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Inositol pyrophosphates modulate hydrogen peroxide signaling.

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List of Abbreviations: IP₃ Inositol(1,4,5)trisphosphates; IP₄ inositol tetrakisphosphates; IP₅ inositol pentakisphosphates, IP₆ inositol hexakisphosphates; IP₇ diphosphoinositol pentakisphosphate; IP₈ bisdiphosphoinositol tetrakisphosphate; PP-IP₄ diphosphoinositol-tetrakisphosphate; (PP)₂-IP₃ bisdiphosphoinositol-trisphosphate; ROS Reactive Oxygen Species; H₂O₂ hydrogen peroxide; IP6K Inositol hexakisphosphate kinase; PP-IP5K or IP7K diphosphoinositol pentakisphosphate kinase; IPK1 inositol phosphate kinase 1; IPMK inositol polyphosphate multi kinase.

Abstract

Inositol pyrophosphates are involved in a variety of cellular functions but the specific pathways and/or downstream targets remain poorly characterized. In this study we use *Saccharomyces cerevisiae* mutants to examine the potential roles of inositol pyrophosphates in responding to cell damage caused by reactive oxygen species (ROS). Yeast lacking *kcs1* (the *S. cerevisiae* IP6K) have greatly reduced IP₇ and IP₈ levels, and display increased resistance to cell death caused by hydrogen peroxide (H₂O₂) consistent with a sustained activation of DNA repair mechanisms controlled by the Rad53 pathway. Other Rad53 controlled functions such as actin polymerization appear unaffected by inositol pyrophosphates. Yeast lacking *vip1* (*S. cerevisiae* PP-IP5K) accumulate large amounts of the inositol pyrophosphate IP₇, but have no detectable IP₈, indicating that this enzyme represents the physiological IP₇-Kinase. Similar to *kcs1*Δ yeast, *vip1*Δ cells showed an increased resistance to cell death caused by H₂O₂, indicating that it is likely the double pyrophosphorylated form of IP₈ ((PP)₂-IP₄) that mediates the H₂O₂ response. However, inositol pyrophosphates are not involved in directly sensing DNA damage, as *kcs1*Δ cells are more responsive to DNA damage caused by pleomycin. We observe *in vivo* a rapid decrease in cellular inositol pyrophosphates levels following exposure to H₂O₂, and an inhibitory effect of H₂O₂ on the enzymatic activity of KCS1 *in vitro*. Furthermore, parallel cysteine mutagenesis studies performed on mammalian IP6K1 are suggestive that the ROS signal might be transduced by the direct modification of this evolutionary conserved class of enzymes.

Introduction

Inositol pyrophosphates in both lower eukaryotes such as yeast as well as in mammalian cells are generated by the sequential phosphorylation of inositol trisphosphate (IP₃) [1, 2] (Figure 1). The inositol polyphosphate multi kinase, IPMK (*Saccharomyces cerevisiae* ARG82 or IPK2) metabolizes IP₃ to IP₄ and IP₅ [3-5]. Subsequent phosphorylation of IP₅ by IPK1 generates IP₆ (phytic acid or inositol hexakisphosphate). The fully phosphorylated ring of IP₆ is then further phosphorylated by the inositol hexakisphosphate kinase KCS1 (*S. cerevisiae* homolog of mammalian IP6Ks) and VIP1 (*S. cerevisiae* homolog of mammalian PP-IP5K or IP7K) to generate the inositol pyrophosphates IP₇ (diphosphoinositol pentakisphosphate) and the double pyrophosphorylated form of IP₈ (also referred to as (PP)₂-IP₄, bisdiphosphoinositol tetrakisphosphate), respectively (Figure 1) [4, 6-8]. It is noteworthy that a triphosphate form of "IP₈" (referred to as PPP-IP₅) has recently been identified, which is produced by the direct action of IP6Ks [9]. Inositol pyrophosphates have been linked to a variety of biological functions ranging from vesicular trafficking to apoptosis and telomere length maintenance (recently reviewed in [1, 10]). The most thrilling characteristic of IP₇ is its recently identified ability to pyro-phosphorylate proteins in a kinase-independent manner. However, only a handful of IP₇ targets have been identified so far, and the signaling pathways involved remain largely unknown. It has previously been reported that expression of the mammalian IP6Ks (IP6K1/2/3) in a range of mammalian cells increases their sensitivity to DNA damaging agents, while knock-down of IP6K2 resulted in protection against apoptosis [11-13].

Eukaryotic cells are constantly exposed to exogenous and endogenous agents that cause DNA damage. This can lead to genomic instability, cell death, or genetic anomalies such as neurodegenerative diseases and cancer. To monitor and respond to DNA damage cells have developed sophisticated checkpoint controls as surveillance mechanisms [14]. These are evolutionarily conserved and consist of sensor proteins, that recognise physical alterations in DNA, and transducers that transmit the damage signal to downstream effectors [15]. When DNA checkpoints are activated by DNA lesions or replication blocks they promote transcription of DNA repair genes, delay cell cycle progression, or initiate apoptosis. In *S. cerevisiae* the sensor and signaling functions are mainly performed by the PI3 kinase-like kinases Mec1 and Tel1 (*S. cerevisiae* homologs of mammalian ATM and ATR). Once activated Mec1/Tel1 trigger a phosphorylation cascade that activates Rad53 (*S. cerevisiae* homolog of mammalian Chk2) [16, 17]. A major source of spontaneous DNA damage is caused by the reactive oxygen species (ROS), which can be formed endogenously as by-products of aerobic respiration or other cellular processes [18]. ROS is a general term that includes, hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻) and the hydroxyl radical (HO•). These molecules are generally regarded as toxic compounds because of their chemical reactivity. However, it is also extensively recognized that ROS have important roles as intracellular signaling molecules [19-22].

In mammalian cells overwhelming evidence indicates that the transient production of H₂O₂ is triggered by ligand-mediated receptor activation including growth factors such as platelet-derived growth factor (PDGF) [23] or epidermal growth factor (EGF) [24]. These observations were instrumental in establishing H₂O₂ as a second messenger [19]. It is now accepted that at low concentrations hydrogen peroxide is an important signaling molecule that controls several physiological processes, while at high concentrations it is regarded as a highly toxic molecule [20, 25]. Consequently, spatial and temporal regulation of H₂O₂ production and degradation ensure a positive role for this molecule as an intracellular messenger [19, 26]. Cells have in fact evolved several mechanisms for removing H₂O₂ including catalase, glutathione peroxidase and peroxiredoxin as a means of protecting against oxidatively induced death [27].

In yeast ROS signaling is usually studied via the exposure to exogenous agents such as H_2O_2 that result in DNA base modification, single and double-strand breaks, and the formation of apurinic/apyrimidinic lesions [28-30]. Limited DNA damage will trigger a DNA Checkpoint response promoting DNA repair mechanisms. In contrast, massive DNA damage will lead to cell death. In this study we used yeast to characterize the IP_7 -mediated response to ROS. As a DNA damaging agent, we used sub-lethal concentrations of H_2O_2 which activate the DNA damage response pathway [31]. Our results indicate that yeast mutants that are deficient in inositol pyrophosphates possess a higher threshold of resistance to the lethal effects of H_2O_2 , but not to other DNA damaging agents. This specific resistance to hydrogen peroxide correlates with a sustained activation of Rad53 and consequently a privileged establishment of a DNA repair mechanism. We further report that H_2O_2 regulates higher inositol metabolism, causing a rapid decrease in cellular levels of inositol pyrophosphates.

Materials and Methods

Reagents

Antibodies against Rad53 and Rad9 were purchased from Santa Cruz the antibody against Hsp60 was purchased from Stressgen. Yeast Media were from Formedium and all the other reagents were from Sigma Aldrich. NuPage 4-12% Bis-Tris gels (Invitrogen) were used for proteins analyses.

Strains, plasmids and media

Yeast (*Saccharomyces cerevisiae*) strains used in this study are listed in table 1 were derived from the BY4741 (MATa, his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0) or the protease-deficient DDY1810 (MATa; MATa leu2-3,112 trp1- Δ 901 ura3-52 prb1-1122 pep4-3 prc1-407) genetic backgrounds. Null mutant yeast of the DDY1810 background were generated using standard homologous recombination techniques [32]. Yeast TAP-tagged KCS1 strain was obtained from Open Biosystems. Mouse IP6K1 and kinase dead IP6K1-K(226)/A [33] were sub-cloned into the pYes2NTA vector (Invitrogen). Cells were grown in synthetic complete (SC) media with 2% glucose at 30°C with aeration and plated on SC plates unless stated otherwise. Cells containing expression plasmids were grown in SC media lacking uracil under the same experimental conditions. Analysis of inositol polyphosphate HPLC profiles, revealed a rescue of normal inositol pyrophosphates level when using the pYes2-IP6K1 construct in glucose containing medium; no rescue is observed when the mutant form pYes2-IP6K1KA is utilized.

