Analysis of All Protein Phosphatase Genes in *Aspergillus nidulans* Identifies a New Mitotic Regulator, Fcp1

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Reversible protein phosphorylation is an important regulatory mechanism of cell cycle control in which protein phosphatases counteract the activities of protein kinases. In *Aspergillus nidulans*, 28 protein phosphatase catalytic subunit genes were identified. Systematic deletion analysis identified four essential phosphatases and four required for normal growth. Conditional alleles of these were generated using the *alcA* promoter. The deleted phosphatase strain collection and regulatable versions of the essential and near-essential phosphatases provide an important resource for further analysis of the role of reversible protein phosphorylation in the biology of *A. nidulans*. We further demonstrate that *nim1* and *bimC* have essential functions required for mitotic progression since their deletions led to classical G2- and M-phase arrest. Although not as obvious, cells with AnpphA and Anmem1 deleted also have mitotic abnormalities. One of the essential phosphatases, the RNA polymerase II C-terminal domain phosphatase Anfcp1, was further examined for potential functions in mitosis because a temperature-sensitive Anfcp1 allele was isolated in a genetic screen showing synthetic interaction with the *cdk1F* mutation, a hyperactive mitotic kinase. The Anfcp1*ts* *cdk1F* double mutant had severe mitotic defects, including inability of nuclei to complete mitosis in a normal fashion. The severity of the Anfcp1*ts* *cdk1F* mitotic phenotypes were far greater than either single mutant, confirming the synthetic nature of their genetic interaction. The mitotic defects of the Anfcp1*ts* *cdk1F* double mutant suggests a previously unrealized function for AnFCPI in regulating mitotic progression, perhaps counteracting Cdk1-mediated phosphorylation.

In many cellular processes reversible phosphorylation/dephosphorylation reactions play important roles by regulating protein activity and subcellular localization. As enzymes counteracting the function of protein kinases, protein phosphatases have been firmly established as key coordinators of diverse biological events. Three main criteria are used to classify protein phosphatases: sequence homology, structure, and catalytic mechanism concerning substrate specificity. According to these features, protein phosphatases are divided into the three main groups: classical serine/threonine phosphatases, protein tyrosine phosphatases, and the aspartate-based catalysis protein phosphatase (reviewed in reference 33).

The classical Ser/Thr phosphatases can be further subdivided into the phosphoprotein phosphatase (PPP) family and the protein phosphatase Mg2+/Mn2+-dependent (PPP) family. All members of the PPP family share high sequence similarities and have in common a conserved phosphoesterase catalytic motif (PP2Ac), although their biological functions range widely from mitotic regulation (PP1) (8), immune response (PP2B) (4), DNA damage response (PP4) (15), and blue-light signaling in plants (PP7) (32). PP2C type protein phosphatases make up the PPP family and are characterized by structural similarity of their catalytic domains to PPPs despite the lack of any sequence similarity (33).

The protein Tyr phosphatase (PTP) superfamily is distinguished by its CX_{5}R catalytic signature motif shared by all of its members. It consists of the classical PTPs, the dual-specificity phosphatases (DSPs), the Cdc25 type phosphatases, and the low-molecular-weight phosphatases. The classical PTPs consist both of receptor and nonreceptor PTPs, with functions related to cell adhesion and cell-cell signaling. While the DSPs belong to the PTP superfamily, they can not only dephosphorylate tyrosine residues but also show phosphatase activities toward Ser/Thr residues. Of the DSPs, the function of Cdc14 (mitotic exit) (56) and PTEN (PI4P signaling) (17) are relatively well understood, while little is known about many other members. The Cdc25 type phosphatases are well known for their essential function in regulating mitotic entry via the activation of cyclin-dependent kinase 1 (Cdk1) (13). Little is known about the function of the low-molecular-weight phosphatases.

The aspartate-based catalysis phosphatases are defined by the shared signature catalytic motif of DXDXTV (CPDs). Its founding member is the FCPI phosphatase with an essential function in coordinating the phosphorylation status of the RNA polymerase II largest subunit C-terminal domain (RNAP II CTD) and thus regulating the activity of the transcription machinery (24).

In addition to these main protein phosphatase families, SSU72 and the tyrosine phosphatase families are considered separate types of protein phosphatases, although both share significant sequence similarities with the PTPs (12, 31).

Like many other biological events, numerous regulatory mechanisms concerning the eukaryotic cell cycle have been
shown to involve reversible protein phosphorylation/dephosphorylation. During mitosis, the function of protein kinases such as Cdk1, Aurora, NIMA, and Polo-like kinases are of critical importance for proper mitotic progression (16, 30, 40). As enzymes countering the effects of kinases, protein phosphatases have naturally been considered as logical candidates to have important cell cycle functions as well. As of now, Cdc25 and Cdc14 of the PTP superfamily and PP1 and PP2A of the classical Ser/Thr phosphatase family are the protein phosphatases whose mitotic functions have been most studied.

Identified for its essential function in mitotic entry (9, 46), Cdc25 functions to activate Cdk1 by removal of the inhibitory Tyr15 phosphorylation of Cdk1. The activity and localization of Cdc25 itself is regulated by phosphorylation as well. It has been shown that Cdc25 is activated by Cdk1 mediated phosphorylation, forming a positive-feedback loop to facilitate rapid entry into mitosis (18). Phosphorylation by Polo-like kinases is important in determining the subcellular localization of Cdc25 (54).

The DSP family member Cdc14 is known mostly for its role of regulating later stages of mitosis. In the budding yeast Saccharomyces cerevisiae, Cdc14p remains sequestered in nucleoli during interphase but is released in anaphase via the FEAR (for Cdc fourteen early anaphase release) and MEN (for mitotic exit network) signaling cascades. Once released Cdc14p localizes to the spindle and spindle pole body to promote mitotic exit by reversing the phosphorylation of mitotic proteins carried out by Cdkds during earlier phases of mitosis (53, 56). The first evidence for PP1 functioning in mitotic regulation were presented from genetic analysis in Aspergillus nidulans and Schizosaccharomyces pombe showing that PP1 was required for the completion of mitosis (6, 8, 37). Further studies in Drosophila melanogaster confirmed the essential function of PP1 in mitotic progression (2). Since the M-phase arrest phenotypes of different PP1 mutations in different organisms show a high degree of heterogeneity, it is generally believed that PP1 has multiple targets during mitosis, which is also consistent with its diverse localization to chromatin, nuclear lamina, nucleolus, and spindle pole body during different stages of mitosis (11, 55).

