Restraint of the G2/M transition by the SR/RRM family mRNA shuttling binding protein SNXA$^{HRB1}$ in \textit{Aspergillus nidulans}


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ABSTRACT

Control of the eukaryotic G2/M transition by CDC2/CYCLINB is tightly regulated by protein-protein interactions, protein phosphorylations, and nuclear localization of CDC2/CYCLINB. We previously reported a screen, in Aspergillus nidulans, for extragenic suppressors of nimX2^cdc2^ that resulted in the identification of the cold-sensitive snxA1 mutation. We demonstrate here that snxA1 suppresses defects in regulators of the CDK1 mitotic induction pathway, including nimX2^cdc2^, nimE6^cyclinB^, and nimT23^cdc2^, but does not suppress G2-arresting nimA1/nimA5 mutations, the S-arresting nimE10^cyclinB^ mutation or three other G1/S phase mutations. snxA encodes the A. nidulans homolog of Saccharomyces cerevisiae Hrb1/Gbp2, nonessential shuttling mRNA binding proteins belonging to the SR (Serine-Arginine Rich) and RRM (RNA Recognition Motif) protein family, and human hnRNP-M, a spliceosomal component involved in pre-mRNA processing and alternative splicing. snxA^Hrb1^ is nonessential, its deletion phenocopies the snxA1 mutation, and its overexpression rescues snxA1 and ΔsnxA mutant phenotypes. snxA1 and a second allele isolated in this study, snxA2, are hypomorphic mutations that result from decreased transcript and protein levels, suggesting that snxA acts normally to restrain cell cycle progression. SNXA^Hrb1^ is predominantly nuclear, but is not retained in the nucleus during the partially-closed mitosis of A. nidulans. We show that the snxA1 mutation does not suppress nimX2 by altering NIMX2^CDC2/NIME^CYCLINB^ kinase activity, and that snxA1 or ΔsnxA alter localization patterns of NIME^CYCLINB^ at the restrictive temperatures for snxA1 and nimX2. Together, these findings suggest a novel and previously unreported role of an SR/RRM family protein in cell cycle regulation, specifically in control of the CDK1 mitotic induction pathway.
INTRODUCTION

Control of the eukaryotic G2/M transition by protein kinases has been widely studied and is highly conserved among all eukaryotes, from the budding and fission yeasts and filamentous fungi to metazoans (for review, see (MA and POON 2011)). The CDK1/CYCLINB protein kinase complex is a major regulator of this transition in all eukaryotes and is responsible for the phosphorylations of numerous proteins, leading to massive nuclear and cytoplasmic reorganizations that regulate mitosis (for review, see (LINDQVIST et al. 2009)). The complex itself is tightly regulated, both temporally and spatially, to allow mitotic entry.

Although CDK1/CYCLINB activity is essential for mitotic entry in all eukaryotes, structural differences in the nucleus in various organisms result in “open” mitosis (more complex eukaryotes) or “closed” mitosis (budding yeasts); these differences likely affect the temporospatial functioning of CDK1/CYCLINB. The partially-closed mitosis of the filamentous fungus *Aspergillus nidulans* is an evolutionary intermediate between open and closed mitoses and provides a system for studying mitotic entry in organisms intermediate between budding yeasts and more complex eukaryotes. The nuclear pore complexes in *A. nidulans* partially disassemble at mitotic entry (they are “partially-closed”), and proteins not specifically retained in the nucleus diffuse out of the partially-closed nuclear pores and may equilibrate across the nuclear envelope (DE SOUZA et al. 2004). In *A. nidulans*, the activity and proper localization of three protein kinases are required for initiation of mitosis: the CDK1/CYCLINB protein kinase complex (NIMX\textsuperscript{CDC2}/NIME\textsuperscript{CYCLINB}), the NIMA kinase, and a polo-like kinase (PLKA), all three of which must be inactivated to allow mitotic exit (BACHEWICH et al. 2005; OSMANI et al. 2006). The activity of CDK1/CYCLINB is tightly regulated by phosphorylation and is part of an
autocatalytic feedback loop (Ye et al. 1995); its activity is inhibited by the ANKAWEE1 kinase and activated by the NIMTCDC25 phosphatase. Furthermore, active NIMA kinase is required for mitotic initiation; in the absence of functional NIMA kinase, cells with fully active CDK1/CYCLINB arrest in late G2 (Osmani et al. 1991). NIMA activity is also regulated by phosphorylation (Ye et al. 1995) and is required for proper localization of CDK1/CYCLINB (Wu et al. 1998) and tubulin (Ovechkina et al. 2003) into the nucleus at the G2/M transition. Specifically, the SONAGLE2 and SONBNUP98 nucleoporins interact with NIMA to regulate the nuclear localization of NIMXCD2/NIMECYCLINB (Wu et al. 1998; De Souza and Osmani 2009). Wu et al. (1998) demonstrated that NIMXCD2 colocalizes in the nucleus with NIMECYCLINB during S and G2, that the G2 arrest which occurs in the absence of NIMA activity occurs with predominantly cytoplasmic NIMXCD2/NIMECYCLINB, and that in nimA1 mutants, the sonA1 suppressor of nimA1 re-establishes nuclear localization of NIMXCD2/NIMECYCLINB and entry into mitosis. These data provide evidence that proper localization of NIMXCD2/NIMECYCLINB is both regulated and essential for controlling mitotic entry during the partially closed mitosis of A. nidulans.

CDK1 localization and cell cycle progression depend upon the localization of CYCLINB (NiGG 1995). NIMECYCLINB localization has been characterized through the cell cycle in A. nidulans (Wu et al. 1998; De Souza et al. 2009); its nuclear localization is closely mirrored by NIMXCD2 localization (Nayak et al. 2010). NIMXCD2 and NIMECYCLINB become visible in the nucleus at or near the G1/S boundary and disappear from the nucleus during mitosis. De Souza et al. (2009) localized NIMECYCLINB in live cells to the nucleoplasm and the spindle pole bodies (SPBs) during interphase and early mitosis; this work demonstrated that the partial disassembly of the nuclear pore complex (NPC) at mitotic prophase allows most of the
NIME\textsuperscript{CYCLINB} to exit the nucleus; however, a nuclear pool remains, concentrated at the SPBs and also in the region of the segregating kinetochores. It is to this pool of NIME\textsuperscript{CYCLINB} that NIMX\textsuperscript{CDC2} presumably remains bound. The nuclear NIME\textsuperscript{CYCLINB} disappears sequentially during mitotic progression. The SPB pool disappears during anaphase, followed rapidly by the pool at the kinetochores. Surprisingly, NIMX\textsuperscript{CDC2} exits the nucleus slightly before the complete destruction of nuclear NIME\textsuperscript{CYCLINB} (NAYAK et al. 2010).

While phosphorylation/dephosphorylation and cell cycle-regulated localization of mitotic proteins have been shown to play integral roles in controlling the transition from G2 into mitosis in \textit{A. nidulans}, much remains to be learned. NIMX\textsuperscript{CDC2} encodes the only known p34\textsuperscript{CDC2} protein kinase in \textit{A. nidulans}, and its activity is required at both G1 and G2 (OSMANI et al. 1994). NIME\textsuperscript{CYCLINB} also functions at both G1 and G2 in \textit{A. nidulans}. While nimE6 causes a G2 arrest at restrictive temperature (O’CONNELL et al. 1992), the nimE10 mutation (originally identified as nimG10) arrests cells in S at restrictive temperature (Figure S1). The G1-specific functions of NIMX\textsuperscript{CDC2}/NIME\textsuperscript{CYCLINB} are not well understood. To better understand cell cycle regulation in \textit{A. nidulans} by NIMX\textsuperscript{CDC2}/NIME\textsuperscript{CYCLINB}, an extragenic suppressor screen to identify genes that interact with NIMX\textsuperscript{CDC2} was undertaken (MCQUIRE et al. 2000). The snxA1 mutation was identified in this screen as an extragenic suppressor of nimX2\textsuperscript{F223L}. snxA1 suppresses the NIMX2\textsuperscript{CDC2} heat-sensitive G2 arrest, allowing cells to enter and exit mitosis at the restrictive temperature for nimX2; additionally, and independent of its suppression of nimX2, snxA1 confers cold-sensitivity, leading to a G1 arrest at its restrictive temperature (MCQUIRE et al. 2000). Thus, as is the case for nimX\textsuperscript{cdc2} and nimE\textsuperscript{cyclinB} mutations, the snxA1 mutation has effects on the cell cycle at both G1 and G2. In this manuscript, we report genetic, cytological, and
molecular analysis of SNXA and demonstrate that it encodes the *A. nidulans* homolog of *Saccharomyces cerevisiae* Hrb1/Gbp2 proteins.

Hrb1/Gbp2 and the more divergent but similar protein Npl3 are nonessential shuttling mRNA binding proteins that are similar to members of the mammalian serine-arginine rich (SR) protein family (ROUGEMAILLE *et al.* 2008). SR proteins are a class of small nuclear ribonucleoprotein splicing factors that harbor two distinct types of domains: RNA recognition motifs (RRMs), responsible for RNA binding, and SR domains, long repeats of serine-arginine or arginine-serine dipeptides that are extensively phosphorylated (MA and POON 2011). Some RRM proteins contain RGG/RG tripeptide motifs that serve as sites for arginine methylation (THANDAPANI *et al.* 2013). In addition to their functions in spliceosome assembly and catalysis (SANFORD *et al.* 2004), SR proteins are also involved in mRNA transcription and export (TREX; reviewed in (REED and CHENG 2005; ROUGEMAILLE *et al.* 2008)).

