Genome-Wide Studies of Rho5-Interacting Proteins That Are Involved in Oxidant-Induced Cell Death in Budding Yeast

Komudi Singh,*1,2 Mid Eum Lee,†2,3 Maryam Entezari,* Chan-Hun Jung,*4 Yeonsoo Kim,* Youngmin Park,* Jack D. Fioretti,* Won-Ki Huh,‡4,5 Hay-Oak Park,*†4,5 and Pil Jung Kang*,5

*Department of Molecular Genetics, †Molecular Cellular Developmental Biology Program, The Ohio State University, Columbus, OH 43210, and ‡Department of Biological Sciences, Seoul National University, Seoul 08826, Republic of Korea

ABSTRACT  Rho GTPases play critical roles in cell proliferation and cell death in many species. As in animal cells, cells of the budding yeast Saccharomyces cerevisiae undergo regulated cell death under various physiological conditions and upon exposure to external stress. The Rho5 GTPase is necessary for oxidant-induced cell death, and cells expressing a constitutively active GTP-locked Rho5 are hypersensitive to oxidants. Yet how Rho5 regulates yeast cell death has been poorly understood. To identify genes that are involved in the Rho5-mediated cell death program, we performed two complementary genome-wide screens: one screen for oxidant-resistant deletion mutants and another screen for Rho5-associated proteins. Functional enrichment and interaction network analysis revealed enrichment for genes in pathways related to metabolism, transport, and plasma membrane organization. In particular, we find that ATG21, which is known to be involved in the CVT (Cytoplasm-to-Vacuole Targeting) pathway and mitophagy, is necessary for cell death induced by oxidants. Cells lacking Atg21 exhibit little cell death upon exposure to oxidants even when the GTP-locked Rho5 is expressed. Moreover, Atg21 interacts with Rho5 preferentially in its GTP-bound state, suggesting that Atg21 is a downstream target of Rho5 in oxidant-induced cell death. Given the high degree of conservation of Rho GTPases and autophagy from yeast to human, this study may provide insight into regulated cell death in eukaryotes in general.

KEYWORDS  yeast knockout strains bimolecular fluorescence complementation oxidative stress regulated cell death autophagy

Rho GTPases regulate diverse cellular processes in species ranging from yeast to humans. In addition to their functions in cytoskeleton organization in various cell types (Hall 2012), Rho GTPases including Rac have been implicated in phagocyte killing and programmed cell death (PCD) in Caenorhabditis elegans (Ravichandran and Lorenz 2007). The evolutionally conserved role of Rac during the engulfment of apoptotic cells in mammals and C. elegans is related to their critical roles in cytoskeleton reorganization (Ravichandran and Lorenz 2007). Rac GTPases are also involved in activation of NADPH oxidases (NOX enzymes), which accept electrons from NADPH to produce the superoxide radical, in neutrophils and non-phagocytic cells (Abo et al. 1991; Pick 2014; Werner 2004). Budding yeast has nine ORFs with sequence similarity to mammalian NADPH oxidases, and some of them are involved in the regulation of the actin cytoskeleton (Rinnerthaler et al. 2012), but their link to Rho GTPase is not known.

A large number of studies have shown that yeast cells undergo ‘regulated cell death’ (RCD) or PCD under various physiological conditions (Carmona-Gutierrez et al. 2018; Strich 2015). Regulation of cell death appears conserved in yeast, sharing some common regulators of cell death in metazoan and other multicellular systems, including the
AAA-ATPase Cdc48/VCP (Madeo et al. 1997; Braun and Zischka 2008; Braun et al. 2006) and metacaspases (Madeo et al. 2002). Yet the mechanisms by which yeast cell death is regulated are not well understood. We previously found that Rho5, which is closely related to Rac GTPases in mammals, is necessary for oxidant-induced cell death in budding yeast (Singh et al. 2008). Since Rho5 interacts with Trt1, thioredoxin reductase, specifically in its active GTP-bound state, we proposed that Rho5 might downregulate the thioredoxin antioxidant system during cell death (Singh et al. 2008). Other studies have suggested that Rho5 downregulates the yeast cell wall integrity pathway (Schmitz et al. 2002) and is involved in osmotic stress response (Annan et al. 2008), although the underlying mechanisms are not clear. Consistent with these previous reports, cells lacking Dck1 and Lmo1, the homologs of mammalian DOCK180 and Elmo, which catalyze the nucleotide exchange of Rac1 or Cdc42 (Brugnera et al. 2002; Cote and Vuori 2002), exhibit hypersensitivity to cell wall stress and hydrogen peroxide (H2O2) (Schmitz et al. 2015).