PP2A is another classical Ser/Thr phosphatase that has confirmed mitotic functions. Although (along with PP1) the mitotic function of PP2A in S. pombe was identified as early as 1990 (23), studies in recent years point to a specific role of PP2A in controlling sister chromatid cohesion by interacting with shugosin proteins at the centromere (52).

In order to analyze the protein phosphatase genes in A. nidulans for potential mitotic functions, we carried out a systematic gene deletion analysis of all protein phosphatase catalytic subunit-encoding genes. In addition to this genomic approach, a novel genetic strategy, engaging the function of the mitotic kinase Cdk1 (known as NimX\text{Cdc25} in A. nidulans) (42), was set up in the form of a synthetic interaction screen to isolate mutations that, combined with a hyperactive cdk1F mutant (61), cause increased temperature sensitivity. This screen identified a conditional mutant allele of the RNAP II CTD phosphatase, AnfpC1, and the function of this gene was examined for its potential role in mitosis in combination with regulated Cdk1 phosphorylation.

**MATERIALS AND METHODS**

**General A. nidulans methodologies.** Standard growth and classical genetic methodologies for A. nidulans were in essence as previously described (44). A strain list is provided (see Table S1 in the supplemental material). Endogenous gene deletions and green fluorescent protein (GFP) tagging was carried out as described previously (35, 59). Identification and analysis of essential genes via heterokaryon rescue was completed according to the method described previously (41).

**Genetic screen.** For the genetic screen, A. nidulans strain FYR24 was used in which a nimX\text{Cdc25} mutant allele had been inserted in the chromosomal locus of nimX\text{Cdc25} to form a tandem repeat of the wild-type nimX\text{Cdc25} and mutant nimX\text{Cdc25} alleles, linked together by the auxotrophic marker pyr4. The screen consisted of two successive selection procedures after the initial introduction of new mutations via UV irradiation (administered at 3.15 × 10\text{4} μJ/cm\text{2} on 500,000 viable spores on 100 plates to yield ~5,000 viable spores/plate at a survival rate of 1.08%). After random UV mutagenesis, surviving cells were tested for initial temperature sensitivity at 42°C. Strains that showed initial temperature sensitive phenotypes were selected for subsequent eviction of the nimX\text{Cdc25} allele. By utilizing the toxicity that hydroxyurea (HU) and 5-fluorouracil (5-FU) confer upon cells expressing nimX\text{Cdc25} and pyr4, respectively, a double counterselection was carried out on the ~5,000 surviving strains by growth on medium containing 8 mM HU and 0.1% 5-FU to recover cells that had evicted both the nimX\text{Cdc25} allele and the pyr4 gene via homologous recombination between the nimX\text{Cdc25} and the wild-type nimX\text{Cdc25} alleles. After verifying the eviction of nimX\text{Cdc25}, the mutant strains were tested for phenotypic change to their sister-conflicting phenotypes within the range of 32 to 42°C. Loss of the temperature sensitivity due to nimX\text{Cdc25} eviction confirmed that the newly introduced mutation and nimX\text{Cdc25} were genetically interacting in a synthetic manner. Mutations identified as showing genetic interactions with nimX\text{Cdc25} in this way were further analyzed genetically to verify whether they were single mutations, and their allelic relationship was examined. Mendelian segregation of the mutant phenotypes was used to confirm the strains contained single mutations. Allelic relationships of the different mutants were verified by crossing the different mutants with each other, whereupon the out segregation of a wild-type phenotype in the F1 progeny determined the given two mutations to have occurred on different genes. Dominance of the mutations were determined by generating diploid strains of wild-type and mutant haploid strains. Only recessive mutations were selected for molecular cloning purposes. Single, recessive mutations were consequently cloned by complementation via transformation with an AMA1 plasmid-based A. nidulans genomic DNA library (38). A single mutant allele of AnfpC1 was recovered and sequenced.

**Conditional growth test conditions.** Strain with null allele of the nonessential phosphatase genes were tested on MAGUU plates for their response to the following conditions: benomyl (0.4 and 0.8 g/ml), sucrose (0.5, 1, and 1.5 M), hydroxyurea (8 and 16 mM), NaCl (0.5, 1, 1.5 M), methyl methanesulfonate (0.025 and 0.05%), 1,2,7,8-diepoxyoctane (0.01 and 0.02%), camptothecin (25 μM), and temperature (29 to 32°C).

**Affinity purifications using the S-Tag affinity peptide.** We have developed the S-Tag affinity purification system for single step protein affinity purification of A. nidulans proteins endogenously tagged with the S-Tag peptide (21). Details have been published elsewhere (29a). Notably, however, the standard protocol was modified by the addition of 320 mM ammonium sulfate (replacing 300 mM NaCl) and 20% glycerol to the extraction buffer. Purified proteins were run on standard Laemmli–sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels using a 3% stacking and 6% separating gel made from a 40% (wt/vol) solution of acrylamide and bis-acrylamide (37:5:1).

In vitro phosphatase reactions and analysis. After purification of a strain via S-Tag affinity purification, beads were treated with 40 μl of λ-phosphatase (New England Biolabs) with or without phosphatase inhibitors (1 mM sodium vanadate plus 50 mM sodium fluoride) after being washed in 2× phosphatase buffer that included MnCl2 and protease inhibitors. The beads were mixed and incubated at 30°C for 30 min (with occasional mixing). Sample buffer was added, and the supernatant was collected and analyzed by SDS-PAGE. For Coomassie blue staining, gels were fixed in 50% ethanol and 10% acetic acid overnight, rinsed three times for 5 min with distilled H2O and stained with Bio-Rad Bio-Safe Coomassie blue solution for 1 h. Background staining was removed by using a water wash for 30 min. Standard methodologies were used for silver staining.

