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MAÍSE GOMES QUEIROZ

Função do gene *NCW2* na reparação de danos causados à parede celular da levedura *Saccharomyces cerevisiae*

Recife

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor em Genética.

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Orientador: Prof. Dr. Marcos Antonio de Morais Junior

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Aprovada em 08/05/2020.

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“Apesar de você, amanhã há de ser outro dia. Você vai ter que ver a manhã renascer e esbanjar poesia. Como vai se explicar vendo o céu clarear, de repente, impunemente. Como vai abafar nosso coro a cantar na sua frente.”

(Chico Buarque)

RESUMO

O gene NCW2 foi recentemente descrito como codificador de uma proteína que auxilia na remodelagem da parede celular (PC) da levedura *Saccharomyces cerevisiae* e na reparação de danos causados pelo polímero PHMB. Em vista disso, o presente trabalho teve como objetivo estender a análise funcional desse gene no contexto do mecanismo de manutenção da integridade da PC, ou mecanismo CWI. Os resultados de expressão gênica mostraram que NCW2 pertence à família CWI e que a sua ausência leva à ativação de um mecanismo compensatório que regula positivamente os genes desta família, reforçando a PC com acúmulo de quitina. Embora Ncw2p não esteja envolvida diretamente na resposta a estresse osmótico, esse acúmulo de quitina parece aliviar a resposta através da via HOG, tornando as células sensíveis ao estresse osmótico. Ncw2p se acumula em regiões da membrana que correspondem aos anéis de brotamento na medida em as células alcançam a fase estacionária, quando Ncw2p está altamente N- manosilada pela proteína Mnn9. Isso é mimetizado na resposta ao PHMB mesmo na fase exponencial. Ncw2p também sofre alterações do O-manosilação pelo complexo Pmt3/5 e Pmt4, sendo ambos complexos relevantes para a resposta ao PHMB. Em conclusão, mostramos que Ncw2p desempenha uma função fisiológica na correta organização da camada de quitina e sua ligação aos demais componentes da parede, interferindo na capacidade de expansão da parede durante o crescimento em resposta a estresse de parede celular.

Palavras-chave: CWI. Manosilação. PHMB. Levedura.

ABSTRACT

The NCW2 gene was recently described as encoding a protein that assists in the remodeling of the cell wall (CW) of the yeast *Saccharomyces cerevisiae* and in the repair of damage caused by the PHMB polymer. Hence, the present study aimed to extend the functional analysis of this gene in the context of the mechanism for maintaining the integrity of CW or CWI mechanism. The gene expression results showed that NCW2 belongs to the CWI family and its absence leads to the activation of a compensatory mechanism that positively regulates the genes of this family, reinforcing the CW with chitin accumulation. Although Ncw2p is not directly involved in the response to osmotic stress, this accumulation of chitin appears to alleviate the response via the HOG pathway, turning the cells sensitive to osmotic stress. Ncw2p accumulates in regions of the membrane that correspond to the budding rings as the cells reach the stationary phase, when Ncw2p is highly N-mannosylated by the Mnn9 protein. This is mimicked in the response to PHMB even at the exponential stage. Ncw2p also undergoes changes in O-mannosylation by the Pmt3 / 5 and Pmt4 complexes, both complexes being relevant to the response to PHMB. In conclusion, we show that Ncw2p plays a physiological role in the correct organization of the chitin layer and its binding to the other components of the wall, interfering with the expansion capacity of the wall during growth in response to cell wall stress.

Keywords: CWI. Mannosylation. PHMB. Yeast.

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LISTA DE ABREVIATURAS E SIGLAS

CFW	Calcofluor White
CR	Congo Red
CW	Cell wall
CWI	Cell Wall Intregrity pathway
CWP	Cell wall proteins
ERAD	Endoplasmic-reticulum-associated protein degradation
EtNP	Ethanolamine phosphate
GFP	Green fluorescent protein
GlcNAc	N-Acetylglucosamine
GPI	Glycosylphosphatidylinositol
HOG	High-osmolarity glycerol pathway
ORF	Open reading frame
PHMB	Poli - hexametileno biguanida
PIR	Proteins with internal repeats
PMT	Protein mannosyl transferase
PM	Plasma membrane
RE	Reticulum endoplasmic
SCCS	Scientific Committee on Consumer Safety
YNB	Yeast-Nitrogen-Base
YPD	Yeast Extract- Peptone -Dextrose

SUMÁRIO

1 INTRODUÇÃO

O Polihexametileno biguanida (PHMB) é um biocida de amplo espectro, amplamente utilizado na indústria farmacêutica devido a sua alta eficácia contra diversos microrganismos de relevância clínica. No ambiente industrial havia sido proposta sua utilização na eliminação de contaminantes do processo de fermentação, mostrando-se útil para o reaproveitamento das células entre uma batelada e outra, eliminando bactérias e leveduras contaminantes, como por exemplo, leveduras da espécie *Dekkera bruxellensis*, muito comum no ambiente industrial. Tal aplicabilidade tornou-se restrita à linhagem industrial utilizada no processo devido à variabilidade no perfil de tolerância de diferentes linhagens industriais de *Saccharomyces cerevisiae*, como foi verificado analisando duas linhagens amplamente utilizadas na indústria, JP1 e PE2, das quais a primeira se mostrou mais tolerante enquanto a segunda mais sensível ao PHMB.

A principal diferença entre essas duas linhagens é que na mais tolerante há uma resposta celular voltada para a manutenção da integridade da parede celular. Essa resposta envolve uma sinalização de dano que leva à expressão diferenciada de genes da via CWI (*Cell Wall Integrity*). Dentre esses genes, foi identificado um grande aumento na expressão do gene NCW2 em células tolerantes em resposta ao PHMB. Havia relatos de sua associação com a parede celular, porém sem descrição da função da sua proteína na estrutura ou remodelagem da parede. Apresentando até o momento poucos fenótipos resultantes da sua deleção, sendo todos relacionados à parede celular.

Além disso, o fator de transcrição Yap1, o principal regulador de genes de resposta a estresse oxidativo, mostrou-se também envolvido na regulação de genes CWI. Sendo que, uma vez verificado que não havia estresse

oxidativo na célula, observado a partir da análise da formação de grupos tióis intracelulares, e que a deleção do gene YAP1 leva a maior sensibilidade ao PHMB, foi constatado que este fator de transcrição poderia estar regulando genes envolvidos na reestruturação da parede celular, sem o envolvimento de estresse oxidativo.

Desta forma, presume-se que há uma sinalização ou resposta de integridade de parede celular ainda não relatada para o agente estressor de PHMB, especialmente pelo fato de Ncw2p não ter sido, até o momento, encontrada no genoma de outros organismos depositados nos bancos de dados, dificultando a identificação baseada em homologia. Apesar da espécie *S. cerevisiae* ser amplamente estudada como modelo celular eucarioto e com forte aplicação industrial, segundo o banco de dados Yeast Genome, 2566 genes estão anotados com função molecular desconhecida.

A proteína Ncw2p, ainda pouco estudada, está relatada na literatura como um componente da parede celular, contudo o seu papel nessa estrutura permanece desconhecido. A parede celular, por sua vez, atua como a principal barreira da célula contra diversas condições de estresse, na seletividade e integridade do envelope celular, cujos mecanismos de estruturação e remodelagem não estão inteiramente elucidados. Sendo assim, a identificação da função de Ncw2p pode revelar mecanismos não elucidados sobre a resposta ao estresse de parede célula. Para isto, se faz importante a identificação de seus aspectos moleculares e como eles influenciam tal resposta.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Identificar o mecanismo celular disparado pela presença de PHMB, bem como a relação que Ncw2p tem com essa resposta, e quais aspectos moleculares desta proteína podem estar envolvidos na via CWI.

1.1.2 Objetivos específicos

1. Verificar o efeito do tratamento por PHMB de células da linhagem parental e mutantes Δ*ncw2* Δ*yap1* quanto ao fenótipo de resistência à lise por uma hidrolase de beta-glucanos;

2. Determinar o perfil de deposição de quitina na parede celular de células parentais e mutantes Δ*ncw2* incubadas em PHMB;

3. Quantificar a expressão dos principais genes da via CWI na ausência do gene NCW2;

4. Avaliar efeito sinergético do PHMB em outros tipos de estresse;

5. Localização subcelular de Ncw2p;

6. Identificação de modificações pós-traducionais de Ncw2 e sua relação com PHMB e crescimento celular;

2 REVISÃO DA LITERATURA

2.1 ESTRUTURA E BIOSSÍNTESE DA PAREDE CELULAR DE *SACCHAROMYCES CEREVISIAE*

A parede celular é uma organela que, juntamente com a membrana plasmática, faz parte do envelope celular e é responsável por determinar a forma e manter a integridade da célula (Gow et al, 2017). Essa estrutura fornece força mecânica contra mudanças na osmolaridade do meio, bem como contra o turgor citoplasmático de *S. cerevisiae* (e outros fungos) que normalmente é alto (Minc et al, 2009; Schaber et al, 2010). Essa estrutura é formada por uma malha constituída por glicoproteínas e polissacarídeos, principalmente quitina e glucanos. As glicoproteínas podem ser modificadas por carboidratos (normalmente oligômeros de manose) ligados a N- ou O-, já os beta-glucanos são predominantemente dos tipos beta-1,3-glucano e beta-1,6-glucano, e a quitina é produzida como cadeias de beta-1,4-N-acetilglicosamina, estando eles interligados covalentemente (Kollar et al, 1997; Lipke and Ovalle, 1998; Bowman and free, 2006).

Estes componentes estão alocados na parede celular formando uma estrutura organizada (Figura 1), na qual é formado um *backbone* constituído por fibras beta1,3-glucano, ao qual os demais componentes se ligam, tornando este o principal glucano (Kopecká et al, 1974; Douglas, 2001), sendo que esta camada fibrilar de beta-1,3-glucano, associada a uma fina camada inferior de quitina (mais próxima da membrana) e ao beta-1,6-glucano formam a camada interna da parede, enquanto que a camada externa é composto por manoproteínas, esta última é conectada à camada interna pela sua ligação covalente às cadeias laterais de beta-1,6-glucano, sendo assim, este é o responsável por conectar as duas camadas (Zlotnik et al, 1984; Alsteens et al, 2008).

Os polissacarídeos quitina e glucanos são sintetizados na membrana plasmática por complexos enzimáticos transmembrana (glucano-sintases e chitina-sintases), utilizam UDP-glicose e UDP-N-acetilglucosamina como substrato, translocando os monômeros para o periplasma, onde ocorre a polimerização dos glucanos e da quitina, respectivamente. Já as proteínas são produzidas, endereçadas ao complexo de Golgi e após serem glicosiladas normalmente com manose, elas são endereçadas à parede celular pela via secretória (Douglas, 2001; Cabib and Arroyo, 2013; Gow et al, 2017).

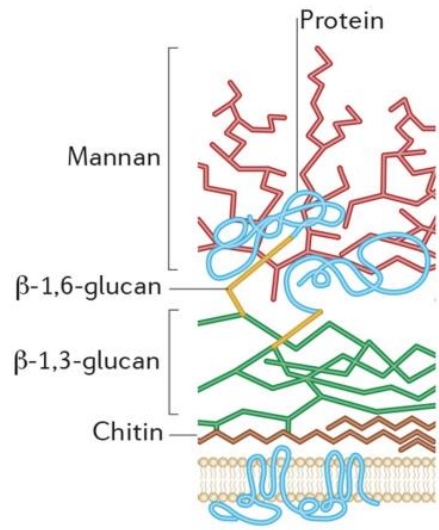

Figura 1: Organização estrutural da parede celular similar entre leveduras dos gêneros Candida e Saccharomyces (Gow et al 2017).

