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Investigation

Cohesin dysfunction results in cell wall defects in budding yeast

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Abstract

Cohesin is a conserved chromatin-binding multisubunit protein complex involved in diverse chromosomal transactions such as sister-chromatid cohesion, chromosome condensation, regulation of gene expression, DNA replication, and repair. While working with a budding yeast temperature-sensitive mutant, *mcd1-1*, defective in a cohesin subunit, we observed that it was resistant to zymolyase, indicating an altered cell wall organization. The budding yeast cell wall is a strong but elastic structure essential for maintenance of cell shape and protection from extreme environmental challenges. Here, we show that the cohesin complex plays an important role in cell wall maintenance. Cohesin mutants showed high chitin content in the cell wall and sensitivity to multiple cell wall stress-inducing agents. Interestingly, temperature-dependent lethality of cohesin mutants was osmoremedial, in a *HOG1-MAPK* pathway-dependent manner, suggesting that the temperature sensitivity of these mutants may arise partially from cell wall defects. Moreover, Mpk1 hyper-phosphorylation indicated activation of the cell wall integrity (CWI) signaling pathway in cohesin mutants. Genetic interaction analysis revealed that the CWI pathway is essential for survival of *mcd1-1* upon additional cell wall stress. The cell wall defect was independent of the cohesion function and accompanied by misregulation of expression of several genes having cell wall-related functions. Our findings reveal a requirement of cohesin in maintenance of CWI that is independent of the CWI pathway, and that may arise from cohesin's role in regulating the expression of multiple genes encoding proteins involved in cell wall organization and biosynthesis.

Keywords: Cohesin; Cell wall; Osmotic stress; cohesion; gene expression; cohesinopathy

Introduction

Yeast cells are encased within a protective cell wall, which acts as a first line of defence against rapid and extreme changes in the environment. The cell wall is a complex structure essential for maintenance of cell shape that protects against osmotic and mechanical stress, and acts as a scaffold for cell-surface proteins (Klis *et al.* 2006; Levin 2011). The budding yeast cell wall is mainly made up of three polysaccharides: polymers of mannose (as mannoproteins), β-glucan chains, and chitin (Klis *et al.* 2006; Lesage and Bussey 2006). In *Saccharomyces cerevisiae*, the cell wall can be divided into two layers, an electron-transparent inner layer and an electron-dense outer layer. The inner layer is composed of glucan polymers and chitin (β-1,4-*N*-acetylglucosamine polymers). β-1,3-glucan chains make up ∼80–90% of the inner layer and the rest consists of polymers of β-1,6-glucan chains (8–18%) and chitin (1–2%) (Klis *et al.* 2006; Lesage and Bussey 2006). The outer layer is mainly composed of glycoproteins represented by glycosylphosphatidylinositol proteins, which are linked to the outer surface of β-1,3-glucan via β-1,6-glucan chains (Lesage and Bussey 2006; Orlean 2012).

The cell wall is a dynamic structure that can adapt to environmental, physiological, and morphological changes. A cell wall compensatory mechanism is activated in response to cell wall perturbing agents or mutations that affect cell wall integrity (CWI). The CWI signaling pathway is a part of this compensatory mechanism whose activation leads to cell wall remodeling and compensatory synthesis of cell wall material to prevent cell lysis (Levin *et al.* 1994; Klis *et al.* 2002).

The CWI signaling pathway consists of a group of plasma membrane proteins, such as Wsc1-3, and Mid2, which act as sensors for the pathway under various stress conditions. Their signal results in the activation of Rho1p GTPase via Rom1/2 GEFs (Guanosine nucleotide exchange factors) and is then further transferred to the Pkc1-controlled MAP kinase cascade. This MAP kinase cascade is a linear pathway consisting of Pkc1, a mitogen-activated protein kinase kinase kinase (MAPKKK) Bck1, a pair of redundant MAPKKs (Mkk1/2), and a MAP kinase (Mpk1/Slt2) (Levin 1994, 2005; Lesage and Bussey 2006). The outcome of this pathway is activation of a transcriptional response to protect the cell from lysis. This is achieved by Mpk1-mediated activation of

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the transcription factors, such as Rlm1p (Jung *et al.* 2002) and SBF (Swi4/Swi6). The majority of the Rlm1-controlled genes are involved in cell wall biogenesis, *e.g.*, *FKS1* and *GSC2/FKS2* (two catalytic subunits of β-1,3-glucan synthase), *CHS3* (chitin synthase 3, involved in chitin synthesis), and various cell wall proteins (CWPs) (Jung and Levin 1999). Mutations in various proteins involved in CWI signaling and cell wall biosynthesis show an osmoremedial cell lysis phenotype (Lee and Levin 1992; Paravicini *et al.* 1992; Levin 1994, 2011), indicating that these mutants have a weakened cell wall and therefore need osmotic support for survival.

Compensatory mechanisms responsive to cell wall perturbation are not restricted only to the CWI pathway. CWI defects may also lead to activation of other pathways, such as calcium/calcineurin, the *HOG1-MAPK* pathway, or Msn2/Msn4 transcription factors (Brewster *et al.* 1993; Martinez-Pastor *et al.* 1996; Estruch 2000; Hohmann 2002; Klis *et al.* 2002; Yoshimoto *et al.* 2002; Levin 2011). There also exists a CWI checkpoint that links cell wall synthesis to the cell cycle (Suzuki *et al.* 2004). Mutations in genes encoding proteins involved in various processes related to cell wall biosynthesis or the CWI pathway lead to activation of the CWI checkpoint and cell cycle arrest with a small bud and duplicated DNA (Levin and Bartlett-Heubusch 1992; Ram *et al.* 1995; Suzuki *et al.* 2004).

Cohesin is one of the structural maintenance of chromosome (SMC) family complexes composed of Smc1, Smc3, Mcd1/Scc1, and Irr1/Scc3. Cohesin binds at regular intervals throughout the chromosome length (Blat and Kleckner 1999; Laloraya *et al.* 2000; Glynn *et al.* 2004) and is involved in various chromosomal transactions, such as sister chromatid cohesion, chromosome condensation, DNA replication and repair, and regulation of gene expression (Guacci *et al.* 1997; Michaelis *et al.* 1997; Donze *et al.* 1999; Sjogren and Nasmyth 2001; Biswas *et al.* 2009; Terret *et al.* 2009; Skibbens *et al.* 2010; Jeppsson *et al.* 2014; Schalbetter *et al.* 2017; Kothiwal and Laloraya 2019). Interestingly, while working with *mcd1-1*, a temperature-sensitive (ts) mutant of the budding yeast cohesin complex, we observed that mutant cells are resistant to zymolyase (a cell wall digesting enzyme) treatment, indicating an altered cell wall structure in the cohesin mutant. This prompted us to study the role of cohesin in cell wall assembly and maintenance. A role for cohesin in CWI was suggested earlier as well (Cena *et al.* 2007), when a heterozygous diploid *irr1-1/IRR1* having a mutation in one copy of the gene encoding the cohesin subunit Irr1, was found to be sensitive to cell wall affecting compounds and showed altered chitin distribution, although a lot of questions remained unanswered. For example, is this a subunit specific effect (limited to Irr1) or a role involving the whole cohesin complex? What are the cell wall defects caused by mutation in cohesin? How does cohesin interact with the known players of the cell wall assembly and integrity pathway? What role does cohesin play in CWI? And how is this new role linked to the known functions of cohesin, etc.? Our study tries to answer some of these important questions.

Here, we show that the cohesin holocomplex is required for CWI as various cohesin subunit ts mutants are highly sensitive to cell wall stress inducing agents and show cell lysis at the restrictive temperature. We further show that ts phenotype of cohesin mutants is osmoremedial and that cohesion and cell wall maintenance are separable functions of cohesin. We report that the cohesin mutant requires an active CWI and *HOG-MAPK* pathway to survive under cell wall stress conditions, suggesting an important role for cohesin in CWI, possibly arising from its effect on gene expression of numerous genes involved in cell wall biosynthesis and maintenance.

Material and methods

Media and reagents

Cells were grown in YPD, YPD plus sorbitol, or NaCl medium as indicated. We purchased hygromycin and zymolyase from US Biologicals, and nourseothricin from Jena Biosciences. G418, calcofluor white, Congo red, tunicamycin, and chlorpromazine were obtained from Sigma. For western blot analysis, 12CA5 anti-HA antibody was purchased from Roche Applied Sciences, anti phospho-p44/42 MAPK from Cell Signalling Technology, anti-Pgk1 antibody (YOL1/34) from Abcam, and HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Bangalore Genei.

Yeast strains and plasmids

The yeast strains and plasmids used or generated in this study are listed in Supplementary Tables S1 and S2, respectively. Oligonucleotides used for construction of strains and plasmids were synthesized by Sigma-Aldrich; sequences of these oligonucleotides are provided in Supplementary Table S3.

Construction of plasmids having cassettes to integrate MCD1 *or* mcd1-1 *at* LEU2 *(pDK29 and pDK30):*

The plasmids pDK29 and pDK30 were created in two steps. First *MCD1* or *mcd1-1* was PCR-amplified from genomic DNA of ROY783 or VG955-7D respectively, using the primer pair SLO505/SLO612 and cloned into EcoRV site of pUC19-*LEU2* (*LEU2* BglII fragment excised from Yep13 inserted in the BamHI site of pUC19) and were named pDK31 and pDK32, respectively. In the second step, a *natNT2* containing EcoRV-PvuII fragment from pYM17 was cloned into Ale1 digested pDK31 or pDK32 to get pDK29 and pDK30, respectively.

Construction of plasmids for the over-expression of PSA1 *and* GUK1*:*

The *PSA1* ORF along with its native promoter was amplified using the primer pair SLO1119/SLO1120 and cloned into the Sma1 site of pRS425 and pRS422 to create pDK42 and pSW1, respectively. Clones were confirmed by the excision of the *PSA1* cassette (2010 bp) from the plasmid upon double digestion with BamH1 and Xho1 restriction enzymes. Similarly, *GUK1* was amplified (using the primer pair SLO1121/SLO1122) and cloned into the Sma1 site of the pRS422 to create pSW2. Confirmation of desired plasmids was done by double digestion with BamH1 and Xho1 restriction enzymes to release the *GUK1* cassette (1378 bp). Both the *PSA1* and *GUK1* fragments were amplified from genomic DNA isolated from the *W3031a* strain.

