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Identification of *Ustilago maydis* Aurora Kinase As a Novel Antifungal Target

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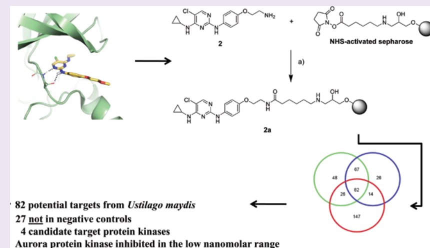
S *Supporting Information*

ABSTRACT: Infestation of crops by pathogenic fungi has continued to have a major impact by reducing yield and quality, emphasizing the need to identify new targets and develop new agents to improve methods of crop protection. Here we present Aurora kinase from the phytopathogenic fungus *Ustilago maydis* as a novel target for N-substituted diaminopyrimidines, a class of small-molecule kinase inhibitors. We show that Aurora kinase is essential in *U. maydis* and that diaminopyrimidines inhibit its activity *in vitro*. Furthermore, we observed an overall good correlation between *in vitro* inhibition of Aurora kinase and growth inhibition of diverse fungi *in vivo*. *In vitro* inhibition assays with Ustilago and human Aurora kinases indicate that some compounds of the N-substituted diaminopyrimidine class show specificity for the Ustilago enzyme, thus revealing their potential as selective fungicides.



Securing global food supply is and will be a major challenge in times of increasing food demand due to changing environments and a growing world population.[1] The Food and Agriculture Organization of the United Nations (FAO) estimates that 1 billion people were undernourished worldwide in 2009, highlighting the need for improved agricultural productivity and increased crop yields. One factor contributing to yield reduction is infestation by harmful organisms such as insects, fungi, and weeds. Between 2001 and 2003, 10% of crop losses were due to fungi and bacteria.[2] Presently, yield losses caused by invasive plant pathogens, most of which are fungi, are estimated at $21 billion per year in the United States alone.[3] Although various effective fungicides are presently available for commercial use and application, frequently emerging resistance requires new strategies to protect crop yield and quality by elucidating novel targets and modes of action.[4]

Protein kinases play an essential role as key mediators in signal transduction in all organisms ranging from simple prokaryotes to plants and humans.[5−8] For instance, deregulated protein kinase activity in humans has causative roles in severe diseases such as cancer, diabetes, and neurological and autoimmune disorders.[9,10]

Numerous small-molecule kinase inhibitors are being employed to inhibit aberrant kinase activity to treat disease in humans[11,12] and have also been suggested as potential agents for fighting human pathogens.[13,14] One of the next steps will be the indication shift of this principle—kinases as targets in pathogens—to aid in crop protection. As kinases have also been found to be essential for invasive hyphal growth and pathogenesis in several phytopathogenic fungi,[15−21] they represent attractive targets for antifungal drug discovery.

In earlier studies, protein kinases of a nonpathogenic fungus have been proposed as potential antifungal targets.[22] Furthermore, the commercial fungicide Fludioxonil has been suggested to interfere with fungal growth partly through improper activation of the MAP kinase cascade.[23] However, definitive proof that targeting protein kinases in phytopathogenic fungi is sufficient to control fungal growth has not been demonstrated so far.

A commonly used model organism for phytopathogenic fungi is *Ustilago maydis*, the cause of corn smut. It is easy to cultivate under laboratory conditions and amenable to genetic manipulation.[24,25] Moreover, its entire genomic sequence is available and well annotated.[26]

In screening campaigns, we identified N-substituted diaminopyrimidines to be inhibitors of fungal growth *in vivo* as well as inhibitors of human protein kinase activity *in vitro*. This class of small-molecule kinase inhibitors are known to possess antifungal

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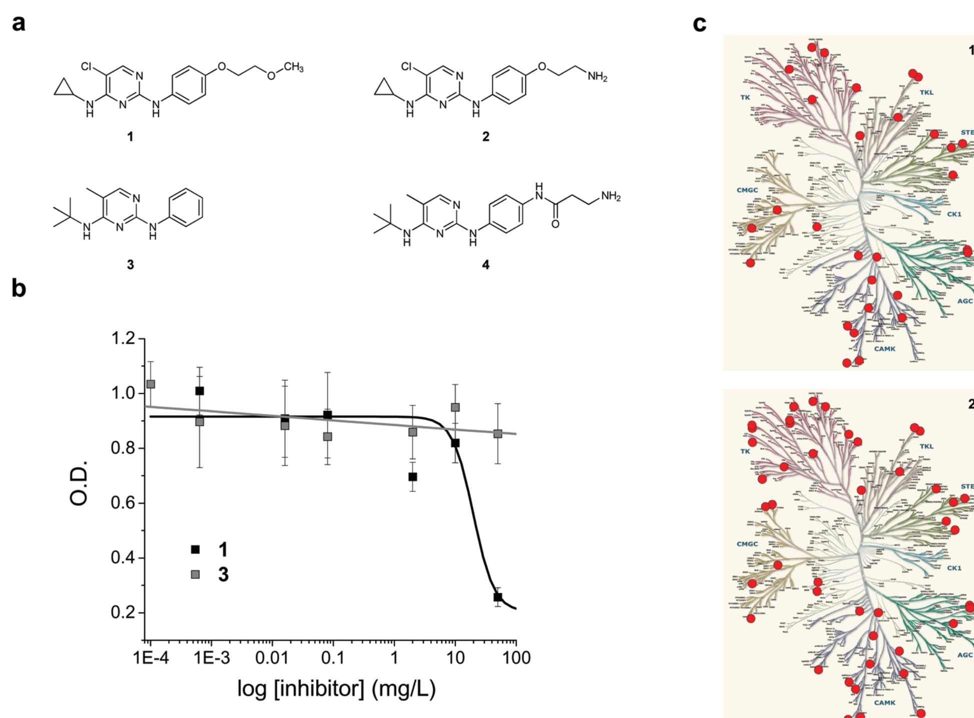

Figure 1. *In vivo* and *in vitro* data of compounds used in this study. (a) **1**: biologically active compound; **2**: derivative of **1** with primary amine for coupling; **3**: free, biologically inactive control; **4**: derivative of **3** with primary amine for coupling. (b) Dose−response curves for *U. maydis* with indicated compounds. OD_{600} was measured after 72 h. (c) Kinase inhibition profiles for compounds **1** and **2**. Red circles indicate kinases that exhibit ≤10% activity at 10 μM inhibitor concentration.

activity[27] and have already been shown to inhibit several human kinases.[28−31] Therefore, the fungal target is suspected to be one or more protein kinases.

