Functional Analysis of the *Aspergillus nidulans* Kinome

Colin P. De Souza¹*, Shah B. Hashmi¹, Aysha H. Osmani¹, Peter Andrews³, Carol S. Ringelberg², Jay C. Dunlap², Stephen A. Osmani¹

¹Department of Molecular Genetics, The Ohio State University, Columbus, Ohio, United States of America, ²Department of Genetics, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, United States of America, ³Institute for Quantitative Biomedical Sciences, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, United States of America

### Abstract

The filamentous fungi are an ecologically important group of organisms which also have important industrial applications but devastating effects as pathogens and agents of food spoilage. Protein kinases have been implicated in the regulation of virtually all biological processes but how they regulate filamentous fungal specific processes is not understood. The filamentous fungus *Aspergillus nidulans* has long been utilized as a powerful molecular genetic system and recent technical advances have made systematic approaches to study large gene sets possible. To enhance *A. nidulans* functional genomics, we have created gene deletion constructs for 9851 genes representing 93.3% of the encoding genome. To illustrate the utility of these constructs, and advance the understanding of fungal kinases, we have systematically generated deletion strains for 128 *A. nidulans* kinases including expanded groups of 15 histidine kinases, 7 SRPK (serine-arginine protein kinases) kinases and an interesting group of 11 filamentous fungal specific kinases. We defined the terminal phenotype of 23 of the 25 essential kinases by heterokaryon rescue and identified phenotypes for 43 of the 103 non-essential kinases. Uncovered phenotypes ranged from almost no growth for a small number of essential kinases implicated in processes such as ribosomal biosynthesis, to conditional defects in response to cellular stresses. The data provide experimental evidence that previously uncharacterized kinases function in the septation initiation network, the cell wall integrity and the morphogenesis Orb6 kinase signaling pathways, as well as in pathways regulating vesicular trafficking, sexual development and secondary metabolism. Finally, we identify ChkC as a third effector kinase functioning in the cellular response to genotoxic stress. The identification of many previously unknown functions for kinases through the functional analysis of the *A. nidulans* kinome illustrates the utility of the *A. nidulans* gene deletion constructs.

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### Introduction

The filamentous fungi have critical ecological roles both as plant symbionts and recyclers of biomass. They have additional economic impact via their beneficial industrial applications and via their detrimental effects as pathogens and agents of food spoilage [1]. The filamentous ascomycete *Aspergillus nidulans* has historically been a productive model system for the discovery of genes involved in fungal specific processes such as secondary metabolite production as well as universal regulators of the cell cycle and cytoskeleton [2–4]. Along with a growing number of *Aspergillus* and other filamentous fungi, the *A. nidulans* genome has been sequenced and the extensive annotation of this organism is being further refined by recently available RNA-Seq data [5–11]. However, despite these advances, the majority of filamentous fungal genes still await characterization. To provide tools to enhance this effort we describe the generation of gene knock-out constructs for 93.3% of the 10,560 predicted *A. nidulans* genes. To demonstrate the utility of these constructs and to better understand fungal kinase biology, we have created and phenotypically characterized gene deletion strains for 128 *A. nidulans* protein kinases.

Reversible protein phosphorylation plays a critical role in the regulation of virtually all eukaryotic biological processes [12–18]. Reflecting this, a significant proportion of eukaryotic genomes encode enzymes regulating phosphorylation. For example, the 131 predicted protein kinases and 28 phosphatase catalytic subunits respectively comprise 1.25% and 0.27% of the *A. nidulans* genome [19]. Based on their catalytic domains conventional protein kinases can be classified into the following groups; AGC (protein kinase A, G or C), CAMK (Ca2+/calmodulin-dependent protein kinases), CK1 (casein kinase 1), CMGC (cyclin-dependent, mitogen-activated, glycogen synthase and cyclin-dependent protein kinase-like kinases), STE (sterile kinases), RGC (receptor guanlylate cyclase kinases), TK (tyrosine kinases), TKL (Tyrosine like kinases), and others [20]. Additional atypical kinases, which show little or no similarity to the above conventional kinases, include the PIKK (Phosphatidylinositol kinase-related kinases), PDHK (pyruvate dehydrogenase kinases), RIO (right open reading frame) and histidine kinases [20]. Interestingly, recent analysis indicates that filamentous fungi contain novel families of kinases not related to the above conventional kinases, include the PIKK (Phosphatidylinositol kinase-related kinases), PDHK (pyruvate dehydrogenase kinases), and other families of kinases not related to the above families of kinases [9–11,14,16].

Cells respond to environmental stimuli through signaling pathways often involving kinase cascades which transmit external signals to downstream effectors. These circuits are highly complex and interconnected, giving rise to a variety of responses. The understanding of the regulatory logic underlying these networks is incomplete. However, recent improvement in methods for genetic manipulation of filamentous fungi such as *A. nidulans* have allowed systematic functional analysis of large numbers of genes and identification of novel kinases with unknown functions [21–23].

The filamentous fungi have critical ecological roles both as plant symbionts and recyclers of biomass. They have additional economic impact via their beneficial industrial applications and via their detrimental effects as pathogens and agents of food spoilage. Protein kinases have been implicated in the regulation of virtually all biological processes but how they regulate filamentous fungal specific processes is not understood. The filamentous fungus *Aspergillus nidulans* has long been utilized as a powerful molecular genetic system and recent technical advances have made systematic approaches to study large gene sets possible. To enhance *A. nidulans* functional genomics, we have created gene deletion constructs for 9851 genes representing 93.3% of the encoding genome. To illustrate the utility of these constructs, and advance the understanding of fungal kinases, we have systematically generated deletion strains for 128 *A. nidulans* kinases including expanded groups of 15 histidine kinases, 7 SRPK (serine-arginine protein kinases) kinases and an interesting group of 11 filamentous fungal specific kinases. We defined the terminal phenotype of 23 of the 25 essential kinases by heterokaryon rescue and identified phenotypes for 43 of the 103 non-essential kinases. Uncovered phenotypes ranged from almost no growth for a small number of essential kinases implicated in processes such as ribosomal biosynthesis, to conditional defects in response to cellular stresses. The data provide experimental evidence that previously uncharacterized kinases function in the septation initiation network, the cell wall integrity and the morphogenesis Orb6 kinase signaling pathways, as well as in pathways regulating vesicular trafficking, sexual development and secondary metabolism. Finally, we identify ChkC as a third effector kinase functioning in the cellular response to genotoxic stress. The identification of many previously unknown functions for kinases through the functional analysis of the *A. nidulans* kinome illustrates the utility of the *A. nidulans* gene deletion constructs.
cellular signals to the nucleus [9,21]. Examples of this include the MAPK (mitogen activated protein kinase) signaling pathways which are found throughout eukaryotes [22,23]. Although absent from mammals, many eukaryotes also utilize two component cell signaling systems consisting of a histidine kinase and a response regulator [24,25]. Relative to yeast, filamentous fungi encode an expansion of histidine kinases which are of considerable interest as anti-fungal targets [23,26]. Unlike other protein kinases which phosphorylate serine, threonine and/or tyrosine residues, histidine kinases transfer phosphate groups between specific histidine and aspartate residues [24,25]. In addition, individual cell signaling pathways can communicate with each other thereby building a complex network to ultimately control gene expression and other cellular functions.

Cell growth and the cell cycle are also in large part coordinated by kinases. For example, the Cdc1 mitotic kinase is kept inactive during G2 by inhibitory phosphorylation carried out the Weel kinase. Removal of Cdc1 inhibitory phosphorylation by the Cdc25 phosphatase activates Cdc1 thereby promoting entry into mitosis [3,27]. In A. nidulans, Magnaporthe grisea and likely other filamentous fungi, activation of the NIMA kinase is additionally required to promote mitotic entry [3,28,29]. Kinases also play central roles following exposure of cells to genotoxic or osmotic stress [30,31]. For example, when DNA is damaged, the ATM and/or ATR PIKK kinases phosphorylate the downstream effector kinases Chkl and Chk2 [31]. These effector kinases then signal to the DNA repair and cell cycle machinery to arrest the cell cycle until DNA repair is completed.

Several kinases have been intensively studied in filamentous fungi including Neurospora crassa Cot-1 and A. nidulans NIMA which are the founding members of their respective kinase families [32,33]. However, despite their importance, only 44 of the 131 kinases encoded by the A. nidulans kinase have been genetically characterized. By phylogenetic analysis we show that this kinase encodes 11 predicted filamentous fungal kinases (Fkls) not found outside the filamentous fungi. Using deletion constructs we describe the generation and phenotypic analysis of mutants for 128 A. nidulans kinases. This analysis defined 25 kinases with an essential function and a further 43 kinases whose deletion lead to at least one phenotype. We have expanded on recent studies of the kinomes of N. crassa [16] and the wheat scab fungus Fusarium graminearum [14] by including the PIKK and histidine kinases, and by utilizing heterokaryon rescue to phenotypically analyze cells lacking the essential A. nidulans kinases. Our phenotypic analysis has implicated A. nidulans kinases in regulating ribosomal biogenesis, mRNA splicing and the unfolded protein response, and revealed previously unknown roles for kinases in the regulation of vegetative growth, septation, polarized growth, the cell cycle, development, secondary metabolite production, the DNA damage response, and the cellular response to osmotic stress.

**Results**

**Global Production of A. nidulans Gene Deletion Constructs**

To generate gene deletion constructs for all A. nidulans genes we utilized a high throughput platform used to generate similar constructs for Neurospora crassa [34]. Constructs were designed to replace the open reading frame (ORF) of each A. nidulans gene with pyrG [A. fumigatus orotidine 5’-phosphate carboxylase which complements the A. nidulans pyrG89 or ΔpyrG mutations] (Figure 1). The pyrG nutritional marker was chosen as it provides a robust means to identify and phenotypically study gene deletions which cause lethality using the heterokaryon rescue technique [35], and additionally allows counter selection using 5-FOA (5-Fluoroorotic Acid) [36]. Deletion constructs consisted of 0.6–1 kb of 5’ sequence flanking the target gene ORF (5’ flank), pyrGΔ, and 0.6–1 kb of 3’ sequence flanking the target gene ORF (3’ flank), and were generated by PCR and yeast recombinational cloning (Figure 1). For each deletion construct, four primers, designated 5f, 5r, 3f and 3r, were designed as described using some modifications [34] (materials and methods). Extensions on the 3r and 5r primers complementary to the pyrGΔ cassette, and on the 5f and 3r primers complementary to a yeast vector, facilitated yeast recombinational cloning (Figure 1). Primer design was successful for 95.4% of the 10,360 ORFs predicted in the Broad A. nidulans database (version 2) and these primers are listed in Table S1 (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm). Design failures were attributable to the close proximity of genes to contig ends or because the sequence flanking the gene was not compatible with the stringent parameters of the design algorithm. The final linear deletion constructs were amplified by PCR with a 95.3% success rate. Of the 263 genes for which yeast recombinational cloning failed, 249 were successfully generated by fusion PCR [37,38] using the same primers. In total, full length deletion constructs were generated for 9,851 A. nidulans genes (93.3%) and are available under request.

