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Involvement of the cyclin-dependent kinase Pho85 in responding to environmental stress



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# Involvement of the Cyclin-Dependent Kinase Pho85 in Responding to Environmental Stress

by

Adam Slade Carroll

DISSERTATION

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Biochemistry and Molecular Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

**For my family**

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Abstract

Involvement of the Cyclin-Dependent Kinase Pho85

in Responding to Environmental Stress

Adam Slade Carroll

Through its association with a family of ten cyclins, the Pho85 cyclin-dependent kinase is involved in several signal transduction pathways in the yeast *Saccharomyces cerevisiae*. The responses mediated by Pho85 include cell cycle progression and metabolism of nutrients such as phosphate and carbon sources. While these responses require the phosphorylation of different substrates and produce different mechanistic consequences through that phosphorylation, all appear to be involved in responses to stressful environmental conditions. Few of the activating signals or regulated targets have been unambiguously identified, but the kinase activity of Pho85 appears to inform the cell that the current environment is satisfactory. In addition to its well-established role in responding to phosphate starvation, Pho85 has been implicated in controlling the synthesis of glycogen. To comprehensively characterize the range of Pho85-dependent gene expression, I used a chemical genetic approach that enabled us to control Pho85 kinase activity with a cell-permeable inhibitor and whole genome transcript profiling. I found significant phenotypic differences between the rapid loss of activity caused by inhibition and the deletion of the genomic copy of *PHO85*. I demonstrate that Pho85 controls the expression of not only previously identified glycogen synthetic genes, but also a significant regulon of genes involved in the cellular response to environmental

stress. In addition, I show that the effects of this inhibitor are both rapid and reversible, making it well suited to the study of the behavior of dynamic signaling pathways. Also, I demonstrate a role for Pho85 in activating the transcriptional response to DNA damage. Pho85 appears to act independently of previously identified factors required for DNA damage-induced transcription in transducing this signal.

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# CHAPTER 1

## Pho85 and signaling environmental stress

**Pho85 and signaling environmental stress**

Adam S. Carroll and Erin K. O'Shea

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**Keywords**

Pho85; Cyclin-Dependent Kinases; *Saccharomyces cerevisiae*; phosphate starvation; Environmental Stress Response; kinase substrate specificity.

**Summary**

Through its association with a family of ten cyclins, the Pho85 cyclin-dependent kinase is involved in several signal transduction pathways in the yeast *Saccharomyces cerevisiae*. The responses mediated by Pho85 include cell cycle progression and metabolism of nutrients such as phosphate and carbon sources. Although these responses require the phosphorylation of different substrates and produce different mechanistic consequences as a result of phosphorylation, all appear to be involved in responses to stressful environmental conditions. Few of the activating signals or regulated targets have been unambiguously identified, but the kinase activity of Pho85 appears to inform the cell that the current environment is satisfactory.

Microorganisms such as the budding yeast *S. cerevisiae* make coordinated, accurate, and robust responses to changes in a complex extracellular environment. They can coordinate such responses by reusing individual factors and motifs for related responses. They must also ensure that the correct response is triggered by the appropriate stimulus when such components are shared. An example of a kinase that is shared between several pathways can be found in the multiple functions and cyclin binding partners of the CDK Pho85 [1].

The past 15 years have seen the extensive characterization of a wide variety of cyclin-dependent kinases (CDKs). These signaling molecules have proven to be central to several cellular processes in addition to the regulation of the cell cycle, the context in which they were first identified. Although we have a basic understanding of CDKs and their activity, this understanding is by no means complete. We understand that the binding of a cyclin partner confers substrate specificity on the kinase [2-4], but we have no systematic way to predict the substrates, regulators, or physiological function targeted by a given cyclin/CDK complex. The budding yeast CDK Pho85 has proven useful for approaching these general questions, due to its nonessential nature, the number of cyclin partners with which it associates, and the experimental tractability of yeast in general.

As we learn more about the functions of Pho85, one theme emerges repeatedly. The functions of Pho85 appear to be involved in transducing signals related to cellular stress. Furthermore, the kinase activity of the relevant Pho85 complex appears to send the message that the current environmental situation is satisfactory; when environmental conditions become stressful, the relevant kinase activities of Pho85 are switched off, resulting in activation of the appropriate stress response.

**Pho80 and phosphate metabolism**

Pho85 is best recognized for its pivotal role in the *PHO* pathway, a signaling pathway that coordinates the responses of yeast to phosphate starvation [5, 6]. Pho85 is directed to this function by its association with the cyclin Pho80 [7]. Pho80/Pho85 kinase activity is regulated in response to phosphate levels by the CDK inhibitor (CKI) Pho81, which remains bound to Pho80/Pho85 in high and low phosphate conditions [8]. In the presence of high levels of inorganic phosphate in the environment, the Pho80/Pho85 kinase is active, phosphorylating and inactivating the transcription factor, Pho4 [9]. Phosphorylation of Pho4 inhibits its transcriptional activity by preventing association with the coactivator Pho2 and the nuclear import receptor Pse1 and by promoting association with the nuclear export receptor Msn5 [10-12]. Each of these associations depends on a particular phosphorylation site or sites, but together, Pho4 phosphorylation causes it to be localized predominantly to the cytoplasm and unable to activate transcription of the phosphate starvation-responsive genes [12, 13]. When phosphate becomes limiting, the kinase activity of Pho80/Pho85 is inactivated, permitting activation of Pho4 and causing the transcription of genes relevant for surviving conditions of phosphate starvation [8].

**Phenotypes of the *pho85*Δ strain**

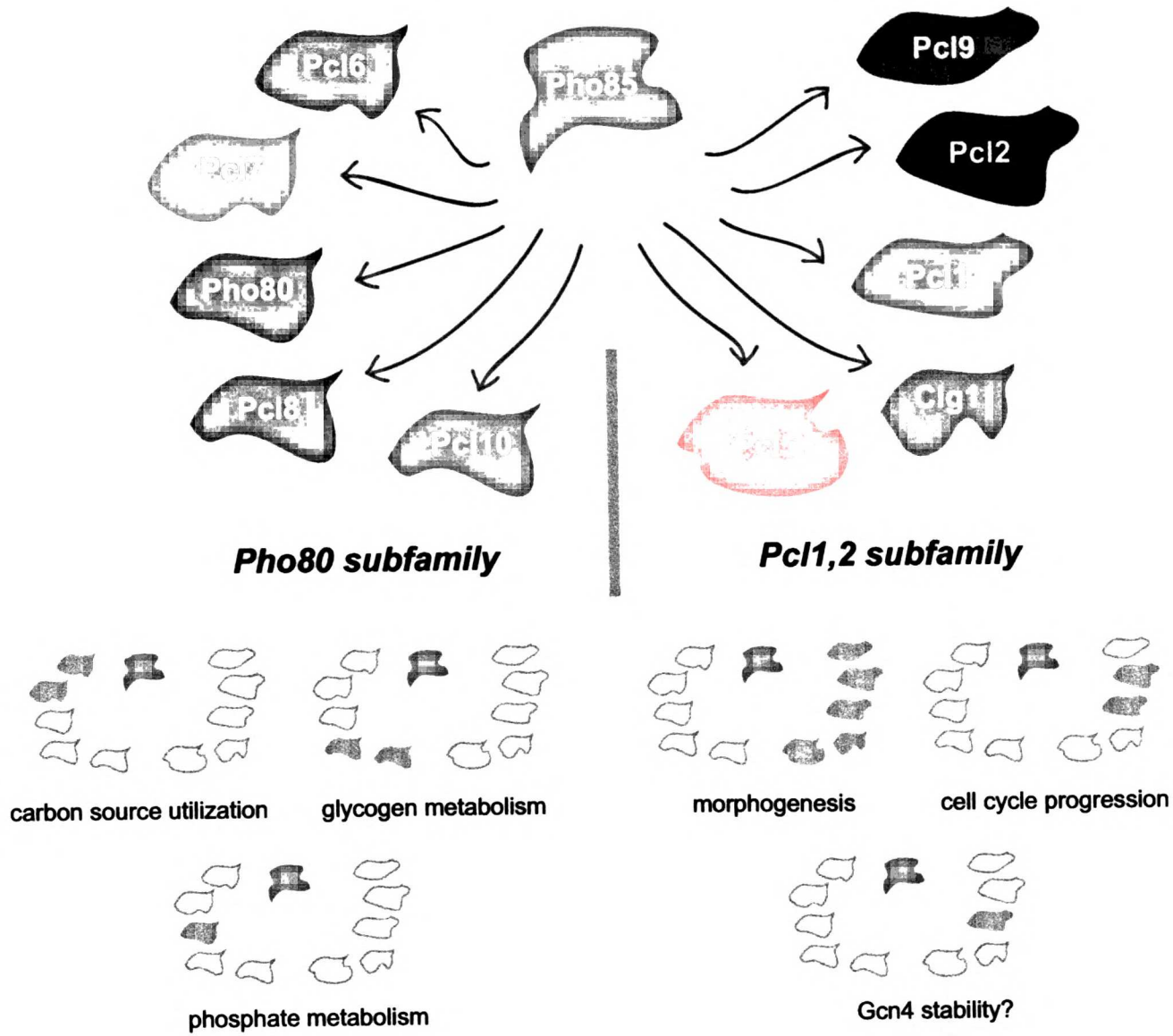
Pho85 has multiple functions, as suggested by the pleiotropic phenotypes caused by its mutation. Deletion of *PHO85* causes not only constitutive expression of phosphate starvation-dependent genes [5], but also slow growth with a G1 delay on rich media, poor

growth on nonfermentable carbon sources, hyperaccumulation of glycogen, abnormal morphology, irregular budding pattern, and sporulation defects [14-17]. Roles have also been proposed for Pho85 in autophagy and proline utilization [18, 19]. Cells lacking *PHO85* are hypersensitive to several chemical treatments including hydroxyurea [20], hygromycin B and G418 [21], and salt [22]. There are also synthetic lethal interactions between *PHO85* mutation and several other genes involved in the cell cycle, morphogenesis, and transcription [20, 23]. In some cases, these phenotypes can be reproduced by deletion of cyclins, but we do not have a comprehensive understanding of which cyclins account for which phenotypes of the *pho85Δ* strain.

**The Pcls**

Pho85 has ten cyclin partners, called Pcls for Pho85 cyclin. The Pcls were identified primarily through sequence homology and two-hybrid screens [16, 24, 25] and have been grouped by sequence homology into two subfamilies of five: the Pcl1,2 subfamily of Pcl1, Pcl2, Clg1, Pcl5, and Pcl9; and the Pho80 subfamily of Pho80, Pcl6, Pcl7, Pcl8, and Pcl10 [16] (Figure 1). Of these ten cyclins, four (*PCL1*, *PCL2*, *PCL7*, and *PCL9*) demonstrate patterns of cell cycle-regulated expression [24-27]. These patterns suggest that some functions of Pho85 have cell cycle dependence or relevance, while others may not. Transcriptional regulation may also be important for those *PCLs* whose expression is not cell cycle controlled. In fact, a number of other *PCLs* show divergent patterns of expression in a comparison of several genome transcript profiling experiments done under different conditions of stress [28, 29]. Such expression data may provide clues to

FIGURE 1. The Pho85 cyclins and their functions. The large graphic denotes the cyclins that belong to each subfamily, as determined by sequence homology. Each small graphic indicates the cyclins that participate in the listed function. The filled-in cyclins participate; the outlined cyclins do not.


Pcl6
Pho85
Pcl9
Pcl2
Pho80
Pcl8
Pcl10
Clg1
Pho80 subfamily
Pcl1,2 subfamily
carbon source utilization
glycogen metabolism
morphogenesis
cell cycle progression
phosphate metabolism
Gcn4 stability?

the function and regulation of Pcls without known molecular functions and allow us to distinguish differences between seemingly redundant cyclins.

The pleiotropic phenotype that results from deletion of *PHO85* provides many possible interpretations for loss of function of an individual cyclin-CDK complex. One long-term goal for the study of Pho85 is to ascribe each of the phenotypes of the *pho85*Δ strain to the loss of function of a particular Pcl or group of Pcls and a resulting change in the activity of the relevant substrate. This review will focus on recent advances in understanding the functions of the non-Pho80 Pcls, with an emphasis on those with proposed molecular functions or substrates.

## Molecular functions of Pho85

### *The cell cycle*

Genetic and gene expression data suggest that some Pcl/Pho85 kinases play a role in the cell cycle. The first identified synthetic lethal interaction for *PHO85* is the inviability of *cln1*Δ *cln2*Δ *pho85*Δ and *cln1*Δ *cln2*Δ *pcl1*Δ *pcl2*Δ strains [24, 25]. *CLN1* and *CLN2* encode cyclins that associate with Cdc28, the yeast CDK required for cell cycle progression [30]. Cln1, Cln2, Pcl1, and Pcl2 are all expressed during the G1 phase of the cell cycle, so the belief is that each of these cyclins contributes information to the decision to pass START and commit to the cell cycle, perhaps through the sharing of substrates between Cdc28-containing and Pho85-containing complexes. Because deletion of *PCL1* and *PCL2* recapitulates the synthetic lethal with *cln1*Δ *cln2*Δ phenotype of the *pho85*Δ strain, we know that these are the only *PCL*s required for this function of

Pho85 (see also [20]). The best model to explain this observation thus far arises from the demonstration that the *in vivo* stability of the CKI Sic1 depends on *PHO85* [31].

*Regulation of Sic1 stability*

Sic1 is responsible for repressing the activity of Clb cyclin-containing Cdc28 complexes until the cell is prepared to leave G1 and commit to progression through the cell cycle [30]. Sic1 degradation has been previously demonstrated to be dependent on the G1 Cln/Cdc28 kinases [32, 33]. A role for Pho85 in controlling Sic1 stability might explain the lethality of the *cln1Δ cln2Δ pho85Δ* and *cln1Δ cln2Δ pcl1Δ pcl2Δ* strains. If Sic1 were hyperstabilized by loss of both Cdc28- and Pho85-dependent mechanisms for its degradation, then the cell would not be able to exit G1.

*In vivo*, the instability of Sic1 depends on the presence of *PHO85*, and the Pho85-dependent instability depends on a particular phosphorylation site on Sic1 [31]. *In vitro* phosphorylation studies have shown that coexpression of Pho85 with the cyclin Pcl1 produces more activity towards Sic1 than coexpression with Pcl2, Pho80, or the Cdc28 cyclins Clb2 and Clb5 [31]. However, at this point there is no evidence to determine whether Pcl1 (or Pcl2) is actually the cyclin required *in vivo* for the observed stabilization of Sic1. The predicted (and parsimonious) explanation for the observed synthetic lethalities would suggest that both Pcl1 and Pcl2 control Sic1 stability, but no concrete link for these cyclins has been established.

If hyperstabilization of Sic1 is the cause of the *cln1Δ cln2Δ pho85Δ* and *cln1Δ cln2Δ pcl1Δ pcl2Δ* synthetic lethalities, then deletion of *SIC1* should restore viability to these strains. One group has reported that deletion of *SIC1* suppresses the synthetic

lethality of a *cln1*Δ *cln2*Δ *pho85*Δ strain, albeit with a substantial decrease in the viability of the strain [31]. It has also been reported, however, that *cln1*Δ *cln2*Δ *pcl1*Δ *pcl2*Δ *sic1*Δ strains are as inviable as a *cln1*Δ *cln2*Δ *pcl1*Δ *pcl2*Δ strain [20]. The resolution of these apparently conflicting genetic differences awaits further investigation. Although deletion of a CDK can certainly have a very different phenotype from deletion of a subset of its cyclins, differences in strain background may provide the simplest explanation for these differences in synthetic lethal interactions.

*Pcl8, Pcl10, and glycogen metabolism*

In most strain backgrounds, deletion of *PHO85* causes hyperaccumulation of glycogen during late log phase growth [15, 26]. This hyperaccumulation occurs because Pho85 acts as a glycogen synthase kinase for Gsy2 [10, 34], the predominant glycogen synthase isoform in yeast [35]. The cyclin partners responsible for directing Pho85 to this well characterized function are Pcl8 and Pcl10. Deletion of these two cyclins produces the same glycogen hyperaccumulation phenotype and hyperactive Gsy2 enzyme as is observed in *pho85*Δ cells [10]. Furthermore, the Pcl10/Pho85 complex is capable of phosphorylating recombinant Gsy2 *in vitro* and demonstrates a 50-fold *in vitro* specificity for Gsy2 over Pho4 [10]. A similar *in vitro* activity for Pcl8/Pho85 complexes has not yet been demonstrated. The *in vivo* mechanism of regulation of this glycogen synthase kinase activity has not been elucidated. The physiological relevance of this regulation to responding to particular environmental or nutritional conditions also remains to be determined. The activity of Pcl10/Pho85 does demonstrate that not all Pcl/Pho85 complexes directly regulate transcription factors and gene expression.

