Inn1 and Cyk3 regulate chitin synthase during cytokinesis in budding yeasts

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Summary

The chitin synthase that makes the primary septum during cell division in budding yeasts is an important therapeutic target with an unknown activation mechanism. We previously found that the C2-domain of the *Saccharomyces cerevisiae* Inn1 protein plays an essential but uncharacterised role at the cleavage site during cytokinesis. By combining a novel degron allele of *INN1* with a point mutation in the C2-domain, we screened for mutations in other genes that suppress the resulting defect in cell division. In this way, we identified 22 dominant mutations of *CHS2* (chitin synthase II) that map to two neighbouring sites in the catalytic domain. Chs2 in isolated cell membranes is normally nearly inactive (unless protease treatment is used to bypass inhibition); however, the dominant suppressor allele Chs2-V377I has enhanced activity *in vitro*. We show that Inn1 associates with Chs2 in yeast cell extracts. It also interacts in a yeast two-hybrid assay with the N-terminal 65% of Chs2, which contains the catalytic domain. In addition to compensating for mutations in the Inn1 C2-domain, the dominant *CHS2* alleles suppress cytokinesis defects produced by the lack of the Cyk3 protein. Our data support a model in which the C2-domain of Inn1 acts in conjunction with Cyk3 to regulate the catalytic domain of Chs2 during cytokinesis. These findings suggest novel approaches for developing future drugs against important fungal pathogens.

Key words: Inn1, Chs2, Cyk3, Cytokinesis, Budding yeasts

Introduction

Fungal pathogens such as the budding yeasts *Candida albicans*, *Cryptococcus neoformans*, *Cryptococcus gattii* and *Ajellomyces dermatitidis* share much of their cell biology with animal cells (Kim and Sudbery, 2011; Kronstad et al., 2011), so that effective therapies are often associated with considerable toxicity. Current approaches aim to target biological processes that are only required for the viability or proliferation of fungi, but the emergence of resistant strains and the lack of effective oral formulations mean that there is a continued need for novel therapies against fungal-specific proteins (Sable et al., 2008).

The biology of cell division provides a potential target. Although both yeasts and animal cells employ a contractile ring of actin and type II myosin to mediate division of the cytoplasm (Oliferenko et al., 2009; Pollard, 2010), in yeast cells the contractile ring serves as a guide for septum formation, which is tightly coupled to ingression of the plasma membrane at the cleavage site (Roncero and Sánchez, 2010). Formation of the septum is an attractive target for therapeutic intervention as mammalian cells lack the enzymes that synthesise cell wall carbohydrates such as chitin.

The budding yeast *Saccharomyces cerevisiae* provides an ideal model system with which to study mechanisms of cell division that are likely to be shared with less well characterised pathogenic species of budding yeasts. The chitin synthase Chs2 is synthesised during mitosis and stored in the endoplasmic reticulum, before being transported in secretory vesicles during late anaphase to the bud-neck (VerPlank and Li, 2005; Zhang et al., 2006; Teh et al., 2009; Chin et al., 2012), where it is inserted into the plasma membrane and deposits the primary septum in a centripetal fashion just behind the contracting actomyosin ring (Cabib, 2004; Roncero and Sánchez, 2010). Following deposition of the primary septum, Chs3 and other enzymes then synthesise secondary septa on either side, so that subsequent digestion of the primary septum leads to cell separation and completes the process of cell division. Interestingly, Chs2 appears to be delivered to the plasma membrane in an inactive form and is then activated *in situ* by a mechanism that has not previously been elucidated, though it might involve a change in structure as it can be mimicked *in vitro* by protease treatment of Chs2 in isolated membranes (Sburlati and Cabib, 1986; Silverman et al., 1988).

Factors associated with the actomyosin ring play a critical but poorly understood role in regulating septation. Soon after the formation of a new bud in the early stages of the cell cycle, the type II myosin Myo1 forms a ring at the bud-neck that subsequently associates with other factors during mitosis, including actin-nucleating and bundling factors such as formins and the IQGAP protein Iqg1, leading to assembly of the contractile actomyosin ring at the end of anaphase (Tolliday et al., 2001; Balasubramanian et al., 2004; Pollard, 2010). Iqg1 also seems to regulate septum formation independently of its role in actomyosin ring assembly, together with other factors associated with the contracting actomyosin ring such as the

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SH3 proteins Hof1 (the orthologue of fission yeast Cdc15; Hof1=Homologue of fifteen) and Cyk3. For example, although the actomyosin ring normally guides formation of the primary septum, increased expression of the *IQG1* or *CYK3* genes suppresses defective cell division in *myo1Δ* cells (Ko et al., 2007). Moreover, high copy plasmids carrying the *HOF1* or *CYK3* genes promote cell division in *iqg1Δ* cells that lack an actomyosin ring, indicating that Hof1 and Cyk3 act downstream of Iqg1 to promote septation (Korinek et al., 2000). But it remained unclear whether Hof1 and Cyk3 regulate the action of Chs2 directly, and neither factor is essential for cell division in cells that can assemble an actomyosin ring (Kamei et al., 1998; Lippincott and Li, 1998; Korinek et al., 2000).

We previously identified the Inn1 protein (required for Ingression) in a screen for new cell cycle proteins in budding yeast and found that it associates with the actomyosin ring and co-purifies with Hof1 and Iqg1 (Sanchez-Diaz et al., 2008). In cells with Inn1 fused to the heat-inducible degron cassette, rapid depletion of Td-inn1 (td=temperature sensitive degron) still allows assembly of an actomyosin ring during mitosis, but cytokinesis is blocked. Contraction of the actomyosin ring is initiated under such conditions, but membrane ingression fails and the primary septum is not formed (Sanchez-Diaz et al., 2008; Nishihama et al., 2009). The contracting actomyosin ring appears to be unstable in the absence of Inn1 and often collapses, similar to the phenotype of cells that lack Chs2 (VerPlank and Li, 2005). Overall, these features suggested that Inn1 acts downstream of Iqg1 and Hof1 to promote septum formation and ingression of the plasma membrane, by a mechanism that remained unclear.

The first 134 amino acids of the Inn1 protein form a 'C2-domain' (originally identified as 'Conserved region 2' of Protein Kinase C), which comprise eight Beta-strands in a sandwich of two sheets, with three loops emerging from one corner of the sandwich (supplementary material Fig. S1). C2 domains are generally found in proteins that act at cell membranes, and positively charged amino acids in the loops often make key contacts with targets that can either be lipids or proteins (Cho and Stahelin, 2005; Cho and Stahelin, 2006; Hurley, 2006). We found that the C2-domain is the major effector of Inn1 function during cytokinesis. The remaining 70% of the protein is principally important for timely localisation of Inn1 at the bud-neck, as artificial fusion of the C2-domain to Hof1 allowed *inn1Δ* cells to grow in a similar fashion to control cells, whereas mutation of two lysines in 'loop 1' of the C2-domain of Inn1 blocked cytokinesis (Sanchez-Diaz et al., 2008). A subsequent study showed that the C2-Hof1 fusion also suppressed the lethality of *iqg1Δ* but not *chs2Δ*, suggesting that recruitment of the C2-domain of Inn1 to the bud-neck by factors such as Iqg1 and Hof1 is a critical step in the regulation of septum formation during cytokinesis in *Saccharomyces cerevisiae* (Nishihama et al., 2009). Nevertheless, the molecular targets and mode of action of the C2-domain were unknown, so we established a screen for mutations in other genes that could suppress cytokinesis defects produced by a mutated version of the C2-domain of Inn1. Our data support an integrated model for the regulation of cytokinesis and septum formation, not just in *Saccharomyces cerevisiae* but also in a range of pathogenic fungi.

Results

Positive charge at a unique site in Loop1 of the Inn1 C2-domain is critical for cytokinesis

By comparing the sequences of Inn1 orthologues from a range of fungal species, we found that Loops 1 and 2 of the C2-domains are the most highly conserved regions and contain a number of invariant residues that might represent important contact points with targets (supplementary material Fig. S1). One such residue in Loop 1 of the Inn1 protein from *Saccharomyces cerevisiae* is lysine 31, which is essential for cytokinesis (Fig. 1A and see below), consistent with our previous observation that simultaneous mutation of K28 and K31 blocked cell division (Sanchez-Diaz et al., 2008). Two other positively charged residues in the C2 domain are also invariant amongst Inn1 orthologues (supplementary material Fig. S1B,C), but R39 in Beta strand 2 of *Saccharomyces cerevisiae* Inn1 (ScInn1) was dispensable for cytokinesis and mutation of R52 in Loop 2 only produced a mild defect in cell division (supplementary material Fig. S2). Moreover, each of the less well conserved lysines and arginines in Loops 1–3 were also mutated, showing that these too were dispensable for cytokinesis (supplementary material Fig. S2 and data not shown). It thus seems that K31 makes a critical contribution to Inn1 function during cytokinesis, and positive charge at this precise position seems to be the key requirement, as the *inn1-K31R* allele is functional, and the defect of *inn1-K31A* cannot be suppressed by introducing a lysine at the preceding residue (Fig. 1A, *inn1-D30K* is functional, but *inn1-D30K K31A* is not).

Overall these data indicated that the positive charge of K31 contributes to an interaction of Loop1 with a presumed target of Inn1 that is critically important for cytokinesis. The *inn1-K31A* allele thus represented a good starting point for suppressor screens that aimed to elucidate the role of the C2 domain during cell division in *Saccharomyces cerevisiae*.

