

Construction of conditional analog-sensitive kinase alleles in the fission yeast *Schizosaccharomyces pombe*

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Published online 15 November 2007; doi:10.1038/nprot.2007.447

Reversible protein phosphorylation is a major regulatory mechanism in a cell. A chemical-genetic strategy to conditionally inactivate protein kinases has been developed recently. Mutating a single residue in the ATP-binding pocket confers sensitivity to small-molecule inhibitors. The inhibitor can only bind to the mutant kinase and not to any other wild-type kinase, allowing specific inactivation of the modified kinase. Here, we describe a protocol to construct conditional analog-sensitive kinase alleles in the fission yeast *Schizosaccharomyces pombe*. This protocol can be completed in about 3 weeks and should be applicable to other organisms as well.

INTRODUCTION

To inactivate essential proteins conditionally, temperature-sensitive alleles, regulatable promoters, degron alleles, conditionally active inteins as well as other strategies have been used^{1–7}. However, these alleles are often leaky, require analysis under non-physiological conditions (high temperature) or their inactivation requires a long period of time. In addition, the molecular mechanism of temperature-sensitive protein inactivation is rarely understood. Small-molecule inhibitors selective for a target protein provide a valuable tool for conditional inactivation of proteins and provide several advantages over genetic approaches. Total as well as partial inhibition of the target protein can be achieved, depending on the amount of inhibitor added. The cell-permeable nature of these inhibitors allows reversible inhibition of the target protein both *in vitro* and *in vivo*. Rapid inactivation of the target protein by addition of the inhibitor leaves the cell with little time to adapt to or compensate for the missing protein activity. Small-molecule inhibitors do not typically alter the expression level of the target protein, nor do they disrupt protein complexes. In many cases, a small-molecule inhibitor and a genetic mutation can perturb a protein's activity in different ways. This may result in different phenotypes, often providing complementary information about the cellular function of a given kinase⁸. Small-molecule inhibitors can therefore reveal new biological functions of proteins that have already been studied genetically⁹.

Numerous small-molecule inhibitors have been discovered and employed in the study of various protein kinases. However, due to the large number of kinases in the genome and their highly conserved active sites, which these inhibitors target, many inhibitors suffer from poor selectivity¹⁰. Moreover, little is known about the specificity of these inhibitors against yeast kinases, as most of these inhibitors were developed and characterized against mammalian kinases. A chemical-genetic strategy for sensitizing protein kinases to small-molecule inhibitors has been developed recently¹¹. A single residue in the ATP-binding pocket, termed the gate-keeper

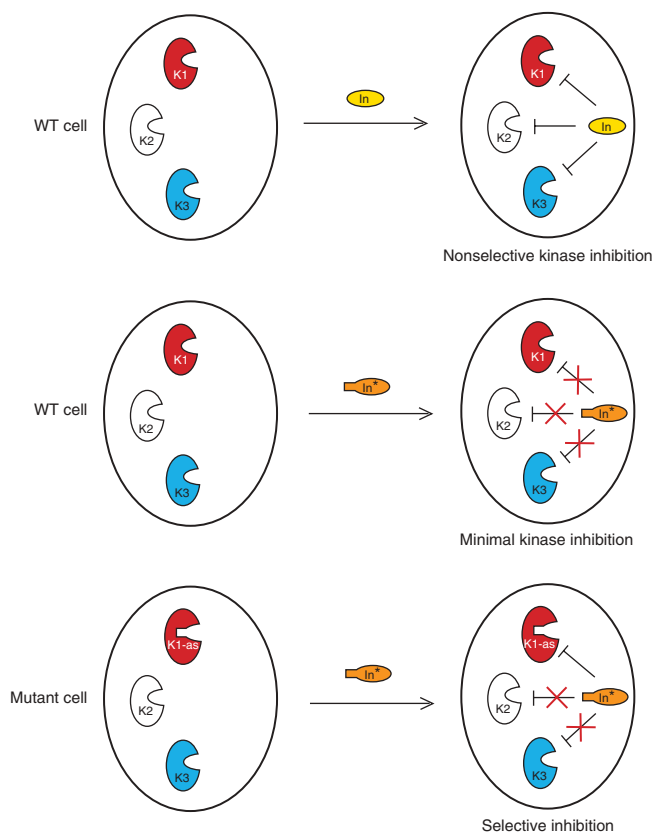


Figure 1 | Strategy for sensitizing protein kinases to small-molecule inhibitors. A conventional small-molecule inhibitor (In) typically blocks multiple kinases in the cell. Mutation of the gate-keeper residue in a protein kinase (K1) creates a new pocket where an enlarged inhibitor (In*) can bind. The inhibitor (In*) cannot bind to wild-type (WT) kinases, allowing a specific inactivation of the mutant kinase.