The participation of Pcl8 and Pcl10 in regulation of reserve carbohydrate levels suggests that these cyclins might be responsible for the phenotype of the *pho85Δ* strain that causes poor growth on non-fermentable carbon sources. Strangely, this is not the case; only *pcl6Δ* and *pcl7Δ* strains exhibit this phenotype [10, 26]. These are the closest homologs of Pcl8 and Pcl10, but their regulation, particularly that of Pcl7, is complex, as described below [26].

*Morphogenesis*

There are three lines of evidence suggestive of a role for Pho85 in controlling cellular morphogenesis. First, *pho85Δ* cells are larger, with more elongated buds and a wider bud neck than isogenic wild-type strains; these morphogenetic phenotypes are more pronounced in **a**/α diploid cells than in haploid cells [16]. The cyclins important for this morphogenetic phenotype are the Pcl1,2 subfamily, as deletion of all five members of this subfamily causes a phenotype very similar to that observed in the *pho85Δ* strain [16]. Also, the *pho85Δ* strain and a strain lacking all five members of the *PCL1,2* subfamily display random patterns of budding [17]; this phenotype is likely related to the abnormal morphology and actin localization observed in those strains [22]. Second, mutations in several genes involved in morphogenesis, including *BCK1*, *MPK1*, *BEM2*, and *CDC42*, display a synthetic lethal phenotype when mutated in strains lacking *PHO85* [20, 23]. Pcl1 and Pcl2 are the cyclins relevant to this phenotype, as deletion of *PCL1* and *PCL2* also causes synthetic lethality with these morphogenetic genes. In the case of *BEM2*, deletion of only *PCL1* also produces the synthetic lethal phenotype, indicating that there are nonredundant functions for these highly homologous cyclins [20]. Third, Pcl2 and

Pcl9 interact physically with Rvs167, the yeast amphiphysin homolog, which is known to be involved in actin cytoskeleton organization, endocytosis, and survival during starvation conditions [22]. Deletion of *PHO85*, like deletion of *RVS167*, causes defects in this set of processes [22]. It is thought that Rvs167 is phosphorylated *in vivo* by Pcl2/Pho85, since maximal *in vivo* phosphorylation of Rvs167 depends on the presence of Pho85 and the Pcl1,2 subfamily [22]. It is not clear, however, that Pcl2/Pho85-dependent phosphorylation of Rvs167 is direct, nor has any effect on the activity or intermolecular associations of Rvs167 been attributed to loss of regulation by Pho85. Although all of this evidence points towards a morphogenetic function for the Pcl1,2 subfamily and Pho85, the precise molecular mechanism of the function remains unknown.

*Regulation of Gcn4 stability*

Pho85 has also been implicated in controlling stability of the transcription factor Gcn4. This function of Pho85 appears to be similar to its role in Sic1 regulation, as both proteins undergo phosphorylation- and ubiquitination-dependent degradation [32, 33, 36]. Gcn4 is required for the increased expression of amino acid biosynthetic genes, such as *HIS4*, upon starvation for amino acids [37, 38]; such starvation causes an increase in the half-life of the protein, as measured by *in vivo* pulse-chase experiments [39]. Similarly, deletion of *PHO85* causes a significant increase in the half-life of Gcn4 [39]. Conditions that stabilize Gcn4 also produce a lower kinase activity in immunoprecipitated Pcl1/Pho85 complexes, although the experiment did not include a necessary control: a measurement of Pcl1 and Pho85 protein quantity in the immunoprecipitation [39]. Gcn4

can also be efficiently phosphorylated *in vitro* by Pho80/Pho85 [39]. Furthermore, no *in vivo* consequences on Gcn4 stability were found for the *pcl1*Δ strain or for several unspecified strains containing deletions of other *PCLs* in addition to *PCL1* [39]. Further studies are required to establish which Pcl is responsible for this stabilization *in vivo*.

An interesting future experiment would be to determine if Pho85 function has an impact on the native promoters of genes known to be under Gcn4 control. One such experiment has shown that deletion of *PHO85* causes a 2.5-fold increase in expression from a derivative of a *HIS4* promoter that is exclusively dependent on Gcn4 [39]. Subsequent experiments have demonstrated that the CDK Srb10 also plays a role in Gcn4 stability [36]. The role of Srb10 is apparently distinct from that of Pho85, as the simultaneous deletion of both *SRB10* and *PHO85* results in a stabilization of Gcn4 greater than that observed for either single mutant alone. When both *SRB10* and *PHO85* are inactivated, Gcn4 may be sufficiently stabilized to result in expression of Gcn4-dependent genes. Alternatively, if transcription of Gcn4-dependent genes is unaffected, the stabilization may be important for establishment of a sensitized state that enables the yeast to better respond to further environmental insult.

*Interaction with Swi5*

Pho85 has also been implicated in regulating the transcription factor Swi5 [40]. Swi5 regulates transcription of several cell cycle-dependent genes, including *PCL9* and *PCL2* [17, 27]. Interestingly, Swi5 and Pcl2 proteins interact in a two-hybrid screen and when translated *in vitro* [40]. Although Pcl2/Pho85 phosphorylates Swi5 *in vitro*, several results call into question the *in vivo* relevance of this observation. First, Pho80/Pho85

also phosphorylates Swi5 *in vitro* to a similar level as Pcl2/Pho85 [40]. Second, the only apparent effect of Pho85 on Swi5 activity *in vivo* is a mild transcriptional defect in expression of two Swi5-dependent genes [40]. To observe this change in expression, the partially redundant transcription factor Ace2 must also be deleted from the strain [40]. Unlike Sic1 and Gcn4, the stability of Swi5 is unchanged by deletion of *PHO85* in synchronized or asynchronized cultures [40]. It is possible that the binding of Swi5 to Pcl2 serves a function other than directing Pho85 to phosphorylate Swi5.

*The Environmental Stress Response*

The ability of the Pho80/Pho85 complex to signal a response to a specific type of cellular stress is well documented. Recently, a more general role for Pho85 in producing a generic response to many types of cellular stress has been suggested by studies using a chemical genetic approach [41]. The rapid loss of Pho85 activity in the absence of any cellular stress causes the induction of a diverse set of genes that has recently been characterized as comprising a generic response to stressful conditions known as the Environmental Stress Response, or ESR ([28], see also [29]). It is not clear if such induction is due to loss of Pho85 function involved directly in induction of the ESR or if loss of a particular non-Pho4-dependent Pho85 function creates ESR inducing conditions. Furthermore, the Pho85 cyclins responsible for this ESR-inducing condition have not yet been identified. It is important to note that these genes are not constitutively expressed when *PHO85* is deleted from the genome, suggesting that activation of the ESR is eventually repressed. Some of the pleiotropic phenotypes and sensitivities caused by deletion of *PHO85* may be explained by the adaptive state that corrects for this induction

of the ESR. The constitutive expression of ESR genes, such as *CTT1* and *GSY2*, caused by deletion of *PHO85* in some strain backgrounds [42] may be due to the failure of these strains to adapt to induction of the ESR.

**Regulation of Pcl/Pho85-containing complexes**

At this juncture, substantially more effort has been directed towards the identification of substrates than the identification of regulatory factors that control Pcl/Pho85 complexes other than Pho80/Pho85. As described above, Pho80/Pho85 complexes are bound and regulated by the CKI Pho81 [8]. Pho81 also binds to Pcl7/Pho85 and regulates its activity based on extracellular phosphate levels, as determined by the ability of immunoprecipitated complexes to phosphorylate Pho4 *in vitro* [26]. Assessing the relevance of this regulation will require identification of a bona fide *in vivo* substrate targeted by Pcl7. Since Pcl7 is regulated by both the cell cycle and phosphate levels and is involved in carbon source metabolism, its functions are likely complex. Strangely, Pcl6, the closest homolog of Pcl7, shares only the carbon source utilization phenotype with Pcl7 [26].

There are several genes in the yeast genome with significant homology to Pho81; it remains to be seen if any of these have a role in regulation of Pcl/Pho85 complexes. Such regulatory domains may be possible to identify given the recent definition of the minimum domain of Pho81 required for proper Pho80/Pho85 regulation [43]. It is entirely possible that some Pcl/Pho85 complexes are regulated by other means, including other CKIs unrelated to Pho81 or by phosphorylation of residues important for CDK catalytic activity.

## Conclusions

Conclusively demonstrating that a protein is an *in vivo* substrate of a Pho85 complex is not an easy task. Given that more than half of the proteins in the yeast genome contain at least one SP or TP dipeptide, the Pho85 phosphorylation site, the range of *in vitro* substrates of Pho85 kinases is likely to be quite large. The range of significant *in vivo* activities is likely much smaller. A strong indication that an *in vitro* substrate of a Pcl/Pho85 complex might have some *in vivo* relevance is a preference for phosphorylation by a specific Pcl/Pho85. At this point, seven of the ten Pcl/Pho85 complexes have been assayed for kinase activity, and all are capable of phosphorylating Pho4 *in vitro* [7, 10, 24-27], whereas only Pho80/Pho85 has any *in vivo* relevance for Pho4 regulation [7]. For example, Pcl10/Pho85 exhibits a 50-fold *in vitro* preference favoring its *in vivo* substrate Gsy2 over the unnatural substrate Pho4 [10]. The experiments that can conclusively demonstrate that an *in vitro* substrate is also an *in vivo* substrate, a tryptic phosphopeptide map of *in vivo* phosphorylated material and comprehensive Ser/Thr substitution mutation analysis, have been performed only with Pho4 thus far [7].

The remaining challenges in the study of the functions of Pcl/Pho85 complexes include understanding issues of specificity, redundancy, regulation, and identifying a theme that binds the functions discussed above together. All of these functions may be thematically related to extracellular stress, possibly the specific stress of starvation for an essential nutrient, such as phosphate or a carbon source. Currently, all of the known functions of Pho85 (summarized in Table 1) involve the kinase activity sending a

message that the current extracellular environment is satisfactory. Perhaps all of the functions of Pho85 will be constitutive in this manner, or perhaps some will require induction of Pho85 kinase activity by the presence of a particular activating stress signal. It is also clear from the existing data that many downstream effects of phosphorylation by Pho85 are possible. Such phosphorylation can regulate the subcellular localization, enzymatic activity, cofactor association, and stability of the targets (Figure 2). Two of the known targets of Pho85 are transcription factors, and, although the activities of Pho85 may have profound transcriptional consequences, there is no guarantee that such effects are direct.

In the future, proteomic approaches may prove fruitful for addressing the abundant unresolved issues with Pho85. *In vitro* kinase assays may enable the identification of novel substrates of Pcl/Pho85 complexes, as well as providing information about substrate specificity and its mechanisms. Eventually, we may be able to assign a function of Pho85 to every Pcl and correlate this assignment with a mechanistic understanding of the effects on the substrate. Furthermore, we hope to understand the regulation mechanism and identify the environmental activating signal for each Pcl/Pho85 complex. Such a comprehensive catalog of functions may reveal advantages of having Pho85 involved in multiple processes related to environmental stress.

FIGURE 2. Consequences of phosphorylation by Pho85. Four well-characterized substrates are shown, with their active condition shown on the top. Question marks indicate that Pcl1 has not been shown to be required for this activity *in vivo*. The degradation of the substrates is phosphorylation-dependent; Pho85 does not degrade these substrates directly.

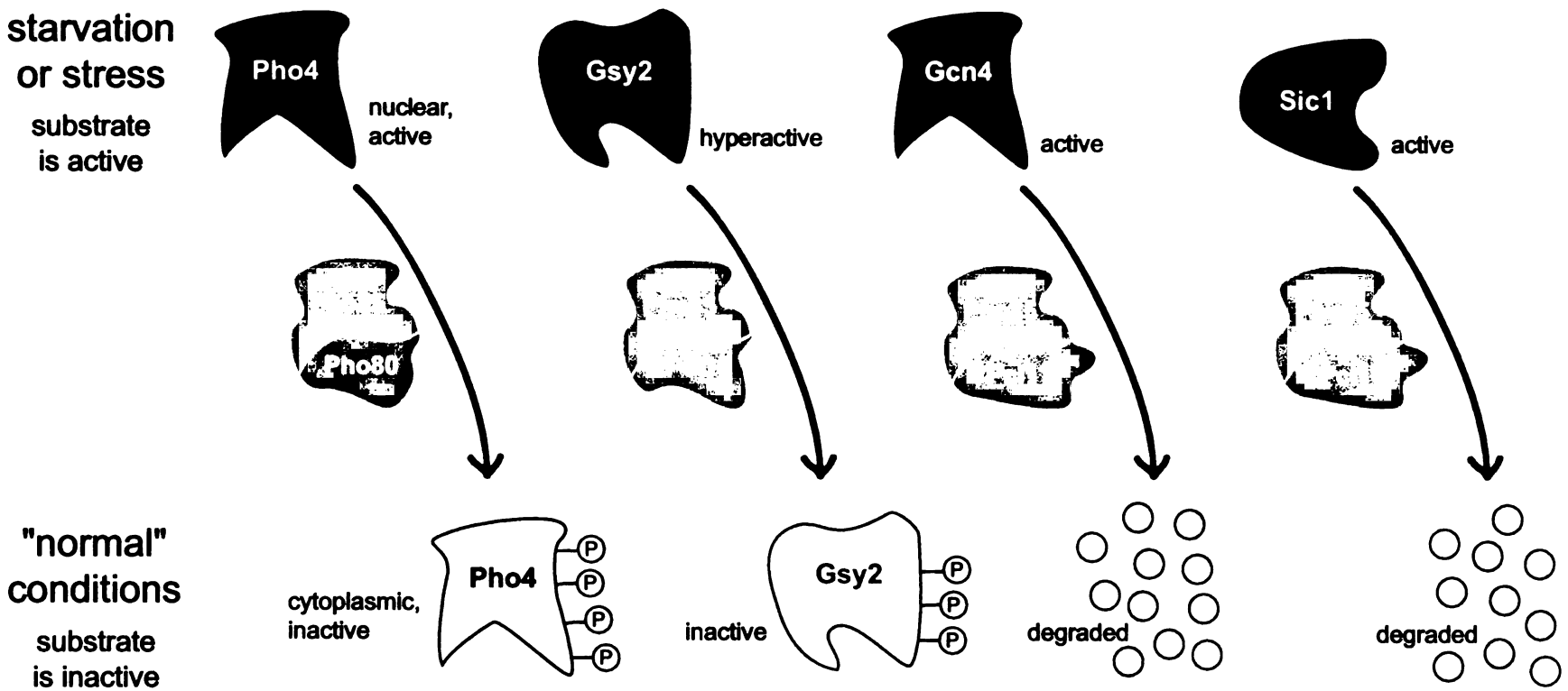

starvation
or stress
substrate
is active
Pho4
nuclear,
active
Gsy2
hyperactive
Gcn4
active
Sic1
active
Pho80
"normal"
conditions
substrate
is inactive
cytoplasmic,
inactive
Pho4
P
inactive
Gsy2
degraded
degraded

**Table 1. Pho85 cyclins and their functions.**

| Cyclin | Expression | Substrate(s) | Functions/Details |
|---|---|---|---|
| Pcl1 | Peaks in late G1 | Sic1 | Sic1 stability/Cell cycle progression? |
| | | Gcn4 | Gcn4 stability? |
| | | | Morphogenesis |
| Pcl2 | Peaks in late G1 | Rvs167 | Morphogenesis |
| | | | Sic1 stability/Cell cycle progression? |
| Pho80 | Constant | Pho4 | Pho4 localization and |
| | | | phosphate-dependent gene expression |
| Clg1 | Constant | | Morphogenesis |
| Pcl5 | Constant? | | Morphogenesis |
| Pcl6 | Constant | | Carbon source utilization |
| Pcl7 | Peaks in mid to | | Carbon source utilization |
| | late S | | Activity regulated by Pho81/Pi levels |
| Pcl8 | Constant | Gsy2 | Glycogen metabolism |
| Pcl9 | Peaks in late M/ | | Morphogenesis |
| | early G1 | | |
| Pcl10 | Constant | Gsy2 | Glycogen metabolism |

# CHAPTER 2

## Chemical inhibition of the Pho85 cyclin-dependent kinase reveals a role in the environmental stress response

## Credits

This work is the result of collaboration between our laboratory and the laboratories of Kevan Shokat and Joe DeRisi. I performed all the experiments described. Anthony Bishop and other members of the Shokat laboratory synthesized the inhibitors used. Joe DeRisi provided guidance for all aspects of the microarray experiments.

Biological Sciences – Genetics

# Chemical inhibition of the Pho85 cyclin-dependent kinase reveals a role in the environmental stress response

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Abbreviations: CDK, cyclin-dependent kinase; WT, wild type; GFP, green fluorescent protein; 1-Na PP1 (4-Amino-1-*tert*-butyl-3-(1'-naphthyl)pyrazolo[3,4-*d*]pyrimidine); ESR, environmental stress response.

