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Modelling and molecular docking studies of the cytoplasmic domain of Wsc-family, full-length Ras2p, and therapeutic antifungal compounds

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Abstract

Saccharomyces cerevisiae, the budding yeast, must remodel initial cell shape and cell wall integrity during vegetative growth and pheromone-induced morphogenesis. The cell wall remodeling is monitored and regulated by the cell wall integrity (CWI) signaling pathway. Wsc1p, together with Wsc2p and Wsc3p, belongs to a family of highly O-glycosylated cell surface proteins that function as stress sensors of the cell wall in *S. cerevisiae*. These cell surface proteins have the main role of activating the CWI signaling pathway by stimulating the small G-protein Rho1p, which subsequently activates protein kinase C (Pkc1p) and a mitogen activated protein (MAP) kinase cascade that activates downstream transcription factors of stress-response genes. Wsc1p, Wsc2p, and Wsc3p possess a cytoplasmic domain where two conserved regions of the sequence have been assessed to be important for Rom2p interaction. Meanwhile, other research groups have also proposed that these transmembrane proteins could support protein-protein interactions with Ras2p. Molecular structures of the cytoplasmic domain of Wsc1p, Wsc2p and Wsc3p were generated using the standard and fully-automated ORCHESTAR procedures provided by the Sybyl-X 2.1.1 program. The tridimensional structure of full length Ras2p was also generated with Phyre2. These protein models were validated with Procheck-PDBsum and ProSA-web tools and subsequently used in docking-based modeling of protein-protein and protein-

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Conflict of interest

The authors declare that they have no conflict of interest.

compound interfaces for extensive structural and functional characterization of their interaction. The results retrieved from STRING 10.5 suggest that the Wsc-family is involved in protein-protein interactions with each other and with Ras2p. Docking-based studies also validated the existence of protein-protein interactions mainly between Motif I (Wsc3p > Wsc1p > Wsc2p) and Ras2p, in agreement with the data provided by STRING 10.5. Additionally, it has shown that Calcofluor White preferably binds to Wsc1p (−9.5 kcal/mol), meanwhile Caspofungin binds to Wsc3p (−9.1 kcal/mol), Wsc1p (−9.1 kcal/mol) and more weakly Wsc2p (−6.9 kcal/mol). Thus, these data suggests Caspofungin as a common inhibitor for the Wsc-family. MTiOpenScreen database has provided a list of new compounds with energy scores higher than those compounds used in our docking studies, thus suggesting these new compounds have a better affinity towards the cytoplasmic domains and Ras2p. Based on these data, there are new and possibly more effective compounds that should be considered as therapeutic agents against yeast infection.

Keywords

Cell wall integrity; Wsc-family; Ras2p; Antifungal compounds; Molecular modeling; Molecular docking

1. Introduction

The fungal cell wall is a dynamic structure that changes during cellular growth and interacts with their environment. Any external stimuli must overcome this layer, thus causing a cell wall stress that is responsible for the induction of a series of stress-responsive factors, which normally engage in phosphorylation cascades. In *S. cerevisiae*, one of the most studied signaling pathways is the mitogen-activated protein kinase (MAPK) that functions in the maintenance of the cell wall integrity (CWI) and in the stress response stimuli. The Pkc1 cell wall integrity (Pkc1-CWI) pathway is activated by different stimuli including cell wall damage, heat shock, oxidizing agents, drugs, nutrient starvation, and other types of environmental stress (Sanz et al., 2017; Valiante, 2017). However, the signaling mechanisms by which the sensors detect and translate the signal to downstream effectors are extremely complex and remain to be completely elucidated. Therefore, current experimental efforts are focused in understanding the communication between different signal transduction pathways and the mechanisms involved in collecting the information from various signaling pathways is mediated by a conciliatory response (Albert et al., 2007). In addition to other pathways, Ras2p can be considered as a possible conciliatory entity because it has shown that can crosstalk with the Pkc1-CWI pathway, thus suggesting an interaction between Ras2p and CWI sensors (Petkova et al., 2010; Fuchs et al., 2009). Hence, conciliatory proteins such as Ras2p are also suitable as therapeutic drug targets due to their distinct functionality.

The Pkc1-MAPK pathway (Fig. 1) also requires the activation of the cell surface sensors Wsc1p, Wsc2p, and Wsc3p (Verna et al., 1997; Levin, 2011). Wsc1p belongs to a family of highly O-glycosylated cell surface proteins together with Wsc2p and Wsc3p, but the last two are less studied. A mutational analysis performed by Levin's group found two conserved motifs within the cytoplasmic tail of Wsc1p important for Rho1-GDP/GTP exchange factor (Rom2p) interaction, which suggests Wsc1p interaction with Rom2p facilitates Rho1p

activation, but is negatively regulated by phosphorylation of serine residues at positions 319, 320, 322 and 323 (Vay et al., 2004). Two essential functions have been identified for Rho1p: 1) it serves as an integral regulatory subunit of the 1,3-beta-glucan synthase complex (GS) that stimulates GS activity in a GTP-dependent manner, and 2) it binds and activates protein kinase C, which is encoded by PKC1 (Heinisch et al., 1999; Levin, 2011). Ras2p could have a similar function in the participating pathways (Fig. 2). Loss of Pkc1p function or of any of the components of MAPK cascade under its control results in cell lysis. Rho1p, in the GTP-bound state, interacts and activates Pkc1p. A module formed by conserved MAPK, transfer the signal to Slr2p (Mpk1p), a Ser/Thr MAPK, which is the final protein kinase of the cascade. The phosphorylated state of Slr2p is monitored as indicative of CWI pathway activation. Slr2p transmits the signal to two known transcription factors, Rlm1p and the heterodimeric SBF complex (formed by Swi4p and Swi6p). These transcription factors regulate the function to initiate the transcriptional up-regulation of cell wall remodeling, cell cycle progression genes and stress-response genes (Levin, 2005).

