

A Bipartite Glucan Synthase-Remodeler Module Organizes Branched Glucan Assembly in the Fungal Cell Wall

Corresponding Author: Dr Kathleen Gould

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript is well written, logically structured, and presents a compelling argument. It convincingly demonstrates that the Ghs2 GH16 domain is responsible for linking the β -1,6 chain onto the β -1,3 chain during cell wall assembly in *S. pombe*, and that this enzyme functions in concert with Bsg3.

While the study is primarily directed toward specialists in fungal cell wall biology, it also illustrates, for a broader audience, an important organizational strategy by which complex biological polymers are synthesized and their morphogenesis regulated.

Overall, the manuscript is thoughtfully organized and clearly presented. That said, some aspects of the manuscript could be improved to enhance readability and better address potential questions from readers:

- In the introduction, a simplified schematic illustrating the composition and morphogenesis of the yeast cell wall would greatly help readers engage with the topic.
- Is there any evidence of *in vitro* activity of the Ghs2 GH16 domain on glycan chains?
- Regarding the AlphaFold model proposing a direct interaction between Ghs2 and Bsg3: this model appears to rely primarily on interactions between their respective transmembrane helices. It is therefore difficult to assess its reliability. While the conservation of residues on the GH16 side is well described, no information is provided on the residues involved on the TM1–17 region of Bsg3. A genomic modification targeting the transmembrane helix of Bsg3 would have strengthened the hypothesis of a direct membrane-mediated interaction.
- Figure 5A: glycan chains H and B are shown, but chain A is missing, making it difficult to follow the nomenclature used in the other panels.
- The change in scale between panels D and E complicates interpretation, especially since the choice of the two regions is neither explained in the legend nor indicated (e.g., with dashed lines) in panel E.
- Regarding the Ghs2 Y478I mutant, it would have been informative to include the NMR spectrum of this strain to confirm its effect on glycan chain synthesis.
- In Figure S4, NMR data show a strong decrease in protein signals in the flexible phase of the mutants, with little change in the rigid phase (Figure S5). Is this reduction in attached proteins confirmed by other methods, and what is its underlying cause?

Finally, on page 9, line 232: is the detection of the flexible domain truly related to T1 (spin–lattice) relaxation effects, or could it instead (or additionally) arise from T2 (spin–spin) relaxation?

Reviewer #2

(Remarks to the Author)

In contrast to β -1,3-glucan it is a long standing question how in a fungal cell wall β -1,6-glucan is synthesized. There is no clearly defined synthase enzyme that both polymerizes and branches. This important unsolved question is addressed in the manuscript.

The authors used the fission yeast *Schizosaccharomyces pombe* as the experimental organism. By using reporter fusions and IP the authors identified Ghs2 which is a glycoside hydrolase 16 (GH16) domain-containing transmembrane protein, as an obligate partner of the β -1,3-glucan synthase Bgs3. They showed that Ghs2 and Bgs3 co-localize at sites of polarized growth and by IP they showed that the proteins physically associate *in vivo*. Structure-guided modeling positioned the Ghs2 catalytic domain proximal to the predicted Bgs3 glucan extrusion pore. This alphafold-based modelling provided a

theoretical frame suggesting coordinated polymer synthesis and remodeling. By applying solid-state NMR analyses it was demonstrated that both Ghs2 and Bgs3 are required for β -1,6-glucan production. By using inhibitors of α -1,6-glucan synthesis and by analysis of generated mutant strains the authors concluded that Ghs2 acts on newly synthesized β -1,3-glucan to introduce β -1,6-linked branch points.

Major comments

The question how β -1,6-glucans in the fungal cell wall are synthesized is unsolved. Thus, the authors addressed an important question for fungal biology. The genetic analysis is very well performed. The authors clearly demonstrated the interdependence of Ghs1 and Bgs3 and also convincingly showed that there is a synthase-modifier pair involved in β -1,6-glucan synthesis.

However, I have some major questions and caveats that I recommend to be addressed:

1. The authors observed that (lines 113-115) 'GFP-Ghs2 expression was reduced to 10 % of wild-type levels in bgs3-1 cells at both temperatures by immunoblotting'. The authors provide no explanation for this surprising observation. How could the enzymes be involved in the expression of other enzymes? I also do not think it is possible to measure expression of genes by immunoblot. Alternatively, did the authors check whether the formation of the heterodimer prevents degradation which also could explain the reduction of proteins.
2. Whereas the results on the interaction of Ghs2 and Bgs3 are convincing, and also that they influence the cell wall and β -1,6-glucan formation, it is less clear to me whether the enzymes are really sufficient to provide the β -1,6-linkage in the cell wall. This caveat is based on the solid state NMR which, to me, is unclear. Even under the non-permissive temperature there apparently is production of β -1,6-glucan. How do the authors explain this contradiction? In the discussion the authors write about reduction of β -1,6-glucan. However, according to their hypothesis the heterodimer should be sufficient for β -1,6-glucan formation and their lack should halt the synthesis.
3. To finally prove that the enzymes are sufficient for production of β -1,6-glucan and also to prove that the enzymes are in fact able to synthesize the carbohydrate polymer, this proof can only be provided by an in vitro assay using the purified enzymes. For example, this was published for the β -1,3-glucan synthase. Without such an assay and taking into account the NMR analysis that provides a strong hint but no proof, the final conclusion that the enzymes are sufficient for β -1,6-glucan synthesis is not given. Such an in vitro enzyme assay is strongly recommended.

Reviewer #3

(Remarks to the Author)

The manuscript by Willet et al. investigates a fundamental unresolved challenge in fungal cell wall biology and biochemistry: the molecular machinery responsible for the formation of β -1,6-glucan linkages within the primary β -1,3-glucan network to form β -1,3-glucan branches. The authors show that the resulting branched β -1,3-glucan is essential for the maintenance and accumulation of β -1,6-glucan in the *Schizosaccharomyces pombe* cell wall. They further suggest that the remodeling protein Ghs2 (which contains a putative glycoside hydrolase 16 catalytic domain) and the β -1,3-glucan synthase Bgs3 constitute an indispensable physical and functional module. Despite certain criticisms, the multidisciplinary approach employed is excellent. The integration of classical genetics, utilizing temperature-sensitive mutants and transmembrane chimera analyses, with structural predictions via AlphaFold3 (AF3) and, most significantly, solid-state Nuclear Magnetic Resonance (NMR) in intact cells, lends substantial experimental weight to the study. The conceptualization of a "synthase-remodeler module" represents a genuine paradigm shift that is expected to have a high impact in fungal microbiology and open new avenues for the rational design of antifungals. However, the manuscript requires the correction of several important fundamental conceptual errors about the biochemical structure of the different types of glucans and the function of the glucan remodeling enzymes, as well as the clarification of specific mechanistic limitations and the inclusion of critical experimental controls, prior to its final publication in a journal such as *Nature Communications*.

MAJOR POINTS

The author's description of the various cell wall β -glucans is currently unclear and wrong. In page 3, lines 69-71, the authors state: "The *S. pombe* cell wall consists primarily of α -1,3-glucans, β -1,3-glucans, β -1,6-glucans, both as side branches on β -1,3 chains and as linear polymers." The assertion that β -1,3-glucans and β -1,6-glucans are both side branches on β -1,3 chains is incorrect. The β -1,3-glucan only contains branches of β -1,3-glucan, β -1,6 is only the linkage to attach the β -1,3-glucan side chain. Furthermore, the diagram depicting β -glucans in Figure 5a is erroneous and leads to confusion. β -1,3-glucans and β -1,6-glucans represent two independent classes of polysaccharides. Alkali-extraction, methylation experiments, NMR studies by other groups, and immunoelectron microscopy have demonstrated that the *S. pombe* cell wall contains two β -glucans: 1) a major branched β -1,3-D-glucan, composed of a β -1,3-D-glucan backbone branched with β -1,6-linked β -1,3-D-glucan side chains; and 2) a minor branched β -1,6-D-glucan (or diglucan), composed of a β -1,6-D-glucan backbone extensively branched with β -1,3-linked β -1,6-D-glucan side chains (References: *Anal. Biochem.* 2005, 336:202; *Carbohydr. Res.* 2004, 339:2255; *J. Electron Microsc.* 2003, 52:237; *Yeast* 2001, 18:433; *Experientia* 1985, 41:748; *J. Gen. Microbiol.* 1974, 81:199). While these β -glucans may be interconnected within the cell wall, the nature of such linkages remains unknown. The fission yeast cell wall also appears to contain trace amounts of linear, unbranched β -1,3-D-glucan (Reference 17 in the manuscript; *Mol. Microbiol.* 2007, 65:201; *iScience* 2024, 27:110477). In addition, the *S. pombe* cell wall consists primarily of just α -1,3-glucans and β -1,3-glucans (up to 80-85% of the cell wall), with the β -1,6-glucans being a minor polysaccharide that only represents 4-5% of the cell wall, even lower than another minor polysaccharide, the mannan, which represents 10-12% of the cell wall.

Based on the established types of β -glucans in *S. pombe*, the description of the results derived from the NMR analysis is

currently inaccurate and difficult to follow. For example, in page 9, lines 215-225, the authors state: "...the signals corresponding to beta-1,3-glucan and beta-1,6-glucan (Fig. 5a) were reduced within the mobile domain... The compositional changes were more pronounced in the bgs3-1 ghs2-2 double mutant, which exhibited an extensive loss of beta-1,3- and beta-1,6-glucans, and near-complete depletion of mobile carbohydrate components." However, the right diagram in Figure 6e does not clearly illustrate an extensive loss of beta-1,3-glucans (Bs) or beta-1,6-glucans (H) in the mobile fraction. It remains unclear whether these beta-glucan signals correspond to backbones, specific branches, or the total polysaccharide population. Similarly, in lines 229-231, the authors describe the 2D NMR results by stating that the rigid core "lacked detectable linear beta-1,6-glucan." Given that all the beta-1,6-glucan in the fission yeast cell wall is natively highly branched, does this "linear" beta-1,6-glucan refer to the backbone, the branches, the nascent beta-1,6-glucan, or the general branched beta-1,6-glucan? The manuscript would benefit significantly from a more precise and simplified rephrasing of the NMR data, explaining how the changes detected in polysaccharide signals correlate with structural alterations in the live cell wall. The authors should consider simplifying the relative molar composition shown in Figure 5f, perhaps adopting the color-coding scheme used in Jacob et al. (PNAS 2025 Vol. 122 No. 29 e2505509122) to improve legibility. Notably, the authors did not detect linear beta-1,6-glucan (H) in the rigid core of the wild-type cell wall in that previous study; the reason for this discrepancy should be addressed. Additionally, the authors should explain in the text the structural difference between beta-1,3-glucans Ba and Bb, and what they represent in the network of branched beta-1,3-glucan in the live cell wall. The manuscript would also benefit notably with a table showing the total amount of each polysaccharide in the cell wall of wild type and each of the single and double mutant strains at both permissive and restrictive temperatures, i.e. linear beta-1,3-glucan, branched beta-1,3-glucan, beta-1,6-glucan, alpha-1,3-glucan, and galactomannoproteins.

In page 10, lines 234-236, the authors affirm that the mobile fraction of the double mutant was "dramatically remodeled... with a marked reduction in beta-1,3- and beta-1,6-glucans." This observation fails to distinguish between the beta-1,6 linkages that anchor beta-1,3-glucan side chains to the backbone and the linkages comprising the beta-1,6-glucan polymer itself. Consequently, the claim that Ghs2 activity is specifically required for the formation of beta-1,6 linkages on beta-1,3-D-glucan side chains appears insufficiently supported. While the reduction of branched beta-1,3-glucan in the bgs3-1 single mutant was accompanied by an increase in linear beta-1,3-glucan, this is not explicitly explained for the double mutant. Since Bgs3 is not a transglycosylase, its loss of function should theoretically reduce beta-1,3-glucan chain length rather than branching frequency. Although erroneously explained, the text assumes that Ghs2 transfers linear beta-1,3-glucan branches to a beta-1,3-glucan backbone via a beta-1,6 linkage. However, the ghs2-2 single mutant was notably excluded from the 2D NMR experiments. Therefore, the current results could possibly suggest that Bgs3 also plays a role in the attachment of beta-1,3-glucan side chains to the backbone.

More importantly, the 2D NMR experiments were conducted using cell walls derived from cells incubated at the restrictive temperature (presumably 36°C) for 4 hours. Consistent with the methodology in Figures 1 and 2, these experiments require essential controls, specifically cell walls from the wild-type, single, and double mutants incubated at the permissive temperature. Similarly, if the 1D NMR analyses were also performed under restrictive conditions, the corresponding permissive-temperature controls are currently lacking and should be presented.

The experiments involving the D75-4590 drug are pivotal for establishing a direct or indirect relationship between Ghs2 and beta-1,6-glucan synthesis. In page 10, lines 249-251, the authors state: "Both bgs3-1 and ghs2-2 mutants, but not the temperature-sensitive allele of bgs1 (cps1-191), were hypersensitive to D75-4590 and exhibited a more severe accumulation of cell wall material than D75-4590-treated wild-type cells." However, the authors do not present standard hypersensitivity assays (e.g., growth inhibition curves of mutant strains compared to wild-type). Instead, they only show that the mutants exhibit a more pronounced phenotype. Comparative susceptibility assays across all four strains are necessary. Furthermore, since D75-4590 has not been previously characterized in fission yeast (with existing studies limited to *Saccharomyces cerevisiae* and *Candida* sp.), the authors should clarify how the drug concentration and time of treatment were determined. It is unclear whether wild-type cells continue to proliferate under these conditions while the mutants do not, or if a Minimal Inhibitory Concentration (MIC) was established. Does a 6-hour treatment with 1 mM induce a maximal phenotype in the wild-type strain? Notably, the bgs1 mutant appears to exhibit more significant cell wall accumulation at the cell poles than the wild-type; the percentage and thickness of this polar accumulation should be quantified for all four strains in Figure 6a, once the suggested susceptibility assays are performed.

The authors conclude that these findings provide evidence that inhibition of beta-1,6-glucan synthesis accounts for the observed cell wall defects. However, the drug's effect on the wild-type is markedly reduced compared to the aberrant cell wall deposition phenotype of bgs3-1 and ghs2-2 mutants alone (Figure 2a). The authors should evaluate a range of concentrations to identify a treatment that faithfully mimics the mutant phenotypes, perform a comparative resistance assay to evaluate if the MIC of D75-4590 is much higher in the Ghs2-Y478I than in the wild-type strain, and subsequently verify if the resistance mutation suppresses the phenotypes caused by the drug. Additionally, analyzing the cell wall accumulation in a bgs3-1 strain carrying the Ghs2-Y478I substitution would provide valuable insights.