Combination of two degrons produces a conditional allele of *INN1* that is ideally suited for suppressor screens

We initially used a colony-sectoring assay to screen visually for suppressors of *inn1-K31A* after mutagenizing cells with ethyl methanesulfonate (see Materials and Methods), but were unable to find any suppressors amongst 38,000 analysed clones. To increase greatly the number of mutated cells that could be tested, it was first necessary to generate a very tight conditional allele of *INN1* with a very low reversion frequency, so that we could then combine this allele with *inn1-K31A* and screen directly for suppressor mutations that restored growth under restrictive conditions. We previously generated a conditional allele of *INN1* by introducing the heat-inducible degron cassette into the *INN1* locus (Dohmen et al., 1994; Labib et al., 2000), so that the Td-inn1 protein (td=temperature sensitive degron) could be depleted rapidly at 37°C following induction of the Ubr1 E3 ligase (Sanchez-Diaz et al., 2008). Degron alleles have not previously been used for suppressor screens, however, and we found that revertants of the *td-inn1* allele arose at a very high frequency under restrictive conditions (Fig. 1C, *td-inn1*; compare growth at 37°C on YPGal medium after 2 days and 3 days), either reflecting incomplete degradation of Td-Inn1 or loss of some component(s) of the degron or N-end rule degradation pathway. We also tested the 'auxin inducible degron' approach in which degradation of the 'aid' cassette (aid=auxin inducible degron) is mediated by the E3 ligase SCF^{Tir1} following addition of auxin to the culture medium of yeast cells expressing the Tir1 F-box protein from rice (Nishimura et al., 2009). The Inn-aid protein was expressed at a low level even under permissive conditions (Fig. 1B) and this facilitated depletion of Inn1-aid under restrictive conditions, but revertants of the degron system still

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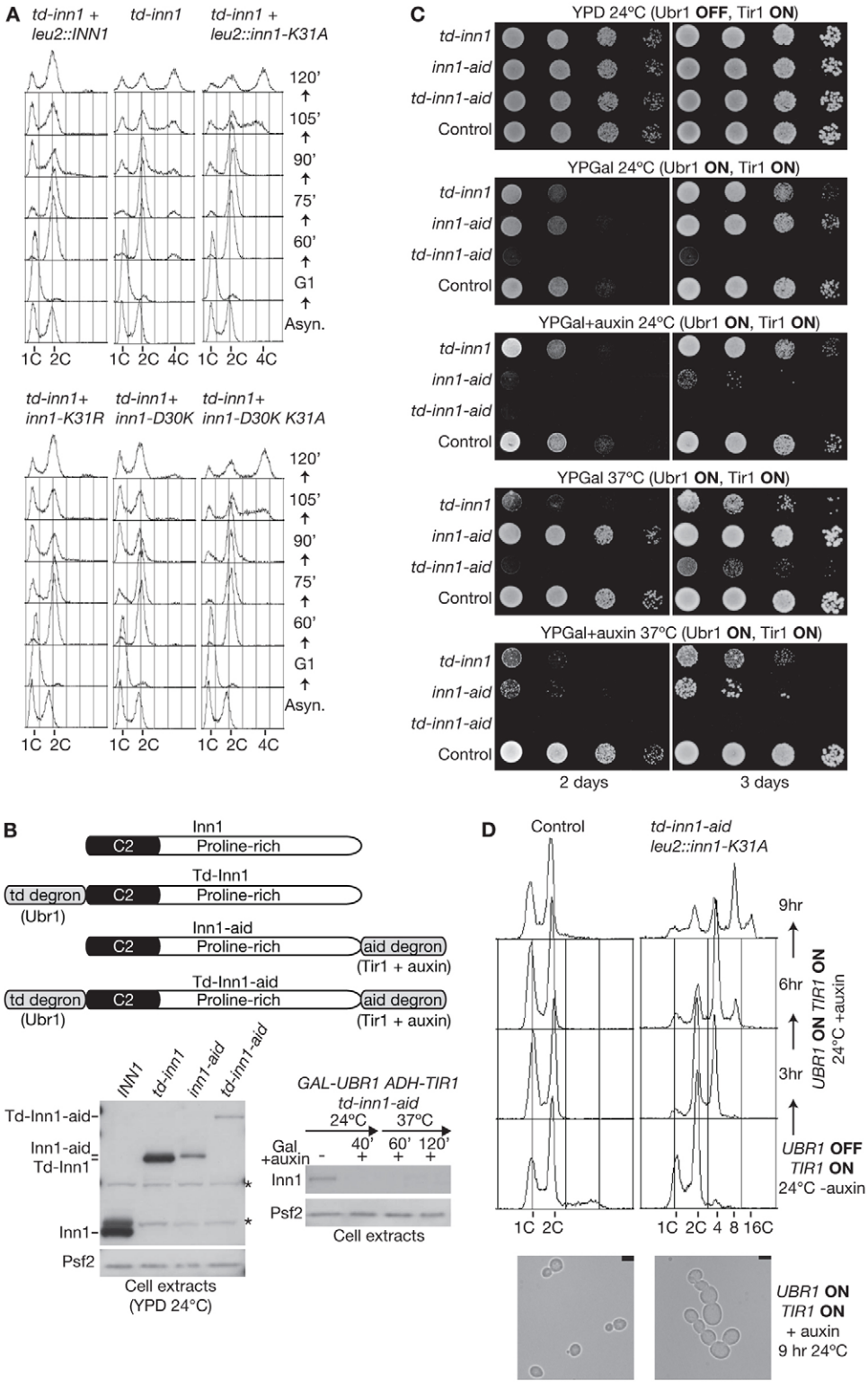

Fig. 1. A novel system with which to screen for suppressors of a lethal defect in the C2-domain of Inn1. (**A**) The indicated strains were grown at 24℃ in YPRaff medium before the cells were arrested in the G1-phase with mating pheromone. After the induction of *GAL-UBR1* for 30 minutes in YPGal medium, the cells were released at 37℃ into fresh YPGal medium lacking mating pheromone. The progression of the cell cycle was monitored at the indicated times by flow cytometry. (**B**) Yeast strains were constructed in which the *INN1* locus was modified by adding the indicated degron cassettes. The level of the tagged proteins at 24℃ in YPD medium was compared to wild-type Inn1 (bottom left panel – the asterisks denote unknown proteins that cross-react with the anti-Inn1 antibody), and the degradation of Td-Inn1-aid was assayed under the indicated conditions (bottom right panel). The Psf2 protein was monitored as the loading control. (**C**) The control cells (*ADH-TIR1 GAL-UBR1*; YAD240), *td-inn1 GAL-UBR1* (YASD522), *inn1-aid ADH-TIR1* (YAD236) and *td-inn1-aid ADH-TIR1 GAL-UBR1* (YAD245) were grown at 24℃ on YPD medium before serial dilutions of 50,000, 5000, 500 and 50 cells were plated on the indicated media and incubated for two or three days. (**D**) Cultures of control cells (W303-1a) and *td-inn1-aid leu2::inn1-K31A* (YAD257) were grown at 24℃ in YPRaff medium before shifting to YPGal + auxin medium for the indicated times. DNA content was monitored throughout the experiment by flow cytometry, and images of cells were captured nine hours after depleting Td-Inn1-aid. Scale bars: 5 μm.

arose at an unacceptably high frequency (Fig. 1C, *inn1-aid*; revertants appeared on YPGal + auxin medium after 2 days growth at 37℃ or 3 days growth at 24℃), probably due to loss of Tir1 or the degron cassette.

To reduce the occurrence of revertants that prevented degradation of Degron-Inn1, a *td-inn1-aid* allele was generated that carried both degron cassettes on the same protein. The *td-inn1-aid* cells grew in a similar fashion to control cells under permissive conditions (see below), despite only having a very low level of Td-inn1-aid protein (Fig. 1B), which could then be depleted further by simultaneous activation of both 'Td' and 'aid' degradation pathways (Fig. 1B). Critically, revertant colonies did

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not arise when cells were plated under restrictive conditions (Fig. 1C, *td-inn1-aid*; compare growth at 24℃ or 37℃ on YPGal + auxin medium after 2 days and 3 days), presumably as cells with revertant mutations in either of the two degron pathways would still be killed by the other. These findings indicated that *td-inn1-aid* was an ideal conditional allele with which to screen for mutations suppressing defects in Inn1 function, and we therefore integrated a plasmid expressing *inn1-K31A* at the *leu2* locus in *td-inn1-aid* (hereafter, '*td-inn1-aid*' refers to '*td-inn1-aid leu2::inn1-K31A GAL-UBR1 ADH-TIR1*' unless otherwise specified), and confirmed that the resultant strain grew in a similar fashion to control cells under permissive conditions (*td-inn1-aid* 24℃, Ubr1 **OFF** Tir1 **ON** - auxin), as in Fig. 1D. Upon depletion of Td-inn1-aid, almost all cells failed to divide in the first cell cycle and then accumulated as multi-budded and multi-nucleate cells with progressively higher DNA contents up to 16C after several generation times (Fig. 1D, *td-inn1-aid leu2::inn1-K31A* 24℃, Ubr1 **ON** Tir1 **ON** + auxin; we used 24℃ as *td-inn1-aid* is sensitive to Ubr1 expression even at this low temperature).

Dominant alleles of *CHS2* suppress the cytokinesis defects of *inn1-K31A*

After mutagenesis of an asynchronous culture of *td-inn1-aid leu2::inn1-K31A GAL-UBR1 ADH1-TIR1* with ethyl methanesulfonate, 2×10^8 cells were grown in the presence of auxin on solid medium that allowed expression of both Ubr1 and Tir1 (see Materials and Methods). After several days growth, 27 clones were isolated that grew in a similar fashion to control cells even under restrictive conditions (Fig. 2A), but still retained all the markers associated with both degron systems. Each strain was then crossed to wild-type cells before sporulation and tetrad analysis of the resultant diploids, revealing that 22 clones had suppressor mutations that segregated independently of *td-inn1-aid*, *leu2::inn1-K31A*, *GAL-UBR1* or *ADH-TIR1* (the remaining five clones were not pursued further as the suppression phenotype co-segregated with *td-inn1-aid* itself). Each of the 22 clones was then crossed to the parental *td-inn1-aid* strain, and analysis of the meiotic progeny showed that two colonies in each tetrad had the suppressor phenotype, indicating that suppression was caused by mutation of a single gene in each case. Finally, a series of pairwise crosses were performed amongst the 22 clones to explore how many different suppressor genes were involved. Strikingly, all four meiotic progeny had the suppressor phenotype in every case, showing that the suppressor genes in all 22 clones were very tightly linked to each other, suggesting the involvement of a single gene. In all clones the suppression was highly efficient, and analysis of liquid cultures showed that *td-inn1-aid* cells with suppressor mutations continued to grow under restrictive conditions without detectable defects in cell division (Fig. 2B), despite efficient depletion of the Td-Inn1-aid protein (Fig. 2C).