Creation of MCD1 *and* mcd1-1 *derivatives of various strains:*

Yeast strains having *MCD1* and *mcd1-1* inserted at the *LEU2* locus were created in two steps. First *mcd1-1* or its corresponding wild-type (WT) was integrated at *LEU2* using the plasmids pDK29 (pUC19 *leu2::MCD1 natNT2*) and pDK30 (pUC19 *leu2::mcd1-1 natNT2*). Both the plasmids were digested with HpaI and after ethanol precipitation approximately 3 μg of the DNA were used to transform the appropriate yeast strains. Transformants were selected on YPD plates having nourseothricin (100 μg/ml). *MCD1* integration at *LEU2* was confirmed by colony PCR using primers SLO791 and LeuR3B that yield a band of ~500 bp upon correct

integration, whereas a band of ~1600 bp is expected with SLO791 and LeuL1F upon *mcd1-1* integration. The second step required creation of an *mcd1* null allele at its original chromosomal location; *MCD1* was deleted from its genomic locus using one-step PCR-mediated gene disruption using SLO604 and SLO874 and pYM variants, pYM16 and pYM22 (Janke *et al.* 2004), as the template. Correct disruption was confirmed by colony PCR using the primers SLO791 and SLO613 that produce a band of ~450 bp.

Creation of a galactose-inducible allele of MCD1 *(pGAL-MCD1):*

To create the galactose-inducible *MCD1* allele, the *MCD1* promoter was replaced by one-step PCR-mediated integration (Janke *et al.* 2004). *GALL* promoter was amplified from pYM-N28 using primer pair SLO874/SLO1039. The yeast strain W303-1a was transformed with ethanol-precipitated PCR product and transformed cells were selected by plating on nourseothricin (100 μg/ml) containing plates. Correct integration of the promoter fragment was confirmed by colony PCR using primer pair SLO505/SLO941 that generates an ~850-bp fragment in desired integrants.

All the deletion mutants and epitope-tagged strains were created by one-step PCR-mediated gene replacement or integration (Janke *et al.* 2004). The *hog1 and mpk1* null alleles were created using the primer pair SLO1047/SLO1048 and SLO992/SLO993, respectively, and pYB239 (a kind gift from Yves Barral) or pYM17 were used as the template. *GAS1* and *FKS1* were deleted using a PCR-amplified replacement cassette produced using pYM17 as the template for PCR amplification using primers SLO1053/SLO1054 and SLO1056/1057, respectively. C-terminal epitope tagging of *CLB2* with 3HA was done using the primer pair SLO1050/SLO1051 and pYM22 as the template. Correct deletions or insertions were confirmed by colony PCR using marker-specific primer (SLO791) and a locus-specific primer, SLO1049 (*HOG1*), SLO896 (*MPK1*), SLO1055 (*GAS1*), SLO1058 (*FKS1*), and SLO1052 (*CLB2*), not overlapping with the disruption cassette.

Spot assay

To assay growth and drug sensitivity, yeast cells were grown in YPD media until they reached an OD_{600} of 0.8–1.0. Then, the cultures were serially diluted 10-fold starting from 1 OD and spotted on YPD plates or on YPD plates having various drugs or osmostabilizers. Plates were incubated at various temperatures (as indicated in the figure or corresponding figure legend) and images were captured at various time points as mentioned in the figures. Two or more independent biological isolates (of integrants or gene deletions) were tested for growth defects and drug sensitivities as mentioned above.

Zymolyase sensitivity assay

The assay was adapted from Krause and Gray (2002). Cells from an exponentially growing yeast culture were pelleted and resuspended in 1 ml of YPD. β-mercaptoethanol (286 μM) was added to the cells that were incubated at 30°C for 30 min. Cells were pelleted and resuspended in 1 ml of 1 M NaN_3. About 150 μl of this suspension was added to 850 μl PBS, to which 0.5 μl Zymolyase 20T (15 mg/ml) was added and an initial OD_{600nm} reading was observed. Subsequently, $OD_{600\ nm}$ readings were measured for a total of 30 min at 5 min intervals. The graph shows a change in the ratio of OD_{600nm} at a particular time to initial $OD_{600\ nm}$ plotted on the Y-axis with respect to time on the X-axis.

Calcofluor white staining

Cells from a log phase culture were pelleted and resuspended in PBS. About 3–4 μl of cells were placed on a cover slip and one drop each of calcofluor white stain (Sigma), and 10% potassium hydroxide were dropped on it. Images were captured using LSM880 (Airyscan) confocal system from Zeiss and were processed using ZEN BLACK 2.1 software.

Sister chromatid cohesion assay

For the sister chromatid cohesion assay, exponentially growing WT and *mcd1-1* cells at 23°C were supplemented with 1% DMSO, for 30 min and nocodazole (SIGMA) was added to a final concentration of 15 μg/ml; cells were incubated at 37°C (2 h) for arresting in G2/M. Metaphase-arrested cells having GFP chromosome tags (Neurohr *et al.* 2011) were analyzed by confocal microscopy, for which 3 μl (1 mg/ml) DAPI was added to 1 ml of cell suspension for staining DNA 10 min prior to harvesting. The experiment was repeated four times and a total of >250 cells were counted for both WT and mutant. Images were captured using Zeiss LSM880 (Airyscan) confocal microscope and were analyzed using ZEN 2.1 (black) software. The unpaired two-tailed Student's *t*-test was used to evaluate the statistical significance of the observed differences in cohesion. *** denotes $P \leq 0.001$.

RT-qPCR

Total RNA was isolated from exponentially growing yeast cells using Qiagen RNeasy minikit. cDNA was prepared from 2 μg of DNase-treated RNA and qPCR was carried out on a Bio-rad IQ5 real-time PCR machine using Bio-rad iTaq universal SYBR green supermix. The primers used for qPCR are listed in Supplementary Table S4. The expression level of test genes in all cases was normalized with respect to *ACT1*. The mean values for $n \geq 3$ experiments are plotted on the Y-axis. Error bars represent the standard error of the mean. The unpaired two-tailed Student's *t*-test was used to evaluate the statistical significance of the observed differences in expression. * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, and *** denotes $P \leq 0.001$.

Preparation of whole cell extracts and western blotting

Cells were grown to mid-log phase and protein extracts were prepared by lysing the cells in trichloroacetic acid using glass beads (Foiani *et al.* 1994). Samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane using Bio-Rad semi dry transfer apparatus in presence of Tris-glycine transfer buffer (39 mM glycine, 48 mM Tris, and 15% methanol). Proteins were detected using the indicated primary antibodies and HRP conjugated secondary antibody (Goat anti-mouse IgG HRP and Goat anti-rabbit IgG HRP). Blots were developed using Biorad Clarity™ Western ECL substrate or Perkin-Elmer chemiluminescence reagents.

Quantitative β-1,3-glucan measurements

Amount of β-1,3-glucan per cell was measured using aniline blue as described earlier (Watanabe *et al.* 2001) with some modifications. 1 OD early log phase cells were harvested, washed, and resuspended in 500 μl TE. About 6 N NaOH was added to the suspension to a final concentration of 1 N and incubated in a water bath at 80°C for 30 min followed by addition of 2.1 ml of AB mix

[0.03% aniline blue (Sigma), 0.18 N HCl, and 0.49 M glycine/NaOH, pH 9.5]. The tubes were vortexed briefly and then incubated for 30 min at 50°C and an additional 30 min at room temperature to allow reaction with the fluorochrome and decolorization of the aniline blue. Fluorescence of β-1,3-glucan was quantified using a spectrofluorophotometer (TECAN Infinite M200 PRO) with a excitation wavelength of 400 nm and emission wavelength of 460 nm.

Colony overlay assay

Overlay assay was essentially performed as described in Paravicini et al. (1992). Freshly patched cells were resuspended in water and 5 μl of 1 OD_{600} cell suspension were spotted on YAPD plates with or without sorbitol. Plates were incubated for 1 day at 30°C and then shifted overnight to 37°C. Cell spots (colonies) were then overlaid with an alkaline phosphatase assay solution containing 0.05 M glycine hydrochloride (pH 9.5), 1% agar, and 10 mM chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate. Colonies which contained lysed cells appeared blue, whereas intact colonies remained white. Images were captured after 100 min of incubation with alkaline phosphatase assay solution at 37°C.

RNA-sequencing

Generation of RNA-seq data and the data analysis pipeline has been described previously (Kothiwal and Laloraya 2019). The RNA-Seq data have been deposited in the Gene Expression Omnibus database (accession no. GSE126364). Genes whose expression was altered in the *mcd1-1* mutant and that are also involved in cell wall organization or biogenesis were identified using the GO slim mapper tool of SGD and are listed in Supplementary Table S7.

Fluorescence-activated cell sorter analysis

For fluorescence-activated cell sorter (FACS) analysis, 0.1–0.2 OD_{600} cells from exponentially growing cultures were fixed in 70% ethanol overnight at 4°C. Cells were washed once with PBS, dispersed by gentle sonication (Branson Sonifier; 20% duty cycle six to seven pulses) and treated with 0.25 mg/ml RNase A overnight at 37°C. Propidium iodide was added to a final concentration of 16 μg/ml, and FACS analysis was performed on a BD FACSVerse. The data were analyzed using BD FACSDiva software.

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Supplementary material is available at figshare DOI: https://doi.org/10.25386/genetics.13308476.

Results

Mutation in cohesin results in alteration of cell wall composition and activation of a compensatory mechanism

We observed that *mcd1-1*-mutant cells require more time to form spheroplasts upon zymolyase treatment relative to WT cells. To quantitatively measure this delay in completion of zymolyase treatment for *mcd1-1* cells, we carried out a zymolyase sensitivity assay. Zymolyase sensitivity or treatment can be monitored by observing a drop in optical density (OD) with respect to time (Krause and Gray 2002). We monitored zymolyase treatment for 30 min by measuring OD_{600} (OD at 600 nm) at the end of every 5 min interval. This assay revealed that the OD of mutant cell cultures dropped more slowly compared to WT (Figure 1A), confirming that *mcd1-1* cells are resistant to zymolyase.

Zymolyase resistance has been linked to increased chitin content in the cell wall (Aguilar-Uscanga and Francois 2003). Hence, we next examined chitin deposition in WT and mutant cells using calcofluor white, a dye which specifically stains chitin and cellulose. We observed increased staining in *mcd1-1* cells, indicating enhanced chitin deposition in the cell wall of the *mcd1-1* mutant compared to WT cells (Figure 1B) and suggesting that the zymolyase resistance of *mcd1-1* cells may be because of increased chitin in the cell wall.

Cell wall stress results in activation of a compensatory mechanism and increased chitin deposition in a *CHS3*-dependent manner (Popolo *et al.* 1997; Garcı'a-Rodriguez *et al.* 2000; Valdivieso *et al.* 2000). Similarly, the expression of *FKS2/GSC2* is also known to increase specifically upon cell wall stress (Zhao *et al.* 1998). To test whether the increased chitin deposition observed in *mcd1-1* cells is a response to cell wall stress, we examined the expression of these two genes, whose expression increases specifically upon cell wall stress. RT-qPCR analysis revealed a significant increase in expression of both *CHS3* and *FKS2* in the *mcd1-1* mutant relative to WT cells (Figure 1C), suggesting elevated cell wall stress and confirming activation of a compensatory mechanism in the cohesin mutant.