1D NMR analysis reveals that beta-1,6-glucan chains in the mobile phase are reduced in the single mutants and extensively depleted in the double mutant. Notably, the ghs2-1 single mutant was excluded from the 2D NMR experiments without justification in the text. Although the authors assert in the abstract and throughout the manuscript that Ghs2 is required for the synthesis/production of beta-1,6-glucan chains, I believe this is incorrect because Ghs2 presents homology with transglycosylases, which are involved in branching activity, and therefore it cannot be simultaneously a glucan synthase (glycosyltransferase). The reduction in beta-1,6-glucan content is likely an indirect effect, as for example a compensatory mechanism downregulating its synthesis, or defect in beta-1,6-glucan attachment to the cell wall being released to the medium. The indirect relationship of Ghs2 with the beta-1,6-glucan has not been sufficiently demonstrated, particularly given the lack of appropriate D75-4590 treatments in wild-type and resistant mutant strains. To date, a specific beta-1,6-glucan

synthase activity has not been identified, nor has the site of beta-1,6-glucan synthesis been localized. Because beta-1,3-glucan side chains may be covalently linked to the beta-1,6-glucan polysaccharide, the observed loss of this polymer could represent an indirect effect; specifically, the absence of beta-1,3-glucan branches might impair the maintenance or retention of beta-1,6-glucan within the cell wall matrix, as proposed by the authors in pages 12–13, lines 309–312. Given that this hypothesis is the basis of the proposed cell wall model, the manuscript would benefit significantly from the additional genetic testing of potential beta-1,6-glucan synthase candidates (e.g., *S. pombe* homologs of the KRE family). Alternatively, the authors should revise their terminology, substituting "synthesis/production" with "accumulation" or "maintenance" throughout the text. The fact that bgs3-1 exhibits a loss of beta-1,6-glucan similar to ghs2-2 further suggests an indirect effect.

Furthermore, while the study relies on AF3 structural models and whole-cell NMR to propose the transglycosylase activity of Ghs2, an in vitro enzymatic activity assay is required to unequivocally confirm the mechanism proposed in Figure 6d. The authors are strongly encouraged to attempt the purification of the extracellular GH16 domain of Ghs2 and conduct transglycosylation assays with oligosaccharide substrates, utilizing the single mutants (T205P, F449L, E414A, or E419A) described in Figure 4. If biochemical reconstitution proves unfeasible, the authors must moderate their conclusions, explicitly stating that the proposed transglycosylase activity is predictive and phenotypic rather than biochemically demonstrated in in vitro assays with isolated Ghs2 protein.

Regarding data visualization, the schemes in Figure 6e attempt to integrate wall thickness measurements with compositional and structural information from solid-state NMR. However, the composition and structure are extremely difficult to compare due to the use of similar color palettes for different beta-glucan chains. The manuscript would benefit greatly from a table or a comparative figure summarizing the distinct differences between the primary polysaccharides in the mobile and rigid fractions across the various strains.

A further issue regarding the schematics in Figure 6e is the methodology used for measuring cell wall thickness in the double mutant (bgs3-1 ghs2-2). The transmission electron microscopy (TEM) data in Figure 2d only displays single mutants, omitting the double mutant entirely. These TEM images must be included in Figure 2 to allow for a direct comparison and to determine if the outer galactomannan layer is more severely affected than in the single mutants. Also, in page 10, lines 235–237, the authors state that: "The mobile fraction of the double mutant was dramatically remodeled compared to wildtype, with a marked reduction in beta-1,3- and beta-1,6-glucans and the loss of alpha-1,2- and alpha-1,6-linked mannose residues, indicating disruption of the outermost galactomannan layer (Fig. 5e, f)." These biochemical observations further accentuate the necessity of including TEM images for the double mutant in Figures 2b to 2e. Furthermore, as cell wall thickness appears to be measured specifically at sites of abnormal deposition, it should be indicated in the figure the site selected to measure the thickness, and this must be explicitly stated in the figure legend for Figure 6e. Additionally, while the legend for Figure 2d mentions a total sample size of $n = 100$, the authors must specify the exact number of cells analyzed for each individual strain.

In page 11, lines 269–275, the authors state: "Here, we propose Ghs2 as the catalytic factor for branched beta-1,6-glucan synthesis in *S. pombe* and show that it partners physically and functionally with the beta-1,3-glucan synthase Bgs3." In my view, the current results do not demonstrate direct catalytic activity for Ghs2 in beta-1,6-glucan synthesis, mainly because Ghs2 shares homology with transglycosilases, and therefore it hardly would be a beta-1,6-glucan synthase (glycosyltransferase) and much less it could be both enzyme types. The fact that Bgs3 exhibits identical phenotypes regarding beta-1,6-glucan suggests an indirect effect, as it has been observed following the loss of the beta-1,3-glucan synthase Fks1 in other yeasts (Yeast 2002, 19:671–90). The authors further suggest that the Ghs2 GH16 domain is "ideally positioned to add beta-1,6 branches to newly synthesized beta-1,3-glucan chains." This remains conceptually problematic. First, the branches of beta-1,3-glucan are not themselves chains of beta-1,6-glucan, as erroneously stated in the text and depicted in the architecture model in Figure 5a. As previously explained, beta-1,3-glucan consists of a beta-1,3-backbone with beta-1,3-side chains; only the single glucose residue connecting the branch to the backbone involves a beta-1,6-linkage. Moreover, it is highly improbable that Ghs2 possesses both a glycosyltransferase activity for beta-1,6-glucan production and a transglycosylase activity for transferring beta-1,3-glucan chains via beta-1,6-linkages to a beta-1,3-backbone, as suggested in Figure 6d. The frequency of such linkages would be residual compared to the extensive chains found in branched beta-1,6-glucan. The authors appear to be mistaking the synthesis of beta-1,6-glucan with the branching of beta-1,3-glucan, which leads to significant confusion. I strongly recommend that the authors describe the direct or indirect effects of the mutations on beta-1,6-glucan and branched beta-1,3-glucan as separate, and distinct phenomena.

MINOR POINTS

In page 2, lines 31–32, the authors state: "we identify Ghs2, a glycoside hydrolase 16 (GH16) domain-containing transmembrane protein." Given that the catalytic activity of Ghs2 has not been biochemically demonstrated, the forms "predicted" or "putative" should be added to this type of sentences. Similarly, in lines 38–39, the authors claim that "genetic and pharmacological evidence further indicate that Ghs2 acts on newly synthesized beta-1,3-glucan to introduce beta-1,6-linked branch points." The experiments involving D75-4590 and the Ghs2-Y478I mutation do not provide evidence to conclude that Ghs2 exhibit a transglycosylase activity responsible for beta-1,6 linkages in branched beta-1,3-glucan. Those data would suggest an indirect relationship between Ghs2 and beta-1,6-glucan synthesis but do not demonstrate transglycosylase activity. The abstract should be revised accordingly to reflect these limitations.

In page 3, lines 73–74, the authors refer to "an outer layer composed mainly of linear beta-1,6-glucans." It is unclear how the authors arrived at this conclusion. All fungal cell wall beta-1,6-glucans are described as branched, no linear beta-1,6-glucan has been described, unless it is considered the nascent beta-1,6-glucan before branching maturation. In *S. pombe*, chemical and glucanase-driven cell wall fractionation and NMR spectroscopy have established that beta-1,6-D-glucan is constitutively branched, consisting of a beta-1,6-D-glucan backbone extensively modified (up to 75% of glucose residues) with beta-1,3-

linked beta-1,6-D-glucan side chains. This terminology should be corrected throughout the text, and a detailed explanation should be included in the introduction, citing the studies by Magnelli et al. (Anal. Biochem. 2005) and Sugawara et al. (Carbohydr. Res. 2004). Furthermore, the author's earlier statement contradicts their subsequent claim in page 4, lines 83-84, that "none has been demonstrated to directly catalyze beta-1,6-glucan branches or chains, and the enzymatic mechanism underlying beta-1,6-glucan assembly has remained elusive." This is not correct, in budding yeast, in addition to all the works around the Kre and related proteins, there are some works regarding the enzymatic activity of the beta-1,6-glucan synthesis, Montijn et al. (J. Bacteriol. 1999, 181(24):7414-7420), Vink et al. (Yeast 2004, 15;21(13):1121-31). The manuscript requires internal consistency regarding what is known versus what is hypothesized and what is demonstrated by their NMR analysis.

In page 4, line 88, the statement "Ghs2 is a single-pass transmembrane protein" should be changed to "Ghs2 is a predicted single-pass transmembrane protein," as its topology has not been experimentally verified. Similarly, in lines 91-92, the claim that "Ghs2 is positioned to directly modify newly synthesized beta-1,3-glucan chains, adding beta-1,6 branches" would be more accurate if phrased as: "Ghs2 is positioned to directly transfer a beta-1,3-glucan chain to another nascent beta-1,3-glucan chain via a beta-1,6-linkage."

In page 5, lines 105-196, the authors state that "Total GFP-Bgs3 protein levels were unchanged in ghs2-2 cells compared to wild type... (Supplementary Fig. 1b)." However, the specific band for GFP-Bgs3 is barely discernible in the provided immunoblot. Consequently, it is difficult to confirm whether total Bgs3 levels are truly equivalent between wild-type and ghs2-2 cells. To improve the detection of the integral membrane protein Bgs3, the authors should perform a direct immunoblot using the specific protocol for integral membrane proteins cited in their methods section, as it has been shown in other works in which Bgs3 and other Bgs proteins are well detected. Furthermore, the immunoblots for GFP-Bgs3 in Figure 3b are of poor quality and should be repeated to ensure reliable detection.

Regarding protein stability, the thermosensitive allele bgs3-1 exhibits undetectable Ghs2 protein levels at both permissive and restrictive temperatures (Supplementary Figure S1). A similar loss of signal is observed for GFP-Ghs2 fluorescence in cells growing at the assumed permissive temperature (Figure 1d). Did the authors assess GFP-Ghs2 localization or stability at lower (19°C) or intermediate (28-30°C) temperatures to determine the threshold of this instability?

Regarding Figure 2a, the phenotypes depicted in the panels must be quantified. The authors utilize Methyl Blue rather than conventional Aniline Blue that has been extensively used and described to bind beta-1,3-D-glucan in budding yeast. It should be clarified which specific polysaccharide this fluorochrome is assumed to bind to, and it should be shown whether Methyl Blue labels the same cell wall structures as the more conventional dye Aniline Blue.

In page 6, lines 134-139, the authors assert: "Given that ghs2-2 and bgs3-1 exhibited the same defects, it was not surprising that the double mutant was synthetically sick... These results suggested that Bgs3 and Ghs2 act in the same pathway..." This interpretation is logically inconsistent. If two proteins function strictly within the same linear pathway, their combined loss-of-function should result in a phenotype identical to the single mutants, rather than an exacerbated or additive defect. The authors should discuss the underlying reasons for this synthetic sickness, as it may suggest partially redundant functions or parallel roles in maintaining cell wall integrity.

Since the target of D75-4590 in other fungal species appears to be Kre6, another GH16 domain-containing protein, it would be highly informative if the authors provided a detailed comparison of the amino acid sequence and structural similarities between Ghs2 and Kre6. Furthermore, it is well-established that *S. cerevisiae* Kre6 primarily localizes to the endoplasmic reticulum (ER), though a fraction is detected at the plasma membrane of the bud and within trafficking vesicles (J. Biol. Chem. 2010, 286:7429-7438). While the exact site of beta-1,6-glucan synthesis remains elusive, Humbel et al. (Reference 17) provided evidence that in *S. pombe*, beta-1,6-glucan could be synthesized within the ER-Golgi system and subsequently exported. In this manuscript, Ghs2 does not appear to localize to the ER, instead it co-localizes with Bgs3 in the plasma membrane and cytoplasmic vesicles. These data suggest further discussion to reconcile the authors' proposed plasma membrane "synthase-remodeler" model with the proposed secretory pathway-mediated synthesis of beta-1,6-glucan.

In Figure 5f, the authors provide the molar composition of the rigid fraction for the bgs3-1 ghs2-2 double mutant but omit the corresponding data for the mobile fraction. This omission is significant, as the authors explicitly state in page 10, lines 235-237, that: "The mobile fraction of the double mutant was dramatically remodeled compared to wild type, with a marked reduction in beta-1,3- and beta-1,6-glucans and the loss of alpha-1,2- and alpha-1,6-linked mannose residues, indicating disruption of the outermost galactomannan layer (Fig. 5e, f)." Despite the reported general disruption of mobile polysaccharides in the bgs3-1 ghs2-2 strain, the molar composition of the residual mobile polysaccharides (specifically Ab1, H1, B1, H2, and B2) depicted in Figure 5e must be quantified and included in Figure 5f. These biochemical observations further accentuate the necessity of including TEM images for the double mutant in Figures 2b to 2e. In addition, the proposed loss of external galactomannan layer in the double mutant should be confirmed in wild type, single and double mutants with specific fluorescent-conjugated lectins like Concanavalin A, which presents affinity for terminal mannosyl and glucosyl residues, or the lectin from *Bandeiraea simplicifolia*, which presents affinity for terminal galactosyl residues.

In page 11, lines 281-282, the authors state: "Our solid-state NMR analyses demonstrated that loss of either Ghs2 or Bgs3 led to reduced beta-1,3-glucan branched beta-1,6-glucan." This statement further mixes the synthesis of beta-1,6-glucan with the branching of beta-1,3-glucan, resulting in significant conceptual confusion. Additionally, the term "beta-1,3-glucan branched beta-1,6-glucan" appears to be a contradiction. Based on the preceding results section dedicated to NMR analysis, the authors do not describe such a species; instead, they refer to beta-1,6-glucan and branched beta-1,3-glucan (BBR). I assume the authors are referring to the former beta-1,6-glucan (H), but this must be clarified. Furthermore, there is a major discrepancy in experimental design: the 2D NMR experiments utilized cell walls from the bgs3-1 single mutant and

the bgs3-1 ghs2-2 double mutant, while the single ghs2-2 mutant was notably omitted. This leads to an ambiguity in the visual evidence, as it is unclear whether the right panel of Figure 6e (labeled as "Bgs3-Ghs2 defective") represents the cell wall architecture and composition of the single mutants bgs3-1 or ghs2-2, independently, or that of the double mutant (bgs3-1 ghs2-2). If the model is intended to represent the single mutants, the authors appear to assume that both bgs3-1 and ghs2-2 possess an identical cell wall architecture. While 1D NMR analyses (Figures S4-S6) suggest their compositions are very similar, this assumption ignores the strong genetic interaction demonstrated in Figures 2a and S2, and the increased cell wall alterations of the double mutant. Furthermore, since the cell wall of the ghs2-2 mutant was not independently examined via 2D NMR, there is no experimental basis to assume its structural identity with bgs3-1. The authors must explicitly label the strains used in each panel to ensure the data accurately support their mechanistic claims.

