TOR complex 2–Ypk1 signaling regulates actin polarization via reactive oxygen species

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ABSTRACT The evolutionarily conserved mTOR complex 2 (mTORC2) signaling pathway is an important regulator of actin cytoskeletal architecture and, as such, is a candidate target for preventing cancer cell motility and invasion. Remarkably, the precise mechanism(s) by which mTORC2 regulates the actin cytoskeleton have remained elusive. Here we show that in budding yeast, TORC2 and its downstream kinase Ypk1 regulate actin polarization by controlling reactive oxygen species (ROS) accumulation. Specifically, we find that TORC2-Ypk1 regulates actin polarization both by vacuole-related ROS, controlled by the phospholipid flippase kinase Fpk1 and sphingolipids, and by mitochondria-mediated ROS, controlled by the PKA subunit Tpk3. In addition, we find that the protein kinase C (Pkc1)/MAPK cascade, a well-established regulator of actin, acts downstream of Ypk1 to regulate ROS, in part by promoting degradation of the oxidative stress responsive repressor, cyclin C. Furthermore, we show that Ypk1 regulates Pkc1 activity through proper localization of Rom2 at the plasma membrane, which is also dependent on Fpk1 and sphingolipids. Together these findings demonstrate important links between TORC2/Ypk1 signaling, Fpk1, sphingolipids, Pkc1, and ROS as regulators of actin and suggest that ROS may play an important role in mTORC2-dependent dysregulation of the actin cytoskeleton in cancer cells.

INTRODUCTION

For cells to adapt to changing conditions, they must be able to respond rapidly to intracellular and environmental cues. Remodeling of the actin cytoskeleton is one method cells use to accomplish this, and its importance is highlighted by its involvement in a number of physiological processes, including cell growth and division, chemotaxis, and neurite extension, as well as in polarized growth in budding yeast (Mammoto and Ingber, 2009; Mooren et al., 2012; Taulet et al., 2012). There are a number of molecules that regulate actin cytoskeletal architecture, including actin-binding proteins such as profilin, the G-proteins Rac and Rho, and mitogen-activated protein kinase (MAPK) signaling cascades, as well as a recently emerging role for reactive oxygen species (ROS; Fiaschi et al., 2006; Moseley and Goode, 2006). A balance of ROS and antioxidant defense systems allows actin to alternate between oxidized and reduced forms, with reactions centered at two highly conserved redox-sensitive cysteine (Cys) amino acid residues, Cys-272 and Cys-374. Oxidation of these residues leads to formation of a disulfide bridge and actin dimers, which can positively affect certain cellular functions, such as motility (Lassing et al., 2007; Taulet et al., 2012). However, defects in the regulation of ROS in conditions such as sickle cell disease lead to actin oxidation and an altered actin cytoskeleton in sickled red blood cells (Shartava et al., 1995). In addition, ROS have been implicated in tumor cell migration and invasion, through regulation of the actin cytoskeleton (Park et al., 2012). Thus determining how ROS is regulated in cells is important for understanding conditions that lead to defects in actin cytoskeletal architecture.

We recently demonstrated that an important regulator of ROS is target of rapamycin complex 2 (TORC2), an evolutionarily conserved regulator of cell growth in eukaryotic organisms (Niles et al., 2014). We observed that TORC2, through its downstream target kinase Ypk1, regulates ROS produced from both mitochondrial and nonmitochondrial sources, including changes in acidification of the vacuole. We demonstrated that maintenance of vacuolar acidification by TORC2/Ypk1 signaling requires both proper levels of sphingolipids.
and regulation of the phospholipid flippase kinase Fpk1 (Niles et al., 2014). A well-characterized phenotype of TORC2 mutants is dysregulation of actin, establishing TORC2 as a promoter of actin polarization in both mammalian and yeast cells (Helliwell et al., 1998a; Jacinto et al., 2004). In yeast, actin is organized into actin cables and cortical actin patches, where patches are normally clustered within the emerging bud tip, and is essential for daughter cell formation (Moseley and Goode, 2006). TORC2-dependent regulation of the actin cytoskeleton is required to maintain the polarized nature of cell growth in budding yeast and is required for endocytosis as well as genome stability in response to DNA damage (deHart et al., 2003; Shimada et al., 2013). However, the mechanism by which TORC2 signaling regulates the actin cytoskeleton remains poorly understood.

In mammalian cells, mTORC2 phosphorylation of protein kinase Cα (PKCa) and PKCζ is required for proper actin cytoskeletal organization and migration (Ikenoue et al., 2008; Li and Gao, 2014). Similarly, Pck1 in yeast is known to play a role in the regulation of actin downstream of TORC2, as overexpression of an activated allele of Pck1 (Pck1C374A) rescues the actin depolarization phenotype of torc2- and ypk1Δ mutants (Helliwell et al., 1998b; Roelants et al., 2002; Schmelzle et al., 2002). However, direct regulation of Pck1 by TORC2 has not been observed in yeast. Instead, Pck1 is activated by the GTPase Rho1, which is itself regulated by a balance of GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs; Bickle et al., 1998; Nonaka et al., 1995). Whereas overexpression of the GEF Rom2 also rescues the growth and actin defects of TORC2 mutants (Schmidt et al., 1997), links between TORC2 or its downstream target kinase Ypk1 and Rom2/Rho1 and Pck1/MAPK signaling have not been identified. Here we address this issue and identify a number of important functional interactions by which TORC2/Ypk1 signaling regulates actin polarization via modulation of ROS, including interactions between Fpk1, sphingolipids, and Pck1/MAPK activity.

