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Generation of a set of conditional analog-sensitive alleles of essential protein kinases in the fission yeast *Schizosaccharomyces pombe*

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The genome of the fission yeast *Schizosaccharomyces pombe* encodes for 17 protein kinases that are essential for viability. Studies of the essential kinases often require the use of mutant strains carrying conditional alleles. To inactivate these kinases conditionally, we applied a recently developed chemical genetic strategy. The mutation of a single residue in the ATP-binding pocket confers sensitivity to small-molecule inhibitors, allowing for specific inactivation of the modified kinase. Using this approach, we constructed conditional analog-sensitive alleles of 13 essential protein kinases in the fission yeast *S. pombe*.

Introduction

It is well-established that reversible protein phosphorylation is one of the major mechanisms by which cellular processes are regulated.[1,2] The analysis of protein kinases and phosphatases has been instrumental to various fields of cell biology. The fission yeast *Schizosaccharomyces pombe* is an excellent model organism for studying eukaryotic biology, because it is amenable to genetic, biochemical and cytological techniques as well as genome-wide approaches.[3-5] The *S. pombe* genome encodes 107 predicted protein kinases, and 17 of them are known to be essential for cell growth. To date, studies of the essential protein kinases in fission yeast have relied mostly on the use of mutant strains carrying conditional temperature-sensitive alleles. However, these alleles are often leaky and require analysis under non-physiological conditions, and their inactivation requires a long period of time. A recently developed chemical genetic strategy for sensitizing protein kinases to small-molecule inhibitors provides an elegant tool for constructing conditional analog-sensitive kinase alleles (**Fig. 1**).[6] In our current study, we used this strategy to create analog-sensitive alleles of *S. pombe* protein kinases that are essential for cell growth.

Results

A recent systematic deletion analysis of genes in the fission yeast *S. pombe* revealed that 18 protein kinases are essential for viability.[7,8] We created heterozygous diploid deletion strains for each of these 18 kinases and analyzed the haploid products after sporulation. We confirmed that 17 kinase genes are essential for viability. However, the sporulation of a heterozygous diploid deletion strain, followed by the dissection of the asci on rich medium, showed that one of the kinases, *ppk28*, is not essential for viability (data not shown). This observation is consistent with previous reports showing that *ppk28* is dispensable for normal cell growth.[9] Of the 17 essential kinases, only one analog-sensitive allele (*ark1 as3*)[10] was available when we started this project. Therefore, we decided to create conditional analog-sensitive alleles for 16 remaining protein kinases essential for viability (**Table 1**).

To create conditional analog-sensitive alleles, we mutated the gate-keeper residue within the kinase ATP-binding site to a glycine (as1) or an alanine (as2). The mutations were expressed in an *S. pombe* strain lacking the wild-type allele of the corresponding kinase according to the protocol of Gregan et al.[11] Out of the 16 kinases with a mutated gate-keeper residue, 13 remained functional as indicated by growth of haploid cells carrying the corresponding gate-keeper mutation in YES medium (**Table 1 and Figs. 3 and S1**). Tetrad analysis of heterozygous diploid strains showed that the remaining three mutants (*cdc7-as*, *hsk1-as* and *sid1-as*) were not functional (**Table 1**). We also observed that two mutants (*cdc2-as1* and *pat1-as*) were sensitive to elevated temperature (32°C), but they grew normally at a low temperature (25°C). The temperature sensitivity of *cdc2-as1* has