In page 12, lines 295-296, the authors state: "A key outstanding question however is how the Ghs2 GH16 domain generates the beta-1,6-glucan branches." This phrasing remains technically imprecise. In the context of beta-1,3-glucan architecture, the "branches" are themselves beta-1,3-glucan side chains that are connected to the main backbone via a single beta-1,6-glycosidic bond at the junction point. Referring to these as "beta-1,6-glucan branches" erroneously implies that the branches consist of beta-1,6-linked glucose polymers, rather than beta-1,3-linked chains. The authors must use more rigorous chemical nomenclature to distinguish between the linkage type at the branch point and the polymer identity of the side chain itself.

In page 12, lines 300-301, the authors state: "If this pattern holds true for *S. pombe*, this architecture implies that Ghs2 activity is usually limited to branch initiation or very short elongation." This statement inaccurately implies that Ghs2 possesses synthase activity, which has not been experimentally demonstrated. It is essential to distinguish between a synthase (glycosyltransferase), which catalyzes the de novo polymerization of glucose residues, and a transglycosylase, which remodels existing polymers by breaking and reforming glycosidic bonds. "Branch initiation" or "elongation" in a biosynthetic context typically refers to the action of a synthase. If the authors propose that Ghs2 functions as a transglycosylase, they should rephrase this to clarify that it involves the transfer of pre-existing glucan fragments rather than the elongation of a new chain.

In page 12 (lines 302-306), the authors suggest: "...beta-1,6 branches introduced by Ghs2 could serve as substrates or nucleation sites for additional enzymes that elongate or reorganize beta-1,6-linked structures." As previously argued, this hypothesis is biochemically imprecise. In the established architecture of beta-1,3-glucan, the "branches" are themselves beta-1,3-glucan chains tethered to the main backbone via a single beta-1,6-glycosidic bond at the junction point. Therefore, these branches, composed of beta-1,3-linked glucose residues, cannot serve as substrates for enzymes specialized in the elongation or reorganization of beta-1,6-linked polymers. For a beta-1,6-linked structure to be elongated, the substrate must possess a beta-1,6-linked terminus.

In page 12, lines 309-310, the authors state: "Alternatively, beta-1,6-branched beta-1,3-glucan may be required for the stable incorporation or retention of linear beta-1,6-glucan within the wall matrix." As previously noted, beta-1,6-glucan in fission yeast is constitutively branched; therefore, the reference to "linear" beta-1,6-glucan should be corrected or demonstrated as a new not yet described polysaccharide for accuracy. Furthermore, the model presented in Figure 6d lacks biochemical precision. It appears to depict Ghs2 joining individual glucose residues (de novo) to the nascent linear beta-1,3-glucan via a beta-1,6 linkage. To accurately represent a potential transglycosylase, the model should instead show Ghs2 catalyzing the attachment of preformed linear beta-1,3-glucan chains to the nascent beta-1,3-glucan backbone via a beta-1,6-linkage of a glucose residue.

In page 13 (lines 332-336), the authors state: "...suggesting that distinct classes of auxiliary factors may regulate different glucan synthases and support a division of labor in cell wall construction: Bgs1 with Sbg1 produces septum-specific beta-1,3-glucan, Bgs4 supplies the lateral wall beta-1,3-glucan, and Bgs3, together with Ghs2, constructs branched networks at the division site and the lateral cell wall." This paragraph is wrong and represents an oversimplification of the physiological roles of Bgs1 and Bgs4. Like Bgs3, both Bgs1 and Bgs4 localize to the cell poles (tips) and the septum, and they are involved in both cell wall, primary septum (Bgs1 and Bgs4) and secondary septum (Bgs4) biosynthesis (e.g., Reference 81 for Bgs1; Reference 20 and J. Cell Biol. 2013, 203:265-82 for Bgs4).

Finally, in page 16, lines 399-405, the authors use the phrasing "30 ODs of cells were lysed..." or "120 ODs of cells..." This terminology is imprecise and wrong. It is unclear whether the cultures were harvested during the logarithmic or stationary phase, or what the actual absorbance and cell count used for the lysates was. The authors should adopt more standard quantitative descriptions, specifying the growth phase and the exact number of harvested cells (e.g., 1×10^8 cells) to ensure experimental reproducibility. In addition, and more important, OD is not accepted biochemically for many years. The correct nomenclature is "Absorbance" and therefore OD595 or any other wavelength used should be changed to A595 or the corresponding wavelength.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I am satisfied with the authors' responses to my initial feedback and with the resulting revisions made to the new manuscript.

I therefore support the publication of this manuscript.

Reviewer #2

(Remarks to the Author)

The revised version has adequately addressed most of my questions. The only remaining issue to be discussed is providing final proof that the enzymes are sufficient to carry out the proposed reactions. The authors have provided several lines of evidence for this, and one way to achieve this would be to purify the enzymes and carry out the reactions *in vitro*. I agree with the authors that such experiments are difficult to carry out because, in addition to purifying the extracellular GH16 domain of Ghs2 for *in vitro* biochemical assays, the reconstitution of the membrane-associated Ghs2-Bgs3 complex is also required. Therefore, at this stage, I believe it is sufficient to emphasize that the final proof is missing. This is now stated in the revised version: The mechanism remains a model supported by the available data, and definitive biochemical reconstitution of Ghs2 and the Ghs2-Bgs3 complex will be required to establish the precise enzymatic activities involved.

Reviewer #3

(Remarks to the Author)

Willet et al. have justified and resolved most of the conceptual errors and mechanistic ambiguities raised in the initial version of the manuscript. Although a few of the requested experimental validations could not be fully completed, the authors have provided alternative justifications that preserve the integrity of their primary claims. Therefore, I recommend this manuscript for publication in Nature Communications, conditional upon the correction of the following conceptual inaccuracy, formatting errors and missing references:

1. In the revised Discussion section, the authors state: “Bgs1, together with Sbg1, plays a central role in linear β -1,3-glucan synthesis for primary septum formation, Bgs4 contributes to β -1,3-glucan synthesis in both the septum and lateral cell wall, and Bgs3, together with Ghs2, promotes the formation of branched β -glucan networks...”

This updated paragraph remains imprecise and incomplete as it still misses the multi-spatial role of these synthases. Experimental evidence has established that Bgs1 does not function exclusively at the septum; it also localizes to active growth poles (cell tips) and plays a fundamental role in maintaining polarized growth (as supported by reference 93 of this current manuscript).

Also, references 39 and 40 describe that the deletion of *sbg1* results in lethality accompanied by severe defects in both polarized growth and division septum assembly. These studies proved that Sbg1 and Bgs1 are mutually dependent for their correct localization at cell tips and the division site (i.e., GFP-Bgs1 is cleared from the cell tip plasma membrane in *sbg1* mutants). This simplification also directly contradicts the authors' own statement: “Sbg1 which associates with the β -1,3-glucan synthase Bgs1 and is required for Bgs1's proper localization and function at sites of polarized growth.”

The authors must rewrite this paragraph to explicitly state that the Bgs1–Sbg1 complex is essential for both primary septum formation and polarized growth at cell tips, rather than restricting its functional narrative solely to the septum.

2. Throughout the text, figures, and supplementary materials, the authors must follow the standard fission yeast genetic nomenclature as depicted in Table S1. All wild-type loci must explicitly include the plus symbol as a superscript (e.g., *bgs3*⁺, *ghs2*⁺, *bgs1*⁺).

In the References section, all the gene names, genera, and species must be always italicized, and references to the different types of cell wall glucans should be uniformized by utilizing Greek symbols (α and β). Furthermore, the typographical error in reference 28 (“cell wall (1 $\rightarrow$ 6)-beta-glucan synthesis”) should be corrected.

In the Introduction, the paragraph “The biosynthesis of linear β -1,3-glucans is carried out by the essential integral membrane synthases Bgs1, Bgs3 and Bgs4 as well as the meiosis-specific Bgs2” is attributed to references 21 to 26. I think that several original references for Bgs1 from the Balasubramanian and Ribas's labs are missing in the main text, despite some being included in Table S1. In opposition, reference 21 relates to the alpha glucan synthase Mok1/Ags1 which is unrelated to the synthesis of beta-glucans described in this sentence. If the authors decide to include an independent sentence for the alpha glucan synthase, then they should also add the reference Satoshi Katayama, S. et al. (1999). Journal of Cell Biology, 144 (6): 1173–1186.

Additionally, the original references for *cps1*-191 and *cwg1*-1 mutant alleles are absent in the manuscript and must be incorporated.

In Supplementary Table S1, the genotype *his3*-1 must be changed to *his3*-D1. In addition, the included references the GFP-Bgs1 and GFP-Bgs3 strains Cortès (Cortés in the main text) et al. 2007 and 2002 lack in the main manuscript text. Similarly, as recommended above, the original strains and references for *cps1*-191 and *cwg1*-1 should be included in the table. Lastly, in Table S3 all references, with the sole exception of Chakraborty, A. et al. 2021, are missing from the main manuscript text.

3. In their rebuttal, the authors justify the poor quality and diffuse nature of the GFP-Bgs3 (Figure S1B) band by stating: “Bgs3 is a large (~210 kDa), heavily glycosylated, low-abundance integral membrane protein, which makes its detection by immunoblotting inherently challenging... To our knowledge, only a single other study includes Bgs3 immunoblots (Martin et al., Eukaryotic Cell 2003) [which] used an overexpression system...”

This affirmation is incorrect and ignores established literature. In several publications from Dr. Ribas's or Dr. Balasubramanian laboratories other Bgs proteins that share similar high molecular weights and probably the same glycosylation profiles of Bgs3 have been resolved as sharper and defined bands under the control of their endogenous promoters without the distinctive "smearing" shown here.

Also, the assertion that physiological levels of Bgs3 have never been blotted or analyzed outside of Martin et al. (2003) again is incorrect. Endogenous physiological levels of Bgs3 have been clearly resolved and published in Cortes, J.C.G. et al. (2007). *Molecular Microbiology*, 65: 201-217 (Specifically Figure 2B) or Munoz, J. et al. (2013). *Journal of Cell Biology*, 203(2): 265–282 (Supplementary Figure S1F).

Although technically the immunoblots could be improved, the similar diffuse band and smears in the four lines, together with the detection of GFP-Bgs3 in both wild-type and ghs2-2 cells, are sufficient to support the corresponding conclusion.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

Reviewer's Comments:

Reviewer #1 (Remarks to the Author):

The manuscript is well written, logically structured, and presents a compelling argument. It convincingly demonstrates that the Ghs2 GH16 domain is responsible for linking the β -1,6 chain onto the β -1,3 chain during cell wall assembly in *S. pombe*, and that this enzyme functions in concert with Bsg3.

While the study is primarily directed toward specialists in fungal cell wall biology, it also illustrates, for a broader audience, an important organizational strategy by which complex biological polymers are synthesized and their morphogenesis regulated.

Overall, the manuscript is thoughtfully organized and clearly presented. That said, some aspects of the manuscript could be improved to enhance readability and better address potential questions from readers:

- In the introduction, a simplified schematic illustrating the composition and morphogenesis of the yeast cell wall would greatly help readers engage with the topic.

While we agree that a schematic of yeast cell wall composition and morphogenesis can be helpful for readers who are new to the field, such diagrams are already readily available in several of the review articles cited throughout the manuscript. Because the primary focus of this study is the identification and characterization of the Ghs2-Bgs3 synthase-remodeler module and its role in β -glucan architecture, we believe that including a general overview of cell wall organization would be outside the scope of the present work and would not substantially enhance the presentation of our findings. We have therefore elected not to add an additional introductory schematic.

- Is there any evidence of *in vitro* activity of the Ghs2 GH16 domain on glycan chains?

We thank the reviewer for this question. To our knowledge, *in vitro* enzymatic activity has not yet been demonstrated for any member of the Ghs2/Kre6 protein family. At present, we do not have direct *in vitro* evidence demonstrating enzymatic activity of the isolated Ghs2 GH16 domain on glycan substrates. We have attempted to purify the extracellular GH16 domain of Ghs2 for biochemical assays, however, despite trying multiple expression and purification strategies, we were unable to obtain sufficient quantities of stable, active protein for *in vitro* assays. These efforts are ongoing.

- Regarding the AlphaFold model proposing a direct interaction between Ghs2 and Bsg3: this model appears to rely primarily on interactions between their respective transmembrane helices. It is therefore difficult to assess its reliability. While the conservation of residues on the GH16 side is well described, no information is provided on the residues involved on the TM1–17 region of Bsg3. A genomic modification targeting the transmembrane helix of Bsg3 would have strengthened the hypothesis of a direct membrane-mediated interaction.

The predicted Ghs2-Bgs3 interaction does not rely solely on a single transmembrane contact. Rather, the AlphaFold model identifies multiple distinct interaction interfaces. The first is mediated by the Ghs2 transmembrane helix contacting two Bgs3 transmembrane helices, the second involves the intracellular regions of Ghs2 and Bgs3 and a third interaction interface involved the Ghs2 GH16 domain and the extracellular loops of Bgs3. We have updated the main text to make this clearer to the readers. Our structure-function analyses indicate that both the transmembrane and intracellular interfaces are required, but neither is sufficient on its own to support the interaction.

To further address the reviewer's concern, we have conducted additional analyses of the interaction interface involving the transmembrane helices and identified the Ghs2 and Bgs3 residues predicted to mediate this interaction (Figure S3d). We also added sequence alignments of the residues predicted to mediate the transmembrane interface within Bgs3 helices 6 and 8 in Figure S3e. To test the model, we generated a *bgs3-5A* allele in which the conserved residues W670, L673, I1334, I1338, and L1341 were mutated to alanine (Figure S3e, f). Consistent with the structural model, *bgs3-5A* did not support growth when expressed in *bgs3-1* cells (Figure S3f). Reciprocally, we mutated the residues in the predicted Ghs2 transmembrane helix that contact these Bgs3 residues (Figure S3d). Specifically, L177, L184, and F185 were changed to alanine to generate the *ghs2-3A* allele (Figure S3f). This construct likewise failed to support growth (Figure S3f).

Together, these reciprocal mutational analyses provide functional support for the predicted membrane-embedded interaction interface and strengthen the conclusion that direct contacts between the transmembrane regions of Ghs2 and Bgs3 are important for complex function.

- Figure 5A: glycan chains H and B are shown, but chain A is missing, making it difficult to follow the nomenclature used in the other panels.

We have updated Figure 5a (now Figure 5d) as recommended.

- The change in scale between panels D and E complicates interpretation, especially since the choice of the two regions is neither explained in the legend nor indicated (e.g., with dashed lines) in panel E.

We have updated the panels shown in Figure 5 and provided better rationale within the main text for showing these spectra.

- Regarding the Ghs2 Y478I mutant, it would have been informative to include the NMR spectrum of this strain to confirm its effect on glycan chain synthesis.

