevated ROS (Fig. 6B); the previously reported progressive decline of respiratory function in these cells may also contribute to the loss of rapamycin resistance. Interestingly, the pretreatment of stationary WT and oma1Δ cultures with antioxidants had an advantageous effect on their growth on rapamycin plates, suggesting that dampening ROS in postdiauxic cells may be beneficial for their survival. Collectively, these data indicate that a transient increase in mitochondrial ROS modulates TORC1-related signaling in oma1Δ cells.

The Oma1 homolog is necessary for TOR signaling-mediated physiological responses in C. albicans. We asked if the impact of Oma1 upon TOR signaling is evolutionarily conserved. To explore this, we examined the role of the Oma1 ortholog in an evolutionarily distant yeast, the human pathogen C. albicans. We deleted both alleles of orf19.3827 (whose products had 45.2% sequence identity to the S. cerevisiae Oma1 protein) to construct a homozygous null mutant in the diploid yeast (Fig. 7A). Like the S. cerevisiae oma1Δ strain, the C. albicans oma1Δ/Δ mutant displayed no obvious phenotype under standard growth conditions. However, consistent with S. cerevisiae data, upon stress with 5 mM H2O2, C. albicans oma1Δ/Δ cells displayed mild growth impairment. Additionally, the survival of the strain was affected by the drug caspofungin (Fig. 7B). Like the S. cerevisiae oma1Δ strain, the C. albicans oma1Δ/Δ mutant displayed no obvious phenotype under standard growth conditions. However, consistent with S. cerevisiae data, upon stress with 5 mM H2O2, C. albicans oma1Δ/Δ cells displayed mild growth impairment. Additionally, the survival of the strain was affected by the drug caspofungin (Fig. 7B). Notably, S. cerevisiae oma1Δ/Δ cells were also found to be caspofungin sensitive in our miniscreen (Fig. 1A). Most significantly, C. albicans oma1Δ/Δ cells exhibited marked resistance to rapamycin (Fig. 7B).

TOR pathway components have been implicated as virulence-mediating factors in C. albicans (42, 43). We therefore examined the virulence of the C. albicans oma1Δ/Δ strain by two independent approaches. First, we tested the survival of WT and oma1Δ/Δ cells upon incubation with human PMNs isolated from three different donors. (C) Survival of WT and oma1Δ/Δ cells. The viable colonies were assessed after 48 h at 30°C. (D and E) Survival of G. mellonella larvae infected with the indicated strains. The larvae were injected with cells or buffer and monitored for 5 days at 37°C. Pathogen cells were reisolated and plated for survival. The viable colonies were assessed after 48 h at 30°C. (D and E) Survival of G. mellonella larvae infected with the indicated strains. The larvae were injected with cells or buffer and monitored for 5 days at 37°C. Pathogen cells were reisolated and plated for survival. The viable colonies were assessed after 48 h at 30°C.

FIG 7 Loss of Oma1 impinges on TOR signaling and promotes virulence in C. albicans. (A) PCR confirming homozygous deletion of orf19.3827/OMA1 in C. albicans strain SC5314. (B) Sensitivity of the SC5314 and oma1Δ/Δ strains to various stressors. Mid-log-stage cells were grown on YPD plates containing 5 mM H2O2, 0.1 μg/ml caspofungin (Caso), or 0.4 μg/ml rapamycin (Rapa). The plates were placed in the 30°C static incubator, and photographs were taken 24 h after spotting. (C) Survival of C. albicans strains in human neutrophils. The indicated strains (1 x 10⁶ cells) were isolated, washed, and cocultured with 1 x 10⁶ RPMI medium-plus-FBS-treated polymorphonuclear granulocytes from 3 unrelated healthy donors. Following 2-h coincubation at 37°C, pathogen cells were reisolated and plated for survival. The viable colonies were assessed after 48 h at 30°C. (D and E) Survival of G. mellonella larvae infected with the indicated strains. The larvae were injected with cells or buffer and monitored for 5 days at 37°C. n = 25 subjects per replicate for each group plus 10 noninjected animals (nonmanipulated control). Three biological replicates of each experiment were conducted, and two independent isolates of the oma1Δ strain were tested. (D) Kaplan-Meier survival plot of animals infected with the indicated strains (the results show the data pooled for a single comparison; n = 75 for each strain). P values were determined using log rank statistics. (E) Complementary data showing the percentage of G. mellonella killing ± SEM; P values were determined as for Fig. 1. * , P < 0.05; ** , P < 0.01 by Student’s t test.