Here we report the identification and validation of Aurora kinase from *U. maydis* as a prominent target for N-substituted diaminopyrimidines using affinity chromatography techniques followed by biological characterization. We describe the expression and purification of recombinant Ustilago Aurora kinase and subsequent *in vitro* inhibition studies. Generation of a conditional knockdown strain revealed that Aurora kinase is essential for growth of *U. maydis*, thus validating this kinase as a prominent antifungal target.

RESULTS AND DISCUSSION

Evaluation and Design of Compounds for Pull-Downs. In screening campaigns, we used a library of known kinase inhibitor scaffolds and tested for growth inhibition of Ustilago. Among the class of N-substituted diaminopyrimidines, we identified **1** as a potent inhibitor of fungal growth (ED_{50} = 21.8 ± 6.1 mg L^{-1}, corresponding to 65 μM), whereas its closely related derivative **3** did not show inhibition (Figure 1, panel a and b). To establish a selectivity profile, **1** was screened against a panel of 114 human protein kinases and was found to be a rather nonspecific kinase inhibitor (Figure 1, panel c and Supplementary Figure 1).

We next chose to make use of affinity chromatography by immobilizing inhibitors in order to pull down target proteins from Ustilago lysates. Subsequent mass spectrometry analysis was then used to elucidate the fungal protein targets of **1**.[32] In order to avoid significant changes in the binding properties or affinity of the compound once bound to the affinity matrix, we first determined a suitable labeling strategy prior to affinity chromatography experiments. We docked **1** into the catalytic domain of a prototypical protein kinase and analyzed its most probable binding mode. Our modeling studies suggested that the pyrimidine core as well as the secondary aromatic amine of the inhibitor form hydrogen bonds with the hinge region of the kinase domain. Such interactions would position the ethoxymethoxy substituent outside the ATP binding pocket toward the solvent (Figure 2, panel a), which would qualify this position as an attachment point for immobilization strategies without introducing any steric clashes with the protein.

On the basis of the kinase-inhibitor models, we chose the ethoxy moiety for design and synthesis of probe molecules **2** and **4**, which were derived from compounds **1** and **3**, respectively (Figure 1, panel a). Compounds **2** and **4** were tested for *in vivo* growth inhibition and neither compound was able to inhibit growth of *U. maydis*. Although this was expected for the negative control **4**, the negative result for **2** was unanticipated. However, the primary amine of this compound might be charged under neutral conditions in culture and thus would render the compound membrane impermeable. Interestingly, investigation of the biochemical activity of **2** revealed no loss of inhibitory potency, and we were able to establish an *in vitro* selectivity profile for this compound. Surprisingly, we found that **2** inhibits several of the profiled kinases more potently than **1** (Figure 1, panel c and Supplementary Figure 1).

These findings and the observation that the methoxy group of **1** can be replaced by a broad range of moieties without significant loss of inhibitory activity suggested that **2** and **4** can be coupled to preactivated sepharose beads *via* their primary amine without disrupting the binding of potential kinase targets.

Target Identification from Cell Lysates. In order to prepare Ustilago cell extracts for a large number of reproducible affinity

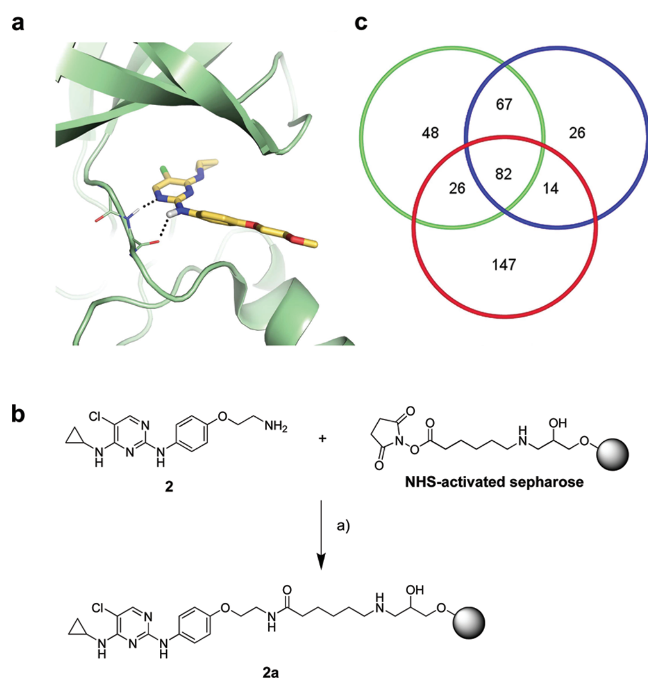

Figure 2. Strategy for affinity chromatography. (a) Model of **1** in complex with the ATP pocket of a protein kinase. The inhibitor adopts a binding mode similar to VX-680 (PDB code 3E5A), an ATP-competitive Aurora inhibitor that also carries a cyclopropyl moiety as well as a pyrimidine core. The site of linker attachment is pointing out of the ATP-binding pocket suggesting that coupling to the affinity matrix will not disrupt kinase binding. (b) Schematic representation of the coupling reaction of **2** to NHS-activated sepharose. (c) Venn diagram of proteins identified in three independent affinity chromatography experiments using **2a**. Each circle represents one experiment, numbers represent identified proteins. Numbers in intersection were considered potential targets.

chromatography experiments, *U. maydis* was grown in a fermenter and harvested while still in the exponential growth phase. We reasoned that most proteins would likely be expressed during that stage of growth; however, it should be noted that not all proteins, such as factors specifically expressed during mating or plant infection,[33] might be present at this stage. Since several grams of *U. maydis* cells were found to be optimal for mass spectroscopic analysis, experiments with material obtained during mating or even plant infection were not feasible due to comparably very low yield of cells. For pull-down experiments, Ustilago lysates containing 60 mg of soluble protein were first incubated with either inhibitor beads **2a** (Figure 2, panel b) or control beads **4a**. Bound proteins were eluted with free compound in the presence of ATP and $MgCl_2$ as this was shown to facilitate the elution of protein kinases.[34] Eluted proteins were then precipitated and separated by standard SDS−PAGE, and Coomassie staining revealing numerous weakly resolved bands. In order not to overlook proteins that are low in abundance, the entire lane of the gel, instead of individual bands, was excised and analyzed by mass spectrometry to elucidate the fungal protein targets of **2a.**[32] In three independent affinity purifications with active inhibitor beads, a total of 410 proteins were identified, of which 82 were found in all three experiments (Figure 2, panel c). Furthermore, 55 of these were also found in one or more of three control experiments using **4a** and were therefore excluded from the analysis. Among the remaining 27 candidate target proteins,

