

Repression of ergosterol level during oxidative stress by fission yeast F-box protein Pof14 independently of SCF

Lionel Tafforeau, Sophie Le Blastier¹,
Sophie Bamps¹, Monique Dewez, Jean
Vandenhoute and Damien Hermand*

Laboratoire de Génétique Moléculaire (GEMO), Unité de Recherche en Biologie Moléculaire (URBM), Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium

We describe a new member of the F-box family, Pof14, which forms a canonical, F-box dependent SCF (Skp1, Cullin, F-box protein) ubiquitin ligase complex. The Pof14 protein has intrinsic instability that is abolished by inactivation of its Skp1 interaction motif (the F-box), Skp1 or the proteasome, indicating that Pof14 stability is controlled by an autocatalytic mechanism. Pof14 interacts with the squalene synthase Erg9, a key enzyme in ergosterol metabolism, in a membrane-bound complex that does not contain the core SCF components. *pof14* transcription is induced by hydrogen peroxide and requires the Pap1 transcription factor and the Sty1 MAP kinase. Pof14 binds to and decreases Erg9 activity *in vitro* and a *pof14* deletion strain quickly loses viability in the presence of hydrogen peroxide due to its inability to repress ergosterol synthesis. A *pof14* mutant lacking the F-box and an *skp1-3 ts* mutant behave as wild type in the presence of oxidant showing that Pof14 function is independent of SCF. This indicates that modulation of ergosterol level plays a key role in adaptation to oxidative stress.

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Introduction

Oxidative stress occurs when cellular defences are unable to cope with existing reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), the superoxide anion (O₂⁻) and the hydroxyl radical (OH⁻). In respiring cells, the primary source of ROS is incomplete reduction of oxygen to water, leading to leakage of electrons from the respiratory chain. ROS are also generated extracellularly by stimulated host phagocytes, exposure to UV and other environmental agents. This causes damage to numerous cellular components, including nucleic acids and lipids, which can result in a number

*Corresponding author. Laboratoire de Genetique Moleculaire, University of Namur, Facultés Universitaires Notre Dame de la Paix, 61 Rue de Bruxelles, B-5000 Namur, Belgium. Tel.: + 32 81 724241; Fax: + 32 81 724297; E-mail: damien.hermand@fundp.ac.be

¹These authors contributed equally to this work

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of cardiovascular diseases, cancer or age-related pathologies (Busciglio and Yankner, 1995; Costa and Moradas-Ferreira, 2001). Therefore, it is not surprising that cells developed a variety of defences, including production of detoxifying enzymes (catalases, peroxidases and superoxide dismutases) and synthesis of molecular scavengers such as glutathione, ubiquinol and vitamins (Moradas-Ferreira and Costa, 2000). The response is adaptative, with exposure to a low dose leading to resistance to a higher dose that would otherwise be lethal (Marini *et al*, 1996).

Although it has been assumed that H₂O₂ could diffuse freely across biological membranes, recent data support the fact that their permeability is under active regulation. A stress-induced decrease in permeability could impose an extra-intracellular gradient attenuating the toxicity of subsequent exposure to a higher dose of ROS, and that could be a major determinant of the adaptative response (Branco *et al*, 2004; Sousa-Lopes *et al*, 2004). However, no molecular mechanism supports this hypothesis, and the strongest evidence that membrane permeability plays a key role in adaptation is that mutants producing altered sterols show higher permeability to and are more sensitive to oxidative stress-generating compounds (Higgins *et al*, 2003; Branco *et al*, 2004; Thorpe *et al*, 2004). The predominant sterol in yeast is ergosterol, which is identical to cholesterol except for the presence of double bonds in C7 and C22 and a methyl group at C28. Its synthesis and modifications are very similar to those occurring in mammalian cells for cholesterol and it likely acts in a similar manner with regard to both membrane fluidity and microdomain functions (Sturley, 2000). All cellular sterols are derived from farnesyl diphosphate (FPP) through the intermediate squalene. The membrane-bound squalene synthase Erg9 therefore represents an ideal site to regulate sterol formation selectively without interfering with other FPP derivatives such as isoprenoids (Robinson *et al*, 1993; Sturley, 2000). Deletion of *erg9* is lethal in budding yeast, except in anaerobic growth where extracellular uptake of ergosterol is possible (Henneberry and Sturley, 2005). In the pathogenic fungus *Candida glabrata*, depletion of squalene synthase is also lethal but growth in mice is not affected due to incorporation of cholesterol from the serum (Nakayama *et al*, 2000). By contrast, many mutations occurring in genes acting downstream of *erg9* in the pathway are not lethal but lead to other final products that can substitute for ergosterol in unchallenging conditions. Ergosterol synthesis is regulated by intracellular sterol and oxygen levels, and some regulations are known to occur at the transcriptional level for many *ERG* genes encoding enzymes implicated in the pathway (Kennedy and Bard, 2001; Hongay *et al*, 2002). However, direct links between that control and the oxidative response have not been reported so far.

The fission yeast *Schizosaccharomyces pombe* responds to a wide range of stresses by a multistep phosphorelay activat-

ing the Sty1 SAPK (stress-activated protein kinase) (Millar *et al*, 1995; Degols *et al*, 1996; Shieh *et al*, 1997; Shiozaki *et al*, 1998; Nguyen *et al*, 2000), similarly to the activation of JNK and p38 stress-activated kinases in metazoans (reviewed in Toone and Jones, 1998). In turn, Sty1 regulates the transcription of stress response genes through a b-Zip transcription factor Atf1 (Takeda *et al*, 1995). However, the *atf1* deletion mutant only displays a subset of the *sty1* deletion phenotypes, suggesting that other factors might act downstream of Sty1 (Toone and Jones, 1999; Toone *et al*, 2001). Although another b-Zip transcription factor, Pap1, does not seem to be a direct target of Sty1, H₂O₂ dose-dependent changes in its subcellular localisation are impaired in a *sty1* deleted strain (Wilkinson *et al*, 1996; Shiozaki *et al*, 1998; Toone *et al*, 1998). The reason appears to be that at a low dose of oxidant, an H₂O₂ induced disulphide bond directly activates Pap1, while at a higher dose, Sty1 needs to be activated first to reach the lower activation range of Pap1 (Vivancos *et al*, 2004). The current model is that Pap1 is primarily required for transcription of target genes in response to low levels of oxidant, while Atf1 becomes predominant at higher doses and only the double inactivation of *atf1* and *pap1* mimics the *sty1* deletion defect in both acute and adaptive responses (Quinn *et al*, 2002).