When each clone of *td-inn1-aid* with suppressor was crossed to the parental *td-inn1-aid* strain, we found that the resultant diploids all showed the suppressor phenotype (Fig. 3A), demonstrating that the suppressor mutations were all dominant. This suggested that the suppressor mutations might compensate for a defective and functionally important interaction between Inn1-K31A and a target protein, and provided a rationale for

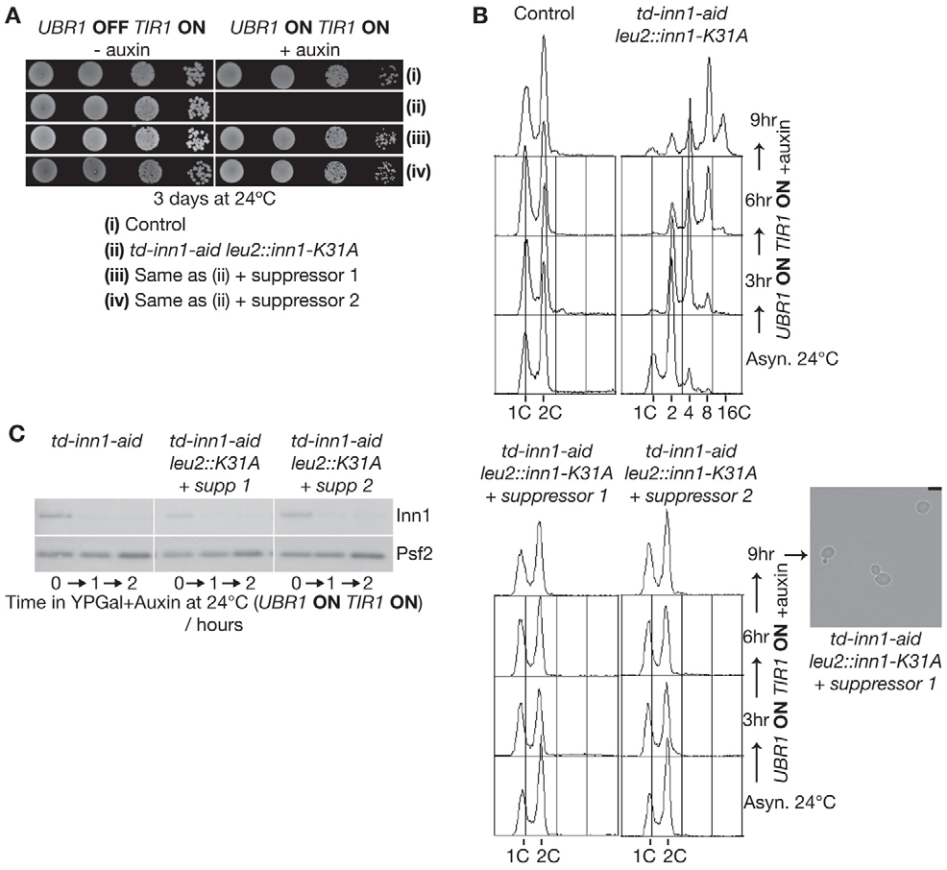

Fig. 2. Highly efficient suppressors of the cytokinesis defects associated with the *inn1-K31A* allele. (**A**) Serial dilutions of the indicated strains were plated on YPD medium (*UBR1* **OFF** *TIR1* **ON**) or YPGal + auxin medium (*UBR1* **ON** *TIR1* **ON**) and incubated for three days at 24℃. (**B**) The indicated strains were grown as described in Fig. 1D to deplete Td-Inn1-aid, and the DNA content was monitored by flow cytometry at the indicated times. Phase contrast images of *td-inn1-aid* + *suppressor 1* (YAD276) were taken nine hours after the depletion of Td-Inn1-aid. Scale bar: 5 μm. (**C**) Depletion of the Td-Inn1-aid was monitored by immunoblotting and compared to the Psf2 protein (i.e. the loading control).

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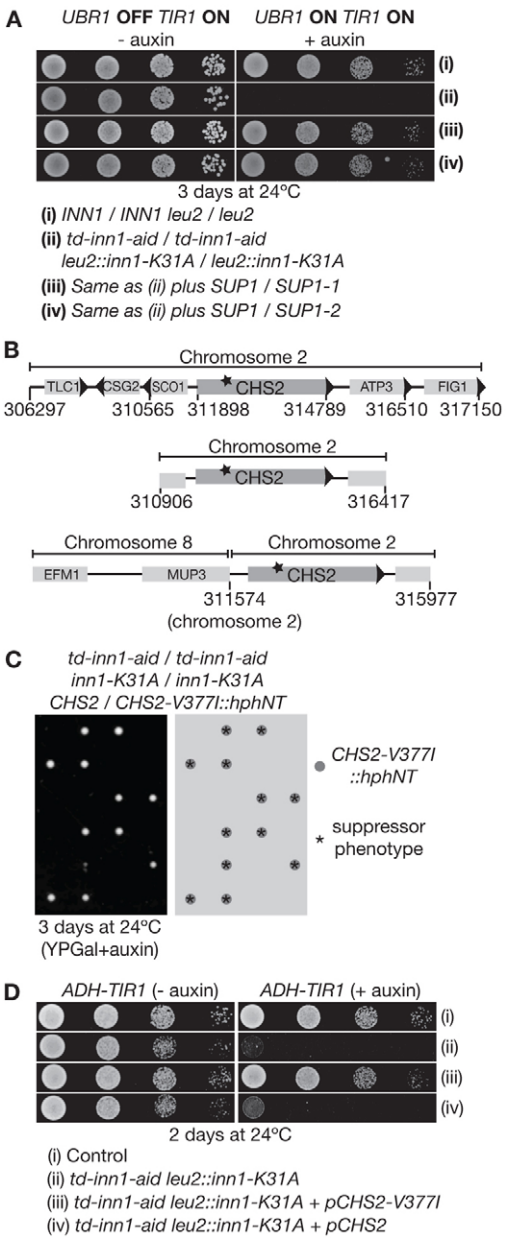


Fig. 3. Dominant alleles of *CHS2* suppress the cytokinesis defects produced by the *inn1-K31A* mutation. (**A**) The indicated diploid strains were treated as described in Fig. 2A. (**B**) After transforming *td-inn1-aid* with a genomic library of plasmids made from a *td-inn1-aid* strain carrying a dominant suppressor mutation, three classes of rescuing plasmids were isolated with the indicated inserts. The numbers correspond to coordinates on chromosome 2. (**C**) Tetrad analysis of the meiotic progeny from the indicated diploid strain in which the *hphNT* marker had been integrated next to the *CHS2-V377I* allele. Spores were grown on YPGal + auxin medium and incubated for 3 days at 24℃, leading to two viable colonies in each tetrad (containing the suppressor of *inn1-K31A*). Replica plating on a medium containing Hygromycin B was then used to show that all viable colonies had the hphNT marker gene. This demonstrated that the suppressor phenotype was tightly linked to *CHS2-V377I*. (**D**) The lethality of *inn1-K31A td-inn1-aid ADH-TIR1* (YAD331) is suppressed by a centromeric plasmid expressing *CHS2-V377I*, but not by an equivalent plasmid expressing wild-type *CHS2*. Cells were treated as in Fig. 2A.

identifying the suppressor gene. We generated a genomic library of DNA fragments from one of the suppressor clones (see Materials and Methods), and transformed the library into a *td-inn1-aid leu2::inn1-K31A ADH-TIR1* strain, which was subsequently grown in the presence of auxin to deplete Td-Inn1-aid. Five transformed colonies were identified that grew well under such conditions, in a manner that was dependent upon the continued presence of the library plasmid. After rescuing the plasmids to *Escherichia coli*, sequence analysis showed that each plasmid contained DNA from the same region of chromosome 2 (Fig. 3B; one plasmid also contained a piece of DNA from chromosome 8). A single intact gene was shared by all of the plasmids, namely the *CHS2* gene that encodes the chitin synthase responsible for synthesising the primary septum during cytokinesis (Silverman et al., 1988; Shaw et al., 1991). To test whether *CHS2* was indeed the suppressor gene, we integrated a marker gene next to the *CHS2* locus in a *td-inn1-aid* strain containing the suppressor mutation, and then crossed this strain to the parental *td-inn1-aid* strain. Tetrad analysis of the meiotic progeny indicated that the marker gene next to *CHS2* always segregated with the suppressor phenotype (Fig. 3C), showing that *CHS2* was very closely linked to the suppressor locus and indicating that they represent one and the same gene.

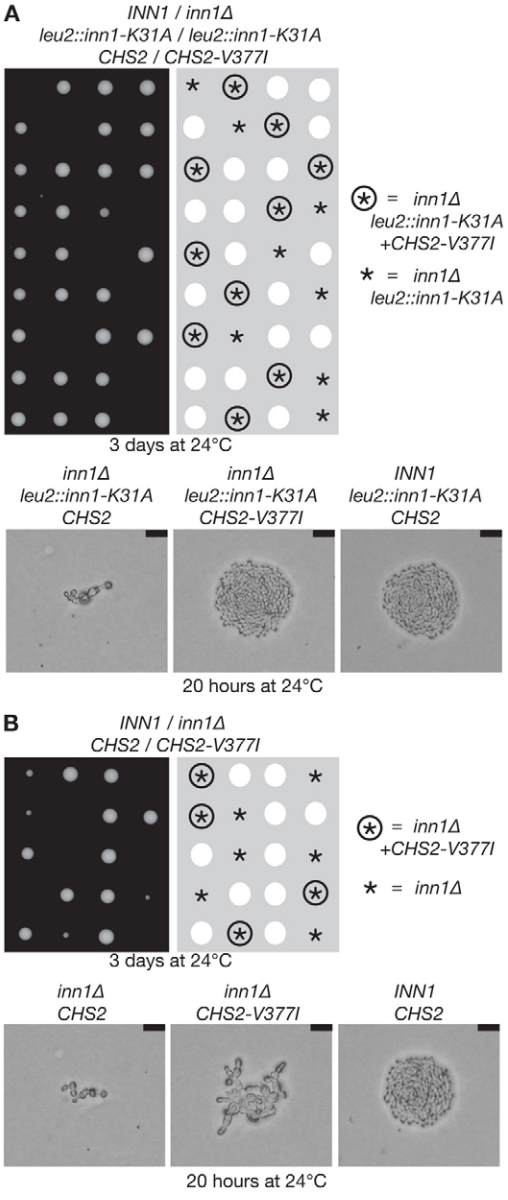
The entire *CHS2* gene was then sequenced in the plasmids that had been rescued from the genomic library, and we also amplified and sequenced the *CHS2* gene from a further six of the 22 suppressor clones. In each case, the *CHS2* gene contained a single mutation relative to the parental strain (we amplified and sequenced *CHS2* from the parental strain as part of the same analysis). In six of the suppressor clones, a G to A mutation at nucleotide 1129 of *CHS2* generated the *CHS2-V377I* allele, whereas one clone had a G to A transition at nucleotide 1114 of *CHS2*, which produced *CHS2-V372M*. As the two mutations are very close to each other within the region of *CHS2* encoding the catalytic domain of the chitin synthase protein (see below), we amplified and sequenced this region from the 15 remaining suppressor clones, and found that 14 had the *CHS2-V377I* mutation, whereas one had *CHS2-V372M*. To confirm that mutation of *CHS2* was indeed responsible for the dominant suppression phenotype, we transformed a *td-inn1-aid leu2::inn1-K31A ADH-TIR1* strain with centromeric plasmids expressing either *CHS2* or *CHS2-V377I*, and showed that only Chs2-V377I was able to support growth upon depletion of Td-Inn1-aid (Fig. 3D). In conclusion, the above data demonstrate that dominant mutations at two neighbouring sites in the catalytic domain of Chs2 are extremely efficient suppressors of the defects in cytokinesis that are produced by the non-functional *inn1-K31A* allele. These findings suggested that the C2-domain of Inn1 might normally be required during cytokinesis to regulate the action of the catalytic domain of Chs2, which is the chitin synthase that deposits the primary septum behind the contracting actomyosin ring.