Table 1. Inhibition Data for Diaminopyrimidines

compound	IC_{50} (μM)		
	GSK3	Kpp2	UmAurora/-UmINCENP$^{1424-1575}$
1	23.4 ± 9.0	33.9 ± 15.7	0.0059 ± 0.0014
2	29.9 ± 8.8	7.5 ± 2.9	0.0139 ± 0.0036
3	>50	36.9 ± 20.3	0.09 ± 0.0334
4	>50	>50	0.0698 ± 0.0265
staurosporine[a]	0.33 ± 0.27	1.0 ± 0.5	0.0021 ± 0.0003

[a] Used as positive control.

four protein kinases were identified which are annotated by the MIPS *U. maydis* DataBase (http://mips.helmholtz-muenchen.de/genre/proj/ustilago): um00560 (probable glycogen synthase kinase 3 alpha, GSK3), um06363 (related to APG1, essential for autophagocytosis, also termed ATG1[35]), um03305 (MAP Kinase Kpp2), and um10119 (probable IPL1, Ser/Thr protein kinase). The remainder of identified candidate targets consisted mostly of abundant housekeeping proteins such as ribosomal proteins and several uncharacterized proteins with yet unknown function (Supplementary Table 1). Since N-substituted diaminopyrimidines have been described as kinase inhibitors in the literature[28−31] and profiling of our compounds revealed that they inhibit a wide range of human kinases, we speculated that the major target responsible for growth inhibition is likely to be a protein kinase and thus chose to focus on the identified kinases in *U. maydis*.

Biochemical Inhibition Studies. GSK3 from *U. maydis* was evaluated as a target by analyzing the inhibitory effect of compounds **1**−**4** on the *in vitro* activity of GSK3 purified as His fusion protein from *E. coli*. GSK3 was active in a phosphorylation assay (Z′Lyte-Assay (Invitrogen)) and was weakly inhibited by compounds **1** and **2**, whereas inhibition by control compounds **3** and **4** was >50 μM (Table 1). Together with the relatively potent growth inhibition of *U. maydis* by **1** (ED_{50} = 21.8 mg L^{-1}, corresponding to 65 μM), this suggests that GSK3 is a target of the N-substituted diaminopyrimidines, albeit not the most relevant target. To evaluate the effects of **1** on the remaining three target kinase candidates, all were expressed as MBP (maltose binding protein) fusions in *E. coli*, purified, and tested for activity in a radioactive phosphorylation assay using myelin basic protein as the kinase substrate as previously employed for *U. maydis* protein kinases.[15] The serine/threonine kinase ATG1 could not be isolated in an enzymatically active form due to instability and degradation of the protein. However, since ATG1 deletion mutants are viable and only weakly affected in growth rate,[35] we reason that this protein is not a prominent target for our compounds. The MBP fusion of Kpp2 efficiently phosphorylated myelin basic protein as previously described[15] and was employed for IC_{50} determinations. Surprisingly, compound **2** inhibited Kpp2 with a significantly lower IC_{50} than compound **1**, while negative control compound **3** showed an inhibition similar to that of compound **1** (Table 1). This might be an indication of a different binding mode of **1** and **2** to Kpp2, since the exchange of the methoxy moiety for a primary amine increases inhibitor affinity, which could also be observed for several human kinases (Figure 1, panel c and Supplementary Figure 1). Still, the observed IC_{50} values in the micromolar range suggest that inhibition of Kpp2 is also not the main factor responsible for the potent growth inhibition by **1**.

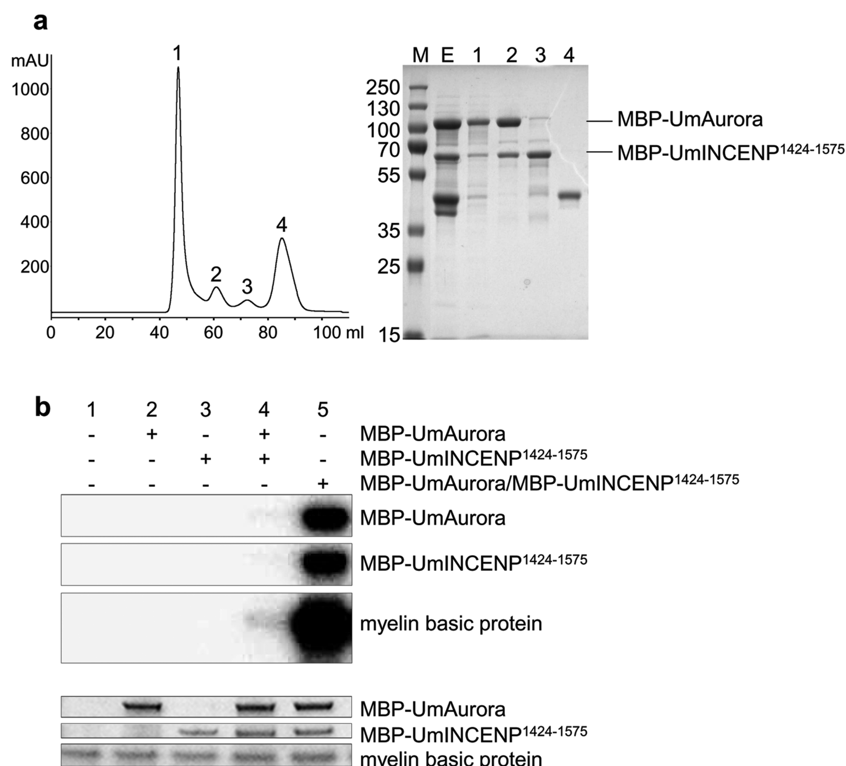

Figure 3. Purification and activity assay of MBP-UmAurora and UmINCENP$^{1424-1575}$. (a) Gel filtration purification of MBP-UmAurora/MBP-UmINCENP$^{1424-1575}$ complex. Left panel: chromatogram of gel filtration run. Four distinct peaks could be observed. Right panel: Coomassie gel of gel filtration run. M: Marker; E: combined eluates from previous affinity chromatography; 1−4: peak fractions eluted from gel filtration column. Fraction 1 contains oligomeric MBP-UmAurora/MBP-UmINCENP$^{1424-1575}$ complex, fraction 2 contains the monomeric complex, fraction 3 contains MBP-UmINCENP$^{1424-1575}$ alone, and fraction 4 contains an impurity (presumably MBP tag). (b) Radioactive MBP-UmAurora activity assay. Purified MBP-UmAurora and/or MBP-UmINCENP$^{1424-1575}$ was incubated with [γ-^{32}P]ATP and myelin basic protein as substrate. The sample was then separated by standard SDS−PAGE, exposed to film, and subsequently stained with Coomassie to visualize all protein. Upper panel: Radioactive signal. Lower panel: Coomassie stain. 1: negative control containing no enzyme. Neither MBP-UmAurora (2) nor UmMBP-INCENP$^{1424-1575}$ (3) alone is able to phosphorylate the substrate, while combination of both proteins in the assay leads to weak substrate- and autophosphorylation (4). Co-purification of MBP-UmAurora and MBP-UmINCENP$^{1424-1575}$ yields a highly active kinase/activator complex with strong activity toward both substrate and itself (5).