In budding yeast, Hrb1/Gbp2 are recruited to and associate co-transcriptionally with mRNA transcripts by physical association with the TREX complex and Ctk1, a member of the cyclin-dependent kinase family that is the catalytic domain of Ctdk-1 (C-terminal domain kinase 1) (Figure S2). Ctdk-1 is a protein kinase required for efficient transcription elongation that phosphorylates the C-terminal domain of RNA polymerase II (RNAPII; (HURT *et al.* 2004)). The TREX complex consists of the THO complex (comprised of Tho2, Hpr1, Tft1, and Thp2), which associates with RNA polymerase II as it transcribes, and Sub2 and Yra1, export factors that bind to THO and function as a platform to recruit Hrb1/Gbp2 (HURT *et al.* 2004). Hrb1/Gbp2 associate both with the genes and the growing, unprocessed mRNA transcripts produced from them, and remain bound to the mRNA transcripts as part of the messenger RNA-protein complex (mRNP). As shuttling proteins, Hrb1/Gbp2 remain bound to the transcript
during export from the nucleus; they also remain associated with the mRNP during translation (Windgassen et al. 2004). Nuclear export of the mRNP requires the THO complex (Hacker and Krebber 2004) and the Mex67-Mtr2 dimer, an export receptor that interacts with nucleoporins (Strasser and Hurt 2000). Yra1 interacts with Mex67 (Strasser and Hurt 2000) and is essential for mRNA export for some, but not all, transcripts (Kim Guisbert et al. 2005). In addition to their functions in mRNA transcription and export, SR-like proteins act as localization signals that aid in delivering the mRNP to the translational machinery (Windgassen et al. 2004). More recently, both Hrb1 and Gbp2 have been shown to associate with the budding yeast spliceosome (Warkocki et al. 2009), with unspliced transcripts close to 5’ intron sequences (Tuck and Tollervey 2013), and to intron sequences and splicing factors (Hackmann et al. 2014).

While Hrb1/Gbp2 remain bound to transcripts during translation, localization studies show that Hrb1 is predominantly nuclear at steady-state and its re-import into the nucleus is mediated by the Mtr10 karyopherin import receptor (Hacker and Krebber 2004). Both Hrb1 and Gbp2 are phosphorylated by the cytoplasmic SR-specific protein kinase Sky1 (Porat et al. 2006; Ma and Poon 2011). This phosphorylation increases the affinity of Hrb1 for the mRNP. However, while Sky1 phosphorylation is required for re-entry of Gbp2 into the nucleus, it is not essential for nuclear import of Hrb1.

Hrb1/Gbp2 appear to have roles in mRNA export; however, deletion of these genes does not disrupt or alter the nucleocytoplasmic distribution of bulk polyadenylated mRNAs (Hacker and Krebber 2004). Interestingly, overexpression of Gbp2 leads to a delay in G1 (Stevenson et al. 2001). As suggested by Rougemaille et al. (Rougemaille et al. 2008), Hrb1/Gbp2 may act as dedicated adaptors that recognize specific sets of transcripts. Hackman et al. (2014) recently
demonstrated that Hrb1/Gbp2 function both to recognize and mediate the elimination of aberrantly spliced transcripts via binding to Mtr4 and to mediate the nuclear export of correctly spliced transcripts via Mex67 (Hackmann et al. 2014).

Given that the A. nidulans Hrb1 homolog, snxA, was identified as a genetic interactor with nimXcdc2, and that the snxA1 mutation arrests cells in G1 at its restrictive temperature, it is possible that SNXAHRB1 interacts with cell cycle-specific transcripts or proteins. The SR/RRM family has not previously been implicated in the regulation of the G2/M transition. These findings may therefore represent a novel regulatory mechanism in cell cycle control of Aspergillus nidulans.

**MATERIALS AND METHODS**

**Strains and general techniques**

Strains used in this study are listed in Table S1. PCR primers used for gene deletion, gene tagging, and molecular diagnoses are shown in Table S2. Phusion Polymerase™ was used for all PCR experiments, and all other DNA-modifying enzymes were from New England Biolabs. Standard methods of Aspergillus culture (Kafér 1977), genetic analysis (Pontecorvo et al. 1953; Kaminskyj 2001), construction and analysis of heterokaryons and diploids (Todd et al. 2007), and Aspergillus transformation (Ballance et al. 1983) were employed. Appropriately supplemented minimal media (1% glucose) were used for all strain construction and genetic mapping. Rich media, composed of minimal medium (Kafér 1977) supplemented with 0.5% yeast extract and containing 2% glucose, were used for kinase assay experiments and to grow cells for DNA-mediated transformation.
**Generation of a new snxA allele by a non-complementation screen**

To generate a new snxA allele, we mutagenized a diploid strain, dSWJ 3693, homozygous for nimX2 and heterozygous for snxA1 (riboA1/+ +/yA2; nimX2 snxA1 wA2/nimX2 + +; +/pyroA4; +/methB3; nicB8/+), in which the snxA1 chromosome is marked by the wA2 white-spored mutation. This diploid is green because it carries a wild-type wA allele, and it expresses the nimX2 ts-lethality at 39 °-44 °. 2 x 10^7 freshly harvested conidia were treated for 30 minutes at 30° with 0.8 or 1.2 µg/ml of the UV-mimetic 4-nitroquinoline-1-oxide (4-NQO), inactivated by treatment with 10% Na2S2O3 for 5 minutes, washed twice in dH2O, then 5x10^6 spores were spread on appropriately supplemented minimal medium and grown for four to six days at 41° to select for strains that gain the ability to grow at the nimX2 restrictive temperature. Because one copy of snxA is defective, we reasoned that new nimX2-suppressing mutations might occur in the remaining wild-type snxA allele. Alternatively, suppression may occur by back-mutation or reversion in a nimX2 allele, or by dominant mutations in genes other than nimX or snxA.

Thirteen candidate TS+ green colonies were recovered, streaked three times and grown at 37° to ensure clonal purity.

To recover the new mutations in a haploid background, a parasexual genetic approach was employed (TODD et al. 2007) to break down the diploid strains into their component haploid strains non-meiotically, i.e., via random assortment of chromosomes without crossing over. Each TS+ strain was streaked in a line on rich media containing the microtubule-destabilizing agent benomyl (Bonide™, Trainor and Bonide Products, Inc.) at 60, 67.5, 75, and 82.5 µg/ml, and grown at 33° for eight days until haploid sectors grew out. Haploids were distinguished from diploids by segregation of yellow (yA2 on Chromosome I) or white (nimX2 snxA1 wA2 on
Chromosome II) sectors, or occasional green sectors, from the green diploid parent. Thirty-five white, yellow, or green haploid sectors were recovered from each TS+ diploid and streaked three times to clonal purity. The haploids were then screened on minimal media for the auxotrophic markers (pyroA4, nicB8, methB3, riboA1) and for temperature sensitivity. As expected, all wA2 sectors were phenotypically nimX2 snxA1. Among the TS+ candidate diploids, representative yellow and green haploids derived from seven of these 13 strains were analyzed further, as reported in Results (below).

Fluorescence microscopy

For fluorescence microscopy, cells were grown in minimal media containing 1% glucose or 50 mM glycerol as the carbon source. To visualize fluorescently labeled proteins, conidia were incubated on coverslips as described (HARRIS et al. 1994) and fixed for 20 minutes in PEMF (80mM PIPES, 1mM EGTA, 1mM MgCl₂, 4% formalin). Nuclear staining with 2,4-diamidino-2-phenylindole (DAPI) following fixation was as described (BRODY et al. 1991). Experiments were performed in duplicate; 200 germlings (containing <8 nuclei/germling) per replicate were visualized and quantitated using a Zeiss Axioimager.M1 microscope equipped with a Photometrics Coolsnap HQ² camera. Exit from mitosis was determined by dissolution of the mitotic spindle in strains harboring both GFP-α–tubulin and SNXA::mRFP. For NIME\textsuperscript{CYCLINB} localization experiments, bulk localization of NIME\textsuperscript{CYCLINB} was quantitated; detecting the localization of the small nuclear pool that remains after mitotic entry and is degraded at mitotic exit is beyond the capabilities of our instrumentation.
**snxA gene deletion, overexpression, and gene tagging**

*snxA* predicted gene structure is complex, with as many as 11 coding exons, and in which the first two exons are very short: the first = 34 nt, containing 30 nt of 5’ UTR and 1-1/3 codons, ATGG; and the second = 16 nt, and encodes 5-1/3 codons (*Aspergillus* Comparative Sequencing Project, Broad Institute of Harvard and MIT ([http://www.broadinstitute.org/](http://www.broadinstitute.org/)). Furthermore, these short ORFs are interspersed with unusually long and GC-rich introns of 766 and 548 nt, respectively. Moreover, an alternative start codon, 42 nt inside the 3’ end of the second intron and in-frame with the third exon, would eliminate the first two short exons and would create a nine-exon protein that differs by 13 N-terminal amino acids from the 11-exon protein.

By one-step gene replacement, *snxA* was deleted by replacing all but the first two short exons and by completely eliminating the alternative nine-exon gene. Three-way fusion PCR was used to create a linear deletion construct (*Yu* et al. 2004) (Table S2) using the *Aspergillus fumigatus pyroA* gene as a selectable marker, followed by nested PCR to amplify a sufficient amount of purified product for DNA-mediated transformation. This nested construct was introduced into a *snxA*+ host strain (tSWJ 2973: *pyrG*89; *pyroA*4 Kutr-L::*pyrG* Af; *nicA*2; *riboB*2) in which the Non-Homologous End-Joining (NHEJ) pathway of DNA repair (*nkuA* gene, encoding Ku70) was transiently disrupted (*Nielsen* et al. 2008) in order to facilitate homologous integration (*Nayak* et al. 2006). Weakly growing *pyroA*+ transformants were selected at 37° for six days, followed by serial streaking to clonal purity. The expected patterns for gene replacement at the *snxA* locus were observed by Southern blotting (data not shown), and two of these deletions were further verified by trans-locus PCR using primers that lie outside of the region covered by the nested deletion construct (Figure S3).