Since Rac is an important player during apoptotic cell death in other cell types, we asked whether a similar mechanism might be involved in Rho5-mediated cell death in yeast. The rho5G12V mutant, which is believed to encode the GTP-locked Rho5 in vivo, exhibits higher sensitivity to oxidants compared to cells lacking Trt1 (Singh et al. 2008), suggesting that Rho5 has additional targets to promote cell death. We thus performed genome-wide screens to identify genes that are closely associated with Rho5 and are likely involved in oxidant-induced cell death. Here, we report that several genes involved in vesicular traffic and organelle organization are important for oxidant-induced cell death. In particular, we found that ATG21, which is known to be involved in the Cvt pathway and mitophagy (Stromhaug et al. 2004), is necessary for cell death mediated by Rho5.

MATERIALS AND METHODS

Plasmids, yeast strains, and growth conditions
The Saccharomyces cerevisiae haploid knockout (YKO) strains (Thermo Scientific Open Biosystem) and wild-type (WT) BY4741 were used to screen for deletion mutants that were resistant to oxidants. A collection of VN (the N-terminal fragment of Venus, a yellow fluorescent protein)-tagged yeast strains (Sung et al. 2013) was used to screen for Rho5-binding proteins by bimolecular fluorescence complementation (BiFC) assays. All yeast strains and plasmids used in this study are listed in Supplemental Table S1 and S2, respectively, with a brief description. Standard methods of yeast genetics, DNA manipulation, and growth conditions were used (Guthrie and Fink 1991). Yeast strains were grown in rich yeast medium YPD (yeast extract, peptone, dextrose) or synthetic complete (SC) containing 2% dextrose as a carbon source, unless stated otherwise.

Growth phenotype and treatment With H2O2 or heat stress
Sensitivity to H2O2 was monitored by plating assays, as previously described (Singh et al. 2008). Since the laboratory WT strains exhibited a varying degree of sensitivity to H2O2, depending on the background (Singh et al. 2008), 1 mM H2O2 was used for all strains in BY4741 background, whereas 3–4 mM H2O2 was used for strains in HPY210 background to test oxidant-induced cell death. All strains were examined in comparison to WT in the isogenic strain background. Treatment with ramped heat stimulus was performed as previously described (Teng et al. 2011): cells in fresh culture (OD600 = 0.5–0.6) were treated by increasing temperature in a PCR machine, first from 25°C to 40°C over 2 min and then 40°C to 51°C during the 10 min period. Cells were then kept at 51°C for 10 min and plated on YPD. Cells grown for 1 day (postdiauxic) were treated by increasing temperature from 25°C to 55°C during the 15 min period and then kept at 55°C for 10 min before plating on YPD.

Cell viability assays
Plasma membrane integrity was monitored by staining cells with propidium iodide (PI) immediately after incubation with 4 mM H2O2 for 4 hr (or mock-treated), as previously described (Kainz et al. 2017). Single z-stack images of PI-stained cells were captured using a Nikon E800 microscope with a 40x objective lens and TRITC/TexasRed filter from Chroma Technology, Hamamatsu ORCA-2 CCD (Hamamatsu Photonics), and Slidebook software (Intelligent Imaging Innovations). The methylene blue reduction test was used to determine metabolically active cells, which convert methylene blue to colorless leucomethylene blue (Bapat et al. 2006). After H2O2 treatment (or mock-treated) as described above, cells were stained with 10 μM methylene blue (Sigma) and then observed by DIC (Differential interference contrast microscopy) using the Nikon E800 microscope with a 100x/1.3 NA oil-immersion objective lens. The same H2O2-treated and mock-treated cells were also plated on YPD plates (~200 cells per plate) to determine the colony forming unit (CFU).

Genome-wide screen for H2O2-resistant mutants
First, the YKO collection in 96-well plates was transformed with a multicopy rho5G12V plasmid to generate a ‘sensitized’ strain collection, and the transformants were selected on SC-Ura plates. Each transformant as well as WT strain carrying the same plasmid organized in the 96-well plates (in duplicate sets) were treated with 1 mM H2O2 for 3 hr or mock-treated, and then plated on SC-Ura plates using a 96-pin metal multi-blot replicator (‘Frogger’, V&P Scientific, Inc.). After two days of incubation at 30°C, deletion mutants that were more resistant to H2O2 than WT were visibly identified. Those potential candidate strains were subjected to retesting under the same conditions except by growing them in individual liquid culture using SC-Ura medium. The authenticity of each deletion that was reproducibly resistant to H2O2 was then tested by genomic PCR. Second, deletions of these candidate genes were generated in another strain background (HPY210) and tested for resistance to oxidants with 4 mM H2O2 (see above).