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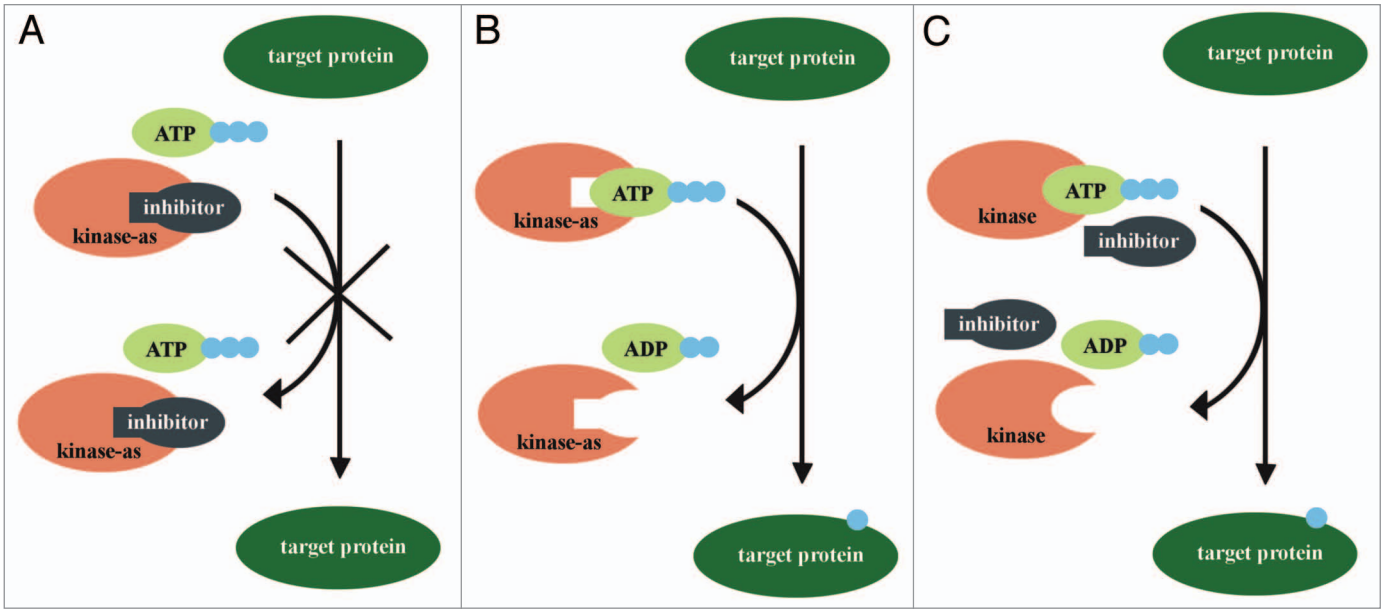

Figure 1. Chemical genetic strategy for sensitizing protein kinases to small-molecule inhibitors. Mutation of the gate-keeper residue in a protein kinase (kinase-as) creates a new pocket where an ATP analog (inhibitor) can bind. Binding of the inhibitor to kinase-as leads to inactivation of the kinase (A). However, the kinase-as mutation typically does not interfere with the kinase function in the absence of the inhibitor (B). The inhibitor cannot bind to wild-type kinases, allowing for specific inactivation of the mutant kinase (C).[6]