A síntese de β-1,3-glucano está associada à família Fks, da qual Fks1 e Fks2 utiliza Rho1 GTPase como subunidade reguladora. Tal atividade é associada a membrana, onde UDP-glucosamina atua como doador sendo estimulado por GTP através de Rho1 (Drgonová et al. 1996). A proteína Rho1 GTPase, essencial como subunidade regulatória para proteínas Fks,

também atua na ativação Pkc1 na via Cell Wall Integrity (CWI), e é necessária para a progressão do ciclo celular e polarização do crescimento (Levin 2011; Orlean, 2012). As cadeias de β-1,3-glucano no momento da síntese são exportas através da membrana, onde então podem ser ligadas as fibras de quitina por Chr1 e Chr2 (Cabib 2009). Depois de exportadas para o espaço periplasmático, essas cadeias podem ser estendidas pela ação de β-1,3-glucanosiltransferases como Gas1p (Mouyna et al. 2000), e suas cadeias laterais são ligadas ao β-1,6-glucano, bem como às proteínas PIR (*putative proteins with internal repeats*) (Ecker et al. 2006).

Aspectos bioquímicos da síntese de β-1,6-glucano ainda não estão muito bem definidos, e sua via de síntese parece ser mais complexa que dos demais polissacarídeos da parede (Cabib and Arroyo, 2013). É possível que o último passo de sua síntese ocorra na membrana plasmática, enquanto que os açúcares de nucleosídeo difosfato, precursores de β-1,6-glucano, são produzidos dentro da célula para então serem direcionados aos locais de síntese na membrana plasmática, onde doam uma unidade de monossacarídeo para a cadeia nascente, que está sendo exportada através da membrana para o espaço periplasmático (Orlean, 2012; Cabib and Arroyo, 2013).

A maioria das proteínas da parede celular (CWP- *cell wall proteins*) é modificada com GPI, uma estrutura glicolipídica que é adicionada a algumas proteínas destinadas à parede e que atua como âncora na membrana, e que conecta essas proteínas indiretamente ao beta-1,6-glucano, e este por sua vez ligado ao *backbone* de beta-1,3-glucano (Klis et al, 2006). No entanto há um pequeno grupo de proteínas, que não são associadas à âncora GPI, e que se liga covalentemente ao beta-1,3-glucano, e uma vez que essa ligação é sensível à álcali moderado, essas proteínas são denominadas ASL (*álcali-sensitive linkage*) – CWPs (Groot et al, 2005; Klis et al, 2006). Esse grupo

proteico inclui as proteínas Pir (*putative proteins with internal repeats*), posteriormente nomeadas proteínas CCW (*covalented linked to cell wall*), as quais além de não possuírem sinal para modificação de GPI, elas apresentam repetições internas de uma determinada sequência de aminoácidos, contendo resíduos de glutamina, pelo qual faz ligação com a fibra de beta-1,3-glucano (Ecker et al, 2006).

A organização estrutural da parede celular, seja pelos tipos de ligações quanto à proporção de cada componente, se dá por um processo dinâmico que está sujeito à remodelagem em resposta a diferentes condições fisiológicas e ambientais como tipo de fonte de carbono, pH, temperatura, dentre outros (Aguilar-Uscanga and François, 2003; Bowman and free, 2006). O dinamismo da construção e remodelagem da parede celular também se apresenta durante a divisão celular, onde a formação do *bud neck* é essencial para ocorrer citocinese e separação do broto e célula-mãe. O *bud neck* por sua vez é uma constrição de uma região da célula, caracterizado pela redução na expansão da parede, para isto, a região do *bud neck* apresenta grande acúmulo de quitina que satura as terminações de ligação do beta-1,3-glucano, de tal forma a impedir a ligação dos oligômeros de beta-1,6-glucano associado associados a manoproteínas ao beta-1,3-glucano (Cabib and Arroyo, 2013).

2.2 VIA DE INTEGRIDADE DA PAREDE CELULAR PKC-CWI (*CELL WALL INTEGRITY - CWI*)

A via de sinalização de integridade da parede celular (CWI) é necessária para remodelagem da parede durante o crescimento celular e é ativada por fatores que desencadeiam danos à parede. A via CWI (Figura 2)

é caracterizada por sensores da superfície celular acoplados a uma pequena proteína G, denominada Rho1, que ativa uma série de efetores. Essa cascata gera um sinal que regula diversos processos relacionados à síntese dos componentes em sítios de remodelagem da parede celular, para tal, ela controla a expressão de genes relacionados à biogênese da parede, à organização do citoesqueleto de actina e o direcionamento de vesículas secretórias aos sítios de crescimento e biossíntese de parede celular (Levin, 2011).

Seja um dano que necessite reparo ou uma condição celular que necessite controle da expansão da parede, estes sinais são percebidos pelos sensores, os quais iniciam a cascata CWI. Dentre eles, há duas pequenas sub-famílias de sensores, um tipo Wsc1/Slg1 e outra composta pelos os sensores Mid2 e Mtl1 (tipo Mid), todos esses sensores possuem poucas semelhanças nas sequencias primárias, no entanto compartilham a mesma estrutura geral (Jendretzki et al, 2011). Ambas as sub-famílias apresentam um domínio transmembrana que conecta o pequeno terminal carboxi citoplasmático com a relativamente grande cauda extracelular, esta última rica em resíduos de serina e treonina altamente O-manosilados. Essas duas sub-famílias se diferenciam pela N-terminal extracelular, nos sensores tipo Wsc1/Slg1, há um domínio adicional rico em cisteína, enquanto no tipo Mid o domínio adicional é caracterizado pela presença de resíduos de asparagina N-glicosilados (Figura 3) (Heinisch and Dufrêne, 2010).

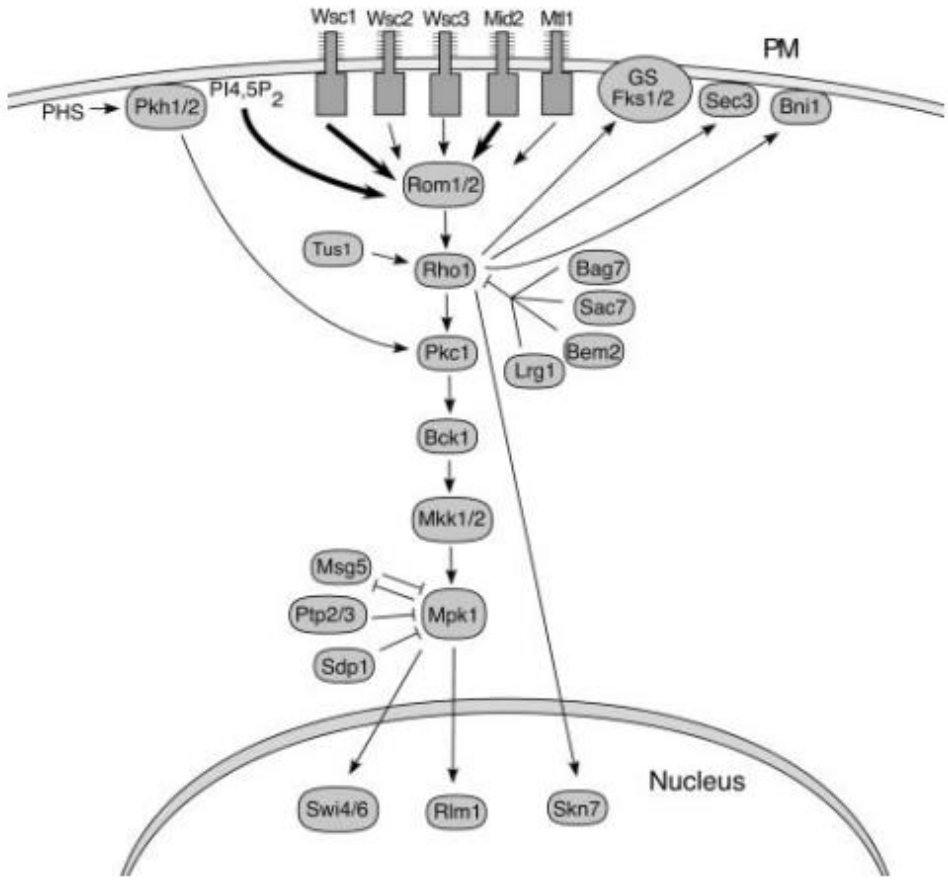

Figura 2: Principais elementos da cascata de sinalização de integridade da parede celular - Cell Wall Integrity pathway (CWI) (Levin, 2011).

A sinalização disparada pelos sensores Wsc1/Slg1 e Mid2 ocorre pelo seu caráter de mecanosensor, de tal forma que alterações na estrutura da parede celular geram deformidade na porção extracelular da proteína (Kock et al, 2015), induzindo alterações conformacionais do terminal citoplasmático, levando à interação deste com os fatores de troca de nucleotídeo de guanina (GEF – *guanine nucleotide exchange factors*) Rom1 e Rom2, os quais ativam Rho1, promovendo a troca de GDP por GTP e assim ativando esta proteína que irá transmitir o sinal para a quinase Pkc1 (Philip and Levin, 2001), a qual através da cascata de sinalização MAP quinase, ativa os fatores de transcrição Rlm1 e Swi4/6 reguladores de genes

relacionados à biossíntese e remodelagem da parede (Levin, 2011). Essa sinalização é amplificada à medida que a parede celular é submetida a um estresse prolongado, devido ao fato de que os sensores Wsc1/Slg1 são distribuídos pela superfície da célula formando regiões de clusterização, uma vez a via CWI estimulada por um dano na parede, há um aumento na concentração dos sensores nesses clusters de maneira dependente dos domínios ricos em cisteína, que se ligam entre si e com a malha de beta-glucano, aumentando assim o sinal transdutor da via PKC/CWI (Heinisch et al, 2010).

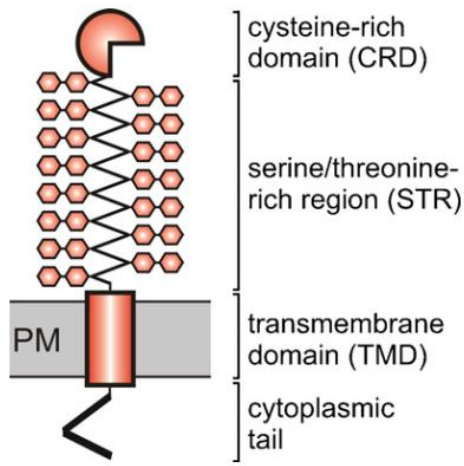

Figura 3: Estrutura geral dos sensores de superfície de parede celular de ambas sub-famílias, tipo Wsc1/Slg1 e tipo Mid. Estão sendo representados os sensores tipo Wsc1/Slg1, devido ao domínio rico em cisteína, crucial para sua função, presente apenas neste grupo, enquanto o tipo Mid possui um domínio de resíduos de asparagina N-glicosilados (Jendretzki et al, 2011).

A resposta disparada por danos na parede, ou até mesmo pela inativação/deleção de genes CWI, percebida pelos sensores que levam à cascata de sinalização, corresponde à expressão de genes CWI, aumento da atividade de sintetases dos componentes da parede, a fim de reparar tais danos de tal maneira a manter a integridade da estrutura da parede celular.

Esse mecanismo compensatório na reconstituição da parede celular normalmente tende a aumentar a produção de quitina, e consequentemente, fortalecer o *backbone* formado pela ligação entre o beta-1,3-glucano e as fibras de quitina (Popolo et al, 2001; Valiante et al, 2015; Gow, 2017).