Cohesin mutants are sensitive to cell wall stress-inducing agents

Increase in chitin content has been observed in various mutants defective in cell wall biosynthesis (Popolo *et al.* 1997; Garcı'a-Rodriguez *et al.* 2000; Valdivieso *et al.* 2000). Cell wall defects can be assessed by testing sensitivity toward cell wall stress-inducing agents. To test for a cell wall defect in the cohesin mutant, we targeted three major components of the yeast cell wall-chitin, β-1,3-glucans and glycoproteins.

First, we tested the sensitivity of three different cohesin subunit mutants (*mcd1-1*, *smc3-1*, and *smc1-2*) toward chitin intercalating agents, calcofluor white and Congo red, which generate cell wall stress by inhibiting formation of chitin fibrils (Elorza *et al.* 1983; Roncero and Duran 1985; Roncero *et al.* 1988). All three mutants were highly sensitive to the presence of chitin intercalating agents (Figure 2A). Similarly, we found that all the mutants were highly sensitive to anidulafungin (an echinocandin family member that causes cell wall stress by inhibiting β-1,3-glucan synthase) (Douglas 2001; Wiederhold and Lewis 2003; Levin 2011) as well (Figure 2B). Majority of the CWPs are transported through the secretory pathway, where they are glycosylated in the endoplasmic reticulum. We next used tunicamycin to inhibit N-glycosylation (Tkacz and Lampen 1975) and observed that *mcd1-1* cells are also sensitive to tunicamycin, albeit to a much lesser extent (Figure 2C). Interestingly, *mcd1-1* cells did not show any sensitivity toward the plasma membrane stretching agent chlorpromazine (Figure 2D) (Sheetz and Singer 1974; Kamada *et al.* 1995), suggesting that cohesin mutants are specifically sensitive to cell wall stress-inducing agents. Altogether, these observations confirm a cell wall defect in cohesin mutants.

Temperature-sensitive phenotype of cohesin mutants is osmoremedial

Mutations in various genes involved in CWI signaling and cell wall biosynthesis pathway show either a cell lysis phenotype that is osmoremedial, or, a cell cycle arrest with a small bud and

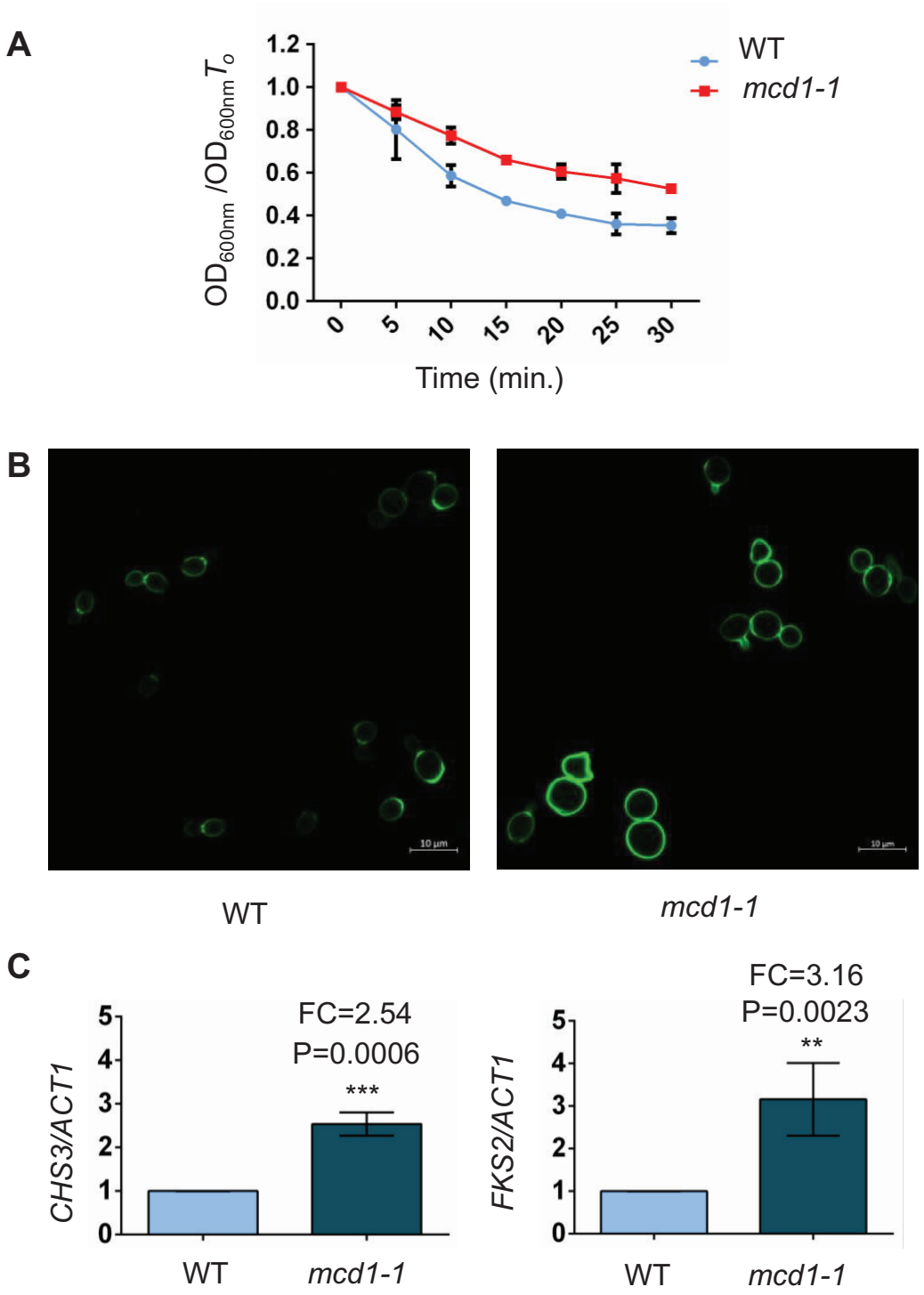

Figure 1 Mutation in cohesin leads to alteration in the cell wall. (A) *mcd1-1* is resistant to zymolyase. Zymolyase sensitivity was measured as decrease in OD_{600} with respect to time at 30°C in WT (SLY2199) and *mcd1-1* (SLY2200) cells. Y-axis: ratio of OD_{600nm} at a particular time to the initial OD_{600nm}. (B) Increased chitin deposition in *mcd1-1*-mutant cells. Exponentially growing cells at 30°C were stained with calcofluor white that stains chitin. Images were captured using LSM880 system from Carl Zeiss. A representative field is shown in each case from two independent biological replicates. (C) Transcript levels of two genes, *CHS3* ($n = 3$) and *FKS2/GSC2* ($n = 4$), whose expression increases upon cell wall stress, were compared in WT(SLY2199) and *mcd1-1*(SLY2200) cells grown at 31°C, by RT-qPCR. The mean values are plotted on the Y-axis, error bars indicate standard deviation (SD); FC ~ fold change. The statistical significance was analyzed using the two-tailed unpaired *t*-test where *** denotes $P \leq 0.001$ and ** denotes $P \leq 0.01$.

duplicated DNA (Levin *et al.* 1994; Levin 2011). Many of the cohesin complex mutants are *ts* and this *ts* phenotype has been linked to defects in sister chromatid cohesion. As we observed cell wall defects in the cohesin mutants, we wondered whether the *ts* phenotype could be a result of a cell cycle arrest (due to activation of the CWI checkpoint) or cell death (due to lysis resulting from defects in the cell wall). To test the activation of the CWI checkpoint, we checked Clb2 protein levels after releasing G1 cells into nocodazole containing medium at the restrictive temperature (37°C). It has been shown that upon activation of the CWI

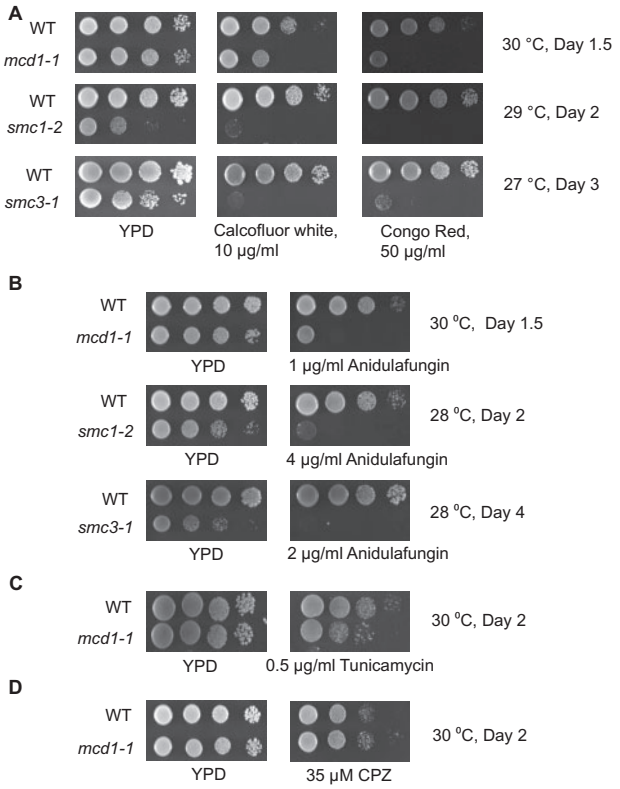

Figure 2 Sensitivity of cohesin mutants to cell wall stress inducers. (A and B) Cohesin mutants are sensitive to chitin intercalating agents and a β-1-3 glucan synthase inhibitor. Exponentially growing WT (VG906-1A), *mcd1-1* (VG955-7D), *smc1-2* (1355-1A), *smc3-1* (ROY1060), and its corresponding WT (ROY951) cells were serially diluted (10 fold) and spotted on YPD plates or YPD plates containing indicated drugs. (C and D) Assessment of *mcd1-1* sensitivity to the N-glycosylation inhibitor (Tunicamycin) and plasma membrane stretching agent (chlorpromazine) using WT (SLY2764) and mutant (SLY2764) strains. Plates were incubated at the indicated temperatures and images were captured at regular intervals.