**Abstract**

**In addition to its well-established role in responding to phosphate starvation, the cyclin-dependent kinase Pho85 has been implicated in a number of other physiological responses of the budding yeast *Saccharomyces cerevisiae*, including synthesis of glycogen. To comprehensively characterize the range of Pho85-dependent gene expression, we used a chemical genetic approach that enabled us to control Pho85 kinase activity with a cell-permeable inhibitor and whole genome transcript profiling. We found significant phenotypic differences between the rapid loss of activity caused by inhibition and the deletion of the genomic copy of *PHO85*. We demonstrate that Pho85 controls the expression of not only previously identified glycogen synthetic genes, but also a significant regulon of genes involved in the cellular response to environmental stress. In addition, we show that the effects of this inhibitor are both rapid and reversible, making it well suited to the study of the behavior of dynamic signaling pathways.**

The protein kinase is an evolutionarily well conserved, ubiquitous, and widely implemented enzymatic activity. Many signaling pathways within cells, from bacteria to mammals, utilize a protein kinase for some aspect of their function [44]. Protein kinases are well adapted to participate in diverse processes by virtue of the fact that they can modify their target proteins rapidly and in a reversible manner, thereby altering the function of the targets. Frequently, functional changes in the kinase are brought about by utilizing additional specificity factors.

The cyclin-dependent kinase (CDK) is a prototypical class of kinase that exemplifies how kinase activity can be specified to multiple and divergent processes [2]. CDK monomers depend on association with a cyclin subunit for stimulation of catalytic activity as well as generation of substrate specificity [2]. The CDK Cdc28, of the budding yeast *Saccharomyces cerevisiae*, illustrates this paradigm clearly. *CDC28* itself is expressed at a constant level throughout the cell cycle [45], yet it is also essential for orderly progression through multiple steps of the cell cycle that differ broadly in their molecular mechanisms. The timely activation of the kinase, as well as the conferment of substrate specificity relevant to each specific phase, is accomplished by a family of cyclin partners that are synthesized when their activities are required and degraded when their phase is completed [45].

We study the diversity of CDK-cyclin partners, functions, and substrates by using the nonessential yeast CDK, Pho85, which is involved in processing information about the nutritive environment of the cell [1, 5, 46, 47]. Pho85 associates with a family of 10 cyclins known as Pcls, which direct the CDK to different functions [1, 4, 48]. The best

studied of the functions of Pho85 is the response to phosphate starvation conferred by the cyclin Pho80 [49]. The Pho80/Pho85 kinase complex regulates the starvation response by controlling the localization and activity of the transcription factor Pho4 [9], which activates transcription of genes such as *PHO5* [50, 51]. More recent studies have identified Gsy2 as a direct target of Pcl8/Pho85 and Pcl10/Pho85 [4] and Sic1 as a direct target of Pcl1/Pho85 [52]. Yet, we still do not know which of the Pho85 cyclins or target proteins are responsible for the molecular functions that account for most of the *pho85*Δ phenotypes. The known phenotypes of the *pho85*Δ strain include: the constitutive expression of the secreted acid phosphatase *PHO5*, slow growth, poor growth on non-fermentable carbon sources, a $G_1$ delay, and morphogenetic defects [14, 47, 53]. Also, deletion of *PHO85* results in hyperaccumulation of glycogen [54, 55], as well as constitutive expression of the glycogen synthase *GSY2* [56] and UDP-glucose pyrophosphorylase *UGP1* [57].

The importance of protein kinases has spurred the development of numerous tools for their study. One recent development has been the creation of sensitized, yet functional, alleles of kinases that can be inhibited by small, cell-permeable drugs [58, 59]. This system is particularly useful because the hydrophobic residue that is mutated to sensitize the kinase is so highly conserved throughout the family that the technique can be applied to virtually any protein kinase [60, 61]. We have chosen this tool to study Pho85 so that the rapid loss-of-function phenotype may be compared to the genetic deletion phenotype. In the latter case, the gene product has been absent for many generations, which may lead to adaptation. We coupled this inhibitor technology with microarray analysis of global gene expression to comprehensively identify the

transcriptional consequences of any previously unidentified constitutive functions of Pho85.

**Materials and Methods**

*Plasmid and Strain Construction.* The F82G mutation was introduced into a *PHO85*-containing plasmid by site-directed mutagenesis using standard techniques [62]. For integration into yeast, the *PHO85* promoter and gene (for both the mutagenized and wild-type versions) were subcloned into pRS304 [63]. The integration was performed by cutting the resulting plasmids [wild type (WT), EB1379; F82G, EB1378] in the *TRP1* locus with *Mfe* I and transforming the linear fragment into yeast (EY0821 or EY0822) by standard techniques [64]. For the bacterial expression vector, the *PHO85* coding sequence was cloned into pQE-60 (Qiagen, Valencia, CA) containing the lacIQ gene (EB1164) as an *Nco* I fragment. The yeast strains EY0821 and EY0822 were manipulated by standard mating and sporulation techniques to obtain a strain that was K699 *MATa pho85*Δ::*LEU2 pho3*Δ *ADE2* and either did (EY0821) or did not (EY0822) contain *PHO4-GFP* (green fluorescent protein) integrated at the *PHO4* genomic locus. The *pho3*Δ allele was generated by unmarking a *LEU2*-marked disruption (generated with EB0888) at the genomic locus of *PHO3*, using a *LEU2* disruption vector containing the *URA3* gene flanked by hisG repeats (EB1005). The integrated *PHO4-GFP* allele was generated by standard pop-in, pop-out techniques [64]. Repair of *ADE2* and deletion of *PHO4* (for EY0837) were performed by using a PCR-based gene replacement system [65].

*Inhibitor Synthesis and Dilution.* The inhibitors used in this study were synthesized and handled as described [66].

*Liquid Phosphatase Assay.* Yeast were diluted from a saturated yeast extract/peptone/dextrose (YEPD) culture and grown overnight at 30°C in YEPD to an $OD_{600}$ of 0.3 to 0.8. Each culture was then split and treated with a 1000X 4-Amino-1-*tert*-butyl-3-(1'-naphthyl)pyrazolo[3,4-*d*]pyrimidine (1-Na PP1) solution or DMSO alone. The zero time point was assayed before treatment. Assays were performed essentially as described [51].

*Microscopy.* All images were captured using a BX60 microscope (Olympus) and a Sensys charge-coupled device camera (Photometrics, Tucson, AZ) using identical exposure settings, and were normalized to the same intensity range using IP Lab Spectrum (Scanalytics, Fairfax, VA). The yeast were grown with shaking in synthetic complete media, supplemented with dextrose (SD/complete), to mid-log phase at 30°C. For static microscopy, the cultures were treated with 1000X 1-Na PP1 or an equivalent volume of DMSO and shaken at 30°C for 15 minutes. For the perfusion chamber experiment, the yeast were adhered for 5 minutes in media to a cover slip [coated in 10 μg/ml ConA (Sigma) for 5 minutes, washed 3 times with water, and allowed to dry] in the perfusion chamber (Warner Instruments, Hamden, CT, RC-21B). The medium in the chamber was then switched to medium supplemented with 10 μM 1-Na PP1, and images were captured every 5 minutes until no further change in GFP localization was observed. The medium was then switched back to medium without 1-Na PP1, and images were captured every 5 minutes until no further change in GFP localization was observed.

*Protein Purification.* The purification of the recombinant kinase complexes and Pho4 were performed as described [49, 67]. The concentrations of protein solutions were determined by using calculated extinction coefficients and measured absorbance at 280 nm in 6 M guanidine hydrochloride [68, 69].

*Kinase Assays.* Kinase assays were performed essentially as described [67]. For the determination of $k_{cat}$ and $K_M$, cold ATP was included at 900 $\mu$M and [$\gamma$-$^{32}$P] ATP at 86 nM. The kinases were diluted to a final concentration of 100 pM, and Pho4 was titrated from 3 $\mu$M to 100 nM. The *in vitro* inhibition experiments to determine the $IC_{50}$ utilized [$\gamma$-$^{32}$P] ATP at a final concentration of 86 nM as the only source of ATP, allowing the measured $IC_{50}$ to serve as an approximation of the $K_I$ [70]. These reactions included the kinase complex at 100 pM (except in the case of Pcl7, where the final concentration was 10 nM), Pho4 at 3 $\mu$M, and a titration of 1-Na PP1 from 4 $\mu$M to 10 nM. All quantitation was performed with a Storm 860 PhosphorImager (Molecular Dynamics).

*Microarray Analysis.* Yeast were grown overnight at 30°C in SD/complete media to an $OD_{600}$ of 0.5. For 1-Na PP1 treatments, either 1000X 1-Na PP1 in DMSO or an equivalent volume of DMSO was added. For the 24-h treatment, the cells were grown to saturation twice before being diluted for growth to an $OD_{600}$ of 0.5, all while in the presence of the appropriate treatment. Cells were harvested by centrifugation, flash-frozen in liquid nitrogen, and stored at -20°C for up to 2 weeks. Total and poly-(A) RNA were isolated as described [71], and poly-(A) RNA was reverse-transcribed with StrataScript (Stratagene) incorporating amino-allyl dUTP (Sigma) at a ratio of 3:2 with dTTP. The resulting cDNAs were labeled by using monofunctional reactive Cy3 and

Cy5 dyes (Amersham Pharmacia) in the presence of sodium bicarbonate. The spot-printed microarrays were fabricated essentially as described [71, 72].

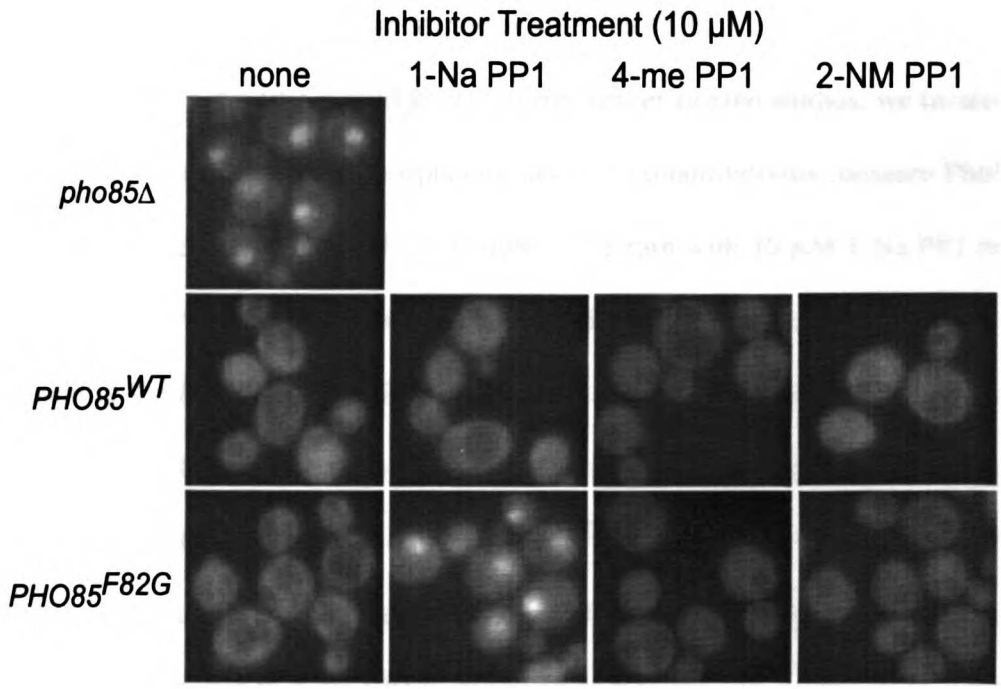
For inhibitor-treated cultures, the DMSO-treated sample (Cy3) was compared directly to the 1-Na PP1-treated sample (Cy5) by hybridization to the same microarray. In the case of other experiments, the Cy5 sample is listed second. The standard deviation from the mean expression ratio of 1 was calculated by using all of the individual gene expression measurements in a given experiment. This value provides an indication of the number and magnitude of changes occurring in that experiment. The analysis and presentation of the data was performed by using several software tools: Cluster and Treeview [73], AMAD (http://www.microarrays.org/software.html), Genepix Pro (Axon Instruments, Union City, CA), and Microsoft Excel (Microsoft).

## Results

**Construction of *PHO85* Allele and Selection of Inhibitor.** As stated above, we wanted to use previously developed chemical genetic methods to study Pho85 function [58, 59]. We mutated a conserved hydrophobic residue, Phe-82, in the ATP-binding pocket of Pho85 to Gly to render the kinase sensitive to appropriately derivatized inhibitors [66].

Monitoring the subcellular localization of Pho4-GFP fusion protein constitutes a simple and rapid method for assessing the activity of Pho80/Pho85 [9]. In conditions of high phosphate, Pho80/Pho85 phosphorylates Pho4, causing Pho4 to be localized to the cytoplasm. Low levels of extracellular phosphate or loss of Pho80/Pho85 activity cause Pho4 to move from the cytoplasm to the nucleus ([9]; Figure 3), where it is required for *PHO5* induction. We found that 1-Na PP1 is effective as an inhibitor of

FIGURE 3. Selection of inhibitor by measuring Pho4-GFP localization with static microscopy. The strains tested [EY0821 (*pho85*Δ), EY0825 (*PHO85*$^{WT}$), and EY0823 (*PHO85*$^{F82G}$)] were analyzed 15 minutes after treatment with inhibitor. A representative field of cells is shown for each condition. 2-NM PP1, 4-Amino-1-*tert*-butyl-3-(2'-naphthylmethyl)pyrazolo[3,4-*d*]pyrimidine); 4-me PP1, (4-Amino-1-*tert*-butyl-3-(1'-naphthyl-4'-methyl)pyrazolo[3,4-*d*]pyrimidine).


Inhibitor Treatment (10 μM)
none
1-Na PP1
4-me PP1
2-NM PP1
pho85Δ
PHO85WT
PHO85F82G

Pho80/Pho85$^{F82G}$ activity, whereas neither 4-Amino-1-*tert*-butyl-3-(2'-naphthylmethyl)pyrazolo[3,4-*d*]pyrimidine nor 4-Amino-1-*tert*-butyl-3-(1'-naphthyl-4'-methyl)pyrazolo[3,4-*d*]pyrimidine demonstrated any ability to affect the localization of Pho4 (Figure 3). When added to a strain with a WT version of Pho80/Pho85, none of the inhibitors tested showed any effect on Pho4-GFP localization (Figure 3).

**Determination of Effective Concentration of 1-Na PP1.** To determine what concentrations of 1-Na PP1 would be useful for further *in vivo* studies, we titrated the inhibitor and performed liquid phosphatase assays to quantitatively measure Pho5 activity. After 2-3 h, treatment of the *PHO85$^{F82G}$* strain with 10 $\mu$M 1-Na PP1 results in approximately 90% of the acid phosphatase activity of the *pho85*$\Delta$ strain (Figure 4). Treatment of the *PHO85$^{F82G}$* strain with 20 $\mu$M 1-Na PP1 does not result in a significant increase in acid phosphatase activity (data not shown), suggesting that the response to the inhibitor is saturated. As expected, the *PHO85$^{WT}$* strain does not demonstrate any sensitivity to 1-Na PP1 even at a concentration of 10 $\mu$M (Figure 4). An approximately half-maximal response to 1-Na PP1 is observed at 500 nM. These results suggest that the low micromolar range constitutes a useful concentration range for *in vivo* studies with 1-Na PP1.

**Determination of the Kinetics of Inhibition *in vivo*.** In order to learn how soon after treatment we could expect to see transcriptional effects *in vivo*, we monitored the localization of Pho4-GFP in cells immobilized in a perfusion chamber in which we could rapidly alter the extracellular concentration of inhibitor. Upon treatment with 10 $\mu$M 1-Na PP1, Pho4-GFP reaches its peak of nuclear localization between 5 and 10 minutes (Figure 5). The treatment can be extended for at least 1 hour, with no further change in

FIGURE 4. Analysis of Pho5 activity induced by inhibitor treatment using the liquid phosphatase assay. Units of activity were calculated by dividing the measured $OD_{420}$ by the $OD_{600}$ of the yeast suspension used in the assay and are displayed on the vertical axis. Time after inhibitor addition is shown on the horizontal axis. The strains and treatments shown are: EY0822 (*pho85*Δ) + DMSO (open squares); EY0824 ($PHO85^{F82G}$) + 10 μM 1-Na PP1 (filled squares), + 5 μM 1-Na PP1 (filled triangles), + 1 μM 1-Na PP1 (crosses), + 0.5 μM 1-Na PP1 (filled diamonds), or + DMSO (open diamonds); and EY0826 ($PHO85^{WT}$) + 10 μM 1-Na PP1 (open triangles). Each point shown is the mean of three independent experiments, and the error bars indicate two standard deviations.