Here we describe a new target of Pap1 regulated transcription in *S. pombe*, namely the F-box protein Pof14 that is required for survival to H₂O₂ stress. F-box proteins constitute a large family of proteins (Cenciarelli *et al*, 1999; Regan-Reimann *et al*, 1999; Winston *et al*, 1999; Jin *et al*, 2004) that are thought to provide substrate specificity to the SCF (Skp1, Cullin, F-box protein) E3 ubiquitin ligases by interacting with the core component Skp1 through their F-box motif, and by recruiting substrates (often after their phosphorylation) to be ubiquitinated (Bai *et al*, 1996; Skowrya *et al*, 1997; Patton *et al*, 1998). A dynamic equilibrium has been proposed to exist because several F-box proteins are degraded by an autocatalytic mechanism (Galan and Peter, 1999). We show that Pof14 exemplifies such an F-box protein that is degraded in a proteasome and SCF-dependent manner. We also show that Pof14 plays an essential, SCF-independent role in the stress response to peroxide by negatively regulating ergosterol synthesis most likely by directly binding the squalene synthase Erg9.

Results and discussion

Pof14 is a component of the SCF ubiquitin ligase

We screened 10⁷ clones from a fission yeast two-hybrid cDNA library using Skp1 as bait. Ninety-four positive candidates representing 19 proteins (Supplementary Table I) were isolated. Unsurprisingly, several previously characterised F-box proteins were recovered, but the most frequently isolated clone encoded an unknown protein (SPAC13D6.01) lacking known motifs. However, dissection of the protein sequence with the help of the Blocks software (Henikoff *et al*, 2000) revealed the presence of a putative F-box between residues 172 and 214. To test this possibility, a Gal4-AD (Gal4 activation domain) fusion protein bearing deletion of the first 14 residues (172–186) of the predicted F-box was constructed and tested against Skp1 in the two-hybrid system. Figure 1A–C shows that in spite of similar expression levels, only the full-length protein retained ability to interact with Skp1. We

therefore named the new protein Pof14 (*S. pombe* F-box protein). We checked the ability of Pof14 to form an SCF complex *in vivo*. Co-immunoprecipitations from fission yeast extract revealed that Pof14 forms a complex with the SCF core components Skp1 and Pcu1 (Figure 1D). Together with the two-hybrid data, this demonstrates that a canonical SCF^{Pof14} complex is present in fission yeast. We noted that Pof14 could precipitate two forms of Pcu1 most likely corresponding to the unmodified and the neddylated forms based on published data (Osaka *et al*, 2000; Harrison *et al*, 2005).

The *pof14* open reading frame is not essential and no obvious phenotype was noted in the deleted strain (data not shown). The protein is very unstable with a half-life of less than 20 min, as determined after cycloheximide addition, which blocks protein synthesis and allows the half-life of the present pool to be estimated (Figure 1E). The instability was fully dependent on the presence of the F-box (Figure 1E) and therefore on the interaction with SCF through Skp1. This suggests that Pof14 could be degraded by an autocatalytic mechanism, as reported for other F-box proteins (Galan and Peter, 1999). This possibility is further supported by the fact that wild-type Pof14 was stabilised by inactivation of either SCF (using the *skp1-3 ts* allele) or the proteasome (using the *mts3-1 ts* allele) (Figure 1F). We conclude that Pof14 is part of an SCF, which regulates its own stability.

Pof14 interacts with the squalene synthase Erg9 independently of SCF

To gain insight into the function of Pof14, a second two-hybrid screen with Pof14 as bait was performed and 61 candidates representing five proteins were isolated (Supplementary Table II). Two clones were recovered at similar high frequency: Skp1, as expected, and Erg9 (Figure 2A and B). Erg9 is the squalene synthase, a membrane-bound enzyme that plays a pivotal role in sterol metabolism. Analysis of overlapping interacting clones revealed that the C-terminal part of Erg9 was sufficient for interaction with Pof14 (Figure 2B).

Pof14 and Erg9 could be co-immunoprecipitated from fission yeast extracts (Figure 2C), supporting the fact that the two-hybrid interaction is physiologically relevant. However, neither Skp1 (Figure 2C) nor Pcu1 (data not shown) could be co-immunoprecipitated with Erg9, raising the possibility that two Pof14-containing complexes might coexist *in vivo*, one of them being a canonical SCF and the other one containing Erg9. To further investigate this possibility, subcellular localisation of both Erg9 and Pof14 was determined by fusing the proteins to CFP and GFP, respectively. As reported for the budding yeast homologue of Erg9 (Kumar *et al*, 2002), SpErg9 associated with vesicle-like and ER (including a nuclear rim) structures (Figure 2D, left panel), consistent with the presence of a conserved transmembrane domain in Erg9 (Figure 2B; Robinson *et al*, 1993). Pof14 displayed a very similar localisation (Figure 2D, central panel), and colocalisation was confirmed (Figure 2D, right panel). No change in Erg9 localisation was observed when *pof14* was absent (Figure 2E). In the reverse experiment, *erg9* was placed under the control of the weak *nmt81* promoter, which is turned off in the presence of thiamine. Upon thiamine addition, Pof14 localisation was largely lost although Pof14 protein level was unchanged (Figure 2E).