The gene product of um10119 has not yet been described but is annotated by MIPS as probable Aurora kinase due to strong sequence similarities to Aurora kinases from several organisms. A BLAST search with the amino acid sequence of um10119 revealed Aurora B kinase Ark1 from *S. pombe*[36] as the closest homologue (E value 5×10^{-99}). The purified MBP fusion of um10119, subsequently referred to as UmAurora, did not phosphorylate myelin basic protein; however, it has been reported for other Aurora B kinases that the IN-box of Aurora-interacting protein INCENP is required for full activity binding.[37−39]

We therefore performed a BLAST search against *U. maydis* proteins with the INCENP/Pic1 sequence from *S. pombe* as the query sequence and identified the uncharacterized protein um03367 as a weak hit (E value 2×10^{-14}). Despite its low sequence homology, um03367 was further investigated as it contains a C-terminal INCENP domain as predicted by NCBI's conserved domain search. A ClustalW alignment of um03367 and the INCENP domain (residues 925−972) from Pic1 revealed several conserved residues in this domain starting with Tyr926 in Pic1 and Tyr1424 in um03367, respectively. Since residues 925−972 of Pic1 are necessary and sufficient for Aurora binding,[36] we chose to clone and express residues 1424−1575 of um03367, subsequently referred to as UmINCENP$^{1424-1575}$, as a MBP fusion protein. As described for *X. laevis* Aurora B, binding of INCENP induces a conformational change in the kinase domain leading to its full activity.[39] We therefore reasoned that it might be advantageous to co-purify separately expressed MBP-UmAurora and MBP-UmINCENP$^{1424-1575}$ to facilitate kinase activation (Figure 3, panel a). Indeed we found that the co-purified MBP-UmAurora/MBP-UmINCENP$^{1424-1575}$ complex displayed strong kinase activity with respect to substrate phosphorylation and UmAurora autophosphorylation (Figure 3, panel b, lane 5). Conversely, separately purified MBP-UmAurora and MBP-UmINCENP$^{1424-1575}$ combined for the radioactive assay resulted in weak substrate and autophosphorylation activity (Figure 3, panel b, lane 4). We furthermore observed that the yield of soluble MBP-UmAurora protein is significantly higher when co-purified with MBP-UmINCENP$^{1424-1575}$, indicating that the activator protein helps to stabilize the kinase domain and ensures proper folding (Supplementary Figure 2). We thus conclude that INCENP binding induces a conformational shift, as has been shown for *X. laevis* Aurora B.[39] This process is given substantially more time during co-purification of the proteins, thus leading to a greater portion of correctly folded, active kinase. The co-purified MBP-UmAurora/MBP-UmINCENP$^{1424-1575}$ complex was subsequently tested in phosphorylation assays (IMAP fluorescence polarization assay). Compound **1** strongly inhibited kinase activity with an IC_{50} of 5.9 nM, and only a small

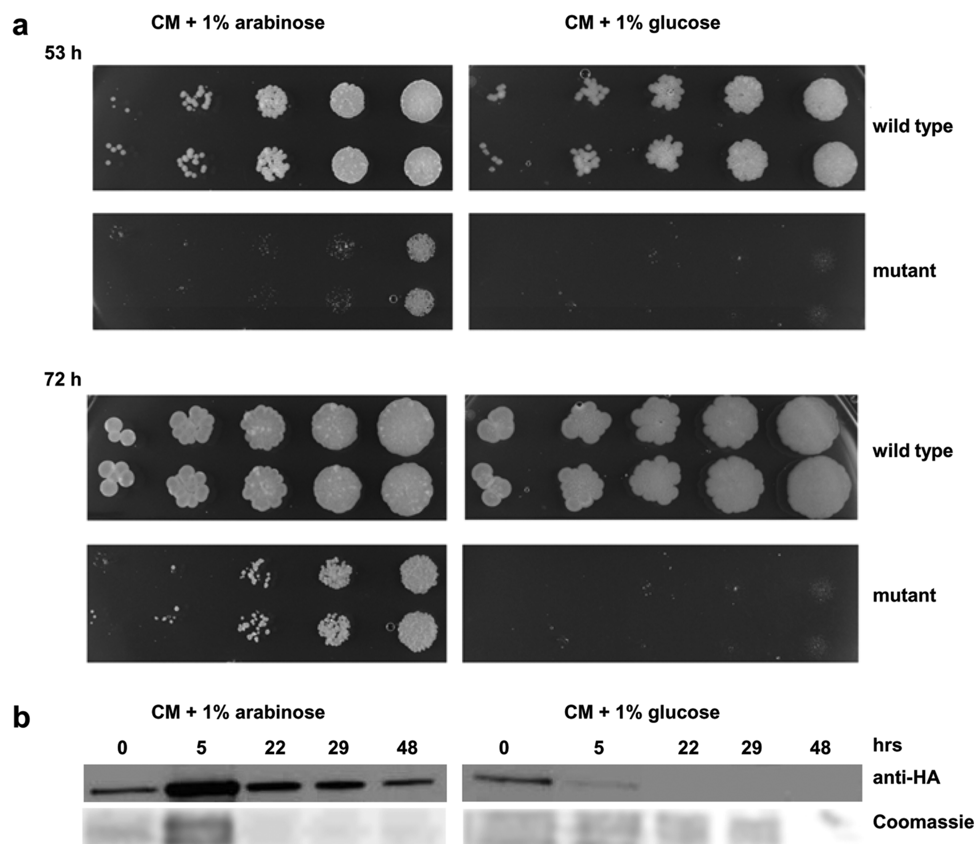

Figure 4. Growth of Ustilago mutant strain with induced and repressed UmAurora expression. (a) Five-fold dilutions of Ustilago liquid cultures were spotted in duplicates on agar plates containing arabinose or glucose, and pictures were taken after 53 and 72 h of growth. On plates containing *crg1*-inducing arabinose the mutant strain exhibited a slight growth defect in comparison to wild type. No growth of the mutant strain was observed on plates containing *crg1*-repressing glucose. (b) UmAurora down-regulation was monitored by Western blot analysis. Upper panel: chemiluminescent signal. Lower panel: Coomassie stain of gel after blotting.

fraction of activity was lost by the exchange of the methoxy for a primary amine in **2**. Although the negative controls **3** and **4** also show inhibition of kinase activity, a significant loss of potency was observed (Table 1). These data suggest UmAurora as a major target of the N-substituted diaminopyrimidines used in this study.