Colony Forming Assay

To observe the effect of H₂O₂ in logarithmically growing cultures yeast were grown to log-phase in SC media at 30°C and were treated with various concentrations of H₂O₂. To assess viability the cultures were serially diluted and plated onto SC plates. Untreated samples were used as a standard for calculating percent viability.

DNA damage response activation

Cultures (2-4 units of OD₆₀₀ 0.2-0.7) in mid-log phase were treated with various concentration of H₂O₂ for the indicated times at 30°C. Cells were collected by centrifugation and rapidly lysed in lysis buffer (20mM HEPES pH 6.8; 1mM EGT; 1mM EDTA; 0.1% CHAPS; 5mM NaF; 5mM DTT, 200 mg/L phenylmethanesulfonyl fluoride; proteinase inhibitor cocktail 1:500 dilution). Ten percent of the extracts, about 20 μ g of protein were used for western blotting with anti-Rad53 (Santa Cruz; sc-6748) or anti-Rad9 (Santa Cruz; sc-6740) antibodies. For western blotting normalization the membrane was probed with anti-Hsp60 (Santa Cruz; sc-13966) or alternatively the blot was stained with coomassie blue.

Mutation frequency assay

The Can^R assay was essentially performed essentially as described previously by Madia et al. [34]. Briefly, yeast cultures were grown to log-phase in SC media at 30° C. The cultures were treated with 1mM H₂O₂ for 3 h, washed with H₂O and approximately 1x10⁷ cells were spread on arginine deficient plates containing 60 mg/ml L-canavanine. Serial dilutions of samples from the same cultures were spread on plates containing only minimal medium in order to calculate the exact number of cells/ml. After 4 days incubation the number of colonies was counted and the mutation frequency was calculated as mutations/10⁶ cells.

Generation of mouse IP6K1 mutants

The bacterial expression plasmid harbouring Gst-IP6K1 was previously described [7]. The conserved cysteine mutants were generated by PCR technology using the following oligos and their complementary partners: Cys48Ala (gaccactgtggccaaacctctc), Cys187Ala (tggagcctgcgggcccacaagcagcag), Cys221Ala (ttcaagtaccccgcggtgctggacttg), Cys221Asp (ttcaagtaccccgatgtgctggacttg), Cys248Ala (cagatgaggaaggctgagcagagcacg), Cys261Ala (ggggtcagggtcgccggcatgcaggtg). The mutagenesis was confirmed by restriction analysis or by direct sequencing. Purification of His-tagged proteins was performed as previously described [7].

IP₆-Kinase assay.

Purification of TAP-protein was performed as previously described [35]. Briefly, yeast KCS1-TAP cells (100ml OD₆₀₀ 0.8-1.2) were harvested by centrifugation and washed with ice cold water. Cells were homogenized using glass beads for 5 min in 5 ml of ice-cold binding buffer: 50 mM HEPES pH 7.4, 150 mM NaCl, 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl₂, 2 mM NaF. The homogenate was centrifuged at 15,000g for 20 min. Calmodulin-sepharose 4B (40 μl) (Amersham) was added to the supernatant and incubated at 4°C for 2 h. The resin was then washed twice with binding buffer containing 500 mM NaCl and then twice with 100 mM NaCl. The resin was then used to perform IP₆ kinases reactions using [³H]-IP₆ (Amersham) as previously described [4] and reactions were analysed by SAX-HPLC.

Determination of yeast inositol polyphosphates levels

The studies of yeast inositol polyphosphate levels, was performed as previously described [36] with a few modifications; yeast were grown in synthetic media without inositol and supplemented with 75 μCi/ml of [³H]inositol (Perkin-Elmer NEN). After drug treatment the yeast were harvested by centrifugation, washed with ice-cold water, and lysed in 0.3 ml of ice-cold lysis buffer (1 M perchloric acid, 3 mM EDTA) with two minutes of glass beads beating. Lysates were centrifuged at 4°C and neutralized with the neutralization solution (1 M potassium carbonate, 3 mM EDTA). Inositol phosphates were resolved by SAX-HPLC using a 4.6×125-mm PartiSphere SAX column (Whatman). The column was eluted with a gradient generated by mixing buffer A (1 mM Na₂EDTA) and buffer B (buffer A plus 1.3 M (NH₄)₂HPO₄, pH 3.8, with H₃PO₄) as follows: 0–5 min, 0% B; 5–10 min, 0–30% B; 10–60 min, 30–100% B; 60–80 min, 100% B. Fractions (1 ml) were collected and counted using 4 ml of Ultima-Flo AP LCS-cocktail (Packard). Inositol polyphosphates species were identified by comigration with standards using [³H]-IP₆ (Amersham), [³H]IP₇ prepared by phosphorylation of [³H]InsP₆ and [³H]PP-IP₄ purified from metabolically labelled *ipk1Δ* cell as described [7, 36].

Results

Yeast deficient in inositol pyrophosphates are resistant to H₂O₂

To study whether inositol pyrophosphates influence yeast survival after exposure to H₂O₂, WT and *kcs1Δ* yeast lacking the IP6K gene, and therefore do not possess inositol pyrophosphates [37, 38] (Figure 4A), were treated with 0.1mM, 0.5mM and 1mM H₂O₂ for 3 hours. We studied the effects of H₂O₂ using two different yeast genetic backgrounds, DDY1810 and BY4741 (Table 1). Differences in genetic background are an established cause of experimental variability [39, 40] and in fact we observe diverse sensitivity to H₂O₂ in the two WT strains (Figure 2A and B effect of the 0.5 mM concentration). However, survival rate evaluated by colony formation assays revealed that the *kcs1Δ* yeast are significantly more resistant to higher concentrations of H₂O₂, when compared to the respective WT strain in both genetic backgrounds (Figure 2A and B). When IP₇ levels were restored in *kcs1Δ* cells by expressing the mammalian IP6K1 enzyme this resistance was reversed, while cells expressing a kinase-dead IP6K1 mutant (IP6K1KA) remained significantly more resistant to the H₂O₂ treatment (Figure 2A). To further address whether inositol pyrophosphate levels correlated with the survival response to H₂O₂, we analysed yeast lacking the IPK1 gene (*ipk1Δ*). As IPK1 is the enzyme responsible for IP₆ synthesis, thus *ipk1Δ* yeast lacks IP₆ and IP₇ but contains large amounts of the inositol pyrophosphate species derived from IP₅ (Figure 1) diphosphoinositol-tetrakisphosphate (PP-IP₄) and bisdiphosphoinositol-trisphosphate ((PP)₂-IP₃) [38]. The *ipk1Δ* yeast showed a sensitivity to H₂O₂ comparable to WT, while the *ipk1Δkcs1Δ* double mutant that once again lacks inositol pyrophosphates remain resistant (Figure 2B). Previous studies showed that although *ipk1Δ* mutant yeast have abnormal inositol phosphate metabolism, their morphology is identical to WT yeast, suggesting that PP-IP₄ and (PP)₂-IP₃ can carry out the functions normally performed by IP₇ and IP₈ [38, 41]. Together these results demonstrated that inositol pyrophosphates play a key role in mediating hydrogen peroxide-induced cell death. Notably, this function might not be restricted to IP₇/IP₈ but could extend to other inositol pyrophosphate species.

Inositol pyrophosphates regulate Rad53 activation following H₂O₂ treatment

We next tested whether Rad53, an important component of the DNA damage response in yeast, was a downstream target of inositol pyrophosphate signaling. Rad53 activation can be observed as an electrophoretic mobility shift caused by its trans-phosphorylation by Mec1 and Tel1 followed by Rad53 auto-phosphorylation [42, 43]. In total, Rad53 is phosphorylated at more than 20 residues [44]. The DNA damage checkpoint response was investigated by exposing WT and *kcs1Δ* mutant yeast to different concentrations (0.1 mM, 0.5mM and 1mM) of H₂O₂ for various times; proteins were then extracted and blotted with Rad53-specific antibodies. We observed a rapid activation of Rad53 in response to 0.5mM H₂O₂ in both DDY1810 WT and *kcs1Δ* cells. Although we failed to observe Rad53 activation when WT cells were exposed to 1mM H₂O₂, this stimulation strongly activated Rad53 in *kcs1Δ* mutants (Figure 3A), correlating with the increased survival of these cells. Similarly, Rad9, which acts upstream of Rad53, was only activated in *kcs1Δ* at high concentrations of H₂O₂ (data not shown). Rad9 is a large protein (148 kDa) that displays a less evident mobility shift upon H₂O₂ treatment. Therefore, Rad53 was used in all subsequent experiments as a read-out of the activation of the DNA damage response. Expression of mammalian IP6K1 in *kcs1Δ* yeast restored the WT phenotype with rescued cells failing to activate Rad53 in response to 1mM H₂O₂. In contrast, cells expressing the kinase-dead IP6K1KA still retained strong Rad53 activation (Figure 3B). These results indicate that inositol pyrophosphates and not the Kcs1 protein itself, mediate Rad53 activation. Consistently, we did not observe Rad53 activation in *ipk1Δ* mutant treated with 1mM H₂O₂, although Rad53 phosphorylation occurred in

ipk1Δkcs1Δ double mutants (Figure 3C).