![Figure 1. Strategy for generation of deletion constructs.](image-url)
The Aspergillus Nidulans Kinome

Although protein kinases are involved in the regulation of essentially all biological processes, many kinases remain poorly studied. The *A. nidulans* genome encodes 131 predicted protein kinases, of which only 44 (33.6%) have been genetically characterized. Analysis of the catalytic domains of these kinases by BLAST comparison with the Salk Institute’s kinome database (http://kinase.com/) allowed 120 to be classified into the following groups of kinases: 13 AGC (protein kinase A, G or C), 15 CAMK (Ca2+/calmodulin-dependent protein kinases), 2 CK1 (casein kinase 1), 27 CMGC (cyclin-dependent, mitogen-activated, glycogen synthesis and cyclin-dependent kinase-like kinases), 12 STE (sterile kinases), 11 atypical, 15 atypical histidine kinases and 25 other (Table 1, Figure 2). We assigned names for previously unnamed kinases based on the most well characterized orthologous kinase present in other organisms (*see Table 1*).

Notably, the above domain analysis identified 11 kinases with catalytic domains displaying only low similarity to the complete set of *S. cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and human kinases within the kinome database. We have called these novel kinases FfK-A-K (filamentous fungal kinase) as they appear to be specific to filamentous fungi. Importantly recently available *A. nidulans* RNA-Seq data generated by the Caddick laboratory (http://www.aspgd.org/) indicates that all 7 SrpkB-G. Importantly, analysis of RNA-Seq data (http://www.aspgd.org/) indicates that all 7 *A. nidulans* SRPKs and 15 histidine kinases are expressed.

Generation of *A. nidulans* Kinase Deletion Null Mutants

To illustrate the utility of the gene deletion constructs and provide tools useful to the research community, we systematically generated gene knock out strains of each *A. nidulans* kinase. Kinase deletion constructs were transformed into a *pyrG* strain, *A. nidulans* recipient strain selecting for growth on media lacking uridine and uracil. The *A. nidulans* genetic background of this strain facilitated a high yield of construct targeting via homologous recombination [43]. For each gene deletion multiple primary transformants were tested to determine if the respective kinase was essential or non-essential using the heterokaryon rescue technique [35]. This basis is on the ability of *A. nidulans* to form heterokaryons containing two genetically distinct nuclei within a common cytoplasm. Following deletion of an essential kinase, primary transformant colonies can maintain a heterokaryotic state with cells containing transformed (kinase-) nuclei providing *PyrG* media on which they grow whereas the kinase deleted phenotype. An important part of this analysis is that conidia streaked on non-selective media should grow since these conidia are uninucleate. Such heterokaryotic colonies are therefore generated 2 types of uninucleate conidia from the *pyrG* strain: one that is a heterokaryotic colony and the other that is a homokaryotic colony. In the former, conidia from primary transformants should grow on selective or non-selective media. Contrasting this, following deletion of a non-essential kinase, conidia from primary transformants should grow similarly on selective or non-selective media (Figure 3A). We found that replica plating of primary transformants using a velvet disk greatly expedited this analysis and allowed both essential and non-essential kinases to be identified.

Differences in the normal repertoire of kinases was in the PIKK family of atypical protein kinases which have critical conserved functions in diverse cellular processes. For example, the Aspergilli and *N. crassa* each only encode a single *Tor* PIKK kinase whereas two *Tor* kinases are present in *S. pombe*, *S. cerevisiae* and humans (Figure 2B in File S1). Even more surprisingly, *A. nidulans* does not encode an orthologue of the PIKK kinase which is essential in both *S. pombe* and *S. cerevisiae* where it has been implicated in the regulation of protein secretion, endocytosis, vacuolar dynamics and cytokinesis [40]. Other Aspergilli also lack a PIKK orthologue although a PIKK kinase (NCU10397) is encoded within the *N. crassa* genome (Figure 2B in File S1). One possibility is that the essential function of PIKK in other organisms is carried out by the related Str4 PIKK kinase in the Aspergilli. As previously reported *A. nidulans* and *N. crassa* encode an expansion of histidine kinases (Figure S3 in File S1) [23, 26, 41]. An expansion of SRPK (serine-arginine protein kinases) kinases within the GMCG kinase group was also present in both *A. nidulans* and *N. crassa*. A similar expansion of SRPK kinases has recently been reported in Dermatophytes [9]. Members of this kinase family are known to phosphorylate serine within RS (arginine/serine rich) domains that are generally found in splicing factors and other proteins involved in mRNA maturation [42]. Whereas *S. pombe* and *S. cerevisiae* each encode a single SRPK family member, Dsk1 and Sky1 respectively, *A. nidulans* encodes 7 and *N. crassa* encodes 5 predicted SRPK kinases (Figure S4 in File S1). We named the *A. nidulans* Dsk1/Sky1 orthologue *SrpkA* [34] and the other *A. nidulans* SRPK kinases *SrpkB-G*. Importantly, analysis of RNA-Seq data (http://www.aspgd.org/) indicates that all 7 *A. nidulans* SRPKs and 15 histidine kinases are expressed.

from the Fungal Genetics Stock Center (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm).
| Systematic name | A. nidulans name | S. cerevisiae orthologue | S. pombe orthologue | N. crassa orthologue | Group/Family/Sub-Family | Deletion | Phenotype |
|-----------------|------------------|--------------------------|--------------------|---------------------|-------------------------|----------|-----------|
| AN4238 SchA     | SCH9             | sck1                     | NCU03200 stk-10    | AGC/Akt             | Viable                  |          |           |
| AN5973 PkcB     | YPK1 (BH)        | gad8                     | NCU07280 stk-50    | AGC/Akt             | Lethal                  | Microcolony |           |
| AN5529 CotA     | CBK1 (BH)        | orb6                     | NCU07296 cot-1     | AGC/NDR             | Lethal                  | Microcolony; brown; Polarity defect |           |
| AN10485 An-Stk21* | CBK1 (BH)         | orb6 (BH)                | NCU03242 stk-21    | AGC/NDR             | Viable                  |          |           |
| AN7572 SnB      | RIM15            | ppk18                    | NCU07378 stk-12    | AGC/NDR             | Viable                  | NaCl      |           |
| AN8751 SidB     | DBF20/2          | sid2                     | NCU09071 dbf-2     | AGC/NDR             | Viable                  | Strong growth defect-septation and conidiation; NaCl^5       |           |
| AN3110 An-Ksg1* | PKH1/2 (BH)      | ksg1                     | NCU03571 stk-23    | AGC/PDK1            | Lethal                  | Microcolony |           |
| AN4717 PkaB     | TPK1 (BH)        | pka1 (BH)                | NCU00682 pka-2     | AGC/PKCA            | Viable                  | Strong growth defect; Suc^res; NaCl^res            |           |
| AN6305 PkaA     | TPK2             | pka1                     | NCU06240 pka-1     | AGC/PKA             | Viable                  | Swollen    |           |
| AN1006 PckA     | PKC1 (BH)        | pck2                     | NCU06544 pck       | AGC/PKC/PKC         | Lethal                  | Moderate growth defect |           |
| AN4980 An-Psk1* | YPK3 (BH)        | psk1                     | NCU03197 stk-11    | AGC/RSK             | Viable                  | NaCl^5    |           |
| AN0144 An-Nrc2* | PFK1/KIN82 (BH)  | ppk14                    | NCU01797 nrc-2     | AGC/RSK             | Viable                  | Moderate growth defect |           |
| AN7537 An-Pgk3* | YPK2 (BH)        | ppk33                    | NCU07062 stk-49    | AGC/YANK            | Viable                  | Moderate growth defect; increased pigment production |           |
| AN2412 CmkA     | CMK1/2           | cmk1                     | NCU09123 camk-1    | CAMK/CAMK           | Viable                  | Moderate growth defect |           |
| AN3065 CmkB     | CMK1/2 (BH)      | cmk1                     | NCU02283 camk-2    | CAMK/CAMK           | Viable                  |           |           |
| AN4483 CmkD*    | RCK1/2 (BH)      | skr1                     | NCU09212 camk-4    | CAMK/CAMK           | Viable                  |           |           |
| AN7695 An-Snf1* | SNF1             | ssp2                     | NCU04566 prk-10    | CAMK/CAMK/AMPK      | Viable                  |           |           |
| AN494 ChkA      | CHK1             | chk1                     | NCU08346 mus-58    | CAMK/CAMK/CHK1      | Viable                  | HU; DEO |           |
| AN1110 An-Gin4* | GIN4             | cdr2 (BH)                | NCU09064 stk-53    | CAMK/CAMK/GIN4      | Viable                  | Early sexual development |           |
| AN1171 An-Kin1* | KIN1 (BH)        | kin1                     | NCU04747 stk-31    | CAMK/CAMK/Kin1      | Viable                  | Moderate growth defect |           |
| AN0822 KsA      | KIN4 (BH)        | ppk1 (BH)                | NCU00914 stk-16    | CAMK/CAMK/Kin4      | Viable                  | Moderate growth defect |           |
| AN5759 An-Stk19* | YPL150W (BH)     | ppk16 (BH)               | NCU02245 stk-19    | CAMK/CAMK/ARK       | Viable                  |           |           |
| AN4536 An-Psk1* | PSK1/2           | ppk6                     | NCU06249 stk-40    | CAMK/CAMK/PASK      | Viable                  |           |           |
| AN6347 An-Stk26* | PRR1 (BH)        | ppk27 (BH)               | NCU04143 stk-26    | CAMK/CAMK/Unique    | Viable                  |           |           |
| AN7737 An-Mek1* | MEK1             | mek1                     | NCU06486 stk-43 (BH) | CAMK/CAMK/Unique | Viable                  |           |           |
| AN1097 An-Nnk1* | NNK1             | kin1 (BH)                | None               | CAMK/CAMK/Unique    | Viable                  |           |           |
| AN4279 ChkB     | DUN1             | cds1                     | NCU02814 prd-4     | CAMK/RAD53          | Viable                  | HU        |           |
| AN7563 ChkC*    | RAD53 (BH)       | cds1 (BH)                | NCU02751 mus-59    | CAMK/RAD53          | Viable                  | HU        |           |
| AN4563 CkA      | HRR25 (BH)       | hhp1                     | NCU00685 ck-1a     | CK1/CK1/CK1-D       | Lethal                  | Short germling arrest |           |
| AN5757 Ckb      | YCK2             | chi1                     | NCU04005 ck-1b     | CK1/CK1/CK1-G       | Viable                  | Strong growth defect |           |
| AN0699 An-Cak1* | CDC28 (BH)       | csk1                     | NCU04426 div-4     | OMGC/CDK/CDC2       | Viable                  | Strong growth defect; Het |           |
| AN4182 NimX^ck-1 | CDC28            | cdc2                     | NCU09778 cdc28     | OMGC/CDK/CDC2       | Lethal                  | Cell cycle |           |
| AN1867 PhoB     | PHO85            | pef1                     | NCU07580 mdk-1     | OMGC/CDK/CDK5       | Viable                  |           |           |