residue, controls sensitivity of protein kinases to designed small-molecule inhibitors. This gate-keeper residue is conserved as a bulky hydrophobic amino acid in the protein kinase superfamily. Mutation of the gate-keeper to a small residue (alanine or glycine) creates a novel pocket that can be uniquely targeted by inhibitors with properly enlarged substituents. The mutation thus confers inhibitor sensitivity but does not interfere with kinase function in the absence of inhibitor (Fig. 1). The ATP-binding pocket of kinases is so conserved that the inhibitor can only bind to the mutant kinase and not to any wild-type kinases due to steric clash of the enlarged substituent and the bulky wild-type gate-keeper residue, hence allowing specific inactivation of the mutant kinase. Importantly, the fact that only sensitized protein kinase is inhibited allows a critical control experiment to be performed in which wild-type cells are treated with the inhibitor, thus revealing any possible off-target effects of the inhibitor. Such a control experiment should be performed in parallel with every experiment involving sensitized protein kinase and the inhibitor. The gate-keeper residue can be easily identified from primary sequence alignments in many protein kinases from various kinase subfamilies (<http://sequoia.ucsf.edu/ksd/>) (see ref. 12). Thus, the same strategy can be used to construct conditional analog-sensitive alleles of a wide variety of protein kinases^{11,13–18}. This technology can also be used for identification of kinase substrates and has multiple applications in the drug discovery process^{19–21}. Moreover, this methodology of generating inhibitors that selectively target a sensitized enzyme is not limited to protein kinases. A similar approach has been successfully used to inhibit proteins from other families, including phosphatases, motor proteins and GTPases^{22–26}.

Despite many advantages, the chemical-genetic strategy of protein kinase sensitization contains a number of limitations. The catalytic activity of the protein kinase may be diminished by mutating the gate-keeper residue. In such cases, a second-site suppressor mutation can be introduced to rescue the kinase activity²⁷. Another limitation is that this approach is only applicable to organisms in which genetic techniques are available to knock out or inactivate the target kinase and introduce the

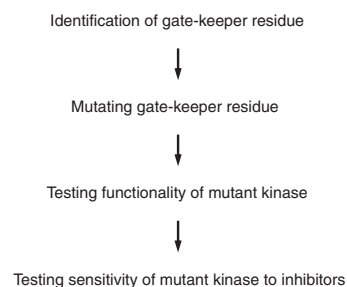


Figure 2 | Flowchart of the protocol.

sensitized kinase allele. Exquisite skills and lengthy procedures are necessitated to carry out gene replacement in high eukaryotes such as *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus*, impeding chemical-genetic analysis of protein kinases from these organisms. However, such limitation does not apply to the fission yeast *S. pombe*, a single-celled fungus, which has been extensively studied and has served as an excellent model organism. Importantly, the genome of *S. pombe* has been sequenced²⁸.

Here, we describe a detailed protocol for the construction of conditional analog-sensitive kinase alleles in *S. pombe* (Fig. 2). Hhp1 and Hhp2 are members of a highly conserved casein kinase 1 family involved in DNA repair in *S. pombe*. Mutants lacking both Hhp1 and Hhp2 proteins grow extremely slowly, which precludes careful analysis of the double mutant phenotype^{29,30}. We therefore constructed an analog-sensitive *hhp1* mutant and combined it with an *hhp2* deletion mutant. This allowed us to grow sufficient amounts of cells in the absence of inhibitor and to harvest them after adding the inhibitor. In this way, we were able to analyze the mutant phenotype in cells with inactivated Hhp1 kinase and lacking the Hhp2 protein³¹. We mutated methionine 84 of *hhp1* to a glycine residue (*hhp1-as*) and expressed it in an *S. pombe* strain lacking wild-type *hhp1*. The mutated gene *hhp1-as* complemented the phenotype caused by deleting the *hhp1* gene and resulted in only a minor growth defect. Importantly, the *hhp1-as* mutant, but not a wild-type strain, was sensitive to the inhibitor 1-NM-PP1 (Figs. 3 and 4) (see ref. 31).