To test if the prolonged activation of Rad53 in *kcs1Δ* mutants was associated with an increase in activation of DNA repair mechanisms, a canavanine resistance (CanR) mutation frequency assay was performed [45]. This assay measures mutation of the *CAN1* gene that encodes the arginine permease involved in the uptake of arginine and its cytotoxic analogue canavanine. Cells that have acquired mutations that inactivate *CAN1* are capable of growing in media containing canavanine. Mutation frequency can be easily measured by counting the number of colonies that are able to grow in canavanine-containing media [34]. The basal rate of mutations in *kcs1Δ* yeast is comparable to WT (Figure 3D). Exposure of WT cells to 1mM H_2O_2 for 30 minutes increased the mutation frequency of WT yeast more than twenty times, while longer treatment (3 hours) induced massive cell death. In contrast, *kcs1Δ* yeast showed a much lower mutation frequency after exposure to H_2O_2 for 30 minutes. The *kcs1Δ* yeast after 3 hours treatment, a condition that was associated with a similar survival rate to WT treated for 30 minutes, showed a still lower increase of mutation frequency (Figure 3D). These data indicate that the privileged activation of RAD53 to DNA damage in yeast deficient in inositol pyrophosphates results in the subsequent activation of a DNA damage response mechanism that ultimately supports the cells' survival.

vip1Δ mutants accumulate large amounts of IP₇, are resistant to H₂O₂, and display prolonged Rad53 activation.

Vip1 was recently identified as an additional IP6-kinase in yeast [6]. However subsequent analysis of the mammalian form suggested that it is an enzyme that more likely converts IP₇ to IP₈ [46, 47]. HPLC analysis of the *vip1Δ* single mutant yeast revealed a substantial accumulation of IP₇ (Figure 4A and Table 2), similar to what has been recently reported [48]. In yeast mutants for inositol kinases, such as IPMK and IPK1, the intracellular accumulation of specific inositol polyphosphates species provides the genetic proof that they are the physiological substrates of the deleted kinases [38, 49]. The HPLC analysis of *vip1Δ* single mutants therefore indicates that cellular IP₇ is produced by Kcs1, while Vip1 is likely responsible for the synthesis of (PP)₂-IP₄ (Figure 1, 4A and Table 2). Interestingly, we found that *vip1Δ* mutants displayed a substantial resistance to H_2O_2 , although *vip1Δkcs1Δ* double mutants were likewise resistant to cell death to an extent similar to *kcs1Δ* yeast (Figure 4B). Moreover, both *vip1Δ* and *vip1Δkcs1Δ* yeast also exhibit Rad53 activation in response to 1mM H_2O_2 (Figure 4C), suggesting that it is the double pyrophosphate form of IP₈ ((PP)₂-IP₄) (Figure 1) that plays a role in mediating the H_2O_2 response.

kcs1Δ cells show normal cell cycle-dependent Rad53 activation and actin cytoskeleton depolarization after treatment with H₂O₂

A previous report by Leroy et al. demonstrated that Rad53 activation after hydrogen peroxide treatment only occurred during the S-phase of the cell cycle [31]. To study whether the different levels of Rad53 activity observed in *kcs1Δ* and WT cells were due to abnormal Rad53 activation throughout the cell cycle, cells were arrested in G2-M phase by nocodazole or in S-phase by hydroxyurea (HU) and treated with H_2O_2 . In both WT and *kcs1Δ* yeast, Rad53 was predominantly activated during the S-phase of the cell cycle indicating that increased Rad53 activation and survival of *kcs1Δ* cells is not cell cycle-dependent (Figure 5A).

A novel function for Rad53 in regulating cell morphology and the actin cytoskeleton has been recently described [50]. Phalloidin staining of *rad53Δ* mutants was similar to WT cells, displaying a polarized actin cytoskeleton and actin cables extending from the mother cell into the bud [50]. Treatment of WT yeast with HU arrested the cells with large buds and a depolarized actin cytoskeleton. In contrast, the actin cytoskeleton of *rad53Δ* mutant cells

remained polarized upon HU treatment. These results suggested that Rad53 plays a role in regulating the actin cytoskeleton polarization upon exposure to some cell stressors [50]. To study whether abnormal Rad53 signaling in *kcs1Δ* yeast might affect the polarity state of actin after exposure to H₂O₂, yeast were stained with rhodamine-labeled phalloidin. In *kcs1Δ* cells the actin cytoskeleton appeared predominately diffused and not organized in filaments, when compared to WT. Interestingly, the diffused phenotype was reversed by introducing mammalian IP6K1 (Figure 5B). The actin behavior in WT and *kcs1Δ* yeast was also analyzed following exposure to 1mM H₂O₂ for 1hour. We observed that in both WT and *kcs1Δ* treated yeast actin becomes depolarized to a similar extent (Figure 5B). This result suggests that Rad53-mediated regulation of the polarity state of the actin cytoskeleton is not influenced by inositol pyrophosphates.

Yeast deficient in inositol pyrophosphates are sensitive to phleomycin treatment

The normal Rad53 activation during the cell cycle and the unaffected ability to polymerize actin in *kcs1Δ* cells in response to H₂O₂ indicated that inositol pyrophosphates only specifically alter Rad53 signaling in relation to cell survival. To test whether inositol pyrophosphate signaling plays a wider role in affecting cell survival, we performed cell survival and Rad53 activation assays following exposure of yeast to the DNA damaging agent phleomycin. The glycopeptide antibiotic phleomycin belongs to the bleomycin family and directly binds to DNA creating double strand breaks [51, 52]. WT and *kcs1Δ* yeast were exposed to 10 μg/ml phleomycin and the survival rate was assessed by colony formation assays (Figure 6A). We observed that *kcs1Δ* yeast was more sensitive to phleomycin, when compared to WT. The hypersensitivity of *kcs1Δ* yeast to phleomycin was reversed by introducing mammalian IP6K1 enzyme (Figure 6A). Rad53 activation analysis did not reveal significant differences between WT and *kcs1Δ* cells in response to different doses of phleomycin (Figure 6B). Similarly, a time course of Rad53 activation using 10μg/ml phleomycin failed to reveal any difference between WT and *kcs1Δ* yeast (Figure 6C). Our results indicated that *kcs1Δ* yeast are more sensitive to phleomycin treatment than WT. Therefore, it is likely that inositol pyrophosphate signaling is not directly sensing DNA damage.

Exposure to H₂O₂, but not to phleomycin, causes a rapid decrease in inositol pyrophosphates

The opposite behaviour of *kcs1Δ* yeast to phleomycin and hydrogen peroxide treatment suggested a differential modulation of inositol pyrophosphate signaling. To study whether either H₂O₂ or phleomycin treatment affected intracellular levels of IP₇ and other inositol pyrophosphates, WT and *ipk1Δ* yeast were labelled with [³H]–inositol and the cellular inositol phosphate levels were analyzed by SAX-HPLC [36] following 20 min exposure to 1mM H₂O₂ or 10μg/ml phleomycin. Treatment with phleomycin does not affected the levels of inositol pyrophosphates of both WT and *ipk1Δ* yeast (Figure 7A and B).

Interestingly, we observed that treatment with H₂O₂ led to a rapid decrease of inositol pyrophosphate levels. The H₂O₂ effect was particularly evident in the *ipk1Δ* yeast, as they contain high levels of the pyrophosphates PP-IP₄ and (PP)₂-IP₃ (Figure 7B). These results suggest that IP₇/IP₈, and possibly other inositol pyrophosphates, play an important role in mediating the cellular responses to H₂O₂. The decrease of inositol pyrophosphates in WT yeast suggest that the cells may be trying to decrease inositol pyrophosphate-mediated signaling, mimicking the situation in *kcs1Δ* yeast, and thus becoming less sensitive to H₂O₂. These yeast data are complementary to the mammalian system where several reports indicate that an increase in inositol pyrophosphate cellular concentration sensitises the cell to apoptotic treatment [11-13].