RESULTS

Ypk1-dependent ROS perturbs actin cytoskeleton organization

In a previous study, we demonstrated that TORC2-Ypk1 signaling suppresses ROS accumulation (Niles et al., 2014). Given the role ROS play in regulating the actin cytoskeleton (Villella et al., 2005; confirmed in Figure 1, A and B, by treating wild-type [WT] cells with 1 mM H2O2), we tested whether ROS accumulation was involved in actin depolarization after inhibition of Ypk1 signaling. Our approach was to inhibit Ypk1 by treating ypk1Δ ypk2Δ cells that expressed an analogue-sensitive allele of Ypk1 (ypk1Δ) with an ATP-analogue inhibitor for 60 min. As reported previously (Niles et al., 2012), inhibiting Ypk1 kinase activity resulted in a majority of cells displaying either completely depolarized or partially polarized actin (Figure 1, A and B). We observed partial improvement in actin polarization when we treated ypk1Δ-AS cells with the ROS scavenger N-acetyl cysteine (NAC; Figure 1, A and B). This partial improvement in actin polarization is consistent with our previous observation that treatment with NAC results in a partial reduction in ROS (Niles et al., 2014). Deletion of the oxidative stress–induced transcription factor Yap1, required for the removal of ROS (Kuge et al., 1997), exacerbated the actin depolarization phenotype of ypk1Δ-AS cells (Figure 1, A and B). Taken together, these results suggest that ROS contribute to depolarization of actin in Ypk1-AS cells. A ROS-induced disulfide bond between two conserved cysteine residues in actin (C285 and C374) has been demonstrated to be responsible for depolarization of actin upon oxidative stress (Farah and Amberg, 2007; Lassing et al., 2007). To test whether direct oxidation of actin is the mechanism that leads to actin depolarization in Ypk1-AS cells, we examined actin polarization of Ypk1-AS cells harboring a mutant allele of Act1, termed act1C374A, which is incapable of forming a disulfide bond in the presence of ROS. We observed that expression of act1C374A, but not WT Act1, was sufficient to largely reverse the oxidation-induced actin depolarization phenotype of Ypk1-AS cells, indicating that increased ROS leads directly to actin depolarization in Ypk1-AS cells (Figure 1, A and B).

Although these data suggested that ROS accumulation is responsible for actin depolarization in Ypk1-AS cells, we tested the reciprocal hypothesis that actin depolarization was the cause of increased ROS, as mutations in actin that decrease actin dynamics reportedly increase ROS (Gourlay et al., 2004). Accordingly, we examined Ypk1-AS act1C374A cells for indirect in vivo levels of ROS with the fluorescent ROS indicator dye 2,7-dichlorofluorescin diacetate (DCF; Lee et al., 2011). Despite rescue of actin polarization by the act1C374A mutation, the level of ROS in Ypk1-AS act1C374A cells was comparable to that in Ypk1-AS cells (Figure 1C). Thus we conclude that actin depolarization is not responsible for accumulation of ROS in Ypk1-deficient cells.

Because Ypk1 is a downstream kinase of TORC2, which also regulates actin polarization, we examined whether this regulation by TORC2 also involves ROS. Using a temperature-sensitive allele of the essential TORC2 subunit AVO3, avo3-30 (here termed torc2-ts), we found that actin depolarization in torc2-ts cells was partially rescued by treatment with NAC. We demonstrated further that expression of an allele of Ypk1 (ypk11024ΔA; Niles et al., 2012) that bypasses the need for TORC2-dependent phosphorylation restored actin polarization to levels comparable to those of wild-type cells (Figure 1D). Thus, upstream of Ypk1, TORC2 mediates suppression of ROS and maintenance of actin polarization.

Ypk1 regulates actin polarization through Fpk1, sphingolipids, and mitochondria-mediated ROS

To identify components involved in TORC2-Ypk1 regulation of actin polarization, we next examined actin polarization when Ypk1-AS cells were combined with mutations that are known to reduce ROS. We showed previously that inhibition of Ypk1 results in ROS accumulation from multiple sources, with one source being aberrant mitochondrial respiration that is dependent on the protein kinase A (PKA) subunit Tpk3. In addition, a second source of ROS results from defects in vacuolar acidification, which is dependent on the phospholipid flippase kinase Fpk1 (Niles et al., 2014). Remarkably, Ypk1-AS rho0, Ypk1-AS fpk1Δ, and Ypk1-AS fpk1Δ rho0 cells all displayed improved actin polarization that correlated precisely with their reduction in ROS (Niles et al., 2014; Figure 2A). In particular, Ypk1-AS fpk1Δ rho0 cells, which have WT levels of ROS (Niles et al., 2014), displayed completely normal actin polarization (Figure 2A). Furthermore, deletion of the phospholipid flippases DNF1, DNF2, and DNF3 or the PKA subunit TPK3 also restored actin polarization within Ypk1-AS cells in a manner that was consistent with their reduction in ROS (Figure 2A).