Overexpression studies were performed with strains carrying one copy of an *alcA*-driven
snxA allele integrated at the argB locus. The alcA::snxA construct was made by amplifying the alternative nine-exon gene from genomic DNA, using PCR primers into which a novel SpeI or BamHI restriction site was incorporated (Table S2). The 2249 nt genomic PCR clone was digested first with SpeI, blunted by treatment with Klenow fragment, and then digested with BamHI. This fragment was cloned into the SmaI and BamHI sites of a plasmid, pSDW194 (James et al. 1999) that contains the inducible-repressible A. nidulans alcA alcohol dehydrogenase gene promoter (Waring et al. 1989) and the A. nidulans argB gene as a selectable marker. Southern blotting with probes against argB and snxA was used to diagnose single argB integrations (Figure S4). alcA::snxA expression was strongly induced in minimal medium containing 200 mM ethanol + 0.04% fructose as the carbon source. Basal expression was achieved using 50 mM glycerol, and alcA::snxA expression was repressed on rich media containing 2% glucose.

GFP and mRFP tags were added to the C-terminus of the wild-type snxA gene by using universal tagging cassettes (Yang et al. 2004). Linear tagging constructs were generated by three-way fusion PCR (Table S2), followed by nested PCR. These constructs were transformed into a snxA+ host (tSWJ 2353: pyrG89; pyroA4 ΔnkuA::argB; nimO18; riboB2). From each transformation five pyrG+ transformants were recovered and streaked three times to clonal purity. Creation of snxA::GFP and snxA::mRFP was verified in all 10 pyrG+ strains by trans-locus PCR (data not shown). Several of these strains were examined by fluorescence microscopy; all produced strong nuclear signals in interphase cells. These strains were outcrossed to remove the nimO18 mutation and to combine the tagged alleles with other relevant markers and mutations, including GFP-α-tubulin, GFP-nimE^{cyclinB}, nimX2^{cdc2}, nimT2^{cdc25}, and nimE6^{cyclinB}. 
A myc9 epitope tag was added to the C-terminus of snxA wild-type, snxA1, and snxA2 alleles by using universal tagging cassettes. This tag contains an 11-amino acid GAGAGAGFDGA N-terminal linker followed by the myc 10-amino acid repeats; each myc repeat is flanked by a GA dipeptide. The myc9 tag was generated de novo using two self-dimerizing primers as described by (NAKAJIMA and YAOITA 1997) (Table S2). The primers were 5’ phosphorylated using T4 Polynucleotide Kinase (New England Biolabs), boiled three minutes to inactivate the kinase, and then used as template to amplify myc tags of varying length via 30 cycles of PCR. AmpliTaq™ DNA Polymerase Stoffel fragment (Applied Biosystems) was used for amplification in 1x Stoffel Buffer with annealing temperature 62° and 10-minute extension at 72°. The largest PCR products were recovered and purified (QIAEX II Gel Extraction Kit, QIAGEN Corp.), cloned into the EcoRV site of pBluescript KS(+), and sequenced. One of these clones, containing a myc9 fragment, was amplified further in order to add, at the 5’ end, a BspDI site followed by an N-terminal GAGAGAGFDGA linker; to the 3’ end a stop codon followed by a HindIII site were added (Table S2). This amplicon was cloned into the BspDI and HindIII sites of pBluescript KS(+), and then sequenced to verify the (GA)$_3$GFDGA-myc9-STOP tag. Finally, three universal myc9 tagging cassettes were constructed by cloning in the pyrG, pyroA, or riboB selectable marker genes from Aspergillus fumigatus. pyrG$^{Af}$ was removed from a plasmid, pCLS366, as an EcoRV - EcoRI fragment, and cloned into the corresponding sites in the myc9 plasmid, to generate, pmyc9-pyrG$^{Af}$. pyroA$^{Af}$ and riboB$^{Af}$ were removed from pTN01 and pTN02, respectively (NAYAK et al. 2006) as HindIII (blunt) – PstI fragments, and cloned into the EcoRV and PstI sites of the myc9 plasmid, to create pmyc9-pyroA$^{Af}$ and pmyc9-riboB$^{Af}$ (Figure S5).
Using three-way fusion PCR followed by one-step gene replacement, the \textit{snxA+}, \textit{snxA1}, and \textit{snxA2} genomic loci were C-terminally tagged with \textit{myc9::riboB}\textsuperscript{Af} (Table S2) in five \textit{A. nidulans} host strains (\textit{snxA+}: tSWJ 2888; \textit{snxA1}: tSWJ 5573/5574; \textit{snxA2}: tSWJ 5583/5584), and diagnosed by trans-locus PCR (data not shown).

**Isolation and characterization of \textit{snxA} cDNAs**

A 1.3 kb PCR fragment corresponding to the AN3739 \textit{snxA} predicted coding region (spanning exons 6 – 11) was used to screen an \textit{A. nidulans} \lambda gt10 cDNA library (generously provided by S. Osmani) (Osmani \textit{et al.} 1988). Of eight independent phage isolates that were amplified and cloned into pBSKS(\(+\), four were judged to be full-length based on their size (1.8 – 2.1 kb). Sequencing revealed two alternative \textit{snxA} cDNAs (two clones of each) that were spliced differently at their N-termini, and composed of either nine or 11 exons (Figure S6). Both of these isoforms correspond to alternatively spliced AN3739 transcripts identified previously by EST sequencing of \textit{A. nidulans} cDNA libraries (Aspergillus Comparative Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)), and as described above. To assess their functions, the cDNAs were fused with the \textit{A. nidulans alcA} promoter in plasmid pSDW194 (James \textit{et al.} 1999). Following transformation of \textit{snxA1} mutants SWJ 3676 and SWJ 3678, Southern blotting was used to identify strains carrying a single copy of \textit{alcA}-driven \textit{snxA} integrated at the \textit{argB} or \textit{snxA} loci. Ability to complement \textit{snxA1} cold-sensitivity was assessed on inducing medium (200 mM ethanol + 0.04\% fructose), medium that allows basal expression (50 mM glycerol), and on repressing medium (2\% glucose).
mRNA Transcript Analysis

RNA isolation and gene expression were determined for wild-type (PCS 439), *snxA1* (SWJ 4030), and *snxA2* (SWJ 5562) strains using qRT-PCR as described (Alam et al. 2012). Randomly cycling vegetative mycelia grown at 32°C were collected by vacuum filtration using Miracloth (Calbiochem), frozen in liquid nitrogen, and lyophilized overnight. Total RNA was extracted using an RNeasy plant kit (Qiagen) per manufacturer’s instructions. RNA concentration and RNA quality were determined using a NanoDrop spectrophotometer to determine 260/280 ratio followed by native agarose gel electrophoresis. Total RNA was diluted to 400 ng/µl and gDNA elimination and reverse transcription were performed using a QuantiTect reverse transcription kit (Qiagen), according to manufacturer’s instructions, including no reverse transcriptase controls. Samples were diluted 1:5 and Quantitative real-time PCR (qPCR) was performed using iQ SYBR Green Supermix (Qiagen) according to manufacturer’s instructions. Appropriate negative controls were used in all experiments (no template DNA; no reverse transcriptase) for each gene. Six replicates of each reaction were performed using a 20 µl total volume in 96 well optical plates in a C1000 thermal cycler using a CFX96 real-time detection system (Bio-Rad). *actA* (actin) was used as the reference gene, using primers actF and actR that amplify across an intron; for *snxA*, either of two forward primers, one straddling intron nine (snxA-q1) and one in exon 10 (snxA-q2), were paired with one reverse primer (snxA-q3), located 80 nt into exon 11 (Table S2). qPCR amplification conditions were: 95°C/3 min for one cycle, 95°C/15 sec, 60°C/15 sec, 72°C/20 sec for 44 cycles, 95°C/ for 10 sec. Melt curve analysis was performed starting at 65°C and rising by increments of 0.5°C every 5 seconds to 95°C. Expression was normalized to *actA* and calculated using the ΔΔCt method (Livak and
Five independent replicate experiments were performed with nearly identical results.

**Western blot analysis and kinase assays**

Protein purification, Western analysis and kinase assays were performed as previously described (Osmani et al. 1991; Liu et al. 2010). Briefly, total protein extracts were generated from lyophilized mycelia as described (Liu et al. 2010). For NIMX detection, 100 µg of extracts were separated by SDS-PAGE, blotted to nitrocellulose, and probed with anti-NIMX E77 antibody (kind gift of Dr. Stephen Osmani). Detection was performed using goat anti-mouse IgG, HRP conjugate (Millipore; #12-349) and GE Healthcare Advance Chemiluminescent western blotting detection kit. For the histone H1 kinase assay (described in (Osmani et al. 1991), anti-NIMX E77 antibody was incubated with 200 µg of extracts and immune complexes were precipitated with protein A/G sepharose (Pierce). Following precipitation, beads were washed four times and then resuspended in 20 µl kinase assay buffer (Osmani et al. 1991), incubated with substrate (Millipore histone H1; #14-155) for 5 minutes, and the reaction was stopped by addition of 20 µl Laemmli sample buffer. After boiling for five minutes, the entire supernatant for each reaction was separated by SDS-PAGE and the dried gel was exposed to autoradiography film.

For SNXA detection, 2.5 µg of extracts were separated by SDS-PAGE (4-20% TBX gels, Bio-Rad), blotted to PVDF, and probed with either myc.A7 mouse mAb at 1:3000 (Abcam ab18185) or DM1A anti-α-tubulin mouse mAb at 1:1000 (Abcam DM1A) followed by 1:5000 anti-mouse IgG HRP (Promega W4021). Protein complexes were detected by one-minute exposure in 5 ml of TMB Stabilized Substrate for HRP (Promega W4121). For GFP-
NIME\textsuperscript{CYCLINB} detection, 2.5 µg of extracts were separated by SDS-PAGE (10% Tris-glycine gels, Bio-Rad), blotted to nitrocellulose, and probed with anti-GFP antibody at 1:1000 (InVitrogen A11122) followed by 1:10,000 anti-rabbit IgG HRP (Santa Cruz Biotechnology Sc-2004). The blots were stripped and re-probed with anti-α-tubulin mouse mAb at 1:1000 (Sigma T9026) followed by 1:10,000 anti-mouse HRP (Pierce 32230).

