Sir2 histone deacetylase prevents programmed cell death caused by sustained activation of the Hog1 stress-activated protein kinase

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Exposure of yeast to high osmolarity induces a transient activation of the Hog1 stress-activated protein kinase (SAPK), which is required for cell survival under these conditions. However, sustained activation of the SAPK results in a severe growth defect. We found that prolonged SAPK activation leads to cell death, which is not observed in nma111 cells, by causing accumulation of reactive oxygen species (ROS). Mutations of the SCFcdc4 ubiquitin ligase complex suppress cell death by preventing the degradation of Msn2 and Msn4 transcription factors. Accumulation of Msn2 and Msn4 leads to the induction of PNC1, which is an activator of the Sir2 histone acetylase. Sir2 is involved in protection against Hog1-induced cell death and can suppress Hog1-induced ROS accumulation. Therefore, cell death seems to be dictated by the balance of ROS induced by Hog1 and the protective effect of Sir2.

Keywords: cell death; Hog1; SCF; SAPK; Sir2

INTRODUCTION

Mitogen-activated protein kinase pathways convert extracellular stimuli into cellular responses. Mitogen-activated protein kinase activation must be appropriately regulated because the biological outcome is dependent on the intensity and duration of the activation (Marshall, 1995). For instance, transient activation of the p38 stress-activated protein kinase (SAPK) leads to cell proliferation, whereas sustained activation leads to apoptosis-like cell death (Williamson et al, 2004; Wagner & Nebreda, 2009). In yeast, transient activation of Hog1 SAPK is essential for cell adaptation and survival of osmostress and controls from cell-cycle progression to gene expression (Clotet et al, 2006; Chen & Thorner, 2007; Hohmann et al, 2007; de Nadal & Posas, 2010). In contrast to its role in cell survival, sustained activation of the SAPK results in a severe growth defect that is prevented by overexpression of protein tyrosine phosphatase 2 (Maeda et al, 1994; Wurgler-Murphy et al, 1997). However, little is known about the molecular basis of this severe growth defect.

Here, we demonstrate that sustained activation of Hog1 induces cell death by promoting high levels of reactive oxygen species (ROS). This is suppressed by mutations of the SCFcdc4 (SCF; Skp1/Cul1/F-box protein) ubiquitin–ligase complex. Accumulation of Msn2 and Msn4 transcription factors induces PNC1, an activator of the Sir2 histone deacetylase.

RESULTS AND DISCUSSION

Sustained activation of Hog1 leads to cell death

Inactivation of SLN1 or overexpression of PBS2DD impairs cell growth (Maeda et al, 1994; Wurgler-Murphy et al, 1997; Fig 1A). We tested whether this was associated with a decrease of cell survival, by assessing colony-forming units. Only 24% of wild-type cells survived 24 h of PBS2DD expression (Fig 1B). A decrease in cell viability was also observed in a cell permeability assay with propidium iodide (supplementary Fig S1A online).