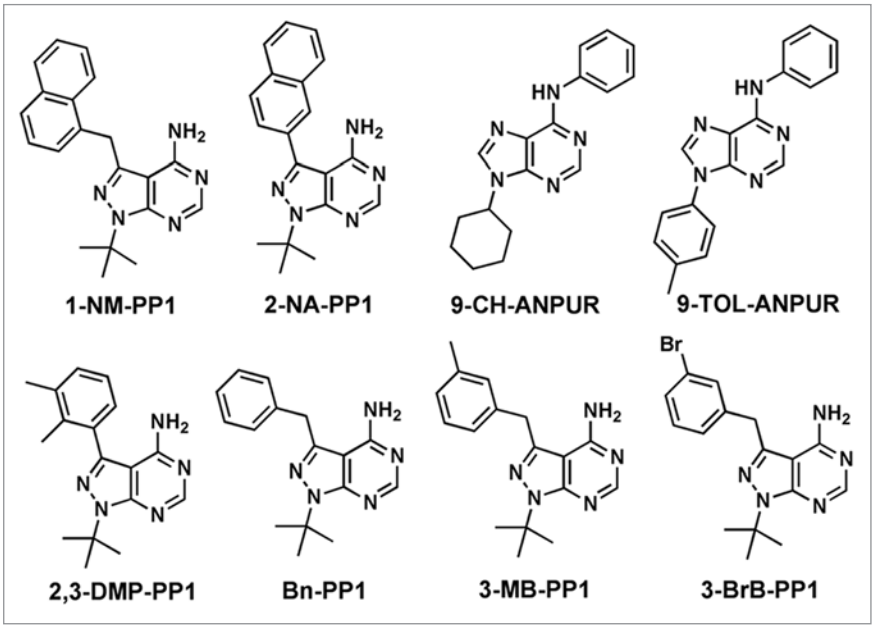

Figure 2. Chemical structures of the inhibitors used in this study.

been described previously in references 12 and 13. Thus, out of the 16 essential kinases, we constructed 13 functional alleles where the gate-keeper residue was replaced by a glycine or an alanine.

To test the sensitivity of the 13 kinase mutants to inhibitors, we synthesized eight different inhibitors (ATP analogs) (**Fig. 2**).

In addition to the mutants created in this study, we included the previously constructed *ark1-as3* mutant in our analysis.[10] Spot test on YES plates showed that eight mutants were highly sensitive to one or more of the tested inhibitors (≤10 μM ATP analog), and six mutants exhibited sensitivity only to higher concentrations of inhibitors (**Table 2 and Fig. 3**). To further characterize the mutant phenotypes, we added an inhibitor to cultures exponentially growing in liquid YES medium and analyzed the effect of inhibitors on cell growth by counting cell number. Four hours after adding the inhibitor, we harvested cells and analyzed the morphology of the cells by microscopy and the cellular DNA content by flow cytometry (**Fig. S1**). These analyses confirmed that all 13 kinase mutants were sensitive to at least one of the tested inhibitors, and the growth of the mutant cells was either inhibited (7 mutants) or completely blocked (6 mutants) in the presence of the inhibitor (**Fig. S1**). Thus, we conclude that analog-sensitive alleles were constructed for 13 out of 16 of the essential kinases. Therefore, a chemical genetic strategy of conditional inactivation of protein kinases by creating mutants sensitive to ATP analogs is a viable option for studying *S. pombe* protein kinases that are essential for cell growth and should encourage wider use of this strategy in yeast as well as other organisms.

Discussion

In this study, we created conditional analog-sensitive alleles of 13 *S. pombe* protein kinases. Four of these mutants have been described by others during the course of this work.[12,14-17] Although seven mutants were highly sensitive to one or more of the tested inhibitors, six mutants exhibited sensitivity only to higher concentrations of inhibitors. In the future, it will be useful to further optimize these mutants to increase their sensitivity to inhibitors. In addition, the three kinases that did not tolerate mutation of the gate-keeper residue may be potentially salvaged by second-site suppressor mutations.[18] A growing number of studies have shown that small-molecule inhibitors selective for target kinases are beneficial for conditional inactivation.[19,20] Therefore, we believe that the analog-sensitive alleles constructed in this study will provide a valuable tool for studying these protein kinases. ATP analogs are usually cell permeable, allowing for rapid and reversible inhibition of the target protein kinase. An important advantage of rapid inactivation of the kinase is that the cell has little time to compensate for the missing kinase activity. In addition, depending on the amount of inhibitor added, total or partial inhibition of the target kinase can be achieved. Finally, a small-molecule inhibitor and a genetic mutation can perturb the activity of a kinase in different ways and may result in different phenotypes. Therefore, analog-sensitive alleles may reveal new biological functions of kinases.[11,19,21]

The analog-sensitive alleles described here not only provide a new tool for studying protein kinases, but they also complement currently available conditional alleles. For example, the analog-sensitive alleles could be combined with temperature-sensitive alleles to create double mutants in which the temperature-sensitive protein can be inactivated by shifting cells to non-permissive temperature, and the analog-sensitive kinase can be independently inactivated by adding inhibitor. Notably, some of the tested ATP analogs inhibit only a subset of kinase mutants (**Table 2**), which raises the possibility that some of the analog-sensitive alleles could be combined in double-mutant strains and separately inactivated with specific inhibitors.