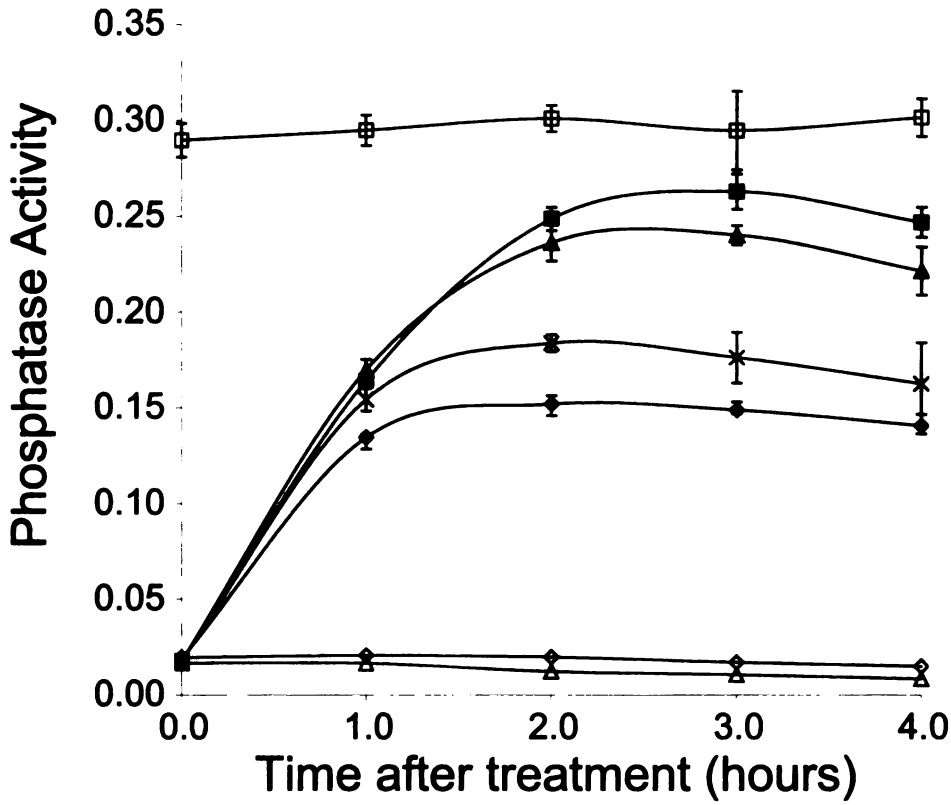
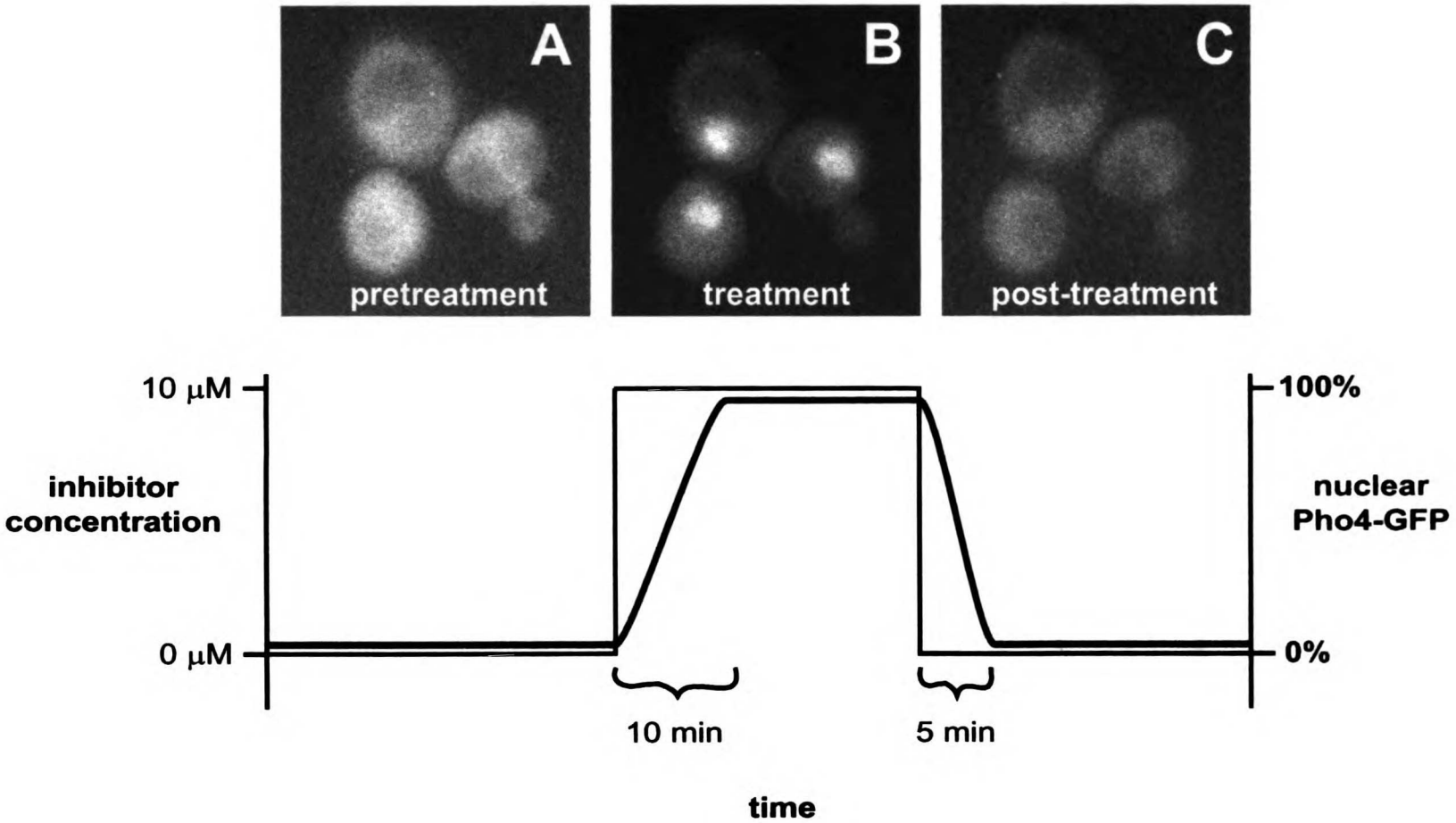

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Phosphatase Activity
0.0
1.0
2.0
3.0
4.0
Time after treatment (hours)

FIGURE 5. Perfusion chamber fluorescence microscopy of Pho4-GFP localization in response to treatment with 10 $\mu$M 1-Na PP1. A single representative field of cells is shown through the course of the experiment. (*A*) Pretreatment. (*B*) After 10 minutes in 10 $\mu$M 1-Na PP1. (*C*) Five minutes after removal of 1-Na PP1. (Magnification: 2000X.) The graph below the images approximates the indicated levels during the course of the experiment.


A
B
C
pretreatment
treatment
post-treatment
10 μM
0 μM
inhibitor concentration
100%
0%
nuclear Pho4-GFP
10 min
5 min
time

fluorescence distribution (data not shown). Similarly, upon removal of 1-Na PP1 by perfusing the chamber with fresh media without inhibitor, the Pho4-GFP fluorescence signal returns entirely to the cytoplasm with similar kinetics (5 minutes). These results suggest that changes in transcription may be observed minutes after raising or lowering the extracellular inhibitor concentration.

**Analysis of Kinetic Parameters and *in vitro* Inhibition of Pho85$^{F82G}$.** To determine whether the F82G mutation had significantly altered the enzymatic characteristics of the kinase, we determined the kinetic parameters of the WT and mutant alleles, using recombinant proteins expressed in *Escherichia coli* [49, 67]. Neither the $K_M$ nor the $k_{cat}$ of the Pho80/Pho85 complex for the substrate Pho4 (308 ± 55 nM, 12.3 ± 1.8 $s^{-1}$) is significantly altered by mutation of Phe82 (350 ± 33 nM, 13.2 ± 1.6 $s^{-1}$), indicating that this mutant allele should be capable of faithfully substituting for all *PHO85*$^{WT}$ functions *in vivo*.

We also determined the *in vitro* concentration at which 1-Na PP1 inhibits Pho85$^{F82G}$ to half of its maximal activity ($IC_{50}$). The $IC_{50}$ for Pho80/Pho85$^{F82G}$ was approximately 360 nM, as estimated by curve-fitting the data for the linear range (100 nM to 1 μM 1-Na PP1) of the experiment (data not shown). This value is approximately the same as determined by the liquid assay, further demonstrating that low micromolar concentrations of 1-Na PP1 have substantial effects on the activity of the sensitized kinase. Only at a concentration of 400 μM did 1-Na PP1 demonstrate any effect on Pho85$^{WT}$ (<20% inhibition; data not shown). The specific cyclin partner is not likely to contribute significantly to the efficacy of the inhibition by 1-Na PP1: the $IC_{50}$ for the

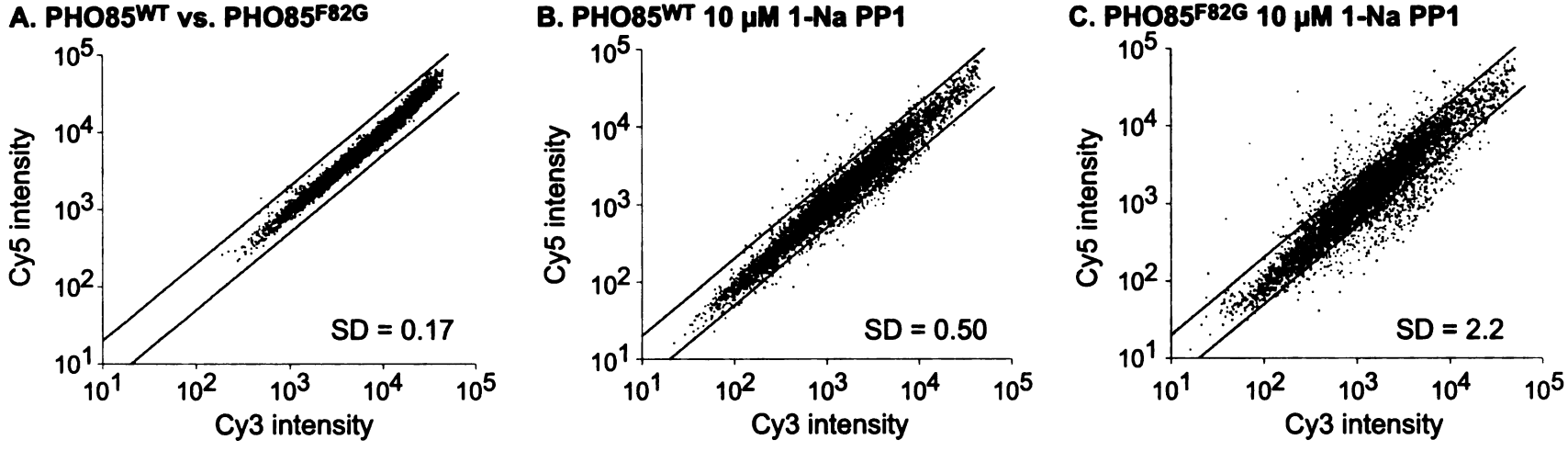
Pcl7/Pho85$^{F82G}$ complex is the same as the $IC_{50}$ for the Pho80/Pho85$^{F82G}$ complex (data not shown).

**Microarray analyses.** Having determined several parameters necessary for the effective use of 1-Na PP1 with this sensitized kinase, we used spotted cDNA microarrays [71, 72] to assess the effects of inhibitor treatment on gene expression. The rapid loss of Pho85 kinase activity may permit the identification of physiological functions of Pho85 that are not revealed in the transcriptional profile of the *pho85*Δ strain.

As a first step, we compared the *PHO85*$^{WT}$ strain to the *PHO85*$^{F82G}$ strain in the absence of any inhibitor treatment. The mutation of Phe82 to Gly has a negligible effect on the expression profile of the yeast, as demonstrated by a standard deviation of 0.17 from the mean ratio of 1 for this experiment (Figure 6). Only 3 genes (*HSP12*, *CHA4*, *YIL102C*) out of more than 6000 change expression by more than 2-fold (Table 2, which is published as supporting information on the PNAS web site, www.pnas.org).

We performed several inhibitor treatments on various strains to identify genes whose regulation is controlled by Pho85. To maximize the probability of identifying a direct transcriptional effect of loss of Pho85 function, we analyzed samples treated for only 10 minutes with 1-Na PP1. We hybridized the treated sample to the same array as a sample treated with DMSO alone to control for the effects of the DMSO solvent in the inhibitor solution, revealing only the inhibitor-specific changes. When a *PHO85*$^{WT}$ strain was treated in this manner with 10 $\mu$M 1-Na PP1, we observed a standard deviation of 0.50 and a 2-fold change in expression for 295 genes (Figure 6), indicating that the inhibitor treatment has some Pho85-independent effects. When the *PHO85*$^{F82G}$ strain is treated with 10 $\mu$M 1-Na PP1, the effects are both more numerous and of a larger

FIGURE 6. Scatter plot showing the effects of the F82G mutation and inhibitor treatment on gene expression. Each point represents a single gene, with the net intensity in the 635-nm channel (Cy5) plotted on the *y* axis and the net intensity in the 532-nm channel (Cy3) plotted on the *x* axis. Two-fold changes, both up and down, from the median intensity of 1 are shown as gray diagonal lines. The standard deviation of the gene expression ratios for each experiment (as described in *Materials and Methods*) is indicated in the graph area. Experiment conditions are described in *Materials and Methods*. The Cy5-labeled sample is listed second. (*A*) EY0826 ($PHO85^{WT}$) versus EY0824 ($PHO85^{F82G}$), both strains untreated (no DMSO, no 1-Na PP1). (*B*) EY0826 ($PHO85^{WT}$) treated with 0.1% DMSO for 10 min versus EY0826 ($PHO85^{WT}$) treated with 10 $\mu$M 1-Na PP1 for 10 min. (*C*) EY0824 ($PHO85^{F82G}$) treated with 0.1% DMSO for 10 min versus EY0824 ($PHO85^{F82G}$) treated with 10 $\mu$M 1-Na PP1 for 10 min.


A. PHO85WT vs. PHO85F82G
B. PHO85WT 10 µM 1-Na PP1
C. PHO85F82G 10 µM 1-Na PP1
Cy5 intensity
Cy3 intensity
SD = 0.17
SD = 0.50
SD = 2.2
10^1
10^2
10^3
10^4
10^5

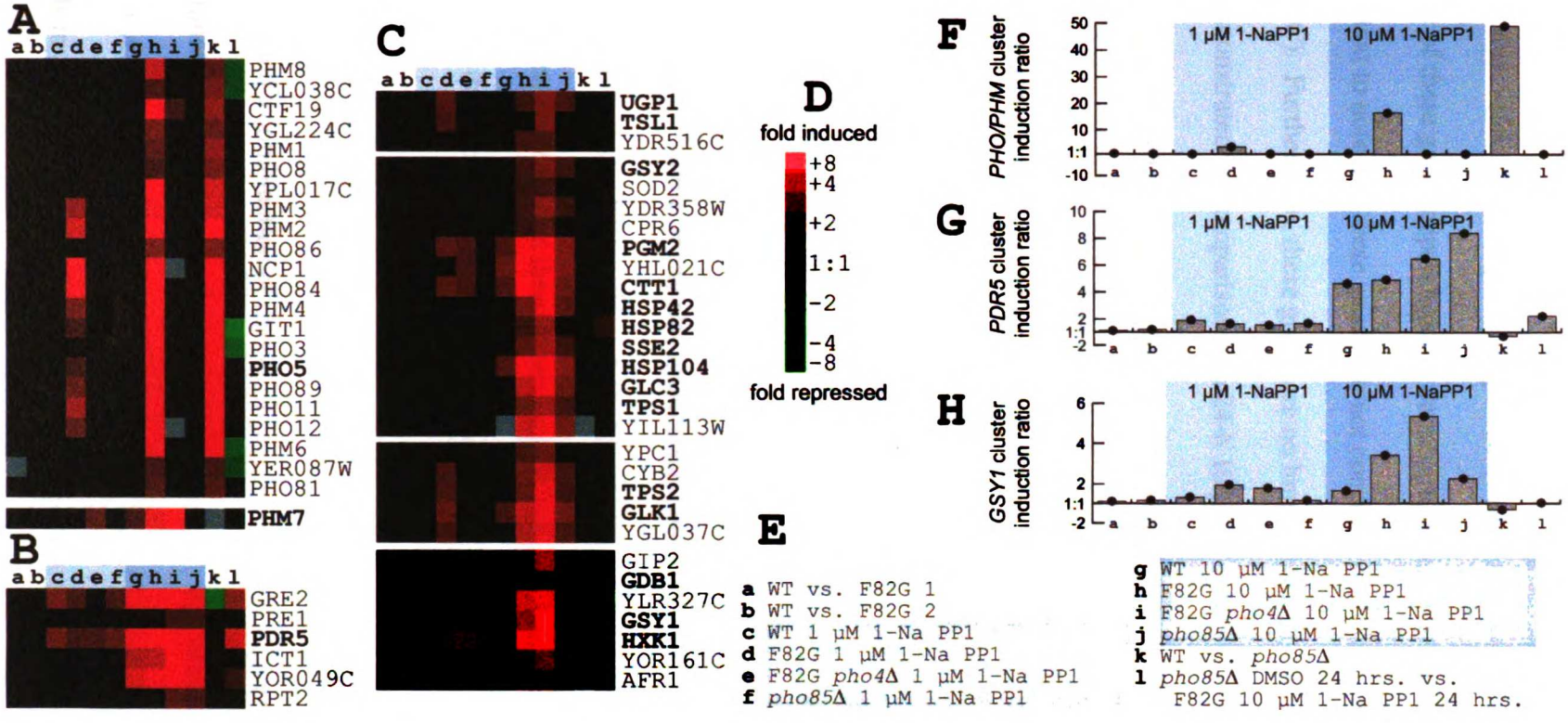
magnitude. A 2-fold change in expression is observed for 853 genes, and the standard deviation is 2.2 (Figure 6). Of these, 332 genes have a greater than 2-fold induction, and 521 genes have a greater than 2-fold repression. We focused our analysis on genes that are induced rather than repressed, because of potential oversights caused by our inability to detect decreases in expression of RNAs with long half-lives.