Activation of p38 has been associated with apoptosis (Dolado & Nebreda, 2008). Yeast can undergo cell death accompanied by features that are diagnostic of apoptosis or programmed cell death (PCD), and it is associated with characteristics of apoptosis (Galluzzi et al, 2009; Madeo et al, 2009). Activation of Hog1 by overexpression of Pbs2DD resulted in 18.2% of Tdt-mediated dUTP nick end labelling-positive cells, whereas only 3.8% of cells...
expressing a control plasmid were positive (Fig 1C). Activation of the SAPK resulted in 19.5% of cells having a SubG1 DNA content, indicating DNA fragmentation (Fig 1D). Correspondingly, sustained activation of Hog1 induced an increase in the number of cells with metacaspase activation in the highly sensitive FAM–FLICA Apoptosis Detection Kit (Immunochemistry technologies) (supplementary Fig S1B online). Thus, several independent assays indicated that activation of the Hog1 SAPK induced cell death that is partly suppressed by SCF\textsuperscript{CDC4} mutations. (A) Mutations on the SCF\textsuperscript{CDC4} complex prevent cell death induced by PBS2\textsuperscript{DD} or SLN1 inactivation. Cells expressing the PBS2\textsuperscript{DD} allele under the GAL1 promoter (p\textsuperscript{GAL1–PBS2DD}) were spotted on glucose (control) or galactose. The \textit{shl1Δ} and \textit{shl1Δ cdc4-1} strains carrying a plasmid expressing the protein tyrosine phosphatase 2 (PTP2) gene under the GAL1 promoter (p\textsuperscript{GAL1–PTP2}). (B) Hog1-mediated cell death is improved in a cdc4-1 mutant strain. Cells as in A were grown in galactose for 24h. Viability was monitored by counting the colony-forming units in glucose plates. Data represent the mean and standard deviation of three independent experiments. (C) DNA single-strand breaks caused by Hog1 activation are reduced in cdc4-1 mutant cells. Cells were processed with the TdT-mediated dUTP nick end labeling (TUNEL) assay (+) and the presence of single-strand DNA breaks was detected by FACS analysis. Data shown are representative of three independent experiments. (D) Induction of the appearance of SubG1 population of cells on Hog1 activation is reduced in cdc4-1 mutant cells. Cells were grown as in A and analysed for SubG1 population by FACS analysis. Data shown are representative of three independent experiments. (E) Cell death caused by sustained PBS2\textsuperscript{DD} expression is mediated by Nma111. The indicated strains expressing the PBS2\textsuperscript{DD} allele were grown in glucose (control) or galactose. (F) Cell viability on permanent Hog1 activation is fully restored in the absence of NMA111. Strains as in E were grown in glucose (control) or galactose for 24h and colony-forming units were assessed in glucose plates. Data represent the mean and standard deviation of three independent experiments. FACS, fluorescence-activated cell sorting.
Fig 2 | Hog1-induced reactive oxygen species accumulation is reduced in a cdc4-1 mutant. (A) Reactive oxygen species (ROS) production causes cell death under sustained Hog1 activation. Indicated strains plated on glucose or galactose, and anaerobiosis (left panel) in the presence of antimycin at 2.5 µg/ml (middle panel) or dinitrophenol at 25 µg/ml (right panel). (B) Hog1-induced ROS accumulation is reduced in a cdc4-1 mutant. Strains were grown in galactose for 12 h. Cells were incubated with DCFH-DA for 30 min and ROS was measured. Data represent the mean and standard deviation of three independent experiments. (C) Hog1 inhibits mitochondrial respiration in response to osmostress. Wild-type and hog1as-mutant strains in YPD plates with or without 0.8 M NaCl were grown for 12 h at 25 °C, and tetrazolium chloride was added as an overlay in the presence (+) of the kinase inhibitor 1NM-PP1 (5 µM). (D) Activation of Hog1 causes a reduction in oxygen consumption independently of SIR2, cdc4-1 and GDP1. Oxygen consumption was measured as in B. Data represent the mean and standard deviation of three independent experiments. DCFH-DA, dichloro-dihydro-fluorescein diacetate.

dehth. Several genes have been involved as mediators of apoptosis-like cell death in yeast (Carmona-Gutierrez et al., 2010). Deletion of NUC1 did not prevent cell death on Hog1 activation. By contrast, deletion of YCA1 partly suppressed cell death and deletion of NMA111—the Omi/HtrA2 homologue (Walter et al., 2006)—completely abolished it (Fig 1E and F). Thus, Hog1 activation leads to PCD mediated by Nma111.

SCF mutations suppress Hog1-mediated cell death
Permissive mutations of the CDC4 gene—the E3 ligase of the SCF-CDC4 complex—suppressed the growth defect associated with HOG hyperactivation (Fig 1A,B; supplementary Fig S1A online). SCF-CDC4 is a complex containing Skp1, Cdc53 and Cdc34 proteins. Permissive mutations in any of these genes suppressed the growth defect caused by activation of Hog1 (Fig 1A), although to different extents. The cdc4-1 cells overexpressing PBS2D3D showed a survival rate almost two times higher than wild-type cells and reduced apoptosis (Fig 1C and D; supplementary Fig S1B online). Thus, mutations of the SCF-CDC4 partly suppress cell death caused by Hog1 activation.

cdc4-1 shows reduced levels of Hog1-induced ROS
The induction of ROS is the most common cause of apoptosis-like cell death in yeast (Madeo et al., 1999). In the absence of O₂, which prevents ROS formation, cell death caused by Hog1 was abolished (Fig 2A, left panel). Antimycin A inhibits respiration and provokes an increase of ROS formation. Interestingly, antimycin A abolished the anti-apoptotic effect of cdc4-1 on Hog1 activation (Fig 2A, middle panel). Incubation of cells with dinitrophenol—which inhibits adenosine triphosphate formation without affecting ROS levels—did not alter the ability of cdc4-1 cells to suppress cell death caused by Hog1 (Fig 2A, right panel). Therefore, cell death caused by Hog1 activation is not due to a deficit of adenosine triphosphate, but is probably due to an increase in ROS formation.