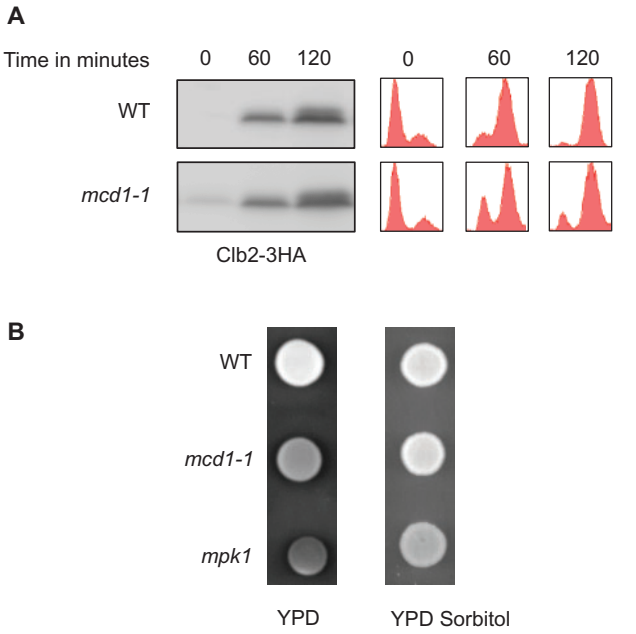

Figure 3 *mcd1-1* cells are lysed at the restrictive temperature. (A) Mutation in cohesin does not lead to activation of the CWI checkpoint. Clb2 -3HA expression in WT (SLY2790) and *mcd1-1* (SLY2792) was compared, at the indicated times, after releasing G1 arrested cells at the restrictive temperature of 37°C in nocodazole-containing YPD medium (left panels). FACS showing cell cycle profile of samples at western timepoints (right panels). (B) WT (SLY2764), *mcd1-1* (SLY2767), and *mpk1*Δ (SLY2869) cells were spotted onto YPD plates without (YPD) or with (YPD plus 1 M sorbitol) osmotic support, grown for 24 h at 30°C, and then incubated overnight at 37°C. The plates were then overlaid with an alkaline phosphatase assay solution and image was captured after 100 min of incubation at 37°C.

checkpoint, cells arrest at a point prior to G2M and hence fail to accumulate Clb2 (Suzuki *et al.* 2004). We did not observe a defect in Clb2 accumulation in *mcd1-1* cells (Figure 3A) suggesting that the *ts* phenotype of this mutant is not due to the activation of the CWI checkpoint.

To examine cell lysis in a cohesin mutant (*mcd1-1*), we carried out a simple colony overlay assay in which leakage of alkaline phosphatase from cells is detected (Paravicini *et al.* 1992). In this assay, colonies or spots having lysed cells appear blue whereas intact cells appear white. *mpk1*Δ cells were used as a positive control for the assay. The overlay assay revealed that *mcd1-1* cells are preferentially lysed at 37 °C compared to WT cells (Figure 3B, left panel). Importantly, this lysis phenotype was osmoremedial (Figure 3B, right panel) as it did not occur in the presence of 1 M sorbitol in *mcd1-1*, suggesting that lysis is indeed related to cell wall defects in this mutant.

We wondered whether increased extracellular osmolarity could suppress the temperature sensitivity of different cohesin subunit mutants. Indeed, increasing extracellular osmolarity by addition of either 1 M sorbitol or 1 M NaCl to the growth medium suppressed the ts phenotype of all three cohesin mutants (*mcd1-1*, *smc3-1*, and *smc1-2*), albeit to varying extents (Figure 4). This suggests that the ts nature of cohesin mutants is osmoremedial.

Cohesin's role in CWI is independent of its function in sister chromatid cohesion

Cohesin has a well-established role in sister chromatid cohesion (Guacci *et al.* 1997; Michaelis *et al.* 1997), and it is believed to be one of the essential functions of this complex. We wondered whether sorbitol-mediated suppression of the ts phenotype of cohesin mutants could result from rescue of the cohesion defect. To test this hypothesis, we analyzed sister chromatid cohesion at 37°C in the presence or absence of sorbitol. Surprisingly, we observed a modest rescue of the cohesion defect in nocodazole arrested *mcd1-1* cells in presence of sorbitol (Figure 5A). We wondered whether this small rescue (~13%) in the cohesion defect could be a reason behind suppression of the ts phenotype. To understand this further, first we compared the protein level of *mcd1-1* in absence or presence of sorbitol. Our western blot analysis showed a very small increase in the protein level of *mcd1-1* at 37°C in presence of sorbitol (Figure 5B), suggesting that the modest rescue of the cohesion defect could be a consequence of this small increase in the level of the *mcd1-1* mutant protein.

To directly test the effect of sister chromatid cohesion on cell wall function of cohesin, we made use of previously described quantized reduction alleles (1 stop and 2 stop alleles) of *MCD1* (Heidinger-Pauli *et al.* 2010); these alleles reduce the Mcd1 protein levels to 30% and 13% of the WT level, respectively and have been shown to have no defect in sister chromatid cohesion either at chromosome arms or centromeres (Heidinger-Pauli *et al.* 2010). Our spot test analysis revealed that cells bearing these alleles are also sensitive to cell wall stress-inducing agents and are ts,

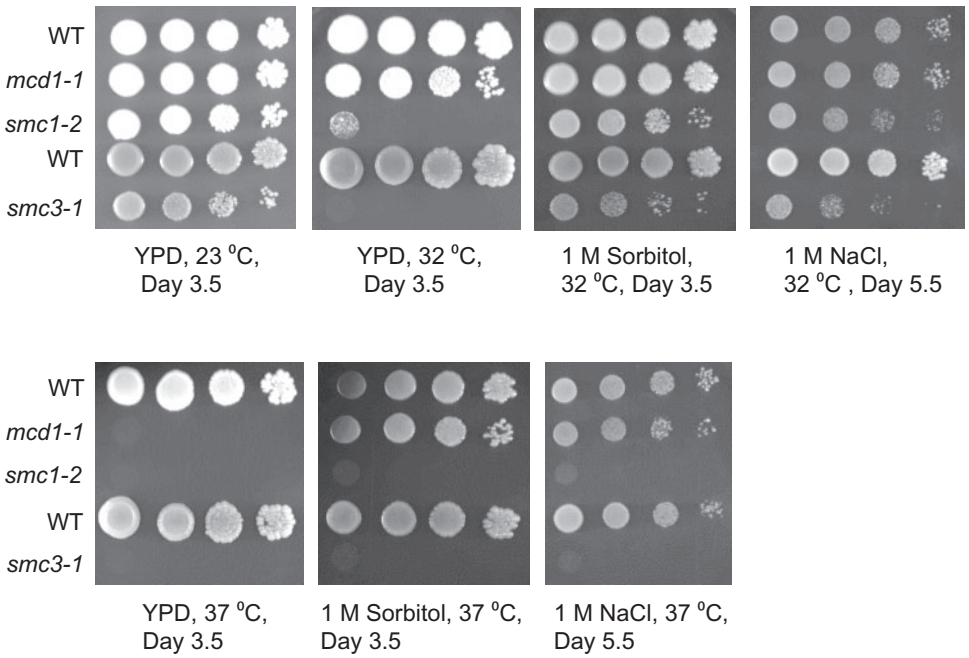

Figure 4 Temperature-sensitive phenotype of cohesin mutants is osmoremedial. Exponentially growing WT (VG906-1A), *mcd1-1* (VG955-7D), *smc1-2* (1355-1A), *smc3-1* (ROY1060), and its corresponding WT (ROY951) cells were serially diluted (10-fold) and spotted on YPD, and YPD containing 1 M sorbitol or NaCl, plates. Plates were incubated at the indicated temperatures.

specially the 2 stop allele (Figure 5C). Importantly, this ts phenotype could be rescued by adding an osmostabilizer (1 M sorbitol) in the growth medium (Figure 5C). Since these alleles do not have a sister chromatid cohesion defect but still show sensitivity toward cell wall stress-inducing agents and an osmoremedial ts phenotype, it is likely that cohesin's role in CWI is independent of its role in sister chromatid cohesion.

Increasing extracellular osmolarity cannot suppress lethality in absence of cohesin

A significant suppression of the ts phenotype of cells bearing 1 stop and 2 stop alleles, and of *mcd1-1* coupled with only a modest rescue of its cohesion defect, in presence of osmostabilizers (Figures 4 and 5, A and C) prompted us to investigate whether cell wall maintenance is an essential function of Mcd1. To address this question, we created a galactose-inducible allele of *MCD1* (*pGAL-MCD1*). As *MCD1* is an essential gene, cells bearing this allele can grow only in the presence of galactose, but not in the presence of glucose due to lack of *MCD1* expression in this growth medium (Figure 6). We reasoned that if cell wall maintenance is the sole essential function of *MCD1*, then cells bearing the galactose-inducible allele should be able to grow on glucose medium containing an osmostabilizer, in which *MCD1* expression is repressed. However, we observed that the strain having *pGAL-MCD1* failed to grow in sorbitol-containing glucose medium (Figure 6), suggesting that cell wall maintenance, is not the only essential function of the cohesin complex, consistent with previous findings suggesting that cohesion and condensation are essential functions of the cohesin complex (Heidinger-Pauli *et al.* 2008; Guacci and Koshland 2012).

The CWI signaling pathway is activated in cohesin mutants

A cell wall defect in a mutant may arise from either a defect in the biosynthesis of the cell wall or in the CWI signaling pathway. To dissect cohesin's role in CWI, we first checked for Mpk1 phosphorylation. Mpk1 is a MAP kinase of the CWI pathway and is known to get phosphorylated upon cell wall stress (Lee *et al.* 1993; Martin *et al.* 1993). We found phosphorylated Mpk1 to be increased in *mcd1-1*, *smc1-2*, and *smc3-1* (Figure 7A) compared to WT cells, consistent with our earlier observation that Mpk1 is hyper-phosphorylated in *mcd1-1* (Kothiwal and Laloraya 2019). To confirm that this hyper-phosphorylation is a result of cell wall stress, we checked Mpk1 phosphorylation status in presence or absence of sorbitol using the *mcd1-1* allele. As expected, Mpk1 was hyper-phosphorylated in *mcd1-1* in the absence of sorbitol (Figure 7B). Importantly, addition of sorbitol brought down the Mpk1 phosphorylation (Figure 7B), suggesting that Mpk1 hyper-phosphorylation is a result of cell wall stress in cohesin mutants.

We also carried out genetic interaction analysis with *MPK1*, which revealed that *mcd1-1 mpk1* double mutant cells exhibit a synthetic sick phenotype (Figure 7C) relative to *mcd1-1* or *mpk1* that is evident at the semipermissive temperatures of 30°C and 32°C. While this growth defect in the double mutant was also osmoremedial in presence of sorbitol, the *mcd1-1 mpk1* double mutant cells do not grow as well as *mcd1-1* or *mpk1* alone in presence of sorbitol at 37°C (Figure 7C). Interestingly, double mutant cells failed to grow in presence of the cell wall stress-inducing agent calcofluor white (Figure 7D). Together, these results suggest that the CWI signaling pathway is active in the cohesin mutants and that its activity is essential for the growth of *mcd1-1* cells under semipermissive conditions and for their survival upon additional cell wall stress.