**Microscopy and image capture.** Fixed samples were examined by using an IX81 Fluorescence Microscope (Nikon, Inc.) with DAPI (6′-diamidino-2-phenylindole), DAPI, or propidium iodide/4′,6-diamidino-2-phenylindole/DAPI, or propidium iodide/4′,6-diamidino-2-phenylindole/isothiocyanate, or Texas Red filters (Omega Optical, Inc.). Image capture was performed by using an UltraPlex digital camera (Life Science Resources, Ltd.). For live cell microscopy, strains were germinated in 5 ml of minimal media in 35-mm glass bottom petri dishes (MatTek Cultureware). Visualization was
TABLE 1. Protein phosphatase catalytic subunit genes in *A. nidulans*

| *A. nidulans* gene | *S. cerevisiae*-human gene | e value | Gene deletion | Domain |
|-------------------|---------------------------|--------|---------------|--------|
| AN0103 Pph3p-Pp4 | | 3e–56 | Viable Viable | PP2Ac |
| AN0164 Pphlp-Pp2Ac | | 2e–109 | Sick Viable | PP2Ac |
| AN0410 bimG | Glc7p-Ppl1a | e–170 | Lethal Lethal | PP2Ac |
| AN0504 sitA | Sit4p-Pp2Ac | e–110 | Viable Viable | PP2Ac |
| AN3793 Ppz1p-Ppl1b | | 2e–145 | Viable Viable | PP2Ac |
| AN6391 pphA | Pph2lp-Pp2a | e–148 | Lethal Viable | PP2Ac |
| AN8820 cnA* | Cmp2p-Ppp3Ca | 3e–173 | Sick Viable | PP2Ac |
| AN10281 Pp1lp-Pp55 | | 5e–110 | Viable Viable | PP2Ac |
| AN0914 Ptc6p-Pm1k | | 3e–16 | Viable Viable | PP2Cc |
| AN1358 Ptc2p-Pmm1a | e–75 | Viable Viable | PP2Cc |
| AN1467 Ptc7p-Pptc7 | | 2e–16 | Viable Viable | PP2Cc |
| AN2472 Ptc2p-Pm1k | | 4e–87 | Viable Viable | PP2Cc |
| AN5722 Ptc5p-Pm2c | | 9e–65 | Viable Viable | PP2Cc |
| AN6892 Ptc1p-Pmm1a | | 4e–44 | Viable Viable | PP2Cc |
| AN1343 Nem1p-Dullard | | 5e–94 | Sick Viable | CPDc |
| AN2902 Fcp1p-Fcp1 | | 3e–61 | Lethal Lethal | CPDc |
| AN10077 Pr1p-Cdps2 | | 7e–61 | Viable Viable | CPDc |
| AN0129 Pps1p-Dusps2 | | 4e–45 | Viable Viable | DSpC |
| AN4419 Yvhlp-Dusp12 | | e–34 | Sick Viable | DSpC |
| AN4544 Ms5p-Dusp9 | | 9e–12 | Viable Viable | DSpC |
| AN5057 Cdc14p-Cdc14 | | 2e–17 | Viable Lethal | DSpC |
| AN10138 Sdp1p-Ssys | | 0.71 | Viable Viable | DSpC |
| AN4896 Ptp1p-Ptpu2 | | e–3 | Viable Viable | PTPc |
| AN6982 Ppy1p-Pp1n1 | | e–34 | Viable Viable | PTPc |
| AN10570 Ltp1p-Acp1 | | 3e–20 | Viable Viable | LMWPc |
| AN3810 Ssu72p-Ssu72 | | 3e–48 | Sick Lethal | SSU72 |
| AN3941 nimT | Mih1p-CDC25 | 2e–27 | Lethal Viable | CDC25 |
| AN4426 Sw14p-Lkhp9428 | | 7e–31 | Viable Viable | Y-phosphatase |

* That is, the designations given by the *A. nidulans* Genome database. Gene names are given for already-cloned genes. * Not deleted in this study but previously identified and characterized (45, 50).

b Values are given for comparison to the *S. cerevisiae* protein at the NCBI.

identified and characterized (45, 50).

**RESULTS**

Identification of 28 protein phosphatase catalytic subunit genes in *A. nidulans*. The protein sequence of all protein phosphatase catalytic subunit genes of the budding yeast *S. cerevisiae* were used for BLAST searches to determine sequence homologues in the *Aspergillus* Comparative Database of the Broad Institute (http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html). In addition, the catalytic domains of different classes of protein phosphatases were used for BLAST searches. A total of 28 different genes predicted to encode protein phosphatase catalytic subunits were thus identified (Table 1). Of the 28 predicted genes, 4 had been identified in previous studies. Originally isolated through genetic screens (34), *nimT* and *bimG* were shown to be required for proper mitotic entry and progression, respectively (8, 36). In addition, *pphA* is thought to regulate hyphal morphogenesis (26), while *cnA* function had been implicated in the regulation of the G<sub>1</sub>/S transition of the cell cycle (45), although recent studies indicate it to be nonessential (50).
heterokaryons containing both parental and deleted alleles. The deletion strains of the other 23 phosphatase genes were confirmed to be viable homokaryons, demonstrating the function of these protein phosphatases to be nonessential. No conditional phenotypes associated with these gene deletions were identified under various experimental growth conditions regarding genotoxic stress, high osmolarity, and high and low growth temperatures. Nevertheless, null alleles of four nonessential protein phosphatases showed significant growth defects. The deletion strains of Anppg1 (AN0164), Annem1 (AN1343), Ansu72 (AN3810), and Anyvh1 (AN4419) exhibited defective growth phenotypes readily distinguishable by the small sizes of their colonies (Fig. 1A). When the diameter of their colonies were measured daily over 5 days, all four showed reduced colony growth rates compared to the control wild-type strains and other deleted phosphatase mutants (data not shown).

**Generation of conditional alleles of protein phosphatase genes utilizing the alcA promoter system.** As a tool to more easily study the protein phosphatase genes whose deletions proved to be lethal, or result in severe growth defect phenotypes, strains containing conditional alleles of these genes were generated. In *A. nidulans*, the targeted regulation of gene expression can be achieved by placing the gene of interest under the control of the promoter of the alcohol dehydrogenase I gene *alcA* (57). As part of the alcohol utilization pathway, *alcA* gene expression is highly induced in response to ethanol and threonine and suppressed in the presence of glucose (3, 28, 29), while fructose, lactose, or glycerol allows a moderate level of expression. The deletion strains of the other 23 phosphatase genes were confirmed to be viable homokaryons, demonstrating the function of these protein phosphatases to be nonessential. No conditional phenotypes associated with these gene deletions were identified under various experimental growth conditions regarding genotoxic stress, high osmolarity, and high and low growth temperatures. Nevertheless, null alleles of four nonessential protein phosphatases showed significant growth defects. The deletion strains of Anppg1 (AN0164), Annem1 (AN1343), Ansu72 (AN3810), and Anyvh1 (AN4419) exhibited defective growth phenotypes readily distinguishable by the small sizes of their colonies (Fig. 1A). When the diameter of their colonies were measured daily over 5 days, all four showed reduced colony growth rates compared to the control wild-type strains and other deleted phosphatase mutants (data not shown).