A selection of induced genes grouped by cluster analysis [73] is shown in Figure 7 (the complete dataset is available in Table 2). The first category contains essentially every known phosphate-responsive gene [74], with the exception of *PHM5* and *PHM7* (Figure 7A). This category contains *PHO4*-dependent genes that are induced by treatment of the $PHO85^{F82G}$ strain with 1-Na PP1 and by deletion of *PHO85* from the genome, but not by treatment of the $PHO85^{WT}$ strain with 1-Na PP1.

The second category includes genes that are induced solely by the presence of the inhibitor and do not depend on the state of *PHO85* (Figure 7B). The prototypical member of this category is *PDR5*, which encodes a small-molecule efflux pump [75]. Other members of this cluster, including *GRE2* and *ICT1*, also have been identified as being coregulated with *PDR5* in a study of *PDR* gene deletions [76]. The induction of these genes constitutes a small-molecule response that depends on the concentration of inhibitor used, but is independent of Pho85 activity (Figure 7B).

We also have identified another large category of genes (Figure 7C) that are induced by 1-Na PP1 treatment, including genes involved in glycogen synthesis (*UGP1*, *GSY2*, *GSY1*, *GLC3*, *GDB1*, *PGM2*), trehalose synthesis (*TPS1*, *TPS2*, *TSL1*), glycolysis (*GLK1*, *HXK1*), oxidoreductive stress (*CTT1*, *GPX1*, *GTT1*), protein folding (*HSP26*, *HSP42*, *HSP104*, *SSE2*), and protein degradation (*UBC5*, *UBC8*, *LAP4*, *PAI3*, *AUT7*,

FIGURE 7. Summary of microarray results. (*A-C*) Clusters of genes from larger tree containing all genes with at least one change greater than 2-fold in at least one of the experiments shown (a-l; see legend for *E*). Genes of interest are indicated in bold. Correlation for each node is indicated below in parentheses. (*A*) *PHO* and *PHM* genes (0.86) and *PHM7*. The *PHO3* induction observed is likely caused by hybridization of *PHO5* cDNA (87% identity), since *PHO3* is deleted in the strains used for these studies. (*B*) *PDR5* cluster (0.98). (*C*) Genes induced by 1-Na PP1 treatment (0.91; individual nodes all greater than 0.96). (*D*) Color key for clusters. Induction value is shown to the right of the corresponding square. A gray square indicates a missing data point. (*E*) Legend of the experiments shown. Experimental design is described in Materials and Methods. Experiments involving treatments with 1-Na PP1 are indicated by a light blue (1 $\mu$M) or darker blue (10 $\mu$M) shaded box. (*F-H*) Graphs of average induction ratios for the clusters shown in *A-C*, respectively. Treatment with 1-Na PP1 is indicated as in *E*.


A
a b c d e f g h i j k l
PHM8
YCL038C
CTF19
YGL224C
PHM1
PHO8
YPL017C
PHM3
PHM2
PHO86
NCP1
PHO84
PHM4
GIT1
PHO3
PHO5
PHO89
PHO11
PHO12
PHM6
YER087W
PHO81
PHM7
B
a b c d e f g h i j k l
GRE2
PRE1
PDR5
ICT1
YOR049C
RPT2
C
a b c d e f g h i j k l
UGP1
TSL1
YDR516C
GSY2
SOD2
YDR358W
CPR6
PGM2
YHL021C
CTT1
HSP42
HSP82
SSE2
HSP104
GLC3
TPS1
YIL113W
YPC1
CYB2
TPS2
GLK1
YGL037C
GIP2
GDB1
YLR327C
GSY1
HXK1
YOR161C
AFR1
D
fold induced
+8
+4
+2
1:1
-2
-4
-8
fold repressed
E
a WT vs. F82G 1
b WT vs. F82G 2
c WT 1 µM 1-Na PP1
d F82G 1 µM 1-Na PP1
e F82G pho4Δ 1 µM 1-Na PP1
f pho85Δ 1 µM 1-Na PP1
g WT 10 µM 1-Na PP1
h F82G 10 µM 1-Na PP1
i F82G pho4Δ 10 µM 1-Na PP1
j pho85Δ 10 µM 1-Na PP1
k WT vs. pho85Δ
l pho85Δ DMSO 24 hrs. vs.
F82G 10 µM 1-Na PP1 24 hrs.
F
PHO/PHM cluster induction ratio
1 µM 1-NaPP1
10 µM 1-NaPP1
50
40
30
20
10
1:1
-10
a b c d e f g h i j k l
G
PDR5 cluster induction ratio
1 µM 1-NaPP1
10 µM 1-NaPP1
10
8
6
4
2
1:1
-2
a b c d e f g h i j k l
H
GSY1 cluster induction ratio
1 µM 1-NaPP1
10 µM 1-NaPP1
6
4
2
1:1
-2
a b c d e f g h i j k l

*APG1*). Although previous work has implicated Pho85 in the transcriptional control of glycogen metabolism [56, 57], this group of genes represents a substantial expansion of that role. None of these genes depend on the transcription factor Pho4 for their induction. It is also important to note that these genes are not constitutively induced in the *pho85*Δ strain (Figure 7C). Furthermore, these genes are no longer induced after 24 hours in inhibitor, as demonstrated by comparison of a 24-h 10 μM 1-Na PP1 treatment of the $PHO85^{F82G}$ strain with a 24-h DMSO treatment of the *pho85*Δ strain (Figure 7). These results indicate that the role *PHO85* plays in the induction of these genes is transient and only exposed immediately after loss of kinase activity, or can be substituted for in the case of its prolonged absence. A larger cluster of 258 genes, with a correlation coefficient of 0.91, relates the clusters representing this category; other genes with similar patterns are not shown. Although the genes in Figure 7C do show significant induction by treatment with both 1 μM and 10 μM concentrations of 1-Na PP1 in the presence of the sensitized $PHO85^{F82G}$ allele, many also show induction to a lesser, but still significant, degree in both the $PHO85^{WT}$ and *pho85*Δ strains when treated with 10 μM 1-Na PP1. This pattern suggests that these genes largely, but not exclusively, depend on Pho85 activity, and that the inhibitor treatment causes some Pho85-independent effects.

**Discussion**

We have used a combination of chemical genetics and whole genome expression profiling to comprehensively characterize the rapid loss of function of the non-essential Pho85 kinase in yeast. The allele of *PHO85* we constructed is functional (Figures 3 and 4) and responds in a dose-dependent fashion to treatment with inhibitor (Figure 4).

Although this general approach has been utilized previously to study an assortment of kinases [59, 70, 77, 78], we were able to use our molecular understanding of Pho85 function in the *PHO* pathway to gain additional information about the characteristics of the inhibitor and the inhibition. For example, we were able to demonstrate that the effects of 1-Na PP1 on Pho80/Pho85 kinase activity *in vivo*, as measured by Pho4 localization, are complete fewer than 10 minutes after addition or removal of the inhibitor (Figure 5). This combination of rapidity and reversibility makes this inhibitor-based methodology well suited to studying the dynamic behavior of signaling pathways in general.

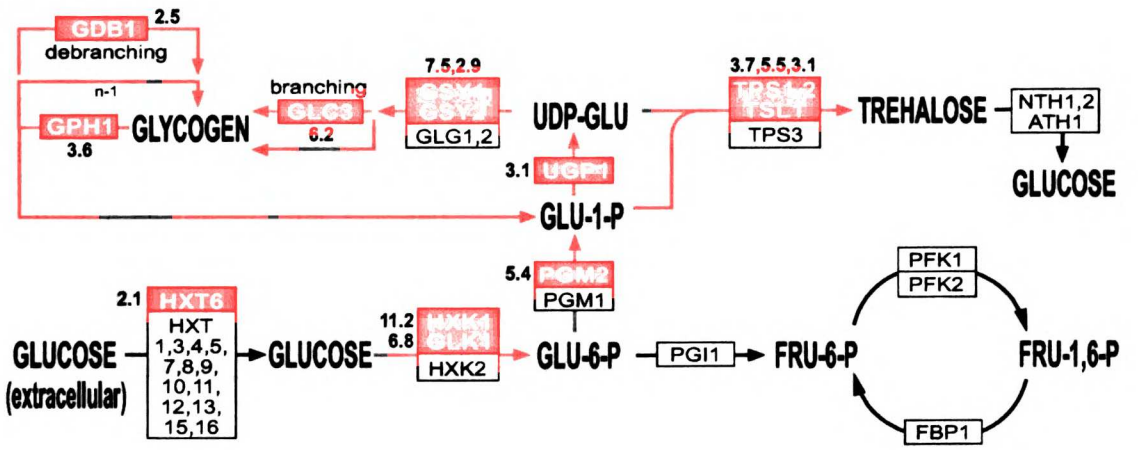
Evaluating the *in vivo* effects of the inhibition using a quantitative biochemical assay rather than a qualitative assessment of microscopic data permitted us to compare the effectiveness of the inhibition *in vitro* to that observed *in vivo*. Surprisingly, we found the $IC_{50}$ for both conditions to be approximately the same: 500 nM *in vivo* (Figure 4) and 360 nM *in vitro*. Previously, inhibitor studies of other kinases had found that a much lower *in vitro* $IC_{50}$ (in the low nanomolar range) corresponded to an effective *in vivo* concentration that was much higher (in the low micromolar range) [59, 77]. This result indicates that the inhibition of $Pho85^{F82G}$ by 1-Na PP1 is not exclusively competitive, as it is unaffected by ATP concentration.

We were able to confirm the effectiveness of the inhibition by identifying all of the genes in the *PHO* regulon (Figure 7A). We also identified some novel features of *PHM* gene regulation. First, we demonstrated that *PHM1-4*, *PHM6*, and *PHM8* all depend on *PHO4* for their transcriptional induction (Figure 7A). Previous work had demonstrated only that these genes had putative Pho4 binding sites in their promoter

regions [74]. Second, *PHM5* is not induced at all in any of the treatment conditions, including our comparison of *PHO85* and *pho85*Δ strains (data not shown). This result stands in contrast to what had been observed previously, but may make some sense in light of the fact that a *phm5*Δ strain has a polyphosphate hyperaccumulation phenotype, not a polyphosphate hypoaccumulation phenotype like that of other *PHM* mutant strains [74]. Third, *PHM7* is the only one of the induced *PHM* genes that does not depend on *PHO4* for its induction (Figure 7A). Most likely, the previously identified imperfect Pho4 consensus binding sites in the regulatory regions of *PHM5* and *PHM7* [74] do not function as Pho4 binding sites *in vivo*.

In this study, we have found a Pho85-dependent gene expression pattern for 250 genes not previously known to be Pho85-dependent. Deletion of *PHO85* is not sufficient to induce this regulon, but treatment of the appropriately sensitized strain with the inhibitor results in a robust induction of several genes (Figure 7C). Twelve of these genes have a role in reserve carbohydrate metabolism: *GSY1*, *GSY2*, *GLC3*, *GDB1*, *GPH1*, *UGP1*, *GLK1*, *HXK1*, *PGM2*, *TPS1*, *TPS2*, and *TSL1* (Figures 7C and 8) [79-88]. Previous work has shown *GSY2* and *UGP1* to be inducible by deletion of *PHO85* in other strain backgrounds [56, 57]. Also, a subset of the genes described above (*GLC3*, *GSY1*, *TSL1*, *TPS2*, *GLK1*, and *PGM2*) appear in a previously reported set of experiments that induce the *PHO* pathway, including phosphate starvation, deletions of *PHO80* and *PHO85*, and constitutive alleles of *PHO4* and *PHO81* [74]; however, none of these genes appears to be significantly induced by deletion of *PHO85* in our strain background (Figure 7C).

FIGURE 8. Induction of genes involved in reserve carbohydrate metabolism by chemical inhibition of Pho85$^{F82G}$. The genes encoding the enzymes for the metabolic processes are shown in boxes. Genes induced 2-fold or greater by chemical inhibition of Pho85 are shown in red boxes with the maximal inhibitor-induced fold induction shown adjacent to the box. Genes shown in white boxes did not have a greater than two-fold change in expression level.


GDB1
2.5
debranching
n-1
GPH1
3.6
GLYCOGEN
branching
GLC3
6.2
7.5,2.9
GSY1
GSY2
GLG1,2
UDP-GLU
3.7,5.5,3.1
TPS1,2
TSL1
TPS3
TREHALOSE
NTH1,2
ATH1
GLUCOSE
3.1
UGP1
GLU-1-P
5.4
PGM2
PGM1
2.1
HXT6
HXT
1,3,4,5,
7,8,9,
10,11,
12,13,
15,16
GLUCOSE
(extracellular)
11.2
6.8
HXK1
GLK1
HXK2
GLUCOSE
GLU-6-P
PGI1
FRU-6-P
PFK1
PFK2
FRU-1,6-P
FBP1

One possible interpretation of these changes in gene expression is that Pho85 plays an expanded role in regulating the expression of genes involved in reserve carbohydrate metabolism, in addition to its role in regulating the transcription of *GSY2* and *UGP1*. Transcription of these reserve carbohydrate metabolism genes also is induced by the diauxic shift, when extracellular glucose concentrations become limiting [71]. Low glucose concentration also causes transcriptional induction of genes involved in gluconeogenesis and the tricarboxylic acid (TCA) cycle and a repression of genes involved in glycolysis. We do not observe any significant change in expression of these genes in our experiments with the inhibitor and the sensitized kinase. This result suggests that the chemical inhibition of Pho85 does not cause the yeast to starve for glucose. If they do starve for glucose, they must be rendered incapable of inducing gluconeogenesis and the TCA cycle by the inhibition of Pho85.

A second possible interpretation of the induction of the reserve carbohydrate metabolism genes also takes into account the remaining 240 genes that have similar expression profiles. A number of these genes are involved in responses to cellular stress of various kinds, including antioxidants, redoxins, *HSP* chaperones, and factors involved in protein degradation and vacuolar function. Recently, whole genome expression profiling experiments have defined an environmental stress response (ESR) comprised of genes whose expression is altered as a general response to a transition to suboptimal environmental conditions [89, 90]. There are many similarities between the genes induced by chemical inhibition of Pho85 and the ESR genes. Of the 258 genes identified by our study with gene expression profiles similar to those in Figure 7C, 46% of these

also can be found in the 283 genes that are induced as part of the ESR [89]. Additionally, 50% of the characterized genes induced when the ESR is activated [89] are also activated by chemical inhibition of Pho85 (data not shown). Furthermore, our data shares with the ESR the same strong preference for the induction of particular isozymes; for example, *HXK1* is induced when the ESR is activated, whereas *HXK2* is not. This is true for *HXK1*, *PGM2*, *GPM2*, *GPD1*, *GTT1*, *GPX1*, *CTT1*, and *TRX2* in both ESR-inducing conditions [89] and chemical inhibition of Pho85 (data not shown).