We do not expect that this allele that changes the sensitivity to an inhibitor but is otherwise like wildtype would have a change in the cell wall composition compared to wildtype. Thus, we did not perform this experiment.

- In Figure S4, NMR data show a strong decrease in protein signals in the flexible phase of the mutants, with little change in the rigid phase (Figure S5). Is this reduction in attached proteins confirmed by other methods, and what is its underlying cause?

Our data instead show a pronounced decrease in protein signal in the rigid fraction (Figure S4), whereas the protein in the mobile fraction (Figure S5) is largely unchanged. We interpret the decrease in protein signals in the rigid core as most likely reflecting the loss or impaired incorporation of cell wall proteins that are initially GPI-anchored and subsequently become covalently crosslinked into the cell wall matrix, typically through β -1,6-glucan linkages (Pittet and Conzelman, *Biochim. Biophys. Acta* 2007; De Groot et al., *Yeast* 2003). Disruption of β -1,6-glucan is therefore expected to reduce the stable incorporation of these proteins into the rigid core. We note that we did not validate this observation with alternative methods, but it is consistent with the well-established role of β -1,6-glucan in mediating the covalent attachment of GPI-anchored proteins to the fungal cell wall (Pittet and Conzelman, *Biochim. Biophys. Acta* 2007; De Groot et al., *Yeast* 2003).

Finally, on page 9, line 232: is the detection of the flexible domain truly related to T₁ (spin-lattice) relaxation effects, or could it instead (or additionally) arise from T₂ (spin-spin) relaxation?

We thank the reviewer for this important point. The selective detection of the mobile domain in the 2D ¹³C DP refocused J-INADEQUATE experiment is primarily governed by ¹³C T₁ (spin-lattice) relaxation. To detect only the mobile components, a short recycle delay of 2s was used, which detects signals from species with faster T₁ recovery while attenuating rigid domains with longer T₁. In contrast, T₂ (spin-spin) relaxation is not expected to contribute significantly to the observed spectral selectivity under these experimental conditions, as the total J-evolution (carbon-carbon) and double quantum (DQ) excitation time are only a few milliseconds (8-10 ms). Therefore, the observed spectral selectivity of detecting mobile or flexible domain is primarily governed by T₁ driven differences in magnetization recovery arising from the short recycle delay of 2s, while any contribution from T₂ relaxation is minimal and does not significantly affect the observed spectra.

Reviewer #2 (Remarks to the Author)

In contrast to β -1,3-glucan it is a long standing question how in a fungal cell wall β -1,6-glucan is synthesized. There is no clearly defined synthase enzyme that both polymerizes and branches. This important unsolved question is addressed in the manuscript.

The authors used the fission yeast *Schizosaccharomyces pombe* as the experimental organism. By using reporter fusions and IP the authors identified Ghs2 which is a glycoside hydrolase 16 (GH16) domain-containing transmembrane protein, as an obligate partner of the β -1,3-glucan synthase Bgs3. They showed that Ghs2 and Bgs3 co-localize at sites of polarized growth and by IP they showed that the proteins physically associate in vivo. Structure-guided modeling positioned the Ghs2 catalytic domain proximal to the predicted Bgs3 glucan extrusion pore. This alphafold-based modelling provided a theoretical frame suggesting coordinated polymer synthesis and remodeling. By applying solid-state NMR analyses it was demonstrated that both Ghs2 and Bgs3 are required for β -1,6-glucan production. By using inhibitors of α -1,6-glucan synthesis and by analysis of generated mutant strains the authors concluded that Ghs2 acts on newly synthesized β -1,3-glucan to introduce β -1,6-linked branch points.

We thank the reviewer for this thoughtful and encouraging assessment of our work. We greatly appreciate the recognition that the synthesis of β -1,6-glucan remains a longstanding and fundamental unanswered question in fungal cell wall biology, and that our study addresses this important gap. We are particularly encouraged that the reviewer finds the genetic analysis to be well executed and that the data supporting the interdependence of Ghs2 and Bgs3, as well as their physical and functional association, are convincing. We also appreciate the acknowledgment of our integrative approach, combining cell biology, biochemical analysis, structural modeling, and solid-state NMR to

develop a mechanistic framework for β -1,6-glucan formation. We have carefully considered these positive comments, which have helped us further refine and clarify our conclusions.

Major comments

The question how β -1,6-glucans in the fungal cell wall are synthesized is unsolved. Thus, the authors addressed an important question for fungal biology. The genetic analysis is very well performed. The authors clearly demonstrated the interdependence of Gsh1 and Bgs3 and also convincingly showed that there is a synthase-modifier pair involved in β -1,6-glucan synthesis.

However, I have some major questions and caveats that I recommend to be addressed:

1. The authors observed that (lines 113-115) 'GFP-Ghs2 expression was reduced to 10 % of wild-type levels in *bgs3-1* cells at both temperatures by immunoblotting'. The authors provide no explanation for this surprising observation. How could the enzymes be involved in the expression of other enzymes? I also do not think it is possible to measure expression of genes by immunoblot. Alternatively, did the authors check whether the formation of the heterodimer prevents degradation which also could explain the reduction of proteins.

We thank the reviewer for this important point. We agree that our original wording was imprecise. We have revised the text to clarify that we measured protein levels, not gene expression, by immunoblotting (lines 111-114). Regarding the reduction in GFP-Ghs2 levels in *bgs3-1* cells, we agree that this observation does not imply that Bgs3 directly regulates Ghs2 expression. Rather, our data are consistent with a post-translational effect. One plausible explanation is that the interaction between Ghs2 and Bgs3 contributes to the stability of Ghs2, and that disruption of Bgs3 function in the *bgs3-1* mutant leads to increased turnover or mis-localization of Ghs2. While we did not directly test whether heterodimer formation protects Ghs2 from degradation, our localization data support this interpretation: despite reduced protein levels, GFP-Ghs2 remains correctly localized at 25°C but becomes largely cytoplasmic at 36°C, consistent with impaired stabilization and/or trafficking under restrictive conditions. We have added text to the manuscript (lines 111-114) to clarify this interpretation and to note that altered protein stability is a likely explanation for the reduced Ghs2 levels in *bgs3-1* cells.

2. Whereas the results on the interaction of Ghs2 and Bgs3 are convincing, and also that they influence the cell wall and β -1,6-glucan formation, it is less clear to me whether the enzymes are really sufficient to provide the β -1,6-linkage in the cell wall. This caveat is based on the solid state NMR which, to me, is unclear. Even under the non-permissive temperature there apparently is production of β -1,6-glucan. How do the authors explain this contradiction? In the discussion the authors write about reduction of β -1,6-glucan. However, according to their hypothesis the heterodimer should be sufficient for β -1,6-glucan formation and their lack should halt the synthesis.

There is actually no contradiction here because the experiment does not measure the production of new material specifically. It measures all material. Thus, even if all new production of β -1,6-glucan ceased at the non-permissive temperature, it exists within the walls before the temperature shift and would not be expected to vanish during the temperature shift. The entire cell wall does not turn over in that amount of time. An additional factor is that the mutants are not null alleles. Thus, some residual synthesis by the mutant proteins may occur. Therefore, persistence of these signals does not contradict our model, but rather reflects the contribution from pre-existing glucan species and possibly residual enzyme activity in the mutants.

3. To finally prove that the enzymes are sufficient for production of β -1,6-glucan and also to prove that the enzymes are in fact able to synthesize the carbohydrate polymer, this proof can only be provided by an in vitro assay using the purified enzymes. For example, this was published for the β -1,3-glucan synthase. Without such an assay and taking into account the NMR analysis that provides a strong hint but no proof, the final conclusion that the enzymes are sufficient for β -1,6-glucan synthesis is not given. Such an in vitro enzyme assay is strongly recommended.

We agree with the reviewer that a successful biochemical reconstitution would provide the most direct test of enzyme sufficiency and would be valuable for defining the precise catalytic activity of the Ghs2–Bgs3 complex. Importantly, our model does not propose that Ghs2 functions as a glucan synthase that polymerizes glucan de novo from nucleotide-sugar substrates. Rather, Ghs2 is proposed to act as a GH16 transglycosylase that modifies glucan chains produced by Bgs3.

We have tried hard to purify the extracellular GH16 domain of Ghs2 for in vitro biochemical assays but were unable to obtain sufficient quantities of stable, active protein for characterization. Likewise, reconstitution of the membrane-associated Ghs2-Bgs3 complex remains technically challenging and is the subject of ongoing work. We note that, to our knowledge, direct biochemical reconstitution of the catalytic activity of any Kre6/Ghs2-family protein has not yet been reported, underscoring the technical difficulty of studying this class of membrane-associated GH16 enzymes.

Overall, our conclusions are based on multiple independent lines of evidence, including genetics, structural modeling, cell biological analyses, pharmacological perturbation, and solid-state NMR, which collectively support a model in which Ghs2 and Bgs3 cooperate in the formation of β -1,6-branched β -1,3-glucan. We have revised the manuscript to more clearly emphasize that this mechanism remains a model supported by the available data and that definitive biochemical reconstitution of Ghs2 and the Ghs2-Bgs3 complex will be required to establish the precise enzymatic activities involved.

Reviewer #3 (Remarks to the Author)

The manuscript by Willet et al. investigates a fundamental unresolved challenge in fungal cell wall biology and biochemistry: the molecular machinery responsible for the formation of beta-1,6-glucan linkages within the primary beta-1,3-glucan network to form beta-1,3-glucan branches. The authors show that the resulting branched beta-1,3-glucan is essential for the maintenance and accumulation of beta-1,6-glucan in the *Schizosaccharomyces pombe* cell wall. They further suggest that the remodeling protein Ghs2 (which contains a putative glycoside hydrolase 16 catalytic domain) and the beta-1,3-glucan synthase Bgs3 constitute an indispensable physical and functional module. Despite certain criticisms, the multidisciplinary approach employed is excellent. The integration of classical genetics, utilizing temperature-sensitive mutants and transmembrane chimera analyses, with structural predictions via AlphaFold3 (AF3) and, most significantly, solid-state Nuclear Magnetic Resonance (NMR) in intact cells, lends substantial experimental weight to the study. The conceptualization of a "synthase-remodeler module" represents a genuine paradigm shift that is expected to have a high impact in fungal microbiology and open new avenues for the rational design of antifungals. However, the manuscript requires the correction of several important fundamental conceptual errors about the biochemical structure of the different types of glucans and the function of the glucan remodeling enzymes, as well as the clarification of specific mechanistic limitations and the inclusion of critical experimental controls, prior to its final publication in a journal such as Nature Communications.

MAJOR POINTS

1. The author's description of the various cell wall beta-glucans is currently unclear and wrong. In page 3, lines 69-71, the authors state: "The *S. pombe* cell wall consists primarily of alpha-1,3-glucans, beta-1,3-glucans, beta-1,6-glucans, both as side branches on beta-1,3 chains and as linear polymers." The assertion that beta-1,3-glucans and beta-1,6-glucans are both side branches on beta-1,3 chains is incorrect. The beta-1,3-glucan only contains branches of beta-1,3-glucan, beta-1,6 is only the linkage to attach the beta-1,3-glucan side chain. Furthermore, the diagram depicting beta-glucans in Figure 5a is erroneous and leads to confusion. beta-1,3-glucans and beta-1,6-glucans represent two independent classes of polysaccharides. Alkali-extraction, methylation experiments, NMR studies by other groups, and immunoelectron microscopy have demonstrated that the *S. pombe* cell wall contains two beta-glucans: 1) a major branched beta-1,3-D-glucan, composed of a beta-1,3-D-glucan backbone branched with beta-1,6-linked beta-1,3-D-glucan side chains; and 2) a minor branched beta-1,6-D-glucan (or diglucan), composed of a beta-1,6-D-glucan backbone extensively branched with beta-1,3-linked beta-1,6-D-glucan side chains (References: Anal. Biochem. 2005, 336:202; Carbohydr. Res. 2004, 339:2255; J. Electron Microsc. 2003, 52:237; Yeast 2001, 18:433; Experientia 1985, 41:748; J. Gen. Microbiol. 1974, 81:199). While these beta-glucans may be interconnected within the cell wall, the nature of such linkages remains unknown. The fission yeast cell wall also appears to contain trace amounts of linear, unbranched beta-1,3-D-glucan (Reference 17 in the manuscript; Mol. Microbiol. 2007, 65:201; iScience 2024, 27:110477). In addition, the *S. pombe* cell wall consists primarily of just alpha-1,3-glucans and beta-1,3-glucans (up to 80-85% of the cell wall), with the beta-1,6-glucans being a minor polysaccharide that only represents 4-5% of the cell wall, even lower than another minor polysaccharide, the mannan, which represents 10-12% of the cell wall.

We thank the reviewer for this important clarification. We agree that our original description of β -glucan architecture was imprecise and did not adequately distinguish between polymer identity and linkage type. In particular, the previous wording incorrectly implied that β -1,6-glucan forms side chains on β -1,3-glucan, rather than correctly describing β -1,3-glucan side chains attached via β -1,6-glycosidic linkages. We have revised the text to accurately reflect the established architecture of the *S. pombe* cell wall, distinguishing between the major branched β -1,3-glucan polymer and the less abundant, structurally distinct β -1,6-glucan. We have also modified the description of cell wall composition to better reflect the relative abundance of these polymers. In addition, Figure 5a (now Figure 5d) has been revised to accurately depict both a single glucosyl unit linked via a β -1,6 bond to a β -1,3-glucan chain and two β -1,3-linked glucosyl units attached to the main chain through a β -1,6 linkage. We have also updated the relevant references to support these representations.

2. Based on the established types of beta-glucans in *S. pombe*, the description of the results derived from the NMR analysis is currently inaccurate and difficult to follow. For example, in page 9, lines 215-225, the authors state: "...the signals corresponding to beta-1,3-glucan and beta-1,6-glucan (Fig. 5a) were reduced within the mobile domain... The compositional changes were more pronounced in the bgs3-1 ghs2-2 double mutant, which exhibited an extensive loss of beta-1,3- and beta-1,6-glucans, and near-complete depletion of mobile carbohydrate components." However,

the right diagram in Figure 6e does not clearly illustrate an extensive loss of beta-1,3-glucans (Bs) or beta-1,6-glucans (H) in the mobile fraction. It remains unclear whether these beta-glucan signals correspond to backbones, specific branches, or the total polysaccharide population. Similarly, in lines 229-231, the authors describe the 2D NMR results by stating that the rigid core "lacked detectable linear beta-1,6-glucan." Given that all the beta-1,6-glucan in the fission yeast cell wall is natively highly branched, does this "linear" beta-1,6-glucan refer to the backbone, the branches, the nascent beta-1,6-glucan, or the general branched beta-1,6-glucan? The manuscript would benefit significantly from a more precise and simplified rephrasing of the NMR data, explaining how the changes detected in polysaccharide signals correlate with structural alterations in the live cell wall. The authors should consider simplifying the relative molar composition shown in Figure 5f, perhaps adopting the color-coding scheme used in Jacob et al. (PNAS 2025 Vol. 122 No. 29 e2505509122) to improve legibility. Notably, the authors did not detect linear beta-1,6-glucan (H) in the rigid core of the wild-type cell wall in that previous study; the reason for this discrepancy should be addressed. Additionally, the authors should explain in the text the structural difference between beta-1,3-glucans Ba and Bb, and what they represent in the network of branched beta-1,3-glucan in the live cell wall. The manuscript would also benefit notably with a table showing the total amount of each polysaccharide in the cell wall of wild type and each of the single and double mutant strains at both permissive and restrictive temperatures, i.e. linear beta-1,3-glucan, branched beta-1,3-glucan, beta-1,6-glucan, alpha-1,3-glucan, and galactomannoproteins.