We then asked whether H₂O₂ directly affected the enzymatic activity of KCS1. Endogenous TAP-KCS1 was purified from yeast and subjected to an enzymatic reaction *in vitro*. Hydrogen peroxide treatment at concentrations as low as 0.1mM dramatically reduced the enzymatic activity of KCS1 (Figure 7C) and completely blocked its activity at higher doses. In contrast, exposure to the reducing agent dithiothreitol at high concentrations, to the contrary, enhanced TAP-KCS1 enzymatic activity (Figure 7C).

The main biochemical effect of hydrogen peroxide is believed to be cysteine oxidation [20]. To identify the possible cysteine targeted by H₂O₂ we analysed mouse IP6K1 enzyme because KCS1 is a polypeptide of 1059 amino acid and its large size complicates the generation and analysis of recombinant enzyme. KCS1 belongs to an highly evolutionary conserved family of enzymes and it is usually acknowledged that mammalian IP6Ks and yeast Kcs1 share the same biochemical characteristics. In fact, *in vitro*, mouse IP6K1 activity is negatively affected by H₂O₂ and enhanced by DTT in a similar fashion to the TAP-KCS1 enzyme (data not shown). For this reason, to identify the possible H₂O₂ target sites, we have utilized mouse IP6K1 enzyme that possesses twelve cysteine residues of which only five are evolutionarily conserved with KCS1 and the other two mammalian isoforms (IP6K2 and IP6K3).

Initially, we mutagenised the five conserved cysteine residues to alanine (Table 3). Analysis of these mutants revealed that the mutation of cysteine 221 to alanine completely abolished enzymatic activity. Interestingly this residue is localized in proximity to the domain important for the catalysis of this class of enzymes [53], as revealed by the crystal structure of the IP₃ 3-Kinase [54] in which the aspartic acid in position 224 has been shown to co-ordinate ATP. Cysteine to alanine mutation, while blocking the role of cysteine in protein functions, doesn't mimic oxidised cysteine that usually evolves to sulfonic acid. Consequently we decided to mutagenise cysteine221 to aspartic acid. Interestingly the mutant Cys221Asp eliminates the enzymatic activity of this class of enzyme (Table 3). These results indicate that it is likely that the cysteine residue (aa221 in mouse IP6K1, aa773 in KCS1) localized inside the inositol binding domain [53, 54] is responsible for the redox regulation of this class of enzymes. However, the analysis of other mutants reveals an important role for another three of the five evolutionarily conserved Cys residues. The mutation cysteine 187 to alanine generated an enzyme only able to convert IP₆ to IP₇ even after a long time of incubation (Table 3). To the contrary, the mutations cysteine 48 or 261 to alanine are more prone to convert IP₆ to a higher inositol pyrophosphate such IP₈ (Table 3). It is possible that this class of enzymes possess an intramolecular disulfide bond and that its destruction generates a more catalytically flexible enzyme. However, further work is required to verify this hypothesis.

Nevertheless, these data suggest that the evolutionarily conserved IP6K enzymes are sensors of the cellular redox status likely through a cysteine residue located in their catalytic site. Oxidative stress signals might be in part directly transduced by inositol pyrophosphates.

Discussion

In this study we used genetically modified yeast with modulated levels of inositol pyrophosphates as a model to investigate the role of inositol pyrophosphates in the oxidative stress response. We found that IP₇/IP₈ and possibly other pyrophosphates derived from IP₅ are required to confer sensitivity to hydrogen peroxide treatment. Cells that were depleted of inositol pyrophosphates showed resistance to this DNA damaging agent and exhibited increased Rad53 activation which ultimately resulted in the avoidance of apoptosis and a dramatic decrease in the mutation rate. However, yeast mutants that do not possess inositol pyrophosphates are more sensitive to DNA damage caused by pleomycin, indicating that inositol pyrophosphate are not involved in directly sensing DNA damage. Instead, there was a rapid decrease of cellular inositol pyrophosphates levels following exposure to H₂O₂ indicating that inositol pyrophosphates act as signaling molecules in response to this genotoxic agent. The inhibitory effect of H₂O₂ on the enzymatic activity of TAP-KCS1 *in vitro* suggests that ROS signal might be transduced by the direct modification of KCS1 enzyme. The comprehensive mutagenesis analysis of the evolutionary conserved cysteine residues of this class of enzyme identified a specific residue (in mouse IP6K1 cysteine 221) localized in the previously characterized inositol binding domain [53, 54] has a likely target of hydrogen peroxide oxidation. This evolutionary conserved enzymatic feature suggests that also in mammalian cells H₂O₂ will elicit a decrease of inositol pyrophosphates. In fact, we observed a reproducible reduction of inositol pyrophosphates levels in HeLa cells after 30 minute of incubation with 1mM H₂O₂ (A.S. unpublished observation). However, the response to oxidative stress in the mammalian system might be cell type dependent because no difference in IP₈ levels was observed in DDT₁ MF-2 using, although, a lower dose of 0.3mM H₂O₂ was used [55].

Hydrogen peroxide is formed naturally through biological processes by the action of oxidases (e.g. in peroxisomes) and by the action of the superoxide dismutase as a by-product of the respiratory chain. Furthermore, the transient production of H₂O₂ is triggered by receptors activation for ligands such platelet-derived growth factor (PDGF) [23] or epidermal growth factor (EGF) [24]. Therefore, H₂O₂ plays an important role as a second messenger in signal transduction and amplification [19, 56, 57]. Hydrogen peroxide signaling cascades require the activation of kinases as well as inhibition of phosphatases (see for Review [20, 58]). The rapid decrease of inositol pyrophosphates after H₂O₂ treatment suggests that inositol pyrophosphates are acting downstream of hydrogen peroxide in one or several pathways. Recently, IP₇ was demonstrated to mediate a novel form of non-enzymatic protein pyrophosphorylation suggesting a potentially preeminent role for the molecule in posttranslational modification of proteins [35, 59]. The differences observed between WT and *kcs1Δ* yeast may thus reflect altered IP₇-mediated protein pyro-phosphorylation; indeed H₂O₂ treatment alters the IP₇ phosphorylation profile (S.N.M.O. and A.S. unpublished observation). The identification of the protein substrates of IP₇ could provide additional insights into the signaling responses triggered by H₂O₂ as well as improve our knowledge of the physiological relevance of IP₇-mediated phosphorylation.

The analysis of *vip1Δ* yeast revealed an accumulation of IP₇ (Figure 5), demonstrating that this protein represents the physiologically relevant IP₇-Kinase. It is important to note that in the cell there are two isomers of IP₈, (PP)₂-IP₄, a double-pyrophosphorylated form generated by the action of IP6K and VIP1, and a second isomer, the triphosphate PPP-IP₅ generated directly by IP6K [9]. The discovery that VIP1 physiologically synthesizes (PP)₂-IP₄ and that in *vip1Δ* yeast there is accumulation of IP₇ prompts led us to reinterpret a recent report indicating that the IP₇ synthesized by VIP1 regulates a cyclin-CDK-CKI transcriptional complex [60]. It is more likely that the absence of the double-pyrophosphorylated form of IP₈

in *vip1* Δ yeast or the large accumulation of Kcs1-synthesized IP₇ is responsible for cyclin-CDK-CKI complex regulation.

In yeast the DNA damage sensing functions are mainly executed by the PIKK Mec1 and Tel1 (mammalian ATM and ATR homologs). Once activated, Mec1/Tel1 trigger a phosphorylation cascade that activates Rad53 (mammalian Chk2 homolog) [16, 17, 61]. Inositol pyrophosphates have previously been identified as negative regulators of Tel1, which apart from playing an important role in the DNA damage response also regulates telomere length as seen by shorter telomeres in *tell1* Δ yeast [41, 62]. However, Mec1/Tel1 activation assayed by the degree of Rad53 phosphorylation after pleomycin treatment appears not to be affected by inositol pyrophosphates. This suggests the presence of normal DNA damage response machinery in the yeast with altered inositol pyrophosphates metabolism, further indicating a specific and definite role of this class of inositols in modulating ROS signaling.