The similarity between these responses, with respect to both the types of genes induced as well as the preference for particular isozymes, suggests that chemical inhibition of Pho85 results in activation of the ESR. Pho85 could be an important component of the signal transduction machinery that regulates the ESR genes in response to adverse changes in the extracellular environment. Alternatively, loss of Pho85 function could result in changes in cellular physiology that induces the ESR indirectly. We cannot, at present, distinguish between these two possibilities. In either case, we do know that the activation of these ESR genes is transient or can be adapted to: after 24 hours in the presence of inhibitor, the induction of these genes is no longer observed (Figure 7C). In our strain, this adaptation correlates with inhibitor-induced accumulation of glycogen in the $PHO85^{F82G}$ strain. Logarithmically growing cultures accumulate significant quantities of glycogen if Pho85 has been chemically inhibited for 24 hours, but no accumulation is observed after 10 minutes of chemical inhibition (data not shown). The slow growth phenotype and inability to grow on non-fermentable carbon sources demonstrated by *pho85*Δ strains might be explained by improper regulation of vital

metabolic responses altered by activation of the ESR, or the adaptive state, including the accumulation of glycogen, that corrects for the misregulation.

Future work should uncover the Pcl cyclin, the transcription factor or factors, and DNA elements that are responsible for directing Pho85-dependent activation of the ESR. Further analysis may identify other Pho85-regulated pathways through further study of the role of Pho85 in responding to various environmental conditions.

**Acknowledgements**

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# CHAPTER 3

## Involvement of Pho85 in the transcriptional response to DNA damage

## Introduction

A unicellular organism must faithfully maintain its genome to evolve and improve its fitness. The genome, like the cell, is under constant stress, both from the act of replication each cell cycle and in the form of induced damage, which is frequently the result of adverse environmental conditions. Many chemicals and forms of radiative energy can create information-altering or information-destroying lesions in the chemical structure of DNA [91]. If the cell is to minimize the rate of mutation, these lesions must be identified and removed or repaired. The potential for any single lesion to be lethal has led the cell to develop many important responses to damage, including both specific responses for a particular type of damage and general responses activated by many kinds of damage [92, 93].

The most important goals of a general response to DNA damage are to provide time and raw materials for the repair processes to function. Providing time, in the form of a delayed or arrested cell cycle, prevents the attempted replication of damaged DNA. If replication proceeds within a damaged region of DNA, the mutation may be permanently fixed in the genome, resulting in segregation of a flawed or incomplete genome to the daughter cells. Providing raw materials ensures that the repair effort the cell undertakes has sufficient access to deoxyribonucleotide triphosphates (dNTPs) and repair enzymes to resynthesize the damaged regions of DNA. These responses are frequently referred to as the cell cycle checkpoint and transcriptional responses to DNA damage.

The transcriptional response to DNA damage was initially appreciated as increased expression of several DNA repair enzymes and the genes encoding subunits of

ribonucleotide reductase in response to DNA-damaging treatments with chemicals or UV radiation [94-99]. Ribonucleotide reductase is the rate-limiting enzyme in the synthesis of dNTPs, the raw material from which the genome is synthesized. It is now appreciated that there are a large number of other genes induced by DNA-damaging treatments [100]. Although some of these induced genes are specific to the type of damage that occurs and others are induced as a general response to stress [89, 90], the RNR subunits are induced as part of this general response.

The minimal expression of *RNR3* in the absence of damage and its robust expression in the presence of damage made this gene an ideal reporter for fruitful genetic selections to identify the factors required for this damage-induced expression. Two categories of mutants were identified using complementary selections for expression of *URA3* under the control of the *RNR3* promoter. The first class of mutants isolated was those with Constitutive *RNR3* Transcription, or *CRT* mutants [101]. These mutants activated transcription from the *RNR3* promoter in both the presence and absence of replicational stress induced by hydroxyurea treatment. *CRT1* encodes a transcriptional repressor that becomes phosphorylated upon DNA damage. *CRT4* and *CRT8* encode the previously characterized transcriptional corepressors Tup1 and Ssn6, respectively. Crt1 binds to Ssn6 and Tup1 *in vitro*. Several other *CRT* mutants (including *rnr1*, *rnr2*, and *rnr4*) that cause constitutive replicational stress and are not involved in the signal transduction were identified. Conversely, the Damage Uninducible, or *DUN*, mutants fail to express the *RNR3* gene under either normal or damaging conditions [102]. Although several *CRT* and *DUN* mutants were identified, only one *DUN* mutant has been cloned and extensively characterized [102]. *DUN1* encodes a protein kinase that is activated

upon DNA damage [102]. Activation of Dun1 kinase activity, like phosphorylation of Crt1, is dependent on *MEC1* and *RAD53*, which encode two essential protein kinases required for transduction of DNA damage signals [92, 103-105].

Although the functions of Dun1 and Crt1 are well characterized and their location in the signal transduction pathway well established, the mechanisms of their regulation have proven more elusive. Genetic and molecular biological experiments have placed these factors downstream of the signaling cascade involving Mec1 and Rad53. *CRT1* is epistatic to *DUN1*, indicating that Crt1 functions below Dun1 in the pathway [106]. This order makes sense for a transcriptional response, given that Dun1 is a protein kinase and Crt1 is a transcription factor regulated by phosphorylation [102, 106]. These results are consistent with the suggestion that Dun1 acts directly on Crt1, but no evidence for this regulation has been presented. The effect of the phosphorylation on the biochemical activities of Crt1 is also unknown. Under conditions of damage, Crt1 is no longer bound to the promoters of the genes it regulates [106]. Whether Crt1 phosphorylation causes changes in its DNA binding affinity, association with Ssn6 or Tup1, or subcellular localization is unknown.

We became interested in elucidating the molecular mechanisms by which this pathway acts, in particular, on a role for the CDK Pho85 in expression of *RNR3* in response to DNA damage. Pho85 has many roles in responding to cellular stress (reviewed in [107]). One phenotype of the *pho85Δ* strain that has been previously identified is hypersensitivity to hydroxyurea, suggesting that these cells are less proficient at responding to DNA damage, including replicational stress [20]. We have characterized the role of Pho85 in activating transcription of *RNR3* in response to DNA damage.

**Materials and Methods**

*Strains and Plasmids*

The plasmid pZZ2 (RNR3-lacZ; EB1056) [101] was a gift from the lab of Sandy Johnson. The plasmid pMH190 (pGAL-3xmyc-CRT1; EB1381) [106] was a gift from Steve Elledge. The Dun1-containing plasmids were cloned from a PCR product amplified from a genomic clone of the *DUN1* genomic region (ATCC #70949). Sites for cloning in epitope tags were generated by standard site-directed mutagenesis [62]. The *crt1*Δ and *dun1*Δ strains used in this study were constructed using disruption constructs (EB0925 and EB1003, respectively) created using a fusion PCR method. Briefly, approximately 500 bp of sequence upstream of the ATG and downstream of the stop codon for each gene were separately amplified by PCR. The amplifying primers were designed to produce an identical 30 bp region containing a unique restriction site in the 3' end of the upstream sequence and the 5' end of the downstream sequence. These products were then used as a template for amplification by the distal primers to produce a fusion of the two regions. The unique restriction site was then be used for the insertion of selectable markers. A linear fragment containing the upstream region, the selectable marker, and the downstream region was transformed into yeast by standard techniques to integrate the disruption construct into the genome [64]. The *pcl*Δ strains were constructed using PCR-based methods [65]. The *pho85*Δ strain (EY0140) comes from the O'Shea lab strain collection; the *pho85*Δ strain (MY136) comes from the strain collection of Marc Lenburg. The inhibitor sensitive strain used for the Crt1

phosphorylation experiment and its isogenic wild type strain are described in Chapter 1 [41].

*RNR3-lacZ Assay*

Strains containing plasmid pZZ2 (EB1056) were grown to an $OD_{600}$ of approximately 0.4 in synthetic dropout media supplemented with dextrose (SD) lacking uracil to select for the plasmid. The cultures were then split and treated with ¼ volume of media either with or without 0.1% MMS (final concentration of 0.02%) and incubated with shaking at 30°C for 3 to 4 h. Generally, the samples without MMS treatment were incubated for 1 h less than the samples with MMS treatment to allow for the longer assay time required for the low expression observed in these samples. A 5 ml aliquot of cells was then pelleted in centrifuge and resuspended in 2 ml of Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, pH 7.0) supplemented with 2.7 µl/ml β-mercaptoethanol (β-ME) (50 mM final concentration). A 100 ml aliquot was used to measure the $OD_{600}$ of the cells suspension and the assay was performed on a 1 ml aliquot. Cells were lysed by addition 100 µl of $CHCl_3$ and 50 µl of 0.1% SDS, followed by vortexing for 20 sec. The samples were then pre-incubated at 30°C for 10 min before starting the assay by addition of 200 µl ONPG (4 mg/ml in 0.1 M $K_3PO_4$, pH 7.0). The reactions were run until sufficient yellow color developed to give optimal signal at $OD_{420}$, generally between 4 and 90 min, and stopped by addition of 500 µl 1 M $Na_2CO_3$. The cellular debris was pelleted in a microcentrifuge for 20 sec and the $OD_{420}$ and $OD_{550}$ of the supernatant were measured. Units of activity were calculated by the following formula (with time expressed in minutes):

$U = (1000 * [(OD_{420}) - (1.75 * OD_{550})]) / [(time) * (OD_{600})]$

*ZZ-Dun1 Kinase Assay*

Yeast strain MY136 (*pho85Δ::HIS3*) was transformed with plasmids containing *DUN1* under the control of the *ADH1* promoter either with (EB1408) or without (EB1407) an N-terminal ZZ tag. These cells also contained pRS316-*PHO85*-myc (EB0005) or empty vector (EB0009). The cultures were grown in SD/-Leu-Ura to an $OD_{600}$ of approximately 0.5, when they were pelleted by centrifugation and resuspended in fresh media with or without 0.1% MMS. The non-MMS-treated cultures were shaken for 1.5 h at 30°C and then were pelleted by centrifugation and frozen in liquid $N_2$. The MMS-treated cultures were shaken for 3 h at 30°C and harvested as above. Cell extracts were prepared by shaking with glass beads in Buffer A (25 mM HEPES pH7.5, 5 mM $MgCl_2$, 10% glycerol, 50 mM KCl, 0.1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine, 0.1 mM $Na_3VO_4$, 30 mM NaF, 1 μg/ml leupeptin, 1 μg/ml pepstatin) essentially as described [103]. The concentration of the extracts was determined by using Bradford assay reagent (Biorad). For each immunoprecipitation (IP), 1 mg extract was diluted to 250 μl in IgG buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween-20, 5 mM $MgCl_2$, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM $Na_3VO_4$, 30 mM NaF, 1 mM PMSF, 2 mM benzamidine, 2.5 mM β-ME) and incubated with 20 μl of a 50% IgG Sepharose bead slurry (washed 2x with IgG buffer, 1x with 0.5M $CH_3COOH$ (pH 3.4), and 3x with IgG, and then resuspended in an equal volume of IgG buffer) for 1 h at 4°C. The success of the IP was evaluated by western blot of the load and flow-through for each sample using rabbit serum (diluted 1:1000) to detect the ZZ tag. The beads were

washed for 3 times for 5 min with HSB (12 mM HEPES pH 7.9, 500 mM NaCl, 2 mM EDTA (pH 8.0), 0.01% Tween-20) and 2 times with kinase buffer (KB) (50 mM Tris pH 7.5, 10 mM $MgCl_2$). A kinase mix (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 250 μM ATP, and either 0.1 μg/μl peptide or no peptide) was added to each of 2 aliquots of beads to begin the reaction. (The peptide (LKKLTRRASFSGQ) was kindly synthesized by Ralph Peteranderl in the laboratory of Alan Frankel; peptide sequence was confirmed by MALDI-TOF mass spectrometry (Voyager, PerSeptive Biosystems).) After 1 h at room temperature, a 30 μl aliquot of the reaction was spotted on a P81 circle (Whatman). The filter circles were dried for 10 min, washed 5 times for 5 min each in 75 mM phosphoric acid with shaking, washed once in 95% ethanol, dried for 10 min, and quantitated in a scintillation counter with 9 ml liquid scintillant.

*Crt1 phosphorylation assay*

This assay was performed using pMH190 (EB1381) essentially as described [106]. Buffer A was made as described above, except for the omission of the $Na_3VO_4$ and the inclusion of 10 nM calyculin A. For inhibitor treatment, a 10 mM stock of 1-Na PP1 in DMSO (for a final concentration of 10 μM) was added immediately before addition of MMS.

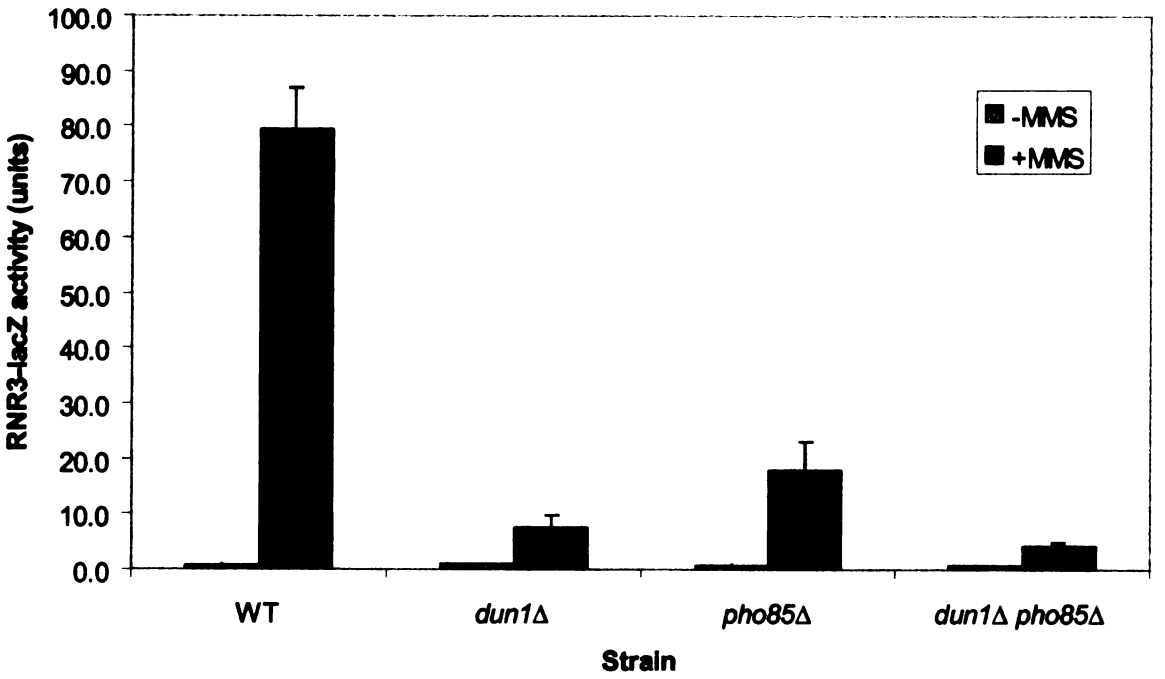
**Results**

Previous experiments demonstrated that loss of *PHO85* results in supersensitivity to treatment with hydroxyurea (HU) [20]. We wanted to identify which molecular processes were deficient and led to this sensitivity. Because deletion of *PHO85* had not

been linked to any defects in checkpoint activation, we reasoned that the observed sensitivity to replicational stress could be produced by an inability to synthesize sufficient levels of dNTPs. With this goal in mind, we examined the expression of an *RNR3*-lacZ fusion construct [101] in several strains treated with the DNA-damaging agent methyl methanesulfonate (MMS). We consistently found that MMS treatment produced more robust activation of activity than HU treatment in this and other assays. Thus, all the experiments described here use MMS as the damaging agent.

We found that deletion of *PHO85* from the genome had a significant effect on the ability of the cells to induce expression of the *RNR3*-lacZ fusion protein when treated with MMS (Figure 9). The strains shown in Figure 9 expressed only about 1 unit of β-galactosidase activity under nondamaging conditions of growth in synthetic media. When a wild-type strain was damaged by a 3 h treatment with 0.02% MMS, this expression increases approximately 80-fold. In contrast, damage of a strain lacking the *DUN1* gene, which is required for the DNA damage-inducible expression of *RNR3*, produced only an 8-fold induction in expression. Similarly, deletion of the *PHO85* gene resulted in decreased expression of the *RNR3*-lacZ reporter, although the decrease was smaller than that produced by *DUN1* deletion. Cells lacking *PHO85* induced *RNR3* expression only 18-fold upon treatment with MMS. This deficiency was highly reproducible. This deficiency might be explained as a secondary effect of the prolonged absence of Pho85 function and accompanying changes in cellular physiology, but an identical result was obtained when Pho85 function is inhibited chemically only during the course of the experiment (Meghan Byrne, data not shown).

FIGURE 9. Pho85 affects *RNR3* expression. The bars reflect the average of at least 4 independent experiments. Assays were performed as described in *Materials and Methods*. The error bars represent one standard deviation.