We also performed genetic interaction analysis with *HOG1*, that encodes a MAP kinase of the high osmolarity glycerol (HOG) pathway. Hyperosmotic shock leads to activation of the HOG pathway, which in turn increases intracellular glycerol levels and hence protects the cell from osmotic shock (Brewster *et al.* 1993; Hohmann 2002). As expected, upon *HOG1* deletion, cells were sensitive to sorbitol (Figure 7E). Interestingly, *mcd1-1 hog1* double

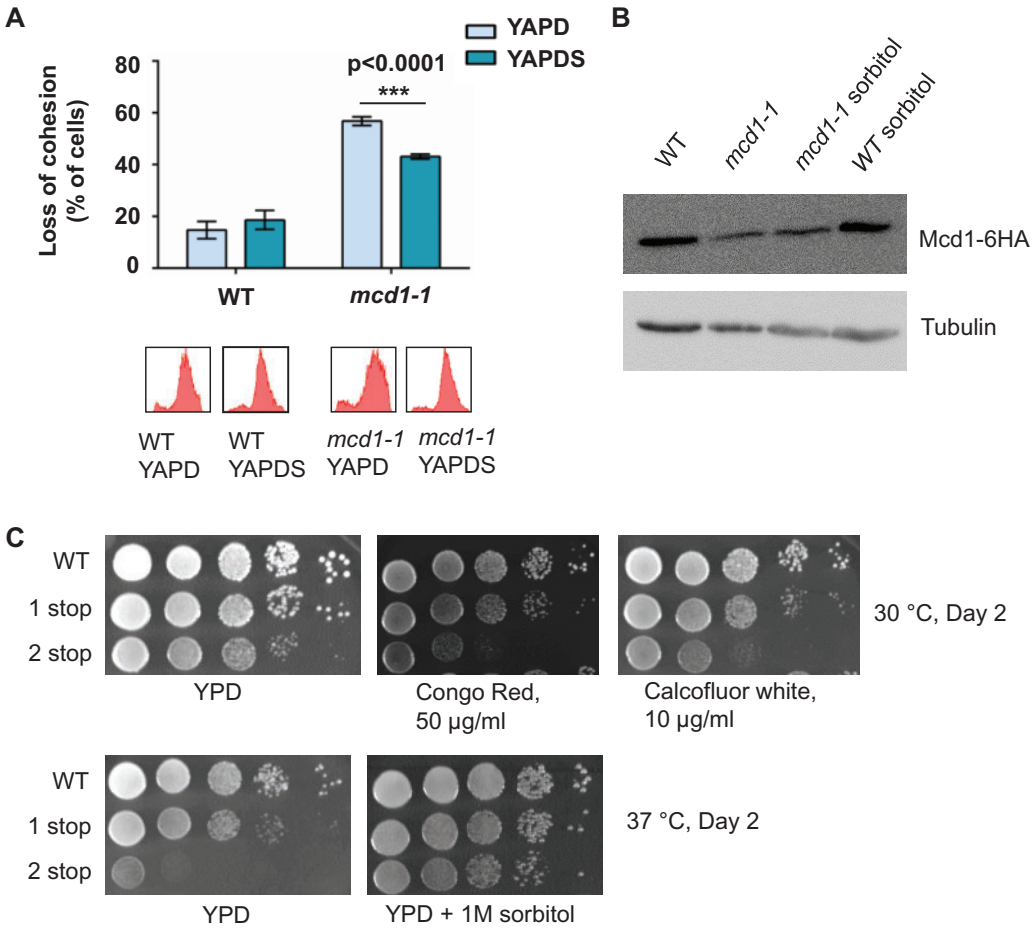

Figure 5 Suppression of cohesion defect in *mcd1-1* and the ts phenotype of quantized reduction cohesin mutants by sorbitol. (A) Sister chromatid cohesion was analyzed on the right arm of chromosome IV in WT and *mcd1-1* cells at 37°C. WT (SLY2553) and *mcd1-1* (SLY2555) cells carrying a chromosomal GFP-tag near *LYS4* were arrested in G2/M using nocodazole and imaged for cohesion by confocal microscopy. The graph shows percentage of cells showing loss of cohesion in WT and *mcd1-1* cells in presence (YAPDS) or absence (YAPD) of sorbitol. The mean values for $n = 4$ experiments are plotted on the Y-axis, ~250 cells were scored in each case. Error bars indicate SD. The statistical significance was analyzed using the two-tailed unpaired *t*-test where *** denotes $P \leq 0.001$. FACS profile of nocodazole-treated WT (SLY2764) and *mcd1-1* (SLY2767) cells is shown below. (B) Western blot to compare Mcd1 levels in WT (SLY2592), *mcd1-1* (SLY2593) cells at 37°C with and without sorbitol. (C) Cells bearing quantized reduction alleles of *MCD1* having reduced Mcd1 levels show an osmoremedial ts phenotype. Cells bearing WT (JH5275b), 1 stop (JH5276b), and 2 stop (JH5277b) *MCD1* alleles were spotted on YPD, YPD containing indicated drugs, and YPD sorbitol plates, incubated at indicated temperatures and images were captured.

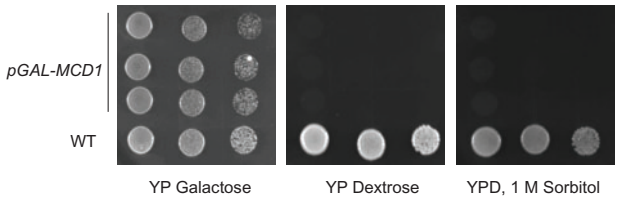

Figure 6 Increased osmolarity cannot bypass the essential function of cohesin. Exponentially growing WT (W303-1a) and *pGAL-MCD1* (SLY2799) cells were serially diluted (10-fold) and spotted on YP galactose, YPD, and YPD containing 1 M sorbitol. Plates were incubated at 23°C and images were captured after 2 days.

mutant cells showed a synthetic sick phenotype at 32 ° C and failed to grow in the presence of sorbitol (Figure 7E), *i.e.*, the osmoremedial effect of sorbitol on *mcd1-1* was no longer evident in the *mcd1-1 hog1* double mutant, suggesting that survival of the cohesin mutant at the restrictive temperature in sorbitol medium depends on the *HOG-MAPK* pathway, and points to the need for osmoprotectants for this suppression.

Comparison of β-1,3-glucan levels in the cohesin mutant with wild-type cells

An active CWI signaling pathway in cohesin mutants suggests that there could be a defect in cell wall biosynthesis in these mutants. Mutation in two genes, *FKS1* (codes for one of the two catalytic subunits of β-1,3-glucan synthase) and *GAS1* (a 1,3-beta-glucanosyltransferase, involved in β-1,3-glucan chain elongation) lead to zymolyase resistance, increased chitin content in the cell wall and sensitivity to various cell wall stress-inducing agents (Popolo *et al.* 1997; Ram *et al.* 1998; Garcı'a-Rodriguez *et al.* 2000; Valdivieso *et al.* 2000). All these phenotypes are also shared by cohesin mutants, indicating that there may be a defect in β-1,3-glucan synthesis and/or elongation, in these mutants. To test this possibility, we directly measured β-1,3-glucan level in the cell wall of *mcd1-1* cells by aniline blue staining (Watanabe *et al.* 2001). As expected, β-1,3-glucan level decreased upon *FKS1* deletion (Figure 8A), but we did not observe any significant reduction in *mcd1-1* cells at 30°C and only a very slight reduction (10.66%; $P = 0.02$, two-tailed unpaired *t*-test) in the mutant at 37°C (grown at 23°C and shifted to 37°C for 3 h) (Figure 8A).