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Table 1: Yeast strains used in this study

Background		Reference
BY4741 <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>		
BY4741 <i>ipk1:kanMX4</i>	<i>ipk1Δ</i>	[38]
BY4741 <i>kcs1:kanMX4</i>	<i>kcs1Δ</i>	[38]
BY4741 <i>ipk1:kanMX4 kcs1:kanMX4</i>	<i>ipk1Δkcs1Δ</i>	[38]
DDY1810 <i>MATa leu2-3,112 trp1-Δ901 ura3-52 prb1-1122 pep4-3 prc1-407</i>		
DDY1810 <i>kcs1:Leu2</i>	<i>kcs1Δ</i>	This study
DDY1810 <i>vip1:kanMX4</i>	<i>vip1Δ</i>	This study
DDY1810 <i>kcs1:Leu2 vip1:kanMX4</i>	<i>kcs1Δvip1Δ</i>	This study

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Table 2: Inositol Pyrophosphate level expressed as % of IP₆

	IP ₇	IP ₈
WT	4.7±1.1	2.4±0.5
<i>kcs1Δ</i>	none	none
<i>vip1Δ</i>	19.7±3.5	2.6±0.7
<i>kcs1Δvip1Δ</i>	none	none

The data represent the average plus minus standard error from two independent experiments run in duplicate of the inositol pyrophosphate levels expressed as percent of IP₆ in DDY1810 WT, *kcs1Δ*, *vip1Δ* and *kcs1Δvip1Δ* yeast.

Table 3: Activity of mouse IP6K1 cysteine mutants

	% IP ₇ 10min reaction	% IP ₈ 10min reaction	% IP ₇ 1h reaction	% IP ₈ 1h reaction
mIP6K1	30±5%	2±1%	70±4%	15±3%
mIP6K1-C47A (KCS1 C350)	33±4%	5±3%	45±7%	31±5%
mIP6K1-C187A (KCS1 C717)	30±3%	1±1%	85±5%	2±1%
mIP6K1-C221A (KCS1 C773)	none	none	none	none
mIP6K1-C221D (KCS1 C773)	none	none	none	none
mIP6K1-C248A (KCS1 C800)	29±6%	2±1%	70±4%	16±2%
mIP6K1-C261A (KCS1 C813)	29±7%	4±4%	51±5%	29±6%

Recombinant IP6K1 proteins (10ng) were incubated with in the presence of 100µm of IP₆ and trace amounts of ³H-IP₆ for the indicated time. The reaction were stopped by adding EDTA and analyzed by SAX-HPLC as described [36]. The data report the percent of the inositol pyrophosphates generated and represent the averages +/- standard deviation from three independent experiments.

Figure Legends

Figure 1: Yeast inositol pyrophosphates metabolism.

Inositol trisphosphate (IP₃) is sequentially phosphorylated to IP₄, IP₅ and IP₆ by serial enzymatic reactions carried by IPMK and IPK1. The fully phosphorylated *myo*-inositol ring of IP₆ is further phosphorylated by KCS1 (mammalian IP6K) to generate the inositol pyrophosphate IP₇. The double-pyrophosphate form (PP)₂-IP₄ of “IP₈” is generated by Vip1 (mammalian PP-IP5K or IP7K). Two other inositol pyrophosphate species, PP-IP₄ and (PP)₂-IP₃ can be generated from IP₅. The kinases that carry out each of these steps are shown above the arrows. A triphosphate form of “IP₈” has recently been identified and it is likely synthesized in yeast by Kcs1.

Figure 2: *kcs1Δ* mutants are more resistant to H₂O₂ than WT cells.

Shown are graphs depicting the yeast survival rate, analyzed by a colony formation assay, after 3 h exposure to none (black), 0.1mM (dark grey), 0.5mM (light grey) or 1mM (white) H₂O₂ for 3 h. **A)** DDY1810 genetic background WT, *kcs1Δ*, and *kcs1Δ* yeast expressing either the mouse IP6K1 (*kcs1Δ*+IP6K1) or the kinase dead IP6K1KA (*kcs1Δ*+IP6K1KA). **B)** BY4741 genetic background WT, *kcs1Δ*, *ipk1Δ*, *ipk1Δkcs1Δ*. The error bars represent the standard deviation from four independent experiments performed in triplicate.

Figure 3: *kcs1Δ* display a sustained Rad53 activation and a decrease mutation rate.

DNA damage response activation was observed by western blotting using anti Rad53 antibody. **A)** Time-dependent activation of Rad53 in the DDY1810 genetic background of WT and *kcs1Δ* yeast after exposure to 0.1mM, 0.5mM and 1mM H₂O₂. At the higher dose, the Rad53 pathway is only activated in *kcs1Δ* yeast. **B)** Mammalian IP6K1 reduced the level of Rad53 activation in *kcs1Δ* cells. After treatment with 1mM H₂O₂ for 30 min Rad53 is activated in *kcs1Δ* and *kcs1Δ*+IP6K1KA but not WT and *kcs1Δ*+IP6K1. **C)** Activation of Rad53 in the BY4741 genetic background. After treatment with 1mM H₂O₂ for 30 min Rad53 is phosphorylated in *kcs1Δ* and *ipk1Δkcs1Δ* but not WT and *ipk1Δ*. Blots were subsequently probed with anti-Hsp60 for gel loading normalization. **D)** Mutation frequency after exposure to H₂O₂ as determined by a CanR Assay [34]. The graph shows the average mutation frequency of three independent experiments in DDY1810 genetic background yeast. The error bars represent the standard deviation of the average values. Also shown is the average survival rate after exposure to H₂O₂.

Figure 4: *vip1Δ* yeast accumulate IP₇ and is more resistant to H₂O₂.

A) HPLC analysis of [³H]-inositol phosphates in DDY1810 WT, *kcs1Δ*, *vip1Δ* and *kcs1Δvip1Δ* yeast, revealed the accumulation of IP₇ in *vip1Δ* yeast. **B)** Vip1Δ is more resistant to H₂O₂ than WT. The graph shows the average survival of WT, *kcs1Δ*, *vip1Δ* and *kcs1Δvip1Δ* cells after 3 h exposure to none (black) 0.1mM (grey) or 1mM (white) H₂O₂. The error bars represent the standard deviation of three independent experiments. **C)** DNA damage response activation as observed by Rad53 phosphorylation. After treatment with 1mM H₂O₂ for 30 min Rad53 is activated in *kcs1Δ*, *vip1Δ* and *kcs1Δvip1Δ*.

Figure 5: Normal cell cycle-dependent Rad53 activation and actin cytoskeleton depolarization in *kcs1Δ* yeast.

A) DNA damage response during the cell cycle. DDY1810 genetic background WT and *kcs1Δ* cells were arrested either in the S-phase of the cell cycle with HU or in the G2-M-phase with nocodazole and subsequently treated with 0.5 mM H₂O₂ for 15 min. In both WT and *kcs1Δ* Rad53 is predominantly activated during the S-phase of the cell cycle.

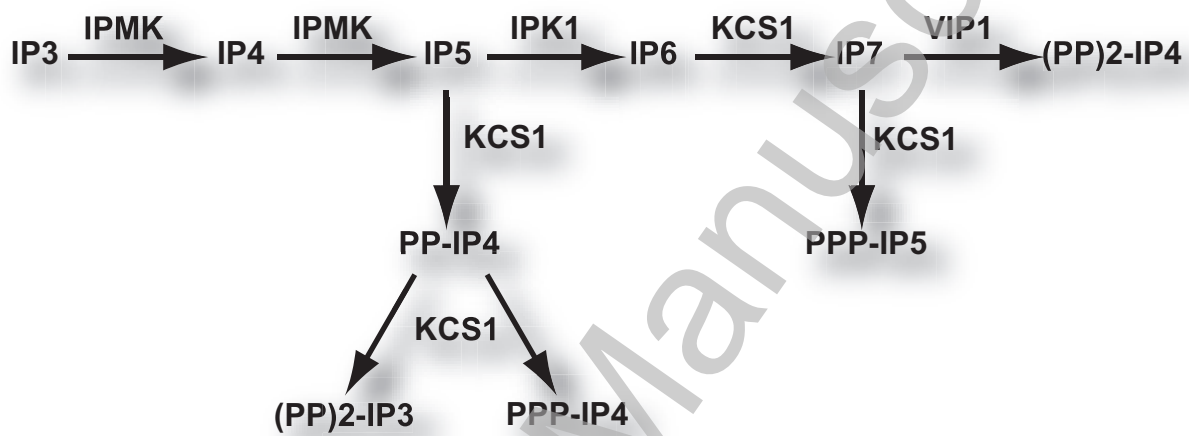
B) Actin staining before and after exposure to H₂O₂. WT, *kcs1Δ* and *kcs1Δ*+IP6K1 were treated with 1mM H₂O₂ for 3 h and the actin cytoskeleton was visualized with rhodamine-labelled phalloidin. Unchallenged *kcs1Δ* cells appear to have a less polymerized actin arrangement. However, after H₂O₂ treatment the actin cytoskeleton depolarises equally well in *kcs1Δ* as in WT and *kcs1Δ*+IP6K1 yeast.

Figure 6: Sensitivity to pleomycin treatment of yeast deficient in inositol pyrophosphates.