Genome-wide screen by BiFC assays
Briefly, a haploid strain HY1029, which expresses VC (the C-terminal fragment of Venus) fused to the N terminus of Rho5, was mated with each VN fusion strain in 96-well plates, followed by selection of diploids on SC-Met-Lys, as previously described (Sung et al. 2013) (see Table S1). Diploid cells were grown to mid-logarithmic phase at 30°C in SC medium, and initial screens were performed using 96-well glass-bottomed microplates (MGP096, Matrical Biosciences) and a Nikon Eclipse E1 microscope and a Plan Fluor 96-well glass-bottomed microplates (MGP096, Matrical Biosciences) and a Nikon Eclipse E1 microscope and a Plan Fluor 2017). Single z-stack images of PI-stained cells were captured using a Nikon E800 microscope with a 40x objective lens and TRITC/TexasRed filter from Chroma Technology, Hamamatsu ORCA-2 CCD (Hamamatsu Photonics), and Slidebook software (Intelligent Imaging Innovations). The methylene blue reduction test was used to determine metabolically active cells, which convert methylene blue to colorless leucomethylene blue (Bapat et al. 2006). After H2O2 treatment (or mock-treated) as described above, cells were stained with 10 μM methylene blue (Sigma) and then observed by DIC (Differential interference contrast microscopy) using the Nikon E800 microscope with a 100x/1.3 NA oil-immersion objective lens. The same H2O2-treated and mock-treated cells were also plated on YPD plates (~200 cells per plate) to determine the colony forming unit (CFU).

Microscopy and image analysis
Since the same split site (154/155) was used to generate both EFYFP and Venus truncated forms for BiFC, candidate VN fusions from the initial screen were tested for nucleotide-specific interactions with Rho5 using YFPc (the C-terminal fragment of YFP) fusions of WT
and Rho5 mutant proteins. Cells were grown in the appropriate synthetic medium overnight and then freshly sub-cultured for 3–4 hr in the same medium prior to imaging. For images shown in Figure S2, 3 z-stack (0.4 μm step) images were captured at room temperature (23–25°C) using a Nikon E800 microscope fitted with a 100x/1.30 NA oil-immersion objective lens and a YFP filter from Chroma Technology (see above). Out-of-focus signals were removed by deconvolution using Slidebook software (Intelligent Imaging Innovations). For images shown in Figure 5A, slides were prepared similarly on an agarose slab, and 3 z-stack (0.4 μm step) images were captured at 22°C using a spinning disk confocal microscope (Ultra-VIEW VoX CSU-X1 system; Perkin Elmer-Cetus) equipped with a 100x/1.4 NA Plan Apochromat objective lens (Nikon); 440-, 488-, 515- and 561-nm solid-state lasers (Modular Laser System 2.0; Perkin Elmer-Cetus); and a back-thinned EM CCD (ImageEM C9100-13; Hamamatsu Photonics) on an inverted microscope (Ti-E; Nikon).

Image processing and analyses were performed using ImageJ (National Institutes of Health). Summed intensity projections of z stacks of representative images were used to generate Figure 5Aa. To quantify BiFC signals, summed intensity projections were analyzed after background subtraction as follows: First, a fluorescence threshold was set above background that was estimated from a control strain (that expressed untagged Rho5 together with Atg21-VN). This threshold mainly selected fluorescent pixels on the vacuolar membrane. About 40–62% of cells expressing YFP-Rho5 or YFP-Rho5G12V had YFP signals above threshold (n = 86 for WT; n = 156 for Rho5G12V). In contrast, less than 10% of cells expressing YFP-Rho5K16N (n = 142) had YFP signal above threshold. To quantify BiFC signals in individual cells, all selected pixels above threshold in each cell were measured by drawing ROI around the cell boundary (based on the synchronized DIC image). The same threshold was also applied to all three strains that were captured and processed using the same conditions. Average integrated density of all ROIs (depicted with each mark on the graph) was calculated for each image set, and mean ± SEM from analyses of 4 independent image sets are shown in Figure 5Ab.

Yeast two-hybrid assay
A two-hybrid assay was carried out as previously described (Gyuris et al. 1993). Atg21 was expressed as an activation domain (AD) fusion protein using plasmid pG4-5-ATG21 (see Table S2). WT and mutant Rho5 proteins were expressed as DNA-binding domain (DBD) fusions using plasmids pEG202-RHO5, pEG202-rho5G12V, and pEG202-rho5K16N, all of which also carry the C328S substitution, as previously described (Singh et al. 2008). The yeast strain EGY48 carrying the LEU2 reporter gene was transformed with pG4-5-ATG21 and each of pEG202-RHO5 plasmids (or empty vector controls), and several independent transformants from each transformation were plated on SC-Gal plates lacking Leu (and Trp and His to maintain the plasmids) to test the Rho5-Atg21 interaction. To compare the levels of WT or mutant Rho5 proteins fused to LexA, yeast extracts were prepared from the strain (EGY48) carrying each PEG plasmid and then were subjected to immunoblotting with rabbit polyclonal anti-LexA antibodies (EMD Millipore) and Alexa Fluor 680 goat anti-rabbit IgG (LI-COR Biosciences).