We then quantitatively assessed ROS production. Overexpression of PBS2D3D caused a 2.6-fold increase in ROS formation in wild-type cells, whereas it was lower than 1.8-fold in a cdc4-1 mutant (Fig 2B). An increase in ROS levels might be caused by inhibition of mitochondrial respiration. We assessed mitochondrial respiration in a plate assay in the presence of triphenyl tetrazolium (Kobayashi et al., 1974) and found that it was strongly inhibited in response to stress (white cells; Fig 2C). Correspondingly, growth on a non-fermentable carbon source was impaired in the presence of osmostress (supplementary Fig S5B online). To establish the role of Hog1 in the inhibition of mitochondrial respiration, we used an analogue-sensitive hog1as strain (Macia et al., 2009) and found that inhibition of hog1as prevented the inhibition of respiration (Fig 2C). Sustained activation of Hog1 by Pbs2D3D resulted in a similar reduction of oxygen consumption in wild-type and cdc4-1 mutant, which was dependent on HOOG1 (Fig 2D). It is noteworthy that deletion of GPD1 did not prevent the reduction of oxygen consumption in response to Hog1 activation, indicating that glycerol metabolism is not the source of the inhibition of respiration (Fig 2D). Therefore, activation of Hog1 decreases mitochondrial respiration and leads to an increase in ROS formation.
SCF<sub>CDC4</sub> restricts Msn2-mediated gene expression

We then assessed HOG signalling. Phosphorylation of Hog1 and its nuclear accumulation were identical in a wild-type and cdc4-1 mutant in response to osmotic stress or PBS<sup>2DD</sup> induction (Fig 3A, B). It is noteworthy that mutations in SCF<sub>CDC4</sub> (cdc4-1, cdc34-2 and cdc53-1) were slightly more resistant to osmotic stress than wild-type cells, despite normal Hog1 signalling (Fig 3C, D).

The Hog1 SAPK is a key regulator of gene expression by controlling several transcription factors and chromatin-associated proteins (de Nadal & Posas, 2010). The SCF<sub>CDC4</sub> complex is involved in control of the turnover of several transcription factors (Pal et al, 2007; Olson et al, 2008). In a cdc4-1 strain, expression of genes under the control of Msn2 and Msn4 transcription factors, but not Sko1 or Hot1 (that is, CTT1 and ALD3 compared with GRE2 and STL1), was stronger than that in wild-type cells (Fig 3E). Therefore, these data indicate that Msn2 and Msn4 might be the target for SCF<sub>CDC4</sub>. It is noteworthy that Msn2 is ubiquitinated in vitro by Cdc4 (Chi et al, 2001).