Effects of Aurora Down-regulation in *U. maydis*. Aurora kinase is essential in numerous fungi such as *S. cerevisiae*,[40] *S. pombe*,[41] or *C. albicans*.[42] This suggested that its loss is also lethal in *U. maydis*. To investigate the effects of lost UmAurora function, we generated a mutant Ustilago strain carrying UmAurora under the control of the glucose-repressible *crg1* promoter[43] with simultaneous loss of UmAurora under its natural promoter. Successful integration of P_{crg1}:UmAurora and replacement of wild-type UmAurora by a hygromycin resistance cassette was verified by PCR and Southern blot (Supplementary Figure 3). Mutant cells were unable to grow in glucose-containing medium, while exhibiting only a slight growth defect in *crg1*-inducing medium containing arabinose (Figure 4, panel a). Down-regulation of UmAurora expression was verified by Western blot analysis. No UmAurora could be detected after 22 h in glucose-containing medium (Figure 4, panel b)

Comparative Aurora Inhibition Studies. To compare the effects of the inhibitor class on Aurora inhibition and inhibition of *in vivo* growth, we determined IC_{50} values for a subset of related compounds that had previously been analyzed for antifungal activity. *In vivo* data were plotted against pI_{50} values (−log IC_{50}) and demonstrated a strong correlation between antifungal effects and inhibition of UmAurora activity (Figure 5, panel a). As product safety is one essential when developing new antifungal agents, we further investigated the selectivity of various N-substituted diaminopyrimidines for Ustilago Aurora kinase in comparison to the human isoforms and thus tested our subset against human Aurora A and B. Most compounds show no selectivity toward either human or Ustilago Aurora; however, we did identify compounds with selectivity toward the human or Ustilago kinase (Figure 5, panel b), which indicates the possibility of developing a fungus-specific Aurora kinase inhibitor.

Co-crystal Structure of 1 with Human Aurora A. To validate our early modeling efforts and to get deeper insights into the binding mode of the inhibitors used in this study, we successfully co-crystallized human Aurora A in complex with **1**. The inhibitor resides in a typical Type I (ATP-competitive) binding orientation and forms hydrogen bonds to the hinge region of the kinase domain, analogous to those previously reported for close structural analogues (PDB codes: 2VGO, 1Z5M, and 2NP8). Briefly, the chloro substituent faces the gatekeeper residue (Leu210) while the cyclopropyl moiety points away from the hinge region toward the substrate binding site. The ethoxy-methoxy group extends toward the solvent, confirming the results of our earlier modeling studies used to determine a tethering point on these compounds for bead attachment. In addition, protein X-ray crystallography using the diaminopyrimidines discussed in this

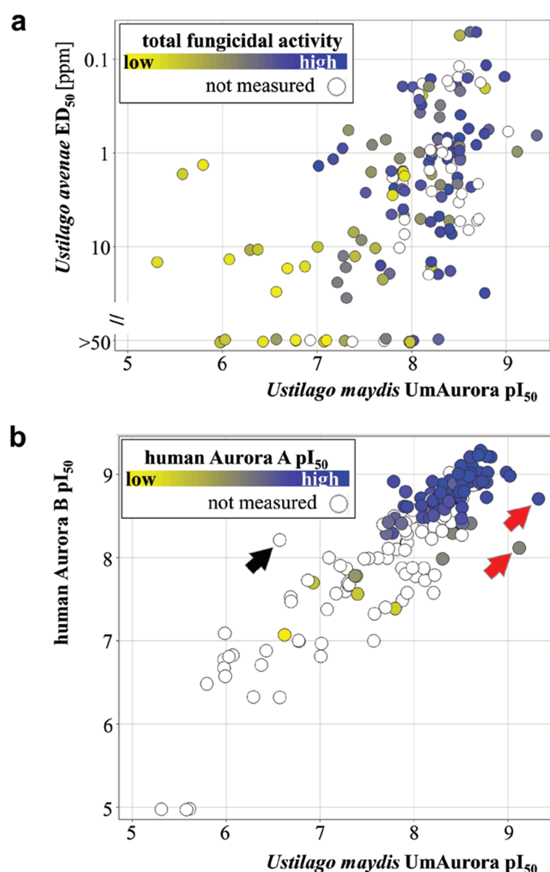

Figure 5. Correlation of inhibition data to *in vivo* observations. (a) Correlation of growth inhibition of *U. avenae* versus *in vitro* inhibition of *U. maydis* UmAurora. Total antifungal activity is represented by color coding. *U. avenae* is a close relative of *U. maydis* and is used in high-throughput screening of inhibitors. Total antifungal activity is expressed as a weighted mean of numerical integrals over dose−response data from diverse phytopathogenic fungi both in liquid culture and on plants (H. Rieck, Th. Pötter, M. E. Beck, unpublished results). Larger numbers mean higher activity. Various diaminopyrimidine compounds spanning a broad range of chemical diversity and biological activity[27] were tested and revealed a good correlation between UmAurora inhibition, growth inhibition of *U. avenae*, and total antifungal activity. (b) Correlation of inhibition of human Aurora kinases A (color), B versus Ustilago UmAurora. Inhibition is shown as pI_{50} values (−log IC_{50}); red arrowheads: compounds showing specificity toward UmAurora; black arrowhead: compound showing selectivity toward human Aurora B.

study may provide crucial insights to support the development of more fungus-specific compounds.

Conclusion. We here present fungal Aurora kinase as a novel target protein in crop protection, although further efforts are needed to reach selectivity toward the fungal kinase over the human isoforms. Yet, the feasibility of targeting highly conserved proteins with concomitant specificity for only a certain class of organisms has already been demonstrated by the success of the widely used insecticide imidacloprid: belonging to the class of neonicotinoids, it targets the highly conserved nicotinic acetylcholine receptors of insects and mammals. Yet the neonicotinoids display an almost 500-fold selectivity toward insects over rats.[44] Therefore, we anticipate that improving our compounds toward a more fungus-specific Aurora inhibitor will initiate the development of a novel fungicide.