As an alternative approach to examine the role of Fpk1 in ROS accumulation and actin polarization, we expressed a hyperactive mutant allele of Fpk1 (Fpk1 3A) that cannot be repressed via phosphorylation by Ypk1 (Roelants et al., 2010). Expression of Fpk1 3A, but not a kinase-dead version of Fpk1 3A (Fpk1 3A KD), both increased ROS (25% of DCF-positive cells) and induced partial depolarization of actin (Figure 2, B and C). Treating Fpk1 3A-expressing cells with NAC both reduced ROS and increased actin polarization (Figure 2, B and C). Moreover, preventing actin oxidation by
We demonstrated previously that Ypk1-AS cells have decreased levels of sphingolipids, and that sphingolipids and Fpk1 function antagonistically within the same pathway to regulate ROS accumulation, in part by influencing vacuolar acidification (Niles et al., 2014).

expressing the act1C374A allele was sufficient to restore actin polarization in Fpk1 3A cells, and yet, as expected, did not reduce ROS (Figure 2, B and C). Together these results confirm that Fpk1-mediated ROS leads to actin depolarization by oxidation of Act1.

We demonstrated previously that Ypk1-AS cells have decreased levels of sphingolipids, and that sphingolipids and Fpk1 function antagonistically within the same pathway to regulate ROS accumulation, in part by influencing vacuolar acidification (Niles et al., 2014).
2-(N-morpholino)ethanesulfonic acid (MES; Figure 2D), both of which restore vacuolar acidification and reduce ROS in myriocin-treated cells (Niles et al., 2014). Although cells deleted for FPK1 have been shown to possess reduced myriocin uptake (Yamane-Sando et al., 2014), we used a concentration of myriocin that is effective even in fpk1△ cells (Roelants et al., 2011). In addition, we were able to restore actin polarization in myriocin-treated cells by directly reducing ROS by treating cells with NAC or, alternatively, by preventing oxidation of actin at C374 (Figure 2D). On the basis of these combined results, we conclude that Ypk1 regulates actin polarization via actin oxidation by ROS produced from multiple sources, including defects in vacuolar acidification mediated by overactive Fpk1 and reduced sphingolipids, as well as by impaired mitochondrial activity.

**Pck1/MAPK activation suppresses ROS and restores actin polarization in Ypk1-deficient cells**

Regulation of actin polarization by TORC2-Ypk1 is known to involve components of the Pck1-MAPK signaling cascade (Helliwell et al., 1998b). In particular, overexpression of Pkc1 or its downstream target Mpk1 rescues actin defects in ypk1ts mutant cells (Roelants et al., 2002; Schmelzle et al., 2002). We sought to determine whether ROS-mediated actin depolarization in Ypk1-AS cells was influenced by Pck1-MAPK signaling. Accordingly, we examined ROS levels in Ypk1-AS cells that expressed an activated allele of Pkc1 (Pkc1R398P; Helliwell et al., 1998b). We observed that expression of Pkc1R398P resulted in a partial but significant reduction in ROS (31 vs. 54% DCF-positive cells; Figure 3A). ROS was further reduced when Pkc1R398P was expressed in Ypk1-AS rho0 cells (22% DCF-positive cells), suggesting that Pkc1 regulates ROS independently of mitochondrial function (Figure 3A). Consistent with these findings, decreased ROS correlated with improved actin polarization (Figure 3B), indicating that Ypk1 regulates actin polarization in part through Pkc1-dependent ROS.

We next tested whether Fpk1 and Pck1 interact functionally to regulate ROS and actin polarization in Ypk1-AS cells. Expression of Pkc1R398P in Ypk1-AS fpk1△ cells did not further decrease ROS and only subtly improved actin polarization (Figure 3, A and B). This suggested that Fpk1 and Pck1 might function within the same pathway to regulate ROS and actin polarization. Accordingly, we tested the possibility that Fpk1 regulates Pck1, by measuring Pck1-dependent phosphorylation of Mpk1 (Slt2), a downstream target of Pck1 signaling (Gustin et al., 1998). Consistent with previous

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**FIGURE 2:** Ypk1 regulates actin polarization by suppression of ROS from multiple sources. (A) Ypk1-WT (PLY1083), Ypk1-AS (PLY1098), Ypk1-AS rho0 (PLY1528), Ypk1-AS tpk3Δ (PLY1529), Ypk1-AS fpk1Δ (PLY1533), Ypk1-AS fpk1Δ tpk3Δ (PLY1534), and Ypk1-AS fpk1Δ rho0 (PLY1536) were grown, fixed, and labeled for actin with rhodamine-phalloidin as in Figure 1A and quantified as in Figure 1B. (B) Act1-WT + Fpk1 3A KD (PLY1629), Act1-WT + Fpk1 3A (PLY1630), and act1 C374A + Fpk1 3A (PLY1631) were grown in SCD-Ura-Leu medium, with 20 mM NAC as noted, and then incubated with DCF as in Figure 1C or (C) fixed and labeled for actin and quantified as in A. (D) WT (PLY062), fpk1Δ (PLY1440), act1 C374A (PLY1628), and Act1-WT (PLY1627) were grown in SCD, or in SCD + MES as noted, and treated with 1.25 μM myriocin (Myr) for 1 h as noted, fixed, and labeled for actin and quantified as in A.