Anormalidades na parede também afetam a proliferação celular, de tal maneira que há um *checkpoint* que acopla síntese da parede com a divisão celular (Suzuki et al., 2004). Esse *checkpoint* da integridade parede modula a progressão do ciclo celular em resposta a danos na parede, através da coordenação entre a via da integridade de parede celular e formação do fuso mitótico (Negishi and Ohya, 2010). Células estagnadas no *checkpoint* da integridade da parede apresentam brotos menores, devido ao fato de que não ocorre remodelagem da parede celular nessa região (Negishi and Ohya, 2010).

Além disso, deleção de genes da via CWI e de genes envolvidos na síntese da parede celular apresentam parada do ciclo celular, uma vez que a ausência de tais genes gera perturbações na parede celular (Ram et al., 1995; Negishi and Ohya, 2010). Esse *checkpoint* de integridade difere dos demais durante a divisão celular, pois ele ocorre após o término da replicação do DNA, e também funciona de maneira diferente dos *checkpoints* de morfogênese ou formação/posição do fuso, uma vez que não requer os principais mecanismos dessa resposta, tais como Swe1p, Mad2p ou Bub2p (Surana et al., 1991). Esse checkpoint disparado por danos na parede é também diferente do causado por danos na membrana plasmática (Kono and Ikui, 2017). Eles diferem no momento de parada da divisão celular. Diferentemente do checkpoint da integridade da parede, no qual a parada do ciclo celular ocorre na fase G2/M após a replicação do DNA, a parada do checkpoint de dano na membrana plasmática ocorre no final de G1 (Suzuki et al., 2004).

Alterações na parede celular também são percebidas pela via SNL1, que responde com aumento de sua atividade principalmente por redução de proteínas ancoradas a GPI ou pela deleção do gene *FPS1,* que codifica um transportador de glicerol (Tao et al., 1999). SNL1 é uma via de transdução de sinal de “dois componentes”, onde componente 1 é uma histidina quinase e o componente 2 é um regulador de resposta (Reiser et al., 2003; Shankarnarayan et al., 2008).

A transdução de sinal dessa via ocorre pela cascata de transferência de grupos fosfato, que começa em resíduos de histidina e aspartato conservados dentro da proteína Sln1, que transfere a uma histidina conservada na proteína Ypd1, que por sua transfere o fosfato para domínios contendo aspartato em dois reguladores de resposta, Ssk1 e Skn7 (Posas et al, 1996; Li et al., 1998). Skn7 é um fator de transcrição também envolvido na resposta ao estresse oxidativo, onde atua de forma independente de Sln1 (Krems et al., 1996; Fassler and West, 2011), formando a via SNL1-SKN7, que é regulada e regula genes envolvidos na via de integridade da parede celular (Cui et al., 2002; Li et al., 1998), atuando em paralelo à via PKC coordenando a resposta a sinais extracelulares e regulando a integridade da superfície celular (Brown et al., 1994).

2.3 MODIFICAÇÃO PÓS-TRADUCIONAL DE PROTEÍNAS DE PAREDE CELULAR; N- E O-MANOSILAÇÃO

Modificação proteica do tipo glicosilação é a mais complexa e mais custosa em termos energéticos. Em *S. cerevisiae*, há dois tipos de glicosilação proteica, N-glicosilação nos resíduos de asparagina e O-manosilação em resíduos de serina e treonina, ambos são iniciados no retículo endoplasmático (Lehle et al., 2006).

O processo de N-glicosilação inicia com síntese $GlcNAc_2Man_9Glc_3$, o chamado *core* oligossacarídeo, a partir da transferência de um α-ligado N-acetilgicosaminafosfato a partir de uniridedifosfato (UDP) – GlcNAc para dolicolfosfato, dando origem a GlcNAc-PP-Dol (Lehle et al., 2006). Em seguida, um segundo resíduo de GlcNAc e cinco grupos manose são transferidos para o lado citosólico do retículo endoplasmático, onde os nucleotídeos de açúcar UDPGlcNAc ou guanosinadifosfato (GDP)-Man atuam doadores de carboidrato, e então $Man_5GlcNAc_2$-PP-Dol é transferido para o lumen do retículo (Helenius et al., 2002).

No lúmen, os resíduos de quatro manoses e três moléculas de glicose são adicionados pelas glicosil transferases (Lehle and Strahl, 2006). A partir disso, esse *core* oligossacarídeo, em estado ativado, é ligado à proteína e modificado de maneira proteína ou tecido - específica pela remoção e/ou adição de resíduos de açúcares (Cheng et al., 2001; Kamemura et al., 2002). A adição da fração de sacarídeo é completamente transferida à cadeia polipeptídica recém sintetizada. A proteína é sintetizada por ribossomos associado ao retículo endoplasmático e translocada através da membrana por um canal transmembrana, o complexo Sec61, para dentro do lúmen do RE. O centro ativo da oligossacaril transferase (OST), responsável pela transferência do oligossacarídeo à proteína, está localizado no lado luminal do RE (Lehle and Strahl, 2006).

Proteínas que serão glicosiladas são reconhecidas por OST, por meio de sequências consenso como asparagina-X-serina/ threonine (N-X-S/T; onde X pode ser qualquer aminoácido, exceto prolina) (Lehle and Strahl, 2006).

O tipo O-manosilação também ocorre no lado luminal do RE, durante a translocação transmembrana da cadeia peptídica. No entanto, o açúcar ativado é provido por Dolicol-P-Man e não por nucleotídeo de açúcar. Em

seguida, manoses adicionais são transferidas in complexo de Golgi, tendo GDP-Man como doador de manosil (Lehle and Strahl, 2006). A manose é transferida do Dol-P-L-D-manose com conversão da sua configuração aos resíduos de serina/treonina das proteínas secretórias formando uma ligação do tipo K-D-manosil (Strahl-Bolsinger et al., 1999). Essa reação é catalisada pela família de proteínas manosiltransferases (PMTs) dependente de dolicol fosfato manose (Willer et al, 2003; VanderVen et al., 2005). A família de PMTs compreende pelo menos seis membros, Pmt1 à Pmt6 (Lommel and Strahl, 2009).

Análise estrutural da Pmt1p em *S. cerevisiae* revela sete domínios transmembrana com extremidade amino e carboxi-terminal localizadas no citosol e no lúmen do RE, respectivamente (Strahl-Bolsinger and Scheinost, 1999). Segundo análises filogenéticas, a família PMT é dividida nas subfamílias PMT1, PMT2 e PMT4, cujos membros incluem transferases proximamente relacionadas a Pmt1p, Pmt2p e Pmt4p de *S. cerevisiae*, respectivamente (Willer et al., 2003). Integrantes da subfamília PMT1 (Pmt1p e Pmt5p) formam complexos heterodiméricos com integrantes da família PMT2 (Pmt2 e Pmt3p), enquanto Pmt4p, único membro da subfamília PMT4, forma complexos homodiméricos. Além do complexo Pmt4p, Pmt1/Pmt2p são responsáveis pela maioria das atividades de transferase em levedura, apesar de formas alternativas dos complexos podem ser formados (Girrbach and Strahl, 2003).

N-glicosilação de proteínas possui um papel decisivo como componentes de sinalização, como por exemplo, tráfego de proteínas lisosomais e controle de qualidade do enovelamento de proteínas secretórias, garantindo que somente proteínas corretamente enoveladas sejam destinadas à via secretória, partindo do lúmen do RE para à superfície celular. Do contrário, uma via dependente de degradação de proteínas

dependente de sacarídeo associada ao RE (ERAD) atua na proteína mal dobrada (Lehle and Strahl, 2006). Em levedura, é necessário haver o mínimo do nível de O-manosilação para a célula ser viável, ou pelo menos proteínas essenciais sejam O-manosiladas. Além disso, O-manosilação também é importante para construção da parede celular e estabilidade proteica, devido ao fato de que na ausência dessa modificação, há ativação da via CWI e na via de resposta a proteínas mal dobradas. Os dois tipos de glicosilação podem em alguns casos se sobrepor na função, e de alguma forma compensar a ausência da outra (Arroyo et al., 2011; Orlean, 2012).

2.4 ÂNCORA GLICOSILFOSFATIDILINOSITOL (GPI)

Glicosilfosfatidilinositol (GPI) são estruturas glicolipídicas adicionadas pós traducionalmente à extremidade carboxi de certas proteínas no lúmen do RE (Ferguson et al., 2009). Elas atuam como âncoras na membrana para muitas proteínas da superfície celular (Baumann et al., 2000).

GPIs possuem um *backbone* consistindo em fosfoetanolamina (EtNP), três manoses, um non-N acetilado glucosamina (GlcN) e fosfolipideo inositol, sendo toda a estrutura EtNP-6Man-2Man-6Man-4GlN-6myoinositol-P-lipideo (Kinoshita et al., 2016). Sua biossíntese é iniciada na face citoplasmática do RE, pela transferência de GlcNAc a partir de UPD-GlcNAc para fosfatidilinositol (PI), gerando o primeiro intermediário GlcNAc-PI, mediada pela GPI-GlcNAc transferase (Watanabe et al., 2000). GlcNAc-PI é N-desacetilada pela enzima Gpi12 para gerar GlcNPI, a qual é translocada para a face luminal do RE (Orlean et al., 2007).

Dentro do RE, a enzima aciltrasnferase Gwt1 adiciona um grupo palmitol na posição 2 do anel de inositol gerando GlcN-(acil)PI (Orlean et al., 2007), que passa por um processo de remodelagem, com adição de dois

resíduos de manose usando dolicol-P-manose como substrato, pela ação de GPI-manosil transferase 1 e GPI manosil transferase 2. Então, além da adição de grupos de manose, ocorre a adição da primeira EtNP à GPI na manose 1, pela ação de Mcd4, e por final, um grupo EtNP é adicionado à manose2 pela enzima EtNP transferase 3 (Pittet and Conzelmann, 2007). Uma vez a estrutura GPI formada, a sua ligação às proteínas se dá entre o grupo carboxil da extremidade C-terminal e um grupo amino do EtNP, em uma reação de transamidação pelo complexo GPI transamidade, para isto é necessário uma clivagem prévia da proteína pela atividade tipo caspase de Gpi8p. Em seguida, é através dos fosfolipídeos de inositol, uma porção de GPI é inserida na face externa da membrana plasmática, enquanto a proteína não é diretamente inserida na membrana (Kinoshita et al., 2016).

A ligação da GPI é essencial para a exportação eficiente de proteínas ancoradas à GPI, saindo do RE. Proteínas precursoras não acoradas não são ativamente incorporadas em vesículas COPII (complexo proteico de revestimento citosólico), pois a maquinaria de exportação do RE não consegue reconhecê-las. No entanto, devido ao fluxo vesicular intenso, proteínas precursoras não ancoradas podem deixar o RE por uma via padrão ou de fluxo em massa, e chegar ao Golgi, de onde podem ser transferidas de volta ao RE, onde serão recicladas, em vesículas revestidas COPI. Tal recuperação é dirigida pelo cargo receptor Rer1 (Sato et al., 2003). O enovelamento apropriado de proteínas secretadas é monitorado por um controle de qualidade sofisticado que pode detectá-las e as enviarem para degradação a fim de preservar a homeostase funcional da via secretória.

Normalmente, proteínas transmembrana e solúveis mal dobradas ficam retidas no RE, e são eliminadas por um processo de degradação associado ao RE (ERAD). Os substratos de ERAD são retro-translocados para o citosol e poliubiquitinados pela ubiquitina ligase Hrd1 e

subsequentemente degradados pelo proteassoma (Wu and Rapoport, 2018). No entanto, em leveduras, proteínas ancoradas à GPI mal dobradas não são destinadas à via ERAD para degradação proteossomal, em vez disso, elas são remodeladas e rapidamente reconhecidas pelo complexo p24 para prevenir sua captura pela Hrd1 e exportadas do RE para serem entregues aos vacúolos para degradação (Fujita et al., 2006; Sikorska et al., 2016).