Gene Ontology (GO) term enrichment analysis
Pathway enrichment analyses of the lists of genes derived from genome-wide studies were performed using the DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics resources (Huang da et al. 2009a, 2009b). The GO for biological processes (BP), molecular functions (MF), cellular components (CC), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were significantly enriched are shown with respective p values (corrected for multiple comparison by Benjamini) in Figure 3C and Tables S3–S5.

Statistical analysis
Data analyses and graph plotting were performed using Prism 6 (Graph-Pad Software). Mean and SEM (error bars) are provided in the bar graphs. A two-tailed student’s t-test was performed to determine statistical differences between two sets of data: ns (not significant) for P ≥ 0.05; *P < 0.05; **P < 0.01; ***P < 0.001.

Data availability
Strains and plasmids are available upon request. The VN fusion library is distributed by the Bioneer Corp (www.bioneer.com). The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and the supplemental files. Tables S1 and S2 contain lists of yeast strains and plasmids used in this study. Tables S3–S5 include all genomics data and GO term enrichment analyses by DAVID. Figures S1 shows spot assays of selected deletion mutants to test resistance to H2O2. Figure S2 shows BiFC analyses of candidate VN fusions with YFP fusion of WT or mutant Rho5. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7294976.

RESULTS AND DISCUSSION
Rho5 is necessary for cell death upon exposure to oxidants and heat stress
While oxidant-induced cell death depends on Rho5 in budding yeast (Singh et al. 2008), it remained unclear how Rho5 promotes cell death. Since yeast medium generally contains high levels of antioxidants, typically a higher concentration of H2O2 is used to introduce oxidative stress in budding yeast than other cell types. In addition, the sensitivity to H2O2 differs significantly depending on strain background (Singh et al. 2008). To investigate regulated cell death and to avoid accidental cell death caused under harsh conditions, it was thus critical to establish a proper experimental condition for each strain background. To ensure the Rho5-mediated cell death conditions, we characterized rho5 mutants by staining with PI or methylene blue (MB) immediately after treating with H2O2 and also determined CFU following the same treatment with H2O2. PI is highly charged and therefore normally cell impermeant, but it penetrates damaged membranes (Stan-Lotter et al. 2006). Prior to H2O2 treatment, less than 3% of WT, rho5Δ, and rho5G12V cells were PI-positive (data not shown), and the percentage of PI-positive cells was slightly increased in the rho5G12V cells after treatment with H2O2 (Figure 1A). Similarly, there was only a minor difference in the percentage of MB-negative cells between WT and rho5Δ (Figure 1B), suggesting the majority of cells have metabolic activity immediately following the treatment with H2O2. Yet the CFU was significantly different among these strains: while over 80% of rho5Δ cells were viable, less than 10% of rho5G12V cells and 30% of WT cells were viable upon treatment with 4 mM H2O2 (Figure 1C). Thus, the majority of these cells likely entered early apoptotic stage (PI) immediately upon exposure to H2O2, and cell death likely occurred during subsequent incubation without further stress. We observed that a lower H2O2 concentration (that caused cell death to a majority of WT cells in S288C background) had little effect on cell survival in the HPY210 background (compare Figures 1C & 5C).
A previous report has shown that a ramped heat stimulus, rather than sudden heat shock, results in reproducible gene-specific survival phenotypes (Teng et al. 2011). By following a similar procedure, we tested viability of rho5Δ when subjected to a controlled heat ramp (see Materials and Methods). We found that the rho5Δ mutant was more resistant to heat stress than WT during the exponential phase of growth and postdiauxic shift (Figure 1D). These observations suggest that Rho5 promotes apoptosis upon exposure to various stresses in addition to oxidants, consistent with previous studies (Schmitz et al. 2002; Singh et al. 2008; Annan et al. 2008; Schmitz et al. 2015).