As sphingolipids are known to regulate actin polarization (Friant et al., 2001), we tested whether this was mediated by suppression of ROS. Indeed, we observed that defects in actin polarization caused by the sphingolipid biosynthesis inhibitor myriocin were restored either by deletion of FPK1 or by treating cells with the buffer 2-(N-morpholino)ethanesulfonic acid (MES; Figure 2D), both of which restore vacuolar acidification and reduce ROS in myriocin-treated cells (Niles et al., 2014). Although cells deleted for FPK1 have been shown to possess reduced myriocin uptake (Yamane-Sando et al., 2014), we used a concentration of myriocin that is effective even in fpk1△ cells (Roelants et al., 2011). In addition, we were able to restore actin polarization in myriocin-treated cells by directly reducing ROS by treating cells with NAC or, alternatively, by preventing oxidation of actin at C374 (Figure 2D). On the basis of these combined results, we conclude that Ypk1 regulates actin polarization via actin oxidation by ROS produced from multiple sources, including defects in vacuolar acidification mediated by overactive Fpk1 and reduced sphingolipids, as well as by impaired mitochondrial activity.
findings that Pkc1 activity is decreased in torc2 ypkr mutants (Kamada et al., 2005), we observed a reproducible reduction in Mpk1 phosphorylation in Ypk1-AS cells (Figure 3C). Of interest, Mpk1 phosphorylation was restored to WT levels in Ypk1-AS fpk1Δ cells (Figure 2C), indicating that overactive Fpk1 in Ypk1-AS cells negatively regulates Pkc1/MAPK signaling. Surprisingly, however, restoring vacuolar acidification by treating Ypk1-AS cells with MES did not restore Mpk1 phosphorylation, suggesting that Fpk1 regulates Pkc1/MAPK signaling independently of either intracellular acidification or ROS (Figure 3C). Taking the results together, we conclude that overactive Fpk1 activity in Ypk1-AS cells regulates ROS by two independent mechanisms, through intracellular acidification defects and also by inhibiting Pkc1/MAPK activity.

On the basis of the similar regulation of actin polarization by both Fpk1 and sphingolipids, we examined whether

**FIGURE 3:** Regulation of ROS by Pkc1 is downstream of Ypk1 and Fpk1. (A) Ypk1-WT (PLY1083), Ypk1-AS (PLY1098), Ypk1-AS + PKC1R398P (1531), Ypk1-AS rho0 + PKC1R398P (PLY1532), Ypk1-AS fpk1Δ (PLY1533), and Ypk1-AS fpk1Δ + PKC1R398P (PLY1538) were grown in either SCD-Ura or SCD-Ura-Leu medium and treated with 0.5 μM 2,3-DMB-PPI for 1 h. ROS was determined and quantified as in Figure 1C. *p values were calculated using Student’s t test; **p between 0.05 and 0.01; ***p ≤ 0.01. (B) Quantification of actin polarization after fixing and rhodamine-phalloidin labeling in the same strains as in A, with at least 100 cells counted for each sample. (C) Ypk1-WT (PLY1083), Ypk1-AS (PLY1098), and Ypk1-AS fpk1Δ (PLY1533) were grown in either SCD-Ura or SCD-Ura + 50 mM MES, pH 6.2, and treated with 0.5 μM 2,3-DMB-PPI for 1 h. Cells were harvested and lysed, and the resulting protein extracts were resolved by SDS–PAGE and immunoblotted with anti–phospho-p44/42 MAPK (for p-Mpk1), anti-Mpk1, and anti-G6PDH antibodies. Quantification below the blot describes the difference relative to Ypk1-WT after normalizing to the anti-p44/42 MAPK signal. (D) WT (PLY062) and fpk1Δ (PLY1440) were grown in SCD medium and treated with 1.25 μM myriocin (Myr) for 1 h as noted and then processed as in C. (E) WT (PLY062) and WT + PKC1R398P (PLY1550) were grown in SCD or SCD-Leu medium and treated as in D. ROS was detected and quantified as in Figure 1C, p values were calculated using Student’s t test; **p ≤ 0.01. (F) Quantification of actin polarization after fixing and rhodamine-phalloidin labeling in the same strains as in E, with least 100 cells counted for each sample.
sphingolipids also played a role in regulating Pkc1 activity. We treated WT cells with myriocin and examined Mpk1 phosphorylation, which we observed was significantly reduced (Figure 3D). Of interest, deletion of FPK1 largely restored Mpk1 phosphorylation in myriocin-treated cells, suggesting that Fpk1 contributes to sphingolipid-dependent regulation of Pkc1 activity. Because sphingolipids regulate actin polarization through ROS, we tested whether Pkc1 contributed to ROS in sphingolipid-depleted cells. Indeed, we found that overexpression of Pkc1T399P in WT cells treated with myriocin partially but significantly reduced ROS (Figure 3E), as well as improved actin polarization (Figure 3F). Taking the results together, we conclude that sphingolipids and Fpk1 cooperate to regulate Pkc1 activity and that this contributes to the suppression of ROS and maintenance of actin polarization.