METHODS

Cultivation of *U. maydis* and Generation Knockdown Strain. *General Growth Conditions. U. maydis* strains 521 or 518[45] were grown at 28 °C in liquid CM[46] or potato dextrose (PD) medium on a rotary shaker at 200 rpm or on solid agar. For repression of *crg1* promoter activity, strains were grown in CM medium containing 1% arabinose, washed with water, and resuspended in CM medium with 1% glucose.

Growth of U. maydis for Affinity Chromatography Experiments. U. maydis strain 518 was grown in CM in a 100 L fermenter at 28 °C to a final $OD_{600} \approx 30$ while still in the logarithmic phase. Oxygen content was monitored and kept at ≥75% through stirring and air flow adjustment. Cells were harvested and stored in aliquots made of 20 g cells (wet weight) at −80 °C.

Generation of Ustilago UmAurora Knockdown Strain. To express UmAurora under the control of the glucose-repressible/arabinose-inducible *crg1* promoter in *U. maydis*, full-length UmAurora (um10119, as annotated by MIPS *U. maydis* DataBase) was amplified from DNA by PCR with primers introducing an EcoO109I and NdeI site at the 5′ terminus (GCCCCATATGGAGTCGCAGCTCGCCAACT) and an AbsI site at the 3′ terminus (GGGACTGAATTTTGCC-ACGAGACG). The PCR product was cloned *via* EcoO109I and AbsI into pHA-Hyg to attach a 3X-HA tag to UmAurora. The UmAurora-3X-HA fragment was excised using NdeI and MaubI and ligated with NdeI/MaubI digested pRU11.[47] The resulting plasmid pRU11_UmAurora contained UmAurora with a 3X-HA tag under the control of the *crg1* promoter and was transformed into *U. maydis* strain 521 as described previously.[48] Endogenous UmAurora was removed using a PCR-based gene replacement method as described earlier.[49] Regions of ∼1 kb flanking the UmAurora locus were amplified with primers LBfor (5′GCTTTGCGGGGGCGATTACAT) and LBrev (5′GACGGTTA-TACGTGTGAGGGCA), RBfor (5′TCCTTGTTACTCGCACCCT-TCTCT) and RBrev (5′GCTTTGTACCCGCCGCTTCC) and fused to a hygromycin B (Hyg) resistance cassette. From the ligation product two overlapping fragments were amplified using primers LBnested (5′ACCGTTCTGTTTTCCAAGTTT) and NLC38 (5′CGTTG-CAAGACCTGCCTGAA), NLC37 (5′GGATGCCTCCGCTCGA-AGTA) and RBnested (5′AAGGCCAATGAAGACCAAGCAGTT). A 2.5 μg portion of each product was transformed into *U. maydis* 521 carrying P_{crg1}: UmAurora-3X-HA. Successful integration was confirmed by PCR analysis. Southern analysis using DIG-labeled UmAurora as a probe was used to confirm both integration of P_{crg1}:UmAurora-3X-HA as well as replacement of endogenous UmAurora by the Hyg resistance cassette.

Inhibitor Affinity Chromatography. *Preparation of the U. maydis Cell Lysate.* Cells (∼20 g wet weight) were resuspended in buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM $Na_4P_2O_7$, 0.2 mM DTT with Complete EDTA-free protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche) and lysed by several passes through a high pressure homogenizer. Lysates were precleared by centrifugation and adjusted to 1.15 M NaCl.

Affinity Chromatography. Inhibitor beads **2a** and **4a**, respectively, were equilibrated in lysis buffer containing 1.15 M NaCl. For each affinity chromatography experiment, 25 μL of beads was incubated with 60 mg (equivalent to 3−4 g of *U. maydis* cells) of high-salt lysate for 2 h at 4 °C. The beads were washed twice with 500 μL of lysis buffer containing 1.15 M NaCl and once with 500 μL of buffer containing 20 mM Hepes (pH 7.5), 150 mM NaCl, 0.25% Triton X-100, 1 mM EDTA, 1 mM EGTA. Bound proteins were eluted in 100 μL of the same buffer containing 0.5 mM free compound **2** or **4**, respectively, 2.5% DMSO, 10 mM ATP, and 20 mM $MgCl_2$ by slow rotation for 2 h at 4 °C. The supernatant was removed and precipitated as described previously.[50]

Preparative Gel Electrophoresis. Precipitated proteins were dissolved in 1X LDS Sample Buffer (Invitrogen) containing 10 mM DTT, heated

to 70 °C for 10 min, and separated on a 4−12% Bis-Tris gel (Invitrogen) for 10−15 min at 200 V. Lanes with Coomassie-stained proteins were divided into 3 equal pieces, and each was individually subjected to analysis by mass spectrometry (see Supplementary Methods).

ASSOCIATED CONTENT

S Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

Accession Codes

Human Aurora A in complex with **1**: PDB-ID code 3QBN.

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Author Contributions

S.T. performed compound coupling and affinity chromatography experiments, overexpressed and purified the kinases, identified and purified the UmINCENP protein, measured activity and determined IC_{50} values, and prepared the Ustilago mutant strain. J.N.G. synthesized the compounds. P.J. and A.B. performed mass spectrometry measurements and data analysis. C.G. overexpressed and purified GSK3 and solved the human Aurora cocrystal structure. J.R.S. expressed, purified, and crystallized human Aurora. O.G. prepared structural models and sequence alignment of Aurora kinases. M.E.B. directed and supervised computational studies at Bayer CropScience. K.T. analyzed inhibition data using Spotfire software. The manuscript was prepared by S.T. with assistance by C.G. and all coauthors. D.R. directed the research and designed the experiments done at the Chemical Genomics Centre. P.H.S. proposed and initiated this study and directed research done by S.T. at Bayer CropScience.

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REFERENCES

(1) Rosegrant, M. W., and Cline, S. A. (2003) Global food security: challenges and policies. *Science 302*, 1917–1919.

(2) Oerke, E.-C. (2006) Crop losses to pests. *J. Agric. Sci. 144*, 31–43.

(3) Rossman, A. Y. (2009) The impact of invasive fungi on agricultural ecosystems in the United States. *Biol. Invasions 11*, 97–107.

(4) Ishii, H. (2006) Impact of fungicide resistance in plant pathogens on crop disease control and agricultural environment. *Jpn. Agric. Res. Q. 40*, 205–211.

(5) Ulrich, L. E., Koonin, E. V., and Zhulin, I. B. (2005) One-component systems dominate signal transduction in prokaryotes. *Trends Microbiol. 13*, 52–56.

(6) Elion, E. A., Qi, M., and Chen, W. (2005) Signal transduction. Signaling specificity in yeast. *Science 307*, 687–688.