\section*{RESULTS}

\textit{snxA1} interacts with the CDK1 mitotic regulatory pathway

To determine if the \textit{snxA1} mutation is allele specific, \textit{snxA1/nimX} double mutants were generated with three heat-sensitive \textit{nimX} alleles (\textit{nimX1}\textsuperscript{G225S}, \textit{nimX2}\textsuperscript{F223L}, and \textit{nimX3}\textsuperscript{Y306H}); colony growth at increasing temperatures was compared for single mutants and double mutants (Figure 1). The \textit{snxA1} mutation conferred cold-sensitivity at 20\degree and reduced colony size at permissive and semi-permissive temperatures (25\degree – 43\degree) in all strains tested. Suppression of heat sensitivity was evident in all three double mutant strains at 37\degree and for \textit{snxA1/nimX1} and \textit{snxA1/nimX2} double mutants at 43\degree.

\textit{snxA1} double mutants were generated with four additional heat-sensitive G2-arresting mutations (\textit{nimE6}\textsuperscript{cyclinB}, \textit{nimT2}\textsuperscript{3dc25}, and \textit{nimA1/nimA5} (O'CONNELL \textit{et al.} 1992)), two S-phase arresting \textit{nim} mutations (\textit{nimE10}\textsuperscript{cyclinB}, Figure S1; and \textit{nimP22}\textsuperscript{DNApol}, S. James unpublished results), and two late G1-arresting mutations (\textit{nimO18}\textsuperscript{dbh4} (JAMES \textit{et al.} 1999) and \textit{nimQ20}\textsuperscript{mcm2} (JAMES \textit{et al.} 1995). The late-G1 arresting \textit{nimO18}\textsuperscript{dbh4} and \textit{nimQ20}\textsuperscript{mcm2} mutations fail to initiate DNA synthesis at restrictive temperature. However, they do undergo mitotic catastrophe by attempting to segregate their unreplicated chromatin; \textit{i.e.}, they undergo a pseudomitosis resulting
from failure to activate a DNA replication checkpoint at the G1/S transition (James et al. 1999). Thus, these mutants are presumed to activate the CDK1 mitotic induction pathway at restrictive temperature. Growth of double mutants was compared to single mutants at increasing temperatures (Figures 2, 3). *snxA1* suppressed the heat-sensitive G2-arrest phenotypes of \( \text{nimX}_2^{\text{cdc2}} \), \( \text{nimE}_6^{\text{cyclinB}} \), and \( \text{nimT}_23^{\text{cdc25}} \) at 37° and 43° (Figure 2a). In contrast, *snxA1* failed to suppress the *nimA1* and *nimA5* G2-M regulatory defects, and instead the double mutants were more growth-impaired than either single mutant at the permissive temperatures of 29° and 33° (Figure 2b). Similarly, *snxA1* exhibited little or no suppression of the *nimE10^{cyclinB}*, and *nimP22^{DNApol}*, S defects, and *snxA1* failed to suppress the heat-sensitive G1 arrest of *nimO18^{dbf4}* and *nimQ20^{mcm2}*. Instead, and contrasting with *snxA1* rescue of CDK1 pathway mutations, growth and temperature defects became more severe in these latter four *snxA1* double mutants, as expected for mutations that operate in unrelated pathways. Therefore, *snxA1* suppression of heat-sensitive cell cycle defects appears restricted to components of the CDK1 mitotic regulatory pathway. Both \( \text{NIM}^{\text{CYCLINB}} \) and \( \text{NIMT}^{\text{CDC2}} \) are specifically required for the activation of \( \text{NIMX}_2^{\text{CDC2}} \) to allow mitotic entry, and NIMA is required for the partial disassembly of nuclear pore complexes, which allows entry of the active \( \text{NIMX}_2^{\text{CDC2/CYCLINB}} \) complex into the nucleus (De Souza et al. 2003; De Souza et al. 2004). The suppression of three different \( \text{nimX}_2^{\text{cdc2}} \) alleles and of mutations in multiple activators of the \( \text{NIMX}_2^{\text{CDC2/CYCLINB}} \) G2-M regulatory pathway but not of mutations affecting G1 or S phase control or the NIMA G2-M regulator strongly suggests a role for SNXA in the regulation of the G2/M transition via the CDC2/CYCLINB regulatory pathway.
SNXA encodes the *A. nidulans* homolog of Hrb1<sup>Se</sup>/Gbp2<sup>Se</sup> and human hnRNP-M

*snxA* (suppressor-of-*nimA*<sup>cdc2</sup>) was previously assigned to Linkage Group II (LGII) via parasexual mapping (McGuire et al. 2000). After several unsuccessful attempts to complement *snxA1* using a LGII-specific cosmid library (Brody et al. 1991)(obtained from the Fungal Genetics Stock Center (FGSC), Kansas City, Missouri, USA) or a total genomic library (Oshirov and May 2000) (obtained from the FGSC), the location of *snxA* within LGII was determined by linkage analysis. The initial discovery of linkage to anB8 (17.4 map units, n = 144) was followed by three-factor crosses involving *snxA1, cnxE16*, and *hisG113*, in which *snxA* localized 10.7 map units rightward of *cnxE* and 1.3 map units rightward of *hisG* (n = 224).

To isolate the wild-type *snxA* gene, a group of five overlapping fosmids (Galagan et al. 2005) (obtained from the FGSC) that span the relevant region were co-transformed into a *snxA1 argB2* strain (SWJ 3676: riboA1; *snxA1*; argB2; pyroA4; chaA1) using the *A. nidulans* autonomously replicating helper plasmid, pDHG25 (Brody et al. 1991) (obtained from the FGSC), which carries *A. nidulans* argB as a selectable marker. Co-transformed cells were grown for 18 hours at 37° to establish thousands of argB+ transformants, then shifted to 20° for 8 days to select for complementation of *snxA1* cold-sensitivity. Only one fosmid, 8201-D10, complemented *snxA1; >100 snxA+ argB+* co-transformants were obtained. 8201-D10 overlapped a non-complementing fosmid (8199-G2) over two-thirds of its length, thereby eliminating 10 out of 16 fosmid-borne genes from further consideration and narrowing the search to a group of 5 protein-coding genes (AN3738 to AN3742) and one leucyl-tRNA in the non-overlapping region of 8201-D10. Four fragments covering this region were subcloned into pBluescript KS(+) and transformed into a *snxA1* strain. Only one plasmid, containing AN3739
and lacking the tRNA gene, complemented *snxA1*, thus identifying AN3739 as the putative *snxA* gene.

Deletion of AN3739/*snxA* was accomplished by one-step gene replacement (Figure S3). Δ*snxA* strains were viable and slightly more impaired than *snxA1* for vegetative growth and conidiation at all temperatures. Deletion of *snxA* phenocopied the *snxA1* mutation, conferring cold-sensitive lethality at 20°, mild heat sensitivity at 43°, and suppressing the heat-sensitivity of *nimX1* and *nimX2* at 37° and 43° (Figure 4a). Conversely, and similar to *snxA1 nimA5* and *snxA1 nimA1* double mutants (Figure 2b), Δ*snxA* did not suppress *nimA5* (Figure 4b); these double mutants remained heat-sensitive and cold-sensitive, with pronounced impairment of growth and conidiation at the permissive temperatures of 29° and 33°, typical of mutations that act through different pathways.

A non-complementation screen was used to generate an additional *snxA* mutation in a diploid strain homozygous for *nimX2* and heterozygous for *snxA1*, as described in Materials and Methods. Thirteen diploid revertants that had regained the ability to grow at the *nimX2* restrictive temperature of 41° were haploidized by treatment with benomyl. Haploid strains derived from seven of the 13 candidates were crossed to *hisG113*, and of these one harbored a cold-sensitive *nimX2* suppressor that was tightly linked to *hisG* (2 recombinants in 60 progeny, 3.3 map units). This mutant was named *snxA2*. To verify the ability of *snxA2* to suppress *nimX2*, *snxA2 nimX2* double mutants were re-synthesized by crossing *snxA2 nimX+* × *nimX2 snxA+*. In these double mutants, *snxA2* suppressed *nimX2* temperature sensitivity at 37° and 43° in a manner similar to *snxA1* (Figure S7). The *snxA2* phenotype differs from *snxA1*. Whereas *snxA1* grows optimally and conidiates strongly at 37°, *snxA2* mutants grow and conidiate
optimally at 33° and exhibit a defect in conidiation at 37°, suggesting that snxA2 harbors a more severe defect than snxA1 and that snxA1 and snxA2 result from different alterations (Figure 5).

To verify the location of the snxA1 and snxA2 mutations, one PCR-derived and two plasmid-derived linear wild-type DNA fragments were used to complement snxA1 in two strains (tSWJ 5573/5574) and snxA2 in two strains (tSWJ 5567/5584). These strains harbored a deletion of the nkuA (Ku70) gene in order to facilitate targeted integration by homologous recombination (Nayak et al. 2006). Each of these transformations yielded hundreds of transformants that efficiently complemented the cold-sensitivity of the mutants (Figure 6). The shortest linear DNA, a 3353 nt EcoRI – SnaBI fragment, begins approximately in the middle of intron 1, and encompasses exons 2 – 11 and 90% of the AN3739 3’UTR (263 out of 291 nt).

Allelism between snxA1, snxA2, and ΔsnxA was assessed in complementation tests using diploids and heterokaryons (Figure S8). In heterozygous diploids, each snxA mutant was fully recessive (Figure S8a). Only one homozygous diploid, snxA1/snxA1, was isolated (Figure S8a). Although balanced heterokaryons harboring ΔsnxA/snxA1 and ΔsnxA/snxA2 were repeatedly established, diploids could not be derived after three independent attempts. Therefore, complementation was examined in these balanced heterokaryons (Figure S8b). snxA1 and snxA2 failed to complement ΔsnxA, conferring cold-sensitive lethality at 20° and growth impairment at 43° similar to haploid snxA mutants. Interestingly, at 43° the growth defect of ΔsnxA/snxA2 heterokaryons was dramatically enhanced, showing greater impairment than ΔsnxA/snxA1 heterokaryons or haploid ΔsnxA and snxA2 single mutants.