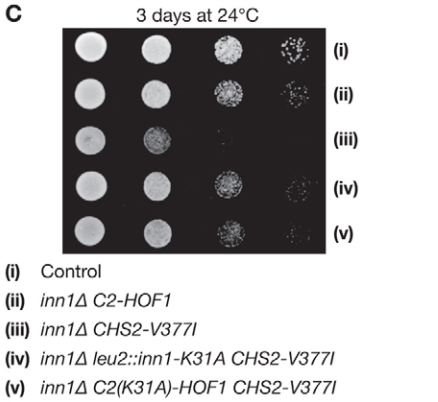
The dominant *CHS2* alleles are best at suppressing specific defects in the Inn1 C2-domain, but also support proliferation in the complete absence of Inn1

To confirm that *CHS2-V377I* was indeed able to suppress the cytokinesis defects associated with Inn1-K31A, in a cell that lacked any other source of the Inn1 protein, we used tetrad analysis to examine the growth of *inn1Δ leu2::inn1-K31A* in the presence of *CHS2-V377I* or wild-type *CHS2*. As expected, spores

with the genotype *CHS2 inn1Δ leu2::inn1-K31A* germinated and then died as a chain of a few cells that failed to divide (Fig. 4A). In contrast, *CHS2-V377I inn1Δ leu2::inn1-K31A* grew in a very similar fashion to control cells (Fig. 4A). In similar experiments, we found that *CHS2-V377I* also restored growth to cells that were completely devoid of the Inn1 protein (Fig. 4B, *inn1Δ CHS2-V377I*), but colony growth was slower in this case and cells were enlarged and formed chains, indicating that cell division was still somewhat aberrant. Although cytokinesis appears to be equally defective in cells that either lack Inn1 entirely or only have Inn1-K31A, these data indicated that *CHS2-V377I* allows cells to proliferate in both cases but only restores very efficient growth in the presence of the Inn1-K31A protein.

Two different mechanisms could explain why *CHS2-V377I* is much better able to suppress the failure of cytokinesis in cells expressing *inn1-K31A*, compared to cells that lack any form of Inn1. Firstly, the C2-domain of Inn1-K31A might be defective in a functionally important interaction with the catalytic domain of Chs2, and the Chs2-V377I mutation might compensate for this defect but still require the C2-domain of Inn1-K31A for optimal activation (even though it appears that Chs2-V377I can also be activated to some degree in the complete absence of Inn1). Secondly, Chs2-V377I might suppress the defective action of the C2-domain of Inn1-K31A, but the remainder of the Inn1 protein after the C2-domain might also contribute to efficient cytokinesis in a manner that is independent of the action of the C2-domain. To distinguish between these possibilities, we took advantage of the fact that the C-terminal 70% of Inn1 becomes dispensable for efficient cytokinesis if the C2-domain is fused artificially to Hof1, providing an alternative mechanism by which the C2-domain can be recruited to the bud-neck (Sanchez-Diaz et al., 2008). Mutation of K31 within Loop 1 of the C2-domain prevented the C2-Hof1 fusion from supporting cytokinesis in the absence of endogenous Inn1 (data not shown), but *CHS2-V377I C2(K31A)-Hof1 inn1Δ* grew almost as well as *CHS2-V377I leu2::inn1-K31A inn1Δ* (Fig. 4C). These data indicated that





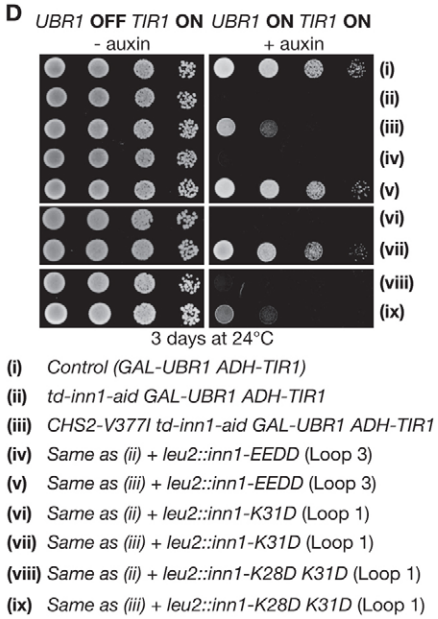


Fig. 4. *CHS2-V377I* is a highly efficient suppressor of specific defects in the loops of the C2-domain of Inn1, and allows cells that completely lack Inn1 to proliferate slowly. (**A**) Tetrad analysis of the indicated diploid strain shows that *CHS2-V377I* allows *inn1Δ leu2::inn1-K31A* to grow as well as control cells. Scale bars: 20 μm. (**B**) *CHS2-V377I* also allows cells to grow in the complete absence of Inn1, but growth remains slow and cell division is impaired. Scale bars: 20 μm. (**C**) If the C2-domain of Inn1-K31A is recruited to the bud-neck by fusion to Hof1, then *CHS2-V377I* allows cells to grow very efficiently in the absence of any other form of the Inn1 protein. Serial dilutions were grown for three days at 24℃ on YPD medium. (**D**) Serial dilutions of the indicated haploid strains were grown for three days at 24℃ under permissive conditions for *td-inn1-aid* (YPD medium − auxin: *UBR1* **OFF** *TIR1* **ON**) or restrictive conditions that induced depletion of Td-Inn1-aid (YPGal medium + auxin: *UBR1* **ON** *TIR1* **ON**).

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CHS2-V377I is able to compensate very effectively for a specific defect in Loop 1 of the C2-domain of Inn1, so that the C2-domain of Inn1-K31A can once again promote efficient cytokinesis.

We also explored the ability of Chs2-V377I to suppress other mutations in Loops 1–3 that block cytokinesis. Loop 3 is less well conserved amongst orthologues of Inn1 than Loops 1–2 (supplementary material Fig. S1B), and ScInn1 contains a run of four positively charged residues from R83 to K86 that can be mutated to Glycine and Alanine without blocking cytokinesis (A.S.D., unpublished data). Nevertheless, mutation of these residues to negatively charged residues creates the *inn1-EEDD* allele that cannot support cytokinesis (Fig. 4D (iv) and data not shown). The *CHS2-V377I* mutation allows *inn1-EEDD* cells to grow in a very similar manner to control cells (Fig. 4D (v)), showing that Chs2-V377I can also suppress defects in Loop 3 of the C2 domain of Inn1, in addition to suppressing the K31A mutation in Loop 1. We also made more severe mutations in Loop 1, by introducing negatively charged aspartate residues in place of K31 and the neighbouring lysine K28. Both *inn1-K31D* and *inn1-K28D K31D* were unable to support proliferation (Fig. 4D (vi) and (viii)). Interestingly, however, *CHS2-V377I* allowed *inn1-K31D* to grow as well as control cells (Fig. 4D (vii)), but only suppressed *inn1-K28D K31D* to the same extent as cells that completely lacked Inn1 (Fig. 4D, compare (iii) and (ix); in other experiments we confirmed expression of Inn1-K28D K31D by immunoblotting). Overall, these findings indicate that *CHS2-V377I* suppresses some but not all defects in Loop 1 and Loop 3 of the C2 domain of Inn1. Taken together with the fact that the suppression is dominant, these findings suggest that the C2 domain of Inn1 uses its loops (and perhaps other regions) to regulate some key function of the catalytic domain of Chs2, which is required during cytokinesis to form the primary septum and thus allow ingression of the plasma membrane.

The catalytic activity of Chs2 is required for suppression of cytokinesis defects produced by mutation of Inn1

Chitin is a polymer of N-acetylglucosamine (Glc-NAc) that is synthesised from an activated nucleotide substrate called UDP-N-actetylglucosamine (UDP-GlcNAc). The catalytic mechanism of chitin synthases has yet to be determined experimentally, but they belong to a larger family of 'inverting glycosyl transferases' (Merzendorfer, 2011). Orthologues of *Saccharomyces cerevisiae* Chs2 (ScChs2) are found in a wide range of fungal species and are very similar to ScChs1 that plays a minor role in repairing damage to the primary septum (Cabib et al., 1989), but are much more distantly related to ScChs3 that makes the bulk of cell wall chitin except the primary septum (Shaw et al., 1991; Valdivieso et al., 1991).