**FIG. 1.** Colony growth of all nonessential phosphatase gene deletion strains. (A) While the colony sizes of all other gene deletion strains are comparable to the wild-type control strains SO451 and TNO2A7, including strains SS140 (ΔAN5057), SS150 (ΔAN4544), SS159 (ΔAN0129), SS154 (ΔAN10077), SS157 (ΔAN10138), SS148 (ΔAN5722), SS133 (ΔAN2472), SS141 (ΔAN1467), SS164 (ΔAN0914), SS167 (ΔAN4896), SS143 (ΔAN6982), SS147 (ΔAN1558), SS143 (ΔAN6892), SS135 (ΔAN3793), SS170 (ΔAN0504), SS125 (ΔAN0103), SS137 (ΔAN4426), SS130 (ΔAN10570), and SS129 (ΔAN10281), colonies of the ΔAnppg1 (SS162), ΔAnnem1 (SS132), ΔAnppg1 (SS153), and ΔAnsu72 (SS160) strains are smaller. The numbers refer to the AN numbers designated by the Broad Institute (http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html). (B) The deletion phenotype of the indicated genes can be mimicked utilizing *alcA* promoter driven conditional alleles. When their gene expression is suppressed on glucose medium, colony growth is impaired similar to the gene deletion strain colonies: *alcA*-ppg1 (SS087), *alcA*-nem1 (SS099), *alcA*-nem1 (SS132), *alcA*-su72 (SS122), Δsu72 (SS160), *alcA*-vhl1 (SS078), and Δvhl1 (SS162). Three controls are the nonessential phosphatase AN0129 gene under *alcA* control, a wild-type (growth control strain SO451), and *alcA*-regulated nimT, which is an essential gene (positive control strain SS124).
of *alcA* expression (10, 43). In the present study, the *alcA* promoter constructs were endogenously inserted immediately upstream of the phosphatase gene loci, generating strains in which the only copy of the given phosphatase is under the control of the *alcA* promoter. When the growth characteristics of these strains were compared to the corresponding gene deletion strains, the suppression of the genes on glucose-containing medium proved to be effective enough for the growth patterns exhibited by the *alcA* inducible strains to mimic that of the gene deletion strains. When grown on suppressing medium *alcA::Annem1* strains showed growth defects closely resembling those of the corresponding gene deletion strain (Fig. 1B). Although the growth defects exhibited by the *alcA::Annppl1*, *alcA::Anyvh1*, and *alcA::Anssu72* strains were not as severe as in the deletion strains, they still produced colonies of smaller size than wild-type strains (Fig. 1B). Suppression of the essential protein phosphatases was highly effective since it yielded complete growth arrests of the *alcA::Anfcp1* and *alcA::BimG* strains, and only minicolonies were formed for the *alcA::pphA* strains (Fig. 2B).

**Germination and growth defects resulting from essential protein phosphatase gene deletions.** To examine the growth characteristics of cells with essential protein phosphatase gene deletions, conidia from the primary gene deletion transformants were streaked on YAG medium plates and grown at room temperature for 3 days. Since the nutritional marker used for the transformation was *pyrG*<sup>4F</sup>, YAG plates are nutritionally selective for undeleted conidia that carried the non-functional *pyrG*<sup>G89</sup> mutant allele. Not meeting the nutritional requirement, it is known that these conidia fail to project a germ tube and do not grow beyond swelling of the conidiospores. In contrast, the conidia which are *pyrG*<sup>+</sup> but deleted of the essential phosphatase genes would grow up to the limit imposed by the lack of the essential phosphatase gene function, enabling the assessment of the terminal growth phenotypes caused by the deletions. Deletion of *BimG* showed the most severe growth defect as the ∆*bimG* spores on YAG medium failed to project germ tubes and nuclei failed to divide (Fig. 3A). Deletion of *Anfcp1* was slightly less severe as ∆*Anfcp1* spores were able to germinate and project very short germ tubes (Fig. 3A). The *nimT* gene deletion had a less detrimental effect on initial germination and polarized germ tube projection (Fig. 3A). Similarly, ∆*pphA* spores were also able to grow into germ tubes comparable to ∆*nimT* spores (Fig. 3A).

**Nuclear defects in cells with the essential phosphatase genes deleted.** To determine potential nuclear and mitotic defects associated with the deletion of essential phosphatase genes, spores of these deletion transformants were suspended into YG liquid medium and inoculated on coverslips, grown for 18 h at room temperature, DAPI stained, and imaged by fluorescence microscopy (as an exception, spores taken from the *bimG* deletion transformant were grown in selective YGUU medium since ∆*bimG* *pyrG*<sup>+</sup> spores and *bimG* *pyrG*<sup>G89</sup> spores were more difficult to distinguish in selective YG medium). Previous studies conducted with the *bimG11* conditional mutant allele had revealed that loss of BIMG activity led to mitotic arrest of the cell with condensed DNA (8). When visualized by DAPI staining the nuclei of ∆*bimG* spores were arrested with DNA remaining condensed, a phenotype consistent with the observations made in *bimG11* mutants (Fig. 3A). DAPI staining of ∆*nimT* cells revealed their nuclei showing a G<sub>2</sub>-phase arrest with nuclei unable to enter mitosis with uncondensed DNA, also confirming the previous conclusions about *nimT* function drawn from studies of the *nimT23* temperature-sensitive mutant (36). Although germlings of ∆*pphA* cells were capable of at least three rounds of mitoses, their nuclei had irregular morphologies and distribution along the germ tube, indicative of potential mitotic defects (Fig. 3A).