AN3739 shares approximately 30% identity with S. cerevisiae Hrb1/Gbp2 (1e^-45 and 2e^-43, respectively), non-essential shuttling mRNA binding proteins containing an N-terminal SR
domain and three RNA recognition motifs (RRMs) (Figure S6a). As the only Hrb1/Gbp2-homologous protein in the *A. nidulans* genome, AN3739 contains a similar overall structure, with three highly conserved RRRMs and an N-terminal SR domain that is slightly more similar to Hrb1 than to Gbp2 (34 identities/8 similarities versus 33 identities/4 similarities). The closest human homolog of AN3739, heterogeneous ribonucleoprotein-M (hnRNP-M, 3e^{-27}), is an abundant spliceosomal component involved in pre-mRNA splicing and alternative splicing (Lleres et al. 2010; Xu et al. 2014). hnRNP-M also contains three conserved RRM motifs but lacks an N-terminal SR domain (Figure S6b). SNXA and hnRNP-M share two features that distinguish them from the budding yeast proteins. First, the sequences between RRM1-RRM2 and RRM2-RRM3 are glycine-rich, containing several runs of between two and seven glycines. Second, SNXA and hnRNP-M contain tripeptide motifs that are commonly methylated on arginine. SNXA contains seven RGG tripeptides, of which five are clustered between RRM2 and RRM3; hnRNP-M has undergone an expansion of this same region, which contains 25 RMG/RVG/RIG/RMA/RMV motifs that are known to be methylated (Hung et al. 2009).

*snxA* function was examined further by overexpressing genomic and cDNA clones. Overexpression of a nine-exon wild-type AN3739/*snxA* genomic DNA, containing exons 3 – 11 and 90% of the 3’ UTR, efficiently rescued *snxA1* cold-sensitivity and abrogated the suppression of *nimX2* by *snxA1* (Figure S4c; Figure S9). In addition, overexpression of this alcA::*snxA*+ gDNA complemented Δ*snxA* defects in growth and cold-sensitivity (Figure S10). Unexpectedly, overexpression of the nine-exon region obtained from *snxA1* mutant gDNA also complemented *snxA1* efficiently (data not shown), suggesting that (1) the *snxA1* mutation lies upstream of exon 3, or (2) overexpression of partially functional *snxA1* mutant protein was able to fully rescue *snxA1* phenotypes. We also generated alcA-driven cDNAs from the nine- and 11-exon
alternatively spliced cDNAs described in the Materials and Methods. The nine-exon cDNA contains 27 nt of 5’ UTR. Three different 11-exon cDNA versions were tested, containing 32, 39, or 162 nt of 5’ UTR sequence. No upstream ATGs occur in these four UTR sequences. Overexpression of each cDNA from a single copy at the argB locus complemented snxA1 to varying degrees (Figure S9).

Typically, an exonic mutation can be complemented by integration of promoter-less cDNAs at the endogenous locus to restore a wild-type allele under control of the native promoter. However, in transformations with plasmid-borne alcA::snxA cDNAs, neither the 11-nor the nine-exon cDNAs were able to complement snxA1 on glucose, i.e., in an ethanol-independent manner, in 167 argB+ transformants with the 11-exon cDNA and in a total of 70 argB+ transformants obtained by using the nine-exon cDNA. There is very little difference in length and sequence of the two cDNAs (Figure S6). The two additional N-terminal exons in the 11-exon cDNA encode only nine amino acids; these two exons are interrupted by unusually long introns of 766 and 548 nt. The nine-exon cDNA begins within the 548 nt second intron (42 nt upstream of the intron 2 3’ splice junction), and encodes 14 N-terminal amino acids by which it differs from the 11-exon cDNA (Figure S6). Given that these two cDNAs are therefore identical except at a small region in the N-terminus, the absence among transformants of constitutive restoration of the wild-type phenotype suggests that (1) the snxA1 mutation may lie within an intron or an upstream regulatory region, (2) the mutation may lie so close to the coding region N-terminus as to preclude the likelihood of a crossover event between the N-terminus of the cDNA and the mutation at the endogenous locus, or (3) the snxA1 alteration may affect chromatin structure and function in the snxA region.
We amplified and sequenced the AN3739 genomic region in *snxA1*, spanning 5,882 nucleotides, from nt 3,224,102 to 3,218,220 (Aspergillus Genome Database: http://www.aspgd.org). The sequenced region begins 85 nt before the AN3738 start codon and 671 nt upstream of the start site of the longest 11-exon *snxA* cDNA (nt 3,213,431). This cDNA start site precedes the exon 1 start codon by 162 nt. The 5’ sequenced region contains a 100 nt long leucyl-tRNA gene that ends 333 nt upstream of the cDNA start site. The 3’ region includes the AN3739 3’ UTR, 3’ flanking intergenic region, and the adjacent gene, AN3740. The *snxA1* sequenced region extends >1000 nt beyond both ends of the 3353 nt sequence that fully complemented *snxA1* and *snxA2* in transformations with a linear wild-type DNA fragment (Figure 6). *snxA2* was sequenced across 4842 nt from nt 3,223,063, which lies 82 nt upstream of the 3353 nt *snxA1/snxA2* linear complementing DNA region, and ends at 3,218,220, as for *snxA1* (above). Three independent PCR products each from one wild-type strain (PCS 439), a minimum of two *snxA1* strains (SWJ 3676, SWJ 3678, SWJ 3404, or SWJ 2862) and two *snxA2* strains (EAK 5496 and EAK 5497) were sequenced on both strands, for a total of at least six *snxA1* and six *snxA2* mutant-derived PCR products at each interval, and compared with the published genomic sequence derived from strain FGSC A4 (*Aspergillus* Comparative Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)). In addition, *snxA1* was sequenced more extensively in the 5’ flank plus exons 1 and 2 and introns 1 and 2, from nt 3,223,572 (141 nt upstream of the startsite of the 11-exon cDNA) to 3,221,861 (1711 nt), over three intervals, with at least 10 templates (20 sequences) and as many as 16 templates (33 sequences) per interval. Without exception, no DNA sequence changes were detected in *snxA1* or *snxA2*. 
Multiple lines of genetic and phenotypic evidence indicate that AN3739 is snxA: First, classical genetic mapping localized the mutation to a small interval on chromosome II. Second, plasmid subclones containing only the wild-type AN3739 DNA complemented the snxA1 mutation. Third, both snxA1 and snxA2 were efficiently complemented by a linear 3.353 kb fragment spanning most of the AN3739 locus. Finally, the fully recessive snxA1 and snxA2 mutations failed to complement a deletion of AN3739, indicating that these three mutations are allelic. Phenotypic support for AN3739 being snxA is evident in that deletion of snxA phenocopies snxA1 and snxA2 both in cold sensitivity and suppression of nimX2. Conversely, overexpression of wild-type and snxA1 mutant genomic clones and alternatively spliced AN3739 cDNAs complemented snxA1 and ΔsnxA when integrated as a single copy at the argB locus; and importantly, overexpression of a snxA+ gDNA abrogated the suppression of nimX2 by snxA1. Therefore, despite the absence of DNA sequence changes in the snxA1 and snxA2 ORFs and their upstream and downstream flanking regions, we conclude that snxA (AN3739) encodes the single A. nidulans homolog of S. cerevisiae Hrb1/Gbp2 and human hnRNP-M.

The snxA1 and snxA2 mutations decrease snxA expression

Because deletion of snxA phenocopies both the snxA1 and snxA2 mutations, we hypothesized that the hypomorphic snxA1 and snxA2 phenotypes are due to decreased snxA expression. mRNA transcript levels were quantitated in randomly cycling vegetative mycelia in snxA1, snxA2, and snxA+ strains using qRT-PCR. Both snxA1 and snxA2 exhibited 5-fold decreased expression relative to wild-type (Figure 7a). To investigate the effects of snxA1 and snxA2 mutations on the levels of protein, the snxA1, snxA2, and snxA+ alleles were tagged with a (GA)3GFDGA-myc9 epitope, and their levels were detected by Western blotting from randomly
cycling, vegetative mycelia grown in liquid media. As predicted, SNXA levels were greatly reduced or absent in both mutants (Figure 7b). Thus, the hypomorphic phenotypes caused by both snxA1 and snxA2 are due to decreased expression of snxA.

**SNXA1 does not affect NIMX2^{CDC2} kinase activity**

To determine if SNXA affects the levels of NIMX^{CDC2} protein or NIMX^{CDC2}/NIME^{CYCLINB} kinase activity, histone H1 kinase assays and western blots were performed using total protein extracts from strains carrying either snxA+/-nimX+, snxA+/-nimX2, snxA1/nimX+, or snxA1/nimX2 grown at 32° or germinated at 32° and then shifted for 3 hours to the nimX2 restrictive temperature of 42° (Figure 8). No differences in NIMX^{CDC2} protein levels were evident in any of the strains at permissive temperature or after shifting to restrictive temperature. However, strains harboring the nimX2 mutation had lower NIMX2^{CDC2}/NIME^{CYCLINB} kinase activity at permissive temperature. Furthermore, NIMX2^{CDC2}/NIME^{CYCLINB} kinase activity decreased in both the snxA+/-nimX2 and snxA1/nimX2 strains after shifting to 42° (Figure 8, lanes 6 and 8). These experiments, which were repeated three times with identical results, strongly suggest that snxA1 suppression of nimX2 does not occur by increasing the activity of NIMX2^{CDC2}/NIME^{CYCLINB}.

**SNXA localizes to the nucleus during interphase**

To localize SNXA^{HRB1} in cells, snxA was C-terminally tagged with green fluorescent protein (GFP) and red fluorescent protein (mRFP) by three-way fusion PCR, followed by one-step gene replacement. A very strong nuclear signal was present in 97% of randomly cycling cells, with diffuse signal in 3% of cells, as demonstrated by co-localization with DAPI-stained
nuclei (Figure 9a). SNXA::mRFP was observed in the nucleoplasm but appeared to be excluded from the nucleolus, as both SNXA::mRFP and DAPI were excluded from this area. In asynchronous cultures, approximately 97% of nuclei were in interphase and 3% were mitotic. The high proportion of cells with nuclear SNXA::mRFP (97%) suggests that SNXA is not concentrated in the nucleus during semi-closed mitosis, during which protein localization has been hypothesized to be regulated by diffusion and localized binding (De Souza et al. 2004). Thus, the observed pattern suggests that SNXA^{HRB1} nuclear localization is not required at mitosis.