Ras2p is a homolog of mammalian Ras proto-oncogenes, which plays a fundamental role in regulating how proteins orchestrate the resources in the synthesis of other proteins; that permits a faster growth than healthy cells and metastasize to other tissues. Structural studies, among others in yeast, have contributed significantly to understanding the mechanism of RAS signaling in humans. Therefore, the similarity between these two protein homologs has encouraged many researchers to employ the yeast model system to recognize new possible ways to control Ras genetic interactions in humans (Kalimutho et al., 2009; Cazzanelli et al., 2018). Ras2p was included in this study to predict its interaction with the Wsc-family sensors due to three main reasons: (1) it is a small monomeric GTP-binding protein primarily localized in the plasma membrane. These monomeric units, cycle between active GTP-bound and inactive GDP-bound states as Rho1, a GAP (GTPase Activating Protein) of the rho subfamily of Ras-like protein involved in the regulation of Pkc1p, (2) in the case of positive regulation of Ras2p, it requires the action of GEFs (Guanine nucleotide exchange factors) that catalyzes the transformation from its inactive GDP-bound state to the active GTP-bound form of Ras2p, similar to Rho1 role, (3) Ras2p plays an essential role in regulating cellular processes such as: sporulation, nutrient sensing, cell proliferation, carbon storage, and stress resistance (Broek et al., 1985). All these roles suggest that Ras2p participate in different signal transduction pathways of importance to maintain the cells alive. Additionally, there is a research study that suggested the role of WSC genes on Ras2p by modulating the activity of adenylate cyclase (or Pkap) and restricting their interaction based on the heat shock response (Verna et al., 1997), and another study where the lack of Ras2p partially suppressed the H₂O₂ hypersensitivity in WSC genes deletion mutants (Zu et al., 2001). All these facts let us to suggest that more experiments are needed to clearly explain how (at the molecular level) Ras2p interacts with the CWI sensors implicated in protecting the cells from damage or stresses. Thus, Ras2p is our experimental focus due to the functional similarities found in Rho1p and active role in various relevant signal transduction pathways (Takai et al., 2001).

The prevalence of systemic fungal infections has significantly increased worldwide. Numerous diagnosed fungal diseases are caused by opportunistic pathogens of normal endogenous flora through *Candida albicans*, meanwhile, other fungal infections are acquired

from the host surroundings through *Aspergillus fumigatus* and *Cryptococcus neoformans* (Badiie and Hashemizadeh, 2014; Boral et al., 2018). Therapeutic antifungal drugs with different structures and scaffolds are shown to be effective for disease treatment. Some of these antifungals drugs are classified as echinocandins (e.g., Caspofungin) or polyenes (e.g., Amphotericin B), whose mode of action is to disrupt the integrity of the cell wall and to bind ergosterol, respectively (Formosa et al., 2013; Roemer and Krysan, 2014; Kami ski, 2014). Other antifungal drugs are classified as azoles (e.g., Fluconazole) that inhibit the sterol synthesis (Roemer and Krysan, 2014). New research studies seek to discover novel antifungals and drug targets to treat invasive fungal infections by providing a chemical scaffold for new drugs discovery and synthesis (Sanglard, 2016; Fuentefría et al., 2018). Pyrimidine analogs are the most recent drug class that is being used to disrupt nucleotide metabolic pathways in DNA synthesis (Bondaryk et al., 2013; Chitty and Fraser, 2017). However, these antifungals have developed drug resistance, produced adverse reactions, and have low effects against fungi spreading (Sanglard, 2002; Perlin et al., 2017; Wiederhold, 2017). Therefore, the discovery of new and more efficient therapeutic antifungal drugs is a challenging task because fungi and humans share the same realm, both are eukaryotic.

Several computational tools and approaches were developed and are now available for protein modeling (Xu et al., 2000; Esposito et al., 2006; Jamkhande et al., 2017) and for the molecular docking of small molecules as ligands of a target protein (Chaudhary and Mishra, 2016; Dar and Mir, 2017). Our approach intends to demonstrate how current modeling tools are useful to explain the mode of interaction between Ras2p and the cytoplasmic tail of the stress sensor proteins members of Wsc-family and to predict novel antifungal drugs from the ligand web-based libraries.

2. Materials and methods

2.1. Interaction network

STRING v.10.5 was used to predict protein-protein association data between Wsc1p, Wsc2p, Wsc3p, and Ras2p. This database tool includes physical and functional interactions through evidence channels of protein sources including experiments, database, text mining, co-expression, neighborhood, gene fusion, and co-occurrence (Szklarczyk et al., 2017). STRING uncovers 9.6 Mio proteins from 2,031 organisms and 1380 Mio interactions. A confidence score of 0.65 was chosen for multiple protein-protein networks. This cutoff score generated a network showing the interaction with the supporting evidence, but also less false positives. No changes in interaction were seen on confidence scores of 0.7 or above.

2.2. Protein modelling of the Wsc1p, Wsc2p, Wsc3p and Ras2p

FASTA files for Wsc1p/Slg1p (accession number (AC): P54867), Wsc2p (AC: P53832), Wsc3p (AC: Q12215), and Ras2p (AC: P01120) were uploaded for homology modeling using the standard and fully-automated ORCHESTAR procedures provided by the Sybyl-X 2.1.1 program (Certara USA, Inc.), and for advance remote homology detection methods provided by the Phyre2 web portal (Kelley et al, 2015), respectively.

2.3. Optimization and validation of homology models

Protein models generated through homology modeling were also optimized with Sybyl-X 2.1.1 program (Certara USA, Inc.). The Powell's method and Kollman United/Kollman all-atom force field and AMBER charges were applied to minimize the protein, with a distance-dependent dielectric constant value of 1.0, gradient convergence value of 0.001 kcal mol⁻¹, and a maximum number of optimization iterations set to 1000. The quality of the generated protein models was assessed considering both geometric and energetic aspects using Procheck-PDBsum (Laskowski et al., 1993) and ProSA-web (Sippl, 1993; Wiederstein and Sippl, 2007). PDB files (.pdb) from the generated protein models were uploaded and, in return, both validation programs provided a stereochemical analysis of the peptide structure found inside. The best-quality protein models (PDB format) were used for subsequent docking experiments (Table 1) once converted into Mol2 format (.mol2) with Sybyl-X 2.1.1 program (Certara USA, Inc.).