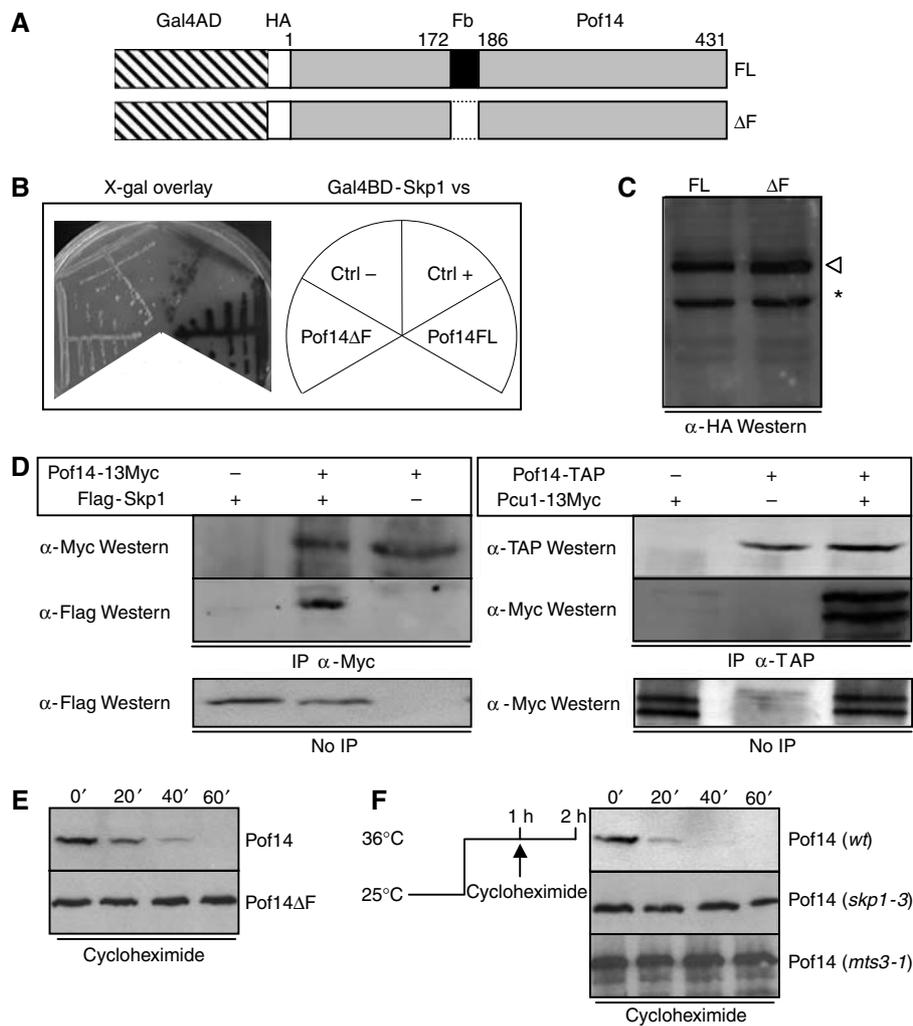


Figure 1 Pof14 is a component of SCF and is degraded by an autocatalytic mechanism. **(A)** Schematic representation of Pof14 deletion mutants. FL: full length, ΔF: deletion of the first 14 residues of the predicted F-box motif (172–186). **(B)** Two-hybrid interaction between Skp1 and Pof14. The MAV103 two-hybrid strain expressing Gal4DB-Skp1 was transformed with pACTII harbouring full-length *pof14* or F-box deletion mutant. Controls consist of MAV103 expressing Gal4DB-Skp1 and transformed with pACTII-*pop2* (Ctrl+) or empty pACTII (Ctrl-). Strains were streaked and overlaid with X-Gal containing medium to test activation of *lacZ* reporter gene. **(C)** Expression of Pof14 variants. Protein extracts from strains used in B were analysed by Western blot using α-HA antibody. An open arrowhead indicates gal4AD-HA-Pof14FL and -ΔF. An asterisk indicates a cross-reaction band. **(D)** Protein extracts from cells expressing the indicated tagged proteins from the endogenous locus (except for Skp1 expressed from a plasmid) were prepared and used for immunoprecipitation using the indicated antibodies. After SDS-PAGE, immunoblotting was performed as indicated. Total extracts are shown as control. **(E)** Cycloheximide was added to cultures expressing HA-tagged Pof14 or a mutant lacking the F-box from the locus. Samples were collected every 20 min and analysed by anti-HA Western blotting. **(F)** Cultures expressing HA-tagged Pof14 in wt, *skp1-3* or *mts3-1* background were shifted for 1 h to 36°C in order to inactivate Skp1 or the proteasome. Cycloheximide was added and samples collected every 20 min. Protein extracts were analysed by anti-HA Western blotting.

We first hypothesised that Erg9 could be a substrate of SCF^{Pof14}. However, the inability of Erg9 to coimmunoprecipitate Skp1 or Pcu1 (Figure 2C) with the fact that neither Skp1 nor Pcu1 present the typical subcellular localisation of Erg9 (D Hermand, unpublished data) did not support the hypothesis. Moreover, inactivation of Skp1 did not affect the level of Erg9 *in vivo* and its half-life was unchanged in a strain deleted for *pof14* (Figure 2F). Altogether, these data led us to consider that Pof14 could regulate Erg9 independently of its proteolysis.

Pof14 and repression of ergosterol synthesis are required for adaptation to hydrogen peroxide

Genome-wide microarray analysis of the stress response in fission yeast showed that transcription of the *pof14* gene is

induced after exposure to 0.5 mM hydrogen peroxide (Chen *et al*, 2003). We expanded these data by analysing the expression of *pof14* during a time course in two oxidative stress conditions: 0.2 and 1 mM based on previous works (Quinn *et al*, 2002). This was performed in a wild-type strain or in strains deleted for the stress response kinase Sty1 or transcription factors Atf1 and Pap1 (Figure 3A). In the conditions tested, an induction peak fully dependent upon the presence of Sty1 and Pap1 was observed within 1 h (Figure 3A), consistent with the published microarray data (Chen *et al*, 2003). We noticed that the *pof14* promoter contains a TCTTTCAT motif reminiscent of the TCTTNCTT consensus of the stress response genes activated independently of Atf1 (Chen *et al*, 2003). The relevance of this motif was not tested experimentally in this study.