To determine if SNXA^{HRB1} is nuclear during interphase but exits the nucleus at mitosis, strains harboring C-terminally tagged SNXA::GFP or SNXA::mRFP were combined with the following heat sensitive mutations: nimX^{2cdc2}, nimE^{6cyclinB}, or nimT^{23cdc25}, which are slightly leaky G2-specific mutations; or nimO^{18dbf4}, a highly heat sensitive G1/S mutation that prevents DNA replication and undergoes mitotic catastrophe, leading to mitotic arrest at restrictive temperature (James et al. 1999). As shown in Figure 9b, localization of SNXA^{HRB1} was predominantly nuclear in nimX^{2cdc2}, nimE^{6cyclinB}, and nimT^{23cdc25} cells at all temperatures. However, as nimO^{18} cells began to enter mitotic arrest at the semi-permissive temperature of 29° and were fully arrested at 37°, nuclear localization of SNXA^{HRB1} decreased to 0%. The entire cell was diffusely stained during mitotic arrest, suggesting that SNXA^{HRB1} exits the nucleus during mitosis.

To demonstrate that SNXA^{HRB1} exits the nucleus during mitosis, a strain with SNXA::mRFP and GFP::α-tubulin was studied. Approximately 97% of randomly cycling cells had nuclear SNXA::mRFP and an interphase array of microtubules (Figure 10). Conversely, in every hypha with mitotic spindles (n>200), SNXA::mRFP no longer concentrated in the nucleus,
but was detected as a diffuse signal throughout the cells. This demonstrates that $\text{SNXA}^{\text{HRB1}}$ exits the nucleus during mitosis, before formation of the mitotic spindle.

**snxA1 and $\Delta snxA$ affect NIME$^{\text{CYCLINB}}$ localization**

As discussed earlier, proper localization of mitotic kinases is essential for mitotic progression. Because $\text{snxA1}$ does not suppress $\text{nimX2}$ by affecting either its protein or activity levels, we suspected that it may affect the localization of $\text{NIMX}^{\text{CDC2}}/\text{NIME}^{\text{CYCLINB}}$. The levels of $\text{NIME}^{\text{CYCLINB}}$ could be monitored using a strain harboring GFP-labeled $\text{NIME}^{\text{CYCLINB}}$ (Nayak et al. 2010) (kind gift of Dr. Berl Oakley). We reasoned that because $\text{NIMX}^{\text{CDC2}}$ is active as a protein kinase only when bound to $\text{NIME}^{\text{CYCLINB}}$ (Osmani et al. 1994), and because its nuclear localization mirrors that of $\text{NIME}^{\text{CYCLINB}}$ (Nayak et al. 2010), if $\text{snxA1}$ affects the localization of $\text{NIME}^{\text{CYCLINB}}$ this could also affect $\text{NIMX}^{\text{CDC2}}/\text{NIME}^{\text{CYCLINB}}$ localization. Wu et al. (Wu et al. 1998) and Nayak et al. (2010) previously characterized $\text{NIME}^{\text{CYCLINB}}$ as having predominantly nuclear localization during interphase (approximately 50%-60% of hyphae had nuclear localization at interphase) and localization during mitosis that was characterized by diffuse staining of the entire hypha. To determine if either $\text{snxA1}$ or $\Delta snxA$ affect the localization of $\text{NIME}^{\text{CYCLINB}}$, we generated strains harboring the GFP-tagged $\text{NIME}^{\text{CYCLINB}}$ together with $\text{snxA}^+$, $\text{snxA1}$, $\Delta\text{snxA}$, $\text{nimX2}$, or $\text{snxA1/nimX2}$ by crossing strain LO1578 (kind gift of Dr. Berl Oakley) into various backgrounds. LO1578 harbors GFP-$\text{NIME}^{\text{CYCLINB}}$ under control of the endogenous nimE promoter; the fusion protein was reported to be fully functional and to cause no apparent mitotic or cell cycle defects (Nayak et al. 2010). We determined the levels of GFP-$\text{NIME}^{\text{CYCLINB}}$ in randomly cycling vegetative mycelia from $\text{snxA}^+$, $\text{snxA1}$, and $\Delta\text{snxA}$.
strains grown at 33° and found that mutation or deletion of snxA does not alter the expression of GFP-NIME\textsuperscript{CYCLINB} (Figure S11). The resulting strains were germinated and the percentage of germlings (containing 2-8 nuclei) with predominantly nuclear GFP-NIME\textsuperscript{CYCLINB} were quantitated at the snxA1 restrictive temperature (20°), nimX2 restrictive temperature (44°), nimX2 semi-permissive temperature (37°), or permissive temperature (32°).

As shown in Figure 11, wild-type germlings (snxA+/nimX+) exhibited ~50%-60% nuclear GFP-NIME\textsuperscript{CYCLINB} at all temperatures tested, in agreement with published data (Wu et al. 1998). Because snxA1 was originally identified as a suppressor of nimX2, we also studied GFP-NIME\textsuperscript{CYCLINB} localization in nimX2 mutants. nimX2/snxA+ germlings exhibited nuclear GFP-NIME\textsuperscript{CYCLINB} similar to that of wild-type germlings at 20°, but the levels increased at permissive and semi-permissive temperatures and approached 85% during the slightly leaky G2-arrest (44°). This increase in nuclear GFP-NIME\textsuperscript{CYCLINB} would be expected as the germlings arrest in G2. As in wild type cells, GFP-NIME\textsuperscript{CYCLINB} localization of nimX+/snxA1 and nimX+/ΔsnxA germlings was consistent over a range of temperatures, but was slightly lower at all temperatures compared to wild-type germlings, ranging from 5% to 20% lower than in wild-type cells. Whereas nimX2/snxA1 germlings generally mirrored wild-type at 20°, 32°, and 37°, 50% fewer cells showed nuclear localization at 44°, relative to wild-type.

Given that NIME\textsuperscript{CYCLINB} exits the nucleus at mitosis, one possible reason for decreased nuclear localization of GFP-NIME\textsuperscript{CYCLINB} in snxA1/ΔsnxA cells could be an increase in mitotic index. To determine if the localization differences were due to an increased percentage of cells in mitosis, which would lead to a drop in the percent of cells with nuclear GFP-NIME\textsuperscript{CYCLINB}, due to its destruction at the metaphase to anaphase transition, the chromosome mitotic index was
assessed in these same strains under the same conditions. Due to fluorescence interference, mitotic index could not be assessed in the same slides as nuclear NIME\textsuperscript{CYCLINB}, thus NIME\textsuperscript{CYCLINB} localization and chromosome mitotic index were measured in separate experiments. The percentage of mitotic cells in all strains at permissive temperatures was between 2\%-4\%, except at 44\(^\circ\), where \textit{nimX2/snxA}+ germlings were 0.5\% mitotic and \textit{nimX2/snxA1} germlings were 6\% mitotic. Although we have conservatively reported a small increase in mitotic index (to 6\%) in the \textit{snxA1/nimX2} double mutant at 44\(^\circ\), repeated observations in these cells (data not shown) gave variable mitotic indices as high as 10-15\%. It is not possible to determine the percent of mitotic cells below 25\(^\circ\) in strains containing deleted or mutated \textit{snxA} due to the accumulation of nuclear abnormalities. These data therefore demonstrate a temperature-dependent perturbation of nuclear NIME\textsuperscript{CYCLINB} localization in \textit{snxA}-deficient strains.

**DISCUSSION**

Multiple lines of evidence reveal that in \textit{Aspergillus nidulans}, SNXA\textsuperscript{HRB1} plays an important role in cell cycle progression, functioning both at G1 and G2. At G2, \textit{snxA1} and \textit{snxA2} affect the \textit{A. nidulans} CDK1, NIMX\textsuperscript{CDC2}. In addition to isolation of the cold-sensitive, G1-arresting \textit{snxA1} mutation as an extragenic suppressor of \textit{nimX2} (McGuire \textit{et al.} 2000), which arrests cells in G2 at elevated temperatures, we demonstrate here that \textit{snxA1} suppression is not allele-specific and that \textit{snxA1} also suppresses mutations in two heat-sensitive, G2-specific regulators of NIMX\textsuperscript{CDC2}: \textit{NIME6\textsuperscript{CYCLINB}} and \textit{NIMT23\textsuperscript{CDC25}}. In contrast, \textit{snxA1} exhibits little or no suppression of the G2-arresting \textit{nimA1} and \textit{nimA5} mutations, the S arresting \textit{nimE10\textsuperscript{cyclinB}}
mutation or of three G1 and S-phase specific mutations. Functional NIMA is required for the partial disassembly of nuclear pore complexes, which allows mitotic regulators to enter the nucleus (De Souza et al., 2004; De Souza et al., 2003); together with the specific suppression of CDK1 pathway mutations, the lack of suppression of nimA mutations by mutation or deletion of snxA suggests that SNXA specifically affects the CDK1 pathway. Deletion of snxA phenocopies the snxA1 and snxA2 mutations in its cold-sensitivity and suppression of nimX2, suggesting that snxA1 and snxA2 are hypomorphic alleles and further supporting the finding that snxA interacts genetically with nimX. That snxA1 and snxA2 are hypomorphic alleles was verified by qRT-PCR and immunoblotting.

Our previous results using double reciprocal hydroxyurea block/release assays showed that at reduced temperatures, snxA1 leads to a non-reversible cell cycle arrest in G1 that causes gross nuclear abnormalities after prolonged incubation at 19°C (McGUIRE et al. 2000). We repeated these experiments with snxA1 strains used herein, with identical results (data not shown). Given that NIMX<sup>cdc2</sup> functions both at G1 and G2 (OSMANI et al. 1994), it is not surprising that a suppressor of nimX2 has effects at G1 and G2.