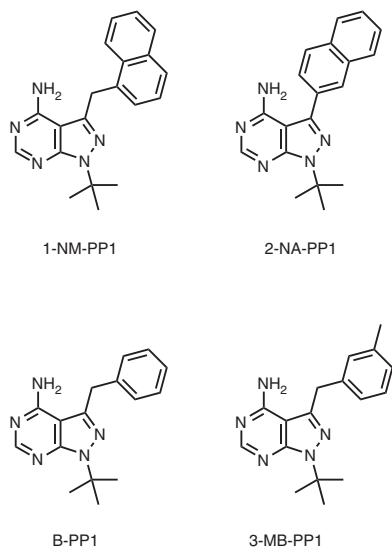


Figure 3 | Chemical structures of commonly used inhibitors for analog-sensitized kinases.

MATERIALS

REAGENTS

- Inhibitors 1-NA-PP1 and 1-NM-PP1 (Calbiochem; <http://www.emdbiosciences.com/product/529579>, <http://www.emdbiosciences.com/product/529581>); other inhibitors are available from Kevan Shokat on request
- PCR kit (Takara; PCR kits from other suppliers should also work); PCR premix (see REAGENT SETUP)
- High-fidelity DNA polymerase (e.g., *PfuTurbo* DNA polymerase; Stratagene)
- Restriction enzymes (Roche; restriction enzymes from other suppliers should also work)
- pCloneHyg1 (EF101286) is the vector used for cloning. It can be requested from Kim Nasmyth (Kim Nasmyth DNA collection number: 4802) or Juro Gregan lab
- *S. pombe* genomic DNA from which the gene can be amplified
- Anion exchange columns (Qiagen) for DNA purification from PCR, restriction digest or agarose gel; products from other suppliers should also work
- Salmon sperm DNA (Stratagene) for use as a carrier for yeast transformation
- Dimethyl sulfoxide (Sigma)
- Agarose (Sigma)

EQUIPMENT

- Benchtop centrifuge
- Incubators
- DNA gel electrophoresis apparatus

PROTOCOL

Figure 4 | Sensitivity of cells expressing Hhp1-as to various inhibitors.

(a) Serial dilutions of *hhp1Δ hhp2Δ* cells expressing either wild-type Hhp1 (K13619) or the analog-sensitive Hhp1-as (K14637) protein were spotted on YES plates containing or lacking inhibitors and grown for 2 d at 32 °C. We noticed that in the presence of 2-NA-PP1, crystals formed on YES plates. (b) DIC (differential interference contrast) images of cells grown on YES plates containing or lacking inhibitors as described in (a).

REAGENT SETUP

Plasmid DNA Prepare a midi-prep (Qiagen) of the cloning vector (pCloneHyg1).

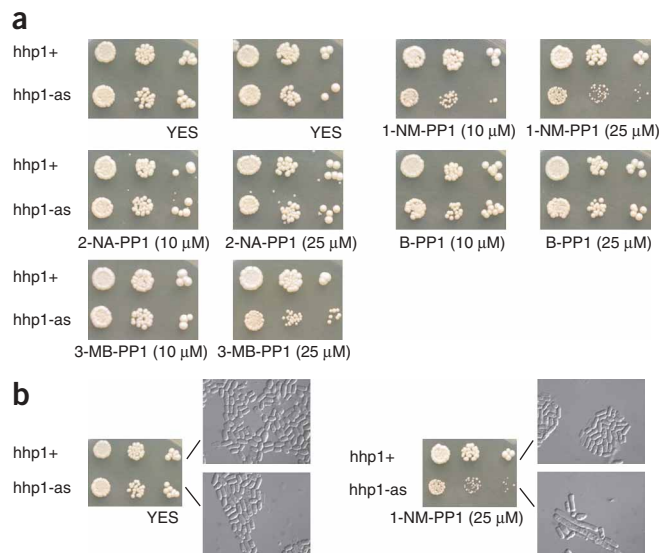
Lithium acetate solution Prepare 0.1 M lithium acetate in 1× TE buffer, pH 7.5.

PEG solution Prepare 40% (wt/vol) PEG 3350 in 1× TE buffer, pH 7.5.

Competent *Escherichia coli* Make transformation-competent *E. coli* DH5α-*pha* using CaCl₂ method³². Alternatively, use commercially available competent *E. coli* cells.

Media Prepare standard 2× bacto-tryptone yeast (TY) medium for *E. coli*. For selection, add ampicillin (100 μg ml⁻¹). For yeast cultivation, prepare standard YES medium supplemented with 0.15g liter⁻¹ adenine and 0.1g liter⁻¹ of each of uracil, L-histidine, L-lysine and L-leucine. For selection, add hygromycin B at 200 μg ml⁻¹.