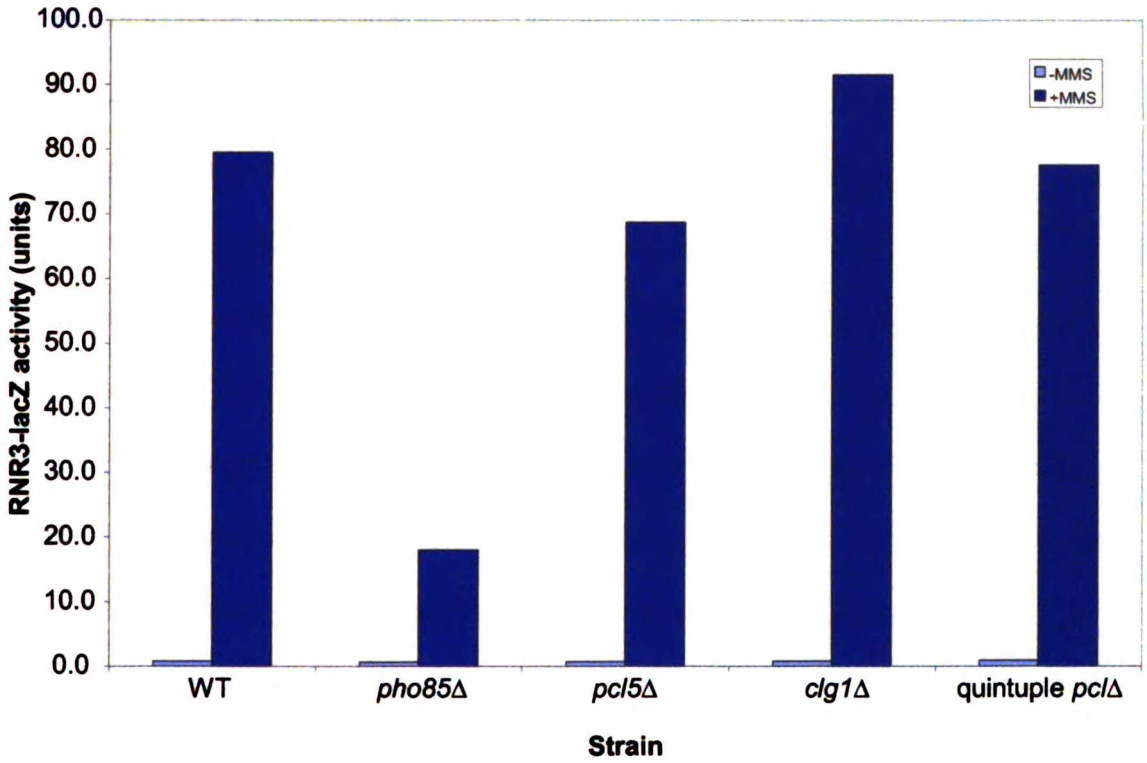

100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
RNR3-lacZ activity (units)
-MMS
+MMS
WT
dun1Δ
pho85Δ
dun1Δ pho85Δ
Strain

We also wanted to determine if *PHO85* and *DUN1* acted through the same pathway to induce *RNR3*-lacZ expression. Measuring the damage-induced expression in a strain lacking both *PHO85* and *DUN1* provides a simple method to assess the additivity of these phenotypes. As shown in Figure 9, the damage-induced expression of such a double mutant was only 4 units, or about a 4-fold increase. The induction in the double mutant is significantly smaller than the induction observed in a *dun1*Δ or a *pho85*Δ strain.

Because Pho85 is a cyclin-dependent kinase, we reasoned that its effect on the MMS-inducible expression of *RNR3* would depend on a cyclin partner. Pho85 binds to 10 known cyclin partners [48]. We focused our analysis on the 5-member Pcl1,2 subfamily, because the expression of *PCL5*, one of its members, is induced 6-fold by treatment with MMS [100]. Although the expression of *PCL5* in this condition suggests a role for the gene in surviving such DNA damage, no effect on MMS-inducible expression of *RNR3*-lacZ was detected in its absence (Figure 10). Furthermore, no effect on MMS-inducible expression of *RNR3*-lacZ was observed when all 5 members of the Pcl1,2 subfamily were simultaneous removed by genetic disruption (Figure 10). The Pho80 subfamily has not been tested for a role in *RNR3* expression.

Having established a role for Pho85 in MMS-inducible expression of *RNR3*, we were interested in determining if Pho85 interacted with any of the other factors known to influence *RNR3* expression. The existing genetic data demonstrate that the damage-inducible protein kinase Dun1 acts upstream of the transcriptional repressor Crt1, which binds directly to the promoters of damage-inducible genes like *RNR3*. Pho85 might exert its effects by acting above, in the middle, or outside of this signal transduction pathway.

FIGURE 10. Deletion of the *PCL1,2* subfamily does not recapitulate deletion of *PHO85* with respect to induction of *RNR3*. Assays were performed as described in *Materials and Methods*. A single representative experiment is shown.


100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
RNR3-lacZ activity (units)
-MMS
+MMS
WT
pho85Δ
pcl5Δ
clg1Δ
quintuple pclΔ
Strain

Although the genetic data described above suggested that Pho85 and Dun1 act in different pathways, we wanted to confirm this suggestion with further experiments.

First, we determined if the damage-inducibility of Dun1 kinase activity depends on *PHO85*. If Pho85 acts upstream of Dun1, its absence would lead to decreased activation of Dun1 upon treatment with MMS. To address this issue, we constructed an epitope-tagged Dun1 that could be immunoprecipitated under various conditions and assayed for kinase activity *in vitro*. For this assay, we used a peptide that has previously been shown to be an *in vitro* substrate of Dun1 [103]. As demonstrated previously using the peptide assay and anti-Dun1 polyclonal antibodies [103], the kinase activity of Dun1 was negligible in the absence of damage, but was strongly induced following a 3 h treatment with 0.1% MMS (Figure 11). The absence of a functional copy of *PHO85* did not cause a decrease in this kinase activity; instead, a small, yet reproducible, increase in damage-inducible Dun1 kinase activity was observed (Figure 11). The absence of *PHO85* caused a slight increase in Dun1 kinase activity in the absence of DNA damage (Figure 11). The status of *PHO85* did not affect the expression of *DUN1* from the *ADH1* promoter or the ability of the IgG Sepharose beads to completely deplete the extract of ZZ-Dun1 (Figure 12). The ZZ-tagged version of Dun1 appeared to be slightly hypomorphic, with respect to its ability to support full MMS-induced *RNR3* expression (Figure 13). It is possible that with a more active kinase, some subtle difference might be elucidated. These results suggest that Pho85 does not affect the damage-inducible kinase activity of Dun1 and likely exerts its effects on *RNR3* expression either downstream of Dun1 or through a separate pathway.

FIGURE 11. Induction of Dun1 kinase activity in response to DNA damage does not depend on *PHO85*. Treatment of cultures with MMS and peptide kinase assay conditions are described in *Materials and Methods*. The bars represent the average of two independent experiments and the error bars represent one standard deviation.

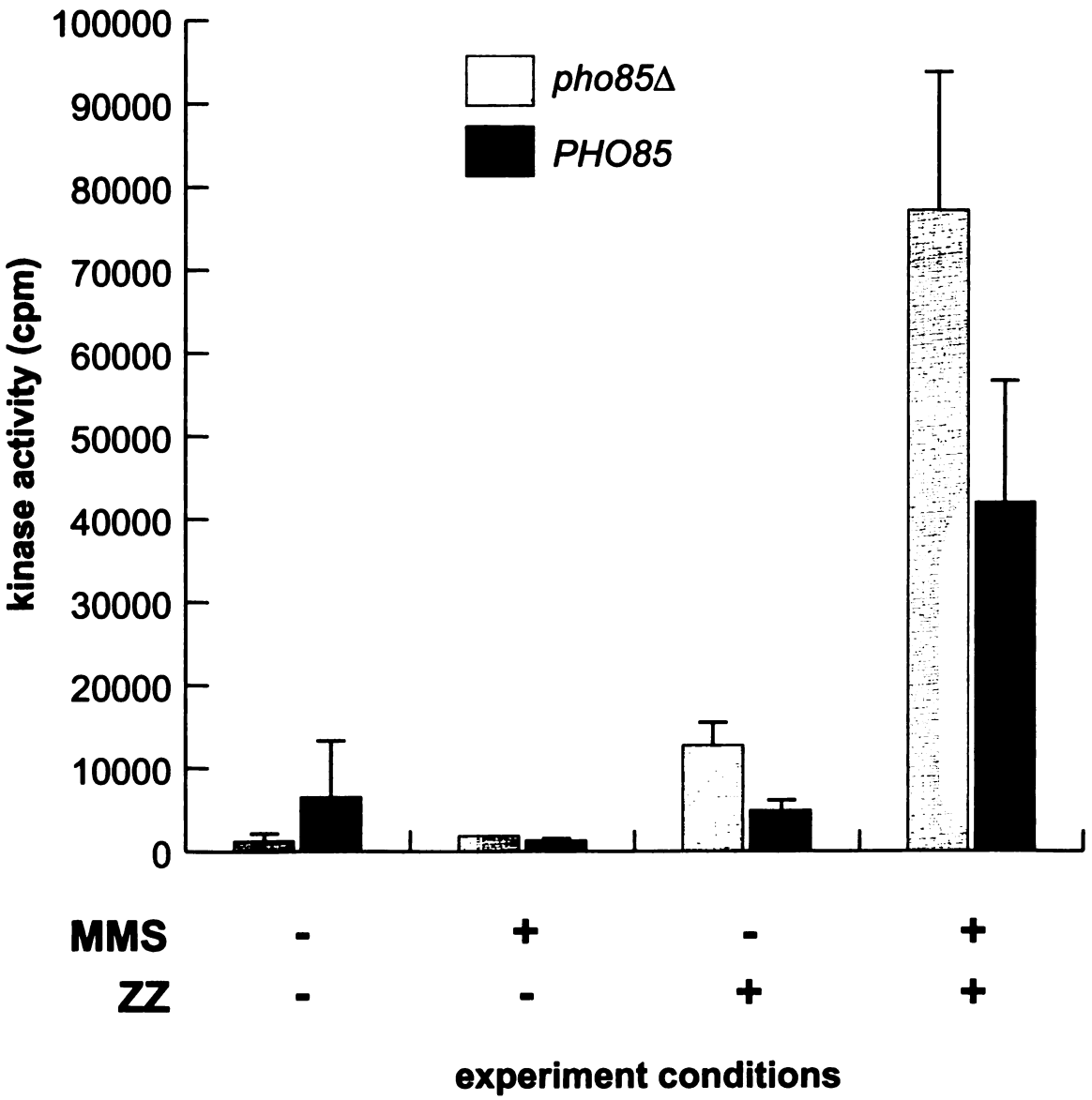

100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
kinase activity (cpm)
pho85Δ
PHO85
MMS - + - +
ZZ - - + +
experiment conditions

FIGURE 12. ZZ-Dun1 is efficiently depleted from extracts. Treatment with MMS and status of *PHO85* are indicated. Immunoprecipitations with rabbit serum were performed as described in *Materials and Methods*. The highest band shown is full-length ZZ-Dun1; the lower bands are likely to be breakdown products. L, Load; F/T, Flow-through.

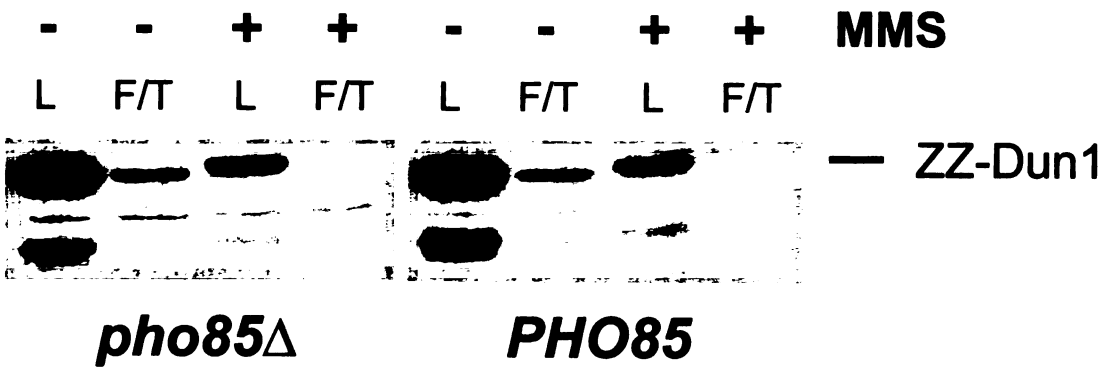
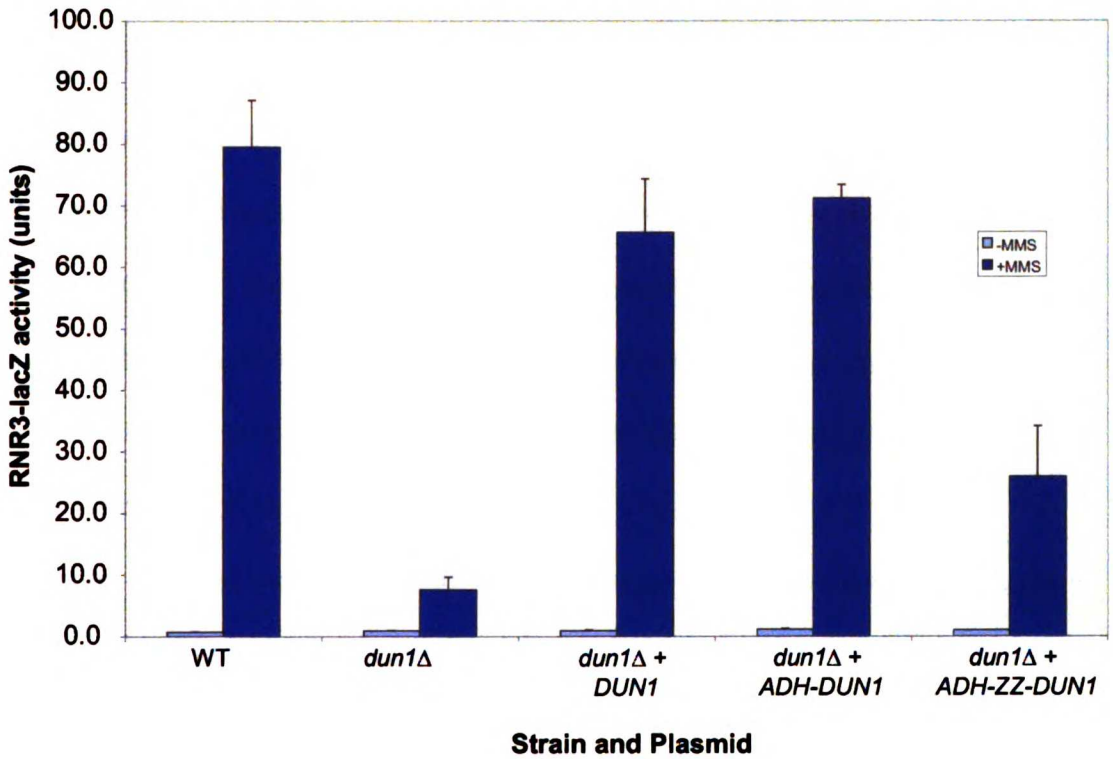

- - + + - - + + MMS
L F/T L F/T L F/T L F/T
— ZZ-Dun1
pho85Δ
PHO85

FIGURE 13. *DUN1* plasmids complement the deletion of *DUN1*. Yeast strain EY0658 (*dun1*Δ) was grown containing the indicated plasmids and assayed as described in *Materials and Methods*.