The nature of the snxA1 and snxA2 defects is at present mysterious. Given that both alleles failed to complement ΔsnxA, we conclude that they are allelic with the deletion. Moreover, a linear wild-type fragment spanning the 3’ half of intron 1 through exon 11 could efficiently complement snxA1 and snxA2 cold-sensitivity. Both alleles were isolated after mutagenesis with the UV-mimetic, 4-nitroquinoline-1-oxide (4-NQO), leading to the expectation that they would harbor point mutations or other DNA sequence alterations. Initially, we found that (1) overexpression from a heterologous locus of an alcA::snxA1 nine-exon genomic clone
complemented *snxA1* as efficiently as *alcA::snxA*+1, and (2) both 9- and 11-exon *alcA*-driven cDNAs, lacking their own promoters, could not rescue *snxA1* in an *alcA*-independent manner, *i.e.*, by crossing over to loop the plasmids into the *snxA* locus. These somewhat incongruous findings could be explained if the mutations were located in the region spanning the 3’ half of intron 1 to a region slightly downstream of intron 2. However, despite extensive sequencing within and beyond the entire *snxA* locus, and exhaustive sequencing of the region containing the promoter, 5’UTR, exons 1–3 and introns 1–2, no DNA sequence changes were found in *snxA1* or *snxA2*.

How, then, to explain the dramatically reduced expression of apparently wild-type SNXA protein in the two mutants, which in turn suppresses defects in the CDK1 mitotic induction pathway? A DNA sequence mutation in a distant *snxA*/AN3739 regulatory element lying outside the sequenced region could lead to reduced expression and failure to complement among *snxA* alleles. However, a distant mutation should not be rescued by linear wild-type DNA fragments corresponding to the *snxA* locus, unless perhaps this mutated element (or gene) alters chromatin structure/function at the *snxA* locus. Nonetheless, to address this possibility a series of 17 overlapping linear fragments (~4 kb each) covering 38,180 nt, extending from AN3735 (nt 3,231,933) to AN4748 (nt 3,193,753) were transformed into *snxA1* and *snxA2* strains. Only AN3739-overlapping fragments complemented the mutations (Figure 6 and data not shown). Although it seems unlikely, we cannot rule out the possibility of a more distant *snxA* regulatory mutation.

More likely is the possibility that *snxA1* and *snxA2* defects may result from altered chromatin structure and function in the *snxA* region, such as by an increase in repressive histone
marks or a decrease in activating marks. *A. nidulans* lacks DNA methylation (Lee et al. 2008) and thus a ChIP approach, using antibodies against a variety of modified histones, may be required to reveal the biochemical nature of *snxA* defects. Of interest in this regard is the recent finding of a functional relationship between the Set1/COMPASS complex (Set1c), an H3K4 methyltransferase, and both the CDK1 and NIMA mitotic kinases, in which Set1c and the two kinases are required for mitotic induction and progression (Govindaraghavan et al. 2014). In general, H3K4 di- and tri-methylation by Set1c has been associated with gene activation in euchromatin, and in humans has been suggested to “bookmark” active genes so that they can resume transcription following exit from mitosis (Blobel et al. 2009; Kelly et al. 2010). In *A. nidulans*, Set1c function has also been shown to mediate repression of certain secondary metabolite gene clusters that occur in subtelomeric regions (reviewed in (Gacek and Strauss 2012)). Given its newly discovered and poorly understood role in promoting mitosis, and the apparent role of *snxA* in restraining nuclear division, it will be of interest to determine what kind of functional relationship may exist between Set1c and *snxA*.

A recent study of Rumplestiltskin (*Rump*), the *Drosophila melanogaster* homolog of *snxA* and hnRNP-M, provides evidence for direct involvement in an epigenetic mechanism affecting higher order chromatin structure through modulation of insulator function (King et al. 2014). *Rump* associates physically with *Drosophila* core insulator proteins, colocalizing to a subset of insulator binding sites. Depletion of *Rump* improved the enhancer-blocking and barrier function of a *gypsy* transposable element, suggesting that *Rump* acts normally to inhibit insulator activity, possibly by competing with insulator-insulator interactions. In addition to this newly discovered role in chromatin dynamics, *Rump* also autoregulates its own expression (King et al. 2014). These findings suggest a possible mechanism for *snxA* in regulating the expression of G2-M
regulators in *A. nidulans*. We show herein that levels of \(\text{NIMX}^{\text{CDC2}}\) and \(\text{NIME}^{\text{CYCLINB}}\) are not affected in \(\text{snxA}\) mutants. However, depletion of SNXA might, for example, improve insulator function to enhance the expression of a positive regulator such as \(\text{NIMT}^{\text{CDC2}}\) or to increase the repression of a negative regulator of G2-M, such as SUC1 or ANKA\(^{\text{WE1}}\).

The role of SNXA\(^{\text{HRB1}}\) in cell cycle progression is further demonstrated by the observed alterations in \(\text{NIME}^{\text{CYCLINB}}\) distributions in populations of \(\text{snxAl}\) and \(\Delta\text{snxA}\) germlings. The depletion of SNXA\(^{\text{HRB1}}\) in these cells diminishes the percentage of cells with nuclear \(\text{NIME}^{\text{CYCLINB}}\). The lowest levels of nuclear \(\text{NIME}^{\text{CYCLINB}}\) occurred in \(\text{nimX2}/\text{snxAl}\) germlings at 37\(^\circ\) and 44\(^\circ\), the temperatures at which \(\text{snxAl}\) suppresses the \(\text{nimX2}\) G2 arrest to permit passage through G2/M. The decreased nuclear \(\text{NIME}^{\text{CYCLINB}}\) localization in the \(\text{nimX2}/\text{snxAl}\) double mutant likely reflects completion of M phase. The consistent and reproducibly lower nuclear \(\text{NIME}^{\text{CYCLINB}}\) localization at 37\(^\circ\) and 44\(^\circ\) that is less than wild-type levels suggests that these cells progress through mitosis, at which time \(\text{NIME}^{\text{CYCLINB}}\) is degraded and thus no longer visible in the nucleus, and that they may be delayed in G1, giving a further reduction in nuclear \(\text{NIME}^{\text{CYCLINB}}\). In other words, the observed decreased nuclear \(\text{NIME}^{\text{CYCLINB}}\) at 37\(^\circ\) and 44\(^\circ\) in \(\text{snxAl}/\text{nimX2}\) mutants might seem counterintuitive, since \(\text{snxAl}\) depletion rescues the G2 arrest of \(\text{nimX2}^{\text{CDC2}}\). This rescue could occur by increased movement of \(\text{NIMX}^{\text{CDC2}}\) and \(\text{NIME}^{\text{CYCLINB}}\) into the nucleus. However, this rescue, by permitting mitotic progression, leads to the destruction of \(\text{NIME}^{\text{CYCLINB}}\) at the metaphase to anaphase transition, and thus can explain why \(\text{NIME}^{\text{CYCLINB}}\) levels are lowest in this \(\text{snxAl}/\text{nimX2}\) strain at 37\(^\circ\) and 44\(^\circ\).

Mitotic regulation via the CDC2/CYCLINB pathway has been shown to ultimately depend on control of CDC2/CYCLINB activity, which is tightly regulated by
phosphorylation/dephosphorylation (Ye et al. 1995), and on proper nuclear localization of the fully active CDC2/CYCLINB complex (Wu et al. 1998). The finding that snxA1 does not increase NIMX2^CDC2 protein levels or NIMX2^CDC2/NIME^CYCLINB activity at the nimX2 restrictive temperature, where snxA1 does suppress the nimX2 G2 arrest, suggests that the effects of SNXA1 on the NIMX2^CDC2/NIME^CYCLINB mitotic initiation pathway occur by a different mechanism, such as proper localization of the active complex. Given that NIMX^CDC2 is active as a protein kinase only when bound to its regulatory subunit, NIME^CYCLINB, our findings that NIME^CYCLINB has altered localization patterns in both snxA1 and ΔsnxA hyphae support this hypothesis. This is particularly interesting, given that snxA is homologous with S. cerevisiae Hrb1/Gbp2, which encode mRNA shuttling binding proteins similar to the mammalian SR-family of proteins. While these proteins have been shown in budding yeast to shuttle mRNAs to the cytoplasm and to remain with the transcript during translation (Windgassen et al. 2004), heretofore no specific cell cycle functions have been suggested for Hrb1/Gbp2, although overexpression of Gbp2 does lead to a delay in G1 (Stevenson et al. 2001). Because deletion of these genes in S. cerevisiae does not disrupt or alter the nucleocytoplasmic distribution of bulk polyadenylated mRNAs (Hacker and Krebber 2004), it is possible that they function either as adaptors for specific subsets of mRNPs, as suggested by Rougemaille (Rougemaille et al. 2008), or, they may confer additional, previously unidentified functions.

With its partially-closed mitosis, A. nidulans represents an evolutionary intermediate between the closed mitosis of budding yeast and the open mitosis of more complex eukaryotes (for review, see (De Souza and Osmani 2009)). It is therefore not surprising that some evolutionarily conserved proteins involved in cell cycle regulation have additional and/or different functions in different organisms. Given that the G2/M effects of SNXA^{HRB1} do not
occur by modulating $\text{NIMX}^{\text{CDC2}}/\text{NIME}^{\text{CYCLINB}}$ activity, but that $\text{snxA}$ mutation or deletion alters $\text{NIME}^{\text{CYCLINB}}$ localization patterns, it is possible that in $A.\text{nidulans}$, SNXA$^{\text{HRB1}}$ regulates the localization of either $\text{NIMX}^{\text{CDC2}}/\text{NIME}^{\text{CYCLINB}}$ or of mitosis-specific proteins that function upstream or downstream of the activation of $\text{NIMX}^{\text{CDC2}}/\text{NIME}^{\text{CYCLINB}}$.