The fission yeast *Schizosaccharomyces pombe* is an excellent model organism for studying various aspects of biology, and it is likely that the analog-sensitive alleles described in this work will become a useful tool for future studies. It is notable that only 3 out of the 16 kinases did not tolerate mutation of the gate-keeper residue. As the gate-keeper residue can be easily identified in most protein kinases (reviewed in ref. 11 and sequoia.ucsf.edu/ksd), we expect that this strategy will also work for many other protein kinases.

Materials and Methods

Strains and general methods. The genotypes of the yeast strains used in this study are listed in **Table 3**. *Schizosaccharomyces pombe* strains were maintained and grown using standard conditions.[22] The transformation of *S. pombe* was performed using the lithium acetate method as previously described in reference 23. Procedures for Hoechst staining (fluorescence microscopy) and Sytox-Green staining (FACS analysis) were followed as previously described in reference 24.

Table 1. Functionality of analog-sensitive kinase mutants as determined by growth on YES plates

Kinase	Gate keeper residue	Functionality of mutants	
		as1 (Gly)*	as2 (Ala)*
Cdc2	F84	yes†	-
Cdc7	L85	no	no
Cdk9	T120	yes†	-
Cka1	F117	yes†	no
Crk1	L87	yes	no
Hsk1	L173	no	no
Ksg1	L177	yes†	-
Nak1	M88	yes	no
Orb6	M170	yes†	-
Pat1	L95	yes	yes
Plo1	L117	yes†	-
Ppk37	M537	yes	yes
Prp4	F238	no	yes
Shk1	M460	no	yes
Sid1	M84	no	no
Sid2	M285	yes	no

*gate-keeper residue mutated to a glycine (Gly) or an alanine (Ala), respectively; †sensitive to ≤10 μM ATP analog.

Deletion of genes encoding for essential kinases. To delete genes encoding for the essential kinases, we used the approach based on knockout constructs that contain 150–800 bp long regions homologous to the target genes cloned into vector-carrying dominant drug-resistance markers.[23] The oligonucleotides were designed according to the database of *S. pombe* deletion constructs (mendel.imp.ac.at/Pombe_deletion). Sequences flanking the genes encoding the essential kinases were PCR-amplified from *S. pombe* genomic DNA using the primers *up-out* and *up-in* for upstream regions and *down-out* and *down-in* for downstream regions (**Table S1**). The resulting PCR products were ligated to each other, and the proximal ends were cloned into a pCloneNat1 vector (EF101285) carrying drug resistance markers for *E. coli* (Ampicillin) and *S. pombe* (Nourseothricin), resulting in pCloneNat1-Δ*kinase* plasmids. The plasmids were amplified in *E. coli*, purified and linearized with restriction enzymes (**Table S2**). The linearized plasmids were used to delete the respective kinases in a diploid strain (JG11315). Successful deletion was confirmed by colony PCR and tetrad analysis.

Construction of analog sensitive (*as*) kinase mutants. The coding sequences of the kinase genes with their promoter and terminator regions were PCR-amplified from *S. pombe* genomic DNA using the forward and reverse primers, *kinase* promotor and *kinase* terminator, respectively (**Table S2**). The PCR products were cloned into a pCloneHyg1 vector (EF101286) carrying drug resistance markers for *E. coli* (Ampicillin) and *S. pombe*