**FIG. 2.** Essential function of protein phosphatase genes analyzed utilizing heterokaryon rescue and *alcA*-driven alleles. (A) Conidia from six primary transformants obtained after transformation with a *bimG* deletion construct were replica plated on selective (YAG) and nonselective (YAGUU) plates and incubated to allow colony formation. The inability of *bimG* conidia and untransformed conidia to grow on selective YAG medium and the ability of untransformed conidia to grow on nonselective YAGUU medium confirms that each primary transformant colony is heterokaryotic, indicating that *bimG* is essential (41). Heterokaryon formation was verified using diagnostic PCR analysis, showing that the wild type (lane 1) contains just the wild-type allele and that the heterokaryon contains both wild-type and deletion alleles (lane 2). (B) Deletion phenotypes of essential phosphatase genes, as indicated, can be reconstituted by suppressing their expression using the *alcA* promoter such that growth can occur when *alcA* is induced (ethanol media) but not when *alcA* is suppressed using glucose media. Strains SS120 (*alcA-fcp1*), SS124 (*alcA-nimT*), SS121 (*alcA-bimG*), and SS123 (*alcA-pphA*) are represented. This both confirms that these genes are essential and provides strains for further functional analysis.
difficult to distinguish individual nuclei, possibly due to mitotic defects.

Cytological abnormalities in nonessential protein phosphatase deletions with growth defects. For the analysis of the nonessential phosphatase gene deletions that cause a decline in colony size, clonal conidium stocks were generated for each of the deletion strains, as well as the alcA-regulated strains. After 6.5 and 9 h of growth in YGUD-rich liquid medium at
32°C, the cells were fixed and stained with DAPI to visualize DNA. Control wild-type cells (strain R153) grown under the same conditions reached, on average, the 4- and 16-nucleus stages, respectively, after 6.5 and 9 h of growth (Fig. 3B), and the growth and cytological defects exhibited by the phosphatase null mutants were compared to them. Deletion of \textit{Anppg1} and \textit{Anssu72} appeared to slightly affect the overall early growth rate without causing any pronounced nuclear defects. At time points of 6.5 and 9 h of growth, both \textit{Anppg1} and \textit{Anssu72} cells had fewer nuclei, as well as shorter germ tubes compared to the wild-type cells, indicating an overall slowdown of growth as a consequence of the gene deletions (Fig. 3B). Although less severe, these phenotype were also observed in the \textit{alc::Anppg1} and \textit{alc::Anssu72} cells grown under suppressing conditions (the same YGCU rich medium used is glucose-based and thus suppresses \textit{alcA}-promoter-driven expression) (data not shown). In the \textit{Annen1} null mutants, although the overall growth rate of the cells was also affected, signs of DNA fragmentation were the most significant deletion phenotype. The nuclei were small in size and of irregular shape, with some appearing to have condensed DNA. A feature more pronounced in the cells that had grown longer (9 h) was the presence of fiber-like structures positively stained by DAPI that appeared to be interlinking the nuclei (Fig. 3B). These phenotypes persist in the \textit{alc::Annen1} strain grown under suppressing conditions, although their growth rate resembles that of the wild-type strain (data not shown). Taken together, these phenotypes suggested defects in mitosis, leading to fragmented DNA masses and nuclei that failed to fully separate from each other. The most severe growth defect phenotype among the nonessential phosphatase gene deletion strains was shown by the \textit{ΔAnyvhl} strain. After 6.5 h of growth, most of the spores had failed to germinate, and many of those cells had not undergone their first mitosis either (Fig. 3B). Even the cells that had managed to initiate germination were small in size, not only in terms of the length of the germ tube but also in the extent of how much the spore had swollen prior to germination. When \textit{ΔAnyvhl} cells were grown for 9 h, the germ tubes were still very short in length (Fig. 3B).

**Genetic interaction of an Anfcp1 mutation with cdk1F.** The systematic gene deletion analysis of the protein phosphatase catalytic subunits revealed the function of \textit{bimG}, \textit{nimT}, \textit{pphA}, and \textit{Anfcp1} to be essential in \textit{A. nidulans}. The severity of nuclear defects, suggestive of mitotic defects, observed in \textit{ΔAnfcp1} cells was interesting, and the results of a novel forward genetic screen provided insight into the potential role of \textit{Anfcp1} in regulating the cell cycle and mitosis. Cdk1 is one of the main regulators determining entry into mitosis. Activation of Cdk1, which triggers the G2/M transition, hinges on the removal of inhibitory phosphorylation from a tyrosine residue in the ATP-binding domain, a mechanism conserved in organisms ranging from fission yeast to humans. However, unlike fission yeast, in \textit{A. nidulans} this mechanism is not the only means through which entry into mitosis is regulated, since mutants that lack the inhibitory phosphorylation (Tyr15) (cdk1F, tyrosine 15-to-phenylalanine mutation) still progress through the cell cycle in the absence of genotoxic stresses. However, this allele does cause sensitivity to DNA-damaging agents and slowed S phase caused by low levels of HU (60, 61). With the aim of identifying additional mitotic regulatory genes that do not function through Tyr15 phosphorylation of Cdk1, a genetic screen was carried out looking for mutations that show genetic interactions with the cdk1F mutant allele. As a result, a loss-of-function conditional mutation of \textit{Anfcp1} was isolated, which showed a synthetic temperature sensitivity phenotype in combination with cdk1F.

The genetic screen utilized an \textit{A. nidulans} strain that has both a wild-type \textit{cdk1} and a cdk1F mutant allele linked in tandem with the nutritional marker \textit{pyr4}. As a hyperactive kinase mutant, the cdk1F mutant allele is dominant over the wild-type counterpart while not causing temperature sensitivity. After UV mutagenesis, mutant strains showing temperature sensitivity were processed to define mutations that depend on the cdk1F mutation for their temperature-sensitive phenotype. This was done by using 5’-FOA to select against \textit{pyr4} and HU to select against cdk1F allowing selection for loop-out of the cdk1F allele via homologous recombination between the wild-type \textit{cdk1} and mutant cdk1F alleles. In this way alleles without cdk1F were selected. Strains obtained in this way were tested for loss of their temperature-sensitive phenotype. Loss of temperature sensitivity as a consequence of cdk1F eviction confirmed that the newly introduce mutations and the cdk1F mutation genetically interact in a negative manner, in combination causing increased temperature sensitivity (Fig. 4A).