**Table 1.** Summary of *A. nidulans* protein kinase classification and phenotypes.
| Systematic name | A. nidulans name | S. cerevisiae orthologue | S. pombe orthologue | N. crassa orthologue | Group/Family/Sub-Family | Deletion | Phenotype |
|-----------------|-----------------|--------------------------|-------------------|---------------------|--------------------------|----------|-----------|
| AN4936          | An-Prp4*        | SGV1 (BH)                | prp4              | NCU01435            | stk-1 CMGC/CDK           | Nulls not generated from heterokaryons |
| AN7185          | SrpkA           | Dsk1*                    | lkh1              | NCU09202            | mdk-2 CMGC/CDK           | Nulls not generated from heterokaryons |
| AN6508          | An-Gsk3*        | RIM11 (BH)               | gsk3              | NCU04185            | gsk-3 CMGC/GSK           | Nulls not generated from heterokaryons |
| AN7104          | An-Yak1*        | YAK1 (BH)                | ppk15             | NCU07872            | prk-2 CMGC/DYRK/YAK      | Viable Moderate growth defect; Yellow condensation defect; Suc Rem; S u |
| Systematic name | A. nidulans name | S. cerevisiae orthologue | S. pombe orthologue | N. crassa orthologue | Group/Family/Sub-Family | Deletion | Phenotype |
|-----------------|------------------|--------------------------|---------------------|----------------------|--------------------------|----------|-----------|
| AN2054          | An-Haspin*       | Alk1/2                   | hrk1                | NCU00407 UN          | Other/Haspin             | Viable   |           |
| AN1665          | An-Stk51*        | IK51 (BH)                | ssr1 (BH)           | NCU08177 stk-51     | Other/IKS                | Viable   |           |
| AN0235          | IreA*            | IRE1                     | ire1                | NCU02202 stk-14     | Other/IRE                | Lethal   | Swollen  |
| AN1015          | An-Pnk1*         | PRK1/ARK1                | ppk30 (BH)          | NCU06202 stk-38     | Other/NAK/NAK            | Viable   | Strong growth defect |
| AN10193         | An-Env7*         | ENV7                     | ppk13               | NCU07399 stk-9      | Other/NAK                | Viable   |           |
| AN9504          | NimA             | KIN3 (BH)                | fin1                | NCU31877 nim-1      | Other/NEK/NEK2           | Lethal   | Cell cycle |
| AN2246          | An-Gcn2*         | GCN2                     | gcn2                | NCU01187 cpc-3      | Other/PEK/GCN2           | Viable   |           |
| AN7321          | An-HriA*         | GCN2 (BH)                | hri1/2              | NCU01187 (BH)       | Other/PEK/HRI            | Viable   |           |
| AN1560          | PIKA             | CDC5 (BH)                | plo1                | NCU09258 cdc5       | Other/PLK                | Viable   | Strong growth defect; Early sexual development; NaCl |
| AN265           | An-Ksp1*         | KSP1 (BH)                | ksp1                | NCU06230 stk-39     | Other/RAN                | Viable   |           |
| AN4935          | An-Ran1*         | VHS1/SKS1                | ran1                | NCU04990 stk-17/45  | Other/RAN                | Viable   |           |
| AN4322          | An-Scy1*         | SCY1                     | ppk32               | NCU04755 stk-32     | Other/SCY1               | Viable   |           |
| AN2927          | An-Mps1*         | MPS1 (BH)                | mph1 (BH)           | NCU00978 UN         | Other/TTK                | Lethal   | Nulls not generated from heterokaryons |
| AN1632          | An-Atg1*         | ATG1                     | atg1                | NCU00188 apg-1      | Other/ULK/ULK            | Viable   |           |
| AN0576          | An-Vps15*        | VPS15                    | ppk19               | NCU06626 stk-45     | Other/VPS15              | Lethal   | Microcolony |
| AN3001          | An-Irs1*         | IRS1 (BH)                | None                | NCU02201 stk-14     | Other/Other-Unique       | Viable   |           |
| AN5822          | An-Wee1          | SWE1 (BH)                | wee1                | NCU04326 stk-19     | Other/WEE                | Lethal   | Cell cycle |
| AN4385          | SepH             | CDC15                    | cdc7                | NCU01335 cdc15      | STE/STE11/CDC15          | Lethal   | Strong growth defect-septation and conidiation; NaCl; Het |
| AN10153         | Ssk8             | SSK2 (BH)                | wts4                | NCU03071 os-4       | STE/STE11/STE11          | Viable   | NaCl; Suc |
| AN2269          | SteC             | STE11                    | byr2                | NCU06182 nrc-1      | STE/STE11                | Viable   | Moderate growth defect; Arrested sexual development |
| AN4887          | BckA             | BCK1 (BH)                | mkk1 (BH)           | NCU02234 mkk-1      | STE/STE11                | Lethal   | Branched germling arrest; NaCF2m; Suc2m |
| AN6339          | An-Pod5*         | KIC1 (BH)                | nak1 (BH)           | NCU11235 pod-6      | STE/STE20                | Lethal   | Microcolony; brown; Polarity defect |
| AN2067          | An-Ste20*        | STE20 (BH)               | shk1                | NCU03894 stk-4      | STE/STE20/PAKA           | Viable   | Increased pigment production |
| AN8836          | An-Clad4*        | CLA4                     | shk2                | NCU00406 vel        | STE/STE20/PAKA           | Viable   | Strong growth defect |
| AN11032         | SepL*            | SPS1 (BH)                | sid1                | NCU04096 prk-9      | STE/STE20/YSK            | Viable   | Strong growth defect-septation and conidiation; NaCl |
| AN5674          | An-Mst1          | KIC1 (BH)                | ppk11 (BH)          | NCU00772 mst-1      | STE/STE20/YSK            | Viable   | Early but incomplete sexual development |
| AN0931          | PbsA             | PBS2                     | wts1                | NCU00587 os-5       | STE/STE7/STE7            | Viable   | NaCl; Suc |
| AN3422          | Ste7             | STE7                     | byr1                | NCU04612 mek-2      | STE/STE7                 | Viable   | Moderate growth defect; Arrested sexual development |
| AN4189          | MkkA             | MKK1                     | pek1                | NCU06419 mek-1      | STE/STE7                 | Lethal   | Branched germling arrest; NaCF2m; Suc2m |
| AN7986          | FRA*             | None                     | None                | NCU06582 (BH)       | Unclassified; Ank        | Viable   |           |
| AN10819         | FKB*             | None                     | None                | NCU06582 (BH)       | Unclassified; Ank        | Viable   |           |
| Systematic name | *A. nidulans* name | *S. cerevisiae* orthologue | *S. pombe* orthologue | *N. crassa* orthologue | Group/Family/Sub-Family | Deletion | Phenotype |
|-----------------|-------------------|---------------------------|----------------------|------------------------|------------------------|----------|-----------|
| AN2373          | FfkC*             | None                      | None                 | NCU06583 (BH)          | Unclassified; Ank      | Viable   |           |
| AN1789          | FfkD*             | None                      | None                 | NCU11331 (BH)         | Unclassified           | Viable   |           |
| AN10869         | FfkE*             | None                      | None                 | NCU11331 (BH)         | Unclassified           | Viable   |           |
| AN4196          | FfkF*             | None                      | None                 | NCU03250              | Unclassified           | Viable   |           |
| AN6192          | FfkG*             | None                      | None                 | NCU05260              | Unclassified           | Viable   |           |
| ANS111          | Fkh*              | None                      | None                 | NCU05638 stk-34 (BH)  | Unclassified           | Viable   |           |
| AN6758          | FkJ*              | None                      | None                 | None                  | Unclassified           | ND       |           |
| AN9302          | Fkk*              | None                      | None                 | None                  | Unclassified           | ND       |           |
| AN10800         | PkpA*             | PKP1                      | Pkp1 (BH)            | NCU11744 stk-58       | Atypical/PDKH          | Viable   |           |
| AN9461          | PkpB*             | PKP2                      | Pkp1 (BH)            | NCU03796 stk-24       | Atypical/PDKH          | ND       |           |
| AN6207          | PkpC*             | PKP2 (BH)                 | Pkp1                 | NCU06760 stk-48       | Atypical/PDKH          | Viable   |           |
| AN4278          | An-Slt4*          | STT4                      | stt4                 | NCU09367 UN           | Atypical/PIKK          | Lethal   | Micro; V; Cell cycle |
| AN4709          | An-Vps34          | VP34                      | pik3                 | NCU00656 UN           | Atypical/PIKK          | Lethal   | Microcolony |
| AN10791         | An-Lsb6*          | LSB6                      | lsb6                 | NCU04355 UN           | Atypical/PIKK          | Viable   | Strong growth defect; Het |
| AN0033          | AtmA              | TEL1                      | tel1                 | NCU00274 mus-21       | Atypical/PIKK/ATM      | Viable   | Camp |
| AN6975          | UvsB              | MEC1                      | rad3                 | NCU11188 mus-9        | Atypical/PIKK/ATR     | Viable   | Moderate growth defect; HU; DEO; Camp; Colony color |
| AN5982          | TorA              | TOR1/2                    | tor1/2               | NCU05608 div-18       | Atypical/PIKK/FRAP     | Lethal   | Short germling arrest; Early septation |
| AN6363          | SudD              | RIO1 (BH)                 | SPAC10F6.10          | NCU 08767 stk-52      | Atypical/RIO/RIO1      | Lethal   | Short germling arrest; Swollen |
| AN0124          | An-Rio2*          | RIO2                      | SPBC1703.05          | NCU07722 rgb-40       | Atypical/RIO/RIO2      | Lethal   | Short germling arrest |
| AN5296          | TcsA              | SKN7 (BH)                 | mak2 (BH)            | NCU07221              | Atypical/HisK          | Viable   |           |
| AN1800          | TcsB              | SLN1                      | mak2 (BH)            | NCU04615              | Atypical/HisK          | Viable   |           |
| AN7945          | hkh-2             | SNL1 (BH)                 | mak2 (BH)            | NCU00939 dcc1         | Atypical/HisK          | Viable   |           |
| AN2581          | hkh-8-1           | SSK1 (BH)                 | mcs4 (BH)            | NCU09520 (BH)         | Atypical/HisK          | Viable   |           |
| AN4113          | hkh-8-2           | SSK1 (BH)                 | mcs4 (BH)            | NCU09520 (BH)         | Atypical/HisK          | Viable   |           |
| AN6820          | hkh-8-3           | SSK1 (BH)                 | mcs4 (BH)            | NCU09520 (BH)         | Atypical/HisK          | Viable   |           |
| AN4818          | hkh-8-4           | SSK1 (BH)                 | mck2 (BH)            | NCU09520 (BH)         | Atypical/HisK          | Viable   |           |
| AN3214          | hkh-8-5           | SKN7 (BH)                 | mck2 (BH)            | NCU09520 (BH)         | Atypical/HisK          | Viable   |           |
| AN2363          | hkh-8-6           | SNL1 (BH)                 | mck2 (BH)            | NCU09520 (BH)         | Atypical/HisK          | Viable   |           |
| AN9048          | hkh-8-7           | SNL1 (BH)                 | mck1 (BH)            | NCU09520 (BH)         | Atypical/HisK          | Viable   |           |
| AN4447          | hkh-9*            | SNL1 (BH)                 | mck1 (BH)            | NCU09520 (BH)         | Atypical/HisK          | Viable   |           |
| AN4479          | NikA              | SKN7 (BH)                 | mck2 (BH)            | NCU02815 nik1         | Atypical/HisK          | Viable   | Strong growth defect |
| AN3102          | PhkA              | SNL1 (BH)                 | mck3 (BH)            | NCU01823              | Atypical/HisK          | Viable   |           |
### Table 1. Cont.

| Systematic name | A. nidulans orthologue | S. cerevisiae orthologue | N. crassa orthologue | Group/Family/Sub-Family | Deletion Phenotype |
|------------------|------------------------|-------------------------|---------------------|------------------------|--------------------|
| AN9008          | FphA                   | SNL1 (BH)              | mak2 (BH)           | Atypical/HisK          | Viable             |

**Group abbreviations:**
- AGC (protein kinase A, G or C)
- CK1 (casein kinase 1)
- CAMK (Ca²⁺/calmodulin-dependent protein kinase)
- CMGC (cyclin-dependent, mitogen-activated, glycogen synthase and cyclin-dependent protein kinase-like kinases)
- STE (sterile kinases)
- HisK (histidine kinase).