2.5 POLIHEXAMETILENO BIGUANIDA (PHMB)

O PHMB é usado em diversas preparações comerciais, tendo aplicação médica com uso dermatológico na desinfecção de feridas e queimaduras, também é usado como desinfectante na limpeza de ambiente industrial e hospitalar, na purificação de água de piscina, na composição de soluções de lente de contato, e como preservante de produtos cosméticos e de higiene pessoal. A segurança da utilização deste composto tem sido avaliada pelo comitê científico da segurança do consumidor (SCCS - Comissão européia), o qual determinou que a concentração de PHMB em produtos cosméticos deve estar abaixo de 0,3% para ser considerada segura para a saúde humana, tendo este órgão verificado que o PHMB não é tóxico pela via dérmica e nem apresenta potencial genotóxico, porém pode ser considerado moderado quanto à toxicidade oral aguda e moderadamente irritante à pele apenas em concentrações altas, como por exemplo, em torno de 20% (SCCS and Bernauer, 2015).

O PHMB é composto por polímeros de biguanidas (Figura 4) que contém grupos catiônicos e de base forte, grupos imino carregados positivamente, os quais interajem com moléculas carregadas negativamente (Ikeda et al, 1984), tal propriedade iônica parece ser o principal fator da sua

atividade antimicrobiana, pois esta pode ser neutralizada pela presença de NaCl, de maneira dependente da concentração (Yanai et al, 2011).

Um de seus mecanismos de ação se dá pela sua interação com os fosfolipídeos, que possuem carga negativa, da membrana plasmática de bactérias (Broxton et al, 1984), e também aos componentes da parede celular, sendo no caso de bactérias Gram-negativas com o peptideoglicano e principalmente com os lipopolisacarídeos (LPS), maiores sítios de adsorção localizados na superfície da membrana externa do envelope, onde atua deslocando cátions do *core* LPS da parede celular de bactérias, e com isto obtém acesso à membrana, levando à perda da fluidez, da separação de fase dos lipídeos (bicamada lipídica) e ruptura da membrana (Gilbert et al, 1990a; Gilbert and Moore, 2005). Em bactérias Gram-positivas ocorre de maneira similar, porém o principal alvo de carga negativa da parede celular são os ácidos teicóicos (Kaehn, 2010).

Outro aspecto do efeito antimicrobiano do PHMB reside nos comprimentos dos polímeros, inicialmente foi descrito que essa atividade era proporcionalmente mais intensa de acordo com o aumento do comprimento do polímero, o qual produziria maiores sítios de adsorção, aumentando o efeito de ruptura da membrana (Broxton et al, 1984). Posteriormente, foi verificado que a eficácia da atividade bactericida do PHMB depende mais da sinergia entre a ação dos polímeros de diferentes comprimentos do que estes isolados, uma vez que os polímeros menores ($n<10$) iniciam a desintegração da parede, permitindo o acesso pelos polímeros maiores ($n>10$) à célula (Gilbert et al, 1990b).

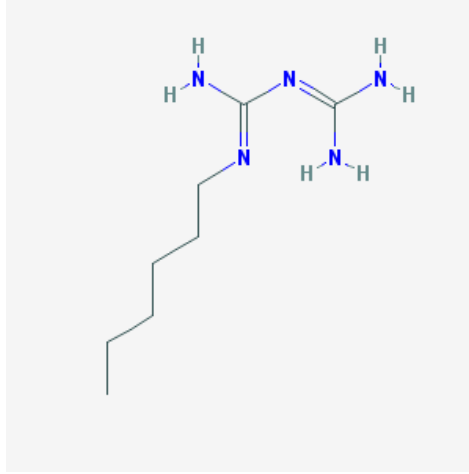

Figura 4: Estrutura química do PHMB. Fonte:https://pubchem.ncbi.nlm.nih.gov/compound/20977#section=Top.

Além deste, há outro mecanismo de ação bactericida apresentado pelo PHMB, cujo caráter catiônico também induz interações eletrostáticas com moléculas de ácidos nucléicos, como foi primeiramente demonstrado *in vitro* por Allen (2004), no qual seus resultados também mostraram que a ligação do PHMB ao DNA ocorre de maneira cooperativa, ou seja, a ligação inicial de uma molécula de PHMB ao DNA promove ligações mais fortes de outras moléculas de PHMB. Essa ligação pode ocorrer com moléculas de fita simples e dupla de DNA, de diferentes comprimentos e também com moléculas de tRNA, com este último mais notavelmente em altas concentrações de PHMB (0,2 mM) (Allen et al, 2004).

A partir disso, o modelo estabelecido do mecanismo de atividade antimicrobiana do PHMB, baseado apenas no efeito de ruptura da membrana, começou a ser reestruturado, uma vez que sob concentrações inibitórias de crescimento células de *Escherichia coli* não lisaram. E então, foi verificado que nessas condições o PHMB foi capaz de entrar em células vivas e fixadas, e dentro das quais levou à condensação cromossômica em células de bactérias Gram-positivas e negativas (Chindera et al, 2016). Ao

contrário do que ocorre em bactérias, em células de mamífero (macrófagos e queratinócitos), os polímeros de PHMB não levam à ruptura da membrana plasmática, que possuem fosfolipídeos neutros e estes são mais fracamente afetados, no entanto eles entram nestas células e são aprisionados em endossomos, o que restringe a entrada no núcleo e assim não se ligam aos cromossomos (Chindera et al, 2016).

A eficácia do PHMB como agente biocida também foi demonstrada para protozoários do gênero *Leishmania*. Para o qual, o PHMB apresentou efeito citotóxico contra ambas as formas promastigotas (circulante) e amastigota (intracelular) do parasita, esta última localizada dentro de células de macrófagos. Ao contrário do observado nos macrófagos, onde não houve entrada dos polímeros no núcleo, nos parasitas houve entrada no núcleo onde os polímeros tiveram acesso ao cromossomo (Firdessa et al, 2015).

Diferentes células de leveduras e até mesmo linhagens da mesma espécie apresentam variabilidade quanto ao fenótipo de sensibilidade ao PHMB. Foi mostrado que algumas espécies de leveduras encontradas no ambiente industrial e que são tidas como contaminantes do processo, como *Dekkera bruxellensis*, são sensíveis ao PHMB, enquanto e a principal fermentadora *Saccharomyces cerevisiae* se mostrou tolerante, quando testada na mesma concentração de PHMB. Essa seletividade em eliminar células, tanto de bactérias quanto de leveduras, que não fazem parte do processo de fermentação industrial deu ao PHMB potencial aplicação na etapa de reaproveitamento do levedo. No entanto, apenas algumas linhagens industriais de *S. cerevisiae* apresentam esse fenótipo de tolerância (Elsztein et al, 2008).

2.6 GENE *NCW2*

O primeiro trabalho reportado sobre o gene NCW2, até então ORF putativa YLR194C, foi de Terashima et al (2000). No qual foi identificado que esta ORF se encontrava superexpresso no mutante com deleção no gene FKS1, que codifica uma subunidade catalítica sintase 1,3-beta-glucano. Nesse mesmo trabalho foi demonstrado que havia uma proteína codificada por esta ORF e esta se ligava a uma âncora GPI, o que estava de acordo com a análise in silico que mostrava a presença de um sítio de clivagem e ligação à âncora GPI, e consequentemente sua função deveria estar associada à parede celular (Terashima et al, 2000).

Este gene também se apresentou superexpresso em linhagens industriais de S. cerevisiae mais tolerantes ao PHMB. Neste caso, a responsividade do gene ocorria sob a ação de um agente estressor de parede celular, visto que maior parte dos genes responsivos, sendo a deleção de alguns letais, nesta condição correspondiam a genes CWI (Elsztein et al, 2011).

Além de verificar que o gene YLR194C também se mostra sobre/super expresso sob tratamento de Zymolyase™, uma enzima que hidrolisa ligação de 1,3-beta-glucano, Elsztein et al (2016) demonstrou que o gene, agora denominado NCW2 (New Cell Wall protein 2), não apenas está mais expresso na presença de PHMB, mas também quando deletado a célula fica mais sensível a este agente. Este trabalho mostrou também que esta deleção leva a um ligeiro aumento na deposição de quitina e aumento na resistência de lise por Zymolyase™, e estas foram as primeiras descrições de fenótipos associados à deleção de NCW2.

3 ARTIGO I

The effects of the Ncw2 protein of Saccharomyces cerevisiae on the positioning of chitin in response to cell wall damage

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3.1 ABSTRACT

The recently described NCW2 gene encodes a protein that is assumed to be located in the cell wall (CW). This protein was proposed to participate in the repair of CW damages induced by polyhexamethylene biguanide (PHMB). However, much of the information on the biological function(s) of Ncw2p still remains unclear. In view of this, this study seeks to extend the analysis of this gene in light of the way its protein functions in the Cell Wall Integrity (CWI) mechanism. Deletion of the NCW2 gene led to constitutive overexpression of some key CWI genes and increased chitin deposition in the walls of cells exposed to PHMB. This means the lack of Ncw2p might activate a compensatory mechanism that upregulates glucan CWI genes for cell protection by stiffening the CW. This condition seems to alleviate the response through the HOG pathway and makes cells sensitive to osmotic stress. However, Ncw2p may not have been directly involved in tolerance to osmotic stress itself. The results obtained definitely place the NCW2 gene in the list of CWI genes of S. cerevisiae and indicate that its protein has an auxiliary function in the maintenance of the glucan/chitin balance and ensuring the correct structure of the yeast cell wall.

3.2 INTRODUCTION

The Cell wall (CW) is a complex membrane compartment of the yeast cells that first makes contact with the external environment, by sensing the conditions of nutrient availability and establishing its physical – chemical composition. Hence, this structure determines the shape, maintains the cell integrity and is the first barrier to many stress agents (Cabib et al. 1988; Gow et al. 2017), since it is able to signalize damage in the cell surface/envelope

under several conditions (Lucena et al. 2012; Heilmann et al. 2013). This sensing mechanism transduces the signal through regulatory cascades that ultimately prepare the cells to adapt to specific conditions. In view of its importance, this cellular component retains its functional role by mechanisms that ensure its correct protein biogenesis, and undertake repairs and re-modelling. One of these mechanisms corresponds to the Cell Wall Integrity (CWI) pathway responsible for the structural and functional maintenance of CW, especially in the presence of CW-damaging agents.

In Saccharomyces cerevisiae, this pathway is activated by mechanosensing proteins such as Wsc1p/Slg1p and Mid2p that sense the damage in the CW and transduce the signal via the transducer protein Rho1p (Kock et al. 2015). It culminates in the activation of protein kinase 1 (PKC) that triggers a phosphorylation cascade through MAP kinases (Philip and Levin 2001; Levin 2011). The last MAP kinase in this cascade is the protein Slt2p that, once phosphorylated, is activated by phosphorylation transcription factors such as Rlm1p and Swi4/6p that regulate the expression of CWI genes. This results in a series of cell responses that initiate repair of cell walls and/or leads to remodelling (Popolo et al. 2001; Levin 2005; Valiante et al. 2015; Gow et al. 2017).

Dozens of genes belong to the CWI regulon which have proteins that act on different levels and have different functions. We identified the gene called *NCW2* (Novel Cell Wall 2) from the S. cerevisiae open reading frame (ORF) YLR194c and suggested its protein should play a part in the construction and remodelling of CW as a result of the stress induced by polyhexamethylene biguanide (PHMB) (Elsztein et al. 2016). PHMB is composed of a mixture of biguanide polymers of variable length, that are used in several clinical applications owing to their broad-spectrum antimicrobial activity (Broxton et al. 1984; Kaehn 2010), which includes killing the yeast

species contaminants during the ethanol fuel fermentation process (Elsztein et al. 2008). Treatment with this compound has revealed some interesting factors about the CWI pathway and cell wall remodelling mechanism, such as the involvement of the Yap1p transcription factor, which is mainly known for regulating oxidative stress response genes, when regulating CWI genes in response to PHMB (Elsztein et al. 2011).