Using a structure-fold prediction server (Kelley and Sternberg, 2009), we found that amino acids 335–625 of ScChs2 are predicted with extremely high confidence to have a similar fold to the catalytic domains of several glycosyltransferases for which the crystal structures have been determined in the presence of substrate, despite very limited primary sequence similarity. These include the human Polypeptide α-N-Acetylgalactosaminyltransferase (pp-GalNAc-T10; estimated precision 100%, E-value=9.5e-12; see Fig. 5A) that transfers N-Acetylgalactosamine (Gal-NAc) from the substrate UDP-GalNAc to glycosylated peptides (Kubota et al., 2006), as well as the SpsA glycosyltransferase from *Bacillus subtilis* (estimated precision 100%, E-value=1.4e-10; see supplementary material Fig. S3) that uses nucleotide-diphospho-sugar donors to synthesise the bacterial spore coat (Charnock and Davies, 1999). Amino acids V372 and V377 of Chs2 (the sites of the dominant mutations that suppress Inn1 defects) are thus located within the predicted catalytic domain of Chs2, at the ends of adjacent secondary structural motifs (Fig. 5A; supplementary material Fig. S3). Moreover, both residues are perfectly conserved in fungal orthologues of ScChs2 (Fig. 5B). These structural considerations suggest that the C2-domain of Inn1 might interact with the catalytic domain of Chs2 during cytokinesis and thus regulate chitin synthase activity, perhaps by inducing a structural change that activates Chs2.

To explore this idea further, we tested the ability of other mutations in this region of Chs2 to act as dominant suppressors of *inn1-K31A*. Similar to V372M and V377I, we found that V372A and V377M could also support growth of *td-inn1-aid leu2::inn1-K31A* under restrictive conditions, but suppression did not result from mutation of N373, D375 or the highly conserved A379 (Fig. 5C). It thus appears that suppression is a specific consequence of mutating sites that are predicted to be at the ends of adjacent structural elements within the catalytic domain of Chs2. In addition, dominant suppression of *inn1-K31A* by *CHS2-V377I* required the catalytic activity of the Chs2-V377I protein (Fig. 5D), as it was blocked by mutation of conserved residues in the catalytic domain (Fig. 5B) that are predicted to be required for catalysis or substrate binding (see Discussion below) and are essential for *in vitro* chitin synthase activity or *in vivo* function (Nagahashi et al., 1995, and our unpublished data). These data suggest that mutation of V372 or V377 of Chs2 induces a structural change that facilitates *in vivo* activation of chitin synthase activity in cells with defective Inn1.

Chs2-V377I is constitutively active *in vitro*

Whereas Chs3 is active in isolated cell membranes, both Chs2 and Chs1 are inhibited by a poorly understood mechanism that can be reversed *in vitro* by protease treatment (Merzendorfer, 2011), presumably reflecting the fact that Chs2 and Chs1 must normally be activated *in vivo* during cytokinesis. The *in vitro* activity of Chs2 and Chs1 can be distinguished by the use of appropriate divalent cations and pH, and we were thus able to measure the activity of Chs2 and Chs2-V377I by isolating membranes from *chs3Δ* cells and performing the assay in the presence of cobalt at pH 8.0 (Choi and Cabib, 1994). As predicted by earlier work, wild-type Chs2 had a low basal level of chitin synthase activity that increased ninefold upon proteolytic treatment (Fig. 5E, V377). Strikingly, Chs2-V377I had six times greater basal activity in isolated membranes than wild-type Chs2 and the activity only increased a further 2.3-fold upon trypsin treatment (Fig. 5E, I377), despite the fact that both proteins are expressed to the same level in cells and have a very similar pattern of localisation (supplementary material Fig. S4). These data indicate that the Chs2-V377I mutation suppresses loss of Inn1 activity by releasing chitin synthase activity from inhibition, suggesting that Inn1 is normally involved in this step during cytokinesis.

Interaction of Inn1 with Chs2

To test whether Inn1 interacts with Chs2 in yeast in cell extracts, we grew cultures of *INN1* and *INN1-TAP* and synchronised cells in the G2-M phase of the cell cycle by addition of Nocodazole, before subsequent release into mitosis so that samples could be

taken when most cells were undergoing cytokinesis (the timing was judged by monitoring the peak time for recruitment of Inn1-GFP to the bud-neck in analogous experiments). After making cell extracts and immunoprecipitation on IgG-beads, we found that Inn1-TAP co-purified specifically with Chs2 but not Chs3 (Fig. 6A; note that Chs2 migrates as a smear of bands, probably

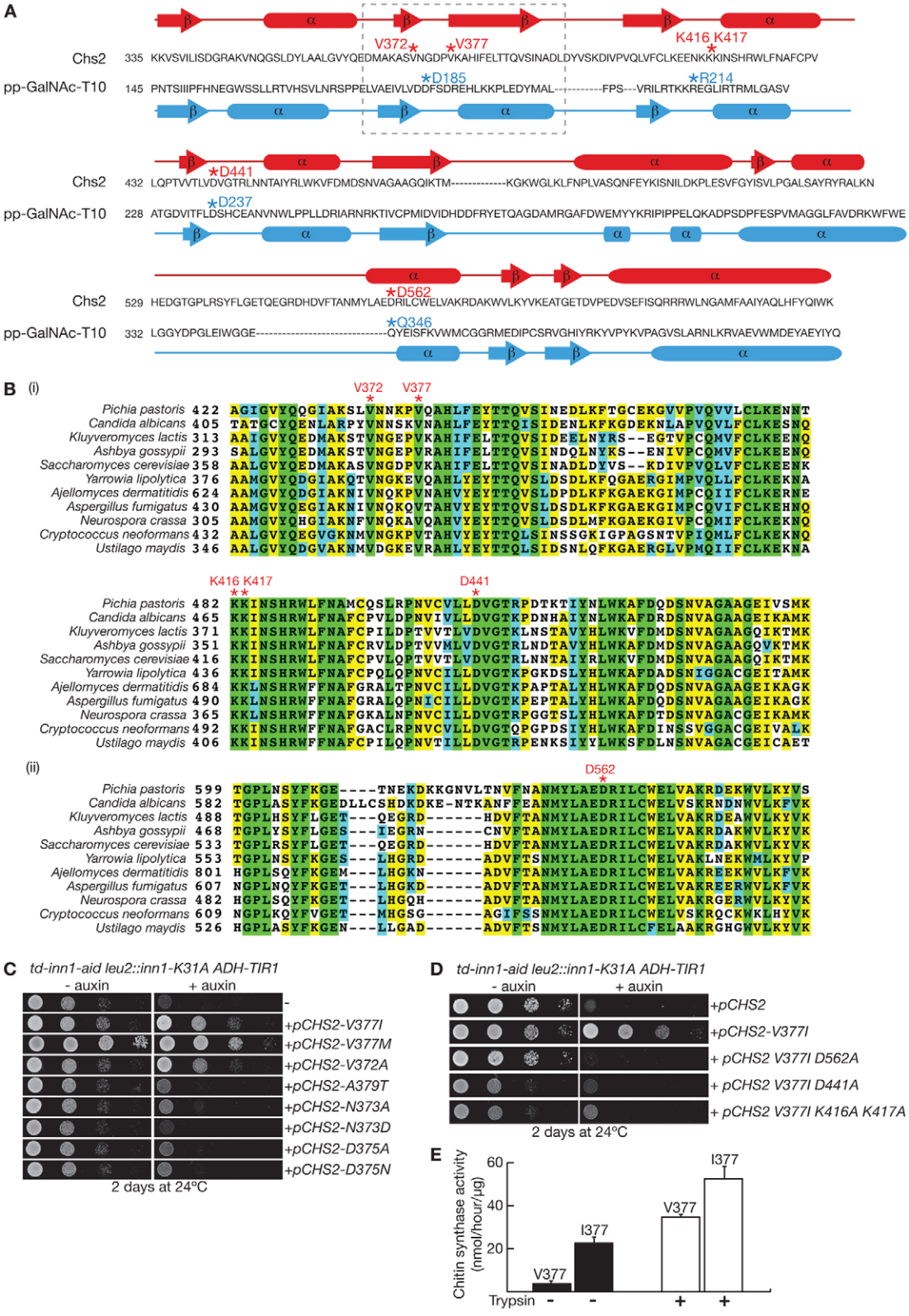

Fig. 5. See next page for legend.

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reflecting post-translational modifications). The interaction of Inn1 with Chs2 probably involves multiple points of contact, as Chs2 also co-purified with Inn1-K31A (Fig. 6B, sample (iii); supplementary material Fig. S5). We also confirmed that Chs2-V377I interacted with Inn1-TAP in a similar manner to wild-type Chs2 (Fig. 6B, sample (iv)). These data support the idea that Inn1 is a direct regulator of Chs2 and indicate that the *inn1-K31A* mutation within the C2-domain stops Inn1 from regulating Chs2, but does not prevent the two proteins from associating with each other. Consistent with this view, we found that both Inn1 and Inn1-K31A interacted specifically in a yeast two-hybrid assay with the N-terminal region of Chs2 that contains the catalytic domain (Fig. 6C).

CHS2-V377I supports cytokinesis in the absence of either Inn1 or Cyk3, but the simultaneous removal of both is still lethal

The Cyk3 protein was identified through its ability, when overexpressed, to support cell division in *iqg1Δ* cells that cannot assemble the actomyosin ring (Korinek et al., 2000). This indicated that Cyk3 acts downstream of Iqg1 to promote some aspect of septum formation, analogous to the role of Inn1. A subsequent study showed that high-copy *CYK3* could also suppress to some degree the growth defects of *inn1Δ* (Nishihama et al., 2009). Most interestingly, the formation of the primary septum was restored under such conditions, indicating that Inn1 and Cyk3 both control a similar step that is important for septation and cytokinesis (Nishihama et al., 2009). Whereas Inn1 is essential or nearly so in various strains of budding yeast, cells lacking Cyk3 have cytokinesis defects but are still able to form colonies, indicating that the two proteins normally act in parallel to each other, with Inn1 being the dominant factor.