**Pkc1/MAPK activity is regulated by Fpk1- and sphingolipid-dependent localization of Rom2**

Pkc1 is activated by Rho1, which, in turn, is regulated by a number of GEFs, including Rom2. Treatment with myriocin is known to disrupt Rom2 localization at the plasma membrane (PM), specifically abolishing its concentration at bud tips (Kobayashi et al., 2005). Because myriocin treatment decreased Pkc1 activity, we tested whether this correlated with mislocalization of Rom2. Indeed, we observed that bud tip recruitment of a green fluorescent protein (GFP)-tagged version of Rom2 was disrupted in Ypk1-AS cells (Figure 4A). On the basis of our finding that sphingolipid levels affect Pkc1 activity, we hypothesized that decreased sphingolipids in Ypk1-AS cells may contribute to the mislocalization of Rom2. We demonstrated previously that addition of the sphingolipid precursor phytosphingosine (PHS) to torc2-ts cells that are deleted for LC8B, the major LC8 kinase, increases synthesis of downstream complex sphingolipids to a level sufficient to restore viability, as well as rescue defects in actin polarization (Aronova et al., 2008). Therefore, we treated Ypk1-AS lcb4Δ cells with PHS and examined Rom2-GFP localization; we observed that bud tip recruitment of Rom2 was significantly restored (Figure 4A). Similarly, we observed that bud/necrotic recruitment of GFP-Rom2 was also improved in Ypk1-AS fpk1Δ cells (Figure 4A). Together these data demonstrate that Fpk1 activity and sphingolipids are critical for Ypk1-dependent regulation of Rom2 localization.

Of interest, treatment with NAC did not restore Rom2 localization at the PM (Figure 4A), suggesting that sphingolipids and Fpk1 do not regulate Rom2 localization via ROS and/or actin polarization.

We next tested whether Rom2 mislocalization contributed to decreased Pkc1 activity in Ypk1-AS cells. Because deleting FPK1 from Ypk1-AS resulted in restoration of Rom2 bud/necrotic recruitment, as well as rescue of Pkc1 activity, we examined whether this rescue of Pkc1 activity required the presence of Rom2. To do this, we deleted ROM2 from Ypk1-AS fpk1Δ cells and examined Pkc1 activity. Indeed, rescue of Pkc1 activity in Ypk1-AS fpk1Δ cells required the presence of ROM2, as Mpk1 phosphorylation was reduced in Ypk1-AS fpk1Δ rom2Δ cells (Figure 4B). Furthermore, the rescue of ROS and actin depolarization that results from deleting FPK1 from Ypk1-AS cells also required ROM2 (Figure 4, D and E). Consistent with these findings, we observed that overexpression of ROM2 in Ypk1-AS cells resulted in increased Mpk1 phosphorylation (Figure 4C), decreased ROS, and improved actin polarization (Figure 4, D and E). Taking these results together, we conclude that restoring Rom2 activity at the PM is crucial for Pkc1 activation, both to rescue ROS and to maintain actin polarization, in Ypk1-deficient cells. In agreement with results of a prior study (Vilella et al., 2005), we observed that loss of Rom2 activity on its own did not result in increased ROS or actin depolarization in rom2Δ cells (unpublished data), suggesting that the Rom2 and Pck1/MAPK branch of the pathway becomes essential within the context of deficient Ypk1 signaling.

**Pkc1/MAPK regulates ROS through cyclin C stability**

Because the MAPK signaling pathway is one of the best-characterized targets of activated Pck1, we tested whether Pkc1 regulation of ROS was mediated by MAPK signaling, first by examining the MAPKK KKK kinase Bck1. We observed that expression of a constitutively active allele of Bck1 (Bck1-20) in Ypk1-AS cells reduced ROS levels similar to that observed by expression of Pkc1T399P, suggesting that Pkc1 regulates ROS through the MAPK signaling pathway (Figure 5A). Of interest, MAPK signaling has been shown to regulate the oxidative stress response by controlling the stability of cyclin C, a transcriptional repressor that inhibits several stress-responsive genes (Krasley et al., 2006). Accordingly, we deleted CNC1, the gene encoding cyclin C, from Ypk1-AS cells and examined ROS levels. Consistent with a role for MAPK in mediating ROS through cyclin C, Ypk1-AS cnc1Δ cells exhibited significant reduction in ROS compared with Ypk1-AS cells (22 vs. 55% DCF-positive cells; Figure 5A). Cyclin C regulates transcription by activation of the cyclin-dependent kinase Cdk8 but has also been shown to regulate ROS independently of Cdk8 (Krasley et al., 2006). No change in ROS was observed in Ypk1-AS cells after deletion of CDK8, indicating that cyclin C is likely to regulate ROS by a mechanism that is independent of Cdk8-mediated transcription (Figure 5A). Consistent with these findings, we observed that actin polarization was improved in Ypk1-AS cells by expression of Bck1-20 or deletion of CNC1 but not by deletion of CDK8 (Figure 5B).