**Cell death mediated by Rho5 may be independent of known apoptotic factors in budding yeast**

A number of orthologs of mammalian genes involved in apoptosis have been implicated in RCD in budding yeast. These include YCA1 (metacaspase) (Madeo et al. 2002), AIF1 (an ortholog of mammalian Apoptosis-Inducing Factor) (Wissing et al. 2004), NUC1 (mitochondrial nuclease, an ortholog of mammalian endoG) (Büttner et al. 2007), and STE20, a PAK (p21-activated protein kinase) (Ahn et al. 2005). We asked whether cell death mediated by Rho5 involves these genes. If any of these gene products function downstream of Rho5 in the same oxidant-induced cell death pathway, we would expect that cells lacking such a gene might be resistant to oxidants even when Rho5G12V was expressed, which caused hypersensitivity to oxidants (Singh et al. 2008). We thus tested deletion mutants of these apoptotic factors after transforming with a plasmid carrying rho5G12V or a vector control. Plate assays using serial dilutions of these transformants indicated that the expression of the GTP-locked Rho5 caused cells to be severely sensitive to H2O2 in these mutants (Figure 2). In addition, all these deletion mutants (which were in the same genetic background as rho5Δ) with the vector control were not as resistant to H2O2 as rho5Δ. These observations suggest that Rho5 promotes cell death independently from these previously known players in yeast cell death.

**Figure 1** Rho5 mediates regulated cell death triggered by H2O2 or heat ramp. A. PI staining of each strain (HPY210 background) right after exposure to 3 mM H2O2 for 4 hr. Average percentage of PI+ cells are indicated from three sets of data (n = 100 for each sample per test). Mock-treated cells showed low numbers of PI+ cells (≤3.5%) for all strains (not shown). B. MB staining of each strain (HPY210 background) after exposure to H2O2 treatment for 4 hr or mock-treated. Mean ± SEM (error bars) are shown from three sets of data (n = 100 for each sample per test). C. CFU was measured for each strain (HPY210 background) after H2O2 treatment for 4 hr. Mean ± SEM (error bars) are shown from three sets of data (n = 100 for each sample per test). D. Cell survival was determined by a fivefold serial dilution of each strain (BY4741 background) grown in YPD after subjecting to heat ramp: (a) cells without treatment; (b) cells from fresh cultures (OD600 = 0.5~0.6) (b); and (c) cells grown 1d (postdiauxic).

**Genome-wide screen uncovers potential downstream mediators of Rho5 involved in oxidant-induced cell death**

To identify additional players that function together with Rho5 in oxidant-induced cell death, we carried out a genome-wide screen for mutants that failed to undergo cell death upon exposure to H2O2. To facilitate identification of genes that function downstream of Rho5 (rather than those that function in oxidant-induced cell death independently of Rho5), we generated a collection of ‘sensitized’ deletion mutants by transforming the rho5G12V plasmid into...
each deletion strain in the haploid YKO collection. A total of 4,506 deletion strains in the ordered arrays that carried the rho5G12V plasmid were successfully recovered and then screened for their resistance to H2O2 (Figure 3A).

From the primary screen of the ‘sensitized’ strain collection, we initially identified 235 candidates that were more resistant to H2O2 than the WT control strain carrying the same plasmid. We tested serial dilutions of each primary candidate without the rho5G12V plasmid and then confirmed correct ORF deletions of those that were reproducibly resistant to H2O2. We also tested the phenotype of additional candidates from those identified from a screen by BiFC assays (see below). Collectively, we identified 67 mutants that were more resistant to H2O2 than WT. To confirm that the phenotype resulted from the expected deletion (and not any other mutation in the strain background), we generated a deletion of each candidate gene in another strain background and then tested its resistance to H2O2. Many deletion mutants in this second test exhibited only little resistance to H2O2 (see Figure 3Bb). The commonly used strain S288C (in which YKO was generated) has a mutated copy of HAP1 (Gaisne et al. 1999), which is involved in carbon catabolite activation of transcription and also gene expression in response to the levels of heme and oxygen (Guarente et al. 1984). S288C also has a variant allele of MIP1, which increases petite frequency (Dimitrov et al. 2009). These factors might have contributed to hypersensitivity of the YKO strains to oxidants. Together from this secondary test, we identified 31 genes whose deletion confers resistance to H2O2 (Figure S1; Table S3).

To gain insight into the biological processes that were represented in the genes identified from our screen, we then performed pathway enrichment analysis using the DAVID bioinformatics resources (Huang da et al. 2009a) (see Materials and Methods). GO terms and KEGG pathways that were significantly enriched could be grouped into main categories of processes associated with GTP binding, intracellular trafficking and the plasma membrane, and metabolism (Figure 3C; Table S3). These results suggest that Rho5 may integrate signals from various intracellular subcomponents to mediate oxidant-induced cell death.