We thank the reviewer for this detailed and constructive feedback. We agree that the description of the NMR-derived polysaccharide signals required clarification, and we have revised the text and figures accordingly to improve clarity. The glucan signals detected by ssNMR correspond to carbon environments within the total polysaccharide population and therefore report on the abundance of specific linkage types across the entire wall, rather than distinguishing individual backbone versus branch positions within a single polymer (Zhao et al, *Solid State Nucl Magn Reson.* 2020; Romaniuk and Cegelski L. *Philos Trans R Soc Lond B Biol Sci.* 2015; Chakraborty et al., *Nat Commun* 2021; Lamon et al *PNAS* 2023). Accordingly, reductions in β -1,3- or β -1,6-glucan signal intensity reflect a decrease in the total abundance of those linkages within a given wall fraction (mobile or rigid), rather than the selective loss of a specific structural subpopulation. We have clarified this point in the Results section.

With respect to the β -1,6-glucan signal, we referred to "linear β -1,6-glucan" because of the presence of β -1,6-linked glucan segments that are not further substituted with branches (B^B) in the rigid core, as defined by their characteristic NMR chemical shifts. While β -1,6-glucan in the fission yeast cell wall is overall highly branched, our data indicate that a small population of unsubstituted β -1,6-linked regions exist within the rigid fraction. In our previous study (Jacob et al., *PNAS* 2025), a small but detectable fraction (~ 2 mol%) of β -1,6-glucan (H) was also identified in the rigid core of wildtype cell walls, and we observe the same fraction in the present study using independent wildtype samples (Fig. 5c). Importantly, the absence of detectable β -1,3,6-glucopyranose-containing branched glucan (B^{Br}) in the rigid core further supports the interpretation that the β -1,6-glucan detected in this fraction is not extensively substituted, consistent with a linear configuration. We have clarified this point in the text. We would like to note that it is possible that the population of β -1,6-glucan in the rigid core represents the β -1,6-glucans that are linked to the cell wall proteins as mentioned in a previous point (Pittet and Conzelman, *Biochim. Biophys. Acta* 2007; De Groot et al., *Yeast* 2003).

We have also revised the text describing the mobile fraction to more accurately reflect the data shown in Figure 5 and updated Figure 6e (now Figure 7b) to ensure that the schematic corresponds specifically to the *bgs3-1* single mutant, rather than the double mutant, which resolves the discrepancy noted by the reviewer.

We have also clarified the interpretation of the β -1,3-glucan signals B^a and B^b . These correspond to distinct conformational or packing states of chemically identical β -1,3-glucan polymers, as resolved by ssNMR. These polymorphic forms likely reflect differences in local organization within the cell wall (e.g., chain packing, intermolecular interactions, or association with other wall components), rather than distinct chemical species (Kang et al., *Nat Commun* 2018; Ehren et al., *Cell Surf.* 2020; Fernando et al., *Front Mol Biosci* 2021; Ankur et al., *Angew Chem Int Ed Engl.* 2025). While their precise molecular distinctions remain incompletely defined, this interpretation is now explicitly stated and discussed in the revised manuscript, with reference to Jacob et al. *PNAS* 2025, where we also discuss polymorphic glucan forms.

To improve clarity and accessibility, we have also updated the color scheme in Figure 5c and 7b to match that used in Jacob et al. *PNAS* 2025 and added new summary tables (Tables S4 and S5) reporting the total abundance of each major polysaccharide class across all strains and condition. Finally, we have also updated Figure 7b to ensure accurate representation of the data shown in Figure 5c.

3. In page 10, lines 234-236, the authors affirm that the mobile fraction of the double mutant was "dramatically remodeled... with a marked reduction in beta-1,3- and beta-1,6-glucans." This observation fails to distinguish between the beta-1,6 linkages that anchor beta-1,3-glucan side chains to the backbone and the linkages comprising the beta-1,6-glucan polymer itself. Consequently, the claim that Ghs2 activity is specifically required for the formation of beta-1,6 linkages on beta-1,3-D-glucan side chains appears insufficiently supported. While the reduction of branched beta-1,3-glucan in the *bgs3-1* single mutant was accompanied by an increase in linear beta-1,3-glucan, this is not explicitly explained for the double mutant. Since *Bgs3* is not a transglycosylase, its loss of function should theoretically reduce

beta-1,3-glucan chain length rather than branching frequency. Although erroneously explained, the text assumes that Ghs2 transfers linear beta-1,3-glucan branches to a beta-1,3-glucan backbone via a beta-1,6 linkage. However, the ghs2-2 single mutant was notably excluded from the 2D NMR experiments. Therefore, the current results could possibly suggest that Bgs3 also plays a role in the attachment of beta-1,3-glucan side chains to the backbone.

We thank the reviewer for raising these important points and agree that our original wording did not clearly distinguish between β -1,6 linkages within the linear β -1,6-glucan polymer and β -1,6 linkages that connect β -1,3-glucan side chains to a β -1,3-glucan backbone. We have revised the text to clarify this distinction. Specifically, our data show a reduction in overall β -1,6-containing material in the mobile fraction, but the ssNMR data alone does not resolve the precise structural context of each β -1,6 linkage.

We also agree that the increase in β -1,3-glucan observed in *bgs3-1* is an interesting finding. Notably, we did not observe a similar increase in *ghs2-2* (Figure 5c), which we determined with the newly added 2D analysis, as requested. At present, we do not have an explanation for this difference. We can only speculate that compensatory pathways may be activated more strongly in *bgs3-1*, potentially including increased activity of other β -glucan synthases. This will be an important area for future investigation.

As mentioned, we have now included *ghs2-2* 2D ssNMR data (Figure 5 and S4-7), which show changes similar to those observed in *bgs3-1*, with a decrease in the branched β -glucan (B^B) in the rigid phase and a decrease in β -1,6-glucan (H) in the mobile and rigid phases. However as mentioned in the previous paragraph, there was no observed increase in β -1,3-glucan in this mutant. We originally prioritized *bgs3-1* for the more time-intensive analyses because it is the stronger and more penetrant temperature-sensitive allele (Figure S2a).

Regarding Bgs3, we agree that its role is unlikely to involve transglycosylation. Rather, our working model is that Bgs3 synthesizes the nascent β -1,3-glucan substrate that is subsequently remodeled by Ghs2. Finally, in the double mutant, the mobile fraction was so extensively disrupted that reliable compositional analysis could not be performed, further underscoring the severe loss of normally flexible wall polymers. We have added text to the results section to explicitly address the lack of the double mutant mobile fraction polymer fractions.

4. More importantly, the 2D NMR experiments were conducted using cell walls derived from cells incubated at the restrictive temperature (presumably 36°C) for 4 hours. Consistent with the methodology in Figures 1 and 2, these experiments require essential controls, specifically cell walls from the wild-type, single, and double mutants incubated at the permissive temperature. Similarly, if the 1D NMR analyses were also performed under restrictive conditions, the corresponding permissive-temperature controls are currently lacking and should be presented.

The ssNMR experiments performed in this study require costly reagents and substantial instrument time, making the acquisition of additional datasets under multiple conditions both time- and cost-prohibitive. We do not think that permissive temperature controls are necessary to support the conclusions of this work. Mutant strains are also typically hypomorphic at “permissive” temperature which is why they display negative genetic interactions. The important comparison to be made is between wildtype and mutant strains at the non-permissive temperature.

5. The experiments involving the D75-4590 drug are pivotal for establishing a direct or indirect relationship between Ghs2 and beta-1,6-glucan synthesis. In page 10, lines 249-251, the authors state: “Both *bgs3-1* and *ghs2-2* mutants, but not the temperature-sensitive allele of *bgs1* (*cps1-191*), were hypersensitive to D75-4590 and exhibited a more severe accumulation of cell wall material than D75-4590-treated wild-type cells.” However, the authors do not present standard hypersensitivity assays (e.g., growth inhibition curves of mutant strains compared to wildtype). Instead, they only show that the mutants exhibit a more pronounced phenotype. Comparative susceptibility assays across all four strains are necessary. Furthermore, since D75-4590 has not been previously characterized in fission yeast (with existing studies limited to *Saccharomyces cerevisiae* and *Candida* sp.), the authors should clarify how the drug concentration and time of treatment were determined. It is unclear whether wild-type cells continue to proliferate under these conditions while the mutants do not, or if a Minimal Inhibitory Concentration (MIC) was established. Does a 6-hour treatment with 1 mM induce a maximal phenotype in the wild-type strain? Notably, the *bgs1* mutant appears to exhibit more significant cell wall accumulation at the cell poles than the wildtype; the percentage and thickness of this polar accumulation should be quantified for all four strains in Figure 6a, once the suggested susceptibility assays are performed.

We thank the reviewer for this insightful suggestion and agree that comparative susceptibility assays strengthen the interpretation of the D75-4590 experiments. As also found in previous studies using D75-4590 and related derivatives, these compounds exhibit limited aqueous solubility, likely due to the hydrophobic steroid-like scaffold of the inhibitor (Kuroyangi et al., 2010). In our hands, D75-4590 precipitates in rich medium (YE) at concentrations >1 mM and in EMM at >4 mM. Above these concentrations, visible precipitation is observed, indicating that the compound had exceeded its solubility limit and that the effective dissolved concentration no longer increased proportionally with the amount added. This limited solubility restricted the ability to perform MIC-0 assays. Thus, we adopted MIC-2 (the concentration causing ~50% growth inhibition), which has also been used in prior studies of D75-4590 derivatives across multiple fungal species (Kuroyangi et al., 2010), as a more reliable metric.

Using this approach, we found that wildtype *S. pombe* cells exhibit an MIC-2 of ~4 mM in EMM (Table 1). We were also able to extend this analysis to the *HA₃-ghs2* and *HA₃-ghs2-Y478I* strains, as described in more detail below (Table 1).

Unfortunately, *ghs2-2* and *bgs3-1* cells did not grow in liquid EMM, and therefore MIC-2 values could not be determined for these strains in the same assay format. However, under the imaging conditions used with the strains grown in YE, both mutants displayed enhanced sensitivity to D75-4590 relative to wildtype, consistent with our original conclusion. As suggested, we have now added quantification of the percentage of cells with abnormal cell wall staining and the size of the cell wall deposits in Figure S9b-c. We also note that *cps1-191* cells were not more susceptible than wildtype and did not exhibit increased abnormal staining or larger deposits relative to wildtype under the same treatment conditions (Figure 9b-c).

Finally, we have revised the main text and Materials and Methods to more clearly explain how the drug concentrations and treatment durations were selected, including the balance between achieving a robust phenotype and remaining within the solubility range of the compound.

6. The authors conclude that these findings provide evidence that inhibition of beta-1,6-glucan synthesis accounts for the observed cell wall defects. However, the drug's effect on the wild-type is markedly reduced compared to the aberrant cell wall deposition phenotype of *bgs3-1* and *ghs2-2* mutants alone (Figure 2a). The authors should evaluate a range of concentrations to identify a treatment that faithfully mimics the mutant phenotypes, perform a comparative resistance assay to evaluate if the MIC of D75-4590 is much higher in the *Ghs2-Y478I* than in the wild-type strain, and subsequently verify if the resistance mutation suppresses the phenotypes caused by the drug. Additionally, analyzing the cell wall accumulation in a *bgs3-1* strain carrying the *Ghs2-Y478I* substitution would provide valuable insights.

We thank the reviewer for these constructive suggestions and have performed several additional experiments to strengthen this section. As suggested, we examined the phenotype of wildtype cells across a range of D75-4590 concentrations (0.125-4 mM) after a 3 hour treatment in EMM medium. These analyses helped better define conditions under which drug treatment more closely phenocopies the *ghs2-2* and *bgs3-1* mutant cell wall defects.

We also performed comparative susceptibility assays using the *HA₃-ghs2* control strain and *HA₃-ghs2-Y478I*. The control strain exhibited an MIC-2 of 0.25 mM (the N-terminally 3xHA tagged *ghs2* is more sensitive than wildtype), whereas *HA₃-ghs2-Y478I* exhibited an MIC-2 of 1 mM (Table 1), indicating substantially reduced drug sensitivity. To further support this conclusion, we performed serial dilution growth assays on agar plates containing 0.25 mM D75-4590. Under these conditions, *HA₃-ghs2-Y478I* grew better than the control strain on drug-containing plates, while both strains grew similarly on DMSO control plates (Fig. S9a).

Based on our optimization of drug solubility, we also repeated the *HA₃-ghs2* and *HA₃-ghs2-Y478I* imaging experiment in EMM and quantified the percentage of cells displaying abnormal cell wall staining, and these data are incorporated into the main text (lines 292-294). Together, these results further support that the *Ghs2-Y478I* substitution confers decreased sensitivity to D75-4590 and is consistent with *Ghs2* being a relevant target of the compound. We did not analyze cell wall composition in a *bgs3-1 ghs2-Y478I* background, as it was unclear how the outcome of that experiment would directly test or refine the current mechanistic model.

7. 1D NMR analysis reveals that beta-1,6-glucan chains in the mobile phase are reduced in the single mutants and extensively depleted in the double mutant. Notably, the *ghs2-1* single mutant was excluded from the 2D NMR experiments without justification in the text. Although the authors assert in the abstract and throughout the manuscript that *Ghs2* is required for the synthesis/production of beta-1,6-glucan chains, I believe this is incorrect because *Ghs2* presents homology with transglycosylases, which are involved in branching activity, and therefore it cannot be simultaneously a glucan synthase (glycosyltransferase). The reduction in beta-1,6-glucan content is likely an indirect effect, as for example a compensatory mechanism downregulating its synthesis, or defect in beta-1,6-glucan attachment to the cell wall being released to the medium. The indirect relationship of *Ghs2* with the beta-1,6-glucan has not been sufficiently demonstrated, particularly given the lack of appropriate D75-4590 treatments in wild-type and resistant mutant strains. To date, a specific beta-1,6-glucan synthase activity has not been identified, nor has the site of beta-1,6-glucan synthesis been localized. Because beta-1,3-glucan side chains may be covalently linked to the beta-1,6-glucan polysaccharide, the observed loss of this polymer could represent an indirect effect; specifically, the absence of beta-1,3-glucan branches might impair the maintenance or retention of beta-1,6-glucan within the cell wall matrix, as proposed by the authors in pages 12–13, lines 309–312. Given that this hypothesis is the basis of the proposed cell wall model, the manuscript would benefit significantly from the additional genetic testing of potential beta-1,6-glucan synthase candidates (e.g., *S. pombe* homologs of the KRE family). Alternatively, the authors should revise their terminology, substituting "synthesis/production" with "accumulation" or "maintenance" throughout the text. The fact that *bgs3-1* exhibits a loss of beta-1,6-glucan similar to *ghs2-2* further suggests an indirect effect.