Fig. 5. The dominant suppressor mutations in *CHS2* are located at conserved sites in the catalytic domain and alter chitin synthase activity. (**A**) The structure-fold recognition server Protein Homology/AnalogY Recognition Engine (PHYRE) (Kelley and Sternberg, 2009) was used to search for structural similarities between Chs2 and proteins of known crystal structure. The region of Chs2 from 335–625 is predicted with very high confidence to have a similar fold to the catalytic domain of human glycosyl transferase pp-Gal-NAc-T10 and bacterial SpsA (see supplementary material Fig. S3). The figure shows the predicted secondary structure for the catalytic domains of Chs2 and pp-Gal-NAc-T10 proteins. The residues marked in blue include sites in pp-Gal-NAc-T10 that touch the substrate (D185, R214 and D237), and the predicted catalytic base Q346. Conserved residues at equivalent sites in Chs2 are marked in red. (**B**) Orthologues of ScChs2 in the indicated fungal species were identified by PSI-BLAST searches, aligned with ClustalW software (http://seqtool.sdsc.edu/CGI/BW.cgi), and displayed by using 'Boxshade'. (i) The putative substrate-binding region showing conserved residues at key sites as in A, together with the sites of the suppressor mutations at V372 and V377. (ii) The conserved region around the predicted catalytic base at D562. (**C**) The indicated alleles of *CHS2* were expressed from centromeric plasmids and tested, as described previously, for their ability to suppress *inn1-K31A*. Suppression was only observed with mutations at V372 and V377I. (**D**) Similar experiments showed that suppression of *inn1-K31A* by *CHS2-V377I* required the predicted catalytic base of Chs2 (D562) and conserved residues at putative substrate-binding sites (D441 and K416-K417). (**E**) The chitin synthase activity of wild-type Chs2 (V377) and Chs2-V377I (I377) was determined in membranes isolated from asynchronous cultures of cells lacking Chs3, as described in the Materials and Methods section.

Together with these findings, our data predicted that Cyk3 might act together with Inn1 to regulate the catalytic domain of Chs2. Consistent with this view, we found that *CHS2-V377I* allowed *cyk3Δ* to grow in a similar fashion to control cells (Fig. 7A). Moreover, Cyk3 became essential in *td-inn1-aid* cells (Fig. 7B), which even under permissive conditions express the Inn1 protein to a much lower level than control cells (Fig. 1B). The *CHS2-V377I* allele was able to suppress the synthetic lethality of cells that lack Cyk3 and express a low level of the Td-inn1-aid protein (Fig. 7C (v) – auxin), but further depletion of Td-Inn1-aid was lethal (Fig. 7C (v) + auxin), showing that Cyk3 is required for Chs2-V377I to suppress the cytokinesis defects produced by Inn1-K31A. Overall these findings suggest that Cyk3 acts in parallel to the C2-domain of Inn1 to regulate Chs2 during cytokinesis. The ability of *CHS2-V377I* to suppress cytokinesis defects produced by mutation of Inn1 or Cyk3 appears to be specific, as *CHS2-V377I* did not suppress the lethal effects of depleting Iqg1 or Myo1 (Fig. 7D).

Fusion of the C2-domain to Chs2 suppresses *inn1Δ*, but cells expressing C2-Chs2 or C2-Hof1 are completely dependent upon Cyk3

We previously showed that fusion of the Inn1 C2-domain to Hof1 supports cytokinesis in *inn1Δ* cells (Sanchez-Diaz et al., 2008), presumably as Hof1 serves to recruit the C2-domain to the bud-neck where it then interacts with Chs2. If the principal role of the C2-domain of Inn1 were to regulate the catalytic domain of Chs2, then direct fusion of the C2-domain to Chs2 might also support cytokinesis in cells that lack the endogenous *INN1* gene. Consistent with this view, *C2-CHS2 inn1Δ* grew at a very similar rate to control cells (Fig. 8A) without any apparent defect in cell division (Fig. 8B).

In the course of crosses to determine whether *C2-CHS2* or *C2-HOF1* might suppress the cytokinesis defects of cells lacking Cyk3, we found that both fusions are actually lethal in combination with *cyk3Δ*, despite the presence of wild-type Inn1 in these cells (Fig. 8C,D). This dominant lethal phenotype suggests that *C2-CHS2* and *C2-HOF1* represent hypermorphic alleles and indicates that the regulation of Chs2 activity by Inn1 and Cyk3 is very finely tuned *in vivo*, to ensure the correct spatial and temporal regulation of chitin deposition during cytokinesis and allow the primary septum to form behind the contracting actomyosin ring.

Discussion

In yeast cells, the action of the contractile actomyosin ring is intimately related to formation of the primary septum, such that the contraction of the ring guides membrane ingression and the centripetal deposition of the primary septum (Schmidt et al., 2002), and septum deposition stabilises the contracting actomyosin ring (VerPlank and Li, 2005). Together with the results of previous studies (Jendretzki et al., 2009; Nishihama et al., 2009), our data support a model whereby proteins associated with the actomyosin ring such as Iqg1 and Hof1 (and perhaps other factors too) serve to recruit Inn1 and Cyk3, which then regulate the catalytic activity of Chs2 at the bud-neck to promote deposition of the primary septum. This model provides a mechanistic explanation for the tight co-ordination between the function of the actomyosin ring and the formation of the primary septum in budding yeast.

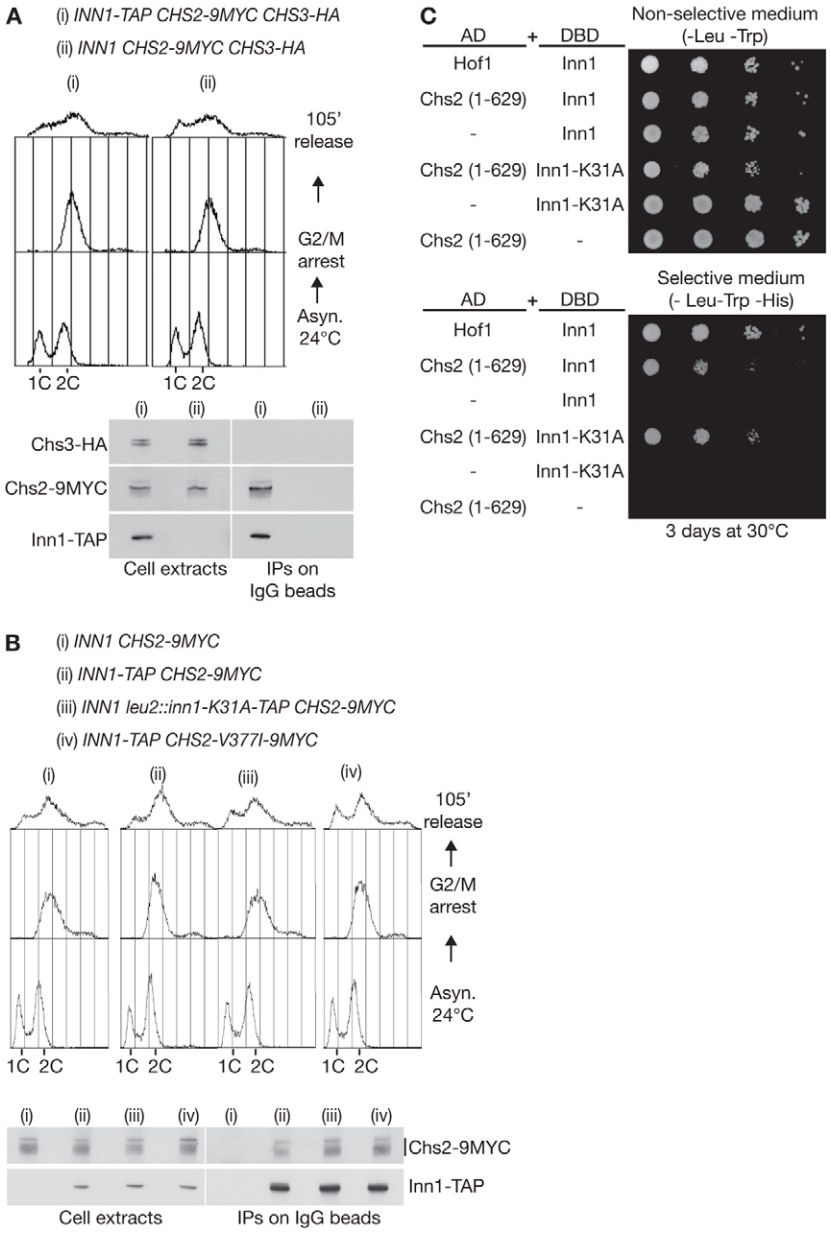

Fig. 6. Inn1 associates with Chs2. (**A**,**B**) The indicated haploid strains were synchronised in G2-M phase with nocodazole and then released for 105′, which corresponded to the peak time for cytokinesis (not shown). Samples were frozen at that point and then used subsequently to make cell extracts before immunoprecipitation with IgG-beads. (**C**) Yeast two-hybrid analysis of interactions between the indicated polypeptides fused to the activation domain (AD) or DNA-binding domain (DBD) of Gal4. Serial dilutions of cells were grown on the indicated minimal media for three days at 30℃.

Inn1 is critical for cell division and the phenotypes of cells lacking either Inn1 or Chs2 are very similar. It appears that the temporal and spatial regulation of Chs2 is extremely important for cells, as the synthesis, localisation, activation and degradation of Chs2 are all regulated during the course of the cell cycle (VerPlank and Li, 2005; Zhang et al., 2006; Teh et al., 2009; Chin et al., 2012; Merzendorfer, 2011).

The catalytic domain of Chs2 is predicted to adopt a very similar fold to other glycosyl transferases including human pp-GalNAc-T10 and bacterial SpsA (Fig. 5A; supplementary material Fig. S3). The crystal structures of the latter two proteins identify the catalytic base as Q346 and D191 respectively, probably equivalent to D562 of Chs2 (Nagahashi et al., 1995). In addition, there are three important contact sites with substrate. Firstly, the ribose component of the substrate is bound by the D237 residue of ppGalNAc-T10, probably equivalent to D441 of Chs2 (Fig. 5A) that is highly conserved (Fig. 5B), essential for chitin synthase activity (Nagahashi et al., 1995) and required for the suppressor activity of Chs2-V377I (Fig. 5D). Secondly, R214 of ppGalNAc-T10 contacts the uracil moiety and we have found that K416 and K417 in the equivalent region of Chs2 are highly conserved (Fig. 5A,B), essential for function *in vivo* (A.D. and K.L., unpublished data) and needed for *CHS2-V377I* to suppress *inn1-K31A* (Fig. 5D). Thirdly, glycosyl transferases such as ppGalNAc-T10 and SpsA have a 'DD motif' that contains a further contact point with the uracil moiety of the substrate (Fig. 5A, D185 of ppGalNAc-T10; supplementary material Fig. S3, D39 of SpsA). Interestingly, the corresponding region of Chs2 lacks the DD motif and is flanked by V372 and V377 (Fig. 5A; supplementary material Fig. S3). Future structural studies of Chs2 will be required to establish whether or not this region contains a substrate-binding site analogous to that of ppGalNAc-T10 and SpsA, but our data raise the possibility that Inn1 (and perhaps also Cyk3) might