(7) Stone, J. M., and Walker, J. C. (1995) Plant protein kinase families and signal transduction. *Plant Physiol. 108*, 451–457.

(8) Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science 298*, 1912–1934.

(9) Blume-Jensen, P., and Hunter, T. (2001) Oncogenic kinase signalling. *Nature 411*, 355–365.

(10) Gschwind, A., Fischer, O. M., and Ullrich, A. (2004) The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat. Rev. Cancer 4*, 361–370.

(11) Fabbro, D., Ruetz, S., Buchdunger, E., Cowan-Jacob, S. W., Fendrich, G., Liebetanz, J., Mestan, J., O'Reilly, T., Traxler, P., Chaudhuri, B., Fretz, H., Zimmermann, J., Meyer, T., Caravatti, G., Furet, P., and Manley, P. W. (2002) Protein kinases as targets for anticancer agents: from inhibitors to useful drugs. *Pharmacol. Ther. 93*, 79–98.

(12) Cohen, P. (2009) Targeting protein kinases for the development of anti-inflammatory drugs. *Curr. Opin. Cell Biol. 21*, 317–324.

(13) Deng, X., Nagle, A., Wu, T., Sakata, T., Henson, K., Chen, Z., Kuhen, K., Plouffe, D., Winzeler, E., Adrian, F., Tuntland, T., Chang, J., Simerson, S., Howard, S., Ek, J., Isbell, J., Tully, D. C., Chatterjee, A. K., and Gray, N. S. (2010) Discovery of novel 1*H*-imidazol-2-yl-pyrimidine-4,6-diamines as potential antimalarials. *Bioorg. Med. Chem. Lett. 20*, 4027–4031.

(14) Krosky, P. M., Baek, M. C., and Coen, D. M. (2003) The human cytomegalovirus UL97 protein kinase, an antiviral drug target, is required at the stage of nuclear egress. *J. Virol. 77*, 905–914.

(15) Mueller, P., Weinzierl, G., Brachmann, A., Feldbruegge, M., and Kahmann, R. (2003) Mating and pathogenic development of the smut fungus *Ustilago maydis* are regulated by one mitogen-activated protein kinase cascade. *Eukaryotic Cell 2*, 1187–1199.

(16) Banuett, F. (1998) Signalling in the yeasts: an informational cascade with links to the filamentous fungi. *Microbiol. Mol. Biol. Rev. 62*, 249–274.

(17) Xu, J. R. (2000) Map kinases in fungal pathogens. *Fungal Genet. Biol. 31*, 137–152.

(18) Castillo-Lluva, S., Alvarez-Tabares, I., Weber, I., Steinberg, G., and Perez-Martin, J. (2007) Sustained cell polarity and virulence in the phytopathogenic fungus *Ustilago maydis* depends on an essential cyclin-dependent kinase from the Cdk5/Pho85 family. *J. Cell Sci. 120*, 1584–1595.

(19) Di Stasio, M., Brefort, T., Mendoza-Mendoza, A., Muench, K., and Kahmann, R. (2009) The dual specificity phosphatase Rok1 negatively regulates mating and pathogenicity in *Ustilago maydis*. *Mol. Microbiol. 73*, 73–88.

(20) Liu, X. H., Lu, J. P., Zhang, L., Dong, B., Min, H., and Lin, F. C. (2007) Involvement of a Magnaporthe grisea serine/threonine kinase gene, MgATG1, in appressorium turgor and pathogenesis. *Eukaryotic Cell 6*, 997–1005.

(21) Jenczmionka, N. J., Maier, F. J., Losch, A. P., and Schaefer, W. (2003) Mating, conidiation and pathogenicity of Fusarium graminearum, the main causal agent of the head-blight disease of wheat, are regulated by the MAP kinase gpmk1. *Curr. Genet. 43*, 87–95.

(22) Pillonel, C. (2005) Evaluation of phenylaminopyrimidines as antifungal protein kinase inhibitors. *Pest Manage. Sci. 61*, 1069–1076.

(23) Kojima, K., Takano, Y., Yoshimi, A., Tanaka, C., Kikuchi, T., and Okuno, T. (2004) Fungicide activity through activation of a fungal signalling pathway. *Mol. Microbiol. 53*, 1785–1796.

(24) Brefort, T., Doehlemann, G., Mendoza-Mendoza, A., Reissmann, S., Djamei, A., and Kahmann, R. (2009) *Ustilago maydis* as a pathogen. *Annu. Rev. Phytopathol. 47*, 423–445.

(25) Steinberg, G., and Perez-Martin, J. (2008) *Ustilago maydis*, a new fungal model system for cell biology. *Trends Cell Biol. 18*, 61–67.

(26) Kaemper, J., Kahmann, R., Boelker, M., Ma, L. J., Brefort, T., Saville, B. J., Banuett, F., Kronstad, J. W., Gold, S. E., Mueller, O., Perlin, M. H., Woesten, H. A., de Vries, R., Ruiz-Herrera, J., Reynaga-Pena, C. G., Snetselaar, K., McCann, M., Perez-Martin, J., Feldbruegge, M., Basse, C. W., Steinberg, G., Ibeas, J. I., Holloman, W., Guzman, P., Farman, M., Stajich, J. E., Sentandreu, R., Gonzalez-Prieto, J. M., Kennell, J. C., Molina, L., Schirawski, J., Mendoza-Mendoza, A., Greilinger, D., Muench, K., Roessel, N., Scherer, M., Vranes, M., Ladendorf, O., Vincon, V., Fuchs, U., Sandrock, B., Meng, S., Ho, E. C., Cahill, M. J., Boyce, K. J., Klose, J., Klosterman, S. J., Deelstra, H. J., Ortiz-Castellanos, L., Li, W., Sanchez-Alonso, P., Schreier, P. H., Haeuser-Hahn, I., Vaupel, M., Koopmann, E., Friedrich, G., Voss, H., Schluter, T., Margolis, J., Platt, D., Swimmer, C., Gnirke, A., Chen, F., Vysotskaia, V., Mannhaupt, G., Gueldener, U., Muensterkoetter, M., Haase, D., Oesterheld, M., Mewes, H. W., Mauceli, E. W., DeCaprio, D., Wade, C. M., Butler, J., Young, S., Jaffe, D. B., Calvo, S., Nusbaum, C., Galagan, J., and Birren, B. W. (2006) Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature 444*, 97–101.

(27) Greul, J. N., Gaertzen, O., Dunkel, R., Mattes, A., Hillebrand, S., Wachendorff-Neumann, U., Dahmen, P., Voerste, A., Schreier, P., Coqueron, P.-Y. (2008) Diaminopyrimidines as fungicides, International Patent WO/2008/107096.