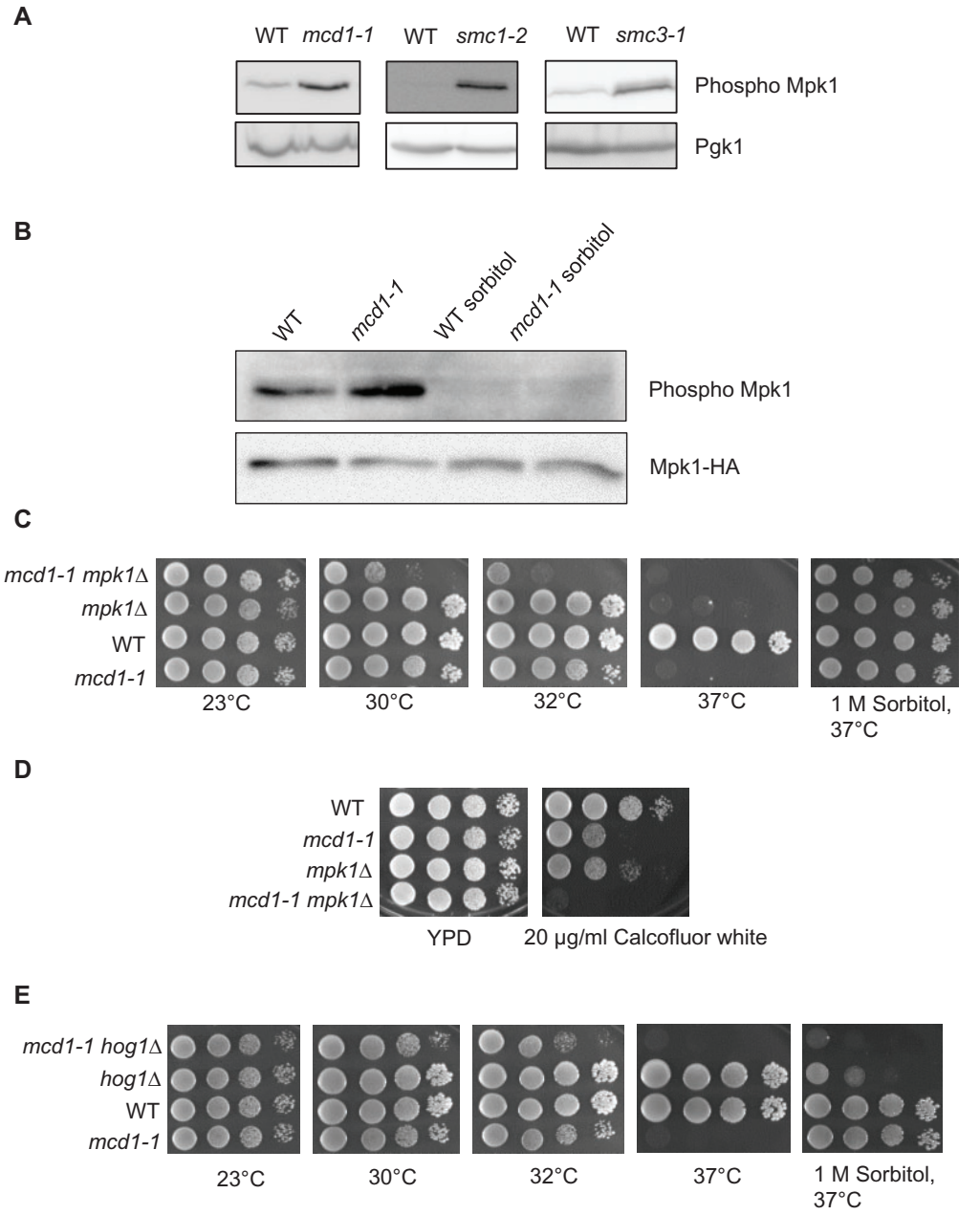

Figure 7 The cohesin mutant requires active CWI and HOG signaling pathways to survive under cell wall stress conditions. (A) Enhanced Mpk1 phosphorylation in cohesin mutants. Western blot showing Mpk1 phosphorylation in WT (VG906-1A), *mcd1-1* (VG955-7D), *smc1-2* (1355-1A), *smc3-1* (ROY1060) and its corresponding WT (ROY951). Phosphorylated form of Mpk1 was detected using anti-Phospho-p44/42 MAPK antibody and Pgk1 was used as a loading control. (B) Mpk1 phosphorylation is decreased in presence of sorbitol. Western blot showing Mpk1 phosphorylation levels in WT (SLY2794) and *mcd1-1* (SLY2796) cells at 30°C without and with sorbitol. (C) Genetic interaction of *mcd1-1* with *mpk1*Δ. Exponentially growing WT (SLY2764), *mcd1-1* (SLY2767), *mpk1*Δ (SLY2869), and *mcd1-1 mpk1*Δ (SLY2871) cells were serially diluted (10-fold) and spotted on YPD and YPD containing 1 M sorbitol plates. (D) A functional CWI pathway is essential for survival of the cohesin mutant upon additional cell wall stress. Exponentially growing WT (VG906-1A), *mcd1-1* (VG955-7D), *mpk1* (SLY2666), and *mcd1-1 mpk1* (SLY2668) cells were serially diluted (10-fold) and spotted on YPD or YPD containing 20 µg/ml calcofluor white, at 30°C. (E) Genetic interaction of *mcd1-1* with *hog1*Δ. Exponentially growing, WT (SLY2764), *mcd1-1* (SLY2767), *hog1*Δ (SLY2865), and *mcd1-1 hog1*Δ (SLY2867) cells were serially diluted (10-fold) and spotted on YPD and YPD containing 1 M sorbitol plates. Plates were incubated at the indicated temperatures and images were captured after 2 days.

We also performed genetic interaction analysis with *FKS1* and *GAS1*. The *mcd1-1 fks1* and *mcd1-1 gas1* double mutant cells were synthetic sick at 30 °C and 32 °C (Figure 8B), and interestingly, upon deletion of these genes, *mcd1-1* cells lost their ability to grow in the presence of sorbitol at the restrictive temperature (Figure 8B), suggesting, that these double mutant cells have an acute defect in the cell wall and even addition of an osmostabilizer such as sorbitol cannot protect them from cell lysis at 37 °C.

Overexpression of CWI signaling pathway genes does not suppress the temperature-sensitive phenotype of the cohesin mutant

Mutants displaying a ts phenotype due to cell wall defects show rescue of the growth defect upon overexpression or hyperactivation of CWI pathway genes (Levin 2005, 2011; Klis *et al.* 2006). We tested for such a possibility in *mcd1-1* by overexpressing various CWI pathway genes (*PKC1*, *MPK1*, *RHO1*, *MID2*, *WSC1*, and *ROM2*),

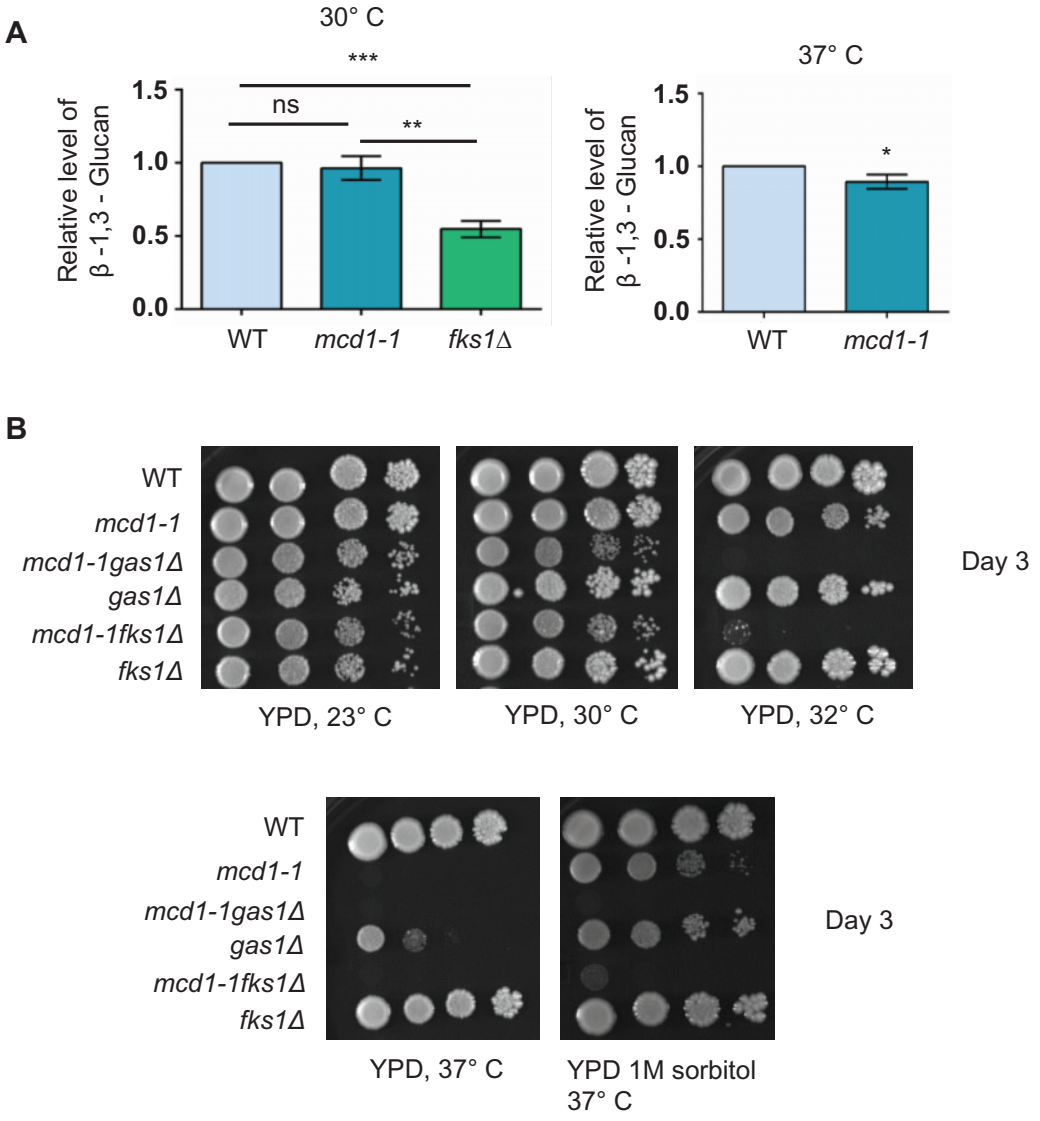

Figure 8 *mcd1-1* is not substantially defective in β-1,3-glucan synthesis. (A) Spectrofluorimetric quantification of the amount of β-1,3-glucan in the cell wall. The amount of β-1,3-glucan in WT (VG906-1A), *mcd1-1* (VG955-7D), and *fks1*Δ (SLY2934) cells was measured using aniline blue staining at 30 °C and 37 °C. Fluorescence intensity was normalized with respect to WT. Error bars represent SD; means of $n = 3$ experiments are plotted. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ (two-tailed unpaired *t*-test), WT values were normalized to 1. (B) Genetic interaction of *mcd1-1* with *fks1*Δ and *gas1*Δ. Exponentially growing, WT (VG906-1A), *mcd1-1* (VG955-7D), *fks1*Δ (SLY2934), *mcd1-1 fks1*Δ (SLY2936), *gas1*Δ (SLY2938), and *mcd1-1 gas1*Δ (SLY2940), cells were serially diluted (10-fold) and spotted on YPD and sorbitol containing YPD plates. Plates were incubated at indicated temperatures.

FKS1 (catalytic subunit of 1,3-beta-D-glucan synthase) and *SKN7* (codes for a transcription factor required during hypo-osmotic shock) using the yeast multicopy 2μ plasmid. Interestingly, we did not observe rescue of the ts phenotype upon overexpression of any of these genes (Figure 9). It has been observed that in some cases, overexpression of these genes is not sufficient for rescue of growth due to loss of activity of key proteins. To test this scenario, we transformed *mcd1-1* cells with a hyperactive form of Rho1 ($Rho1^{Q68L}$) (Sekiya-Kawasaki *et al.* 2002) or Pkc1 ($Pkc1^{R398P}$) (Helliwell *et al.* 1998), but we still failed to observe any rescue in the growth (Figure 9). Together, these results show that the cell wall defect in cohesin mutants cannot be rescued by overexpression or hyperactivation of these CWI pathway genes.