The first report about the function of the putative protein encoded by the ORF YLR194c (NCW2) indicated that its protein was localized in the CW using HA-tag fusion and suggested it should be attached to a GPI anchor using CW-enriched protein extract (Terashima et al. 2000). Furthermore, an *in silico* analysis revealed the presence of putative cleavage sites and the attachment of a GPI anchor at the C-terminal and a signal peptide at the N-terminal (Terashima et al. 2000). Later on, we reported an increase in the readouts of *NCW2* when the yeast cells are exposed to the PHMB, together with several genes of the CWI regulon (Elsztein et al. 2011).

In addition, this kind of PHMB-dependent overexpression is present in the PHMB-resistant industrial JP1 strain, while it is not found in PHMB-sensitive strains such as the industrial PE-2 strain and the mutant yeast cells lacking Yap1p (Elsztein et al. 2011). It was shown that PHMB causes damage to the CW compartment and these results meant that *NCW2* could be included in the set of genes responsive to damage in the yeast CW, although no evidence has yet been provided for the function of its putative protein. Terashima et al. (2000) showed it had a higher constitutive production of the fks1Δ mutant than its parental cells. That mutant lacks one of the catalytic subunits of the glucan synthase complex involved in the CW remodelling.

Besides, the *NCW2* gene is induced in conditions in which the CW is affected, such as in cells treated with caspofungin (Reinoso-Martín et al. 2003) and caffeine (Kuranda et al. 2006) and experiencing high hydrostatic

stress (Iwahashi et al. 2005). It was also reported that *NCW2* is overexpressed when yeast cells are exposed to an acidic environment (pH 2.5) by sulphuric acid (Lucena et al. 2012). This responsiveness is eliminated in strains carrying deletions within the *SLT2* and *HOG1* genes, where the proteins regulate the PKC/CWI pathway and the osmoregulatory stress response, respectively (Lucena et al. 2012). Since medium acidification also causes damage to the CW component (Lucena et al. 2012), these results, together with those obtained from the PHMB-damaged cells, showed that *NCW2* is also a part of the responsive circuit of the CWI pathway. As well as being anchored to GPI, it seems that Ncw2p is O-mannosylated in two sites, which is a characteristic of extracellular and cell -surface attached proteins (Neubert and Strahl 2016).

Recently we embarked on a study to characterize the *NCW2* gene and to define the biological function of its protein in the context of the CWI mechanism. At that time, no clear phenotype had been assigned to the ncw2Δ deletion mutant when it was shown its moderate sensitivity to PHMB and resistance to lysis by zymolyase; this was attributed to the slight increase in the chitin content of the CW of mutant cells (Elsztein et al. 2016). Moreover, *NCW2* expression responded to CW-damaging agents, but not directly to oxidative or osmotic stress. Thus, these results placed this gene within the CWI regulon (Elsztein et al. 2016). However, much of the information on the biological function(s) of Ncw2p remains unclear. In view of this, the aim of this study has been to extend the analysis of this gene to include the role of its protein in the CWI mechanism. In this way, the study of Ncw2p could provide new insights into mechanisms that have not yet been revealed in the CW construction, repair or remodelling process, as well as the existence of new mechanisms.

3.3 MATERIALS AND METHODS

3.3.1 Strains and growth conditions

The strains used in the present study are listed in Table 1. Yeast cells were grown in YPD (10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone and 20 $g.L^{-1}$ dextrose) with agitation at 30°C. Solid media contained 15 g L^{-1} agar. Synthetic defined (SD) medium contained Yeast Nitrogen Base (YNB) at 1.7 $g.L^{-1}$ supplemented with glucose (20 $g.L^{-1}$) and ammonium sulphate (5 $g.L^{-1}$) and the appropriate amino acid drop-out (CSM) solution. Pre-cultures were prepared by inoculating yeast cells from fresh YPD plates in YPD broth and cultivated overnight at 30°C and 150 rpm. These cells were used to prepare seed cultures by re-inoculating YPD broth with pre-cultures to 0.1 units of absorbance at 600 nm (0.1 A600nm) and incubating (as above) until they reached 0.5 A600nm. These seed cells were used for all the experiments which are described as follows.

3.3.2 Cell lysis assay

Seed cells of the parental BY4741 strain and its isogenic mutants (ncw2Δ and yap1Δ) were collected by centrifugation and suspended to the same cell density in YPD (control) or in YPD containing PHMB at 0.0005% (5 $mg.L^{-1}$). Following incubation for 4 h at 30°C with agitation, the cells were collected by centrifugation, washed three times with 0.85% saline solution and concentrated to 1.0 A600nm in CE buffer (0.1 M sodium citrate and 10 mM EDTA, pH 6.5) with 2.25 $U.mL^{-1}$ lyticase (Sigma-Aldrich Co.). The cell suspensions were incubated at 30 °C with agitation and the cell density was

measured at 1-h intervals. Relative absorbance was calculated in relation to zero time and plotted against time.

3.3.3 Chitin staining

Seed cells were treated with PHMB, washed with sterile saline (0.85%) and stained with Calcofluor White/KOH on a glass slide. The cells were analysed in a fluorescence microscope (Leica a DFC 340FX), using a DAPI filter. The images were processed with the aid of Leica CW 4000 software (Elsztein et al. 2016).

3.3.4 Viability and adaptation to different stresses

The seed cells were diluted to 0.1 A600nm either in YPD (non pre-exposed cells) or in YPD containing 0.005% (50 $mg.L^{-1}$) PHMB (pre-exposed cells) and incubated for 30 min at 30°C. Afterwards, the cells were recovered by centrifugation, washed with YPD broth and suspended to the same cell density in YPD or in YPD containing one of the stress agents: ethanol, KCl or calcofluor white (CW). The cultivations were performed on microtitre plates in Synergy HT Multidetection reader (BioTek, Luzern, Switzerland) for 24 h with continuous and fast shaking at 30°C, and automatic absorbance readings. The growth curves were obtained from the average of biological and technical triplicates. In making an assessment of cell viability, samples were taken at the end of the cultivations, diluted to 10^6 cells mL^{-1} and 3 μL of dilutions were spotted on YPD agar plates. The plates were incubated at 30°C for 24 h.

3.3.5 NCW2-GFP protein fluorescence detection

The yeast strain ATCC 20,138 containing integrated genome Ncw2p-GFP fusion cassette (Huh et al. 2003) was purchased from ThermoFisher Scientifics (USA). Seed cells were prepared as described above and suspended in YPD or YPD containing PHMB at 20 mg.L^{-1} and incubated for 4 h at 30C with gentle agitation. Afterwards, the cells were collected by centrifugation, washed with 0.85% saline solution and suspended in this solution. Drops of 5 μL of cell suspension on glass slides were mixed with the same volume of DAPI (40,6-diamidino-2-phenylindole) solution, covered with a coverslip and analyzed in a fluorescence microscope (Leica DFC 340FX) using a DAPI filter and FITC filter for GFP fluorescence in accordance with the manufacturer's instruction. Images were taken and analyzed using software Leica CW 4000.

Table 1: Yeast strains used in this work.

Strain	Genotype	Reference
BY4741	*MATa his3Δ leu2Δ met15Δ ura3Δ*	Elsztein et al. (2011)
ncw2Δ	BY4741 *NCW2*::KanMX	Elsztein et al. (2016)
yap1Δ	BY4741 *YAP1*::KANMX	Elsztein et al. (2011)
GFP-Ncw2	BY4741 *NCW2*-GFP-*HIS3MX6*	Huh et al. (2003)

3.3.6 Gene expression analysis

Seed cells were collected by centrifugation and suspended from 0.5 units of A600nm in YPD or YPD containing 0.002% (20 mg. L^{-1}) PHMB. Following one-hour of incubation at 30 °C and constant agitation, the cells

were recovered for total RNA extraction using the GE Healthcare Illustra RNA spin Mini Kit, following the manufacturer's protocol. RNA integrity was checked in 1% agarose gel electrophoresis and quantified in Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). Primers design, cDNA synthesis and the Quantitative Real-Time PCR were performed according to a previously described methodology and its parameters (Elsztein et al. 2011, 2016; Lucena et al. 2012). The following genes involved in the CWI mechanism were analyzed: *CHS1*, *FKS1*, *GAS1*, *KRE6* and *MNN9*.

3.4 RESULTS AND DISCUSSION

3.4.1 Ncw2p is effective for the cell response to damage caused by PHMB

Our recent report showed there was increased resistance of yeast cells that lack the NCW2 gene, to lysis by zymolyase (Elsztein et al. 2016), a commercial enzyme preparation that mainly contains 1,3-β-glucanase activity responsible for the cleavage of the main component of the yeast cell wall (CW) structure, the 1,3-β-glucan polymer. This layer acts as a scaffold attachment region for the other CW constituents. In addition, this mutant strain also had a slight increase in chitin deposition compared with its parental strain BY4741 (Elsztein et al. 2016).

In this study, we extended this analysis by pre-treating ncw2Δ mutant cells and their parental strain with PHMB before submitting them to cell lysis with lyticase, a commercial enzyme with the same glucanase activity as zymolyase. Initially, the results showed that the lysis profile of both the parental and mutant strain with lyticase (Figure 1) was similar to what was observed with zymolyase (Elsztein et al. 2016). This confirmed the equivalence of both enzymes and showed the resistance profile of the mutant.

Hence, we were able to corroborate the phenotypic resistance of this mutant. Pre-treatment with PHMB made the yeast cells more resistant to cell lysis than to untreated cells (Figure 1). This shows that PHMB induced structural changes in the CW structure, as previously predicted (Elsztein et al. 2011), and thus makes it more resistant to glucanase activities.

Additionally, the absence of Ncw2p makes the yeast cells even more resistant to this injury (Figure 1). It must be the result of a compensatory mechanism displayed by mutants that affects the glucan network biosynthesis, as discussed below. The *NCW2* gene is upregulated by exposure to PHMB and this expression is somehow regulated by Yap1p (Elsztein et al. 2011, 2016).

Moreover, the Yap1p function was also extended to the control of the CWI mechanism (Elsztein et al. 2011) as well as its well-known function in oxidative stress response. In view of this genetic interaction, we also tested the yap1Δ mutant for lysis by lyticase. However, no effect was observed for this mutant that was different from the parental strain (Figure 1), regardless of its preexposure to PHMB. This led us to raise the hypothesis that the lysis resistance profile was the result of the increased chitin deposition in the ncw2Δ mutant, even in the absence of CW damage, when compared with the parental strain (Elsztein et al. 2016).

This hypothesis was tested by staining PHMB pre-exposed cells with calcofluor white. The parental yeast cells showed the same chitin deposition in the CW, regardless of the presence of PHMB (Figure 2a,b). A similar result was observed for the yap1Δ mutant, regardless of the pre-exposure of PHMB (data not shown). On the other hand, a more intense deposition of chitin was visualized on the surface of the ncw2D cells, especially in the budding sites (Figure 2c).