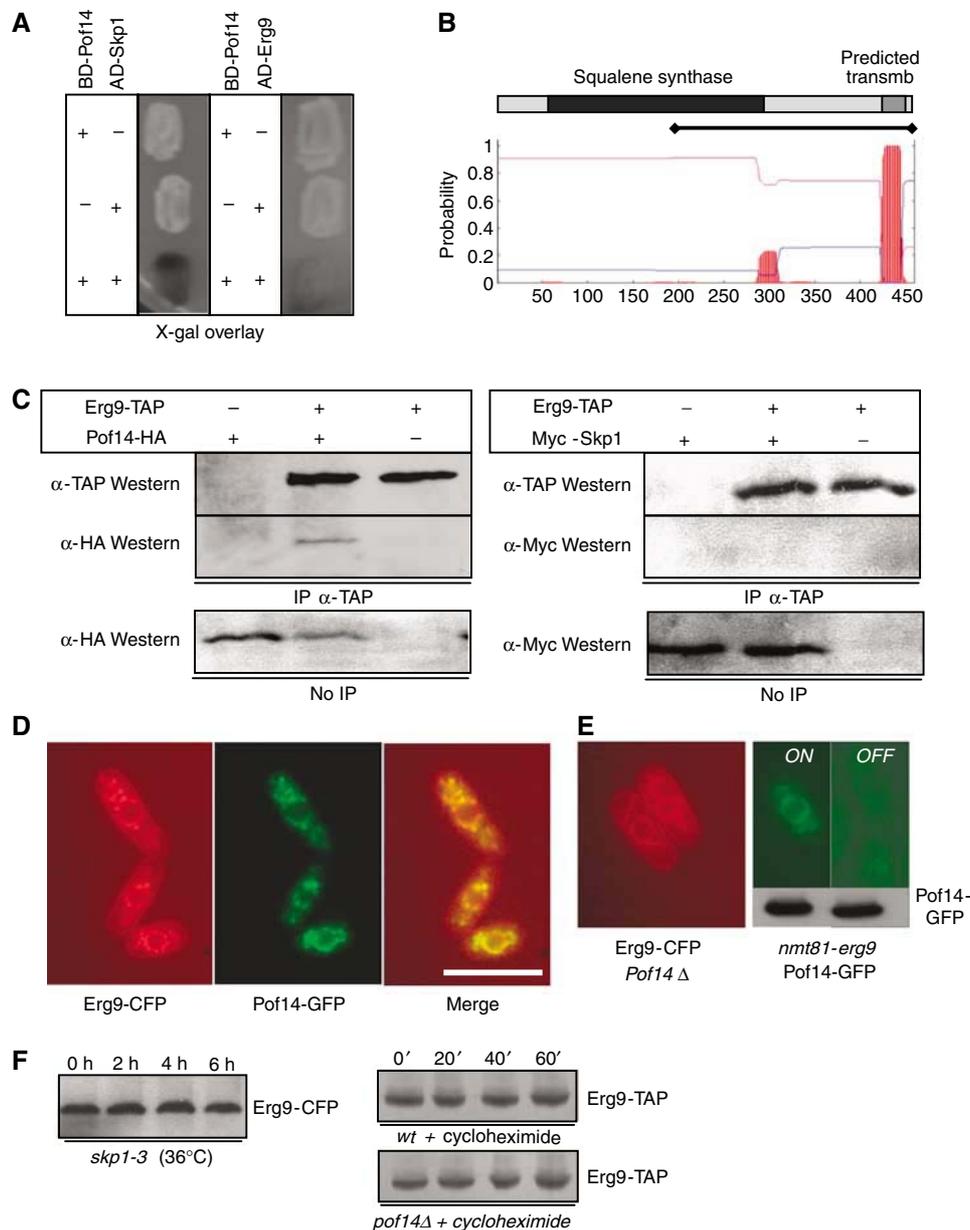


Figure 2 Pof14 interacts with Erg9 in a membrane bound complex. **(A)** Two-hybrid interaction between Pof14 and Erg9. The MAV103 two-hybrid strain expressing Gal4DB-Pof14 was transformed with pACTII harbouring *skp1* (left) or *erg9* (right). Strains were patched and overlaid with X-Gal containing medium to test activation of *lacZ* reporter gene. **(B)** Schematic representation of Erg9 catalytic domain (squalene synthase) and predicted transmembrane domain by a hydropathy plot (www.cbs.dtu.dk/services/TMHMM/). The bar indicates the fragment of Erg9 interacting with Pof14 in the two-hybrid screen. **(C)** Protein extracts from cells expressing the indicated tagged proteins from the endogenous locus (except for Skp1 expressed from a plasmid) were prepared and used for immunoprecipitation using the indicated antibodies. After SDS-PAGE, immunoblotting was performed as indicated. Total extracts are shown as control. **(D)** Cells expressing Pof14-GFP and Erg9-CFP were grown exponentially, patched on EMM agarose pads and observed for fluorescence microscopy. Bar: 10 μ m. **(E)** Left: Cells deleted for *pof14* and expressing Erg9-CFP were grown exponentially, patched on EMM agarose pads and observed for fluorescence microscopy. Right: identical except *nmt81-erg9 pof14-GFP* were grown in the absence (ON) or presence (OFF) of thiamine to repress *erg9* expression. Bottom: Western blot with anti-GFP antibodies. **(F)** Left panel: cultures expressing CFP-tagged Erg9 in *skp1-3* background were shifted for 6 h to 36°C in order to inactivate Skp1. Samples were collected every 2 h and protein extracts were analysed by anti-CFP Western blotting. Right panel: cycloheximide was added to cultures expressing TAP tagged Erg9 in *wt* and *pof14 Δ* backgrounds and samples collected every 20 min. Protein extracts were analysed by anti-TAP Western blotting.

Western blot analysis and quantification of Pof14 protein under several stress conditions (Figure 3B) was also performed and a similar induction was observed. Moreover, the half-life of Pof14 was increased under stress conditions (Figure 3C), raising the possibility that beside transcriptional induction, Pof14 stability is also regulated in these circumstances to increase its level further. This might be achieved

through dissociation of Pof14 from the core SCF as reported for the F-box protein Met30 (Barbey *et al*, 2005). However, it is not likely to be the case because when immunoprecipitating Skp1 in the presence or absence of stress (40 min, 0.2 mM H₂O₂), a similar amount of Pof14 was co-precipitated (Figure 3D), although the total Pof14 concentration was elevated as shown above (Figure 3B). This rather suggests