Deregulation of cell wall-related genes in the cohesin mutant *mcd1-1*

Cohesin has a well-established role in the regulation of gene expression in budding yeast (Donze *et al.* 1999; Biswas *et al.* 2009; Skibbens *et al.* 2010; Kothiwal and Laloraya 2019). As we did not observe any rescue in the ts phenotype of *mcd1-1* upon overexpression of selected cell wall biosynthesis and CWI pathway genes, we wondered whether cohesin dysfunction may be affecting expression of multiple cell wall-related genes and the observed defect in CWI is a combined effect of this misregulation. To test this possibility, we made use of our differential gene expression (DGE) RNA sequencing data of WT and *mcd1-1* strains (Kothiwal and Laloraya 2019). Analysis of RNA-seq data with regard to functions ascribed to affected genes (in the Saccharomyces Genome Database), revealed that many genes involved in cell wall organization and biogenesis are deregulated in the cohesin mutant *mcd1-1* (Table 1, Supplementary Tables S5, S6, and S7). Gene ontology (GO) analysis of RNA-seq data for affected biological processes revealed that the genes that are misregulated in the cohesin mutant *mcd1-1* fall under 96 different GO terms (Supplementary Table S5). Interestingly, nearly half of the affected genes fall under just six GO terms, of which the GO

term cell wall organization and biogenesis is the fourth maximally affected group (Figure 10A, Supplementary Table S5) consisting of 69 genes that represent 23.08% of total genes within this category (Supplementary Tables S5 and S7). GO analysis for affected cellular components also revealed that 59 cell wall genes (that represent 42.14% of total cell wall component genes) were misregulated in the mutant (Supplementary Figure S1 and Supplementary Tables S6 and S7). Interestingly, in the cellular component search, while the GO term cell wall represents the ninth most affected category, when percentage of affected cell wall genes are compared to total affected genes (Supplementary Figure S1A and Table S6), it represents the second most highly affected category (after ribosome, that has 47.3% affected genes) when the percentage of affected genes is calculated with respect to the total genes within the cell wall category (42.14%) (Supplementary Figure S1B and Table S6). Moreover, the expression of cell wall genes in each case (GO Set: process or component) appeared to be preferentially altered relative to the percentage of all affected genes, which is ~14.7% ($P < 0.0001$, Chi-squared test) (Table 1). The affected cell wall genes are functionally diverse encoding proteins involved in biosynthesis of cell wall components, cell wall assembly, signaling, etc. (Supplementary Table S7).

We confirmed deregulation of two such genes *PSA1* and *GUK1* by RT-qPCR analysis. Both genes are involved in cell wall biosynthesis and are downregulated in both RNA-seq and RT-qPCR analysis (Figure 10B). We then overexpressed these genes to test for suppression of the *ts* phenotype of *mcd1-1*. Neither individual nor combined overexpression of these genes could suppress the *ts* phenotype (Figure 10C), suggesting that the cell wall defect in cohesin mutants may arise from deregulation of multiple cell wall-related genes, of which many may contribute toward the defect that cannot be remedied merely by restoring the expression of one or a small subset of these genes.

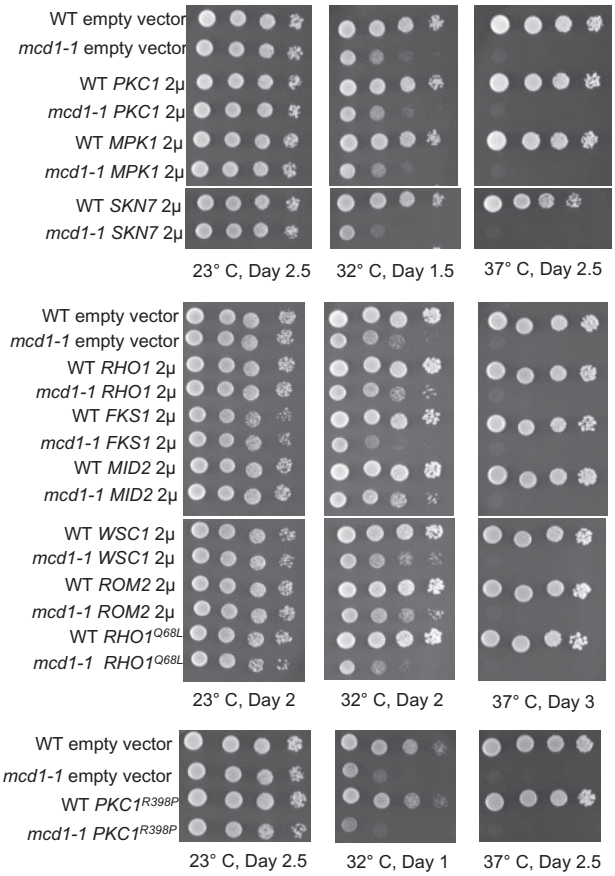

Figure 9 Overexpression of CWI signaling pathway genes does not suppress the ts phenotype of the *mcd1-1* mutant. Various CWI signaling pathway genes, *SKN7* and *FKS1*, were overexpressed using multicopy (2 μ) plasmids. Hyperactive mutants of *RHO1* and *PKC1* were also expressed from a *CEN*-vector. Exponentially growing cells containing indicated constructs were serially diluted (10-fold) and spotted on SC-URA plates. Plates were incubated at the indicated temperatures.

Discussion

The cell wall is a strong but elastic structure which protects yeast cells from changes in the external environment. Any perturbation in the cell wall due to environmental or physiological changes, or mutation in genes involved in cell wall biosynthesis, results in activation of a compensatory pathway and massive deposition of chitin in the lateral walls (Popolo *et al.* 1997; Garcia *et al.* 2004; Levin 2011). Our finding that *mcd1-1* cells are resistant to zymolyase and have high chitin content in the cell wall indicates a cell wall defect in this mutant. Increase in chitin content is a protective measure taken by cells with cell wall defects. Growing these cells in the presence of a chitin intercalating agent inhibits their growth due to loss of the protective chitin layer that was indeed seen for various cohesin subunit mutants (*mcd1-1*, *smc1-2*, and *smc3-1*), confirming the cell wall defect in these mutants. We found that the *mcd1-1* mutant cells also lysed

Table 1 Expression of cell wall genes is preferentially altered in *mcd1-1*

GO category	% of total affected	Number of genes in GO term	Number of genes affected in GO term	% of genes affected within GO term	χ^2	Two-tailed P-value
GO set: yeast GO slim process	—	—	—	—	—	—
Cell wall organization or biogenesis (GO:0071554)	7.26	299	69	23.08	16.39	<0.0001
All the genes	100	6439	951	14.77	—	—
GO set: yeast GO slim component	—	—	—	—	—	—
Cell wall (GO:0005618)	6.22	140	59	42.14	83.65	<0.0001
All the genes	100	6430	948	14.74	—	—

Summary of RNA-Seq data of DGE in *mcd1-1* mutant analyzed for effects on genes that may be involved in the process of cell wall organization or biogenesis [top, GO term: cell wall organization or biogenesis (GO: 0071554)], and on genes encoding cell wall components [bottom, GO term: cell wall (GO: 0005618)]. The number of genes was obtained using the GO slim mapper tool of SGD, with indicated GO sets. The Chi-square test was used to determine that the percentage of genes affected (including up- and down-regulated) in the cell wall category is significantly different from the overall percentage of genes affected in *mcd1-1*, as per the RNA seq data. The two-tailed *P*-value in both cases is <0.0001; by conventional criteria, this difference is considered extremely statistically significant.

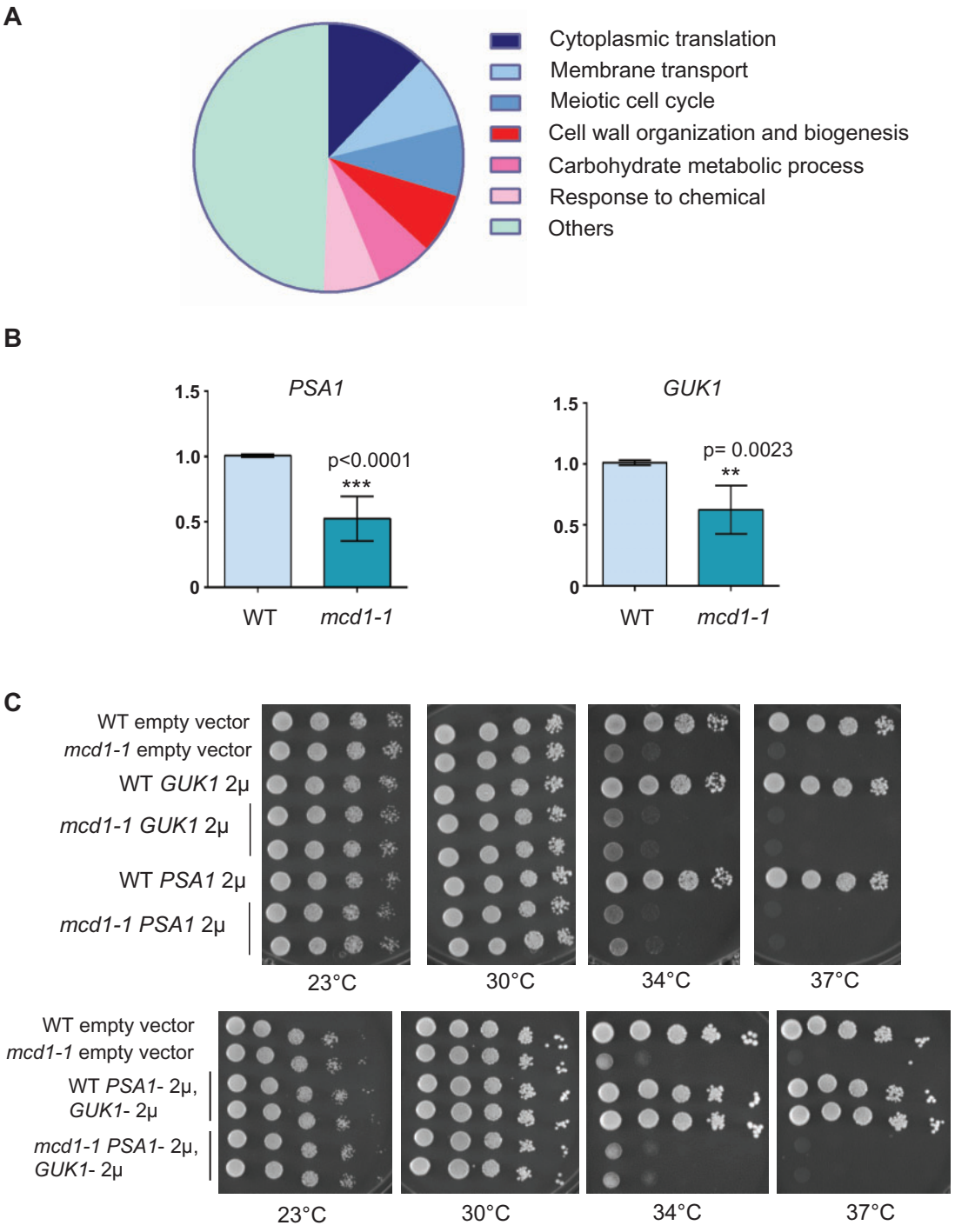

Figure 10 Misregulation of cell wall-related genes in the cohesin mutant. (A) Pie chart showing distribution of GO terms with respect to biological processes affected from RNA-seq analysis of cohesin mutant *mcd1-1*. Nearly half of the misregulated genes fall into six GO terms, which includes cell wall organization and biogenesis. (B) Confirmation of downregulation of *PSA1* and *GUK1* transcript levels by RT-qPCR. Transcript levels of *PSA1* and *GUK1* were compared in WT(SLY2764) and *mcd1-1*(SLY2767) cells at 31°C by RT-qPCR. The mean values for $n \geq 3$ experiments are plotted on the Y-axis. Error bars indicate SD. The statistical significance was analyzed using the two-tailed unpaired *t*-test where ** denotes $P \leq 0.01$ and *** denotes $P \leq 0.001$. (C) Single and co-overexpression of *PSA1* and *GUK1* does not suppress the ys phenotype of the *mcd1-1* mutant. *PSA1* and *GUK1* were overexpressed using multicopy (2 μ) plasmids, pRS422 (for single overexpression) and, pRS425 and pRS422 (Co-overexpression), respectively. Exponentially growing cells containing indicated constructs were serially diluted (10-fold) and spotted on SC-ADE (single overexpression) or SC-LEU-ADE (co-overexpression) plates. Plates were incubated at the indicated temperatures for 2 days.

preferentially relative to WT cells at the restrictive temperature as evidenced by enhanced leakage of alkaline phosphatase enzyme from these cells; this defect was osmoremedial.