A) Analysis of WT, *kcs1Δ*, and *kcs1Δ* yeast expressing mouse IP6K1 (*kcs1Δ*+IP6K1) exposed to none (black) or 10μg/ml (white) pleomycin for 1 hour revealed the sensitivity of *kcs1Δ* to this drug. Error bars represent the standard deviation from three independent experiments. **B)** Rad53 phosphorylation analysis did not reveal significant differences between WT and *kcs1Δ* cells exposed for 1 h to different doses of pleomycin. **C)** Similarly, a time course of Rad53 activation using 10μg/ml of pleomycin, did not reveal significant differences between WT and *kcs1Δ*.

Figure 7: Hydrogen peroxide decreases the levels of inositol pyrophosphates in WT and *ipk1Δ* yeast.

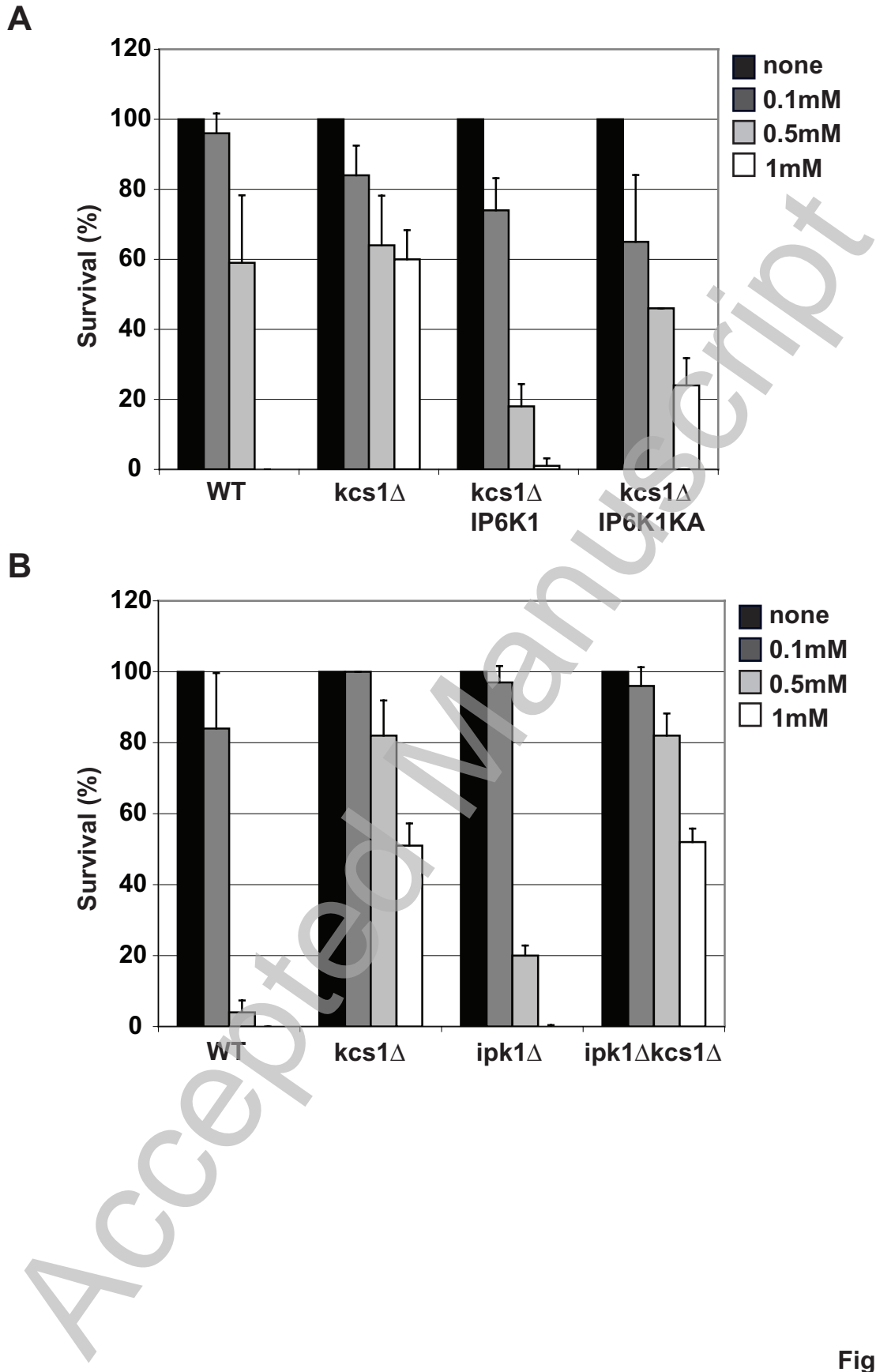
Representative HPLC analysis of [³H]-inositol polyphosphates profile (left) of early logarithmic growing WT (**A**) and *ipk1Δ* (**B**) yeast untreated or treated with 1mM H₂O₂ or 10 μg/ml pleomycin for 20 minutes. Average inositol pyrophosphate levels expressed as ratio of their precursor IP₆ for WT and IP₅ for *ipk1Δ* yeast (right). The error bars represent the standard deviation of three independent experiments. **C)** Effect of H₂O₂ on KCS1-TAP enzymatic activity. Purified proteins were incubated with ³H-IP₆ in presence of none, 0.1, 1, or 10 mM H₂O₂ or 10 mM DTT. The graph represents percent of substrate converted to inositol pyrophosphates after a 15 min reaction. Error bars represent the standard deviation from three independent experiments.