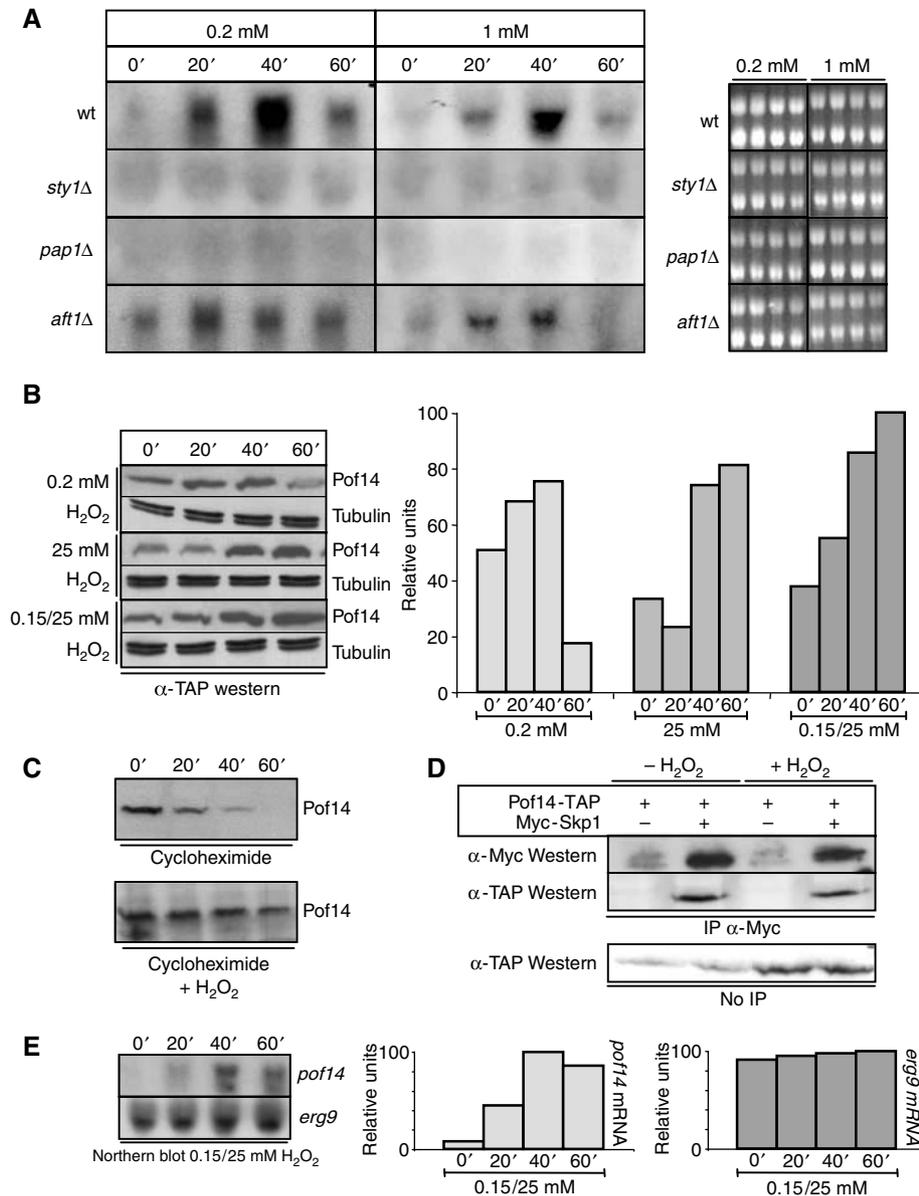


Figure 3 Pof14 expression is induced during oxidative stress. (A) Cultures of wt cells or cells deleted for either *sty1*, *pap1* or *aft1* were grown exponentially and H₂O₂ was added at 0.2 or 1 mM. Samples were taken every 20 min and RNA prepared. After separation on agarose gels, the blots were hybridised with a probe corresponding to *pof14* open reading frame. An ethidium bromide staining of the gels is shown on the right as loading control. (B) Samples from wt cultures expressing Pof14-TAP and treated as indicated were collected and protein extracts were analysed by anti-TAP Western blotting. Anti-tubulin Western blotting is used as loading control. Quantification of Pof14 expression is shown on the right. (C) Cycloheximide was added a culture expressing HA-tagged Pof14 after a 20 min treatment with 0.2 mM H₂O₂. Control cells grown in the absence of H₂O₂ (identical to Figure 1E) are also presented. Samples were collected every 20 min and analysed by anti-HA Western blotting. (D) Protein extracts from cells expressing tagged Pof14 from the endogenous locus (except for Skp1 expressed from a plasmid) and grown in the presence or absence of H₂O₂ (40 min; 0.2 mM) were prepared and used for immunoprecipitation using the indicated antibodies. After SDS-PAGE, immunoblotting was performed as indicated. Total extracts are shown as control. (E) The blotting membrane used in Figure 3A was stripped and rehybridised with a probe corresponding to *erg9* open reading frame. The result with a *pof14* probe (identical to part of Figure 3A) is also shown. Right panel: quantification of the signal.

that the SCF might be saturated when Pof14 is induced, therefore leading to a stabilisation of the Pof14 pool present in the cell. Pof14 could then only reach its normal level after transcriptional induction has been turned off.

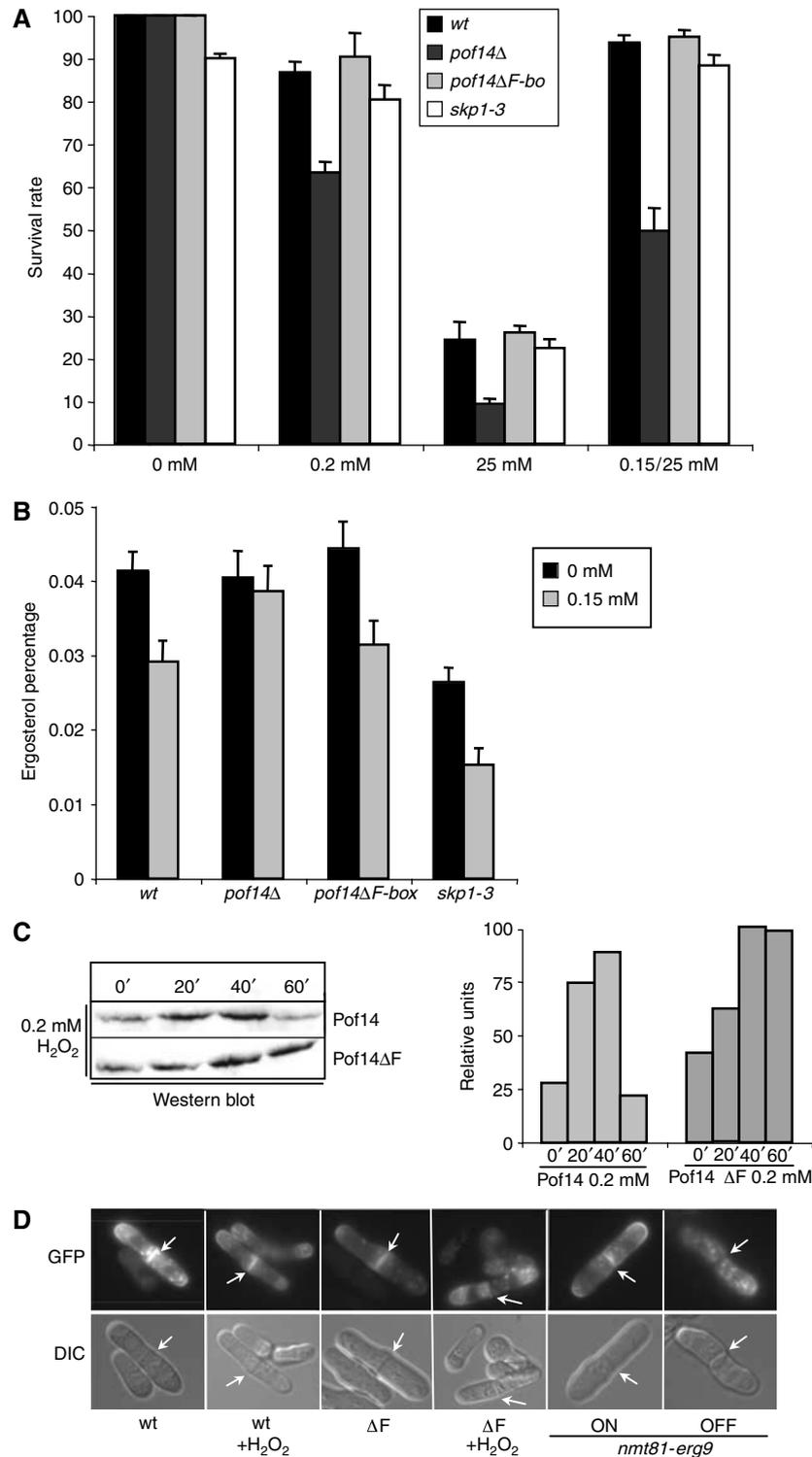
We next compared the effect of hydrogen peroxide addition on Erg9 transcription, and Figure 3E shows that Erg9 does not appear to be regulated at this level. The Erg9 protein level was also stable under these conditions (data not shown).