We thank the reviewer for this thoughtful and constructive comment. We agree that our original use of "synthesis/production" was not precise given the available data and the known homology of *Ghs2* to GH16

transglycosylases. Accordingly, we have revised the text throughout to avoid implying direct synthase activity. We also now explicitly acknowledge that the reduction in β -1,6-glucan observed in the mutants may be indirect within the discussion. We agree that identifying the enzyme(s) responsible for β -1,6-glucan synthesis and defining their site of action remain important open questions. In this regard, analysis of candidate KRE-related genes in *S. pombe* is currently underway in our laboratory and will be the focus of future studies.

8. Furthermore, while the study relies on AF3 structural models and whole-cell NMR to propose the transglycosylase activity of Ghs2, an *in vitro* enzymatic activity assay is required to unequivocally confirm the mechanism proposed in Figure 6d. The authors are strongly encouraged to attempt the purification of the extracellular GH16 domain of Ghs2 and conduct transglycosylation assays with oligosaccharide substrates, utilizing the single mutants (T205P, F449L, E414A, or E419A) described in Figure 4. If biochemical reconstitution proves unfeasible, the authors must moderate their conclusions, explicitly stating that the proposed transglycosylase activity is predictive and phenotypic rather than biochemically demonstrated in *in vitro* assays with isolated Ghs2 protein.

We thank the reviewer for this important suggestion. We agree that direct biochemical reconstitution of Ghs2 activity would provide the most definitive test of the proposed mechanism. We attempted to purify the extracellular GH16 domain of Ghs2 for *in vitro* enzymatic assays; however, despite multiple expression and purification strategies, we were unable to obtain sufficient quantities of stable, active protein for biochemical characterization. These efforts are ongoing.

Importantly, we note that direct biochemical reconstitution of catalytic activity has not, to our knowledge, been reported for any member of the Kre6/Ghs2 family. This likely reflects the substantial technical challenges associated with studying these proteins, which are large membrane-associated enzymes that have proven difficult to express, purify, and reconstitute in an active form. Consequently, while biochemical validation remains an important long-term goal, it is currently a major unresolved challenge for the field as a whole rather than a limitation specific to this study.

In light of this, we have revised the manuscript to more clearly state that the proposed transglycosylase activity of Ghs2 is inferred from genetic, cell biological, structural, pharmacological, and ssNMR evidence and has not yet been directly demonstrated using purified protein *in vitro*. We have also moderated the language throughout the text and figure legends to emphasize that the proposed mechanism remains a model that will require future biochemical reconstitution and validation.

9. Regarding data visualization, the schemes in Figure 6e attempt to integrate wall thickness measurements with compositional and structural information from solid-state NMR. However, the composition and structure are extremely difficult to compare due to the use of similar color palettes for different β -glucan chains. The manuscript would benefit greatly from a table or a comparative figure summarizing the distinct differences between the primary polysaccharides in the mobile and rigid fractions across the various strains.

We thank the reviewer for this helpful suggestion. To improve clarity and facilitate comparison across strains and wall fractions, we have added summary tables (Table S4 and S5) that quantitatively report the relative abundance of the major polysaccharide classes in both the mobile and rigid fractions for all strains examined. These tables enable direct comparison of β -1,3-glucan, β -1,6-glucan, α -1,3-glucan, and galactomannoproteins across strains and complements the schematic representations in Figure 5c and 7b. In addition, we have revised the color scheme in Figures 5c and 7b to improve visual distinction between different β -glucan populations.

10. A further issue regarding the schematics in Figure 6e is the methodology used for measuring cell wall thickness in the double mutant (bgs3-1 ghs2-2). The transmission electron microscopy (TEM) data in Figure 2d only displays single mutants, omitting the double mutant entirely. These TEM images must be included in Figure 2 to allow for a direct comparison and to determine if the outer galactomannan layer is more severely affected than in the single mutants. Also, in page 10, lines 235–237, the authors state that: “The mobile fraction of the double mutant was dramatically remodeled compared to wildtype, with a marked reduction in β -1,3- and β -1,6-glucans and the loss of α -1,2- and α -1,6-linked mannose residues, indicating disruption of the outermost galactomannan layer (Fig. 5e, f).” These biochemical observations further accentuate the necessity of including TEM images for the double mutant in Figures 2b to 2e. Furthermore, as cell wall thickness appears to be measured specifically at sites of abnormal deposition, it should be indicated in the figure the site selected to measure the thickness, and this must be explicitly stated in the figure legend for Figure 6e. Additionally, while the legend for Figure 2d mentions a total sample size of $n = 100$, the authors must specify the exact number of cells analyzed for each individual strain.

While TEM analysis of the double mutant could provide additional structural detail of the cell wall, we have not included these time- and resource-intensive data because they would not alter our conclusions. Our existing TEM, ssNMR, and genetic analyses already demonstrate defects in branched β -glucan incorporation and cell wall organization, and the proposed experiment would primarily assess phenotype severity rather than provide new

mechanistic insight. We have clarified the methods we used to quantify cell wall thickness and added measurements at deposits and non-deposits for the mutants in Figure 2d.

11. In page 11, lines 269-275, the authors state: "Here, we propose Ghs2 as the catalytic factor for branched beta-1,6-glucan synthesis in *S. pombe* and show that it partners physically and functionally with the beta-1,3-glucan synthase Bgs3." In my view, the current results do not demonstrate direct catalytic activity for Ghs2 in beta-1,6-glucan synthesis, mainly because Ghs2 shares homology with transglycosylases, and therefore it hardly would be a beta-1,6-glucan synthase (glycosyltransferase) and much less it could be both enzyme types. The fact that Bgs3 exhibits identical phenotypes regarding beta-1,6-glucan suggests an indirect effect, as it has been observed following the loss of the beta-1,3-glucan synthase Fks1 in other yeasts (Yeast 2002, 19:671–90). The authors further suggest that the Ghs2 GH16 domain is "ideally positioned to add beta-1,6 branches to newly synthesized beta-1,3-glucan chains." This remains conceptually problematic. First, the branches of beta-1,3-glucan are not themselves chains of beta-1,6-glucan, as erroneously stated in the text and depicted in the architecture model in Figure 5a. As previously explained, beta-1,3-glucan consists of a beta-1,3-backbone with beta-1,3-side chains; only the single glucose residue connecting the branch to the backbone involves a beta-1,6-linkage. Moreover, it is highly improbable that Ghs2 possesses both a glycosyltransferase activity for beta-1,6-glucan production and a transglycosylase activity for transferring beta-1,3-glucan chains via beta-1,6-linkages to a beta-1,3-backbone, as suggested in Figure 6d. The frequency of such linkages would be residual compared to the extensive chains found in branched beta-1,6-glucan. The authors appear to be mistaking the synthesis of beta-1,6-glucan with the branching of beta-1,3-glucan, which leads to significant confusion. I strongly recommend that the authors describe the direct or indirect effects of the mutations on beta-1,6-glucan and branched beta-1,3-glucan as separate, and distinct phenomena.

We have modified the text as outlined: "Here, we show that Ghs2 partners physically and functionally with the β -1,3-glucan synthase Bgs3, and propose that it functions as a remodeling enzyme which introduces branches onto linear β -1,3-glucan chains in *S. pombe*. Ghs2-related proteins exist in other fungi, but they have not been demonstrated to directly couple to a synthase enzyme. Bgs3 are strictly interdependent for localization, co-purify, and structural modeling suggests that they form a complex with the Ghs2 GH16 domain ideally positioned to introduce β -1,3-glucan branches onto newly synthesized β -1,3-glucan chains as they are extruded into the periplasmic space by Bgs3 (Fig. 7a)."

MINOR POINTS

12. In page 2, lines 31-32, the authors state: "we identify Ghs2, a glycoside hydrolase 16 (GH16) domain-containing transmembrane protein." Given that the catalytic activity of Ghs2 has not been biochemically demonstrated, the forms "predicted" or "putative" should be added to this type of sentences. Similarly, in lines 38-39, the authors claim that "genetic and pharmacological evidence further indicate that Ghs2 acts on newly synthesized beta-1,3-glucan to introduce beta-1,6-linked branch points." The experiments involving D75-4590 and the Ghs2-Y478I mutation do not provide evidence to conclude that Ghs2 exhibit a transglycosylase activity responsible for beta-1,6 linkages in branched beta-1,3-glucan. Those data would suggest an indirect relationship between Ghs2 and beta-1,6-glucan synthesis but do not demonstrate transglycosylase activity. The abstract should be revised accordingly to reflect these limitations.

The abstract has been updated to reflect the suggested changes.

13. In page 3, lines 73-74, the authors refer to "an outer layer composed mainly of linear beta-1,6-glucans." It is unclear how the authors arrived at this conclusion. All fungal cell wall beta-1,6-glucans are described as branched, no linear beta-1,6-glucan has been described, unless it is considered the nascent beta-1,6-glucan before branching maturation. In *S. pombe*, chemical and glucanase-driven cell wall fractionation and NMR spectroscopy have established that beta-1,6-D-glucan is constitutively branched, consisting of a beta-1,6-D-glucan backbone extensively modified (up to 75% of glucose residues) with beta-1,3-linked beta-1,6-D-glucan side chains. This terminology should be corrected throughout the text, and a detailed explanation should be included in the introduction, citing the studies by Magnelli et al. (Anal. Biochem. 2005) and Sugawara et al. (Carbohydr. Res. 2004). Furthermore, the author's earlier statement contradicts their subsequent claim in page 4, lines 83-84, that "none has been demonstrated to directly catalyze beta-1,6-glucan branches or chains, and the enzymatic mechanism underlying beta-1,6-glucan assembly has remained elusive." This is not correct, in budding yeast, in addition to all the works around the Kre and related proteins, there are some works regarding the enzymatic activity of the beta-1,6-glucan synthesis, Montijn et al. (J. Bacteriol. 1999, 181(24):7414-7420), Vink et al. (Yeast 2004, 15;21(13):1121-31). The manuscript requires internal consistency regarding what is known versus what is hypothesized and what is demonstrated by their NMR analysis.

We thank the reviewer for this important clarification. We have revised the description of *S. pombe* cell wall composition to reflect that β -1,6-glucan is extensively branched, and we have incorporated the suggested references (Magnelli et al., 2005; Sugawara et al., 2004) to describe the cell wall composition more accurately.

We agree that prior work, including Montijn et al., 1999 and Vink et al., 2004, established important genetic and biochemical frameworks for studying β -1,6-glucan production, including the development of in vitro assays that detect β -1,6-glucan synthesis activity. However, these studies do not identify the enzyme(s) directly responsible for catalyzing β -1,6-glycosidic linkage formation, nor do they define the specific enzymatic mechanism underlying branch formation versus chain extension. We have also revised the manuscript to ensure consistency in terminology and to avoid overstatement.

14. In page 4, line 88, the statement “Ghs2 is a single-pass transmembrane protein” should be changed to “Ghs2 is a predicted single-pass transmembrane protein,” as its topology has not been experimentally verified. Similarly, in lines 91-92, the claim that “Ghs2 is positioned to directly modify newly synthesized beta-1,3-glucan chains, adding beta-1,6 branches” would be more accurate if phrased as: “Ghs2 is positioned to directly transfer a beta-1,3-glucan chain to another nascent beta-1,3-glucan chain via a beta-1,6-linkage.”

We made both suggested wording changes in the manuscript.

15. In page 5, lines 105-196, the authors state that “Total GFP-Bgs3 protein levels were unchanged in ghs2-2 cells compared to wild type... (Supplementary Fig. 1b).” However, the specific band for GFP-Bgs3 is barely discernible in the provided immunoblot. Consequently, it is difficult to confirm whether total Bgs3 levels are truly equivalent between wild-type and ghs2-2 cells. To improve the detection of the integral membrane protein Bgs3, the authors should perform a direct immunoblot using the specific protocol for integral membrane proteins cited in their methods section, as it has been shown in other works in which Bgs3 and other Bgs proteins are well detected. Furthermore, the immunoblots for GFP-Bgs3 in Figure 3b are of poor quality and should be repeated to ensure reliable detection.

Bgs3 is a large (~210 kDa), heavily glycosylated, low-abundance integral membrane protein, which makes its detection by immunoblotting inherently challenging because of its broad and diffuse banding upon SDS-PAGE. To our knowledge, only a single other study includes Bgs3 immunoblots (Martin et al., *Eukaryotic Cell* 2003) and although these bands are much sharper, they used an overexpression system (confirmed via personal communication with Dr. Yolanda Sanchez), which enhanced signal intensity but did not report on endogenous protein levels. Further, it is unknown if the over-produced protein is properly modified. In contrast, all Bgs3 IPs and immunoblots presented here were performed on endogenous Bgs3 and all alleles of it were constructed at the endogenous *bgs3* locus. This was important for accurately assessing protein levels and interactions. In addition, exposure conditions for the Bgs3 immunoblots were selected to avoid saturation of other bands, which can reduce the apparent intensity of the GFP-Bgs3 signal. Importantly, GFP-Bgs3 is clearly expressed and properly localized at the plasma membrane under permissive conditions, as demonstrated by fluorescence microscopy (Fig. 1a), providing orthogonal evidence that the protein is produced and functional.

All immunoblot and co-immunoprecipitation experiments were performed using membrane-enriched fractions, as described in the Methods, and were independently repeated at least twice with consistent results. The interaction between Ghs2 and Bgs3 was confirmed in three independent experiments, including reciprocal co-immunoprecipitations in both HA and GFP directions.

16. Regarding protein stability, the thermosensitive allele *bgs3-1* exhibits undetectable Ghs2 protein levels at both permissive and restrictive temperatures (Supplementary Figure S1). A similar loss of signal is observed for GFP-Ghs2 fluorescence in cells growing at the assumed permissive temperature (Figure 1d). Did the authors assess GFP-Ghs2 localization or stability at lower (19°C) or intermediate (28-30°C) temperatures to determine the threshold of this instability?