On oxidative stress, cyclin C is degraded to promote activation of oxidative stress responses (Krasley et al., 2006). On the basis of our foregoing results, we tested whether Ypk1-AS cells exhibited a defect in the regulation of cyclin C stability. Specifically, we examined cyclin C protein levels after treatment with H2O2, using a myc-epitope tagged version of cyclin C (Cooper et al., 1997). In agreement with previous findings (Krasley et al., 2006), cyclin C levels were reduced in WT cells by treatment with H2O2 but not in mpk1Δ cells (Figure 5C). This finding confirmed a requirement for Pck1-MAPK signaling in ROS-mediated cyclin C degradation. Significantly, Ypk1-AS cells exhibited increased cyclin C levels compared with Ypk1-WT cells, and, in addition, treatment with H2O2 failed to decrease cyclin C levels, consistent with Ypk1-AS cells possessing reduced Pck1 activity. Furthermore, we observed that restoring Pck1 activity in Ypk1-AS cells, either by deleting FPK1 or by treating Ypk1-AS lcb4Δ cells with PHS, resulted in cyclin C degradation after treatment with H2O2 (Figure 5C). Together these results suggest that Pck1-mediated degradation of cyclin C is important for the regulation of ROS and actin polarization in Ypk1-deficient cells.

**DISCUSSION**

Our data presented here identify ROS as a crucial mediator of TORC2/Ypk1 regulation of actin polarization. Our findings support a model in which TORC2/Ypk1 regulation of Fpk1, sphingolipids, and mitochondrial respiration combines to prevent ROS-induced oxidation of critical cysteine residues on actin (Figure 6). We also find that Fpk1 and sphingolipids regulate Pck1 activity by influencing the localization of Rom2 at the PM, and Pck1 in turn regulates ROS through MAPK-dependent destruction of cyclin C protein. Thus, whereas Pck1-MAPK signaling is known to function downstream of TORC2 to regulate actin polarization, our findings demonstrate that ROS is a critical determinant within this pathway.
Because Rom2 is required for activation of Pkc1, our observation that Rom2 is mislocalized in Ypk1-AS cells can account for this defect in Pkc1 activation. In addition, our findings that restoring sphingolipid levels or deletion of \textsc{fpk1} is sufficient to restore Rom2 localization suggests that Pkc1/MAPK signaling is induced after oxidative stress and is an important part of the cellular response to ROS (Pujol-Carrion et al., 2013; Vilella et al., 2005). By contrast, we observed that increased ROS correlates with decreased Pkc1 activity in Ypk1-deficient cells.
indicate that membrane lipid composition is an important factor for
Rom2 activity. Rom2 localization is dependent on phosphatidylinosi-
tol 4,5-bisphosphate (PIP$_2$) at the PM, and sphingolipids have been
shown to regulate PIP$_2$ levels by regulating the activity of the phos-
Indian muscle (Saito et al., 2007). Because Rom2 regulates
Pck1 via activation of Rho1, which also localizes to bud tips, another possibility is that
Fpk1-dependent modulation of phospholipid distribution regulates Rho1 directly. Of
interest, a recent study implicated Pck1 in the regulation of membrane fluidity by de-
termining phospholipid acyl group composition (Lockshon et al., 2012). Thus one intrigu-
ging possibility is that phospholipid composition and distribution within the lipid bilayer are interconnected by functional in-
teractions between Pck1 and Fpk1 and that this is critical for maintenance of membrane
homeostasis.

Regulation of ROS by Pck1 provides an explanation for the observation that overex-
pression of Pck1 restores actin polarization in torc2/ypk1 mutants (Helliwell et al., 1998a; Roelants et al., 2002). Previous studies
showed that MAPK signaling is required to regulate the cellular re-
sponse to oxidative stress by modulating cyclin C protein levels. We extended these findings by showing that misregulation of cyclin C
degradation leads to an increase in ROS in Ypk1-deficient cells. MAPK phosphorylation is necessary for cyclin C nuclear-to-cytoso-
plasmic translocation, where cyclin C destruction occurs (Cooper et al., 2012; Jin et al., 2014). Cyclin C is known to repress the activity of
stress response genes, including catalase and several protein chap-
erones (Cooper et al., 1997; Holstege et al., 1998), whose absence
could lead to an increase in ROS. However, we found that deleting
the gene for Cdk8, the cyclin-dependent kinase that partners with
cyclin C, does not affect ROS. Thus either cyclin C represses tran-
scription of target genes independently of Cdk8 or cyclin C regu-
lates ROS by a mechanism that is distinct from transcription.