2.4. Theoretical Calculations

A method for predicting ligand-binding sites in all validated protein models for the antifungal compounds under study was developed using ligand-protein molecular docking calculations. The study included six antifungal compounds, all of which constitute the data set.

2.5. Ligand structures optimization

The 2D structures of the antifungal compounds Amphotericin B (CID: 5280965), Calcofluor White (CID: 6108780), Caspofungin (CID: 16119814), FK506 (CID: 445643), Fluconazole (CID: 3365) and Menadione (CID: 4055) were uploaded as json files (.json), converted into Mol2 format (.mol2) with Sybyl-X 2.1.1 program, and used for the subsequent docking experiments.

2.6. Generation of a protomol of Wsc1p, Wsc2p, Wsc3p and Ras2p

Four protomols were generated (Fig. 3) using proto-threshold of 0.50, a proto-bloat of 0, and other parameters set as default, with standard and fully-automated Surflex-Dock Geom (SFXC) procedures using Sybyl-X 2.1.1 program (Certara USA, Inc.). The protomol is an idealized computational representation of the protein surface that serves as a template that is able to interact with ligands within predicted binding sites. The largest cavity (depicted in red color) for each protein model is shown (Fig. 3).

2.7. Molecular docking calculations with Surflex-Dock

The cytoplasmic domain of Wsc1p, Wsc2p and Wsc3p were docked on each one of the target antifungal compounds (Amphotericin B, Calcofluor White, Caspofungin, FK506, Fluconazole, and Menadione), and Ras2p was docked on each one of the target key motifs (Fig. 4) using the Surflex-Dock module of Sybyl-X 2.1.1 program and visualized with Chimera 1.13 (Pettersen et al., 2004). All docking calculations were set to equal parameters (10 poses each), only using the highest ranked pose by Surflex-Dock. The total score (pKd) calculated by Surflex-Dock was used to determine the affinity of each antifungal compound

or key motif against the cytoplasmic tail of each Wsc-family of sensors and Ras2p, respectively.

2.8. Protein-small ligand database docking

MTiOpenScreen (Labbé et al., 2015) is an open web server program that facilitates the screening of new bioactive compounds in order to improve the effectiveness of drug discovery processes for potential targets. Prior to start, grid boxes with a size of $27 \times 27 \times 27$ (Å) were generated according to the three key motifs found in each of the cytoplasmic domains of Wsc1p, Wsc2p, and Wsc3p, and another grid box with a size of $30 \times 30 \times 30$ (Å) was generated for Ras2p (Table 2). The database chosen for the analysis was the inhibitors of the Protein-Protein Interactions Library (iPPI-Lib). The criteria applied in order to obtain a rough list of novel compounds for each one of the target sequences was as follows: Molecular weight < 567 g/mol, Partition coefficient (xlogp) < 13, Rotatable bonds < 35, Hydrogen bond donors < 24; Hydrogen bond acceptors < 18 and Polar surface area < 370. A total of 1,000 compounds were screened in each run and those compounds with an MTiOpenScreen Energy Score (kcal/mol) of -6 or higher were only considered for this study.

2.9. Antifungal Susceptibility Testing

Antifungal susceptibility tests were performed using 1×10^8 cell/mL aliquots taken from cultures at OD600 between 0.5 – 0.8. The strains bearing null mutations for single genes encoding bait sensor proteins, their corresponding protein partners, or double mutant strains containing both deletions were tested with wild-type strain BY4742 as a control. For agar plate assays, 3 µL drops taken from 1/10 serial dilutions of the working culture (ranging from 10^8 to 10^2 cell/mL) were inoculated in triplicate on Complete Synthetic Medium (CSM) agar plates under the following conditions: normal conditions (30 °C), plasma membrane stress (0.75 µg/mL Amphotericin B), and cell wall stress (150 µg/mL Calcofluor White; 75 ng/mL Caspofungin; 0.5 µg/mL FK506; 30 µM Menadione; 100 µM Fluconazole). Plates were incubated at 30 °C for 2–3 days. Relative growth on agar plates was quantified by comparing the extent of growth displayed by the wild-type strain across all dilutions with the extent of growth displayed by the mutant strains. Relative growth measurements were classified as: equivalent to wild-type (WT), greater than wild type by two or more serial dilutions (R), less than wild-type by two or more serial dilutions (S).

3. Results and discussions

3.1. Molecular Model Building

Due to the lack of a crystal structure for the proteins under study, several bioinformatics procedures were applied to predict its molecular structures. The goal here is to understand what kind of protein-protein or protein-compound interaction might occur in order to find novel therapies. The standard and fully-automated ORCHESTAR procedures provided by the Sybyl-X 2.1.1 program showed various potential templates for molecular modeling purposes. Only few files of crystallographic or NMR data were found certain (99% or more of confidence) and likely (95% or more of confidence) for all the protein sequences under this study. Among them, Arabidopsis Thaliana At3g22680 (PDB ID: 1VK5), an unknown

protein called TT1977 (PDB ID: 2DST), malate synthase G (PDB ID: 1D8C) and human Kras (PDB ID: 4OBE) were selected as templates for Wsc1p, Wsc2p, Wsc3p and Ras2p, respectively (Table 1). Generated molecular structures began with an alignment of the sequence to be modeled (the target) with the relative known 3D structures (the template). About four models of each protein target were generated and the best four structures were chosen for protein structure validation.

3.2. Protein Structure Validation

The outputs generated by Procheck-PDBsum show all secondary structures found in each protein model (Fig. 5). At first glance, these results suggest that the cytoplasmic tail of the three members of the Wsc-family are considerably different. Ramachandran plots were also provided, one for each protein model (Fig. 6). For Wsc1p, the analysis has estimated that 83.0% of the amino acids, that were not Proline or Glycine, are found in the most favored regions [A, B, L]. Meanwhile, 15.4% of the amino acids are found in other allowed regions. For Wsc2p, the analysis has estimated that 70.6% of the amino acids, that were not Proline or Glycine, are found in the most favored regions [A, B, L]. Meanwhile, 26.1% of the amino acids are found in other allowed regions. However, only 1.5% of these amino acids were found in a disallowed region. As for Wsc3p and Ras2p, the analysis has estimated that 81.7% and 86.0% of the amino acids, respectively, that were not Proline or Glycine, are found in the most favored regions [A, B, L]. Meanwhile, 16.3% and 10.8% of the amino acids (respectively) are found in other allowed regions. For Wsc3p, only 1.6% of these amino acids (2 aa) were found in a disallowed region. The results provided by Procheck-PDBsum are based on the analysis of 118 protein structures with a resolution of at least 2.0 Å and an R-factor not greater than 20%. They have estimated that a protein model should have over 90% of its amino acids in the most favored regions to be considered a good quality model. Thus, all predicted models met this requirement in order to be validated as a quality model. Table 1 shows a summary of the analyses generated by Procheck-PDBsum.