The induction of *pof14* transcription by hydrogen peroxide prompted us to analyse the Pof14–Erg9 interaction under oxidative stress conditions (H₂O₂ 0.2 mM for 40 min). An increased amount of Pof14, proportional to its elevated level in total cell extract, was co-precipitated by Erg9, suggesting that after induction, the newly synthesised Pof14 proteins bind to Erg9 (Supplementary Figure 1).

To test the physiological importance of *pof14* induction, we compared the sensitivity of a wild type and *pof14* deletion

treated with 0.2 mM H₂O₂, 25 mM H₂O₂ (acute stress) or 1 h with 0.15 mM H₂O₂ followed by 25 mM H₂O₂ (adaptative stress). Deletion of *pof14* led to a marked decrease in viability in both acute and adaptative oxidative stress (Figure 4A). Surprisingly, the Pof14 mutant lacking the F-box could maintain viability similarly to the wild type, indicating that this function of Pof14 is independent of SCF. An *skp1 ts* mutant incubated at restrictive temperature during oxidative shock

also maintained viability, confirming this conclusion (Figure 4A). In that case, we also checked that the heat shock did not affect the wild-type response (data not shown). Together with the fact that Pof14 and Erg9 form a complex that does not include Skp1 and Pcu1 (Figure 2), this suggests a link between the requirement of Pof14 to maintain viability in oxidative stress and ergosterol metabolism through its interaction with Erg9. To test this, ergosterol



level was measured in the same strains used in Figure 4A. In the wild type, exposure to a low level of peroxide (0.15 mM) enabling adaptation led to a 25% decrease in ergosterol content within 1 h, indicating that cells repress ergosterol metabolism and decrease their ergosterol content to adapt to oxidative stress (Figure 4B). The loss of viability in the *pof14* deletion in the same conditions correlates with stable ergosterol content while a mutant lacking the F-box or a mutant affected in SCF behaved as wild type.

The behaviour of the F-box mutant raised the question of the physiological significance of Pof14 instability. To investigate this further, we compared the level of wild-type versus F-box mutant during oxidative stress. Although total Pof14 protein level was elevated in the mutant, the main feature is persistence (even at the 80 and 100 min time point, data not shown) of the F-box mutant at a high level after transcriptional induction is switched off (Figure 4C). We reasoned that a long-term decrease in the ergosterol level, consequent to high level of Pof14, might be deleterious for the cell. This hypothesis was suggested by recent data establishing a central role for ergosterol in correct and efficient cell surface delivery in yeast (Proszynski *et al*, 2005). Defective ergosterol synthesis results in inhibition of trafficking and sorting of proteins to the cell surface as exemplified by defects in the targeting of the tryptophan permease Tat2 to the cell surface in the absence of ergosterol (Umebayashi and Nakano, 2003). We tested a potential effect of long-term stabilisation of Pof14 on the delivery of the previously characterised cell surface marker ScFus1-GFP, a raft-associated protein that is missorted in mutants defective in ergosterol synthesis (Proszynski *et al*, 2005; Tanaka *et al*, 2005). In fission yeast, the reporter localises mainly to the septum (Tanaka *et al*, 2005). As shown in Figure 4D, the addition of oxygen peroxide (100 min, 1 mM) did not modify Fus1 localisation. By contrast, when the Δ F-box mutant was used, proper delivery of Fus1 to the septum was inhibited and instead it accumulated intracellularly in dot-like structures (Figure 4D and Supplementary Figure 2). Interestingly, inhibition of *erg9* synthesis using a repressible promoter (*nmt81*) leads to similar defects (Figure 4D) in the absence of stress. However, the Δ F-box mutant does not show obvious phenotype, suggesting that in the conditions tested, cells might tolerate this effect.

Taken together, these data suggest that a short-term down-regulation of the activity of Erg9 and the synthesis of ergosterol is required to set up an adaptative response to oxidative stress, and this requires Pof14. If this model is correct, transcriptional induction of *pof14* in the absence of peroxide

might mimic the adaptation following exposure to low dose. To test this, *pof14* was overexpressed from a plasmid under the control of the strong *nmt1* promoter, which is turned on in the absence of thiamine. Figure 5 shows that induction of Pof14 expression led to a decrease in ergosterol content and to a marked increase in viability following an acute stress with 25 mM H₂O₂. We also wanted to test the effect of higher level of *erg9* on the response, but overexpression from the *nmt1* promoter turned out to be toxic for the cell (Supplementary Figure 3).