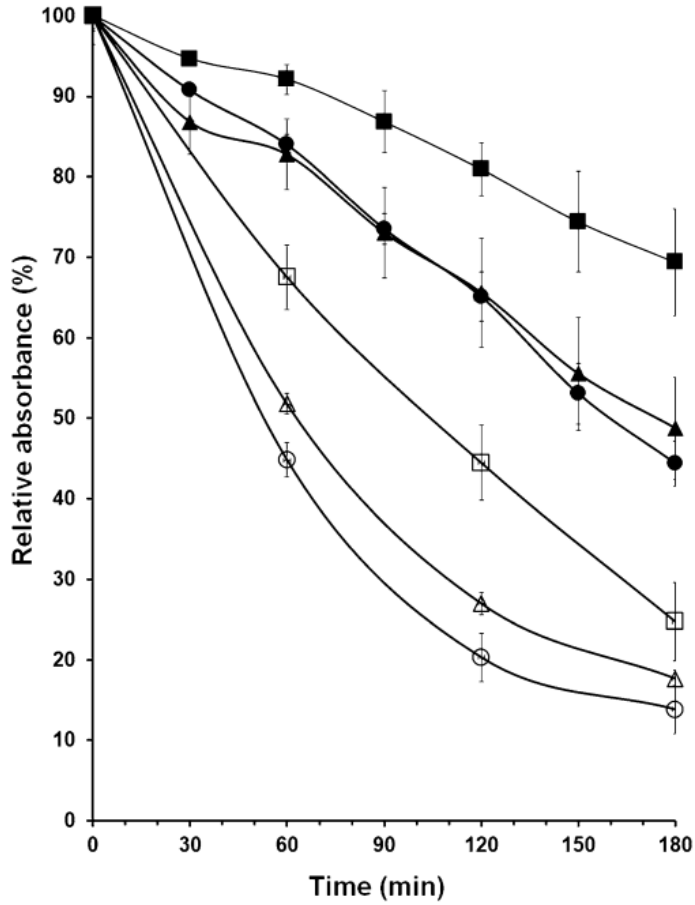

Figure 1: Kinetics of cells lysis by lyticase of Saccharomyces cerevisiae parental strain BY4741 (circles) and its isogenic mutants with deletion in the genes NCW2 (squares) and YAP1 (triangles).The yeast cells were previously incubated in YPD medium (open symbols) or in YPD containing 0.0005% PHMB (closed symbols).

The novelty here is that there is a much more intense chitin deposition on the mutant CW upon pre-exposure of PHMB (Figure 2d). As a result, this combination of an absence of Ncw2p and CW damage caused by PHMB amplified a signal for chitin biosynthesis and, very likely, its spread to other sites in the CW. High chitin deposition is a typical response to genetic modifications that disturb the CW structure, such as mutations in the *FKS1* and *CHS1* genes (Kapteyn et al. 1997; Ram et al. 1998; Lesage et al. 2005).

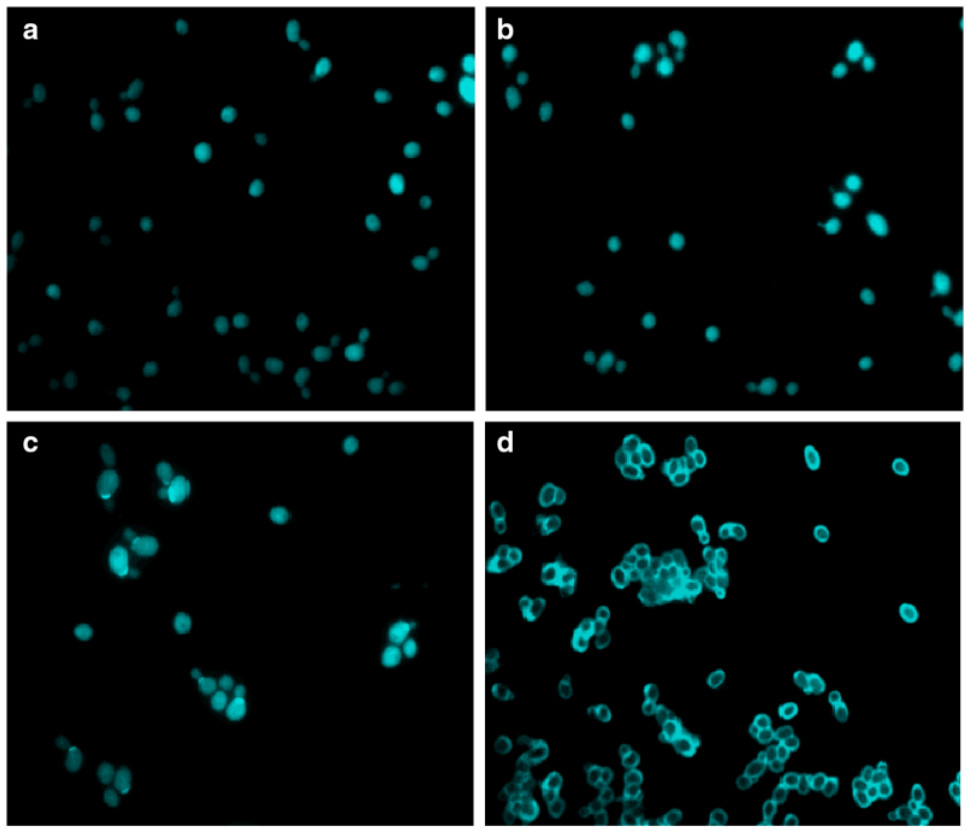

Figure 2: Staining of Saccharomyces cerevisiae cells with calcofluor white for analysis of chitin content in the cells wall.The parental strain BY4741 (a, b) were compared with their isogenic mutants with deletion in the NCW2 genes (c, d). The yeast cells were previously incubated in YPD medium (a, c) or in YPD containing 0.0005% PHMB (b, d).

Hence, as this profile is shared with mutation in the *NCW2* gene, it can be argued that at least these three proteins (Fks1, Chs1 and Ncw2) are involved in the correct balance and distribution of the glucan/chitin structure for the CW. Treatment of yeast cells with zymolyase lead to over-induced localization of Crh1p in the cell envelope (Bermejo et al. 2008), a transglycosylase encoded by the CRH1 gene that catalyses the crosslinkages between chitin and the β-glucan network and correctly positions the chitin layer in the CW scaffold (Cabib 2009). In addition, the fks1Δ and gas1Δ mutants display reduced levels of glucan on the cell wall

and increased chitin deposition as an alternative to the crosslinks between the chitin and glucan in order to strengthen the CW (Kapteyn et al. 1997; Ram et al. 1998; Lesage et al. 2005).

However, gas1Δ cells are highly sensitive to zymolyase while fks1Δ has the same phenotype as the parental strain (García et al. 2015). Moreover, the uth1Δ mutant has a lower chitin deposition, increased glucan content and shows resistance to zymolyase treatment (Ritch et al. 2010). Uth1p is involved in biological processes related to mitophagy, oxidative stress response and cell longevity (Kennedy et al. 1995; Austriaco 1996), as well as CW biogenesis (Ritch et al. 2010). On the other hand, it is disrupted in cells harbouring the *SMI1/KNR4* gene, which encodes an intrinsically disordered protein (Martin-Yken et al. 2016) that regulates the PKC/ CWI pathway through its interaction with Slt2p (Martin-Yken et al. 2003). It also has an increased chitin deposition (Lesage et al. 2005), decreased 1,3-β-glucan synthesis and shows resistance to zymolyase (Hong et al. 1994). This means that while some CWI mutants that display high chitin content in the wall also show resistance to zymolyase, other mutants with high chitin content display sensitivity to zymolyase.

The current results show that the ncw2Δ mutant falls in the first group. As well as this, they suggest that chitin content is not solely responsible for the resistance to zymolyase. This deposition is intensified through treatment with PHMB (Figure 2d) which heightens its resistance to glucanases (Figure 1). Hence, all these results with the different CWI mutants indicate that the phenotypes linked to lysis by glucanases are more closely related to the distribution of chitin and how the cross-links between β-glucans and chitin chains are established rather than the number of its components per se.

3.4.2 The lack of Ncw2p triggers a CWI response even in the absence of CW damage

Furthermore, we tested the production of Ncw2p after treatment with PHMB by tagging this protein with GFP protein, and creating the GFP-Ncw2 fusion protein. In this case, no labelling was observed in the untreated cells (Figure 3b). However, it is unacceptable that no Ncw2p is produced at all in the absence of PHMB, but rather, its physiological content should be below the level of detection by this method. The low abundance of Ncw2p in CW-undamaged yeast cells was reported by means of a high-throughput analysis (Ho et al. 2018). We made use of the available public databases, which were combined so that a comparison could be made of protein abundance, and to calculate the abundance of Ncw2p relative to four GPI-anchored proteins (Ho et al. 2018). The results of mid-log phase cells cultivated in YPD using western blot and quantitative mass spectrometry indicated that Ncw2p was less abundant than Gas1p (-18 9 and -94x, respectively), Dcw1p (-4x and -15x), Yps1p (-8x and -13x) and Dfg5p (-4x and -10x). In contrast, green fluorescent labelling was clearly observed when the cells were treated with PHMB (Figure 3d), which suggests not only that the *NCW2* gene is overexpressed in the presence of this agent (Elsztein et al. 2011, 2016) but also that Ncw2p is over-produced in response to this CW stress agent.

In addition, the relationship between *NCW2* and other CWI regulon genes was tested by measuring the expression of five of these representative genes in the absence of Ncw2p upon treatment with PHMB (Figure 4).

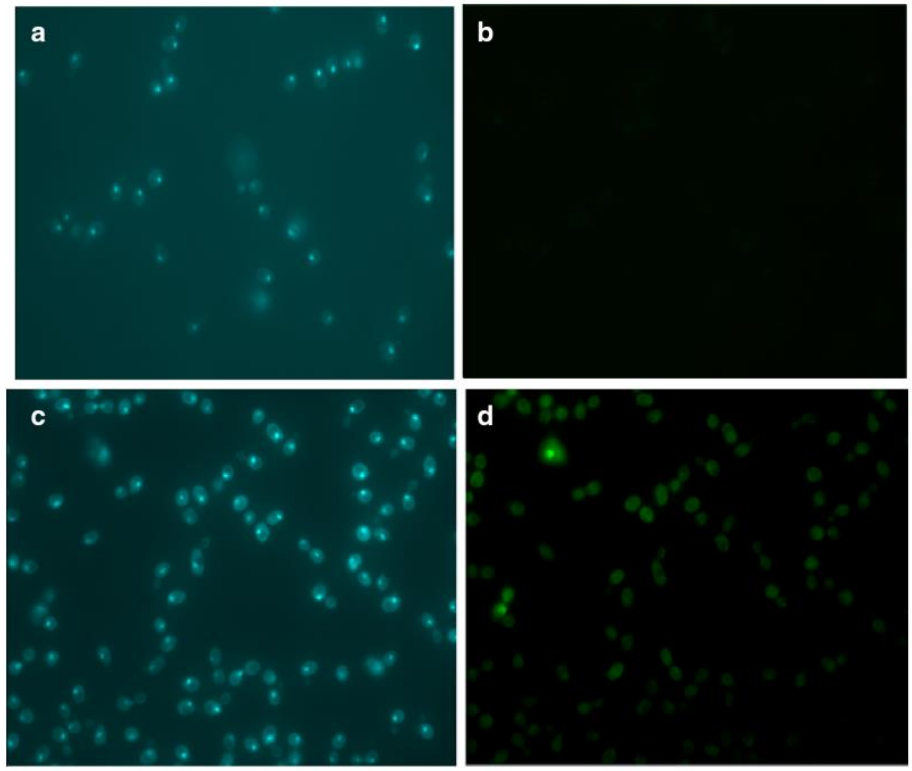

Figure 3: Fluorescence microscopy analysis of Saccharomyces cerevisiae harbouring Ncw2p-GFP fusion protein.The cells were incubated in YPD (a, b) or YPD containing 0.002% PHMB (c, d) and visualized with DAPI filter for nucleus identification (a, c) and with an FITC filter for GFP fluorescent labelling (b, d).