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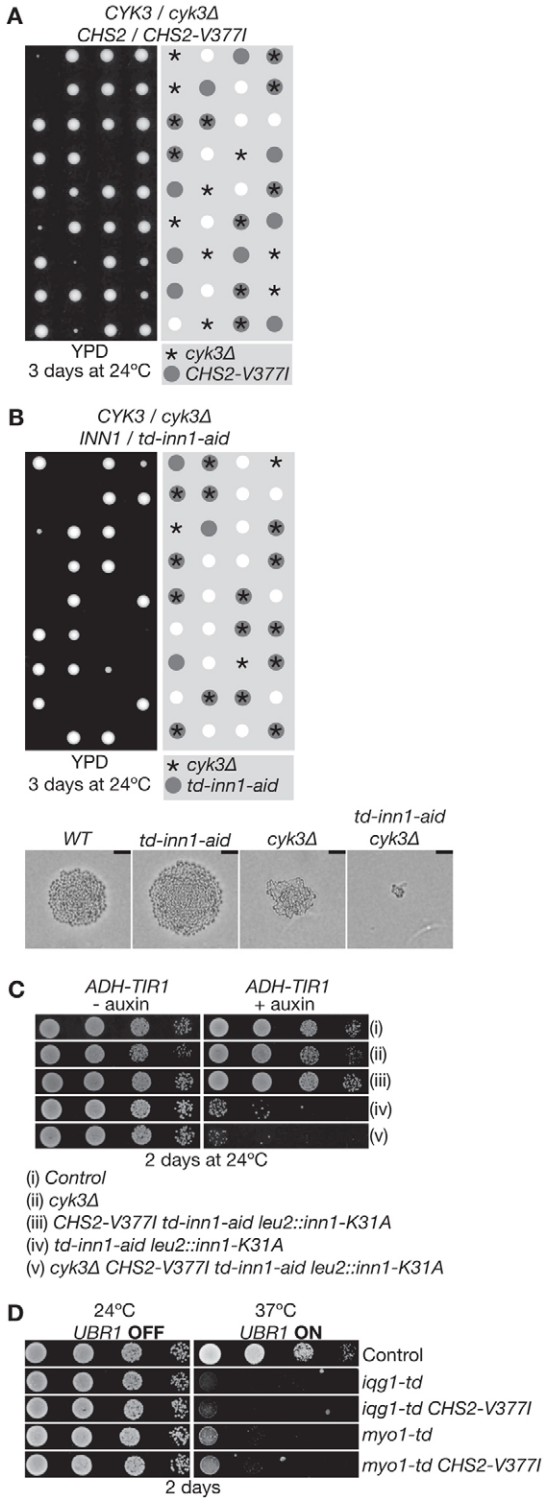

Fig. 7. *CHS2-V377I* is a specific suppressor of *cyk3Δ* or *inn1-K31A*, but cannot support cytokinesis if both *cyk3Δ* and *inn1-K31A* are combined in the absence of any other source of Inn1 protein. (**A**) *CHS2-V377I* suppresses the defects in cell proliferation produced by *cyk3Δ*. (**B**) Loss of Cyk3 is lethal in *td-inn1-aid* cells that have a very reduced level of Inn1. Scale bars: 20 μm. (**C**) The indicated strains were treated as in Fig. 3D. (**D**) The indicated strains were grown for 2 days under permissive conditions (YPD 24℃, *GAL-UBR1* **OFF**) or restrictive conditions (YPGal 37℃, *GAL-UBR1* **ON**) for *myo1-td* and *iqg1-td*.

interact with this region of Chs2, perhaps serving to regulate substrate binding during cytokinesis or control Chs2 catalytic action in some other way.

The dominant suppressor mutations at V372 or V377 might partially mimic the putative structural change mediated by the C2-domain of Inn1. Accordingly, we found that Chs2-V377I is constitutively active *in vitro*, even in the absence of proteolytic treatment (Fig. 5E). Nevertheless, *CHS2-V377I* is much better at suppressing *inn1-K31A* than *inn1Δ*, and it thus seems that the Chs2-V377I protein has deregulated chitin synthase activity but is still partially dependent upon Inn1. It will be very interesting in future studies to try and reconstitute *in vitro* the activation of wild-type Chs2 by Inn1 and Cyk3, and our data indicate this should be achievable.

The model whereby Inn1 and Cyk3 regulate the catalytic domain of Chs2 predicts that other eukaryotic species with orthologues of Chs2 should also have orthologues of Inn1 and Cyk3. By a combination of PSI-BLAST searches and secondary structure prediction, we have found that a wide variety of fungal species have orthologues of ScChs2 (and the related ScChs1 protein), and each of these species also has a single orthologue of Inn1 (supplementary material Fig. S1B,C), though in most cases these have yet to be characterised experimentally. Almost all of these species also have a single orthologue of Cyk3, though the pathogenic yeasts *Cryptococcus neoformans*, *Cryptococcus gattii* and *Ustilago maydis* seem to lack Cyk3 and have a more divergent orthologue of Inn1 that has an additional loop within the Beta-sandwich (supplementary material Fig. S1C). In most cases, the importance of these proteins for cell division has yet to be determined, but will presumably correlate with the proportion of chitin in the primary septum, which varies between species.

Interestingly, orthologues of ScChs2-Inn1-Cyk3 are found not just in budding yeasts but also in the fission yeast *Schizosaccharomyces pombe* that only has very little chitin in the primary septum, as well as in some mycelial fungi including the opportunistic human pathogen *Aspergillus fumigatus*. The role of Chs2-Inn1-Cyk3 in mycelial fungi remains to be explored in the future, but the fission yeast proteins Chs2, Fic1 (the orthologue of Inn1: Fic1='Fifteen interacting C2-domain' as it interacts with the Cdc15 orthologue of ScHof1) and Cyk3 are known to be present at the cleavage site (Martín-García et al., 2003; Roberts-Galbraith et al., 2009; Pollard et al., 2012), though they only have a minor role in cytokinesis (Mishra et al., 2004; Martín-García and Valdivieso, 2006; Roberts-Galbraith et al., 2009; Pollard et al., 2012).

In contrast, it seems very likely that orthologues of ScChs2-Inn1-Cyk3 will be of great importance for cytokinesis in a range of pathogenic budding yeast species. Traditional antifungal therapies are effective but toxic to patients, and so there has been considerable interest in developing novel treatments that exploit fungal-specific biology such as synthesis of the primary septum. A variety of natural and synthetic chitin synthase inhibitors have been described recently, including several compounds with potency *in vitro* against orthologues of ScChs2, and the clinical potential of such compounds is currently under investigation (Calugi et al., 2011). Our data suggest that Cyk3 and the interaction of the Inn1 C2-domain with Chs2 represent alternative targets for the development of novel small molecule inhibitors of cytokinesis in pathogenic yeasts. In support of this idea, the orthologues of ScChs2 and Cyk3 have been shown to be essential in *Candida albicans* (Mio et al., 1996;

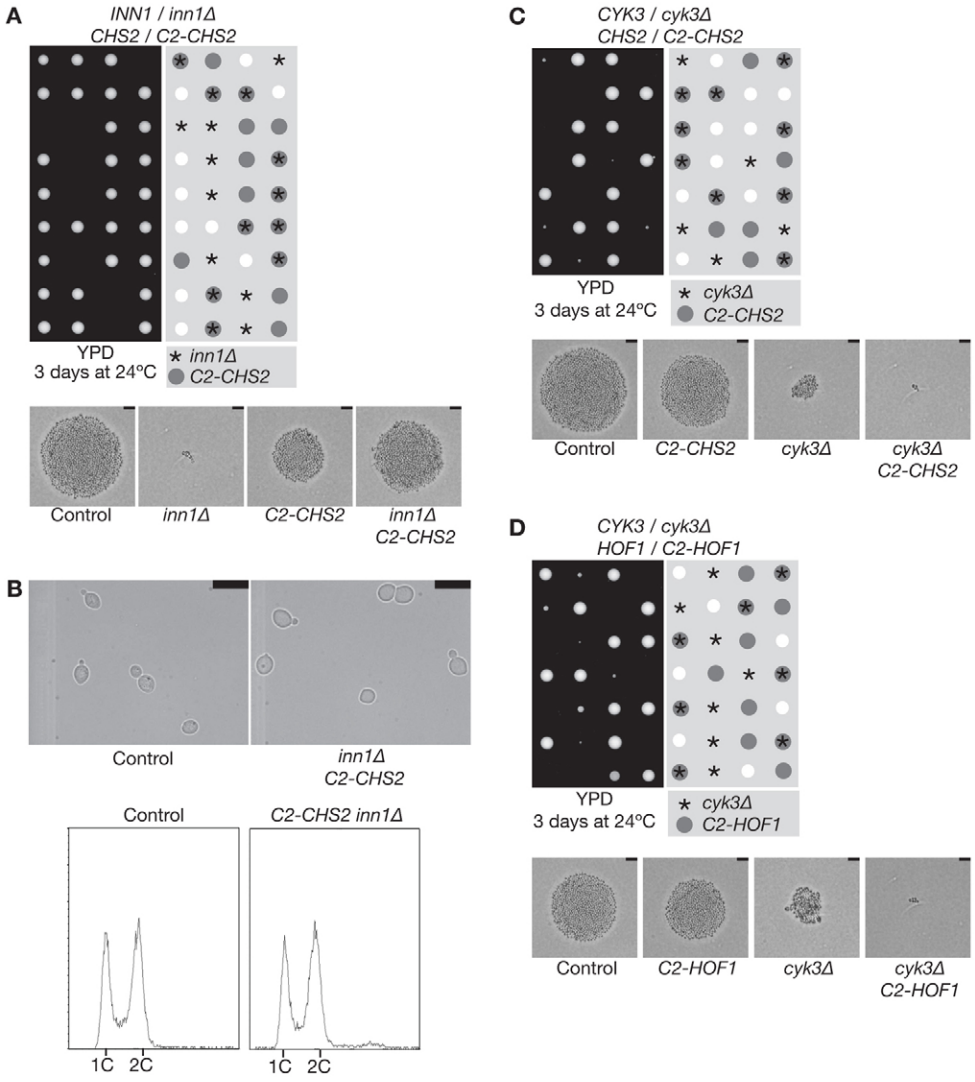

Fig. 8. Direct fusion of the C2-domain of Inn1 to Chs2 is enough to allow *inn1Δ* cells to grow as well as control cells, but the C2-Chs2 or C2-Hof1 fusions appear to be hypermorphic *INN1* alleles that are dominant lethal in the absence of Cyk3. (**A**) Tetrad analysis of the indicated diploid strain (YAD41) shows that the C2-Chs2 fusion allows *inn1Δ* cells to grow as well as control cells. Scale bars: 20 μm. (**B**) Control and C2-CHS2 cells were grown in liquid culture at 24℃ before phase contrast microscopy (scale bar: 10 μm) and flow cytometry. (**C**) Tetrad analysis of the indicated diploid strain (YAD82) shows that the C2-Chs2 fusion is lethal in the absence of Cyk3, even in cells expressing wild-type Inn1. Scale bars: 20 μm. (**D**) The C2-Hof1 fusion is similarly lethal in the absence of Cyk3, even in cells expressing wild-type Inn1. Scale bars: 20 μm.