ProSA-web was also used to generate a quality score of our four protein models in the context of all known protein structures, which only chains with less than 1,000 residues and a Z-score 10 are shown. Authors who designed ProSA-web have established that for a protein model provided, the knowledge-based energy is mostly found under the value of 0.0 (Wiederstein and Sippl, 2007). Their first quality model validation provided was the Overall Quality Model which compare the Z-score of a given model to those found in the protein database (Fig. 7). A second plot was also obtained, thus providing the Local Model Quality that compares the knowledge-based energy to the sequence position (Fig. 8). In general, a positive Z-score commonly means an erroneous or erratic section found in the generated 3D protein model. The Z-score obtained for each protein model generated is located within the region for those protein models found in the protein database (PDB) for the same number of residues. These results might suggest that our protein models are somewhat close to those that have been determined experimentally. In addition, the knowledge-based energy values estimated for the protein models generated for the C-terminal domain of Wsc1p and Wsc2p, and N-terminal of Ras2p were mostly found to be beneath 0.0 (Fig. 8) contrary to Wsc3p, where the N-terminal region of the cytoplasmic tail is mostly disfavored mainly due to high number of serine residues and by its proximity to membrane surface. In fact, some research

groups have described that the N-terminal region of the cytoplasmic tail of Wsc3p susceptible to phosphorylation reaction for control regulation among Rom2p (Vay et al., 2004).

3.3. Predicted protein-protein interactions between Wsc1p, Wsc2p, Wsc3p and Ras2p

Research groups have explained that when the Pkc1-CWIP is activated by an external stimuli, the signaling mechanisms by which the sensors detect and translate the signal to downstream effectors are extremely complex and remain to be completely elucidated (Verna et al., 1997; Levin, 2011). They also have recognized the importance of the members of the Wsc-family at these early stages of the signaling cascade. In order to find more evidence to support this hypothesis, a preliminary network analysis using STRING 10.5 (Szklarczyk et al., 2017) was performed. The results retrieved from STRING 10.5 (Fig. 9A) suggest that Wsc1p, Wsc2p, and Wsc3p are involved in protein-protein interactions with each other, but when Ras2p is also included in the network analysis (Fig. 9B), a new protein-protein interaction is observed between each member of the Wsc-family and Ras2p. These three sensors have in common the mechanism that induces a Rho1-GDP/GTP dependent signaling transduction pathway and other biological processes. This mode of action is relevant since Ras2p have a similar GTPase function as with Rho1p (Cazzanelli et al., 2018).

In order to explain how these protein-protein interactions might occur, several molecular docking analyses were performed, but using only three conserved regions of sequence among all three (Fig. 4). The conserved regions are located within the cytoplasmic domain and were labeled as Motif I (Fig. 4B), Motif II (Fig. 4C) and Motif III (Fig. 4D), respectively. A mutational analysis performed for Wsc1p have revealed that the conserved residues found in Motif I and in Motif III have been implicated in interactions with Rom2p (Lodder et al., 1999; Vay et al., 2004), and were also assessed to be important for Wsc1p function and its regulation. Motif II is a Serine-rich region that could be susceptible to a reversible covalent modification or a drug target. Therefore, a ligand-based screen docking analysis using small molecules was also performed in order to identify novel inhibitors of these protein-protein interactions between the three members of the Wsc-family of sensors.

3.4. Surflex-Dock studies of Ras2p with the conserved regions of sequence of each member of the Wsc-family

In order to understand what type of affinity has each conserved region of the cytoplasmic domain of the three members of the Wsc-family, primary docking calculations were performed with the Surflex-Dock module of Sybyl-X 2.1.1 program (Certara USA, Inc.). The three conserved regions used, labeled as Motif I (Fig. 4B), Motif II (Fig. 4C) and Motif III (Fig. 4D), have been implicated in the regulation of Rom2p during the activation of Pkc1 pathway (Vay et al., 2004). Thus, if any of these motifs could bind another GTP-binding protein such as Ras2p, then the Wsc-family of sensors might be also involved in the regulation of Ras2p as well as of Ras2p-dependent pathways.

The largest cavity of the generated protomol of Ras2p (Fig. 3D) was found to be composed by mostly polar residues included Gly-82, Gly-84, Tyr-104, Gln-144, Arg-169, Asp-173, Glu-174, Gly-176, Thr-181, Ser-291, Lys-312, Gly-317 and Cys-319, and by the non-polar