One of the mutant alleles isolated in this screen was \textit{Anfcp1}ts. When the mutant allele was cloned and sequenced, a mutation that converted a conserved leucine residue, immediately upstream of the “DXDXDT” metal-assisted phosphotransferase signature motif, to a serine residue was identified (Fig. 4B). Designated \textit{Anfcp1}ts, this mutant allele causes a certain level of temperature sensitivity by itself, but in combination with the cdk1F mutant causes a stronger temperature-sensitive phenotype, underscoring the genetic interaction of \textit{Anfcp1} and \textit{cdk1} (Fig. 4A).

\textbf{AnFCP1 is a protein phosphatase which dephosphorylates RNA polymerase II.} Both in \textit{S. cerevisiae} and in humans, the protein phosphatase Fcp1 had been shown to dephosphorylate the C-terminal domain of the largest subunit of RNA polymerase II (1, 24). To verify whether this function of AnFCP1 is conserved, its putative target RNAP II was endogenously tagged C terminally with the affinity S-Tag. This was done since antibodies targeting specific serine residues within the Y-S-P-T-S-P-S heptapeptide repeats of the RNAP II CTD were ineffective, presumably because these repeats are only loosely conserved in \textit{A. nidulans} (51; data not shown). The S-Tag version of RNAP II is functional since RNAP II is essential (data not shown) and strains carrying the S-Tagged version were viable and displayed no growth defects. Using the S-Tag protein purification system (21), RNAP II was affinity purified from both wild-type and Anfcp1ts strains incubated at the permissive (32°C) and restrictive (42°C) temperatures of the Anfcp1ts conditional mutation. RNAP II purified from the wild-type strain separated into subspecies with a size range of ~200 to 250 kDa at both 32 and 42°C. Unlike in the wild-type strain, RNAP II purified at 42°C from the Anfcp1ts strain showed the loss of subspecies with molecular masses lower than ~250 kDa, suggesting the loss of hypo- and unphosphorylated isoforms of RNAP II CTD (Fig. 4C). An in vitro phosphatase treatment confirmed that the differences in electro-
phoretic mobility shown by different RNAP II isoforms were indeed due to differential phosphorylation (data not shown).

Synthetic interaction of Anfcp1 and cdk1. As a hyperactive protein kinase and an inactivated protein phosphatase paired together led to a synthetic mutant phenotype (enhanced temperature sensitivity), we hypothesized that this interaction may be mediated by an abnormally elevated phosphorylation state of a common protein substrate shared by Cdk1 and AnFcp1. In addition to being the substrate of the phosphatase Fcp1, RNAP II CTD had been shown in previous studies to be phosphorylated by Cdk1 in vitro (14) and undergo mitotic specific hyperphosphorylation (58). This suggests RNAP II CTD as a candidate common substrate of Fcp1 and Cdk1. To examine this possibility, endogenously S-tagged RNAP II was purified from a strain with the fcp1ts mutation (SS031) or a wild-type strain (SS025) grown at 32 or 42°C. Purified proteins were separated via SDS-PAGE, and the gel was stained with Coomassie blue. The region of the gel corresponding to RNAP II is shown.

**FIG. 4.** Genetic interaction between the Anfcp1 phosphatase and the activated cdk1F allele of the Cdk1 mitotic kinase. (A) Colony growth at 32°C (left plate) and 38.5°C (right) of strains containing the following mutations: 1, fcp1ts cdk1F (SS101); 2, cdk1F (SS105); 3, fcp1ts (SS002); 4, wild type (TNO2A7); 5, fcp1ts cdk1F cdk1; and 6, cdk1F cdk1 (FRY24). (B) Domains of AnFcp1 and the mutation causing the fcp1ts allele, which changes the indicated conserved leucine to a serine. An, *A. nidulans*; Sce, *S. cerevisiae*; Spo, *S. pombe*; Hu, human. (C) S-Tagged RNAP II was purified from a strain with the fcp1ts mutation (SS031) or a wild-type strain (SS025) grown at 32 or 42°C. Purified proteins were separated via SDS-PAGE, and the gel was stained with Coomassie blue. The region of the gel corresponding to RNAP II is shown.
The synthetic effect of the two mutations was most pronounced at 40°C. Although this was the range of temperature where the severe level of temperature sensitivity compared to the single mutant strains, the difference of the phosphorylation statuses differed only marginally. A similar result was obtained when the cultures of the two strains were grown entirely at 37°C (data not shown). These data indicate that the synthetic interaction of Anfcp1ts and cdk1F are at best only partially mediated through the phosphorylation status of RNAP II.

Localization of RNAP II through the cell cycle. RNAP II was endogenously tagged with GFP and its localization examined throughout the cell cycle by time-lapse confocal microscopy. In wild-type cells, RNAP II located to nuclei, with a partial exclusion from the nucleolus indicated by a darker area within each nucleus (Fig. 5, −1.5°). At certain time points, the fluorescence intensity within nuclei dissipated evenly into the entire cell, remained dispersed for 4 to 5 min, and recondensed back into the nuclei that in the mean time had doubled in numbers. The doubling of the number of nuclei and the time frame of the RNAP II staying dispersed indicated that while RNAP II localized to the nuclei during interphase, it dispersed throughout the cell when mitosis started and relocalized to daughter nuclei once mitosis was completed (Fig. 5). Since A. nidulans is known to undergo a partially open mitosis, the mitotic specific dispersal of RNAP II indicates that it does not remain associated with nuclear components during mitosis.

To determine whether the genetic interaction of the cdk1F and Anfcp1ts mutations affected mitosis, endogenously GFP tagged RNAP II was followed in the wild type and in cdk1F, Anfcp1ts, and Anfcp1ts cdk1F mutants growing at 38°C. Concerning the length of germlings, the number and distribution of the nuclei and the duration of mitoses (i.e., dispersal of RNAP II::GFP from nuclei, due to NPC partial disassembly) (7), the wild-type and cdk1F strains did not appear to have a notable difference, a finding consistent with the fact that at 37°C neither of the strains showed visible growth defects. The Anfcp1ts strain showed a slightly longer duration of RNAP II::GFP dispersed though no major cytological defects were observed. The most striking phenotypes were displayed by Anfcp1ts cdk1F double mutants. Many of these cells displayed nuclei that were near to each other with nuclei appearing to be joined (Fig. 6D).