**Genome-wide screen by BiFC assays uncovers potential targets of Rho5**

In parallel to the screen of the yeast deletion library described above, we carried out another genome-wide screen by BiFC assays to identify proteins associated with Rho5. We screened the diploid library in ordered arrays, which were generated by mating each VN strain in the VN fusion library (Sung et al. 2013) with a strain expressing VC-Rho5 (Figure 4A). In addition, we tested those positives that were identified only from the screen of the deletion library (see above) by BiFC assays. Collectively, we identified 44 genes, whose VN fusions exhibited positive BiFC signals (Figure 4B; Table S4), including ATG21 and CWP1, which were also identified from the screen for H2O2-resistant mutants. These positive candidates may associate closely with Rho5, although they may be necessarily involved in oxidant-induced cell death.

To uncover the cellular processes that Rho5 may control via these potential target genes, we performed pathway enrichment analysis and found that the 44 genes identified from the BiFC-based screen were significantly enriched in pathways associated with metabolism, cell wall processes, and intracellular trafficking (Table S4). When we
compared genes identified from both genome-wide screens, we found 19 genes that were positive in both categories (Figure 4C), with gene enrichment (with $P < 0.05$) in transport and organelle organization (Figure 4D; Table S5). Our initial screen with VN-Rho5, instead of VN-Rho5G12V, might have missed a specific Rho5 effector that might interact transiently with the GTP-bound Rho5. However, BiFC assays facilitate detection of transient protein-protein interactions often by resulting in irreversible bimolecular fluorescent complex formation (Miller et al. 2015), and thus a Rho5 effector could be easily trapped with Rho5-GTP. Some of these candidates exhibited GTP-dependent interactions with Rho5, while others appeared less specific to the GTP-bound state of Rho5 (see below).

A subset of positives identified from both genome-wide screens may interact with Rho5 preferentially in its GTP-bound state

Since small GTPases often interact with their downstream targets in a GTP-dependent manner, we tested by BiFC analyses whether the 16 candidates that were identified from both genome-wide screens associate with Rho5 in a nucleotide-specific manner. By examining strains expressing each candidate VN fusion together with
YFP\textsuperscript{C}-fusions of WT or mutant \textit{Rho5}, we identified seven VN fusions that exhibited stronger fluorescence signals with YFP\textsuperscript{C}-\textit{Rho5\textsuperscript{G12V}} or YFP\textsuperscript{C}-\textit{Rho5\textsuperscript{WT}} compared to YFP\textsuperscript{C}-\textit{Rho5\textsuperscript{K16N}}, which is in either GDP-locked or nucleotide-empty state \textit{in vivo} (see Figures 5 & S2), as discussed below. Since YFP\textsuperscript{C}-fusions of WT and mutant \textit{Rho5} proteins are expressed at about the same level (Singh \textit{et al.} 2008), these observations suggest that these 7 candidates interact preferentially with \textit{Rho5-GTP}.

Interestingly, a major group of genes that were identified from both genome-wide screens included eisosome components. Eisosomes are protein-based structures associated with specific lipid domains on the plasma membrane furrows, which are known as the MCC (membrane compartment containing \textit{Can1}) domains in yeast (Walther \textit{et al.} 2006; Karotki \textit{et al.} 2011; Douglas and Konopka 2014). Lsp1, a core component of eisosome (Walther \textit{et al.} 2006), showed positive BiFC signals with WT \textit{Rho5} and \textit{Rho5\textsuperscript{G12V}} but little signal with \textit{Rho5\textsuperscript{K16N}}. Other proteins closely associated with eisosomes, \textit{Sur7} and \textit{Nce102}, also exhibited BiFC signals with \textit{Rho5}, although their interactions were not specific to its nucleotide-bound state (Figure S2). We found that deletion mutants of the eisosome components, \textit{lsp1\Delta}, \textit{nce102\Delta}, \textit{sur7\Delta}, and \textit{pil1\Delta}, were resistant to cell death, albeit moderately, when exposed to oxidants or when subjected to controlled heat ramp (Figure S1, A & B). These findings suggest that \textit{Rho5-GTP} might signal to eisosomes via Lsp1 under the conditions triggering apoptotic cell death.