Primers Dissolve oligonucleotide primers in TE buffer to 100 μM concentration. The oligonucleotides required to amplify the kinase gene are *hhp1BHIterm2*, ATATGGATCCGATTATTAGCAAATGTACTAATAT and *hhp1XhoIprom2*, ATATCTCGAGAATATTATTAGATTTTGTATATAG. The mutagenic oligonucleotides required are h1AntisM84G, GGACCCAATAAATCCCCACCATTAGCGTTG and h1M84G, CAACGCTATGGTGGGGGATTTATTGGGTCC. The oligonucleotides required to check correct integration are *hhp1ATG-*



check2, ATCCACTGCCAATTTTACGACC and *hhp1pchk2*, ACAGCTTTTATTTTCGTCTGAG.

PCR premix Contains 1× PCR buffer (containing 1.5 mM MgCl₂, 0.2 mM of each dNTP mixture, 0.5 μM of each primer, 2 U per 100 μl *Taq* DNA polymerase and template DNA).

PROCEDURE

Identification of gate-keeper residue

1| Search the kinase sequence database (<http://sequoia.ucsf.edu/ksd/>) to identify the gate-keeper residue. The gate-keeper position, which is used to introduce space-creating mutations, is highlighted in red in the sequence. Other residues contacting ATP in the active site are marked green. **Supplementary Table 1** shows *S. pombe* protein kinases whose gate-keeper residue can be identified. The gate-keeper residue can be easily identified from primary sequence alignments.

? TROUBLESHOOTING

Mutating the gate-keeper residue

2| Amplify the *hhp1* gene together with its promoter region using oligonucleotides (*hhp1BHIterm2*, ATATGGATCCGATTATTAGCAAATGTACTAATAT and *hhp1XhoIprom2*, ATATCTCGAGAATATTATTAGATTTTGTATATAG) and high-fidelity DNA polymerase (e.g., *PfuTurbo* DNA polymerase; Stratagene). Use *S. pombe* genomic DNA as a template. Prepare 50 μl of reaction mixture for PCR containing 0.2 μM oligonucleotides, 0.2 mM dNTP mix, about 100 ng template DNA, 1× polymerase reaction buffer and 2.5 U DNA polymerase. Run a PCR: 3 min at 94 °C; 33 cycles of 50 s at 94 °C, 50 s at 50 °C, 90 s at 72 °C; 5 min at 72 °C.

▲ **CRITICAL STEP** The promoter region must contain a unique restriction site that will be used to linearize the construct for integrating it to the genome (Step 9).

3| Clone the *hhp1* into plasmid pCloneHyg1 (see ref. 33) using *XhoI* and *BamHI* restriction sites. The resulting plasmid is pCloneHyg1-*hhp1*. It is possible to use other integrative vectors carrying a suitable selection marker.

4| Verify the cloned *hhp1* gene by sequencing.

5| Design mutagenic oligonucleotide primers according to instructions given in the QuikChange II site-directed mutagenesis kit (Stratagene). It is possible to use a QuikChange primer design program (www.stratagene.com). To introduce mutation met84gly (*hhp1-as*) use oligonucleotides (h1AntisM84G, GGACCCAATAAATCCCCACCATTAGCGTTG and h1M84G, CAACGCTATGGTGGGGGATTTATTGGGTCC).

6| Use a QuikChange II site-directed mutagenesis kit (Stratagene) and the plasmid pCloneHyg1-*hhp1* to mutate the gate-keeper residue according to the manufacturer's instructions. The QuikChange II site-directed mutagenesis kit allows site-specific mutation in virtually any double-stranded plasmid. The expected colony number is between 10 and 1,000 colonies. More than 80% of the *E. coli* colonies should contain the desired mutation. It is also possible to use mutagenesis kits from other manufacturers.

▲ **CRITICAL STEP** Ensure that the plasmid DNA template is isolated from a *dam⁺E. coli* strain (the majority of the commonly used *E. coli* strains are *dam⁺*).

? TROUBLESHOOTING

- 7| Isolate plasmid DNA using QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions. It is possible to use mini-prep kits from other manufacturers (e.g., GeneJET plasmid miniprep kit; Fermentas).
- 8| Sequence the insert of interest (*hhp1* gene) to verify that selected clones contain the desired mutation and do not contain unwanted second-site mutations. The resulting plasmid is pCloneHyg1-*hhp1-as*.