residues Val-119, Phe-149 and Val-171. Motif I gave the highest energy score (Table 3) compared to Motif II and Motif III. Thus, Motif I of the cytoplasmic tail of the Wsc-family is capable to interact more closely to Ras2p, in the following decreasing order Wsc3p > Wsc1p > Wsc2p (Fig. 10). All of these tetrapeptide sequences have in common the amino acid sequence K(H/E/Q)YQ, thus any difference in affinity towards Ras2p should rely on the chemical properties of the second residue in the sequence. All residues are polar in nature but, Histidine (H) is a basic residue (positively charged) meanwhile Glutamate (E) and Glutamine (Q) are acidic (negatively charged) and polar (neutral) residues, respectively. Therefore, the largest cavity of the generated protomol is more suitable for those basic residues, such as Histidine, where many hydrogen bonding are allowed for those side chains exposed by the polar residues of Ras2p (Fig. 10A) including Thr-113, Asn-146, Glu-174, Lys-298, and Asn-306. Meanwhile, the Motif I of Wsc1p (Fig. 10B) and Wsc2p (Fig. 10C) have shown a few hydrogen bonding with [Glu-83, Thr-113 and Tyr-178], and [Lys-180 and Thr-181], respectively. Motif II is the second region with some affinity towards Ras2p, in the following decreasing order of Wsc2p > Wsc1p > Wsc3p. All these pentapeptide sequences are Serine/Threonine-rich, suitable for post-translational modification, but most commonly for phosphorylation/dephosphorylation reactions. In fact, mutational studies have shown that the Serine/Threonine-rich region of Wsc1p has been implicated in a negative regulatory role with Rom2p after phosphorylation by a MAP kinase (Vay et al., 2004). Consequently, the phosphorylation of these serine residues should abrogate any direct interaction with either Rom2p or Ras2p. Although Motif II has less affinity for Ras2p (Table 3) than Motif I, it could be considered as a second option for its binding. Motif III, which comprised mainly by 10 amino acid residues, is located at the extreme C-termini of the Wsc-family. This region is also important for Wsc1p in order to promote protein-protein interaction with Rom2p. Motif III includes a highly conserved tetramer (-VNPD-) at the center of its sequence which induces a beta-turn supported by the Proline residue. The binding of this motif to Ras2p produces the lowest Energy Scores, in the following decreasing order of Wsc2p > Wsc3p > Wsc1p. Thus, it seems that His-469 (Wsc2p) helps the motif to stabilize the binding better than Gln-535 (Wsc3p) and Thr-370 (Wsc1p).

Thus, our docking experiments have shown that each motif located in the cytoplasmic domain of the Wsc-family is capable to interact with Ras2p in a unique and specific fashion, in agreement to the chemical properties of the amino acid residues involved. Although these experiments were performed independently one from another, it is important to recall that the location of the three motifs (Fig. 11) occur near each other, especially in Wsc1p (Fig. 11A) and Wsc2p (Fig. 11B). Thus, these motifs constitute only a part of the cytoplasmic domain of these membrane proteins, and the rest of the protein might be expected to interact with Ras2p. This hypothesis has been supported with a new experimental data obtained where the interaction between Ras2p and Wsc1p (Santiago-Cartagena et al., 2018), and between Ras2p and Wsc2p or Wsc3p (Vélez-Segarra, 2018) is shown.

3.5. Antifungal Susceptibility Testing

In order to address how these members of the Wsc-family of sensors might have a meaningful function on protecting the cell wall integrity, antifungal susceptibility testing was performed and the results are shown in Table 4. The yeast strains used contain single

and double gene deletions for Wsc1p (*wsc1*), Wsc2p (*wsc2*), Wsc3p (*wsc3*) and Ras2p (*ras2*). In the testing, six commercially available therapeutic compounds were used including Amphotericin B, Calcofluor White, Caspofungin, FK506, Fluconazole, and Menadione. All these compounds have been considered as stressors of the yeast cell wall through its different mechanisms of action in the CWI signaling pathway (Table 4) and were used as standard antifungal drugs to test susceptibility in different fungi strains (Nesbitt et al., 2018). In our study, yeast cells were most susceptible to all compounds with the double mutant *wsc2 wsc3* strain. These results suggest that Wsc1p might work with Wsc2p (or Wsc3p) as coupled sensors. Meanwhile, the single mutant *ras2* strain has a WT response with either Amphotericin B, Calcofluor White, Caspofungin or FK506. Thus, these results suggest that the three members of the Wsc-family of sensors can support yeast survival in the absence of Ras2p, and their action might depend directly or indirectly on the presence of Ras2p (or even with Ras1p) for antifungal efficiency, especially when the budding yeast *Saccharomyces cerevisiae* has two highly conserved RAS genes (Ras1p and Ras2p have a 59% sequence identity) which are redundant for viability (Mösch et al., 1999). Additionally, when the double mutant *ras2 wsc2* strain is exposed to Calcofluor White, Caspofungin and FK506, it becomes resistant to all these compounds. Hence, these results suggest that Wsc3p (in the absence of Wsc2p or Ras2p) might support Wsc1p (or vice versa) during the early stages of the stress-induced signaling. Other variations of double mutant strains such as *wsc1 wsc2* , *wsc1 wsc3* , and *ras2 wsc3* were not used in this study. These preliminary studies have also shed some light on how yeast strain variation can be affected by the presence of therapeutic compounds. However, an extensive analysis is needed to understand the mechanisms involved in preserving cell wall integrity under stressed environments. Our next step is to answer whether the therapeutic compounds used in susceptibility testing could also have the ability to interact with the cytoplasmic domain of the members of the Wsc-family and recognize (if so) which of these compounds has the strongest affinity against their protein sequences. Docking studies of antifungal compounds and the cytoplasmic domain of the Wsc-family

In order to explain whether the therapeutic compounds used for susceptibility testing might be able to bind the cytoplasmic domain of the three members of Wsc-family of sensors, primary docking calculations using the protomol generated for each sensor (Fig. 3) were performed with the Surflex-Dock module of Sybyl-X 2.1.1 program (Certara USA, Inc.). Full-length Ras2p was also used in our docking analyses, in spite of none of the therapeutic compounds used herein have been reported as effectors or inhibitors of Ras2p. As shown in Table 5, the energy scores (in kcal/mol) calculated for Calcofluor White and Caspofungin suggests that these two therapeutic compounds bind preferably to Wsc1p (−9.5 kcal/mol) and Wsc3p (−8.7 kcal/mol), and to Wsc3p (−9.1 kcal/mol) and Wsc1p (−9.0 kcal/mol), respectively. Caspofungin also binds, but more weakly, to Wsc2p (−6.9 kcal/mol). Additionally, it seems that Amphotericin B binds to Wsc1p (−6.9 kcal/mol) and Wsc2p (−6.5 kcal/mol), but these two interactions are too weak compared to Calcofluor White and Caspofungin. As expected, no binding was observed with Ras2p, whose energy scores were equal or less than −2.8 kcal/mol. In an attempt to understand how these compound-protein interactions might occur, an open window within each region of interest was generated (Fig. 12). Most of the residues involved were found in hydrogen bonding or electrostatic

interactions. The cluster of residues involved in interactions with Calcofluor White were [Tyr-303, Gly-339, Asn-367] for Wsc1p (Fig. 12A). The cluster of residues involved in interactions with Caspofungin were [His-389, Asp-486, Gly-487, Asp-488, Asp-489] for Wsc2p (Fig. 12B) and [Leu-492, Gln-493, Asp-494, Arg-519] for Wsc3p (Fig. 12C), respectively. The results suggest that an interaction could exist between the cytoplasmic domain of the yeast sensors and three therapeutic compounds under study (Caspofungin, Calcofluor White and Amphotericin B), although previous reports have shown that these three compounds have a different mode of action as a therapeutic agent against yeast. Our results have also provided some insight regarding its effectiveness as a strategic scaffold for a new drug design.