Potential involvement of eisosome components in \textit{Rho5-mediated} cell death is surprising, because previous studies in yeast and other fungi have suggested that the components of the MCC/eisosomes may play multiple roles in resistance to stress (Foderaro \textit{et al.} 2017). Another previous study, however, also reported that deletion of \textit{PIL1} and \textit{LSP1} enhances heat stress resistance (Zhang \textit{et al.} 2004), consistent with our findings. Although their molecular functions are controversial, recent studies have revealed that eisosome proteins play a role in the control of signaling pathways, especially TORC2 activation (Fröhlich \textit{et al.} 2014; Kabche \textit{et al.} 2014; Berchtold \textit{et al.} 2012). TORC2 serves as a stress sensor to induce various cellular responses including actin polarization, sphingolipid biosynthesis, and stress-related gene transcription (Loewith and Hall 2011). Given the possibility of eisosomes as a link between different signaling pathways, it will be interesting to further investigate the involvement of eisosome proteins in \textit{Rho5} signaling pathway.
Cwp1 is another potential player in Rho5-mediated cell death pathway. Interestingly, CWP1 was previously identified as one of the genes that were essential for cell death induced by the expression of human apoptosis initiator caspase-10 in budding yeast (Lisa-Santamaria et al. 2012). The Cwp1-Rho5 BiFC signals appeared at endomembranes including the endoplasmic reticulum (ER) (Figure S2), consistent with the localization of Cwp1-GFP and GFP-Rho5 (Tkach et al. 2012; Huh et al. 2003; Singh et al. 2008) but not at the cell wall or birth scar as reported (Smits et al. 2006). However, the location of BiFC signals may not necessarily reflect the site of interaction of the two proteins because of the possibility of irreversible bimolecular fluorescent complex formation. Further studies are required to validate the subcellular location where these proteins interact with each other and to determine the functional significance of this interaction.

Other candidates that exhibit GTP-dependent BiFC signals with Rho5 include Msp1 and Tom70 (Figure S2), which act in sorting
and transport of mitochondrial proteins (Nakai et al. 1993). Since the mitochondrion is an intracellular platform integrating cell death and energy production (Eisenberg et al. 2007; Balaban et al. 2005), it will be interesting to investigate further to understand the physiological significance of their interactions with Rho5. Vph1 and Sm11 (also known as Knr4) displayed GTP-dependent BiFC signals with Rho5 (Figure S2), although their deletions exhibited relatively small resistance to H$_2$O$_2$ (Figure S1). Vph1 is a subunit of vacuolar ATPase V$_0$ domain, which is located on the vacuolar membrane (Manolson et al. 1992). Since V-ATPase has been shown to be involved in activation of TORC1 in response to cytoplasmic pH, which in turn depends on glucose (Dechant et al. 2014), this result suggests a possible link between Rho5 and TORC1 signaling. Sm11 encodes a protein localized to the bud neck with a proposed role in coordinating cell cycle progression with cell wall integrity (Martin-Yken et al. 2003), but any functional link between Sm11 and Rho5 remains unknown.

Unlike these candidates discussed above and Atg21 (see below), several other candidates did not exhibit a preferential interaction with Rho5-GTP (see Figure S2). While these proteins may possibly interact with a Rho5 domain that does not undergo a drastic conformational change depending on its GDP- or GTP-bound state, further investigation is necessary to validate these interactions with Rho5 in vivo. Nevertheless, it is interesting to find that genes involved in glucose metabolism are enriched in our screens. Hxt1 and Hxt3 are low affinity glucose transporters (Lewis and Bisson 1991; Ozcan and Johnston 1999), and Hxk2, which is a predominant hexokinase during yeast growth on glucose, is involved in regulation of expression of HXT1 (Ozcan and Johnston 1999; Johnston 1999). A large number of studies have shown that glucose metabolism can regulate cell death in variety of cell types (Gottlob et al. 2001; Zhao et al. 2008a; Zhao et al. 2008b; Rathmell et al. 2003; Matsuura et al. 2016). Interestingly, a recent study suggests that Rho5 relocates from the plasma membrane to mitochondria upon glucose starvation (Schmitz et al. 2018). Although the functional significance of this observation is yet to be determined, it is tempting to speculate that there may be a crosstalk between Rho5-mediated cell death pathway and glucose signaling.

**Rho5 may promote cell death via Atg21 Under oxidative stress**

Among the candidates that might function in Rho5-mediated cell death, we looked at Atg21 more closely because atg21Δ mutant exhibited strong resistance to H$_2$O$_2$ to a similar extent as rho5Δ in two strain backgrounds used in this study and even when Rho5G12V was expressed (see Figures 3Bb, 5C & 5D). BiFC assays with specific Rho5 fusions suggested that Atg21 interacts preferentially with Rho5-GTP. The BiFC signal was mainly observed on the vacuolar membrane with some puncta near and/or on the vacuolar membrane (Figure 5Aa), consistent with the localization of Atg21 (Stromhaug et al. 2004) and a part of Rho5 localization (Singh et al. 2008). Quantification of the BiFC signals indicated that there were strong fluorescence signals in cells expressing YFP$^\text{C}$-Rho5G12V and slightly weaker signals in cells expressing YFP$^\text{C}$-Rho5WT, whereas only minor background level of fluorescence was observed in cells expressing YFP$^\text{C}$-Rho5K16N (Figure 5Ab).