We would like to clarify that Ghs2 is not undetectable in the *bgs3-1* mutant at permissive temperature. Rather, Ghs2 levels are reduced compared to wildtype, as shown in Figure S2b. Consistent with this, GFP-Ghs2 fluorescence is diminished but still detectable under these conditions (Figure 1d). We did not assess GFP-Ghs2 localization or stability in *bgs3-1* cells at additional temperatures as it is not required to support the conclusions of this study.

17. Regarding Figure 2a, the phenotypes depicted in the panels must be quantified. The authors utilize Methyl Blue rather than conventional Aniline Blue that has been extensively used and described to bind beta-1,3-D-glucan in budding yeast. It should be clarified which specific polysaccharide this fluorochrome is assumed to bind to, and it should be shown whether Methyl Blue labels the same cell wall structures as the more conventional dye Aniline Blue.

We thank the reviewer for this helpful suggestion. We have now quantified the phenotypes shown in Figure 2a by measuring both the number and size of Methyl Blue-positive deposits (Fig. S2d). Regarding the use of Methyl

Blue, we clarify that Methyl Blue is a principal component of Aniline Blue, which is itself a mixture of dyes (Methyl Blue and Water Blue) (Kotak et al., *Dyes and Pigments* 1997, Conn, H. J. (1977). *Conn's Biological Stains: A Handbook of Dyes, Stains and Fluorochromes for Use in Biology and Medicine*. 9th ed. Williams & Wilkins). In practice, many reagents labeled as "Aniline Blue" are predominantly or entirely Methyl Blue, and these dyes are therefore often used interchangeably in cell wall staining applications. Consistent with this, Methyl Blue has been widely used to label β -glucan-containing cell wall structures. In addition, Methyl Blue staining has been extensively used in the Gould laboratory for over 35 years as a robust and reproducible readout of cell wall organization in *S. pombe*. We have now clarified in the revised figure legend and text that Methyl Blue recognizes β -glucans.

Importantly, in this study Methyl Blue staining is used as a qualitative indicator of cell wall organization rather than a linkage-specific or compositional readout. For this reason, and to directly address glucan identity and architecture, we performed detailed ssNMR analyses, which provides quantitative and linkage-resolved measurements of cell wall polymers. Because our central conclusions rely on these biochemical data, we have not performed additional comparative staining with Aniline Blue. Instead, we have clarified the scope and interpretation of the Methyl Blue staining in the revised manuscript.

18. In page 6, lines 134-139, the authors assert: "Given that ghs2-2 and bgs3-1 exhibited the same defects, it was not surprising that the double mutant was synthetically sick..." These results suggested that Bgs3 and Ghs2 act in the same pathway..." This interpretation is logically inconsistent. If two proteins function strictly within the same linear pathway, their combined loss-of-function should result in a phenotype identical to the single mutants, rather than an exacerbated or additive defect. The authors should discuss the underlying reasons for this synthetic sickness, as it may suggest partially redundant functions or parallel roles in maintaining cell wall integrity.

We understand the genetic argument that the reviewer is making. However, an additive phenotype does not necessarily exclude genes/proteins from acting in the same pathway when the alleles are hypomorphic rather than null. Indeed, synthetic sick or lethal interactions have been observed between genes encoding components of the same biological pathway when partial loss-of-function alleles are combined. Examples of such negative genetic interactions are found among mutants of the *S. pombe* Septation Initiation Network (SIN), wherein combinations of hypomorphic alleles result in enhanced or even lethal defects despite the proteins functioning in a linear signaling pathway (Balasubramanian MK et al. *Genetics* 1998, Salimova et al., *JCS* 2000, Fournier et al., *Arch Microbiol* 2001). Thus, because *ghs2-2* and *bgs3-1* are hypomorphic alleles, the synthetic sickness of the double mutant is quite consistent with a further reduction in the output of a shared cell wall assembly pathway and does not exclude a model in which Bgs3 and Ghs2 function together. We have re-written the sentence to clarify this logic.

19. Since the target of D75-4590 in other fungal species appears to be Kre6, another GH16 domain-containing protein, it would be highly informative if the authors provided a detailed comparison of the amino acid sequence and structural similarities between Ghs2 and Kre6. Furthermore, it is well-established that *S. cerevisiae* Kre6 primarily localizes to the endoplasmic reticulum (ER), though a fraction is detected at the plasma membrane of the bud and within trafficking vesicles (J. Biol. Chem. 2010, 286:7429–7438). While the exact site of beta-1,6-glucan synthesis remains elusive, Humbel et al. (Reference 17) provided evidence that in *S. pombe*, beta-1,6-glucan could be synthesized within the ER-Golgi system and subsequently exported. In this manuscript, Ghs2 does not appear to localize to the ER, instead it co-localizes with Bgs3 in the plasma membrane and cytoplasmic vesicles. These data suggest further discussion to reconcile the authors' proposed plasma membrane "synthase-remodeler" model with the proposed secretory pathway-mediated synthesis of beta-1,6-glucan.

We thank the reviewer for this thoughtful comment. To address the request for a detailed comparison between Ghs2 and Kre6, we have expanded our analysis in Fig. S8 to include pairwise sequence alignments of the full-length proteins (Fig. 8b), as well as an alignment across many fungal homologs highlighting conservation at the inhibitor resistance site (Fig. 8a). In addition, we now include structural superposition of AlphaFold-predicted GH16 domains, demonstrating that these proteins adopt highly similar folds (C α RMSD = 0.42 Å over 331 residues) and that the residues corresponding to Ghs2 Y478 and Kre6 F552 are predicted to occupy structurally equivalent positions at the entrance of the predicted substrate-binding cleft (Fig. 8c).

We agree that early studies, including Humbel et al., 2001, Roemer et al., 1994 and Li et al., 2002 suggested that β -1,6-glucan formation may involve the ER-Golgi system. Importantly, other work has shown that Kre6 is not confined to the ER and is also detected in secretory vesicles and at the cell surface (Montijn et al. 1999). We have revised the Discussion to explicitly address these prior models and to clarify how our data support a cell surface-associated mechanism.

In *S. pombe*, our data show that Ghs2 co-localizes with Bgs3 at sites of polarized growth at the plasma membrane and in cytoplasmic puncta. While overexpression results in accumulation within internal membranes consistent with ER localization, functional tagged Ghs2 expressed near endogenous levels localizes predominantly to the plasma membrane, supporting its site of action at the cell surface. We did notice that over-production of GFP-Ghs2 did accumulate in cellular structures consistent with the secretory pathway. These observations support a model in which Ghs2 traffics through the secretory pathway but functions at the plasma membrane in coordination

with the β -1,3-glucan synthase Bgs3. Finally, we note that Kre6 is non-essential in *S. cerevisiae*, which can complicate interpretation of localization studies, whereas Ghs2 is essential in *S. pombe*. The use of functional tagged constructs in our study provides confidence that the observed localization reflects the position of the active protein.

20. In Figure 5f, the authors provide the molar composition of the rigid fraction for the bgs3-1 ghs2-2 double mutant but omit the corresponding data for the mobile fraction. This omission is significant, as the authors explicitly state in page 10, lines 235–237, that: “The mobile fraction of the double mutant was dramatically remodeled compared to wild type, with a marked reduction in beta-1,3- and beta-1,6-glucans and the loss of alpha-1,2- and alpha-1,6-linked mannose residues, indicating disruption of the outermost galactomannan layer (Fig. 5e, f).” Despite the reported general disruption of mobile polysaccharides in the bgs3-1 ghs2-2 strain, the molar composition of the residual mobile polysaccharides (specifically Ab1, H1, B1, H2, and B2) depicted in Figure 5e must be quantified and included in Figure 5f. These biochemical observations further accentuate the necessity of including TEM images for the double mutant in Figures 2b to 2e. In addition, the proposed loss of external galactomannan layer in the double mutant should be confirmed in wild type, single and double mutants with specific fluorescent-conjugated lectins like Concanavalin A, which presents affinity for terminal mannosyl and glucosyl residues, or the lectin from Bandeiraea simplicifolia, which presents affinity for terminal galactosyl residues.

We thank the reviewer for this important and thoughtful suggestion. The relative molar composition analysis presented in Fig. 5f (now Fig. 5c) was derived from integrated cross-peak intensities in the 2D ^{13}C DP refocused J-INADEQUATE (SQ-DQ) spectra. For each polysaccharide population, the composition was estimated by averaging the intensities of representative carbon-carbon correlations (C1-C6), while intentionally restricting the analysis to well-resolved and minimally overlapping resonances to ensure reliable quantitative interpretation.

In the mobile fraction of the bgs3-1 ghs2-2 mutant, however, most mobile polysaccharide signals were absent, consistent with the extensive remodeling described in the manuscript. Only a small number of weak residual resonances corresponding to B1/B2 (β -1,3-glucan), H1/H2 (β -1,6-glucan), and Ab1 (α -1,3-glucan) remained detectable. Importantly, the residual β -1,3-glucan and β -1,6-glucan correlations were highly overlapped, particularly at the C1 and C2 resonances that prevent us from accurate integration of the individual polysaccharide populations.

Because quantitative molar composition analysis in our approach relies on multiple independent and well resolved correlations for each polysaccharide, the severely reduced chemical shift signals of mobile polysaccharides and extensive spectral overlap of the remaining resonances in the bgs3-1 ghs2-2 mutant prevented statistically reliable quantification of the residual mobile fraction. Under these conditions, quantitative integration of the remaining highly overlapped resonances would not provide sufficiently reliable compositional values for rigorous interpretation. Therefore, we chose not to include a molar composition analysis for the mobile fraction in Fig. 5f (now Fig. 5c), as we believe such quantification would overinterpret the experimental data. Instead, we limited the quantitative compositional analysis to the rigid fraction, where the spectral resolution and signal-to-noise ratio permitted more robust measurements.

We have now included the following explanation within the manuscript for the lack of molar ratios for the mobile phase of the double mutant: “The mobile fraction of the double mutant was dramatically remodeled compared to wildtype. The absence of 2D ^{13}C DP-INADEQUATE signals in the double mutant indicates that the flexible fraction of the cell wall is largely lost. This reflects a substantial reduction or immobilization of β -1,3- and β -1,6-glucans and the loss of α -1,2- and α -1,6-linked mannose residues, which were readily detected in wildtype, with the outer galactomannan layer disrupted, resulting in insufficient mobile material to produce detectable through-bond correlations. Consequently, calculation of relative molar composition of mobile polysaccharides for the double mutant was not feasible.” Thus, because the residual mobile material was below the threshold required for reliable spectral assignment and quantification, direct calculation of relative molar composition for species such as Ab1, H1, B1, H2, and B2 could not be performed.

Regarding the suggested imaging experiments, as mentioned previously we have opted to not include the double mutant TEM as the results from this experiment would not inform our hypothesis. Likewise, while lectin-based staining of outer wall glycans is an interesting suggestion, we believe it falls outside the central mechanistic scope of the current study, as the ssNMR data already provide direct biochemical evidence for substantial disruption of the mobile galactomannan-rich outer wall fraction in the double mutant.

21. In page 11, lines 281-282, the authors state: “Our solid-state NMR analyses demonstrated that loss of either Ghs2 or Bgs3 led to reduced beta-1,3-glucan branched beta-1,6-glucan.” This statement further mixes the synthesis of beta-1,6-glucan with the branching of beta-1,3-glucan, resulting in significant conceptual confusion. Additionally, the term “beta-1,3-glucan branched beta-1,6-glucan” appears to be a contradiction. Based on the preceding results section dedicated to NMR analysis, the authors do not describe such a species; instead, they refer to beta-1,6-glucan and branched beta-1,3-glucan (BBr). I assume the authors are referring to the former beta-1,6-glucan (H), but this must be clarified. Furthermore, there is a major discrepancy in experimental design: the 2D NMR experiments utilized cell walls from the bgs3-1 single mutant and the bgs3-1 ghs2-2 double mutant, while the single ghs2-2 mutant was notably omitted. This leads to an ambiguity in the visual evidence, as it is unclear whether the right panel of Figure 6e (labeled as “Bgs3-Ghs2 defective”) represents the cell wall architecture and composition of the single mutants bgs3-1

or *ghs2-2*, independently, or that of the double mutant (*bgs3-1 ghs2-2*). If the model is intended to represent the single mutants, the authors appear to assume that both *bgs3-1* and *ghs2-2* possess an identical cell wall architecture. While 1D NMR analyses (Figures S4-S6) suggest their compositions are very similar, this assumption ignores the strong genetic interaction demonstrated in Figures 2a and S2, and the increased cell wall alterations of the double mutant. Furthermore, since the cell wall of the *ghs2-2* mutant was not independently examined via 2D NMR, there is no experimental basis to assume its structural identity with *bgs3-1*. The authors must explicitly label the strains used in each panel to ensure the data accurately support their mechanistic claims.

We thank the reviewer for this careful and constructive comment. As suggested, we have amended the discussion to clarify our interpretation and avoid terminology that could conflate β -1,6-glucan synthesis with β -1,3-glucan branching. We also changed the right-hand label in Figure 7b to more accurately state "*bgs3-1*." In addition, we have now included *ghs2-2* 2D ssNMR analyses in Figures 5 and S7 and revised the accompanying text to clearly describe these data. We originally did not analyze the 2D NMR data for the *ghs2-2* allele because it is a weaker temperature-sensitive allele, as evidenced by the growth assays in Figure S2a. However, given the reviewer's point, we agree that direct structural comparison was important.

The 2D NMR analysis revealed that *ghs2-2* exhibits changes similar to, but less severe than, *bgs3-1* relative to wildtype, including a reduction in the branched β -glucan signal in the rigid core and a reduction in the β -1,6-glucan signal in both cell wall domains. Interestingly, unlike *bgs3-1*, the *ghs2-2* mutant did not display an expansion of the β -1,3-glucan signal in the rigid core. This difference may suggest that compensatory pathways are activated more strongly in *bgs3-1* than in *ghs2-2*, which represents an interesting direction for future study. Together, these revisions now explicitly identify the strains represented in each panel and provide direct experimental support for the distinctions between the *bgs3-1*, *ghs2-2*, and double-mutant cell wall phenotypes.

22. In page 12, lines 295-296, the authors state: "A key outstanding question however is how the Ghs2 GH16 domain generates the beta-1,6-glucan branches." This phrasing remains technically imprecise. In the context of beta-1,3-glucan architecture, the "branches" are themselves beta-1,3-glucan side chains that are connected to the main backbone via a single beta-1,6-glycosidic bond at the junction point. Referring to these as "beta-1,6-glucan branches" erroneously implies that the branches consist of beta-1,6-linked glucose polymers, rather than beta-1,3-linked chains. The authors must use more rigorous chemical nomenclature to distinguish between the linkage type at the branch point and the polymer identity of the side chain itself.