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Figure 1



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Figure 2

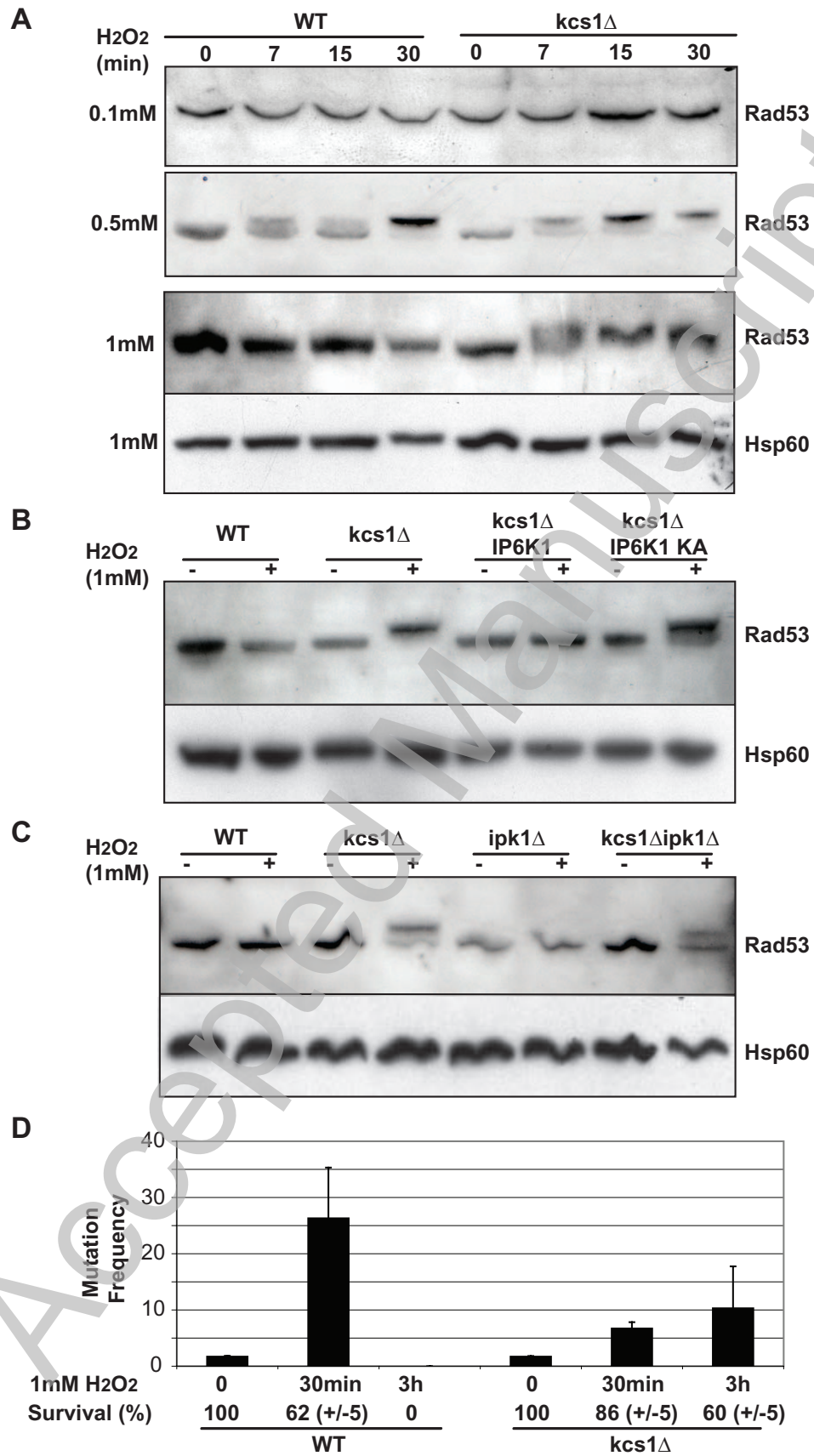
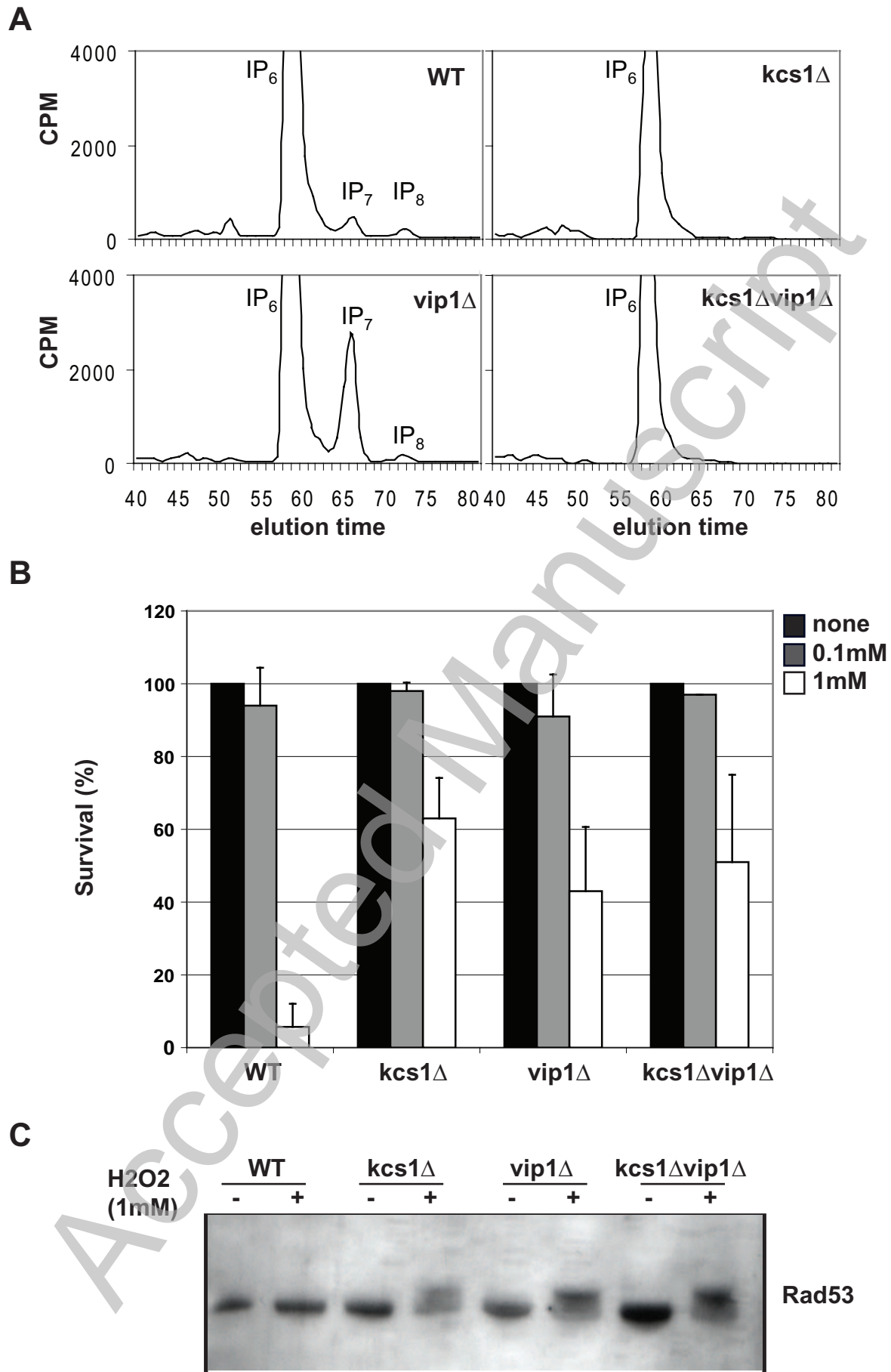
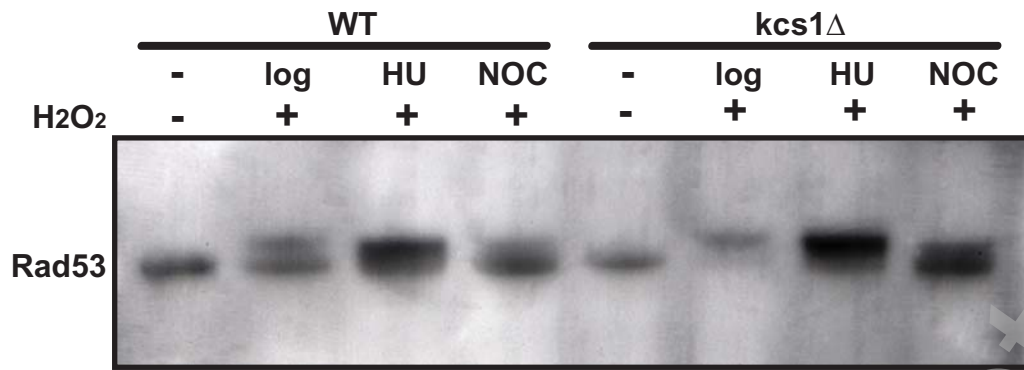