Degradation of Msn2 in response to osmotic stress and by overexpression of PBS<sup>2DD</sup> was slower in a cdc4-1 strain than in the wild type (Fig 3F; supplementary Fig S2 online). Correspondingly, Msn2 occupancy at the CTT1 promoter, analysed by chromatin immunoprecipitation (ChIP), was extended in a SCF<sub>CDC4</sub> mutant on osmotic stress. Cells as in Fig 3F were treated with 0.4 M NaCl. Chromatin immunoprecipitation of Msn2 was assessed by immunoprecipitation with HA antibody. Binding to CTT1 promoter was determined by real-time polymerase chain reaction. Data represent the mean and standard deviation of three independent experiments. (H) Hog1 phosphorylates Msn2 in vitro. Msn2-HA was phosphorylated in an in vitro kinase assay using Hog1 as or without the presence of the kinase inhibitor 1NM-PP1 (5 μM). In both cases, radioactively labelled PE-adenosine triphosphate (ATP) was used. GFP, green fluorescent protein; OD, optical density; PE, phycoerythrin.
**Fig 4** | **MSN2/MSN4, PNC1 and SIR2 counteract Hog1-mediated cell death.** (A) Deletion of MSN2/MSN4 and PNC1 eliminates the ability of cdc4-1 mutation to prevent cell death. Cells were spotted onto glucose or galactose plates. Viability was monitored by counting colony-forming units in glucose plates (lower panel). Data represent the mean and standard deviation of three independent experiments. (B) cdc4-1 mutant shows an increase in PNC1 gene expression in response to osmostress. Strains were treated with 0.4 M NaCl and total RNA was probed with PNC1 and ACT1. (C) Mutation in the SCFCDC4 complex increases Pnc1 protein levels in response to osmostress. Pnc1 and Hog1 were assessed in cells treated as in B. (D) SIR2 deletion eliminates the ability of cdc4-1 mutation to prevent cell death. Strains were grown in the presence of resveratrol in the plates (5 μM). Cells that expressed haemagglutinin (HA)-tagged Sir2 under the GAL1 promoter (pGAL1–SIR2–HA) and those that expressed PBS2DD were spotted on glucose (control) or galactose plates. (E) Hda2 and Hst1 deacetylases do not mediate the effect of cdc4-1. Strains were spotted onto glucose or galactose plates. (F) Sir2 prevents Hog1-induced apoptosis-like cell death. Strains were grown from raffinose to galactose for 24 h. DNA content was assessed by flow cytometry. Data shown are representative of three independent experiments. (G) Tentative model that depicts the effect of Hog1 and Sir2 in dictating cell-fate determination. Hog1 inhibits mitochondrial respiration, which results in an increase in reactive oxygen species (ROS) accumulation that leads to cell death. In parallel, Hog1 induces PNC1 expression through Msn2 and Msn4 transcription factors, which are regulated by SCFCDC4. Pnc1 activates Sir2, which mediates a decrease of ROS accumulation. SIR2 activation by the stress-activated protein kinase Hog1 relieves the Hog1-induced oxidative stress to prevent apoptotic cell death. ROS, reactive oxygen species.
at stress-responsive promoters, which leads to an increase in MSN2-dependent gene expression.

**cdc4-1 suppression on PNC1 expression**

Cell death due to Hog1 was not suppressed in a cdc4-1 msn2 msn4 strain (Fig 4A). Correspondingly, overexpression of MSN2 under the ADH1 promoter promotes cell death in Hog1 activation in wild-type cells (Fig 4A). Expression of PNC1 is induced in response to osmostress by Msn2 and Msn4 (Posas et al., 2000; Causton et al., 2001), and the role of Msn2 and Msn4 in cell longevity is mediated by PNC1, an activator of Sir2 (Bitterman et al., 2002; Anderson et al., 2003; Gallo et al., 2004; Medvedik et al., 2007). Either PBS2ΔD overexpression or osmostress induced PNC1 expression that was stronger and more extended in a cdc4-1 mutant (Fig 3B, C; supplementary Fig S3A, B online). Similarly to a cdc4-1 msn2 msn4 strain, overexpression of PBS2ΔD induced cell death in a cdc4-1 pnc1 strain (Fig 3A). Correspondingly, overexpression of PNC1 prevented cell death in response to Hog1 activation (Fig 3B). Thus, Pnc1 mediates the effect of Msn2 and Msn4 to prevent cell death in response to sustained Hog1 activation.

**Sir2 activation suppresses Hog1-mediated apoptosis**

A cdc4-1 sir2 strain was unable to prevent cell death caused by Hog1 activation (Fig 4D). This effect was specific for Sir2, as deletion of HDA2 or SIR2 did not abolish the effect of cdc4-1 (Fig 4E). Correspondingly, cell death, as measured by the presence of SubG1 cells, was partly suppressed in a cdc4-1 strain, but not in a cdc4-1 sir2 strain (Fig 4F).

We then analyzed whether resveratrol, a drug that was suspected to induce Sir2 activity, improved cell growth (Howitz et al., 2003). Resveratrol did not prevent cell death in a sir2 strain. Correspondingly, overexpression of Sir2 suppressed cell death on Hog1 activation (Fig 4D). The cdc4-1 sir2 strain showed an increase in ROS levels on Hog1 activation, which was similar to that of the wild-type strain and twofold higher than that of the cdc4-1 strain (Fig 2B). There was a slight increase of ROS on 2 h incubation in the presence of NaCl in the wild type, which was further increased in a sir2 strain (supplementary Fig S5 online). Therefore, Sir2 is required for protection from Hog1-induced cell death, by preventing Hog1-induced ROS accumulation.