Table 2. Sensitivity of analog-sensitive kinase mutants to various ATP analogs

	Sensitivity to inhibitors [μM]							
	1-NM-PP1	2-NA-PP1	9-CH-ANPUR	9-TOL-ANPUR	2,3-DMP-PP1	Bn-PP1	3-MB-PP1	3-BrB-PP1
ark1-as3	5.0	5.0	n.s.	40.0	5.0	10.0	10.0	10.0
cdc2-as1	0.5	5.0	5.0	5.0	5.0	5.0	5.0	5.0
cdk9-as1	10.0	20.0	20.0	10.0	n.s.	n.s.	10.0	10.0
cka1-as1	n.s.	n.s.	5.0	30.0	n.s.	n.s.	n.s.	40.0
crk1-as1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	30.0
ksg1-as1	5.0	20.0	20.0	10.0	n.s.	20.0	5.0	5.0
nak1-as1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	40.0
orb6-as1	10.0	10.0	20.0	10.0	10.0	10.0	10.0	5.0
pat1-as2	10.0	20.0	n.s.	10.0	30.0	30.0	n.s.	30.0
plo1-as1	30.0	n.s.	n.s.	n.s.	n.s.	n.s.	40.0	10.0
ppk37-as1	30.0	n.s.	n.s.	n.s.	n.s.	n.s.	30.0	30.0
prp4-as2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	30.0
shk1-as2	n.s.	n.s.	n.s.	30.0	n.s.	n.s.	n.s.	20.0
sid2-as1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	40.0

n.s., not sensitive.

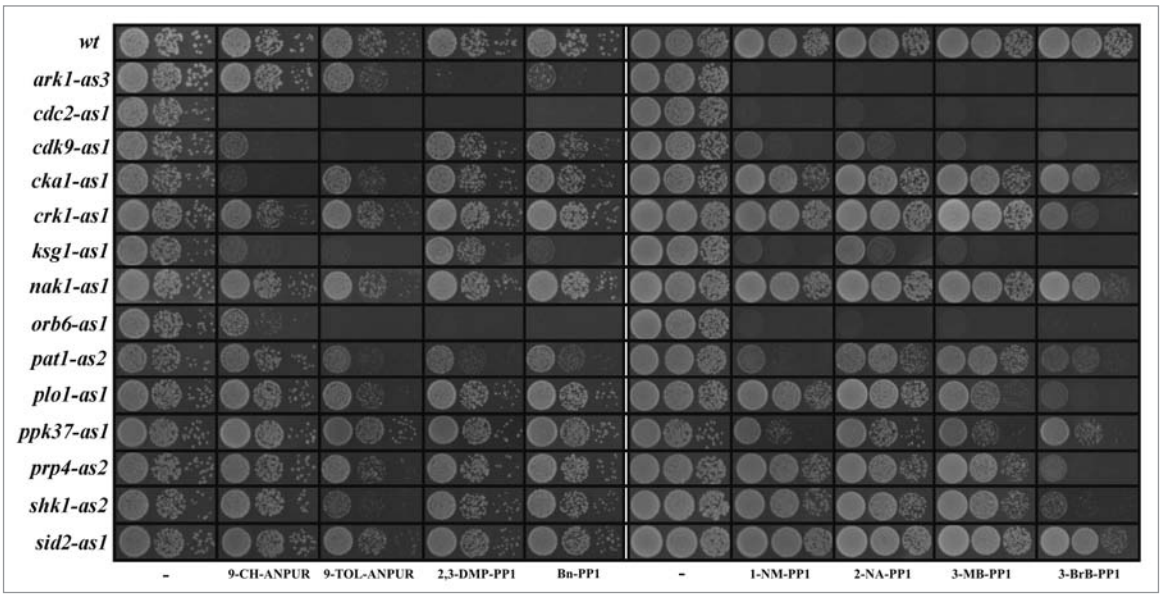

Figure 3. Sensitivity of cells expressing analog-sensitive kinase alleles to various inhibitors. Serial dilutions of wild-type cells or cells expressing indicated analog-sensitive kinase alleles were spotted on YES plates containing or lacking the indicated inhibitors and grown at 32°C for 3–4 days, with the exception of the temperature-sensitive mutants cdc2-as1 and pat1-as2, which were grown at 25°C for 4 days. Concentrations of inhibitors were as described in the **Table 2.**