Intriguingly, we observed that the ts nature of cohesin mutants is osmoremedial (Figure 4); the *ts* phenotype of *mcd1-1* at 37°C could be suppressed by increasing extracellular osmolarity using 1 M Sorbitol or NaCl. The suppression of the growth defect of *mcd1-1* in presence of osmostabilizers raised a possibility that cell wall maintenance could be an essential function of Mcd1. However, a galactose-inducible *MCD1* allele, which grew

well in presence of galactose but failed to grow in glucose could not be rescued by inclusion of sorbitol in the glucose medium, suggesting that cell wall maintenance is not the sole essential function of cohesin. This is consistent with our observation that osmostabilizers could suppress the growth defect of *smc3-1* and *smc1-2* at 32°C but not at 37°C. These observations can be interpreted in light of an excellent study from the Koshland group where they reduced the expression of *MCD1* in a step wise manner and showed that chromosome condensation, rDNA stability, and DNA repair are compromised by decreasing cohesin levels to 30% of WT levels, whereas even 13% of cohesin is sufficient to support viability and sister chromatid cohesion (Heidinger-Pauli *et al.* 2010). We propose that partial inactivation of cohesin (in the ts mutants) leads to a cell wall defect and ts phenotype, which is osmoremedial (32°C for *smc3-1* and *smc1-2* or 37°C for *mcd1-1*), whereas complete inactivation as in the case of the glucose repressible *GALL-MCD1* (when grown on glucose containing medium) or at 37°C for *smc3-1* and *smc1-2*, also drastically affects other essential functions of the cohesin complex such as cohesion and condensation, that cannot be bypassed by an increase in extracellular osmolarity.

Surprisingly, we observed a modest rescue of the cohesion defect upon addition of sorbitol to the growth medium (perhaps owing to stabilization of the misfolded mutant protein) complicating the interpretation of suppression of the *ts* phenotype in presence of sorbitol. To resolve this issue, we made use of the previously described 1 stop and 2 stop alleles that reduce the Mcd1 protein levels to 30 and 13% of the WT level respectively, without affecting the sister chromatid cohesion function (Heidinger-Pauli *et al.* 2010). Interestingly, these mutants were also sensitive to cell wall stress-inducing agents and showed an osmoremedial *ts* phenotype, suggesting that cohesin's role in CWI is independent of its function in sister chromatid cohesion. Although our results suggest that suppression of the ts phenotype by sorbitol may be partially due to stabilization of the cell wall, sorbitol likely has other effects also such as stabilizing mutant proteins, as shown here for *mcd1-1* (Figure 5B), perhaps by correcting misfolding directly or by affecting chaperone availability. As a result, sorbitol may also suppress cohesion (Figure 5A) and perhaps condensation defects in cohesin mutants, and this suppression of chromosome organization defects is also likely to contribute to the observed suppression of the ts phenotype by sorbitol.

Mpk1 hyper-phosphorylation in cohesin mutants suggests that the CWI signaling pathway has been activated in the cohesin mutants. Moreover, the synthetic sick genetic interaction of *mcd1-1* with *mpk1*Δ at the semi-permissive temperature and its enhancement in presence of calcofluor white revealed that this pathway is essential for the cohesin-defective mutant's survival under cell wall stress conditions. To understand the role of cohesin in CWI, we extended the genetic interaction analysis of *mcd1-1* by deleting the *HOG1*, *FKS1*, and *GAS1* genes. Failure of *mcd1-1* cells to survive in sorbitol medium upon deletion of *HOG1* suggests that the osmoremedial phenotype is dependent on the HOG pathway (potentially via its role in generation of intracellular osmoprotectants) and further re-enforces the conclusion that the cell wall is defective in cohesin mutants. *HOG1*-deleted cells are sensitive to the presence of sorbitol due to low intracellular osmolarity but survive because of a robust cell wall. Our observation that *mcd1-1 hog1* double mutant is inviable in presence of sorbitol suggests that defective cell wall and a low intracellular osmolarity in these cells may lead to efflux of water out of the cell, resulting in cell death.

Mutations in *FKS1* or *GAS1* genes that are required for β-1,3-glucan chain formation lead to increased chitin content in the cell wall and sensitivity to cell wall stress inducing agents (Popolo *et al.* 1997; Ram *et al.* 1998; Garcı'a-Rodriguez *et al.* 2000; Valdivieso *et al.* 2000). A functional CWI signaling pathway and phenotypes similar to *FKS1* and *GAS1* mutants suggested that cohesin may be required for β-1,3-glucan synthesis. However, we did not observe any significant difference in β-1,3-glucan levels (Figure 8A) in the cohesin mutant with respect to WT cells at 30°C, and only a marginal (∼10%) reduction in the cohesin mutant at 37°C. Although it is conceivable that a very small decrease in β-1,3-glucan levels may result in a cell wall defect to some extent, the synthetic sick genetic interaction of *mcd1-1* with *fks1*Δ and *gas1*Δ mutants and no suppression of ts phenotype upon overexpression of *FKS1* (Figure 9) suggested that cohesin may also affect some aspect of cell wall biogenesis other than β-1,3-glucan synthesis or elongation.

Our observation that neither overexpression nor hyperactivation of CWI pathway proteins can rescue the *ts* phenotype of the cohesin mutant suggests that cohesin affects an aspect of cell wall which cannot be compensated by the CWI signaling pathway. Retention of the *ts* phenotype upon overexpression of *SKN7* (Figure 9) indicates that the cohesin mutant might not have a defect in β-1,6-glucan, as *SKN7* has been shown to be a multicopy suppressor of mutants having a defect in β-1,6-glucan synthesis (Brown *et al.* 1993), though at this point, we cannot completely rule out a possibility of cohesin's role in β-1,6-glucan synthesis.

Cohesin also has a well-established role in the regulation of gene expression, and our RNA-seq analysis revealed that multiple genes involved in cell wall biosynthesis, assembly, and maintenance (69) or encoding cell wall components (59) are preferentially ($P < 0.0001$, Chi-squared test) misregulated in *mcd1-1* relative to other genes (Table 1). Furthermore, this deregulation of cell wall genes is unlikely to merely be a consequence of activation of Mpk1, since Mpk1 activation has been shown to alter the expression of about 25 genes of which only 20 are cell wall genes (Jung and Levin 1999), whereas in the *mcd1-1* mutant, many more (∼69) cell wall genes are deregulated. Since substantial defects in synthesis of specific cell wall components such as β-1,3-glucan could not be detected in *mcd1-1*, and cell wall defects (as deduced by sensitivity to cell wall stress inducers and enhanced lysis) were found to be uncoupled from a defect in cohesion, these observations collectively suggest that the cell wall defect in cohesin mutants is most likely to arise from changes in gene expression of multiple cell wall-related genes.

The GO analysis of the DGE RNAseq data for *mcd1-1* has revealed other interesting insights regarding other potential cellular defects in this mutant. The genes within the process cytoplasmic translation, and the related cellular component ribosome, represent the most highly deregulated set of genes in the data set, in both the GO searches done by us (55.83% and 47.30% of genes corresponding to the biological process cytoplasmic translation and the cellular component ribosome respectively are misregulated in *mcd1-1*). This is consistent with results from an earlier microarray analysis of DGE (Bose *et al.* 2012) using the *eco1-W216G* mutant that is defective in Eco1, an acetyltransferase that modifies the cohesin subunit Smc3 (Rolef Ben-Shahar *et al.* 2008; Unal *et al.* 2008; Zhang *et al.* 2008) and other nuclear proteins (*e.g.*, Mps3) (Ghosh *et al.* 2012). The budding yeast *eco1-W216G* mutation disrupts the acetyltransferase activity and is the equivalent of a Roberts syndrome human cohesinopathy mutation. GO analysis of the microarray data of the *eco1-W216* mutant also revealed misregulation of genes related to ribosome

biogenesis as a prominent category, including biogenesis of ribosomal proteins and processing of RNAs needed for ribosome assembly (Bose *et al.* 2012), similar to our results with the cohesin subunit *mcd1-1* mutant, although Eco1 is a cohesin modifier with a few other targets as well. In our study as well, in addition to cytoplasmic translation, rRNA processing has the seventh most affected genes (59) of the total, from 96 affected categories. The yeast *eco1-W216G* and *smc1-Q843Δ* mutants display defects in translation, ribosome biogenesis and formation of rRNA; defects in translation and rRNA production are also observed in Roberts syndrome fibroblasts harboring the *eco1-W216G* mutation (Bose *et al.* 2012). Our results from RNAseq analysis of DGE of *mcd1-1* reveal various other processes that are also affected that can result in ribosome and protein translation defects such as ribosome small and large subunit biogenesis, ribosome assembly, regulation of translation, and ribosomal subunit export from the nucleus, all of which could contribute to the overall translation defect. While DGE with a cohesin subunit mutant was not done in the earlier detailed microarray study, our results with *mcd1-1* share some overlap with that of the cohesinopathy mutant *eco1-W216G* defective in the cohesin modifier Eco1, supporting the possibility that cohesin deficiency mediated deregulation of genes required for ribosome biogenesis and translation may cause defects in ribosomes resulting in translation defects seen in cohesinopathy mutants.

Altogether, we present a comprehensive study showing that the cohesin complex is important for CWI in budding yeast. This study suggests that the cell wall defect in cohesin mutants is independent of cohesin's role in sister chromatid cohesion, and also independent of the CWI signaling pathway. The cell wall defect in cohesin mutants, characterized by increased chitin deposition, susceptibility to cell wall stress inducing agents, and resulting in enhanced cell lysis that is osmoremedial, represents a phenotypic manifestation of a budding yeast cohesinopathy. Our study revealing cohesin's requirement for CWI that may be mediated by its requirement in regulating the expression of many cell wall related genes, adds yet another role to cohesin's expanding repertoire of functions.

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Conflicts of interest

None declared.

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