A main role for Sir2 in cell survival is the suppression of ribosomal DNA recombination and the formation of toxic extrachromosomal ribosomal DNA circles in the nucleus of mother cells (Sinclair & Guarente, 1997). Sir2 also affects lifespan by increasing silencing at telomeric regions (Dang et al., 2009). Interestingly, deletion of NET1, a component of the ribosomal DNA-localized Sir2 complex (Straight et al., 1999), abolished the effect of cdc4-1 on Hog1 activation (supplementary Fig S4B online). By contrast, deletion of either SIR4 or deletion of HM loci (Aparicio et al., 1991) did not affect cell viability (supplementary Fig S4B online). Therefore, although this is genetic evidence that will require further characterization, our data indicate that the function of Sir2 at ribosomal DNAs might dictate the level of cell death on Hog1 activation.

Activation of SAPK signalling is essential for cell adaptation to stress. However, sustained activation of the pathway unrolls a more-complex hypothesis. When it is not restricted, SAPK activation causes an inhibition of mitochondrial respiration, which results in an increase of ROS formation that can only be counteracted by the Hog1-dependent activation of Sir2 and the lifespan extension pathway (Fig 4G). Although a decrease in mitochondrial respiration might be important for cell adaptation, an extended reduction of respiration leads to excessive ROS formation. To prevent cell damage, Hog1 induces PNC1 gene expression and concomitantly activates Sir2 to balance excessive ROS accumulation. Therefore, cell fate is dictated by the balance between ROS induced by Hog1 SAPK and the protective effects of Sir2.

**METHODS**

**Yeast strains and plasmids.** A complete list is included in the supplementary information online. The strains used in this study showed similar growth rates when grown in YPD. Expression of the Pbs2ΔD protein was similar in all strains tested (supplementary Fig S4A online).

**Northern blot analysis.** Yeast cultures were grown to early log phase (optical density at 660 nm of 0.6–0.8). Cells were either subjected to stress (0.4 M NaCl, indicated times), shifted to galactose or untreated. Total RNA was probed by using radio-labelled polymerase chain reaction fragments containing labelled CTT1 (1.7 kb), ALD3 (1.5 kb), GRE2 (1.1 kb), STL1 (1.7 kb), PNC1 (0.8 kb) and ACT1 (1.4 kb). Signals were quantified by a Typhoon 8600 phosphorimagur and the ImageQuant software.

**Chromatin immunoprecipitation.** ChIP was performed as described previously (Alepuz et al., 2003). Yeast cultures were grown to early log phase before osmostress (0.4 M NaCl). For crosslinking, yeast cells were treated with 1% formaldehyde for 20 min at 25°C. Primer mixes were adjusted for balanced signals.

**PCD measurements.** Apoptosis-like cell death was measured by using FLICA assay adapted to flow cytometry, a TdT-mediated dUTP nick end labelling assay adapted to flow cytometry or following the presence of a SubG1 population of cells by flow cytometry, as described in the supplementary information online.

** Colony-forming unit assay.** Yeast cultures were grown to early log phase in raffinose and then shifted to galactose or glucose for 24 h before plating. The number of colonies was determined after 3 days at 25°C in replicas. Data are the result of three independent experiments with replicas.

**ROS and mitochondrial respiration assays.** ROS amounts in liquid growing cells were detected by using 2’,7’-dichloro-dihydro-fluorescein diacetate (H2DCF-DA) from Invitrogen (Carlsbad, CA). In plate, mitochondrial analysis was performed using a tetrazolium overlay assay. Measurement of oxygen consumption was recorded by direct measurement using oxygen measurer, as indicated in the supplementary information online.

**Supplementary information** is available at EMBO reports online (http://www.emboreports.org).

**ACKNOWLEDGEMENTS**

We thank L. Subirana, S. Ovejas and O. Fornas for technical support; G. Ammerer, J. Clotet, X. Escote and G. Mas for their helpful advice and contribution; and E. de Nadal for constant advice and support. This work was supported by grants from the Ministerio de Ciencia y Innovacion (BIO2009-07762) and Consolider Ingenio 2010 programme (grant CSD2007-0015). UNICELLSYS (from FP7, as well as supported by Fundacion Marcelino Botin. F.P. is the recipient of the Institucio Catalana de Recerca i Estudis Avanc`ats Academica (Generalitat de Catalunya). D.A.S. is supported by grants from the National Institute on Aging.
Sir2 prevents Hog1-mediated cell death in yeast
A. Vendrell et al

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