(Hygromycin B), resulting in integrative pCloneHyg1-*kinase* plasmids. The plasmids were amplified in *E. coli*, purified and sequenced to exclude the clones with PCR-introduced mutations. To create mutant strains carrying conditional analog-sensitive alleles, we employed site directed mutagenesis (QuikChangeII, Site Directed Mutagenesis Kit*)* and mutated the predicted gatekeeper residues to small amino acid residues (**Table 1**, sequoia.ucsf.edu/ksd).[11] The sequences of primers used for mutagenesis are listed in **Table S3**. The resulting plasmids pCloneHyg1-*kinase-as1* and pCloneHyg1-*kinase-as2* were linearized with a unique restriction enzyme that cuts within the promoter region of the kinase gene (**Table S2**) and transformed into a diploid strain heterozygous for the deletion of the respective kinase. The selected transformants were sporulated at 25°C, and haploids carrying analog-sensitive kinase alleles were selected based on resistance to Nourseothricin and Hygromycin. Correct integration of mutant kinase alelles was confirmed by colony PCR. The presence of mutant alleles in the selected haploid strains was verified by sequencing of the PCR-amplified kinase alleles from genomic DNA.

Table 3. Yeast strains used in this study

Strain	Genotype
JG15459	*h-*
JG15401	*h^+ ark1-as(L166A, S229A, Q28R, Q176R)-HphR*
JG11315	*h^+/h^- leu1–32/leu1–32 ura4-D18/ura4-D18 ade6-M210/ade6-M216*
JG15442	*h^- leu1–32 ura4-D18 ade6-M216 cdc2::ClonNatR cdc2-as(F84G)-HphR*
JG15172	*h^- leu1–32 ura4-D18 ade6-M210 cdk9::ClonNatR cdk9-as(T120G)-HphR*
JG15446	*h^- leu1–32 ura4-D18 ade6-M216 cka1::ClonNatR cka1-as(F117G)-HphR*
JG15452	*h^- leu1–32 ura4-D18 ade6-M210 crk1-as(L87G)-HphR*
JG15433	*h^- leu1–32 ura4-D18 ade6-M210 ksg1::ClonNatR ksg1-as(L177G)-HphR*
JG15448	*h^- leu1–32 ura4-D18 ade6-M210 nak1-as(M88G)-HphR*
JG15439	*h^- leu1–32 ura4-D18 ade6-M216 orb6::ClonNatR orb6-as(M170G)-HphR*
JG15404	*h^- leu1–32 ura4-D18 ade6-M216 pat1::ClonNatR pat1-as(L95G)-HphR*
JG15403	*h^- leu1–32 ura4-D18 ade6-M210 pat1::ClonNatR pat1-as(L95A)-HphR*
JG15431	*h^- leu1–32 ura4-D18 ade6-M216 plo1::ClonNatR plo1-as(L117G)-HphR*
JG16103	*h^- leu1–32 ura4-D18 ade6-M216 ppk37::ClonNatR ppk37-as(M537G)-HphR*
JG16101	*h^- leu1–32 ura4-D18 ade6-M210 ppk37::ClonNatR ppk37-as(M537A)-HphR*
JG15934	*h^- leu1–32 ura4-D18 ade6-M210 prp4::ClonNatR prp4-as(F238A)-HphR*
JG15927	*h^- leu1–32 ura4-D18 ade6-M210 shk1::ClonNatR shk1-as(M460A)-HphR*
JG15450	*h^- leu1–32 ura4-D18 ade6-M210 sid2-as(M285G)-HphR*

Analysis of sensitivity of kinase mutants to ATP analogs. To test if the strains with a mutated gate-keeper residue are sensitive to ATP analogs, the dilution spot test was used. Serial dilutions of cells were spotted on YES plates supplemented with various ATP analogs or DMSO, and grown at 32°C for 3–4 d, with the exception of the temperature-sensitive mutants, *cdc2-as1* and *pat1-as2,* which were grown at 25°C for 4 d. The determination of growth rates and cellular morphology and FACS analysis were performed with cells growing in liquid YES medium supplemented with various ATP analogs.[25]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at:
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