Figure 3

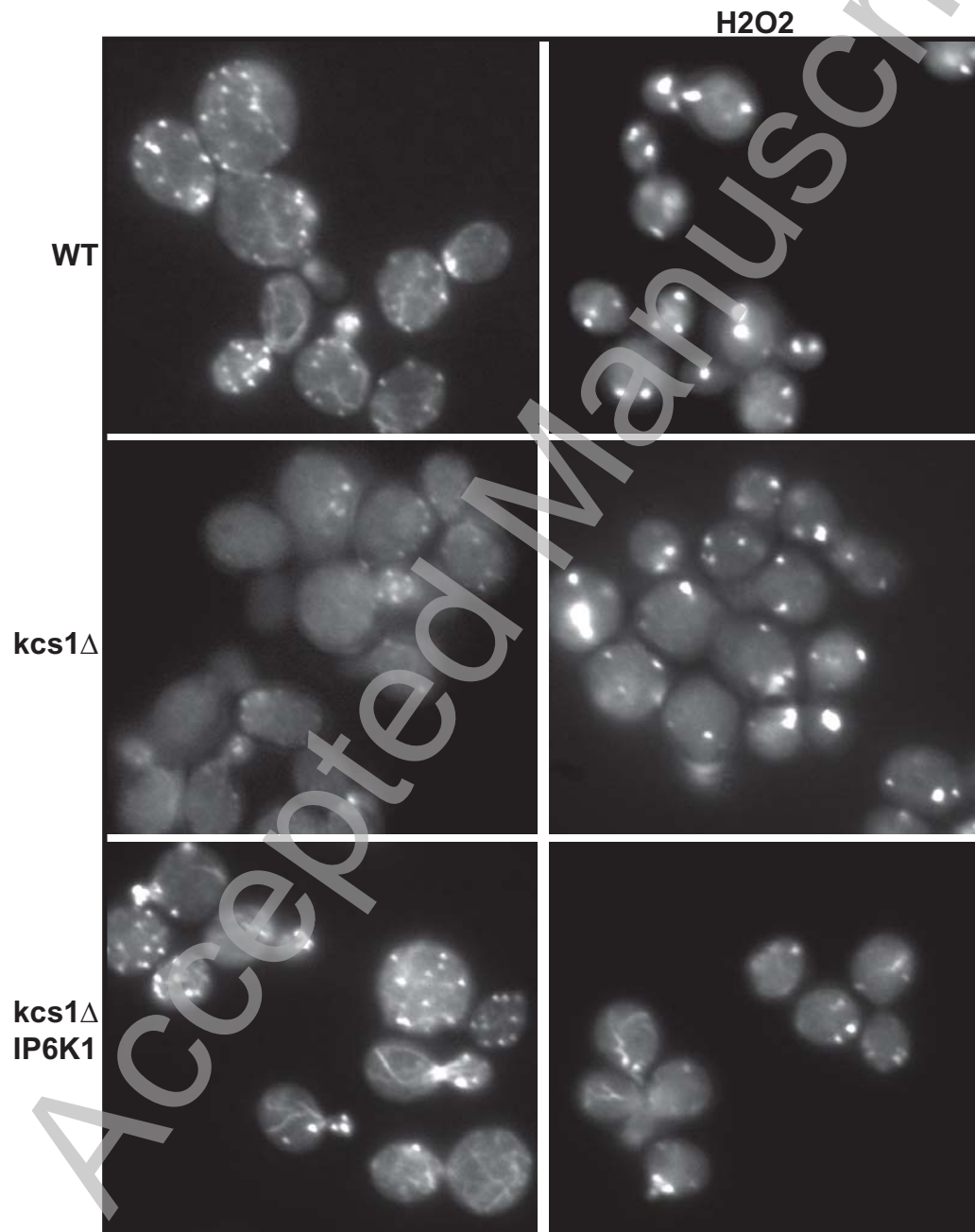


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A



B



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Figure 5

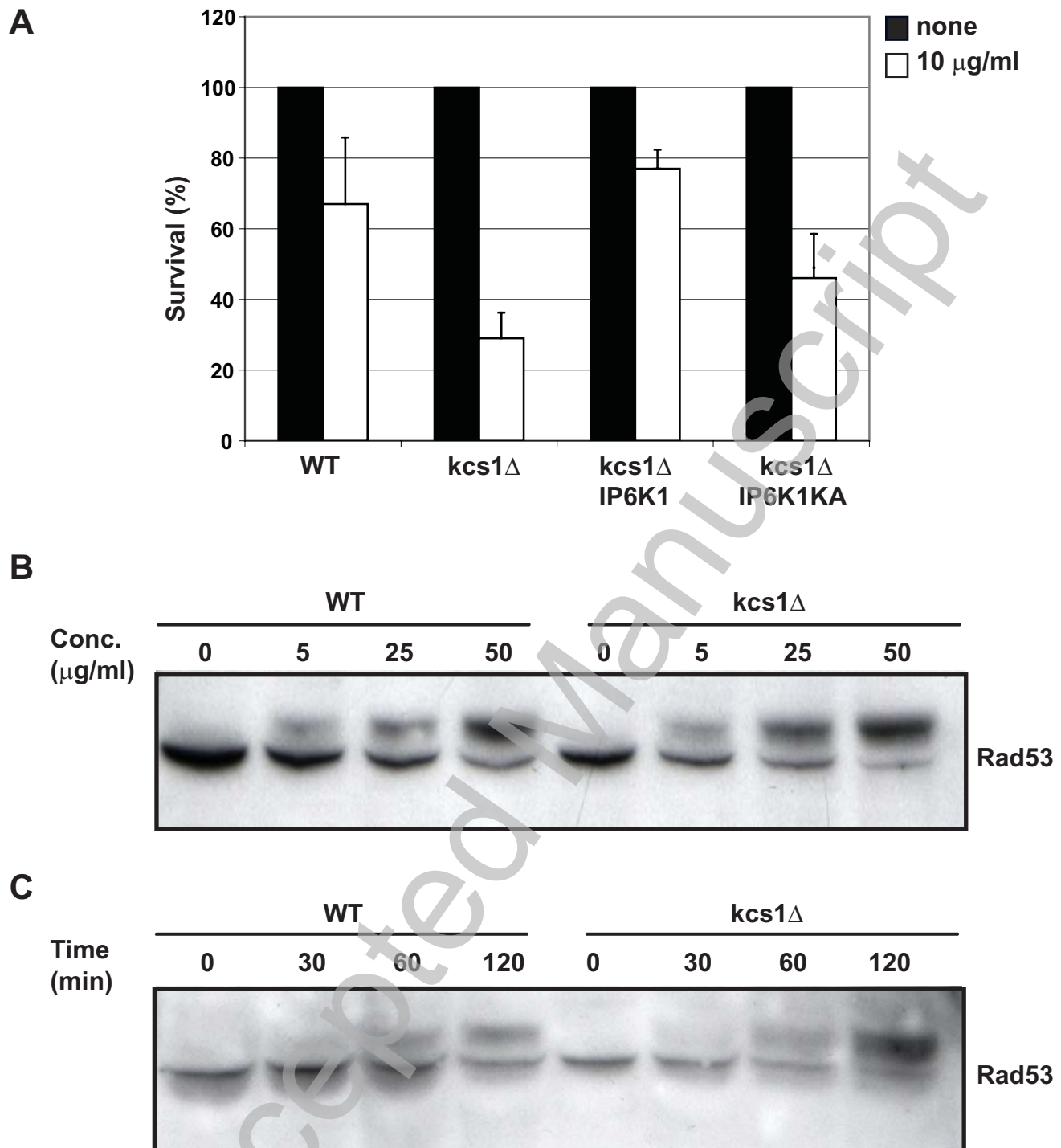


Figure 6

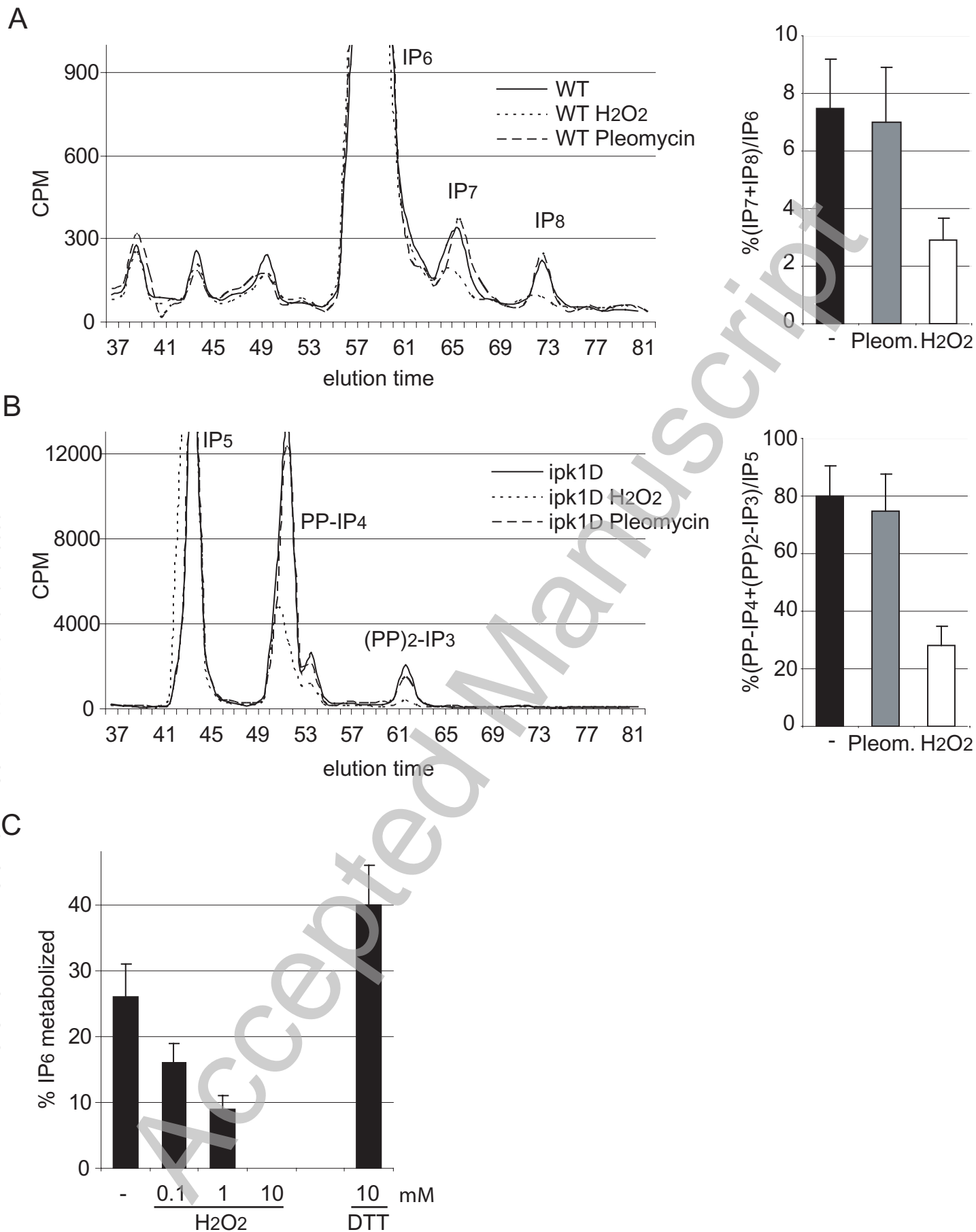


Figure 7