**Family abbreviations:**
- Akt (oncogene protein of v-akt)
- NDR (nuclear Dbf2 related)
- PDK1 (phosphoinoside-dependent protein kinase)
- PKA (protein kinase A)
- PKC (protein kinase C)
- RSK (ribosomal S6 kinase)
- YANK (yet another novel kinase)
- CAMKL (CAMK-like kinase)
- CK (casein kinase)
- CDK (cyclin-dependent protein kinase)
- CLK (CDK-like kinase)
- DYRK (dual-specificity tyrosine phosphorylation-regulated kinase)
- GSK (glycogen synthase kinase)
- MAPK (mitogen-activated protein kinase)
- RCK (related to murine RCK)
- SRPK (serine-arginine protein kinase)
- AUR (Aurora)
- CAMKK (CAMK kinase)
- CDC (cell division cycle)
- HAL (halotolerance)
- IRE (inositol-requiring protein)
- NAK (NF-B-activating kinase)
- NEK (NIMA-related kinase)
- PEK (pancreatic alpha-subunit of eukaryotic initiation factor kinase)
- PLK (polo kinase)
- RAN (Ran GTPase kinase)
- SCY1 (related to Scy1 kinase)
- ULK (Unc-51-like kinase)
- VPS (vacuolar protein sorting)
- WEE (small)
- IKS (Ira1 kinase suppressor)
- PDHK (pyruvate dehydrogenase kinase)
- PIKK (phosphatidyl inositol kinase-related kinase)

**Other abbreviations:**
- Ank = contains ankyrin repeat domains
- HU = HU sensitive
- Camp = Camptothecin sensitive
- DEO = DEO sensitive
- Ben = benomyl sensitive
- NaCls = growth inhibition in the presence of NaCl
- Sucs = growth inhibition in the presence of 1.5 M sucrose
- NaClRem = defects remediated on NaCl
- SucRem = defects remediated on sucrose
- Colony color = Abnormal colony color
- Brown = brown pigment produced by colonies/microcolonies
- Yellow = yellow pigment production
- Cell cycle = cell cycle defect
- k = kinase domain deletion
- V = variability in phenotype
- Het = also recovered heterokaryons

**Phenotypic Analysis of Non-essential Kinase Mutants**

**Non-essential kinase mutants displaying general growth defects.** We analyzed colony formation of non-essential kinase mutants at 20°, 32°, 37° and 42° to determine if these kinases had roles in vegetative growth and development. This identified 29 mutants, including 14 previously uncharacterized kinases, with growth defects and/or poor conidiation (Figure 4). Of these kinase mutants, 14 displayed moderate but reproducible growth defects while 15 displayed strong growth defects. In some cases the growth defect was so severe that we also initially obtained heterokaryons following transformation (Table 1). However, as haploid nulls for these kinases could be propagated as genetically stable haploids on selective media they were classified as non-essential.

**A. nidulans encodes 3 septation initiation network kinases.** Deletion of either the sepL<sup>H<sub>15</sub></sup> (AN4385) or sidB<sup>A2</sup> (AN8751) kinases, which are required for septation, resulted in the expected phenotype of extremely poor conidiation with little effect on radial growth as indicated by colony diameter [44–46]. Unlike S. cerevisiae, S. pombe encodes a third kinase called Sid1 which is required for septation as part of the septation initiation network (SIN). AN11032 (previously called AN8033 in version 2 of the A. nidulans genome) encodes a predicted Sid1 orthologue which, although not experimentally studied, has been termed sepL<sup>H</sup> [47] (Figure S5 in File S1). The colonies of sepL<sup>A2</sup> mutants were phenotypically identical to the sepL<sup>H</sup> and sidB<sup>A2</sup> mutants, strongly suggesting that SepL<sup>A2</sup> is required for septation and conidiation (Figure 4D). To confirm this, we stained the cell walls of sepL<sup>A2</sup> mutant germlings with calciluor which revealed an almost complete absence of septa even in cells with over 32 nuclei (Figure S5B and C in File S1). Thus, SepL<sup>A2</sup> is a third kinase required for septation in A. nidulans.

**Kinase mutants with defects in development and secondary metabolism.** As A. nidulans is homothallic (self-fertile) sexual development can occur in the absence of a mating partner [4]. The onset of sexual development is often indicated by changes in colony color due to the formation of yellow Hulle cells surrounding red pigmented fruiting bodies called cleistothecia and the production of secondary metabolites secreted into the medium.
After 3 days growth, 8 non-essential kinase mutants formed colonies displaying a color distinctly different from the white parent strain (Figure 5A). These color differences correlated with a dark appearance of the underside of colonies grown on minimal media at 37°C (Figure 5A), conditions which promote sexual development. Further examination indicated that the aberrant colony color of the \textit{plkA} (AN1560), \textit{An-gin4} (AN11101) and \textit{An-mst1} (AN5674) mutants was associated with visible morphological hallmarks of sexual development, as previously reported for \textit{plkA} mutants [49]. By 4 days, wild type colonies contained large numbers of asexual conidiophores with little or no evidence of sexual development. Contrasting this, \textit{plkA}, \textit{An-gin4} and \textit{An-mst1} mutant colonies displayed reduced asexual development but increased sexual development as indicated by vast numbers of Hulle cells which often surrounded pigmented nascent cleistothecia (Figure 5B). However, whereas \textit{plkA} and \textit{An-gin4} mutants continued sexual development and formed mature cleistothecia containing ascospores, \textit{An-mst1} mutants arrested in the early stages of cleistothecia formation and only produced immature nascent cleistothecia which did not contain ascospores (Figure 6).

For other kinase mutants, the aberrant colony color was not associated with visible morphological signs of sexual development.
An-yak1 (AN7104) mutant colonies were unique in that they produced a yellow pigment whereas the other mutants produced a brown pigment which was most obvious for the slow growing ΔAn-gsk3 (AN6508) colonies grown on rich media (Figure 5A). These ΔAn-gsk3 colonies produced very few asexual spores as has recently been shown for F. graminearum gsk3 mutants [14]. The secreted brown pigment of the cmkA (AN2412) mutant was most dramatic and spread forming a halo around the colony edge (Figure 5A). A similar pigment was produced by the uvsBATR (AN6975) and sldAbub1/R1 (AN3946) mutants but apparently in lower quantities.

The so called MAPK (mitogen activated protein kinase) module consists of the SteC Ste11 (AN2269), Ste7 (AN3422) and MpkB fus3 (AN3719) kinases and functions in a phosphorylation cascade required for sexual development [50,51]. Consistent with this, the 3 MAPK module mutants displayed an identical moderate growth defect (Figure 4B, Figure S6 in File S1) and, as has previously been reported, did not form cleistothecia [4,51–53]. However, although the MAPK module is predicted to be activated by the An-Ste20 (AN2067) kinase [50], An-ste20 mutants displayed a distinct phenotype from the MAPK module mutants. Most notably, unlike the MAPK module mutants, An-ste20 mutants were able to undergo sexual development resulting in the formation of mature cleistothecia containing ascospores (Figure 6). MAPK module mutants and An-ste20 mutants also displayed significant differences.

Figure 3. Identification of essential and non-essential kinases by heterokaryon rescue. (A) A primary transformation plate for the pkaA kinase deletion construct (left). Conidia from the indicated colonies were tested for their ability to form colonies on selective (YAG) or non-selective (YAGUU) media by either replica streaking or replica plating using a velvet disk. Growth of all 6 tested transformants on selective media suggests that pkaA is non-essential. (B) Diagnostic PCR indicates that 3 tested transformants are haploid nulls confirming that pkaA is non-essential. (C and D) As for (A and B) but showing analysis of an essential gene. Following transformation of the An-mps1 deletion construct, conidia from 4 of 6 tested transformants were unable to form colonies on selective media, strongly suggesting that An-mps1 is essential. Diagnostic PCR indicates that 3 of these heterokaryotic transformants contain both the wild type and deleted allele, confirming that An-mps1 is essential.

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A

Wild Type

B

Moderate

C

Strong

D

Strong - Septation

Wild Type

ΔhahA^{attd} (AN8830)
ΔAn-kin1 (AN1171)
ΔAn-yak1 (AN7104)
ΔAn-ssn3 (AN2489)
ΔsteC^{ste11} (AN2269)
ΔAn-ste7 (AN3422)
ΔmpkB^{fus3} (AN3719)
ΔcmkA (AN2412)
ΔkfsA (AN0822)
ΔAn-nrc2 (AN0144)
ΔsldA^{bub1/Rl} (AN3946)
ΔuvbA^{ATR} (AN6975)
ΔmeB (AN6243)
ΔAn-srpkA^{dsk1} (AN7185)
ΔpIkA^{dc5} (AN1560)
ΔAn-prk1 (AN10515)
ΔAn-Ikh1 (AN0988)
ΔnikA (AN4479)
ΔAn-gsk3 (AN6508)
ΔpkaA (AN6305)
ΔAn-stk47 (AN8190)
ΔAn-cla4 (AN8836)
ΔckiB (AN5757)
Δlsb6 (AN10791)
ΔptkA (AN8865)
ΔAn-cak1 (AN0699)
ΔsepL^{sid1} (AN11032)
ΔsepH^{dc5} (AN4385)
ΔsidB^{sid2} (AN8751)
in the apparent production of secondary metabolites. For example, when colonies were grown on minimal media, An-ste20 mutants secreted a brown pigment on their surface which was not produced by the 3 MAPK module mutants (Figure S6B in File S1). Interestingly, production of this surface brown pigment by An-ste20 mutants was apparent on minimal media but not rich media (Figure S6A-B in File S1), suggesting a role for An-Ste20 in nutrient sensing. Contrasting this, when the MAPK module mutants where grown on rich media in top agar cultures they secreted a dark pigment into the medium which was not apparent in An-ste20 or wild type cultures inoculated at the same density (Figure S6C in File S1). The production of this pigment by the MAPK module mutants is likely a result of their arrest at an early stage of sexual development [4,51–53]. These data are consistent with the regulation of sexual development and secondary metabolite production by the MAPK module [4,51]. However, while the 3 MAPK module kinases are required for sexual development, the predicted upstream An-Ste20 kinase is not

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**Figure 4. Kinase deletion mutants with vegetative growth defects.** Shown is colony formation of the indicated strains after 2 days at 32°, 37° or 42°, or 5 days at 20°. (A) An isogenic wild type control. (B) Kinase deletion mutants with a moderate but reproducible colony growth defect. (C) Kinase deletion mutants with a strong colony growth defect. (D) Septation deficient kinase deletion mutants display a strong growth phenotype characterized by extremely poor conidiation. Colonies are shown as negatives to more clearly visualize the hyphae.