These genes were chosen because of their responsive profile to PHMB and/or to sulphuric acid, and are regulated by Slt2p, Hog1p or Yap1p (Elsztein et al. 2011; Lucena et al. 2012). Three (*FKS1*, *KRE6* and *MNN9*) out of five genes analysed were upregulated in the parental strain after PHMB treatment (Figure 4, white columns), as was previously reported (Elsztein et al. 2011). This responsiveness to PHMB was maintained for the *MNN9* gene in the ncw2Δ mutant, but not for *KRE6* and *FKS1* genes (Figure 4, grey columns). This can be explained by the fact that these two genes were already upregulated in ncw2Δ mutant cells relative to their parental gene even in the absence of PHMB-treatment (Figure 4, black columns). In this case, we assumed that at least a part of the CWI pathway has already been activated

in ncw2Δ cells as a compensatory mechanism for the absence of Ncw2p, and no further increment of *KRE6* and *FKS1* was trigged by PHMB.

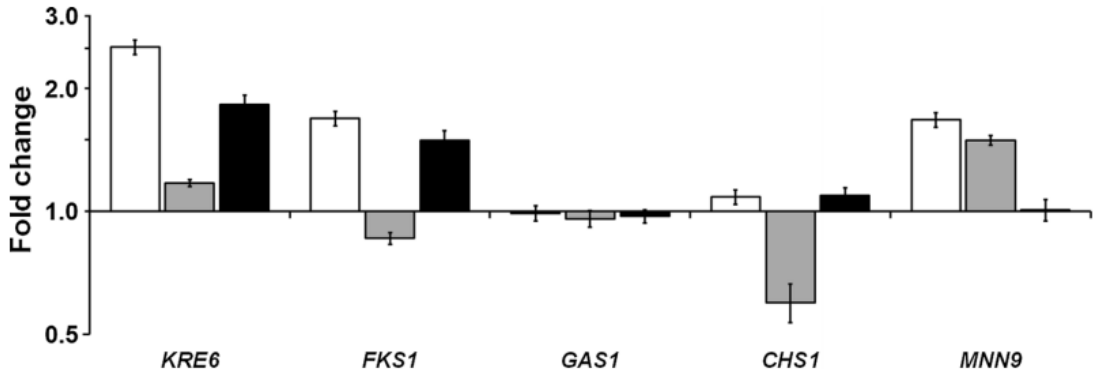

Figure 4: Relative gene expression of five Saccharomyces cerevisiae genes involved in the CWI pathway in the parental strain BY4741 (white columns) and in the ncw2Δ mutant (grey columns) in response to treatment with 0.002% PHMB for untreated cells. Expression of these genes in untreated ncw2D mutant over untreated parental strain (black columns) was shown. The results represent the average of three biological replicates (±SD).

The *KRE6* gene encodes a Type II integral membrane protein required for 1,6-β-Δ-glucan biosynthesis (Roemer and Bussey 1991), while the *FKS1* gene encodes a catalytic subunit of the 1,3-β-D-glucan synthase complex in which the deletion results in a three times greater chitin deposition relative to the parental cells (Lesage et al. 2005). In contrast, the *GAS1* gene that encodes a 1,3-β-D-glucanosyltransferase did not respond either to the PHMB treatment or to *NCW2* deletion (Figure 4). Hence, it was assumed that the overexpression of *FKS1* in the ncw2Δ mutant, relative to parental strain, somehow prevented the induction of the *GAS1* gene. This could be attributed to the fact that Fks1p and Gas1p overlap in their biological activities (Lesage et al. 2004). This is one more piece of evidence that points to a compensatory response mechanism (triggered by the absence of Ncw2p) that keeps the CWI mechanism under constant alert, i.e., glucan synthases (Fks1p) and

transglycosylase (Kre6p) activities, leading to the resistance of the ncw2Δ mutant to cell lysis (Figure1; Elsztein et al. 2016).

Furthermore, the *CHS1* gene was not responsive to PHMB in the parental cells, nor did it respond to the absence of Ncw2p (Figure 4). However, this gene was downregulated almost twofold when ncw2Δ cells were treated with PHMB relative to the untreated mutant cells (Figure 4). This gene encodes the chitin synthase I involved in bud separation during cytokinesis, along with the cell growth, although it is not essential for the maintenance of a normal level of chitin in CW (Bulawa et al. 1986; Cabib et al. 1992; Ziman et al. 1996).

Although Chs1p has been annotated as a CW-repair chitin synthase, deletion of its gene does not cause significant changes in the amount of chitin in the CW (Lesage et al. 2005). The absence of Ncw2p produced a slight increment in the amount of chitin on the CW (Elsztein et al. 2016). This increment is even higher when the mutant cells are treated with PHMB (Figure 2d). This was achieved despite the reduction in the *CHS1* expression (Figure 4). In view of this, we believe that in the combination of reduced *CHS1* expression and absence of Ncw2p under PHMB treatment, there is a need to intervene in the correct positioning of chitin from bud sites so that it can spread to lateral CW, by cell-induced stiffening of the surface with reduction of cell growth (Elsztein et al. 2016) and increasing resistance to lysis (Figure 1).

Lastly, we confirmed that MNN9 gene was upregulated by PHMB, regardless of the presence of Ncw2p (Figure 4). This gene encodes a subunit of the mannosyltransferase complex located in the Golgi compartment that mediates the elongation of the polysaccharide mannan backbone which is more common under CW stress. This protein can function by enabling the transport of the CWI protein to repair the damage in the cell envelope. Thus,

the damage caused by the PHMB leads to an increase in the trafficking of CWI proteins through the Golgi complex to repair the cell wall.

3.4.3 The absence of Ncw2p retards the repair of CW

To test whether an adaptive mechanism is also triggered by PHMB, yeast cells were pre-exposed to this agent and then further cultivated in the presence of different stress agents such as ethanol (CW and membrane stress), KCl (osmotic and membrane stress) and calcofluor white (CW stress) (Figure 5).

Pre-exposure with PHMB did not significantly affect the further growth of the parental strain in fresh YPD media (Figure 5a; Table 2). This suggested that the damage caused by PHMB on CW during the pre-display period were rapidly repaired when the cells were re-inoculated into fresh YPD medium. Regarding the ncw2Δ mutant, pre-exposure with PHMB reduced the maximal growth rate in fresh YPD by 42%, relative to the parental strain (Figure 5a; Table 2). Hence, the damage caused by PHMB to the CW was not completely repaired when Ncw2p was not present in the cell envelope.

Ethanol added at 10% (v/v) in the YPD medium, reduced the maximal growth rate of the parental strain by 53% (Figure 5b; Table 2). It should be noted that in this case, ethanol acted as a stress agent rather than a carbon source owing to the presence of glucose in YPD, which triggers the mechanism of Glucose Catabolite Repression (GCR) that impairs ethanol metabolization. In a previous study, we showed that treatment with ethanol at 9% (v/v) increased cell surface roughness while reducing the CW rigidity, and that this led to lower stiffness of the yeast cells without changing the content of b-glucan and chitin (Schiavone et al. 2016). This shows that ethanol influences the nanomechanical properties of the yeast CW, rather than its

chemical composition, which might result from the reduced thickness of the cell membrane caused by this agent (Schiavoni et al. 2016).

In addition, we detected overexpression of the CWI genes (*FKS1*, *GAS1*, *MNN9* and *NCW2*) caused by exposure to ethanol (Schiavoni et al. 2016). In this study, ethanol reduced the maximal growth rate of the ncw2Δ mutant even more (by 69%) relative to untreated cells (Figure 5b; Table 2). The final viability of the cell population at 10% ethanol was similar in both strains (data not shown) and shows that ethanol at 10% retarded the cell progression of the mutant strain. However, the cell viability of the ncw2Δ mutant was seriously affected at 12% ethanol, which represents a cytotoxic effect that is only detected in the parental strain at 14% ethanol (data not shown).

This led to a novel phenotype of this mutant since it was sensitive to high concentrations of ethanol and meant updating our previous result which showed its insensitivity at 8% ethanol (Elsztein et al. 2016). Lower cell stiffness is also the phenotype displayed by double crh1Δcrh2Δ mutant cells that are treated with ethanol (Dague et al. 2010), and is a means of linking this feature to the correct positioning of the chitin layer in the glucan network (Cabib 2009). In light of the effect of ethanol on the nanomechanical properties of the CW discussed above, it can be assumed that the absence of Ncw2p is a cause of perturbations in these properties, and makes the mutant cells more susceptible than the parental ones.

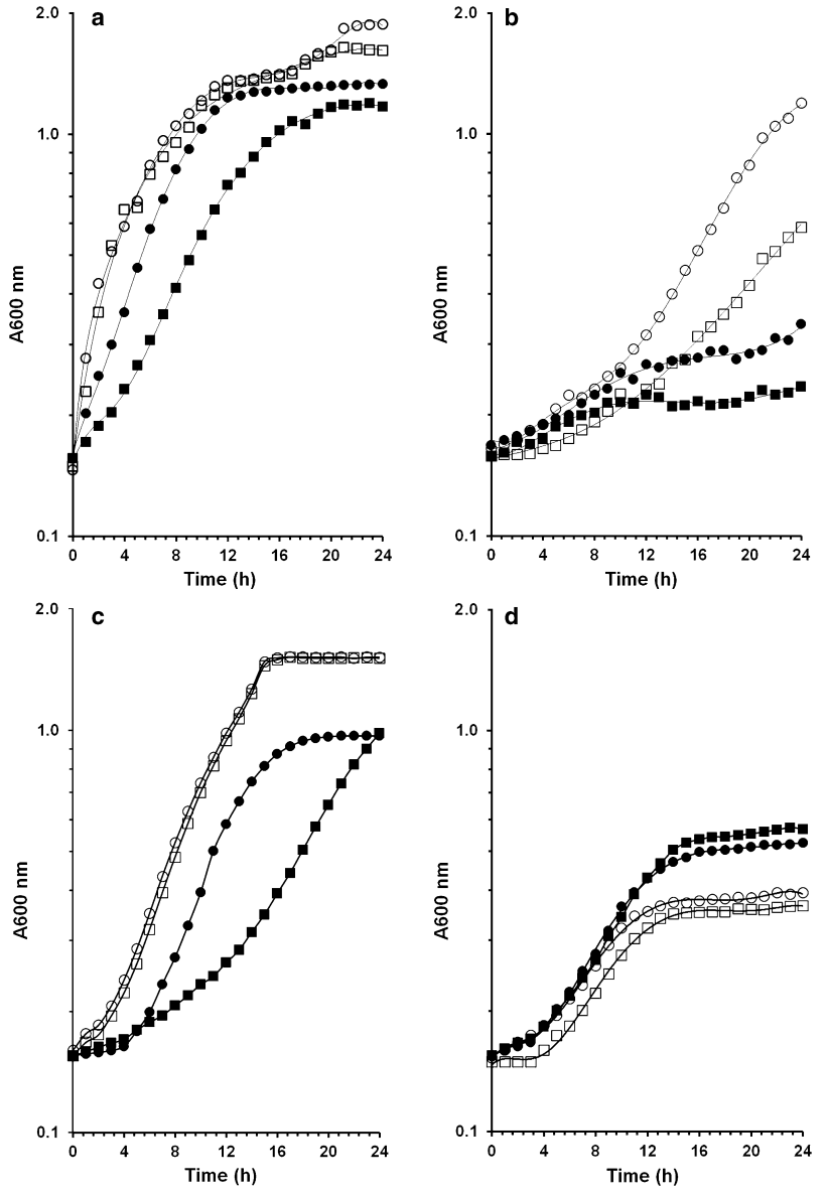

Figure 5: Growth kinetics of Saccharomyces cerevisiae parental strain BY4741 (circles) and ncw2Δ mutant (squares).The yeast cells were cultivated in YPD to mid-log phase, collected and resuspended in YPD (open symbols) or in YPD containing 0.005% PHMB (closed symbols) and incubated for one hour at 30°C. The cells were collected, washed in saline and transferred to YPD medium (a) or YPD in the presence of 10% ethanol (b), 1.2 mmol/L KCl (c) or 15 lg mL^{-1} calcofluor white (d).