Testing functionality of mutant kinase

- 9| Linearize the plasmid pCloneHyg1-*hhp1-as* with the restriction enzyme *SfuI* and integrate into the *hhp1* promoter of a strain where *hhp1* open reading frame has been deleted. Use the yeast transformation protocol described by Gregan *et al.*³³. As a control, integrate the plasmid pCloneHyg1-*hhp1* containing wild-type *hhp1*.
- 10| Confirm the integration by colony PCR³³ using oligonucleotides (*hhp1ATGcheck2*, ATCCACTGCCAATTTTACGACC and *hhp1pchk2*, ACAGCTTTTATTTTCGCTGAG).
- 11| Analyze the functionality of the *hhp1-as* mutant by comparing it with *hhp1* knockout strain and strain carrying a wild-type *hhp1* allele (use a strain containing *hhp2Δ* mutation). *hhp1Δ hhp2Δ* double knockout mutant grows extremely slowly, forms very small colonies and cells display abnormal morphology (elongated cells). This phenotype can be rescued by introducing a wild-type *hhp1* allele as well as *hhp1-as* mutant (Fig. 4).

? TROUBLESHOOTING

Testing sensitivity of mutant kinase to inhibitors

- 12| Dissolve each candidate inhibitor in dimethyl sulfoxide to 5 mM final concentration.
- 13| Add each candidate inhibitor to autoclaved YES medium containing 2% agar (wt/vol) to 25 μM final concentration.
- 14| Prepare serial dilutions of freshly grown cells expressing *hhp1-as*, wild-type *hhp1* and *hhp1* knockout cells. Grow the cells on YES plates containing 25 μM of each candidate inhibitor for 2 to 3 d at 32 °C. Choose effective and mutant-specific inhibitors for further analysis.

● TIMING

The entire protocol can be completed in about 3 weeks.
 Steps 1–4, cloning of the gene: 3 d
 Steps 5–8, mutagenesis: 5 d
 Steps 9–10, yeast transformation: 5 d
 Step 11, testing functionality: 4 d
 Steps 12–14, testing sensitivity: 4 d

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	The kinase of interest could not be found in the kinase sequence database	The database is incomplete and does not contain this kinase	Align the primary sequence of the kinase to kinases with known gate-keeper, such as PKA, CDK2 or c-Src. Identify its gatekeeper from the alignment
	It is not possible to identify the gate-keeper residue	Atypical kinase, which does not align to typical kinases well	Use multiple sequence alignments of closely related kinases. Take a candidate approach and mutate several amino acid candidates for the gate-keeper residue. Alternatively, use a different approach to inactivate the kinase
6	Low transformation efficiency or low mutagenesis efficiency	Amount of DNA template in the mutagenesis reaction not sufficient	Visualize the DNA template on a gel to verify its quantity and quality. Ensure that sufficient DNA template is used in the mutagenesis reaction. See also the QuikChange II site-directed mutagenesis kit (Stratagene) manual



TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	Low transformation efficiency or low mutagenesis efficiency	Formation of secondary structures may be inhibiting the mutagenesis reaction	Increase the annealing temperature (up to 68 °C). See also the QuikChange II site-directed mutagenesis kit (Stratagene) manual
11	Mutating the gate-keeper to glycine residue results in non-functional protein	A bulky residue is required at the gate-keeper position for the kinase to be functional	Mutate the gate-keeper to some other small residue (e.g., alanine). Alternatively, introduce a second-site suppressor mutation to rescue the kinase activity ²⁷

ANTICIPATED RESULTS

This protocol can be used to engineer protein kinases sensitive to cell-permeable inhibitors. Here we describe the construction of the first analog-sensitive allele of an *S. pombe* kinase (Fig. 4). As the gate-keeper residue can be identified in most protein kinases, we expect that our protocol will also work for other *S. pombe* protein kinases as well as other organisms. Analog-sensitive kinase alleles have been successfully created and studied in various organisms including the budding yeast *Saccharomyces cerevisiae*, mouse and human cells^{11,34,35}.

Note: Supplementary information is available via the HTML version of this article.

ACKNOWLEDGMENTS This work was supported by the Austrian Science Fund (P18955-B03). L.C. was a recipient of EMBO and FEBS short-term fellowships. We thank Mark Petronczki and Maria Siomos for helpful discussions and comments on the manuscript.

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