3.7. MTiOpenScreen database for discovering new compounds

MTiOpenScreen database (Labbé et al., 2015) was also used to predict at least ten new compounds with equal affinity or binding to the three cytoplasmic domains of the Wsc-family of sensors, similar to what it was observed with Caspofungin during the docking-based analysis (Table 5). The outputs retrieved from MTiOpenScreen including compound CID, energy scores in kcal/mol, and average energy scores are summarized in Table 6. Based on these results, CID17480242 has the highest average energy score (−9.1 kcal/mol) followed by a pool of compounds with an average energy score from −8.7 to −8.4 kcal/mol. Hence, compound CID17480242 should be considered as a good candidate for an equal inhibitory effect against the three sensors. These kinds of inhibitors shall permit to run further experimental studies, thus mimicking a mutant strain with a triple gene deletion or by “shutting off” three of the five sensors involved in the CWI pathway. Additionally, it seems that these compounds have a slightly more affinity towards Wsc2p. Table 7 summarizes the results of the first ten unknown compounds with the highest energy scores (−8.0 kcal/mol or higher), as well as include those new compounds that could bind Ras2p. This data supports the new hypothesis that novel and more effective compounds should be considered as therapeutic agents against yeast infection. However, more computational and *in vivo* experimental studies are needed to support this hypothesis.

4. Conclusion

Saccharomyces cerevisiae possesses a cell wall which integrity is monitored and regulated by the cell wall integrity (CWI) signaling pathway. This pathway is initiated by several highly O-glycosylated transmembrane proteins including Wsc1p, Wsc2p and Wsc3p (the Wsc-family). These sensors induce stimulation of the small G-protein Rho1p, protein kinase C (Pkc1p) and a MAP kinase cascade that activates transcription factors of stress-response genes through Rho1p. Wsc1p (as well as Wsc2p and Wsc3p) uses its cytoplasmic domain for Rho1-GDP/GTP exchange factor (Rom2p) interaction, which suggests Wsc1p interaction with Rom2p facilitates Rho1p activation. Even when these three members of the Wsc-family are relevant for this process, their tridimensional structures have not been elucidated. Thus, the tridimensional structures of the cytoplasmic domain of these three members of the Wsc-family were generated using the standard and fully-automated ORCHESTAR procedures provided by the Sybyl-X 2.1.1 program. Meanwhile, the tridimensional structure of full-length Ras2p was generated with Phyre2. Computational assessment using discrete

coulombic charges and the evaluation of motifs were completed using Procheck-PDBsum simulations for their secondary structures, and the quality of these results were assessed using ProSA-web tools. The results retrieved from STRING 10.5 suggest that the Wsc-family is involved in protein-protein interactions with each other and with Ras2p. Additional experimental approaches are also summarized in this work, pursuing insight of the hierarchical impact related to the sensors studied. Docking-based studies also validated the existence of protein-protein interactions mainly between Motif I (Wsc3p > Wsc1p > Wsc2p) and Ras2p, in agreement with the data provided by STRING 10.5. Additionally, it has shown that Calcofluor White preferably binds to Wsc1p (−9.5 kcal/mol), meanwhile Caspofungin binds to Wsc3p (−9.1 kcal/mol), Wsc1p (−9.1 kcal/mol) and more weakly Wsc2p (−6.9 kcal/mol). Thus, these data suggest Caspofungin as a common inhibitor for the Wsc-family. MTiOpenScreen database was used to predict at least ten new compounds with equal affinity or binding against the Wsc-family and Ras2p. Based on the data retrieved from MTiOpenScreen, new compounds are available and can show even more affinity towards the Wsc-family. These targeted compounds should be considered as potential new therapeutic agents against yeast infection. However, more computational and experimental studies are needed to support this hypothesis.

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Highlights

- Wsc-family is involved in protein-protein interactions with each other and with Ras2p.
- Motif I of the cytoplasmic tail of the Wsc-family is capable to interact more closely to Ras2p.
- Yeast cells were most susceptible to all compounds with the double mutant *wsc2 wsc3* strain.
- Calcofluor White preferably binds to Wsc1p, meanwhile Caspofungin binds to Wsc3p, Wsc1p and more weakly to Wsc2p.
- MTiOpenScreen predict new compounds with equal affinity or binding to the three cytoplasmic domains of the Wsc-family.

Fig. 1. Schematic representation of the Pkc1-MAPK pathway under normal-no activation (A) and stress-activation (B) conditions in yeast (Valiante, 2017; Levin, 2011; Levin, 2005). In the middle, a brief description of the structural domains assigned to the three cell wall integrity sensors: Wsc1p, Wsc2p, and Wsc3p. CW = cell wall; PM = plasma membrane; NM = nuclear membrane.

Fig. 2.
Schematic representation of a new possible interaction between the three members of the Wsc-family and Ras2p (Cazzanelli et al., 2018; García et al., 2017; Tamanoi, 2011).

Fig. 3.