(28) Jani, J. P., Arcari, J., Bernardo, V., Bhattacharya, S. K., Briere, D., Cohen, B. D., Coleman, K., Christensen, J. G., Emerson, E. O., Jakowski, A., Hook, K., Los, G., Moyer, J. D., Pruimboom-Brees, I., Pustilnik, L., Rossi, A. M., Steyn, S. J., Su, C., Tsaparikos, K., Wishka, D., Yoon, K., and Jakubczak, J. L. (2010) PF-03814735, an orally bioavailable small molecule aurora kinase inhibitor for cancer therapy. *Mol. Cancer Ther. 9*, 883–894.

(29) Mesguiche, V., Parsons, R. J., Arris, C. E., Bentley, J., Boyle, F. T., Curtin, N. J., Davies, T. G., Endicott, J. A., Gibson, A. E., Golding, B. T., Griffin, R. J., Jewsbury, P., Johnson, L. N., Newell, D. R., Noble, M. E., Wang, L. Z., and Hardcastle, I. R. (2003) 4-Alkoxy-2,6-diaminopyrimidine derivatives: inhibitors of cyclin dependent kinases 1 and 2. *Bioorg. Med. Chem. Lett. 13*, 217–222.

(30) Lum, C., Kahl, J., Kessler, L., Kucharski, J., Lundstrom, J., Miller, S., Nakanishi, H., Pei, Y., Pryor, K., Roberts, E., Sebo, L., Sullivan, R., Urban, J., and Wang, Z. (2008) 2,5-Diaminopyrimidines and 3,5-disubstituted azapurines as inhibitors of glycogen synthase kinase-3 (GSK-3). *Bioorg. Med. Chem. Lett. 18*, 3578–3581.

(31) Argiriadi, M. A., Ericsson, A. M., Harris, C. M., Banach, D. L., Borhani, D. W., Calderwood, D. J., Demers, M. D., Dimauro, J., Dixon, R. W., Hardman, J., Kwak, S., Li, B., Mankovich, J. A., Marcotte, D., Mullen, K. D., Ni, B., Pietras, M., Sadhukhan, R., Sousa, S., Tomlinson, M. J., Wang, L., Xiang, T., and Talanian, R. V. (2010) 2,4-Diaminopyrimidine MK2 inhibitors. Part I: Observation of an unexpected inhibitor binding mode. *Bioorg. Med. Chem. Lett. 20*, 330–333.

(32) Daub, H. (2005) Characterisation of kinase-selective inhibitors by chemical proteomics. *Biochim. Biophys. Acta 1754*, 183–190.

(33) Basse, C. W., Stumpferl, S., and Kahmann, R. (2000) Characterization of a *Ustilago maydis* gene specifically induced during the biotrophic phase: evidence for negative as well as positive regulation. *Mol. Cell. Biol. 20*, 329–339.

(34) Godl, K., Wissing, J., Kurtenbach, A., Habenberger, P., Blencke, S., Gutbrod, H., Salassidis, K., Stein-Gerlach, M., Missio, A., Cotten, M., and Daub, H. (2003) An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. *Proc. Natl. Acad. Sci. U.S.A. 100*, 15434–15439.

(35) Nadal, M., and Gold, S. E. (2010) The autophagy genes ATG8 and ATG1 affect morphogenesis and pathogenicity in *Ustilago maydis*. *Mol. Plant Pathol. 11*, 463–478.

(36) Leverson, J. D., Huang, H. K., Forsburg, S. L., and Hunter, T. (2002) The Schizosaccharomyces pombe aurora-related kinase Ark1 interacts with the inner centromere protein Pic1 and mediates chromosome segregation and cytokinesis. *Mol. Biol. Cell 13*, 1132–1143.

(37) Kang, J., Cheeseman, I. M., Kallstrom, G., Velmurugan, S., Barnes, G., and Chan, C. S. (2001) Functional cooperation of Dam1, Ipl1, and the inner centromere protein (INCENP)-related protein Sli15 during chromosome segregation. *J. Cell Biol. 155*, 763–774.

(38) Honda, R., Koerner, R., and Nigg, E. A. (2003) Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis. *Mol. Biol. Cell 14*, 3325–3341.

(39) Sessa, F., Mapelli, M., Ciferri, C., Tarricone, C., Areces, L. B., Schneider, T. R., Stukenberg, P. T., and Musacchio, A. (2005) Mechanism of Aurora B activation by INCENP and inhibition by hesperadin. *Mol. Cell 18*, 379–391.

(40) Francisco, L., and Chan, C. S. (1994) Regulation of yeast chromosome segregation by Ipl1 protein kinase and type 1 protein phosphatase. *Cell. Mol. Biol. Res. 40*, 207–213.

(41) Petersen, J., Paris, J., Willer, M., Philippe, M., and Hagan, I. M. (2001) The S. pombe aurora-related kinase Ark1 associates with mitotic structures in a stage dependent manner and is required for chromosome segregation. *J. Cell Sci. 114*, 4371–4384.

(42) Roemer, T., Jiang, B., Boone, C., Bussey, H., Ohlsen, K. L. (2002) Gene disruption methodologies for drug target discovery, International Patent WO/2002/053728.

(43) Bottin, A., Kaemper, J., and Kahmann, R. (1996) Isolation of a carbon source-regulated gene from *Ustilago maydis*. *Mol. Gen. Genet. 253*, 342–352.

(44) Tomizawa, M., and Casida, J. E. (2005) Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu. Rev. Pharmacol. Toxicol. 45*, 247–268.

(45) Holliday, R. (1961) The genetics of *Ustilago maydis*. *Genet Res Camb 2* 204–230.

(46) Holliday, R. (1974) *Ustilago maydis*, in *Handbook of Genetics* (King, R. C., Ed.), pp 575−595, Springer, New York, NY.

(47) Brachmann, A., Weinzierl, G., Kaemper, J., and Kahmann, R. (2001) Identification of genes in the bW/bE regulatory cascade in *Ustilago maydis*. *Mol. Microbiol. 42*, 1047–1063.

(48) Schulz, B., Banuett, F., Dahl, M., Schlesinger, R., Schaefer, W., Martin, T., Herskowitz, I., and Kahmann, R. (1990) The b alleles of U. maydis, whose combinations program pathogenic development, code for polypeptides containing a homeodomain-related motif. *Cell 60*, 295–306.

(49) Kaemper, J. (2004) A PCR-based system for highly efficient generation of gene replacement mutants in *Ustilago maydis*. *Mol. Genet. Genomics 271*, 103–110.

(50) Wessel, D., and Fluegge, U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem. 138*, 141–143.