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**Figure 5. Kinase deletion mutants with developmental defects.** (A) Colony color after 3 days growth of the indicated strains and conditions. *Images for the MMUU series at 37° are also shown from the underside of the colony to more clearly show pigments produced by the colonies. (B) The left column shows colonies point inoculated with 10⁵ spores after 4 days growth at 37°. The ΔPlkA polo, ΔAn-mst1 and ΔAn-gin4 strains produce few asexual conidia but show advanced sexual development. The right column shows cells collected from the indicated region of each colony at higher magnification. In contrast to the asexual conidiophores (c) and enormous numbers of asexual conidia of the wild type colony, the ΔPlkA polo and ΔAn-mst1 mutant colonies contain numerous Hulle cells (H) surrounding apparent nascent cleistothecia (n). The ΔAn-gin4 mutant also contained Hulle cells and apparent nascent cleistothecia, although more conidiophores were apparent relative to the other mutants. Bar ~ 100 μm.

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always required indicating that the regulation of this pathway is more complicated than predicted.

Drug Sensitivities of Kinase Deletion Mutants

**Kinases required for the cellular response to genotoxic stress.** In response to genotoxic stress the conserved PIKK kinases ATR and ATM phosphorylate serine or threonine within the SQ/TQ cluster domains (SCDs) of the downstream effector kinases Chk1 and Chk2 [31]. In *A. nidulans* these 4 kinases are encoded by *usiB*ATR (AN6975), *atmA*ATM (AN0038), *chkA*chk1 (AN3494) and *chkB*chk2 (AN4279) [54,55]. As previously reported, *usiB*ATR mutants were sensitive to a wide variety of genotoxic agents including the DNA alkylating agent DEO, the DNA double strand break inducing agent camptothecin and the DNA replication inhibitor HU [56,57]. Contrasting this, the *atmA*ATM mutant was only strongly sensitive to camptothecin, consistent with ATM kinase functions being more specific for double strand DNA breaks [31,58,59] (Figure 7). Both the *chkA*chk1 and *chkB*chk2 effector kinase mutants were sensitive to HU but not the DNA damaging agents tested (Figure 7). As HU sensitivity was previously reported for *chkA*chk1 but not *chkB*chk2 mutants [59], we tested the viability of these mutants at different concentrations of HU (Figure S7 in File S1), confirming their HU sensitivity.

Notably, in addition to the *usiB*ATR, *chkA*chk1 and *chkB*chk2 mutants, deletion of the previously uncharacterized ChkC (AN7563) kinase also resulted in HU sensitivity (Figure 7A). Importantly the HU sensitivity of *chkC* mutants was independent of the presence of the Δ*uVA*mut mutation (Figure S8 in File S1). To determine if Δ*uVA* HU sensitivity was due to loss of cell cycle checkpoint regulation, we germinated cells in the presence or absence of 10 mM HU and followed their entry into mitosis. The presence of HU delayed mitotic entry of *chkC* mutants in a manner similar to wild type cells (Figure 7B). This indicates that the checkpoint which delays mitotic entry in the presence of unreplicated DNA is intact and that the HU sensitivity of *chkC* mutants is due to a defect in another aspect of the DNA damage response. Although *chkA*chk1, *chkB*chk2 and ChkC are each CAMK kinases, the kinase domain of ChkC is more closely related to ChkB chk2 (35.7%) than to ChkA chk1 (28.6% identity) (Figure S9 in File S1). Moreover, ChkC displays structural similarity to Chk2 kinases and is an orthologue of mus-59 which encodes a second Chk2 related kinase in *N. crassa* [60]. Similar to ChkB chk2 and other Chk2 kinases, ChkC contains a forkhead associated domain (FHA) which potentially mediates phosphorylation dependent protein-protein interactions (Figure 7C, Figure S9 in File S1). ChkC kinases are present in other filamentous ascomycetes but are
Figure 7. *A. nidulans* kinase deletion mutants with sensitivities to genotoxic agents. (A) Colony formation of the indicated strains with or without the indicated genotoxic agents. Images were taken after 2 days at 32°C except for the 8 mM HU series which is after 3 days. (B) *chkC* mutants do not enter mitosis prematurely in the presence of HU. Wild type and Δ*chkC* conidia were inoculated in the presence or absence of 10 mM HU and monitored for mitotic index over time. (C and D) Schematic representation of the *chk1* and *chk2* kinases with conserved domains indicated.
the chromosome mitotic index (CMI) of DAPI stained cells determined at each time point. Benomyl (2.4 μg/ml) was included in the media to help maintain a mitotic arrest once cells entered mitosis [118]. (C) Schematic diagrams showing the domain structure of the indicated kinases. (D) ClustalW alignment (http://workbench.sdsc.edu/) of the N-terminals of ChkC kinases. Identical (*), conserved strong groups (:), and conserved weak groups (.) are indicated. Di-peptide SQ and TQ motifs are indicated in red. Accession numbers for the sequences are: A. nidulans (AN75563; EAA62143), A. clavatus (ACLA_072560; EAW14223), A. fumigatus (ACFA_072560; XJ_755825), A. oryzae (AO09012000405; BA6E5087), A. flavus (EED54418), A. terreus (ATEG_07832; EUA32904), A. niger (AN15g03280; CAK42403), P. marneffei (EAA23567.1), F. oxysporum (FOX8_01143, EGU88344), F. graminearum (Fg00433), M. oryzae (EHAS53924), N. crassa (NCU02751; Mus-59).

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absent from basidiomycetes including U. maydis which remarkably does not encode any Chk2 related kinases [61]. Notably, the Aspergillus ChkC kinases each contain three conserved SQ/TQ sites, two of which are clustered together in the N-terminus including at least one that is conserved in the more distantly related filamentous ascomycetes N. crassa, P. marneffei, Fusarium oxysporum, F. graminearum and Magnaporthe oryzae (Figure 7D). Thus ChkC kinases encode an N-terminal SCF domain containing potential sites of phosphorylation by the ATM and/or ATR kinases and in A. nidulans ChkC is involved in the cellular response to replicative stress.

**Kinases responsive to osmotic stress.** The growth of 16 kinase mutants was reproducibly inhibited in the presence of 1 or 1.5 M NaCl (Figure S8). This included null alleles of the previously characterized osmotic stress response kinases hog1Δmut (AN1017), phkAΔmut (AN9031) and ssk1Δmut (AN10153), which encode orthologues of the budding yeast HOG-MAPK (high-osmolarity glycerol response mitogen activated protein kinase) pathway [30]. In addition to reduced growth in the presence of NaCl, phkAΔmut and ssk1Δmut mutants also displayed reduced growth in the presence of 1.5 M Sucrose (Figure 8C). However, increased osmolarity did not inhibit growth of pkaBΔmut histidine kinase (AN1800) or steCΔmut (AN2269) kinase mutants, even though these kinases encode HOG-MAPK pathway orthologues, confirming previous studies in A. nidulans [30]. Also confirming a previous report, growth of halA mutant (AN8830) deleted strains was inhibited in the presence of NaCl [62].

Our analysis also identified mutants whose growth was reproducibly inhibited to a similar or greater level as the above bona fide osmotic stress response kinase mutants. These included deletion mutants of the cmkA (AN8927), phkA (AN8261), sldΔmut (AN3946), pkaBΔmut (AN1560), ssk1Δmut (AN4385), ssk2Δmut (AN8751) and srpBΔmut (AN7572) kinases for which sensitivity to osmotic stress has not been previously reported (Figure 8). Although the pkaBΔmut and pkaA mutants displayed strong growth defects in the absence of osmotic stress, other kinase mutants with similar strong growth defects were not similarly inhibited (Figure 8B). We also identified 4 previously uncharacterized kinases, SrpKAΔmut (AN7185), An-Stk47 (AN8190), An-Ppk33 (AN7537), and SepLΔmut (AN11032), whose deletion resulted in growth inhibition in the presence of NaCl (Figure 8). Notably, the 3 SIN kinase mutants each displayed marked sensitivity to osmotic stress (Figure 8B).

Interestingly, the most significant growth defect of the phkA (AN6305) mutant was significantly remediated by increased osmolarity (Figure 8B and C). Along with PkaB (AN4717), PkaA is one of two cAMP-dependent protein kinase catalytic subunits in A. nidulans. As phkA is partially redundant with pkaB [63], one possibility is that under conditions of osmotic stress phkA is up-regulated thereby suppressing the lack of pkaA. Sucrose also partially remediated the poor conidiation of the An-gsk3 (AN6308) mutant even though it did not improve the poor radial growth of this mutant (Figure 8D, Figure S10 in File S1).

**A. nidulans encodes a single Bub1/BubR1 kinase required for the spindle assembly checkpoint.** The majority of organisms encode two proteins related to the Bub1 spindle assembly checkpoint kinase. Humans encode the Bub1 and BubR1 kinases, while budding and fission yeast encode a Bub1 kinase and the related Mad3 protein which lacks a kinase domain [64]. A recent study has found that this complex organization of paralogues is the result of 9 distinct gene duplications combined with the subfunctionalization of the duplicated genes [65]. As part of this remarkable example of parallel evolution, the kinase function of one parologue is almost always lost. This can occur by either kinase domain deletion as for the Mad3 proteins, or by mutation of the kinase domain resulting in a pseudokinase as has been argued for human BubR1 [65]. In A. nidulans SldΔBub1/R1 (AN3946) is the only member of the Bub1/BubR1/Mad3 family and similarly only a single orthologue is present in other Aspergilli and N. crassa (Figure S11D in File S1). This indicates that bub1 gene duplication has interestingly not occurred in these filamentous ascomycetes and suggests that SldΔBub1/R1 must perform all Bub1/BubR1/Mad3 functions. Consistent with this SldΔBub1/R1 contains all the functional domains of the Bub1/BubR1/Mad3 family including a kinase domain which is more similar to the Bub1 kinase than the BubR1 pseudokinase (Figure S11B and C in File S1).

As expected given its function in the spindle assembly checkpoint, deletion of sldΔBub1/R1 resulted in marked sensitivity to the microtubule poison benomyl as previously shown [Figure S11A in File S1] [66,67]. Interestingly however, sldΔmut (AN3946) mutants also displayed moderate growth defects and osmotic stress sensitivity which was not displayed by Δanak spinele assembly checkpoint mutants (Figure S11A in File S1). This suggests that SldΔBub1/R1 has functions in addition to its role in the spindle assembly checkpoint.