These data constitute the first molecular evidence supporting that membrane composition is altered to adapt permeability to H₂O₂. The two-hybrid interaction and the colocalisation are in favour of a direct effect of Pof14 on Erg9, although the way in which Pof14 affects Erg9 activity remains to be determined. To gain further insight on this aspect, we set up an activity assay for Erg9 in fission yeast based on previous works (Jennings *et al*, 1991; Shechter *et al*, 1992; Kribii *et al*, 1997). It consists in isolating Erg9 bound microsomes and measuring the amount of radiolabelled squalene produced *in vitro* in the presence of {³H} FPP and required co-factors. We first determined the squalene synthase activity in wild-type strain grown in the presence or absence of oxygen peroxide. Remarkably, a decrease in activity consistent with the reduction in ergosterol content (Table I) was observed after treatment with H₂O₂ and this

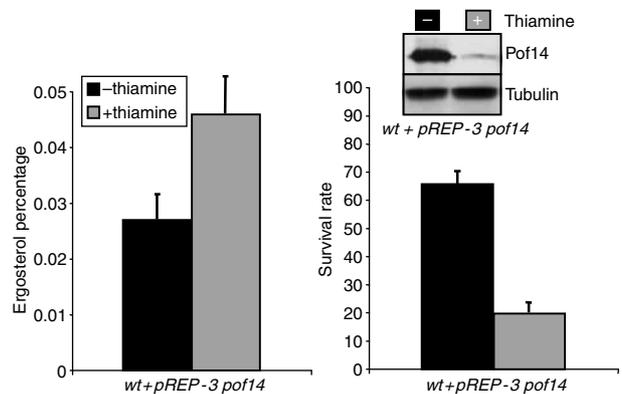


Figure 5 Pof14 directly regulates Erg9 activity. A strain harbouring a *pREP-3 pof14* vector allowing overexpression of *pof14* in the absence of thiamine was grown in the presence or absence of thiamine for 14 h and both ergosterol content and survival rate were determined following an acute stress with 25 mM H₂O₂ as in Figure 4A and B. Right panel: A Western blot showing Pof14 levels, anti-tubulin was used as loading control.

Figure 4 Pof14 mediates decrease in ergosterol content following oxidative stress. (A) The sensitivity of a wild-type strain (wt), a strain deleted for *pof14* (*pof14Δ*), a strain expressing a *pof14* mutant lacking the F-box (*pof14ΔF*) and an *skp1 ts* mutant (*skp1-3*) were analysed. After incubation of the cultures for 1 h with indicated H₂O₂ treatment, cells were diluted and plated. In the case of *skp1-3*, temperature was raised to 36°C at the time of peroxide addition. Survival was measured as a percentage of colony number of untreated cells. Mean survival rate is from three experiments. (B) Ergosterol content expressed as a percentage of wet weight was measured from a wild-type strain (wt), a strain deleted for *pof14* (*pof14Δ*), a strain expressing a *pof14* mutant lacking the F-box (*pof14ΔF*) and an *skp1 ts* mutant (*skp1-3*) grown for 1 h in the presence or absence of 0.15 mM hydrogen peroxide. In the case of *skp1-3*, temperature was raised to 36°C at the time of peroxide addition. Mean ergosterol content is from three experiments. (C) Samples from strains expressing integrated tagged Pof14 and Pof14 Δ F and treated for indicated times with 0.2 mM H₂O₂ were collected and protein extracts were analysed by Western blotting. Quantification of Pof14 expression is shown on the right. (D) 1–4: Indicated strains expressing the cell surface marker ScFus1-GFP from *pREP41* were grown in the presence or absence of 0.2 mM H₂O₂ for 100 min and observed by fluorescence microscopy (GFP) or DIC. 5–6: *nmt81-erg9* cells expressing the cell surface marker ScFus1-GFP from *pREP41* were grown in the presence (OFF) or absence (ON) of thiamine for 6 h and observed by fluorescence microscopy. Arrows indicate the septum.

Table I Squalene synthase activity assay in microsomal and cytosolic fractions

Strain	Specific activity (nmol min ⁻¹ mg ⁻¹)		Relative activity (microsomes)
	Cytosol	Microsomes	
wt	0.08	0.32	100
wt + H ₂ O ₂	0.1	0.21	66
<i>pof14</i> Δ	0.005	0.32	100
<i>pof14</i> Δ + H ₂ O ₂	0.005	0.29	90
wt + GST 1 ng	ND	0.35	109
wt + GST-Pof14 1 ng	ND	0.32	100
wt + GST 10 ng	ND	0.34	106
wt + GST-Pof14 10 ng	ND	0.25	78
wt + GST 100 ng	ND	0.31	97
wt + GST-Pof14 100 ng	ND	0.17	53

Fractions were assayed for squalene synthase activity in triplicate assays with standard deviation within a 0.03 range. The mean specific activity found in wild-type strain (0.32) was assigned a relative activity of 100. ND: not determined.

was not observed in a *pof14*Δ strain. To assess a direct effect on Erg9 activity *in vitro*, GST or GST-Pof14 was expressed and purified from bacteria (Supplementary Figure 4) and increasing amounts of the recombinant proteins were added to the squalene synthase assay. As shown in Table I, addition of GST-Pof14 but not GST alone inhibited Erg9 *in vitro*. With the physical association detected *in vivo*, these *in vitro* data strongly support that Pof14 can directly affect Erg9 activity.

In this study, we report the role of the F-box protein Pof14 in directly regulating the activity of a key enzyme in sterol metabolism as a way for the cell to maintain viability following oxidative stress. This constitutes a molecular mechanism for the change in plasma membrane permeability induced by exposure to oxygen peroxide (Branco *et al*, 2004). Although the ability of the *Pof14*ΔF mutant and the *skp1-3 ts* mutant to behave as wild type shows that the role of Pof14 in this context does not require association with SCF, we do not rule out the possibility that SCF^{Pof14} regulates the stability of proteins other than its own.

Materials and methods

Yeast strains, media and techniques

All standard protocols for fission yeast have been described previously (Moreno *et al*, 1991). Cycloheximide (Sigma) was used at 100 µg/ml.

Two-hybrid screens

The budding yeast strain used for the two-hybrid screen is MAV103 (*MATa*, *gal4*Δ*gal80*Δ, *SPAL*₁₀::*URA3*, *GAL1-LacZ*, *lys2*::*GAL1-HIS3*, *ade2-101*, *ura3-52*, *leu2-3,112*, *trp1-901*, *his3*Δ*200*; Vidal *et al*, 1996). The two-hybrid screens were performed according to the 'TRAF0' protocol (see the Gietz lab website <http://www.umanitoba.ca/faculties/medicine/units/biochem/gietz/2HS.html>) based on the Matchmaker-II Two-Hybrid system (see the Clontech website and handbooks for complete protocols). Sequences and maps of the bait vectors harbouring Skp1 and Pof14 are available upon request. Expression was checked by Western blot using anti-Gal4DB antibody (Clontech).