Cytoskeletal organization in mammalian cells is regulated by
mTORC2 and involves Rho GTPases and PKC (Jacinto et al., 2004; Li and Gao, 2014), which leads us to speculate that TORC2/
Ypk1 regulation of actin organization by ROS is likely to be con-
served. Consistent with a role for mTORC2 in the regulation of the actin cytoskeleton, mTORC2 is required for neutrophil migra-
tion toward chemoattractants (He et al., 2013). Of importance,
mTORC2 regulation of actin has also been associated with in-
creased cancer cell migration and invasion (Gupta et al., 2013).
Because ROS is also associated with regulation of cell motility,
our findings provide novel insight that may be useful in under-
standing the mechanisms involved in mTORC2-dependent cell
migration and provide new targets for preventing or limiting invasion of cancer cells.

**MATERIALS AND METHODS**

**Strains, media, and plasmids**

Yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Culture medium used was synthetic complete dextrose (SCD; 0.8% yeast nitrogen base without amino acids, pH 5.5, 2% dextrose) supplemented with amino acids as described previously (Sherman, 1991). All yeast transformations were conducted using a lithium acetate procedure (Geitz and Woods, 1998). Strains were made respiratory deficient (rho0) by treating with 25 μg/ml ethidium bromide for 16 h, as described in Fox et al. (1991). Construction of deletion strains by replacement of complete open reading frames (ORFs) with a selectable marker was performed as described previously (Dilova et al., 2004) or by replacement of the ORF with the reusable Kan’ marker as described in Guldener et al. (1996). Construction of expression plasmids was performed by PCR amplification, with mutations introduced by PCR SOEing. pPL602 rom2::KanMX was amplified from two yeast strains in pRS315Met25. (1990). Construction of expression plasmids was performed by PCR amplification, with mutations introduced by PCR SOEing. pPL602 rom2::KanMX was amplified from two yeast strains in pRS315Met25.

**Actin labeling and fluorescence microscopy**

Actin labeling and detection in yeast cells was performed as described previously (Aronova et al., 2008). For quantification of status of actin polarization, at least 100 small- and medium-budded

### Table 1: Saccharomyces cerevisiae strains used in this study.

| Strain | Genotype | Source |
|--------|----------|--------|
| PLY062 | W303α    | Nasmyth et al. (1990) |
| PLY517 | W303α, except mpk1::KanMX | Aronova et al. (2007) |
| PLY1083| W303α, except ypk1::TRP1 ypk2::His3 + [pPL216] | Niels et al. (2012) |
| PLY1098| W303α, except ypk1::TRP1 ypk2::His3 + [pPL220] | Niels et al. (2014) |
| PLY1134| W303α, except avo3-30-MYC:TRP1 | Niels et al. (2012) |
| PLY1440| W303α, except fpk1::KanMX | Niels et al. (2012) |
| PLY1527| W303α, except ypk1::TRP1 ypk2::His3 yap1::KanMX + [pPL220] | Niels et al. (2014) |
| PLY1528| W303α, except ypk1::TRP1 ypk2::His3 rho0 + [pPL220] | Niels et al. (2014) |
| PLY1529| W303α, except ypk1::TRP1 ypk2::His3 tpk3::KanMX + [pPL220] | This study |
| PLY1531| W303α, except ypk1::TRP1 ypk2::His3 + [pPL220] + [pPL474] | This study |
| PLY1532| W303α, except ypk1::TRP1 ypk2::His3 rho0 + [pPL220] + [pPL474] | This study |
| PLY1533| W303α, except ypk1::TRP1 ypk2::His3 fpk1::KanMX + [pPL220] | Niels et al. (2014) |
| PLY1534| W303α, except ypk1::TRP1 ypk2::His3 fn1::KanMX + [pPL220] | Niels et al. (2014) |
| PLY1536| W303α, except ypk1::TRP1 ypk2::His3 fn1::KanMX + [pPL220] | Niels et al. (2014) |
| PLY1538| W303α, except ypk1::TRP1 ypk2::His3 fn1::KanMX + [pPL220] | This study |
| PLY1550| W303α + [pPL474] | This study |
| PLY1556| W303α, except ypk1::TRP1 ypk2::His3 lcb4::KanMX + [pPL220] | Niels et al. (2014) |

**Strains, media, and plasmids**

Yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Culture medium used was synthetic complete dextrose (SCD; 0.8% yeast nitrogen base without amino acids, pH 5.5, 2% dextrose) supplemented with amino acids as described previously (Sherman, 1991). All yeast transformations were conducted using a lithium acetate procedure (Geitz and Woods, 1998). Strains were made respiratory deficient (rho0) by treating with 25 μg/ml ethidium bromide for 16 h, as described in Fox et al. (1991). Construction of deletion strains by replacement of complete open reading frames (ORFs) with a selectable marker was performed as described previously (Dilova et al., 2004) or by replacement of the ORF with the reusable Kan’ marker as described in Guldener et al. (1996). Construction of expression plasmids was performed by PCR amplification, with mutations introduced by PCR SOEing. pPL602 rom2::KanMX was amplified from two yeast strains in pRS315Met25. (1990). Construction of expression plasmids was performed by PCR amplification, with mutations introduced by PCR SOEing. pPL602 rom2::KanMX was amplified from two yeast strains in pRS315Met25.