**Functional Analysis of Essential Kinases by Heterokaryon Rescue**

*A. nidulans* offers the advantage that essential gene phenotypes can be readily studied using heterokaryon rescue [35]. Using this technique we determined the phenotype of cells lacking An-Aurora (AN5815) or An-Mps1 (AN2927) function as the respective heterokaryons did not apparently generate conidia containing the deleted kinase allele. As both An-Aurora and An-Mps1 encode predicted cell cycle regulators, it is possible that severe cell cycle defects in conidiophores are preventing the generation of the respective kinase deleted conidia from the heterokaryons. For the other essential kinases, kinase deleted cells were identified by their ability to undergo limited growth on selective media, contrasting the kinase wild type conidia from the same heterokaryon which did not form germ tubes due to lack of pyrG function. The phenotypes of cells lacking essential kinases were further classified based on their nuclear and cellular morphology as well as the extent to which they were able to grow.

**Kinase mutants which terminally arrest as very short germings.** Kinase deletions with the most severe phenotypes formed only short germings after two days growth indicating that
they play vital roles in essential cellular processes (Figure 9B). Among these was the An-ppk33 (AN7537) mutant which encodes an orthologue of the S. pombe Prp4 kinase required for mRNA splicing [68]. The torA (AN5982), ckaAT25 (AN4563), sulDvi1 (AN6363) and An-rio2 (AN0124) mutants also arrested growth as short germlings (Figure 9B). Interestingly, based on the functions of their budding

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Figure 8. Kinase deletion mutants whose growth is reduced or enhanced by osmotic agents. Shown are the indicated deletion strains grown with or without the indicated concentrations of NaCl or sucrose. Growth is after 3 days at 32°C except for the 1.5 M NaCl series which is after 4 days. Mutants were classified as having (A) moderate sensitivity to osmotic stress or (B) strong sensitivity to osmotic stress. (C) Kinase mutants whose colony formation was inhibited by 1.5 M sucrose. (D) Kinase mutants whose defects were partially remediated by sucrose. r = remediation of growth, c = remediation of conidiation.

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yeast orthologues, each of these 4 kinases is predicted to play a key role in ribosomal biosynthesis [69–71]. While this suggests that the terminal phenotype of each of these mutants is due to a defect in ribosomal biosynthesis, these kinases also have other predicted cellular functions [72–74]. Interestingly, cells lacking TorA kinase function displayed a second phenotype in which short germings with 2 or fewer nuclei frequently displayed septa, a phenotype never seen in wild type cells (Figure 9B). This phenotype is seemingly unrelated to known functions of yeast Tor kinases as regulators of protein synthesis, ribosome biogenesis, autophagy and the transcriptional response to cellular stresses or nutrient availability [70], although chemical inhibition of Tor kinase in Podospora anserina interestingly also leads to increased septation [75].

**Kinases with cell cycle functions.** In *A. nidulans* preventing cell cycle progression blocks nuclear division but does not prevent polarized growth, causing cells to arrest as elongated germings containing single nuclei [76]. Depending on the cell cycle stage in which the gene has an essential function, mutants arrest with either a never in mitosis (NIM) phenotype or a blocked in mitosis (BIM) phenotype. As expected, we observed a NIM phenotype following deletion of the essential NIMA (*AN9504*) kinase [28,77,78] (Figure 9D). A single interphase nucleus was also apparent in nimXcdk1 (AN4182) deleted cells but this nucleus was often obscured by brightly staining mitochondrial DNA (Figure 9D) [77]. To more clearly examine these nuclei, we deleted nimXcdk1 in a Histone H1-mCherry strain. This confirmed that nimXcdk1 nulls arrest with a NIM phenotype and indicated that during a prolonged interphase arrest nuclei continued to enlarge resulting in a diffuse nuclear chromatin signal (Figure S12A in File S1).

Cells lacking An-Cdc7 (AN3450) kinase function contained a single nucleus which in ~24% of cells was mitotically condensed (Figure 9D). This is significantly higher than the mitotic index of wild type cells which is typically ~5%. We confirmed that such ΔAn-cdc7 cells were mitotic by deleting An-cdc7 in a GFP-Tubulin and Histone H1-mCherry strain which revealed that cells often displayed monopolar spindles (Figure S12B in File S1). The predicted An-Cdc7 binding partner NimO Dbf4 is required to initiate DNA replication and NimO Dbf4 mutants are also defective in the activation of the checkpoint which prevents mitotic entry before completion of DNA replication [79]. Examination of An-cdc7 mutants indicated that they displayed an identical phenotype to nimOdbf4 mutants resulting in cells entering mitosis with unreplicated DNA (Figure S12C in File S1), as occurs for Cdc7 mutants in other systems [80]. Thus although An-Cdc7 is essential for DNA replication, cells display a mixed NIM and BIM phenotype because cells which fail to initiate DNA replication are also defective in checkpoint regulation over mitotic entry.

The primary function of Wee1 kinases to carry out an inhibitory phosphorylation of the Cdk1 kinase is not essential in *A. nidulans* as non-Wee1-phosphorylatable nimXcdk1 mutants are viable [81]. However, we surprisingly found that An-Wee1 (AN3822) has an essential function which is thus likely distinct from its role in phosphorylating NimXcdk1 (Figure 9D). Cells lacking An-Wee1 predominantly arrested with branched germ tubes and displayed a high frequency of nuclei with an irregular size, shape and distribution along germ tubes (Figure 9D; Figure S12D in File S1). Nuclei were also often connected by strings of stretched DNA caused by a failure in mitotic DNA segregation. Enlarged nuclei of cells lacking An-Wee1 also often displayed multiple spindles consistent with these nuclei being polyploid and containing multiple spindle pole bodies (Figure S12D in File S1). These phenotypes are consistent with lethal defects in cell cycle regulation. However why Wee1 is essential in *A. nidulans* and *U. maydis* [82] but is not essential in *N. crassa* [16] is unclear.

**Kinase mutations which effect cellular integrity.** Deletion of the PkcA (AN0106), IreA (AN0235) or An-Cdk7 (AN0203) kinases resulted in differing degrees of cellular swelling (Figure 9C) which was not suppressed by increased osmolarity (data not shown). The most striking swollen phenotype was for ΔAn-cdk7 mutants which were so swollen by 40 hr that it was difficult to determine if cells had originated from a single spore. In contrast to individual cells lining up in a row (Figure 9C), time lapse brightfield microscopy revealed that ΔAn-cdk7 germlings initially appeared normal but, after septation, underwent drastic swelling during further growth (Figure 10). Interestingly cells separated by septa underwent swelling to different extents with the most pronounced swelling generally occurring in sub-apical cells. In these cells the extent of cellular swelling corresponded with the size of the vacuoles which were often massively enlarged (Figure 10). Another hallmark of this phenotype was that the septal cell wall did not appear to expand in the same manner as the rest of the cell wall leading to the cell restrictions observed between the swollen cells.

As previously reported, cells lacking PkcA kinase function underwent swelling and occasionally lyzed (Figure 9C) [83,84]. PkcA is also predicted to regulate a basic three kinase module consisting of BckA (AN4887), MkkA (AN4189) and Mkpb (AN5666) which is analogous to the *S. cerevisiae* cell wall integrity pathway [50]. Cells deleted for the BckA, MkkA or Mkpb kinases each displayed an identical phenotype in which mutants formed microscopic branched colonies with an irregular morphology after 40 hr (Figure 9E). As the lethal phenotype of the equivalent budding yeast mutants is remediated by high osmolarity [85], we determined if this was also the case for the *A. nidulans* mutants. The presence of either 1 M sucrose or 1 M NaCl had a dramatic effect whereby bcka, mkka and mkpb mutants now formed conidiating colonies after two days, although pcka mutants still failed to form colonies under these conditions (Figure S13 in File S1). We conclude that BckA, MkkA and Mkpb function in a pathway analogous to the *S. cerevisiae* cell wall integrity pathway and the PkcA likely has other functions in addition to regulating this pathway.

The swelling displayed by cells lacking IreA kinase function was most pronounced at the cell tips (Figure 9C). This swelling is presumably related to the well established function of this family of kinases as mediators of the unfolded protein response [86]. In addition to pcka, ireA and An-cdk7, the An-ppk4 and An-cdc7 kinase mutants also displayed moderate cellular swelling although this was not the primary defect of these mutants (Figure 9).

**Kinase mutants defective in polarized growth or vesicular trafficking can form microcolonies.** A less severe phenotype which permitted the formation of microcolonies was displayed by 8 different kinase deletion mutants (Figure 11). We classified these kinases as essential as mutants only formed microcolonies that did not produce viable spores. Most strikingly, cells lacking either CotA (AN5329) or An-Pod6 (AN6339) kinase function formed morphologically identical microcolonies which secreted a brown pigment (Figure 11B, Figure S14C in File S1). Both An-pod6 and cotA nulls displayed a high frequency of germ tubes emanating from a swollen spore (Figure 11B), consistent with the known function of CotA in maintaining cellular polarity [87]. *N. crassa* Cot-1 is orthologous to CotA and the phenotype of cot-1 mutants can be remediated under conditions of high osmolarity [88]. However 1 M sucrose or 1 M NaCl did not remediate the microcolony phenotype of either cotA or An-pod6 deleted cells, although it interestingly decreased pigment production (Figure S14A and B in File S1). The identical
polarity defect of cotA and An-pod6 mutants is consistent with the functions of the orthologous kinases in *N. crassa* and *P. pombe* to regulate polarized growth [89–91]. This has been best studied in *P. pombe* in which Nek1, the likely orthologue of An-Pod6, functions upstream of Orb6, orthologous to CotA, as part of the MOR (morphogenesis Orb6) network which regulates actin location to promote polarized growth [92]. Following the MOR regulated location of actin to sites of polarized growth, the *P. pombe* Cka1 casein kinase II regulates a subsequent step in polarized growth [93]. The role for casein kinase II in regulating polarized growth is conserved in *S. cerevisiae* [94] and also likely *A. nidulans* as cells lacking An-Cka1 (AN1485) arrested with a microcolony phenotype (Figure 11C). Based on the *P. pombe* data and the phenotypes of the *A. nidulans* mutants, we predict that An-Pod6 and CotA function in a network analogous to the MOR and that An-Cka1 performs a yet to be defined role in polarized growth.