We changed the referenced sentence to say, "A key outstanding question however is how the Ghs2 GH16 domain generates the β -1,6-glycosidic linkages that attach β -1,3-glucan side chains to the β -1,3-glucan backbone."

23. In page 12, lines 300-301, the authors state: "If this pattern holds true for *S. pombe*, this architecture implies that Ghs2 activity is usually limited to branch initiation or very short elongation." This statement inaccurately implies that Ghs2 possesses synthase activity, which has not been experimentally demonstrated. It is essential to distinguish between a synthase (glycosyltransferase), which catalyzes the de novo polymerization of glucose residues, and a transglycosylase, which remodels existing polymers by breaking and reforming glycosidic bonds. "Branch initiation" or "elongation" in a biosynthetic context typically refers to the action of a synthase. If the authors propose that Ghs2 functions as a transglycosylase, they should rephrase this to clarify that it involves the transfer of pre-existing glucan fragments rather than the elongation of a new chain.

We thank the reviewer for this important point. We agree that the original wording could be interpreted as implying that Ghs2 has synthase activity. We have revised the discussion to use more precise language.

24. In page 12 (lines 302–306), the authors suggest: "...beta-1,6 branches introduced by Ghs2 could serve as substrates or nucleation sites for additional enzymes that elongate or reorganize beta-1,6-linked structures." As previously argued, this hypothesis is biochemically imprecise. In the established architecture of beta-1,3-glucan, the "branches" are themselves beta-1,3-glucan chains tethered to the main backbone via a single beta-1,6-glycosidic bond at the junction point. Therefore, these branches, composed of beta-1,3-linked glucose residues, cannot serve as substrates for enzymes specialized in the elongation or reorganization of beta-1,6-linked polymers. For a beta-1,6-linked structure to be elongated, the substrate must possess a beta-1,6-linked terminus.

We thank the reviewer for this important clarification. We agree that our original wording did not adequately distinguish between the β -1,6 linkage at the branch point and the β -1,3-linked structure of the side chain, and in doing so, incorrectly implied that these branches could serve as substrates for β -1,6 polymer elongation. We recognize that this interpretation is not biochemically supported. We have therefore revised the text to remove this implication and instead emphasize that β -1,6 linkages introduced by Ghs2 likely contribute to cell wall organization by generating structural features that facilitate the incorporation, retention, or remodeling of β -1,6-glucan components within the matrix. This section of the discussion has been re-written to improve the clarity.

25. In page 12, lines 309-310, the authors state: “Alternatively, beta-1,6-branched beta-1,3-glucan may be required for the stable incorporation or retention of linear beta-1,6-glucan within the wall matrix.” As previously noted, beta-1,6-glucan in fission yeast is constitutively branched; therefore, the reference to “linear” beta-1,6-glucan should be corrected or demonstrated as a new not yet described polysaccharide for accuracy. Furthermore, the model presented in Figure 6d lacks biochemical precision. It appears to depict Ghs2 joining individual glucose residues (de novo) to the nascent linear beta-1,3-glucan via a beta-1,6 linkage. To accurately represent a potential transglycosylase, the model should instead show Ghs2 catalyzing the attachment of preformed linear beta-1,3-glucan chains to the nascent beta-1,3-glucan backbone via a beta-1,6-linkage of a glucose residue.

The assignment of the linear β -1,6-glucan (H) signal is based on its characteristic chemical shifts reported in previous studies (Lowman et al., *PLoS One* 2011; Sugawara et al., *Carbohydr Res.* 2004; Zhang et al., *Bioactive Carbohydrates and Dietary Fibre* 2022), as well as the carbon-carbon correlations observed in the 2D ^{13}C - ^{13}C spectra of *S. pombe*, which selectively probe rigid cell wall components through dipolar coupling. Specifically, the resonances at C3 (76.44 ppm), C2 (74.14 ppm), and C4 (70.42 ppm) exhibit C3-C2 and C3-C4 correlations that are consistent with published ssNMR assignments of linear β -1,6-glucan, including those reported by the Wang group and in our previous ssNMR analysis of the *S. pombe* cell wall (Jacob et al., *PNAS* 2025).

C1	C2	C3	C4	C5	C6
102.96	73.16	76.32	69.79	75.22	68.23

Lowman et al., *PLoS One* 2011. doi:10.1371/journal.pone.0027614

C1	C2	C3	C4	C5	C6
103.00	73.21	76.37	69.89	75.28	68.30

Sugawara et al., *Carbohydr Res.* 2004. doi : 10.1016/j.carres.2004.05.033 (fraction 5, beta-1,6-glucan)

C1	C2	C3	C4	C5	C6
103.63	73.85	76.69	70.51	75.96	69.07

Storseth et al., *Carbohydr Res.* 2006. doi: 10.1016/j.carres.2006.05.005

C1	C2	C3	C4	C5	C6
103.59	73.07	77.01	69.65	73.26	69.19

Zhang et al., *Bioactive Carbohydrates and Dietary Fibre* 2022. doi: 10.1016/j.bcdf.2022.100311

Importantly, in this study, the linear β -1,6-glucan signal represents a minor component of the rigid cell wall fraction, accounting for approximately 2% of the rigid core in the wildtype cell wall. Notably, these measurements were obtained from intact whole cell wall preparations without prior chemical or enzymatic fractionation, thereby reflecting the native organization of the rigid cell wall matrix (Sugawara et al., *Carbohydr Res.* 2004).

Taken together, the chemical shift patterns and observed ^{13}C - ^{13}C correlations are fully consistent with established β -1,6-glucan assignments in both solid-state and solution-state NMR studies, supporting the identification of this signal as a linear β -1,6-glucan population within the rigid phase of the cell wall even though it represents only a minor component of the overall rigid core. We have revised the text (lines 342–352) to clarify that, in this context, “linear β -1,6-glucan” refers to β -1,6-linked glucan segments that are not further substituted with β -1,3 branches, as defined by their characteristic NMR signatures. While β -1,6-glucan in the fission yeast cell wall is generally highly branched, our data and prior work indicate that unsubstituted β -1,6-linked regions exist, and we have updated the manuscript to make this distinction explicit in the discussion.

We also agree that the precise biochemical mechanism and substrate of Ghs2 remain unresolved, and that a transglycosylase could, in principle, act on preformed β -1,3-glucan chains. However, we have chosen to retain the current schematic representation because it is intended to illustrate the introduction of β -1,6-linked branch points rather than to depict a specific catalytic mechanism.

In particular, prior structural analyses of the *S. cerevisiae* cell wall (Manners et al., 1977) indicate that many β -1,6-linked branches consist of single glucose residues, supporting a model in which branch formation can occur through the addition of minimal units. At the same time, our data do not distinguish whether Ghs2 utilizes pre-existing β -1,3-glucan chains or acts directly on nascent chains produced by Bgs3.

To address this uncertainty, we have revised the figure legend and accompanying text to clarify that the model is a simplified schematic and does not specify the precise nature of the donor substrate. We now explicitly state that Ghs2 may act on either nascent or preformed β -1,3-glucan chains. We believe this revision more accurately reflects the current state of knowledge while avoiding overinterpretation of the catalytic mechanism.

26. In page 13 (lines 332–336), the authors state: “...suggesting that distinct classes of auxiliary factors may regulate different glucan synthases and support a division of labor in cell wall construction: Bgs1 with Sbg1 produces septum-specific beta-1,3-glucan, Bgs4 supplies the lateral wall beta-1,3-glucan, and Bgs3, together with Ghs2, constructs branched networks at the division site and the lateral cell wall.” This paragraph is wrong and represents an oversimplification of the physiological roles of Bgs1 and Bgs4. Like Bgs3, both Bgs1 and Bgs4 localize to the cell poles (tips) and the septum, and they are involved in both cell wall, primary septum (Bgs1 and Bgs4) and secondary septum (Bgs4) biosynthesis (e.g., Reference 81 for Bgs1; Reference 20 and J. Cell Biol. 2013, 203:265–82 for Bgs4).

We have changed the wording to state: "Bgs1, together with Sbg1, plays a central role in linear β -1,3-glucan synthesis for primary septum formation, Bgs4 contributes to β -1,3-glucan synthesis in both the septum and lateral cell wall, and Bgs3, together with Ghs2, promotes the formation of branched β -glucan networks at the division site and along the lateral cell wall."

27. Finally, in page 16, lines 399-405, the authors use the phrasing "30 ODs of cells were lysed..." or "120 ODs of cells..." This terminology is imprecise and wrong. It is unclear whether the cultures were harvested during the logarithmic or stationary phase, or what the actual absorbance and cell count used for the lysates was. The authors should adopt more standard quantitative descriptions, specifying the growth phase and the exact number of harvested cells (e.g., 1×10^8 cells) to ensure experimental reproducibility. In addition, and more important, OD is not accepted biochemically for many years. The correct nomenclature is "Absorbance" and therefore OD₅₉₅ or any other wavelength used should be changed to A₅₉₅ or the corresponding wavelength.

We changed the wording as suggested and the text now reads: "In brief, cells were grown to mid-log phase ($A_{595} = 0.3-0.5$), and cultures corresponding to 120 units of A₅₉₅ (approximately 10^9 cells) were harvested." The wording was also changed on page 18 lines 445-446 and page 19 line 477-478.

1. In the revised Discussion section, the authors state: “Bgs1, together with Sbg1, plays a central role in linear β -1,3-glucan synthesis for primary septum formation, Bgs4 contributes to β -1,3-glucan synthesis in both the septum and lateral cell wall, and Bgs3, together with Ghs2, promotes the formation of branched β -glucan networks...”

This updated paragraph remains imprecise and incomplete as it still misses the multi-spatial role of these synthases. Experimental evidence has established that Bgs1 does not function exclusively at the septum; it also localizes to active growth poles (cell tips) and plays a fundamental role in maintaining polarized growth (as supported by reference 93 of this current manuscript).

Also, references 39 and 40 describe that the deletion of *sbg1* results in lethality accompanied by severe defects in both polarized growth and division septum assembly. These studies proved that Sbg1 and Bgs1 are mutually dependent for their correct localization at cell tips and the division site (i.e., GFP-Bgs1 is cleared from the cell tip plasma membrane in *sbg1* mutants). This simplification also directly contradicts the authors' own statement: “Sbg1 which associates with the β -1,3-glucan synthase Bgs1 and is required for Bgs1's proper localization and function at sites of polarized growth.”

The authors must rewrite this paragraph to explicitly state that the Bgs1–Sbg1 complex is essential for both primary septum formation and polarized growth at cell tips, rather than restricting its functional narrative solely to the septum.

We have not suggested elsewhere in the manuscript that Bgs1-Sbg1 functions solely at the septum. So, as recommended by the reviewer, we have now explicitly stated that the Bgs1-Sbg1 complex is essential for both primary septum formation and polarized growth at cell tips. Specifically, the sentence has been corrected to: “Bgs1, together with Sbg1, plays a central role in linear β -1,3-glucan synthesis for primary septum formation and are also important for polarized growth at the cell tips,.” The relevant reference has also been added.

2. Throughout the text, figures, and supplementary materials, the authors must follow the standard fission yeast genetic nomenclature as depicted in Table S1. All wild-type loci must explicitly include the plus symbol as a superscript (e.g., *bgs3*⁺, *ghs2*⁺, *bgs1*⁺).

The correct yeast genetic nomenclature has been used throughout the paper and figures. We have corrected the supplementary material.

In the References section, all the gene names, genera, and species must be always italicized, and references to the different types of cell wall glucans should be uniformized by utilizing Greek symbols (α and β). Furthermore, the typographical error in reference 28 (“cell wall (1-->6)-beta-glucan synthesis”) should be corrected.

All species names and gene names have been italicized in the reference list, “+” has been made superscript, and reference 28 (now 27) has been fixed.

In the Introduction, the paragraph “The biosynthesis of linear β -1,3-glucans is carried out by the essential integral membrane synthases Bgs1, Bgs3 and Bgs4 as well as the meiosis-specific Bgs2” is attributed to references 21 to 26. I think that several original references for Bgs1 from the Balasubramanian and Ribas’s labs are missing in the main text, despite some being included in Table S1. In opposition, reference 21 relates to the alpha glucan synthase Mok1/Ags1 which is unrelated to the synthesis of beta-glucans described in this sentence. If the authors decide to include an independent sentence for the alpha glucan synthase, then they should also add the reference Satoshi Katayama, S. et al. (1999). Journal of Cell Biology, 144 (6): 1173–1186.

We removed the Ags1 reference from the introduction sentence mentioned. All references from Table S3 have been added to the main text.

Additionally, the original references for *cps1-191* and *cwg1-1* mutant alleles are absent in the manuscript and must be incorporated.

References for *cps1-191* and *cwg1-1* were added to the strain table and main text.

In Supplementary Table S1, the genotype *his3-1* must be changed to *his3-D1*. In addition, the included references the GFP-Bgs1 and GFP-Bgs3 strains Cortès (Cortés in the main text) et al. 2007 and 2002 lack in the main manuscript text. Similarly, as recommended above, the original strains and references for *cps1-191* and *cwg1-1* should be included in the table. Lastly, in Table S3 all references, with the sole exception of Chakraborty, A. et al. 2021, are missing from the main manuscript text.

Table S1 *his3-D1* has been fixed. We added references to the main text for GFP-Bgs1 and GFP-Bgs3 (and RFP-Ags1). All references from Table S3 have been added to the main text.

3. In their rebuttal, the authors justify the poor quality and diffuse nature of the GFP-Bgs3 (Figure S1B) band by stating: “Bgs3 is a large (~210 kDa), heavily glycosylated, low-abundance integral membrane protein, which makes its detection by immunoblotting inherently challenging... To our knowledge, only a single other study includes Bgs3 immunoblots (Martin et al., Eukaryotic Cell 2003) [which] used an overexpression system...”

This affirmation is incorrect and ignores established literature. In several publications from Dr. Ribas’s or Dr. Balasubramanian laboratories other Bgs proteins that share similar high molecular weights and probably the same glycosylation profiles of Bgs3 have been resolved as sharper and defined bands under the control of their endogenous promoters without the distinctive “smearing” shown here.

Also, the assertion that physiological levels of Bgs3 have never been blotted or analyzed outside of Martin et al. (2003) again is incorrect. Endogenous physiological levels of Bgs3 have been clearly resolved and published in Cortes, J.C.G. et al. (2007). Molecular Microbiology, 65: 201-217 (Specifically Figure 2B) or Munoz, J. et al. (2013). Journal of Cell Biology, 203(2): 265–282 (Supplementary Figure S1F).

Although technically the immunoblots could be improved, the similar diffuse band and smears in

the four lines, together with the detection of GFP-Bgs3 in both wild-type and ghs2-2 cells, are sufficient to support the corresponding conclusion.

We stand corrected. However, this comment (#3) does not require any text changes as noted in the next comment.