**Actin labeling and fluorescence microscopy**

Actin labeling and detection in yeast cells was performed as described previously (Aronova et al., 2008). For quantification of status of actin polarization, at least 100 small- and medium-budded
cells were counted for each condition. Cells were considered as polarized if actin patches were concentrated in the bud and five or fewer patches were found in the mother cell. Cells were considered as depolarized if actin patches were distributed in both the bud and the mother cell. DA and 5(6)CFDA imaging was performed using a Nikon E600 fluorescence microscope as described (Niles et al., 2012). Fluorescent protein imaging was performed using the spinning-disk module of a Marianas SDC Real Time 3D Confocal-TIRF microscope (Intelligent Imaging Innovations, 3i) as described (Niles et al., 2012). Image capture and processing was done using SlideBook5 software (3i) and Photoshop (Adobe).

**Cyclin C immunoprecipitation**

Yeast strains expressing myc-tagged cyclin C were grown in 0.5-L cultures at 30°C to 0.5 OD<sub>600</sub>/ml in SCD without leucine and treated with 0.2 mM H<sub>2</sub>O<sub>2</sub> as noted. Cells were pelleted and washed in H<sub>2</sub>O and then in extract buffer (YEB; 50 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid-KOH, pH 7.1, 100 mM β-glycerolphosphate, 50 mM NaF, 5 mM ethylene glycol tetraacetic acid, 5 mM EDTA, 10% glycerol, 0.25% Tween 20, and 150 mM KCl). Pellets were resuspended 1:1 (wt/vol) in YEB containing protease inhibitors (cocktail tablet; Roche), 2 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride, and cell lysates were frozen into pellets by dripping into liquid nitrogen. These pellets were then beaten in a freezer mill (6970EFM; SPEX Sample Prep) three times for 1 min. On thawing, the lysate was spun two times for 20 min at 14,000 x g at 4°C. Two milligrams of total protein was incubated with α-myc antibody (9E10; Covance) and rotated for 2 h at 4°C before incubation with YEB-washed Protein G Sepharose beads. Bound beads were resuspended in SDS-sample buffer and boiled to remove bound protein. “Input” samples of 50 μg of total yeast protein and “precipitation” samples were separated by 10% SDS–PAGE, followed by Western blotting using the same anti-myc antibody (1:1000) and anti-glucose-6-phosphate dehydrogenase (G6PDH; 1:100,000; Sigma-Aldrich).

**Western blotting**

Protein extracts from at least three separate experiments were prepared using the NaOH cell lysis method (Dilova et al., 2004), loaded onto SDS–PAGE gels, and transferred to nitrocellulose membrane. Membranes were probed with anti–phospho-p44/42 MAPK (1:1000; Cell Signaling Technology), anti–Mpk1 (1:1000; Santa Cruz Biotechnology), and anti-G6PDH (1:100,000; Sigma-Aldrich) primary antibodies and visualized using the appropriate secondary antibodies conjugated to IRDye (1:5000; LI-COR Biosciences) on the Odyssey Infrared Imaging System (LI-COR Biosciences). Images were quantified using ImageQuant software (GE Healthcare).

**Statistical analysis**

Averages are presented with means ± SD. The p values were calculated using Student’s t test; *p between 0.05 and 0.01, **p ≤ 0.01.

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**TABLE 2**: Plasmids used in this study.

| Plasmid | Parent vector | Insert/ORF | Source |
|---------|---------------|------------|--------|
| pRS315  | Sikorski and Heiter (1989) | Sikorski and Heiter (1989) | Sikorski and Heiter (1989) |
| pRS315Met25 | Niles et al. (2012) | Niles et al. (2012) | Niles et al. (2012) |
| pRS316  | Sikorski and Heiter (1989) | Sikorski and Heiter (1989) | Sikorski and Heiter (1989) |
| pRS425  | Sikorski and Heiter (1989) | Sikorski and Heiter (1989) | Sikorski and Heiter (1989) |
| pYO2518 | ROM2-GFP | Abe et al. (2003) | Abe et al. (2003) |
| pAS32  | ROM2-HA | Schmidt et al. (1997) | Schmidt et al. (1997) |
| pLR101 | CNC1-myc | Cooper et al. (1997) | Cooper et al. (1997) |
| pPL216 | pRS316 | YPK1 | Niles et al. (2012) |
| pPL220 | pRS316 | YPK1<sub>1424G</sub> | Niles et al. (2014) |
| pPL250 | pRS315 | YPK1 | Niles et al. (2012) |
| pPL251 | pRS315 | YPK1<sub>1424G</sub> | Niles et al. (2012) |
| pPL474 | pRS425 | PKC1<sub>R398P</sub> | This study |
| pPL586 | pRS425 | BCK1<sub>R520P</sub> | This study |
| pPL592 | pRS316 | ACT1 | This study |
| pPL593 | pRS316 | ACT1<sub>C374A</sub> | This study |
| pPL602 | pRS315Met25 | FPK1<sub>S481A</sub> T244A | This study |
| pPL603 | pRS315Met25 | FPK1<sub>S481A</sub> T244A | This study |

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