Cells lacking the *An-vps34* ([AN4709] or *An-vps34*) ([AN0576]) kinases formed morphologically identical microcolonies consistent with their common function in endosomal trafficking (Figure 11D) [95]. Although these microcolonies displayed highly segmented and branched tip cells, the initial growth of these *vps* kinase mutants was relatively normal. Contrasting this, both *An-ksg1* ([AN3110]) and *pkcB* ([AN5973]) kinase deleted cells initiated an irregular pattern of growth and branching before arresting as microcolonies (Figure 11E). This phenotypic similarity is consistent with budding yeast Sc-Pkh2 (orthologue to *An-Ksg1*), regulating Sc-Ypk1 (orthologue to PkcB) in a sphingolipid-mediated signaling pathway involved in endocytosis [96]. It was also noticeable that the early growth morphology of *An-stt4* mutants was similar to that of *An-ksg1* and *pkcB* mutants, suggesting that the predicted An-Stt4 function in sphingolipid biosynthesis contributes to this phenotype (Figure 11F). However, budding yeast Stt4 has many functions [97] and *An-stt4* mutants displayed phenotypes in addition to their growth defect. We consistently observed that as An-Stt4 cells grew individual nuclei stained more brightly for DAPI, suggesting a cell cycle defect potentially related to the G2/M arrest phenotype of budding yeast *stt4* mutants (Figure 11F; the DAPI stained cells are from the same field) [98]. However, while many *An-stt4* mutant cells arrested growth as short branched cells with an apparently abnormal DNA content, others formed aconidial microcolonies (Figure 11F). Microscopic examination of the *An-stt4* mutants indicated that colony sectors with defective polarized growth occasionally resumed somewhat normal growth (Figure 11F, arrow). This suggests that mutations which suppress the *An-stt4* mutant phenotype might be arising spontaneously and permitting further growth. Similarly, the microcolonies of the *vps* kinase mutants also occasionally formed sectors with improved growth suggesting the appearance of suppressor mutations in these cells (Figure S15 in File S1). Similar spontaneous suppressors have recently been isolated from several other *vps* mutants [95], suggesting that such suppression is a relatively frequent event in this class of mutants. This highlights the importance of maintaining essential gene deletions with a relatively weak arrest phenotype as heterokaryons in which there will be no selection for such suppressor mutations.

**Discussion**

Utilizing gene deletion constructs generated here for the majority of *A. nidulans* genes, we have generated kinase deletion strains for 128 kinases and identified phenotypes for 68 of these mutants. One advantage of large scale genetic analysis is that mutants generated in the same genetic background have the potential to be phenotypically grouped as likely regulators of the same process when similar phenotypes are uncovered. Given that kinases often function together in signaling pathways, this phenotypic grouping provided experimental evidence that previously uncharacterized kinases function in the SIN, cell wall integrity and MOR kinase signaling pathways, as well as in pathways regulating vesicular trafficking and the cellular response to replicative stress. This analysis also confirmed previously known kinase functions in the MAPK module and the HOG-MAPK pathway. Importantly however the phenotypes of some kinase mutants differed from those defined for orthologues in other systems. This is not unexpected as, for example, it is known that kinases which are essential in one organism can be non-essential in other systems. This holds true also within the filamentous fungi as orthologues of the essential *A. nidulans* kinases, An-Prp14, Wee1, NIMA, An-Vps15 and An-Cka1, are non-essential in *N. crassa* [16].

**Kinases Specific to Filamentous Fungi**

Of considerable interest *A. nidulans* encodes 11 Ffks (filamentous fungal kinases) unrelated to the recently identified Funk1 fungal specific kinase family. Seven of these Ffks, including the likely paralogues FfkD and FfkE, appear to have originated from a common ancestor. Potential functional redundancy among related Ffks might in part explain why we did not identify phenotypes for these mutants. However, as Ffks are present in some Aspergilli but not others, it is likely that these kinases have very specialized functions which might be difficult to define. It will be important to determine if Ffks are active kinases or if they potentially encode pseudokinases which may compete with other kinases for substrates. Interestingly, among the sequenced Aspergilli, *A. terreus* is unique in that it encodes a member of the Funk1 kinase family (ATEG_09985). In striking contrast to its absence from other Aspergilli however, kinases highly related to ATEG_09985 are present in many other fungi with the most similar (74% query coverage at an E-value of 0.00) occurring in the entomopathogenic fungus *Metarhizium anisopliae*. This interesting phylogenetic distribution suggests that the presence of the ATEG_09985 Funk1 kinase in *A. terreus* might be the result of horizontal gene transfer. A common theme among filamentous fungal specific kinases such as the Ffks and Funk1 kinases is that they have often been expanded or lost during the evolution of individual filamentous fungal species. This suggests that these kinases are potentially involved in regulating the diverse array of developmental programs which occur in these organisms [39]. In the long term, defining the function of filamentous fungal specific kinases is important as orthologues present in pathogenic fungi are potential candidates for the development of specific antifungal agents.
Figure 10. The extreme swelling of An-cdk7 mutants correlates with a massive enlargement of vacuoles. (A and B) Time lapse images of conidia inoculated from the heterokaryon in media selective for growth of only the An-cdk7 deleted cells. Cells separated by septa (arrowheads) are numbered sequentially as they form. Cells initially appear normal but undergo extreme swelling which appears to be potentially mediated by an enlargement of vacuoles (v). Note that septa restrict swelling and following lysis (L) appear to seal the junction between adjacent cells. Bar – 50 μm. doi:10.1371/journal.pone.0058008.g010
Figure 11. Essential kinase deletion mutants which form microcolonies. Uninucleate conidia from heterokaryons were streaked on selective media (top 3 rows) or inoculated and fixed for DAPI staining (bottom row). After 4 days growth at 32°C these kinase mutants formed microcolonies which could not be propagated as genetically stable haploids by streaking. (A) Wild type pyrG+ and pyrG89 strains. (B) An- pod6 and cotA nulls display an identical brown microcolony phenotype. Insets show over 5 germ tubes emerging from an enlarged spore body indicative of a defect in the establishment of polarized growth. (C) An-chk1 nulls initiate polarized growth normally but arrest as small microcolonies. (D) The An-yps3+ and An-yps34 nulls display an identical phenotype. Insets show highly segmented cells at colony edges. (E) The An-ksq1 and pckB nulls initiate a nearly identical irregular pattern of growth and branching before arresting as microcolonies. (F) The An-stt4 kinase domain deletion mutant displays a range of phenotypes. The arrow indicates a section of a microcolony which appears to have resumed normal growth. The bottom row shows 2 cells from the same field which have grown to different extents and display distinctly different DNA content. Bar ~ 50 μm.

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Also noteworthy are the significant expansions of histidine kinases and SRPK kinases in A. nidulans relative to the yeasts S. cerevisiae and S. pombe. The expansion of histidine kinases in filamentous fungi has been suggested to help these organisms respond to a broad range of environmental stimuli [23,26,41] although these signaling pathways are presently poorly understood. The expansion of SRPK kinases and potential SRPK pseudokinases in filamentous fungi is not understood [9]. However, as histidine kinases are regulators of mRNA processing, one possibility is that the SRPK expansion reflects an increased importance of splicing in filamentous fungi relative to S. cerevisiae whose genes contain few introns. Consistent with this, S. pombe does not encode a Prp4 mRNA splicing kinase which is essential in A. nidulans and in S. pombe [68]. As was the case for the Fiks, many A. nidulans histidine kinases and SRPKs are closely related and potentially function in a redundant manner.

Intriguingly, we have identified ChkC as a second Chk2 like kinase present in the filamentous ascomycetes. Although ChkC is orthologous to N. crassa Mus-59, their mutations cause sensitivity to different types of genotoxic stress, ChkC to replicative stress, and Mus-59 to DNA double strand breaks [60]. Thus, as for the Chkl and Chk2 effector kinases [31,59,60], the requirement for ChkC orthologues in response to genotoxic stress varies in different organisms. In addition, it is known that functional redundancy exists between effector kinases and it will be important to determine if ChkC has overlapping functions with ChkA<sub>Chk1</sub> and/or ChkB<sub>Chk2</sub>.

Kinases Involved in Maintaining Cellular Integrity

Our analysis indicates that the BckA, MkkA and MpkA kinases function in a pathway analogous to the cell wall integrity pathway of S. cerevisiae, as the lethality of the respective mutants was suppressed by high osmolarity. Although the PkcA kinase is predicted to function upstream of the cell wall integrity pathway, cells lacking pckA displayed a more severe phenotype which was not suppressed by high osmolarity. This suggests that PkcA has regulatory targets in addition to the cell wall integrity pathway, possibly at the growing cell tip or at septa where PkcA localizes [83,84].

After an initial period of normal polarized growth, cells lacking An-Cdk7 underwent dramatic swelling which correlated with a massive enlargement of vacuoles. An-Cdk7 is part of a kinase family including budding yeast Kin28 and fission yeast Mec6 which regulate RNA pol II transcription and in many organisms have a second function as a CDK activating kinase (CAK) [99]. In S. pombe mec6 mutants fail to separate following septation, most likely as a consequence of changes in their transcriptional profile [100]. It is thus interesting that in An-cdk7 mutants the most severe swelling occurred in sub-apical cells after septum formation. Thus one possibility is that An-Cdk7 regulates transcriptional changes in sub-apical cells following septation. Potentially sub-apical cells lacking An-Cdk7 may express an inappropriate profile of genes leading the pronounced vacuolar and cellular swelling in these cells.

Our results regarding orthologues of the budding yeast HOG-MAPK pathway which responds to osmotic stress are consistent with previous studies [30]. Importantly however, our findings also suggest unknown functions for the CnkC, PhoA, SltA<sup>Bol1/K1</sup>, PkaA<sup>Pol1</sup>, Pka, SepkA<sup>Dkh1</sup>, An-Slt47, An-Pyk3/3 and SepB<sup>Rim15</sup> kinases in the cellular response to osmotic stress. The pronounced osmotic stress sensitivity of the sepf<sup>LH1</sup>, sepf<sup>LH15</sup> and sidB<sup>Pol2</sup> septation deficient SIN kinase mutants supports the concept that septa help maintain cellular integrity by compartmentalizing cells as they grow and explore the environment. Contrasting the lack of septation in the SIN mutants, torA kinase mutants underwent precocious septation. This suggests that in addition to their well established essential functions, Tor kinases play a yet to be defined role as negative regulators of septation in filamentous fungi [75].

Kinases Regulating Sexual Development and Secondary Metabolite Production

Relative to an isogenic wild type control strain, 14 kinase mutants displayed altered secondary metabolite production and/or sexual development. However, it is important to note that all strains in this study, and most strains historically used for experimental study of A. nidulans, contain a mutation in the Vea protein (veA<sup>1</sup>), a key coordinator of secondary metabolite production and sexual development [4,101]. Thus in future studies it will be important to determine if kinase mutant phenotypes are altered in a veA+ background. For example, it is known that imeB (AN6243) kinase mutants display increased sexual development in a veA+ but not veA<sup>1</sup> background [102]. Consistent with this the the AnimeB mutant in this study did not display advanced sexual development.

As expected the steC<sup>ste11</sup>, ste7 and mpkB<sup>Pm3</sup> MAPK module mutants displayed few signs of sexual development and did not form cleistothecia [4,51–53]. Most surprisingly however, cells lacking the predicted upstream An-Ste20 PAK (p21 activated kinase) kinase formed mature cleistothecia containing ascospores. Phenotypic differences between F. graminearum FgMAPK module mutants and the FgSte20 mutant have also been recently reported [14]. Thus it will be interesting to determine if in these and other filamentous fungi Ste20 does indeed activate the MAPK module and/or if it has additional regulatory targets involved in developmental regulation. Notably however, deletion of An-Mst1, a GCK (germinal center kinase) STE20 related kinase, resulted in incomplete sexual development. Further, An-Mst1 orthologues in N. crassa and F. graminearum also display developmental defects [14,103,104]. Thus one interesting possibility is that An-Mst1, whose kinase domain is similar to An-Ste20 (2e−54), contributes to the regulation of the MAPK module during sexual development in filamentous fungi.