A protomol of the cytoplasmic domain of Wsc1p (**A**), Wsc2p (**B**), Wsc3p (**C**), and of full-length Ras2p (**D**) generated by Surfex-Dock using Sybyl-X 2.1.1 program. The idealized computational representation of the cartoon representation (depicted in grey color) and the largest cavity in the protein surface (depicted in red color) was visualized with Chimera 1.13 (Pettersen et al., 2004). The position of the amino acid residues found in the protomol of Wsc1p are Lys-301, Asn-344, Gly-355, Asn-359, Gly-364, Asn-367, Leu-369, Val-371 and Val-372, in the protomol of Wsc2p are Ile-379, Pro-391, Asn-394, Thr-396, Tyr-409, Asp-410, Phe-454, Leu-459, Gly-481, Val-484, Asp-486 and Lys-495, in the protomol of Wsc3p are Thr-480, Pro-503, Phe-507, Asn-511, Thr-512, Asn-514, Asn-523, Pro-526 and Pro-542, and in the protomol of Ras2p are Gly-82, Gly-84, Tyr-104, Val-119, Gln-144, Phe-149, Arg-169, Val-171, Asp-173, Glu-174, Gly-176, Thr-181, Ser-291, Lys-312, Gly-317 and Cys-319.

Fig. 4.

Three key motifs (**A**) used as ligands for Surflex-Dock experiments with Ras2p. Molecular models for the Motif I (**B**) of (1) Wsc1p (KEYQ), (2) Wsc2p (KQYQ) and (3) Wsc3p (KHYQ). Molecular models for the Motif II (**C**) of (1) Wsc1p (SSFSS), (2) Wsc2p (SASST) and (3) Wsc3p (SSGTT). Molecular models for the Motif III (**D**) of (1) Wsc1p (LTVVNPDEAD), (2) Wsc2p (LHIVNPD) and (3) Wsc3p (LQVVNPDPNPD). All ligands were drawn (input) and visualized (output) with Chimera 1.13 (Pettersen et al., 2004).

Fig. 5.

The outputs generated by Procheck-PDBsum showing all secondary structures predicted for the cytoplasmic domains of Wsc1p (78 aa), Wsc2p (157 aa) and Wsc3p (151 aa), and for the full-length Ras2p (322 aa). For clarity purposes, the cytoplasmic domain of Wsc1p (A) starts at Lys-301 and ends at Asp-370, of Wsc2p (B) starts at Trp-347 and ends at Arg-503, and of Wsc3p (C) starts at Arg-406 and ends at Ala-556

Fig. 6. Screenshot of Ramachandran plots generated by Procheck-PDBsum. The plot of predicted protein models for the cytoplasmic domain of Wsc1p (A), Wsc2p (B) and Wsc3p (C), and for the full-length Ras2p (D) are shown.

Fig. 7.

ProSA-web Z-scores of all protein chains in PDB determined by X-ray crystallography (light blue) or NMR spectroscopy (dark blue) with respect to their length. The plot shows only chains with less than 1,000 residues (x axis) and a Z-score ranging from -20 to 10 (y axis). The Z-score values for the cytoplasmic domain of Wsc1p, Wsc2p and Wsc3p, and for the full-length Ras2p are highlighted as a black dot at -2.24 (A), -3.43 (B), -2.82 (C) and at -3.63 (D), respectively.

Fig. 8. Screenshot of ProSA-web plot of residue scores. Energy plots for the predicted protein model of the cytoplasmic domain of Wsc1p (A), Wsc2p (B) and Wsc3p (C), and of the full-length Ras2p (D). The plot compares the knowledge-based energy against the amino acid position

Fig. 9.

Screenshots of the network analysis retrieved from STRING 10.5 (Szklarczyk et al., 2017) between the Wsc-family of sensors either without (**A**) or with (**B**) Ras2p. Each node represents a protein suspected to be involved in protein-protein interactions. Edges are drawn in colored lines to describe the evidence used in predicting these associations: *pink*-experimentally determined; *green*-gene neighborhood; *lavender*-protein homology, *yellow*-text mining and *cyan*-curated databases. The protein-protein interaction network was set to a confidence score of 0.65. No changes in interaction were seen on confidence scores of 0.7 or above.

Fig. 10. Docking-based modeling of protein-protein interfaces between Ras2p and the Motif I of Wsc3p (A), Wsc1p (B) and Wsc2p (C). All complexes were visualized with Chimera 1.13 (Pettersen et al., 2004). Those amino acid residues from Ras2p involved in hydrogen bonding or hydrophobic interactions with each motif are also shown.

Fig. 11.

Modeled tridimensional structures of the cytoplasmic domain of each member of the Wsc-family and full-length Ras2p. The ribbon model shows the N-terminal region in green color (the first three amino acids) for Wsc1p (**A**), Wsc2p (**B**), Wsc3p (**C**), and Ras2p (**D**). The ball and stick model shows the location of the three motifs. All protein structures were visualized with Chimera 1.13 (Pettersen et al., 2004).

Fig. 12. Protein-compound docking results of Wsc1p with Calcofluor White (A), Wsc2p with Caspofungin (B), and Wsc3p with Caspofungin (C). All complexes were visualized with Chimera 1.13 (Pettersen et al., 2004).

Table 1
 PROCHECK results for the four generated protein models: Wsc1p, Wsc2p, Wsc3p and Ras2p.

Protein Model	Total Residues	Ramachandran Plot Statistics (%)				Disallowed [XX]	Homology	References
		Most Favored [A, B, L]	Additional Allowed [a, b, l, p]	Generously Allowed [-a, -b, -l, -p]				
Wsc1p, C-terminal	78	83.0	15.4	0.6		1.0	Profile family: 1vk5 Z-score: 6.11 Confidence: Certain	Allard et al., 2005
Wsc2p, C-terminal	157	70.6	26.1	1.8		1.5	Profile family: 2dst Z-score: 4.58 Confidence: Likely	Xie et al., 2007
Wsc3p, C-terminal	151	81.7	16.3	0.4		1.6	Profile family: 1d8c Z-score: 4.29 Confidence: Likely	Howard et al., 2000
Ras2p, Full length	322	86.0	10.8	1.4		1.7	Profile family: 4obe Z-score: 4.79 Confidence: Certain	Hunter et al., 2014

Table 2
Parameters of each grid box generated for MTiOpenScreen analyses (Labbé et al., 2015).

Protein	Center (X)	Center (Y)	Center (Z)	Grid box size (X)	Grid box size (Y)	Grid box size (Z)
Wsc1p, C-terminal	-7	13	-16	27	27	27
Wsc2p, C-terminal	10	-5	-7	27	27	27
Wsc3p, C-terminal	65	85	72	27	27	27
Ras2p	3.389	-3.639	12.917	30	30	30

Table 3
Protein-protein docking results from the Surflex-Dock module of Sybyl-X 2.1.1 program (Certara USA, Inc.).