Munro et al., 2001; Reijnst et al., 2010), although the orthologue of Inn1 has yet to be characterised. Moreover, the orthologue of Inn1 in *Cryptococcus neoformans* is important for cell division at 37℃, and even a hypomorphic allele blocks virulence in a mouse model of yeast infection (Fox et al., 2003).

Our data suggest new approaches that could be used to develop cell-based screens for small molecules targeting the activities of the Inn1 C2-domain or Cyk3. For example, a drug that inhibits Cyk3 function should block cytokinesis in *C2-CHS2* and *C2-HOF1* but not in control cells, whereas a drug that interferes with the action of the C2-domain of Inn1 should block cytokinesis in control cells but be less effective in *CHS2-V377I*. Future studies could use *Saccharomyces cerevisiae* as a model system for such screens, for example by replacing the Chs2-Inn1-Cyk3 axis with orthologues from pathogenic species. This would facilitate the genetic analysis of such Chs2-Inn1-Cyk3 orthologues along the lines of this study, and the subsequent development of specific inhibitors that target fungal-specific biology in pathogenic species.

Materials and Methods

Yeast strains and growth

The budding yeast strains used in this study are based on the W303 background and are listed in supplementary material Table S1. Cells were grown in rich medium (1% Yeast Extract, 2% Peptone) supplemented with 2% sugar (glucose, galactose or raffinose; producing medium called YPD, YPGal and YPRaff, respectively). Cells were synchronised in G1 phase or G2-M phase by incubation with 7.5 μg/ml alpha-factor or 5 μg/ml nocodazole respectively, for at least one generation-time. We grew *td-inn1-aid* strains at 24℃ and induced degradation of the Td-Inn1-aid protein by switching cells to medium containing 500 μM auxin (1-napthaleneacetic acid or indole-3-acetic acid). In the case of *td-inn1-aid ADH-TIR1* strains the growth medium was based on YPD, whereas *td-inn1-aid ADH-TIR1 GAL-UBR1* strains were initially grown in YPRaff medium, before shifting to YPGal + auxin to induce degradation of the Td-Inn1-aid protein.

Point mutations in Inn1 or Chs2 were created by PCR in integrative or centromeric plasmid vectors as necessary (Sikorski and Hieter, 1989). Integrative vectors were linearised within the marker gene to drive homologous recombination with the corresponding chromosomal locus.

The *C2-CHS2* strain was made by a one-step PCR approach that modified the endogenous *CHS2* locus to introduce the C2-domain and marker gene while preserving the native *CHS2* promoter.

Screens for suppressors of *inn1-K31A*

We screened for suppressors of *inn1-K31A* in two ways, the first of which was a colony-sectoring assay. An *inn1Δ leu2::inn1-K31A ade2 ade3* strain was kept alive with a centromeric plasmid expressing *INN1* and *ADE3*, producing red colonies without white sectors (as cells losing the plasmid would die due to lack of Inn1 function). Cells were mutagenised with 3% ethyl methanesulfonate for 25′ at 24℃, corresponding to 50% survival, before plating on YPD medium at a density of 1000 cells per plate. A total of 38,000 colonies were screened to find ones with white sectors, but these were all found to be due to mutations in the *ADE3* gene in the plasmid, and did not represent cells with suppressors of *inn1-K31A*.

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The second screen involved mutagenesis as above of *td-inn1-aid leu2::inn1-K31A ADH-TIR1 GAL-UBR1*. After mutagenesis, cells were allowed to recover in YPD medium for three hours at 24℃, before 2×10^8 cells were plated on YPGal + auxin medium at a density of 10^7 cells per plate. After incubation overnight at 24℃, cells were replica plated to fresh YPGal + auxin medium and incubated at 24℃ for a further 2–3 days. Individual colonies were streaked on selective medium before subsequent analysis as described in the text.

Making a genomic library of DNA fragments from a *td-inn1-aid* strain with a dominant suppressor mutation

To clone the dominant suppressor gene, a genomic library of DNA fragments was generated from one of the suppressor clones. Genomic DNA was prepared from a 400 ml culture using the Qiagen Genomic DNA kit with 500/G tips (Qiagen 10262), before partial digestion with Sau3A restriction enzyme. The digested DNA was purified and precipitated before size selection of fragments between 3 kb and 10 kb in a 0.8% agarose gel. The digested fragments were purified and ligated to the pRS313 vector (Sikorski and Hieter, 1989) that had previously been digested with *Bam*HI and treated with calf intestinal phosphatase. The ligation reactions were transformed into competent DH10B *E. coli* cells by electroporation and transformants were grown overnight at 37℃. Cells were then scraped off the plates and used to purify plasmid DNA. The resultant library of genomic fragments contained about 35,000 clones with an average insert size of about 4 kb.

Measuring the chitin synthase activity of Chs2 and Chs2-V377I

Asynchronous cultures of *chs3Δ CHS2* (YMF56) and *chs3Δ CHS2-V377I* (YMF58) were grown at 24℃ in YPD medium to a density of 2×10^7 per ml. Cells were pelleted in pre-chilled bottles and then washed twice with 50 ml of ice-cold 50 mM Tris-HCl pH 8.0, before storage at −80℃. Subsequently the pellets were thawed on ice, resuspended in 500 µl 50 mM Tris pH 8.0 and broken with glass beads in the presence of ice-cold 50 mM Tris-HCl pH 8.0. After dilution up to 8 ml with 50 mM Tris-HCL pH 8.0, the extracts were centrifuged for 7 minutes at 4800 ***g*** to remove debris, and then total cell membranes were isolated by centrifugation for 35 minutes at 25,000 ***g***. The membranes were then resuspended in 500 µl of ice-cold 50 mM Tris-HCl, pH 8.0 containing 33% glycerol, and the protein content was determined using the Bradford assay (BioRad).

The chitin synthase activity of Chs2 was measured according to the method of Choi and Cabib (Choi and Cabib, 1994). The addition of 5 mM cobalt acetate and the use of pH 8.0 served to inhibit Chs1 activity, and both strains lacked Chs3. Reactions were performed in a total volume of 44 µl (20 µl membranes, 3 µl 50 mM Tris-HCl pH 8.0, 5 µl 50 mM CoAc, 5 µl [^{14}C] UDP-N-acetyl-D-glucosamine (Amersham), 11 µl dH_2O) with or without addition of trypsin as follows: (i) No addition; (ii) 2 µl 1 mg/ml Trypsin (Sigma); (iii) 2 µl 2 mg/ml Trypsin; (iv) 2 µl 3 mg/ml Trypsin.

Samples were incubated for 15 minutes at 30℃ before making the following additions to the corresponding reactions: (i) 2 µl 0.8M N-acetyl glucosamine (Sigma); (ii) 2 µl 0.8 M N-acetyl glucosamine, + 2 µl 4.5 mg/ml trypsin inhibitor (Sigma); (iii) 2 µl 0.8 M N-acetyl glucosamine, + 2 µl 4.5 mg/ml trypsin inhibitor; (iv) 2 µl 0.8M N-acetyl glucosamine, + 2 µl 4.5 mg/ml trypsin inhibitor

The samples were then incubated for a further 90 minutes at 30℃, before the reactions were stopped by addition of 2 ml of 10% TCA and precipitation for 60 minutes at 4℃. The reaction mixtures were passed through glass fibre filters, which were then washed two times with 10% TCA and once with 96% ethanol before drying. The incorporated radioactivity was then measured with a scintillation counter, and the specific activity expressed as nanomoles of N-acetyl-D-glucosamine incorporated per hour per milligram of protein.

Each reaction was performed in duplicate, and the whole experiment was also carried out twice, before calculation of an average activity. The data in Fig. 5E relate to the samples without trypsin or with addition of 1 mg/ml trypsin.

Preparation of cell extracts and immunoprecipitation of Inn1-TAP

To monitor the level of Inn1 fused to the 'td' or 'aid' degron cassettes, we made cell extracts in the presence of Trichloroacetic acid (Foiani et al., 1994).

To isolate Inn1-TAP, native cell extracts containing 100 mM potassium acetate were made from 250 ml mid-log culture as described previously (Gambus et al., 2009; Morohashi et al., 2009). After centrifugation at 25,000 ***g*** for 30 minutes and then 100,000 ***g*** for 60 minutes, immunoprecipitations were performed with magnetic beads coupled to rabbit IgG (S1265, Sigma). Inn1 was detected with sheep polyclonal antibodies and Chs2-9MYC was detected with the 9E10 monoclonal antibody (C.R. UK).

Microscopy

Images of cells and colonies on agar plates were taken using a Nikon eclipse e400 microscope, with×10 Plan Fluor lens 0.30 NA.

Fluorescence microscopy was performed as described previously (Sanchez-Diaz et al., 2008), using a Zeiss Axiovert 200M microscope and a Cool Snap HQ camera (Photometrics). The objective lens was a Zeiss alpha plan×100 1.45 NA, and we used a Chroma ET GFP filter set (49002). The illumination source was the Sutter 300w xenon system with liquid light guide, and we used Metamorph acquisition software (Molecular Devices). Nine z-sections were examined with a spacing of 0.375 µm. Images were viewed using Imaris (Bitplane) software and at least 100 cells were examined for each sample.

Flow cytometry

DNA content was measured by flow cytometry as described previously (Kanemaki et al., 2003).

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