Similar to An-mst1, the An-gnk1 and An-pkaA kinase mutants also displayed a decrease in asexual spore production and the early onset of sexual reproduction. This confirms a recent finding for PkaA [49] and suggests that these kinases negatively regulate the developmental switch from asexual to sexual reproduction. PkaA is
a polo like kinase and members of this family have developmental functions in mammals, flies and worms [105–106]. Therefore, understanding how PlkA negatively regulates sexual development remains an important area for future study. One possibility based on the known cell cycle related functions for PlkA and Gin4 kinase family members [109,110], is that the developmental defects of An-gin4 and plkA mutants reveal an important link between cell cycle control and development. The recent finding that F. graminearum gin4 mutants also display developmental defects provides further evidence that Gin4 is a regulator of sexual development in filamentous fungi [14]. Interestingly however, whereas An-gin4 mutants undergo early sexual development and form ascospores, F. graminearum gin4 mutants undergo incomplete sexual development and form asexual structures such as microcolonies [95]. Varying degrees of pigment production without obvious signs of sexual development were observed following deletion of the An- pod6, CotA, UvdBTR, SldA, CinA, An-Yak1 or An-Gsk3 kinases. Interestingly one of the functions of the budding yeast An-Gsk3 orthologue, Rim11, is to induce the expression of meiosis specific genes [111]. Thus one possibility for the increased pigment production in An-gsk3 mutants is that altered meiotic gene expression has uncoupled secondary metabolite production from sexual development. An alternative explanation for increased pigment production is that the normal response to environmental stimuli is defective in certain kinase mutants. It is thus informative that brown pigment production by An-pod6 and cotA mutants was suppressed by increased osmolarity. Thus pigment production of these mutants may be related to the proposed function for N. crassa cot-1 in an environmental stress response [112]. It will also be interesting to determine if the yellow pigment produced by An-yak1 mutant colonies is related to the glucose sensing function observed for the S. cerevisiae orthologue Yak1 [113].

Kinases Essential for Growth

We identified 25 kinases which had an essential function preventing the null allele being propagated as a genetically stable haploid. Importantly, we were able to further classify these kinases based on the terminal phenotype of mutants determined by heterokaryon rescue. Kinase mutants with a predicted essential function in vital processes such as ribosome biogenesis displayed the strongest growth arrest while kinase mutants with predicted functions in maintaining polarized growth or vesicular trafficking were able to form very slow growing microcolonies. For the latter, maintaining the deleted allele in heterokaryons removed selection for faster growing cells and prevented the selection of mutations which suppressed the microcolony phenotype. At least two features of these microcolonies likely contribute to the relatively frequent appearance of these suppressor mutations. Firstly the increased number of nuclei in these cells increases the probability of these mutations arising and, secondly, the microcolony arrest phenotype can relatively easily be compensated for by mutations in other genes as demonstrated for vps20, vps23, vps27 and vps36 mutants [95].

Our finding that the IreA kinase is essential strongly suggests that the unfolded protein response is an essential process in A. nidulans. S. cerevisiae Ire1 locates to the ER and responds to the accumulation of unfolded proteins by regulating the transcriptional activation of genes encoding proteins involved in protein folding [114]. In filamentous fungi, it has been suggested that protein secretion during polarized growth increases the demand for protein folding thereby triggering the unfolded protein response [115]. Thus the accumulation of unfolded proteins which would normally be secreted might explain the swollen phenotype of ireA mutants. Interestingly this apparent requirement for the unfolded protein response, or how it is regulated, varies in different fungi as Ire1 is essential in A. nidulans, A. niger and F. graminearum but non-essential in A. fumigatus, S. pombe and S. cerevisiae [14,115–117]. An alternative explanation is that Ire1 has an unknown essential function/s in some but not all fungi. Similar arguments can be made for other kinases which are essential in some, but not all filamentous fungi.

Kinases Essential for the Cell Cycle

At least four A. nidulans kinases have essential cell cycle functions, the previously uncharacterized An-Cdk7 kinase and the NIMA, NimXCDK1 and Wee1 kinases. It is also likely that the An-Aurora and An-Mps1 kinases have essential cell cycle functions although we were unable to define the phenotype of cells lacking these kinases. Our finding that An-Wee1 has an essential function that is likely independent of NimXCDK1 regulation was most surprising. However, although nimXCDK1 mutants which cannot be phosphorylated by An-Wee1 are viable, they lack functional interphase cell cycle checkpoints and are highly sensitive to DNA damage or perturbed DNA replication [81,118]. When DNA replication is inhibited in these nimXCDK1 mutants they undergo aberrant mitosis resulting in irregular nuclei often connected by strands of DNA [56]. Interestingly, Δan- wee1 cells frequently displayed a highly similar phenotype during otherwise unperturbed cell cycles. In this regard it is interesting that recent unbiased screens have revealed that human Wee1 has an additional function during DNA replication [119,120]. If this was also the situation in A. nidulans, cells lacking An-Wee1 might take longer to complete replication and additionally fail to activate the checkpoint preventing mitotic entry in response to the incompletely replicated DNA. Thus, independent defects in DNA replication and checkpoint regulation might explain why cells lacking An-Wee1 undergo aberrant mitosis.

Perspectives

Consistent with the vast array of cellular functions carried out by kinases, A. nidulans kinase mutants display a wide range of phenotypes from almost no growth to relatively subtle or no effects on vegetative growth. It is likely that further characterization of this kinase deletion set will reveal additional new phenotypes. Utilization of a similar deletion set of A. nidulans protein phosphatases [19], together with the kinase deletion mutants generated in this study, should advance the understanding of protein phosphorylation in filamentous fungi. Together with the expanding database of A. nidulans mutants (http://www.aspgd.org/), phenotypes defined here should aid in the classification of mutants of as yet uncharacterized genes generated using deletion constructs made available as part of this project. This expanding mutant library should help provide the basis for systems level biology in A. nidulans.

Materials and Methods

Deletion Construct Generation

Deletion construct primers were designed using software as described with modified criteria [34]. For most genes the deletion construct was designed such that pyrGα would replace the target gene within 30 bp (+/- of the start codon (89.3%) and stop codon (85.4%). When primers could not be designed in this region, the region before or after the start and stop codon was increased to 75 bp. Design of deletion construct primers was successful for 10,079 genes (95.4%) and primers for each deletion construct are listed in Table S1. The pyrGα cassette was amplified from plasmid pCDS60 using primers CDS164 and CDS165. Synthesis of the 5′
and 3’ flanking region of each gene was carried out by a Biomek NX robot (Beckman) from A. nidulans genomic DNA using LA Taq (TaKaRa) as described [34]. To generate full length deletion constructs, yeast strain FY834 was transformed with the 3’ and 5’ flanking pieces, the pyrG cassette and plasmid pRS426 (digested with XbaI and EcoRI), and yeast DNA prepared as described [34]. Gene specific primers 5f and 3r were used to amplify the full length deletion constructs. For some kinases, deletion constructs were generated by fusion PCR [37,38].

Bioinformatic and Phylogenetic Analysis

Orthologues of A. nidulans kinases present in other Aspergilli were identified by BLAST search at the AspgD [122] (http://www.aspgd.org/). Kinase domains were identified by BLAST comparison with the Salk Institute’s kinase database (http://kinase.com/)[20] or using a Batch CD search at the NCBI [121]. Phylogenetic analysis was carried out using ClustalW (http://workbench.sdsc.edu/) or http://www.phylogeny.fr). Trees were visualized using MEGA version 5 [123] or Biology Workbench (http://workbench.sdsc.edu/).

Generation of Kinase Deletion Strains

Deletion constructs were transformed into strain SO451 (pyrG89; u43; argB2; AnkuA470; Apyr44; dE15 nic14 che41 fic11) which contains the nbsdA-700 gene deletion to facilitate high frequency homologous recombination [43]. Transformation of 20 μl of SO451 protoplasts with 2 μl of the robotically generated deletion construct generated sufficient transformants for further analysis for most kinases. When necessary, the deletion construct DNA was concentrated or re-amplified prior to transformation to determine the terminal phenotype of essential kinases, uninucleate conidia were inoculated in YG media at 32°C for at least 7 days to germinate and form hyphae. When germination failed to occur, plates were stored at −80°C [35]. For kinase deletion constructs which did not generate heterokaryons, three independent transformants were streaked to single colony prior to further analysis. After the final streak, conidia were inoculated in YG liquid media to generate mycelia which were lyophilized for DNA extraction as described [124]. For putative heterokaryons, mycelial excised from the growing colony edge was inoculated into YG media, grown and similarly processed.

Transformants were tested for site specific integration of the deletion construct by diagnostic PCR using primers (Table S1) situated external to the targeting sequence for each kinase deletion construct. In most cases the size difference between the wild type and null allele was sufficient to distinguish them on a gel. When necessary alleles were distinguished using restriction enzymes which cut within pyrG of the deleted allele but did not cut the wild type allele. The presence of both the wild type and null allele in transformants successfully streaked on selective media indicated that diploids had formed during the transformation and these were discarded. Confirmed non-essential kinase haploid deletion strains and heterokaryons of essential kinases, have been deposited at the FGSC (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm) and are listed in Table S2.

Deletion of An-cdc7, nimXaA and An-aeel1 was also carried out by transformation of HA365 (pyrG89; pyruv44 AnkuA::argB; argB/argB2; Histone H1-mCherry;gyru41D; GFP-nikA) selecting and verifying heterokaryons as above.

Phenotypic Analysis of Kinase Deletion Strains

Two independent deletion strains for each non-essential kinase were phenotypically characterized in an initial test for colony growth at 20°C, 32°C and 42°C, and on MAGUU plates containing sucrose (1 M), NaCl (1 and 1.5 M), the ribonucleotide reductase inhibitor Hydroxurea (4 and 8 mM), or the microtubule poison benomyl (0.4 μg/ml). Kinase deletion mutants with similar phenotypes were restested together to allow phenotypic comparison and for figure generation. Heterokaryons generated for essential kinases were identified as described [35]. To determine the terminal phenotype of essential kinases, uninucleate spores generated from heterokaryons were inoculated in selective YG media at 32°C for 24 hours. The growing colony was then inoculated into YG media at 32°C for 3 days and incubated with germination confirmed visually using a 60×1.40 NA Plan Apochromatic objective. Heterokaryons formed during the transformation were retested together to allow phenotypic comparison for each kinase deletion construct.

Supporting Information

Table S1 Deletion primers and diagnostic primers.

Table S2 Kinase deletion strains.

File S1 Supplemental Figures S1–S15.

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Author Contributions

Conceived and designed the experiments: CPD SAO JCD. Performed the experiments: CPD SBH AHO PA CSR. Analyzed the data: CPD SBH AHO SAO. Wrote the paper: CPD SAO.
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