Protomol Protein	Cytoplasmic tail	Motif	Energy Scores (kcal/mol)
Ras2p	Wsc3p	I	-10.9
Ras2p	Wsc1p	I	-8.4
Ras2p	Wsc2p	I	-7.6
Ras2p	Wsc2p	II	-6.5
Ras2p	Wsc1p	II	-6.2
Ras2p	Wsc2p	III	-5.6
Ras2p	Wsc3p	III	-5.6
Ras2p	Wsc3p	II	-4.8
Ras2p	Wsc1p	III	-4.4

Table 4

Antifungal susceptibility testing results on single and double mutant strains for Wsc1p, Wsc2p, Wsc3p, and Ras2p.

Strains	Antifungal Susceptibility Testing					
	Amphotericin B ¹	Calcofluor White ²	Caspofungin ³	FK506 ⁴	Fluconazole ⁵	Menadione ⁶
<i>Wsc1</i>	WT	S	S	-	-	-
<i>Wsc1 wsc2</i>	-	-	-	-	-	-
<i>Wsc1 wsc3</i>	-	-	-	-	-	-
<i>wsc3 wsc2</i>	S	S	S	S	R	S
<i>ras2</i>	WT	WT	WT	WT	S	R
<i>ras2 wsc1</i>	-	-	S	-	-	-
<i>ras2 wsc2</i>	S	R	WT	R	S	WT
<i>ras2 wsc3</i>	-	-	-	-	-	-

WT = wild-type; S = susceptible; R = resistant

¹ Polyene antifungal drug that binds to ergosterol, the principal sterol in the fungal plasma membranes forming a complex that causing leakage of the intracellular contents (Hamill, 2013; Stone et al., 2016).

² Compound that inhibits binding chitin in the cell wall and disrupts cell wall integrity (Roncero & Durán, 1985; Harrington & Hageage Jr., 2003).

³ Echinocandin, antifungal that inhibits the enzyme (1,3)- β -D-glucan synthase, by blocking cell wall synthesis and disturbing the integrity of the fungal cell wall (McCormack & Perry, 2005; Song & Stevens, 2016).

⁴ An immunosuppressive drug that inhibits the phosphatase activity of calcineurin when forming a complex with FKBP-12, calcium, calmodulin, and calcineurin (Butcher & Schreiber, 2003).

⁵ An azoles antifungal drug that inhibits fungal ergosterol synthesis by inhibiting a P-450 enzyme (C-14 α -demethylase) that demethylates lanosterol to form ergosterol (Zervos et al., 1994; Enache-Angoulvant & Hennequin, 2005).

⁶ Chemotherapeutic agent that promotes cell injury by imposing massive oxidative stress (Kwolek-Mirek & Zadrag-Tecza, 2014; Yamashoji, 2016).

Protein-compound docking results from the Surflex-Dock module of Sybyl-X 2.1.1 program (Certara USA, Inc.). Highest energy scores are highlighted in bold. Caspofungin is also in bold because it shows high affinity against the three members of the Wsc-family.

Table 5

Therapeutic Compound	Energy Scores (kcal/mol)			
	Wsc1p C-domain	Wsc2p C-domain	Wsc3p C-domain	Ras2p
Amphotericin-B	-6.9	-6.5	-5.6	-0.9
Calcofluor White	-9.5	-6.0	-8.7	-2.8
Caspofungin	-9.0	-6.9	-9.1	+6.6
Fluconazole	-4.5	-5.3	-4.5	-2.7
FK506	-2.4	-3.7	-3.2	-0.7
Menadione	-3.1	-4.9	-3.5	-0.8

Energy scores for the first ten (10) potential new compounds that can bind the cytoplasmic domain of each member of the Wsc-family of sensors. PubChem substance accession numbers (CID) were retrieved from MTiOpenScreen (Labbé et al., 2015).

Table 6

Compound CID	MTiOpenScreen Energy Score (kcal/mol)			
	Wsc1p	Wsc2p	Wsc3p	Average
17480242	-8.5	-10.8	-8.0	-9.1
124948355	-8.2	-9.9	-8.0	-8.7
24291193	-8.4	-9.9	-7.9	-8.7
24384935	-8.1	-10.0	-7.9	-8.7
14728891	-8.4	-9.6	-7.9	-8.6
24325108	-8.2	-10.0	-7.8	-8.7
24351984	-9.2	-9.2	-7.6	-8.7
24805332	-8.0	-8.9	-8.3	-8.4
24785981	-7.7	-9.8	-8.0	-8.5
26539518	-8.7	-9.1	-7.8	-8.5

Table 7

Energy scores for the first ten (10) potential new compounds that can bind the cytoplasmic domain of the Wsc-family and Ras2p. PubChem substance accession numbers (CID) were retrieved from MTiOpenScreen (Labbé et al., 2015).

MTiOpenScreen Energy Score (kcal/mol)									
Compound CID	Wsc1p	Compound CID	Wsc2p	Compound CID	Wsc3p	Compound CID	Ras2p	Compound CID	Ras2p
24351984	-9.2	17480242	-10.8	24308238	-8.3	49729862	-9.0		
26662905	-9.1	24325108	-10.0	24805332	-8.3	24290532	-8.8		
17505792	-9.0	24384935	-10.0	57288056	-8.2	26614289	-8.8		
26653430	-9.0	47202310	-9.9	24347499	-8.1	24366176	-8.8		
24355782	-9.0	99456029	-9.9	24302748	-8.1	24375371	-8.8		
29215520	-9.0	24291193	-9.9	17509486	-8.1	24332333	-8.6		
17504894	-8.9	124948355	-9.9	24375539	-8.1	24805332	-8.6		
24320657	-8.8	22402942	-9.8	24837488	-8.1	17504660	-8.6		
24335388	-8.8	125267701	-9.8	17440595	-8.0	17471374	-8.6		
24339291	-8.8	24286115	-9.8	26535875	-8.0	161004162	-8.6		