Using time-lapse imaging of cyclin B-GFP, Nayak et al (2010) demonstrated that cyclin B becomes visible in the nucleoplasm and at spindle pole bodies (SPBs) ~40% of the way through interphase, during S-phase, and confirmed observations by De Souza et al. (2009) that the majority of this cyclin B exits the nucleus when the NPC partially disassembles, leaving a sub-pool of cyclin B concentrated at SPBs and on the spindle. This sub-pool disappears gradually as mitosis progresses. Although it is beyond our abilities to detect, it is possible that mutation of $\text{snxA}^{\text{Hrb1}}$ allows this sub-pool to remain in the nucleus longer; if so, this could allow threshold levels of $\text{NIMX2}^{\text{CDK1}}$ protein to accumulate in $\text{nimX2}$ mutants, resulting in suppression of the heat-sensitive phenotype. Alternatively, it is possible that nuclear transport is altered in $\text{snxA}$ mutants, leading to the accumulation or retention of threshold levels of CDK1/Cyclin B. An additional possibility is that SNXA$^{\text{HRB1}}$ affects proteins involved in mitotic exit; a defect in inactivation of the Anaphase Promoting Complex/Cyclosome (APC/C), as Nayak et al. (2010) reported in $\text{mipAD159}$ mutants ($\gamma$-tubulin), could both allow threshold levels of $\text{NIMX2}^{\text{CDC2}}$ activity to be reached and delay G1, resulting in lower nuclear $\text{NIME}^{\text{CYCLINB}}$. We are currently investigating these alternatives.

The dramatic decrease in SNXA$^{\text{HRB1}}$ nuclear localization during the highly heat-sensitive pseudo-mitotic arrest of the $\text{nimO18}$ mutation underscores the nature of the partially-closed mitosis of $\text{Aspergillus nidulans}$. At mitosis, the nuclear pore complexes partially disassemble,
and proteins not specifically retained in the nucleus diffuse out of the partially-closed nuclear pores and may equilibrate across the nuclear envelope (De Souza et al. 2004); those proteins destined to return to the nucleus are likely specifically re-imported once the nuclear pores completely re-assemble. SNXAHRB1 appears to be one of these proteins.

The identification of snxA as the A. nidulans homolog of Hrb1/Gbp2, the G1 arrest phenotype of snxA1 mutants, the exit of SNXAHRB1 from the nucleus during mitosis and its subsequent re-import after mitotic exit, the NIMX^{CDC2}/NIME^{CYCLINB} activity-independent effects of snxA on the NIMX^{CDC2}/NIME^{CYCLINB} G2/M pathway, and the altered localization of NIME^{CYCLINB} suggest a novel function for the SNXAHRB1 SR family protein in eukaryotes: a function in cell cycle regulation. Moreover, the fact that reducing or eliminating snxA function alleviates heat-sensitive defects in nimX^{cd2} mutants and in mutant regulators of nimX^{cd2} activity (nimE6^{cyclinB} and nimT23^{cd25}) suggests that snxA may normally act to restrain mitotic induction by the CDC2/CYCLINB regulatory pathway.

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Figure 1. *snxA1* suppresses multiple alleles of *nimX^cdc2*. Growth phenotypes of single and double mutants on minimal medium at the indicated temperatures. Days of growth at each temperature as follows: 19°, 6 days; 29/33/37/43°, 2.5 days.
Figure 2. *snxA1* suppresses mutations in multiple components of the CDC2/CYCLINB cell cycle regulatory pathway but not mutations in *nimA*. Growth phenotypes of single and double mutants on minimal medium at the indicated temperatures. Days of growth at each temperature as follows: 20°, 5 days; 29°/33°/37°, 2.5 days; 43°, 3.5 days. (A) Growth phenotypes of single and double mutants in the CDC2/CYCLINB pathway; (B) growth phenotypes of single and double mutants in *nimA*. 
Figure 3. *snxA1* is not a general suppressor of cell cycle defects. Growth phenotypes of single and double mutants on rich medium at the indicated temperatures. Three G1 or S phase cell cycle mutations (*nimO18, nimP22, and nimQ20*) were tested for suppression by *snxA1*, and compared with the G2-M cell cycle mutation, *nimX2*. Days of growth at each temperature as follows: 20°, 7 days; 29°/33°/37°/43°, 2.5 days.
Figure 4. $\Delta snxA$ suppresses $nimX^{cd2}$ mutations but does not suppress the $nimA5$ G2-M defect. (A) Growth phenotypes of $nimX$ single and $nimX$ $\Delta snxA$ double mutants on rich medium at the indicated temperatures. Days of growth at each temperature as follows: 19º, 6 days; 29º/33º/37º/43º, 3 days. (B) Growth phenotypes of three independent $nimA5$ $\Delta snxA$ double mutants at the indicated temperatures. Days of growth: 22º, 5 days; 29º, 2.5 days; 33º, 37º, and 43º, 2 days.
Figure 5. The *snxA2* defect is more severe than *snxA1*. *snxA1* strains harboring white (*wA2*) and chartreuse (*chaA1*) spore color mutations (top row), and green *snxA2* strains (bottom row) were streaked onto minimal medium and grown for three days at 37°C. The brownish coloration of the *snxA2* strains reflects the absence of green conidia, demonstrating weaker growth at 37°C.
Figure 6. Complementation of *snxA1* and *snxA2* by linear DNA fragments. *snxA1* strains tSWJ 5573/5574 and *snxA2* strains tSWJ 5567/5584, containing a deletion of *nkuA* (Ku70) were transformed with a PCR-derived linear DNA fragment of 3897 nt (A), and two plasmid-derived linear DNA fragments of 3530 nt (B), and 6686 nt (C). Transformants were grown at 21°C for 11 days to select for complementation of cold-sensitivity. (A) Complementation results. Single arrows denote genes in the *snxA* region. Red lines indicate linear fragments used for transformation. Green line indicates the region deleted in Δ*snxA* strains. Numbers in red indicate the number of *snxA2*-complementing transformants per µg of DNA. Blue double-arrow indicates the region sequenced in *snxA1* and *snxA2* mutants. (B) *snxA2* transformants complemented by the shortest, 3530 nt linear DNA fragment.
Figure 7. *snxA* mRNA and protein levels are reduced in *snxA* mutants. (A) To determine relative *snxA* mRNA expression, qRT-PCR analysis of randomly cycling vegetative mycelia was performed on *snxA*+, *snxA1*, and *snxA2* strains. Relative expression was normalized to *actA* and calculated using the ΔΔCt method. Data shown are the average relative normalized expression from six replicates. Bars = SEM. (B) To determine relative SNXA protein levels, colorimetric western analysis of total protein extracts from randomly cycling vegetative mycelia of myc9-tagged *snxA*+, *snxA1*, and *snxA2* strains was performed. Blots were probed with mAb mycA7, washed, and re-probed with anti-α-tubulin mAb as a loading control.
Figure 8. SNXA does not affect NIMX\textsuperscript{CDC2} histone H1 kinase activity. To determine if \textit{snxA1} suppresses \textit{nimX2} by affecting NIMX protein levels or activity, \textit{snxA+/nimX+} (lanes 1,5), \textit{nimX2} (lanes 2, 6), \textit{snxA1} (lanes 3,7) and \textit{snxA1/nimX2} (lanes 4,8) were grown at the \textit{nimX2} permissive temperature (32\degree C) or germinated at 32\degree C and shifted to restrictive temperature (42\degree C) for 2 hr. Histone H1 kinase assays were performed on anti-NIMX immunoprecipitates followed by SDS-PAGE. 100 mg total protein was separated by SDS-PAGE, blotted to nitrocellulose, and detected with anti-NIMX antibody E77.
Figure 9. **SNXA localizes to the nucleus in 97% of randomly cycling cells.** Three-way fusion PCR was used to C-terminally tag SNXA by one-step gene replacement. Following transformation of *A. nidulans* with PCR products, gene replacement with *snxA::mRFP::pyrG* or *snxA::GFP::pyrG* was confirmed by trans-locus PCR and southern blot. Fluorescence microscopy was performed to visualize SNXA::mRFP and SNXA::GFP. Strains harboring various cell cycle mutations were crossed with transformants to obtain strains with either SNXA::mRFP or SNXA::GFP and specific cell cycle mutations (*nimO18, nimE6, or nimX2*).

(A) Fluorescence micrographs showing nuclear (top panels) and non-nuclear (bottom panels) localization of SNXA::mRFP at 32°C. Germlings were stained with DAPI after fixation to co-localize SNXA::mRFP to nuclei. Arrows indicate nucleoli. (B) SNXA::mRFP or SNXA::GFP strains with indicated cell cycle mutations were inoculated into liquid minimal media on coverslips, incubated at the indicated temperatures for 16 hours, and visualized by fluorescence microscopy. 200 germlings per strain were counted and scored for nuclear vs. non-nuclear SNXA::mRFP or SNXA::GFP localization.
**Figure 10. SNXA::mRFP is absent from the nucleus during mitosis.** A strain carrying SNXA::mRFP was crossed with a strain harboring *alcA::GFP::α*-tubulin. A double-tagged strain was inoculated into minimal media onto coverslips and incubated 14 hours at 29°C, followed by fixation, DAPI staining, and visualization by fluorescence microscopy. 100% of hyphae with mitotic spindles (n>200) were devoid of nuclear SNXA::mRFP and exhibited a diffusely stained cytoplasm.
Figure 11. *snxA1* and Δ*snxA* strains have decreased nuclear NIME<sub>CYCLINB</sub>. Strains harboring the GFP-tagged NIME<sub>CYCLINB</sub> together with *snxA<sup>+</sup>*, *snxA1*, Δ*snxA*, *nimX2*, or *snxA1/nimX2* were grown to germling stage (2–8 nuclei) at the indicated temperatures and the number of germlings with predominantly nuclear GFP-NIME<sub>CYCLINB</sub> was quantitated. The means of 4–6 replicates per strain are shown, with 100 germlings scored per replicate. Bars = SEM.