Expression vectors, gene deletion and tagging

Flag-tagged Skp1 was expressed from pAAUN. Flag-tag sequence: MDYKDDDDDK.

Myc-tagged Skp1 has been described previously (Hermand *et al*, 2003). pREP41-ScFus1-GFP is a kind gift from Kaoru Takegawa (Tanaka *et al*, 2005).

Deletion, locus-specific integration of the regulatable weak *nmt81* and strong *nmt1* promoters and C-term tagging were performed by the PCR method exactly as described (Bahler *et al*, 1998). All primer sequences are available upon request. For selection of *natMX6*, clonNAT was purchased from Werner Bioagent. All *pof14* deletion mutants were created by combining PCR products; all primer sequences and detailed cloning sites are available upon request.

Western blotting, immunoprecipitations and GST-fusion proteins purification

For Western blotting, boiled extracts were prepared as described (Moreno *et al*, 1991). Cells were broken using 1 ml glass beads (Sigma) and a Fastprep device (Qbiogen). For immunoprecipitation, soluble protein extracts were prepared from log phase cultures, as described (Bamps *et al*, 2004). For IgG precipitation (IP-TAP), 20 µl of IgG agarose (Sigma A2909) was added to 1 mg of soluble extracts prepared with 1% Triton X-100. For Myc immunoprecipitation, 1 µl of anti-Myc (9E10, BabCo) coated to 50 µl of proteinA-sepharose beads (Sigma) was added to 1 mg of soluble extracts prepared with 1% Triton X-100. After three washes, proteins were separated on SDS-PAGE and blotted onto nitrocellulose membranes. Western blotting using anti-Myc (BabCo 9E10), anti-Flag (M5 monoclonal antibody, Stratagene), PAP (Peroxidase anti-peroxydase, Sigma P1291), anti-GFP isoforms (JL8, BD Biosciences), antitubulin (Sigma) and anti-HA (16B12, BabCo) were performed according to the manufacturer's instruction (ECL, Amersham Pharmacia Biotech). NIH Image (National Institutes of Health, Bethesda, MD) was used for quantification of protein levels after immunoblotting. The GST-Pof14 fusion was expressed from pGEX-4T-1 in the *Escherichia coli* BL21 strain according to the manufacturer's instructions (GE Healthcare). Purified proteins were concentrated on vivaspin (VivaScience).

Hydrogen peroxide sensitivity tests

For H₂O₂ sensitivity stress, overnight cultures were diluted at OD₅₉₅ = 0.1. After 1 h recovery, H₂O₂ was added to cultures at indicated concentration. Cells were taken at various time points, diluted, then plated on YES plates to determine CFU, expressed as a percentage of CFU of untreated cells.

Ergosterol quantification method

Ergosterol level was measured using the method described by Arthington-Skaggs *et al* (1999). Briefly, cells were centrifuged and washed with water. The net weight of the cell pellet was determined, 3 ml of 25% alcoholic potassium hydroxide solution was added to each pellet and vortex mixed for 1 min. Cells were incubated at 85°C for 1 h and allowed to cool to room temperature. Sterols were extracted by the addition of a mixture of 1 ml of water and 3 ml of *n*-heptane followed by vortexing for 3 min. The heptane layer was recovered, diluted fivefold in ethanol and scanned spectrophotometrically between 240 and 300 nm. The presence of ergosterol was detected at 281.5 nm and ergosterol content was calculated as a percentage of the wet weight as reported (Arthington-Skaggs *et al*, 1999).

Yeast microsomal extracts and squalene synthase activity (Jennings *et al*, 1991; Shechter *et al*, 1992; Kribii *et al*, 1997)

Yeast cells grown in EMM were harvested by centrifugation, washed in 50 mM potassium phosphate pH 7.5 and resuspended in breakage buffer (50 mM potassium phosphate pH 7.5, 1 mM dithiothreitol and protease inhibitors cocktail). Cells were disrupted by vigorous vortexing in glass beads and the extract was centrifuged at 12 000 g for 30 min. The supernatant was centrifuged again at 105 000 g for 40 min. The supernatant (cytosolic fraction) was collected and the pellet (microsomal fraction) was resuspended in breakage buffer and centrifuged again at 105 000 g for 40 min. The pelleted microsomes were resuspended in 50 mM potassium phosphate pH 7.5 at a concentration of 1 mg/ml.

SQS activity was assayed for 30 min at 30°C in a reaction mixture (0.5 ml) containing 50 mM potassium phosphate pH 7.5, 5 mM

MgCl₂, 10 mM KF, 1 mM NADPH, 4 mM glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase and 25 μM (48 nCi, 20 μCi/mmol) {1-³H} farnesyl-P₂ (farnesyl pyrophosphate triammonium salt, Perkin Elmer) and either microsomal or cytosolic fractions (100 μg protein) supplemented by various amounts of either GST or GST-Pof14 as indicated. The reaction was stopped by adding 0.25 ml 40% KOH and 0.25 ml ethanol. Saponification was allowed to proceed for 30 min at 60°C the nonsaponifiable lipids were extracted three times with 1 ml hexane. The radioactivity present in the hexane extract was quantified by liquid scintillation counting. The lower detection limit under these conditions was 0.002 nmol of squalene/min/mg protien, which corresponds to about 10 dpm above background.

In vivo fluorescence imaging

Cells expressing *pof14-GFP* and *erg9-CFP* were cultured overnight, washed twice with PBS and resuspended in 100 μl PBS. Two microlitres of cells were placed on EMM-agarose pads and covered with a coverslip as described in Hermand *et al* (2003). Living cells were then observed with a Nikon microscope (Eclipse E1000), using a ×100/1.40 Nikon oil immersion objective lens. Images were obtained on a cooled CCD camera (Hamamatsu ORCA-ER) with an exposure time of 2 s. Images were processed using Simple PCI software.

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Northern blots

Aliquots (50 ml) of cultures were collected and centrifuged, and then rapidly frozen. Total RNA was extracted using a hot-phenol protocol (Chen *et al*, 2003). Thirty micrograms were separated on a 1.2% agarose-formaldehyde gel and blotted on a nylon membrane. RNA was probed with ³²P-labeled DNA fragments (obtained by a radioactive PCR) specific to the target mRNA. NIH Image (National Institutes of Health, Bethesda, MD) was used for the quantification of mRNA level after revelation.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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