100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
RNR3-lacZ activity (units)
-MMS
+MMS
WT
dun1Δ
dun1Δ + DUN1
dun1Δ + ADH-DUN1
dun1Δ + ADH-ZZ-DUN1
Strain and Plasmid

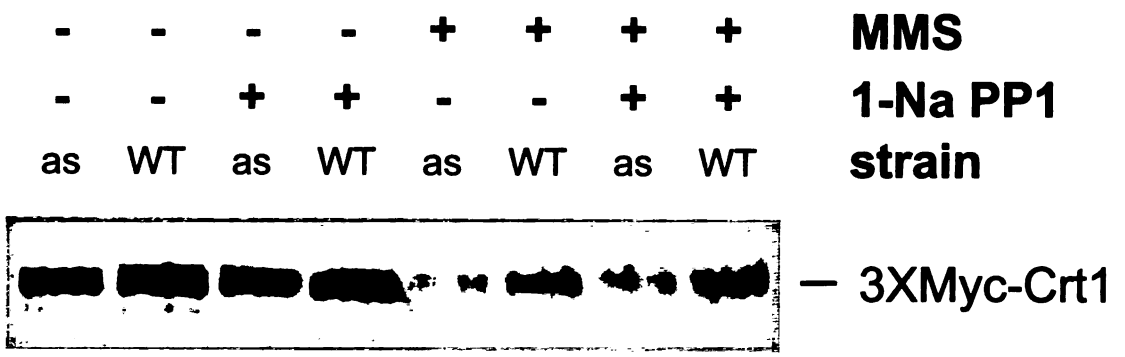
Next, we examined the *in vivo* phosphorylation of Crt1 for dependence on Pho85 [106]. For this assay, we expressed Crt1 from the galactose-inducible *GAL1* promoter. Under nondamaging conditions, Crt1 acts as a repressor of its own transcription, so obtaining sufficient material for biochemical experiments such as those described below with the native promoter was prohibitively difficult. Unfortunately, deletion of *PHO85* resulted in decreased expression from the *GAL1* promoter upon the addition of galactose to the media (data not shown). To circumvent this problem we used a chemical genetic strategy to inhibit Pho85 activity [41] without the adverse effects on Crt1 expression.

As described previously, upon treatment with MMS, 3xMyc-Crt1 became phosphorylated and underwent an SDS-PAGE mobility shift (Figure 14) [106]. Furthermore, the same mobility shift was observed when we added the cell-permeable small molecule 1-Na PP1 at the same time as the MMS treatment (Figure 14). In a strain harboring the $PHO85^{F82G}$ ("analog sensitive") mutation, the addition of 1-Na PP1 results in the rapid and near-total inhibition of Pho85 kinase activity. This result suggests that phosphorylation of Crt1 in response to DNA damage does not depend on Pho85.

**Discussion**

Genetic selections have proven useful in identifying the predominant factors involved in the transcriptional response to DNA damage [101, 102]. These *DUN* and *CRT* genes have sufficiently robust effects on transcription to create selectable change in the expression of *RNR3*. However, genetic inactivation of this pathway does not lead to a complete abrogation of activation of *RNR3* transcription by DNA damage. We have

FIGURE 14. 3xMyc-Crt1 gel mobility is unaffected by inhibition of Pho85. 3Xmyc-Crt1 was expressed from a galactose-inducible promoter as described in *Materials and Methods*. Treatments with MMS and 1-Na PP1 inhibitor are also described in *Materials and Methods*. WT, wild type Pho85; as, "analog sensitive," $Pho85^{F82G}$.

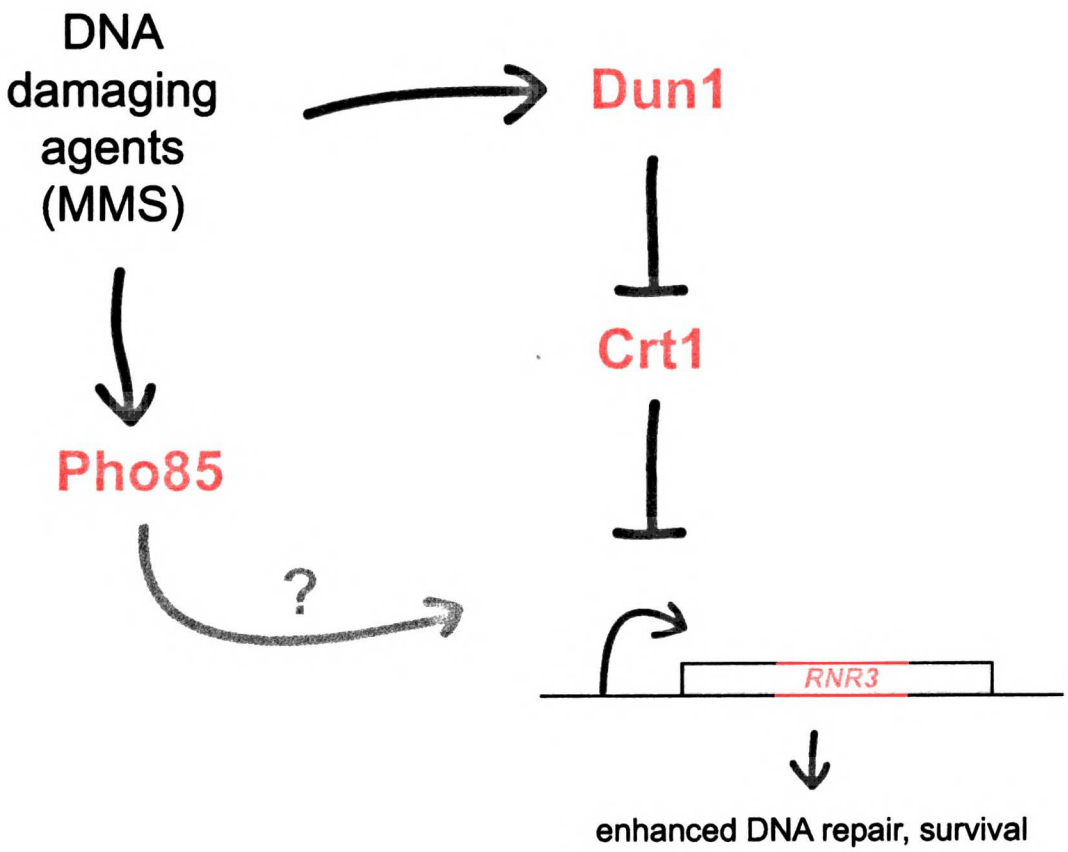

- - - - + + + + MMS
- - + + - - + + 1-Na PP1
as WT as WT as WT as WT strain
— 3XMyc-Crt1

identified an additional signaling pathway that impinges on *RNR3* transcription. Deletion of *PHO85* causes a decreased induction of *RNR3*-lacZ activity upon DNA damage. This decrease is not as severe as that observed when *DUN1* is deleted, suggesting that the *PHO85*-independent expression still exceeds a threshold that may have prevented the identification of *PHO85* as a *DUN* mutant in the original selection. The original selections also isolated single alleles of 4 other *DUN* genes; because the phenotypes of these genes were less robust than the *dun1* phenotype, these genes were never cloned, so *PHO85* may be among them.

Our experiments suggest that Pho85 functions in an *RNR3*-activating pathway distinct from that requiring *DUN1* and *CRT1*. First, the MMS-inducible activation of Dun1 kinase activity did not require *PHO85* (Figure 11). Second, the MMS-inducible phosphorylation of Crt1 was not affected by inhibition of Pho85 activity (Figure 14). Third, the simultaneous deletion of both *DUN1* and *PHO85* resulted in a reduced *RNR3*-inducibility phenotype more severe than that of either single mutant alone (Figure 9). It remains possible that Pho85 participates directly in the regulation of one of these factors, perhaps regulating the specificity rather than the activity of Dun1 or phosphorylating Crt1 in a manner that does not affect its SDS-PAGE mobility. In the absence of conclusive experiments, such as the examination of the phosphorylation of a bona fide *in vivo* Dun1 substrate or the generation of a tryptic phosphopeptide map of Crt1, our experiments suggest that Pho85 acts independently of these other factors (Figure 15).

Interestingly, the function of Pho85 in *RNR3* transcription appears to involve the induction of Pho85 kinase activity by adverse environmental conditions. All of the

FIGURE 15. A hypothetical model for Pho85 in the DNA damage-induced activation of *RNR3* expression. The data suggest that Pho85 participates in *RNR3* induction in response to DNA damage through factors other than Dun1 and Crt1. The mechanism of this activation, with respect to both Pho85 activity and transcriptional activity, is unknown.


DNA
damaging
agents
(MMS)
Dun1
Crt1
Pho85
?
RNR3
enhanced DNA repair, survival

functions of Pho85 identified thus far have the converse mechanism: the kinase activity of the Pho85 is inhibited by a transition to less favorable environmental conditions, like starvation for phosphate. The identification of the cyclin partner or partners that direct Pho85 to this function will be necessary to study the mechanism of this activation. Presumably, this cyclin is a member of the Pho80 subfamily, as deletion of all the members of the Pcl1,2 subfamily failed to produce a *pho85*Δ-like phenotype.

Having established a role for Pho85 in *RNR3* expression, we would like to understand the molecular mechanism in which it participates. Several models for this function are plausible. The simplest model is that Pho85 regulates a sequence-specific DNA-binding transcription factor that acts at the *RNR3* promoter. Such regulation is analogous to both the role of Pho85 in regulating the expression of phosphate-responsive genes through Pho4 (reviewed in [107]) and the role of Dun1 in regulating Crt1. Such a hypothetical factor could either repress transcription in nondamaged conditions, like Crt1, or activate transcription in the appropriate condition, like Pho4. Alternatively, Pho85 may affect a component of the general transcription machinery or transcriptional cofactor that effects the transcription of many genes, such as *RNR3*, that utilize those factors. An example of such a cofactor is the Ssn6/Tup1 complex, the transcriptional corepressor that associates with Crt1. Of course, Pho85 could function well upstream of the factors that have direct transactions with DNA.

The implication that Pho85 functions in a pathway distinct from that of Dun1 and Crt1 predicts that *RNR3* induction by DNA damage would be reduced in a *pho85*Δ *crt1*Δ strain compared to a *crt1*Δ strain. If the induction by DNA damage were unaffected by deletion of *PHO85* in the absence of *CRT1*, then the factors responsible for the

hyperinduction of *RNR3* expression in the absence of *CRT1* do not depend on *PHO85*. An *RNR3*-lacZ expression assay would provide a solid genetic argument for the model proposed here, which suggests a *CRT1*-independent role for Pho85.

Based on the hypothesis that Pho85 acts through a transcription factor other than Crt1, it would be of interest to identify this transcription factor. If such a factor is a sequence-specific DNA-binding protein, it may be useful to mutate the promoter region of *RNR3* to identify a region required for the *PHO85*-dependent effects. Such a region could also be identified by using bioinformatic techniques if a group of co-regulated genes could be identified. Comparing whole-genome transcript profiles of yeast responding to DNA damage in the presence and absence of *PHO85* might reveal several other DNA damage-inducible genes with *PHO85*-dependence like *RNR3*. The identity of such genes might be informative and perhaps allow us to construct a hypothesis regarding the signal to which Pho85 responds in this context. Our current studies have focused exclusively on MMS as a DNA-damaging agent, but *pho85*Δ cells are known to be sensitive to hydroxyurea as well. Further experiments exploring the sensitivity of the *pho85*Δ strain to other DNA-damaging agents could reveal what types of DNA damage can produce signals that Pho85 detects. Pho85 has recently been shown to have a role in the environmental stress response (ESR) [41, 89], which mediates a broad response to many adverse environmental conditions. The defects we observe in *RNR3* transcription in the *pho85*Δ strain may relate to the role of Pho85 in the ESR.

In the future, proteomic approaches may prove useful in elucidating the mechanism of DNA damage-dependent *RNR3* induction. The direct substrates of both Pho85 and Dun1 remain unidentified. A proteome-scale search for the direct substrates

may be the most rapid and effective method for their discovery. The identity of such substrates will be essential to elucidate the mechanisms of action for Pho85 and Dun1 and to demonstrate that these mechanisms are distinct from one another. The kinase that hyperphosphorylates Crt1 is also unidentified, although Dun1 remains a plausible candidate. An attempt to phosphorylate Crt1 *in vitro* using immunoprecipitated Dun1 did not result in any phosphorylation of Crt1, although the Dun1 kinase activity had been successfully induced, as determined by using the peptide assay (data not shown). The mapping of the Crt1 phosphorylation sites used *in vivo* will be a necessity to verify that a candidate kinase is responsible for Crt1 regulation *in vivo*. Also, the identification of the Crt1 phosphorylation sites will permit the study of the mechanisms of Crt1 regulation. The knowledge of the Crt1 phosphorylation sites and their influence on Crt1 activity will permit conclusive demonstration that the role of Pho85 in *RNR3* induction in response to DNA damage does not depend on Crt1 phosphorylation.

**Table 3. Strains used in Chapter 3**

| *Strain* | *Genotype* |
|---|---|
| K699 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 GAL*$^{+}$ |
| EY0057 | K699 |
| EY0140 | K699 *pho85*Δ::*LEU2* |
| EY0634 | K699 *crt1*Δ::*HIS3* |
| EY0658 | K699 *dun1*Δ::*TRP1* |
| EY0694 | K699 *dun1*Δ::*TRP1 pho85*Δ::*LEU2* |
| EY0704 | K699 *pcl5*Δ::*HIS3* |
| EY0708 | K699 *clg1*Δ::*TRP1* |
| EY0752 | K699 *pcl1*Δ::*HIS3 pcl2*Δ::*LEU2 pcl5*Δ::*HIS3 pcl9*Δ::*TRP1 clg1*Δ::*TRP1* |
| EY0824 | K699 *pho85*Δ *ADE*$^{+}$ *pho3*Δ *trp1*::*PHO85*$^{F82G}$::*TRP1* |
| EY0826 | K699 *pho85*Δ *ADE*$^{+}$ *pho3*Δ *trp1*::*PHO85*$^{WT}$::*TRP1* |

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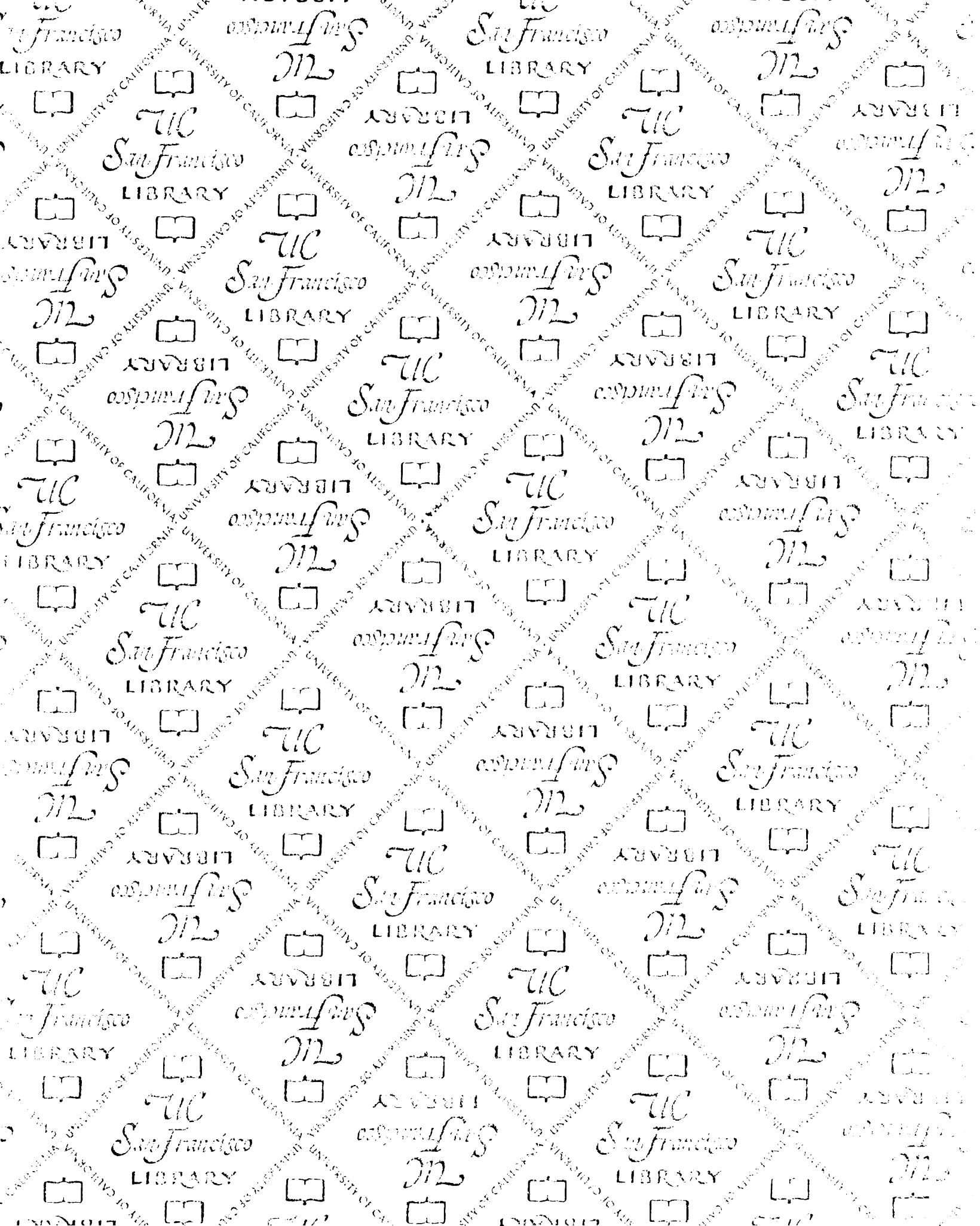
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