Time lapse imaging revealed (Fig. 7A) that such cells had nuclei that were difficult to define from each other and which changed in shape and conformation through time. In fact, nuclei often appeared to merge into one continuous nucleus containing multiple nucleoli (see Fig. 7A at 48', for example). These surprising changes can be seen clearly in a full-time course video file (see Video 1S in the supplemental material). When RNAP II::GFP was seen to disperse from such apparently joined nuclei, indicating entry into mitosis, the number of nuclei was not always increased when RNAP II::GFP returned to nuclei after completion of mitosis (Fig. 7B). Additionally, we observed some nuclei in the double mutant that appeared to complete mitosis, generating two daughter nuclei capable of importing RNAP II::GFP but which then remerged in an apparent reversal of mitosis (Fig. 7C). At time zero’ (Fig. 7C), the cell contains two nuclei and a micronucleus. At 8’ the cell enters mitosis and RNAP II::GFP starts to disperse from nuclei to throughout the cell. At 22’ the cell exits mitosis and daughter nuclei reimport RNAP II::GFP, and at 24’ four nuclei are apparent. However, as the cell progresses further into the cell cycle these four nuclei merge into two nuclei, each with a single nucleolus. The micronucleus can also be seen. Thus, although this cell undergoes a mitotic event the net result is regenera-
tion of the same nuclear configuration (compare the 0' to 40' images in Fig. 7C). Finally, cells were observed in which the RNAP II::GFP signal remained dispersed after entering mitosis for an extended period of time, presumably due to their inability to exit mitoses.

In nuclei where the RNAP II::GFP signals dispersed during mitosis and reconcentrated to the nuclei upon its completion, it took significantly longer for this cycle to be completed (compare the timing of RNAP II-GFP dispersal in Fig. 5 to 7 for example). To rule out the possibility that this may merely be due to an overall slowed-down cell cycle, the frequency of dispersed RNAP II::GFP signals in all four strains was measured in fixed cells grown at 37°C (Table 2). The increased percentage of cells with dispersed RNAP II::GFP (Table 2) support the findings of longer mitoses in the Anfcp1ts and especially the Anfcp1ts cdk1F mutants. The severity of the cytological defects observed in the Anfcp1ts cdk1F double-mutant cells might help explain the basis of the synthetic nature of the genetic interaction between the Anfcp1ts and the cdk1F mutations. While the Anfcp1ts and cdk1F single mutants...
showed moderate or no noticeable defects compared to wild-type cells, the Anfcp1TS cdk1F double mutant exhibited phenotypes that went far beyond the simple addition of that of the two single mutant strains.

**DISCUSSION**

**Essential protein phosphatases and cell cycle regulation.** This study showed the phosphatase genes bimG, nimT, Anfcp1, and pphA to encode proteins with essential cellular functions. The fact that two of these genes were identified previously in genetic screens for cell cycle mutants (34) and that overexpression of a dominant-negative form of PphA affects growth and mitosis (26) and the identification of AnFcp1 as a potential mitotic regulator in the screen described in this study clearly demonstrate the essential importance of phosphatase-mediated protein dephosphorylation in regulating cell cycle progression. The terminal phenotypes displayed by cells with bimG and nimT deleted confirmed their functions to be critical in mitotic regulation, which is consistent with the findings from studies of the conditional mutant alleles bimG11 and nimT23. While Anfcp1 and pphA deletions did not yield phenotypes as clearly demonstrative of mitotic defects as bimG and nimT deletions, their defective nuclear DNA morphologies suggested that there are potentially important functions of these genes in mitotic regulation as well. The availability of the conditional Anfcp1TS mutant allele, as well as strains in which Anfcp1 and pphA are under the inducible promoter alc4, should prove to be useful in further studies of the functions of these essential genes.

One noticeable essential phosphatase, the Cdc14 phosphatase, that is required for the regulation of S. cerevisiae mitosis is not essential in A. nidulans. In fact, the null AnCdc14 strain grows and develops normally. This suggests that Cdc14 phosphatases might not be essential components of mitotic regulation in all organisms, and recent deletion studies indicate Cdc14B is dispensable for human mitotic regulation (5).

**Gene deletion phenotypes of nonessential protein phosphatases.** The gene deletion of four protein phosphatases did not cause lethality but had physiological effects detrimental enough to cause strong growth defects. Of these four phosphatase genes, AnSSU72 is functionally linked to the essential phosphatase AnFcp1 since the physiological target of both phosphatases had been shown to be the C-terminal domain of RNAP II (27). This may reflect the importance of maintaining a proper phosphorylation status of RNAP II CTD for the cell to engage in proper transcriptional activity, by itself an activity absolutely vital for cellular survival. Unlike AnSSU72, the phosphatase AnNEM1 is linked to AnFcp1 by belonging to the same protein phosphatase family that shares the CDP type catalytic domain. Defined first by the discovery of Fcp1, the CDP family is made up of protein phosphatases that engage in aspartate-based catalysis of target proteins and have in common the signature catalytic motif of DXDXT/V. While only three different CDP phosphatases are present in A. nidulans, one of them is essential (Anfcp1) and one causes severe growth defects upon deletion (Annem1), indicating the importance of the biological function conserved in this phosphatase family. Within the context of cell cycle regulation, Annem1 is of added interest since its deletion not only caused an overall decrease of cellular growth but was also marked by noticeable nuclear structure phenotypes, a strong indication that it may play an important role in mitotic regulation and/or nuclear structure. This finding is consistent with the functions of Nem1p (S. cerevisiae) and Dullard (humans), which are known to regulate nuclear membrane biogenesis (22, 47, 49).

**Genetic interaction of Anfcp1 and cdk1 and RNAP II CTD phosphorylation.** The identification of Anfcp1TS to cause increased temperature sensitivity in combination with cdk1F revealed a genetic interaction between Anfcp1 and cdk1. The fact that a hyperactive kinase (cdk1F), together with an inactivated phosphatase (Anfcp1TS), caused a synthetic mutant phenotype suggests the effects of those two mutations may converge into a hyperphosphorylated state of a common protein substrate. While potential targets of cdk1 phosphorylation are numerous, the only substrate of protein phosphatase Fcp1 convincingly shown thus far is the RNAP II CTD. In contrast to our expectations, the overall level of RNAP II CTD phosphorylation did not show a significant elevation when endogenously S-tagged RNAP II was purified and analyzed by SDS-PAGE under conditions where the synthetic temperature sensitivity of the Anfcp1TS cdk1F double mutant is most pronounced. Although this result shows that the bulk amount of RNAP II CTD phosphorylation does not proportionately mediate the synthetic temperature sensitivity of the Anfcp1TS cdk1F double mutant, it should be noticed that the A. nidulans RNAP II CTD contains 44 serine-proline dipeptide sequences, all potential targets of phosphorylation. It is possible that the synthetic phenotype may be attributed to the phosphorylation of specific RNAP II residues which are not readily detectable by mobility shifts during SDS-PAGE. Notably, we were unable to