The Oma1 homolog is necessary for TOR signaling-mediated physiological responses in C. albicans. We asked if the impact of Oma1 upon TOR signaling is evolutionarily conserved. To explore this, we examined the role of the Oma1 ortholog in an evolutionarily distant yeast, the human pathogen C. albicans. We deleted both alleles of orf19.3827 (whose products had 45.2% sequence identity to the S. cerevisiae Oma1 protein) to construct a homozygous null mutant in the diploid yeast (Fig. 7A). Like the S. cerevisiae oma1Δ strain, the C. albicans oma1Δ/Δ mutant displayed no obvious phenotype under standard growth conditions. However, consistent with S. cerevisiae data, upon stress with 5 mM H2O2, C. albicans oma1Δ/Δ cells displayed mild growth impairment. Additionally, the survival of the strain was affected by the drug caspofungin (Fig. 7B). Notably, S. cerevisiae oma1Δ/Δ cells were also found to be caspofungin sensitive in our miniscreen (Fig. 1A). Most significantly, C. albicans oma1Δ/Δ cells exhibited marked resistance to rapamycin (Fig. 7B).
different donors. Surprisingly, the oma1ΔΔ cells exhibited increased resistance to PMN killing (Fig. 7C). This may be due to the higher metabolic plasticity of C. albicans and the evolutionary “rewiring” of the TOR pathway at the level of the Msn2/Msn4 node, because, unlike S. cerevisiae, these transcription factors do not play a significant role in the pathogen’s responses to oxidative stress (44, 45). Next, we tested the virulence of the oma1ΔΔ strain in the established G. mellonella larval model of systemic Candida infection (46). The larvae injected with rapamycin-resistant oma1ΔΔ cells displayed increased virulence, particularly at day 5 of infection (Fig. 7D and E). Together, our data indicate that, as in S. cerevisiae, loss of Oma1 in C. albicans impinges on TOR signaling, which impacts on specific physiological properties of the pathogen, including its virulence.

**DISCUSSION**

Here, we show that loss of the conserved protease Oma1 in yeast reduces signaling via the TORC1-Rim15-Msn2/Msn4 axis, thereby interfering with oxidative-stress responses. These data support a recent model (11, 38, 39) postulating the existence of a transient, adaptive ROS signal necessary for survival in stationary phase in TOR signaling mutants. Our novel finding is the identification of an upstream, mitochondrion-localized, modulator of TOR signaling. Cells lacking Oma1 display (i) elevated resistance to rapamycin, (ii) increased respiration and ROS production during logarithmic growth, and (iii) reduced phosphorylation of the TORC1 effector protein Npr1. These traits are characteristic of strains with reduced TOR activity (11, 28, 29, 47). The loss of the TORC1 subunit Tor1 or its downstream target kinase, Rim15, in oma1Δ cells abolishes these traits. Epistatic interactions between OMA1 and SCH9 or RTG1 suggest that these branch point signaling elements are not directly involved. Moreover, Tor1 appears to be a limiting factor in these genetic interactions. The signaling deficit in oma1Δ cells is also not due to attenuation of PKA or Ras signaling. Two factors appear to be critical for oma1Δ phenotypes.

In line with previous reports (11, 37), transient accumulation of ROS in the actively growing oma1Δ strain is likely a key contributor to altered TOR signaling. Reducing ROS in logarithmic-phase oma1Δ cells with antioxidants, or by genetically blocking respiration, remedies malfunctioning TORC1 signal transduction. The enhancement in the respiratory capacity of log-phase oma1Δ cells likely defines the specificity of the phenotype, because the strains defective in other IM proteases (yme1Δ, yta10Δ, and pcp1Δ) are, to various degrees, respiratory deficient. Consistent with this postulate is the lack of rapamycin resistance in the oma1Δ yta10Δ mutant, wherein loss of the Yta10 subunit of the m-AAA protease results in the complete loss of respiration. It is also noteworthy that the results of our antioxidant pretreatment experiments are in line with the model whereby elevated levels of ROS may be advantageous in younger cells but become detrimental in aged cells (48).

How does suspended TORC1-Rim15 signaling relate to vulnerability of oma1Δ cells to stress? Reportedly, alterations in TORC1 cues attenuate the transcriptional activity of Msn2/Msn4 via several factors, including PP2A phosphatase (17, 36). Our study reveals that, despite the enhanced nuclear localization of Msn2 (and presumably Msn4) in response to acute oxidative stress, the expression of Msn2/Msn4-controlled genes is not significantly changed in oma1Δ cells. This effect is specific to oxidative insults and appears to underpin the reduced oxidative stress tolerance and rapamycin resistance of oma1Δ cells (Fig. 8). Remarkably, inactivation of PP2A alleviates both phenotypes. It is noteworthy that in the W303 genetic background used in this study, oxidation/nuclear retention of another oxidative-stress-responsive redox-sensitive transcriptional factor, Yap1, is largely Hyr1/Gpx3 independent and mediated via the Msn2/Msn4-controlled Srx1-Tsa1 peroxiredoxin relay (49, 50). This situation may therefore potentiate the effect of impaired Msn2/Msn4 function and further contribute to increased sensitivity of oma1Δ cells to H2O2.
The association of Oma1 with TOR signaling is not restricted to baker’s yeast. Deletion of the OMA1 ortholog in C. albicans results in marked resistance to rapamycin, indicating suspended TOR signaling. However, the modulatory effect of Oma1 loss in C. albicans differs from that in S. cerevisiae, likely due to higher metabolic plasticity of C. albicans and the “rewiring” of interactions between TOR and Msn2/Msn4 signaling in the pathogen (44, 45). Consistent with TOR signaling being reduced in the C. albicans oma1Δ/Δ mutant, this strain shows significant enhancement of its resistance to phagocytic killing and its virulence.

Beyond the physiological relevance, our findings have biomed-ical importance. First, we identify Oma1 as a novel factor that integrates mitochondrial function with TOR signaling. Our data may provide explanations for some unexpected metabolic pheno-types in Oma1 knockout mice (51). Second, the enhanced rapa-myacin resistance and virulence observed in Oma1-deficient C. albicans cells reveals a new potential role of Oma1 as a therapeutic target/risk factor in systemic candidiasis. It is also possible that additional mitochondrial proteins, whose deletion can exert similar modulatory effects on TOR signaling, exist. Further studies are warranted to address these exciting questions.

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