As an alternative way to test the GTP-dependent interaction between Rho5 and Atg21, we performed a yeast two-hybrid assay. An AD-Atg21 fusion was expressed together with a DBD fusion of WT or mutant Rho5 in a strain carrying the LEU2 reporter. Growth of the cells expressing AD-Atg21 specifically with DBD-Rho5G12V, but not with DBD-Rho5K16N, on a plate lacking Leu indicated that Rho5-GTP interacts with Atg21 (Figure 5Ba), despite the similar level of DBD fusions of WT and mutant Rho5 proteins (Figure 5Bb). The positive interaction between Rho5WT and Atg21 was more evident by BiFC assays compared to the two-hybrid assays. This is likely because the bimolecular fluorescent complex of YFP$^\text{C}$-Rho5 and Atg21-VN might be more stable or irreversible compared to the interaction between DBD-Rho5WT and AD-Atg21 in a two-hybrid assay.

Consistent with our initial screen by spot assays, an atg21Δ mutant exhibited higher cell survival upon exposure to H$_2$O$_2$ (Figure 5C). Because the WT ATG21 strain undergoes little cell death in the absence of Rho5 and vice versa, and because atg21Δ cells exhibit little cell death even when rho5G12V is expressed (see Figure 3Bc), it is likely that Rho5 and Atg21 function together to promote cell death upon exposure to oxidants. Moreover, the interaction between Rho5-GTP and Atg21 described above suggest that Atg21 may function downstream of Rho5. Atg21 is known to be involved in the CVT pathway and autophagy of mitochondrion (Kim and Klionsky 2000). Interestingly, a previous report suggests that cells deleted for RHO5 or its exchange factor (dck1 or lmo1) exhibit reduced mitophagy (Schmitz et al. 2015), supporting the idea that Rho5 may be activated upon exposure to oxidants and promote cell death via Atg21. ATG18 and ATG21 are paralogous genes that encode WD-repeat proteins, similar to human WIPI2 (WD repeat domain, phosphoinositide interacting 2) (Dove et al. 2004; Stromhaug et al. 2004). However, this role of Atg21 in oxidant-induced cell death is unlikely to be shared with Atg18, since atg18Δ expressing the GTP-locked Rho5 did not exhibit resistance to H$_2$O$_2$ (Figure 5D).

The interconnection between apoptosis and autophagy is still poorly understood. Autophagy is generally known to be a cell survival mechanism, but recent studies suggest that autophagy-dependent cell death (ACD) is a regulated cell death mechanism that requires components of the autophagy machinery instead of other cell death pathways. Although it is more likely that ACD depends on components of the autophagic machinery rather than autophagic responses, increasing evidence suggests that these pathways are intricately connected (Cooper 2018; Bialik et al. 2018; Carmona-Gutierrez et al. 2018). Previous studies have shown that the expression of human apoptosis initiator caspase-8 or caspase-10 is toxic to budding yeast by inducing a lethal phenotype with hallmarks of apoptosis and autophagy (Lisa-Santamaría et al. 2009). Interestingly, this caspase-10-induced cell death in yeast requires genes involved in autophagy (Lisa-Santamaría et al. 2012). While further investigation is required to fully understand how Rho5-GTP promotes cell death via Atg21, our findings suggest an interesting possibility that a Rho GTPase may utilize components of the autophagy machinery to promote cell death under oxidative stress.

In summary, our genome-wide studies reported in this study suggest that Rho5-mediated cell death signaling might impact cellular functions involving vesicle-mediated transport and the plasma membrane. These findings further substantiate the possibility that the oxidant-induced cell death process is complex and requires an interplay of a wide range of intracellular processes. It is tempting to speculate that common cellular components or pathway such as autophagy or eicosomes might be utilized either for cell survival or for death depending on upstream signaling molecules and/or the level of stress. Indeed, despite the similar structure of Rho GTPases, Rho1, which is involved in the cell integrity pathway (Levin 2011), is necessary for survival under oxidative stress (Lee et al. 2011), whereas Rho5 is necessary for cell death (Singh et al. 2008; this study). To fully understand the
functional significance of these interactions and the complex signal integration for survival and death will require further investigation.

**ACKNOWLEDGMENTS**

We thank J. Konz for preliminary screen of YKO collection and members of the Park and Huh laboratories for discussions and comments on the manuscript. This work was supported by research grants from the National Institutes of Health/National Institute of General Medical Sciences (GM76375 and GM114582) and from the National Institutes of Health/National Institute on Aging (AG060028) to H.-O. P., and from the National Research Foundation of Korea (2018R1A2B2009169) to W.-K. H.

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