**TABLE 2.** Frequency of RNAP II::GFP dispersal in the wild-type, cdk1F, Anfcp1TS, and Anfcp1TS cdk1F strains at 37°C

| Expt | Wild type | cdk1F | Anfcp1TS | Anfcp1TS cdk1F |
|------|-----------|-------|---------|----------------|
|      | Dispersed | Total | Dispersed | Total | Dispersed | Total | Dispersed | Total |
| 1    | 8         | 234   | 9       | 220  | 43       | 726   | 76       | 797   |
| 2    | 19        | 515   | 22      | 530  | 46       | 571   | 46       | 543   |
| 3    | 31        | 812   | 31      | 826  | 30       | 851   | 81       | 822   |
| Total| 58 (3.7)  | 1561  | 62 (3.9)| 1,576| 119 (5.5)| 2,148 | 203 (9.4)| 2,162 |

* Cells of individual strains were grown for 8 h at 37°C and fixed. The cells in which the RNAP II::GFP signal was dispersed were counted, as well as the total number of cells. The experiments were repeated three times independently. Student t test analysis indicated that only the difference between the wild-type and the Anfcp1TS cdk1F strains was significant (P < 0.0005).
detect an obviously hyperphosphorylated form of RNAP II during mitosis, whereas such a form has been shown to exist during higher eukaryotic mitosis (58).

Localization of RNAP II during A. nidulans mitosis. Since mitosis in A. nidulans is partially open (7, 39), nuclear proteins that are not attached to the nuclear interior during mitosis, on chromosomes, for example, can disperse into the cytoplasm via passive diffusion once nuclear pores open at the beginning of each mitotic phase. In contrast, proteins such as histones, which remain associated with chromatin during mitosis, are not released from nuclei during mitosis. The predominant nuclear localization of endogenously GFP-tagged RNAP II in growing cells confirmed that it remains in the nucleus during interphase since it is engaged in transcriptional activities. The complete dispersal of RNAP II during mitosis suggests that its association with DNA may be actively disrupted during mitosis, effectively disabling it from continuing transcription. During the transcription cycle, it had been shown that the CTD of RNAP II has to be completely dephosphorylated for the RNAP II to form a preinitiation complex and reengage in transcriptional activity (25, 48). In mammalian cells the RNAP II CTD is hyperphosphorylated during mitosis, and this is thought to be regulated by Cdk1-mediated phosphorylation (58). Although in A. nidulans this mitosis-specific CTD hyperphosphorylation event could not be observed, the fact that RNAP II completely disperses during mitosis suggests that there still may be a mitotic specific phosphorylation pattern of RNAP II CTD in A. nidulans capable of actively dissociating RNAP II from chromatin and thus contributing to its mitosis-specific dispersal. The work described here has demonstrated that cdk1F shows a strong genetic interaction with the RNAP II CTD phosphatase gene Anfcp1. Taken together with the fact that Cdk1 is a major mitotic protein kinase which in other systems is capable of phosphorlating RNAP II CTD in vitro, the Cdk1 kinase could be considered a compelling candidate to promote such a mitosis-specific phosphorylation event of RNAP II CTD. Intriguingly, the mitotic hyperphosphorylation of RNAP II CTD in higher eukaryotes is dependent on the Pin1 peptidyl-prolyl isomerase (58). Pin1 has been shown to both inhibit Fcp1 and activate Cdk1, thus promoting mitotic hyperphosphorylation of RNAP II CTD. It will thus be interesting to investigate the potential role of Pin1 (PinA in A. nidulans [20]) in mediating the genetic interactions and mitotic phenotypes (see below) we have uncovered while studying Fcp1 and Cdk1. One prediction is that increased expression of Pin1 might phenocopy the fcp1ts cdk1F double mutant (by inhibiting Fcp1 and activating Cdk1).

Mitotic defects in the Anfcp1ts cdk1F double mutant. When the localization pattern of RNAP II was examined in the Anfcp1ts, cdk1F, and Anfcp1ts cdk1F strains at 38°C and compared to wild-type cells, the Anfcp1ts cdk1F double mutants revealed by far the most severe defects. The various pleiotropic defects observed in the Anfcp1ts cdk1F double mutants strongly indicates that the synthetic interaction between the Anfcp1ts and cdk1F mutation may impact cells in ways that might go beyond the immediate disruption of the RNAP II-driven transcription cycle. Although the RNAP II CTD is thus far the only known substrate of Fcp1, it cannot be ruled out that FCP1 and Cdk1 may share other common substrates. Since the effects of mutations of Anfcp1 and cdk1 would be complemented in a pleiotropic manner if they were to share many substrates, this could explain why the difference of RNAP II CTD phosphorylation between cdk1F, Anfcp1ts, and Anfcp1ts cdk1F mutants is only moderate compared to the severity of the cytological defects observed in the double mutant. Alternatively, the effects of the double mutant on the fidelity of transcription could potentially preferentially affect gene expression specific to cell cycle transitions, thus causing the mitotic defects observed. It is also possible Cdk1 and FCP1 do not directly share a common substrate but function in different cellular pathways. The interaction between Anfcp1 and cdk1F is genetic in nature, and Anfcp1ts and cdk1F could still show their synthetically interacting effects if the separate biological pathways in which they function merge onto common cellular targets that provide important activities concerning mitotic regulation. In fact, reports suggesting the involvement of Fcp1 in DNA damage responses (19) indicate that Fcp1 may functionally relate to Cdk1 in a manner more directly linked to mitotic regulation. However, we could not detect obvious DNA damage response defects in the fcp1ts allele.

Overall, the severe mutant phenotypes observed in the double mutant compared to that exhibited by the Anfcp1ts and cdk1F single mutants confirms the relevance of the genetic interaction between Anfcp1ts and cdk1F and provides important evidence that the conserved Fcp1 phosphatase might play a role in mitotic regulation by counteracting Cdk1-mediated phosphorylation.

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