Pre-exposure to PHMB sensitized the yeast cells to such an extent that they hardly grew when ethanol was present. This made the damage caused by this agent more toxic to the yeast cells, regardless of the presence of Ncw2p (Figure 5b; Table 2). However, these effects were more powerful on the ncw2Δ mutant. These results indicated that the presence of ethanol reduces the efficiency of repairs to the damage caused by PHMB in a way that seems to be more closely related to changes in the CW biophysics than in the CW composition.

Treatment with 1.2 mol.L^{-1} KCl imposed osmotic stress and affected the parental and ncw2Δ strains in a similar way (Figure 5c) relative to untreated cells (Figure 5a), and reduced their maximal growth rates by 27% (Table 2). Increasing the concentration of this agent caused a proportional reduction in cell growth of both strains until the complete absence of growth at 1.6 mol.L^{-1} (data not shown). Therefore, we subsequently considered the concentration of 1.2 mmol L^{-1} as sub-MIC that causes osmotic stress without impeding cell growth. These results indicated that Ncw2p is not directly involved in the osmotic stress response to this agent. Pre-exposure with PHMB extended by four times the period of the lag phase of the cell population of both strains in the presence of KCl at sub-MIC dosage (Figure 5c), which suggested that the repair of the PHMB damage would also be impaired when the yeast cells underwent osmotic stress. After this adaptation period in which the parental cells adapted to the presence of stressor (KCI), they started to achieve the same growth rate as that calculated for cells not pre-exposed to PHMB (Figure 5c; Table 2).

However, the cell cultures anticipated the entry into the stationary phase (Figure 5c). According to our previous data, the concentration of PHMB used in this experiment was very cytotoxic to the hog1Δ mutant (Elsztein et al. 2011) and together these data forge a connection between the HOG

pathway and the CW repair mechanism, which might be triggered during the adaptive lag phase. This growth recovery was seriously affected by the deletion of the *NCW2* gene, and resulted in a delayed entry into the exponential phase, with a concomitant reduction in the maximal growth rate of 37% relative to the parental strain (Figure 5c; Table 2). However, the same final biomass was achieved by both strains as the cultivations last longer (Figure 5c).

In view of this, the adaptation mechanism discussed above for the parental cells might be less efficient when Ncw2p is absent. Finally, yeast cells were also cultivated in the presence of calcofluor white (CFW) (Figure 5d), which binds to chitin and impairs the appropriate assembly of chitin polymers on the glucan layer (Elorza et al. 1983; Roncero and Durán 1985; Munro 2013).

Table 2: Maximal growth rates (h-1) of Saccharomyces cerevisiae parental strain BY4741 and its isogenic ncw2Δ mutant preincubated in YPD without PHMB (-PHMB) or with PHMB (+PHMB) and reinoculated in YPD or in YPD containing stress agents.

Treatment	BY4741		*ncw2*Δ	
	− PHMB	+ PHMB	− PHMB	+ PHMB
YPD	0.27	0.25	0.26	0.15
Ethanol (10% v/v)	0.12	0.04	0.08	0.03
KCl (1.2 mmol L^{-1})	0.19	0.18	0.19	0.12
CFW (25 µg mL^{-1})	0.11	0.11	0.10	0.11

Exposure to CFW triggers PKC/CWI and calcineurin pathways in the yeast cells, and leads to increased synthesis and deposition of chitin and reduced susceptibility to caspofungin (Walker et al. 2008; Lee et al. 2012). Similarly, exposure to PHMB also triggers the PKC/ CWI pathway (Elsztein et al. 2011). Hence, induction of the PKC/CWI pathway by PHMB could be

an adaptive mechanism for mitigating the toxic effects of CFW. Both parental and mutant strains showed a reduction of 60% in the maximal growth rate in the presence of CFW, regardless of pre-exposure to PHMB (Figure 5d; Table 2). This reveals the absence of a cross-adaptation mechanism between CFW and PHMB and that the damage caused by these two CW damaging agents are different. In addition, Ncw2p is not involved in the tolerance of CFW (Elsztein et al. 2016).

Later, it was found that pre-exposure to PHMB did not affect the growth rate of parental and mutant strains during the CFW treatment (Table 2). However, it extended the log growth, and this resulted in a higher final biomass than was the case with cells not pretreated with PHMB (Figure 5d). This result was unexpected for the ncw2Δ mutant because of the high deposition of chitin observed in its cells upon preexposure to PHMB (Fig. 2d). Roncero et al. (1988) reported that mutant strains displaying higher chitin content are more sensitive to CFW and raised the hypothesis that the ncw2Δ mutant would be more sensitive to CFW after pre-exposure to PHMB.

However, the amount of chitin in the CW does not seem to be the only factor that might determine resistance to CFW. García-Rodriguez et al. (2000) found that HOG-deficient cells are more tolerant to CFW and that cells pre-treated with CFW acquire tolerance to osmotic stress. Hence, susceptibility to CFW depends on the balance between increased chitin synthesis and the effective functioning of the HOG pathway in so far as yeast cells are more resistant to CFW when the HOG pathway is not active. In this study, we showed that the absence of Ncw2p leads to a more rigid cell surface by an increased amount of chitin deposited (Figure 2d) at the same time that it increased cell resistance to lysis (Figure 1).

On the other hand, the mutant was more sensitive to osmotic stress (Figure 5c). Hence, it seems that Ncw2p acts indirectly as an intermediary

factor in the induction of the HOG pathway, by allowing the correct deposition of chitin in the yeast cell wall (CW) that is necessary for a suitable induction of the osmotic stress response. Thus, the more rigid the CW is, the less effective the induction of the HOG response becomes. In conclusion, this work has sought to provide new insights into the function of Ncw2p and its influence in the CWI pathway and CW structure in conditions of stress, especially those caused by PHMB. The results show that the NCW2 gene can indeed be regarded as a part of the CWI regulon. It encodes a protein that seems to serve as an auxiliary regulator by acting on the spatial organisation of the CW, with regard to the correct chitin deposition and its links to the glucan network.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Publisher's Note

4 ARTIGO II

Structural analysis of Ncw2 protein of Saccharomyces cerevisiae involved in maintenance of yeast cell wall upon damaging with PHMB

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4.1 ABSTRACT

The biocidal agent polyhexamethyl biguanide (PHMB), considered to eliminate contaminant microorganisms in the industrial fermenting process, induces cell wall (CW) remodeling in yeast triggering the CW integrity pathway (CWI), whose efficient response is triggered in some tolerant strains of *Saccharomyces cerevisiae.* The NCW2 gene has been recently described as part of the CWI pathway, and its function is related to chitin deposition and PHMB cell response, however, the molecular aspects and protein features of Ncw2p in those biological process are still unclear. In the present work, we have demonstrated that Ncw2p is localized in the membrane through a GPI anchor, which is not linked to the CW, and it accumulates in the bud scars, where Ncw2p has a role in specific chitin-glucan linkages, during the budding process. For that, Ncw2p is O- and N-mannosylated, both modification types change in response to PHMB. N-mannosylation is strikingly increased in response to PHMB. Besides, even though Ncw2p is constitutively O-mannosylated by Pmt3/Pmt5 complex, under PHMB response it might involve more than one PMT complex. Therefore, Ncw2p is regulated at its post-translational level, and it is recruited to the cell surface to act on the cell wall remodeling, where it has a role in specific chitin-glucan linkages, especially in response to PHMB.

4.2 INTRODUCTION

The presence of contaminant yeasts and bacteria in the industrial process for production of fuel ethanol is thought to imposes a series of operational interventions to ensure the stability and productivity (Basilio et al. 2008; Lucena et al. 2010). The use of biocidal agents is always considered,

although sometimes cause damages to the fermenting yeast *Saccharomyces cerevisiae*. The effectiveness of the biocidal agent polyhexamethyl biguanide (PHMB) in killing the main contaminants by eliminating bacteria and yeast strains that are not part of the industrial fermenting process (Elsztein et al. 2008). However, some *S. cerevisiae* strains can also be affected by this agent, which in turn would make difficult its widespread use among the distilleries (Elsztein et al. 2008).

In order to get information on the biological action of this agent, we had performed experiments that indicated its potential to harm the cell wall structure, which lead to yeast cells to respond to the damages by trigging the cell wall integrity (CWI) mechanism regulated by the PKC signaling pathway (Elsztein et al. 2011). Many of the CWI genes encode enzymes responsible for β-glucan network and chitin synthesis of the cell wall, while other genes encode auxiliary proteins that act as mechanical sensors, in the protein trafficking and export and cell surface proteins, among other functions (Free 2013). Amongst these genes, the most responsive to the exposure to PHMB was the ORF YRL194c, whose protein was described as an GPI-anchored protein based on its amino acid sequence, due to the presence of a signal peptide and a cleavage site for GPI attachment (Terashima et al. 2000). Later on, this ORF was named *NCW2*, and this gene included in set of CWI genes (Elsztein et al. 2016). This gene was also responsive to sulfuric acid, used as biocidal agent to control bacterial population in the industrial processes in a way that its expression was dependent not only from the PKC/CWI signaling pathway but also the high-osmolarity response HOG pathway (Lucena et al. 2012).

The main physiological aspects regarding to the absence of *NCW2* gene was the sensitive phenotype to PHMB, slight increase in chitin content and higher resistance to lysis by glucanase enzymes (Elsztein et al. 2016).

Even the Calcofluor white or Congo Red, the most unknown cell wall stressing agents, were as effective as PHMB in *ncw2*Δ cells (Elsztein et. al 2016). For that reason, we further investigated the synergistic aspects of *NCW2* deletion and PHMB stress. For example, the results revealed that this combination increased even more the spread deposition of chitin in the yeast cell wall and increased the resistance to cell lysis by lyticase (Queiroz et al. 2020). Together with other evidences, these results lead us to propose the function of Ncw2p as an auxiliary of the CWI mechanism to maintain the correct architecture of the yeast cell wall and for the repair of damages. (Queiroz et al. 2020).

Nevertheless, there is still a lack of information on the molecular aspects of Ncw2p, which would give a better overview about its role in the cell wall stress response. Studies by fluorescence microscopy had reported the localization of this protein on the cell surface that were supported by computational analysis of its primary structure showing the presence of a signal peptide (SP) at its N terminus for export and an omega site at is C terminus for attachment to a GPI anchor at the outer face of the cell membrane. For such localization, Ncw2p might be directed to the Golgi complex, processed for SP removal and glycosylation and ultimately linked to GPI. In the present work, we seek to determine the mechanistic pathway that makes possible Ncw2p to play its biological role.

4.3 MATERIALS AND METHODS

4.3.1 Yeast Strains and Culture Conditions

The *S. cerevisiae* strains used in the present study are listed in Table 1. Yeast cells were grown in YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone

and 20 g L^{-1} dextrose) with agitation at 30ºC. Solid media were prepared as YPD contained 15 g L^{-1} agar. Pre-cultures were prepared by inoculating yeast cells from fresh YPD plates in YPD broth and cultivated overnight at 30ºC and agitated by 150 rpm. These cells were re-inoculated in a fresh YPD broth to 0.1 units of absorbance at 600 nm (0.1 A600nm) and incubating (as above) until they reached 0.5 A600nm, exponentially growing cells, or around 1.0 A600nm, early stationary phase. These cultures were used for all the experiments which are described as follows.

Table 1: Yeast strains used in this work.

Strain	Genotype	Reference
BY4741	MATa *his3Δ leu2Δ met15Δ ura3Δ*	Euroscarf
ncw2Δ	BY4741 NCW2::KanMX	Euroscarf
TEF2prmCherryNcw2	BY4741 NAT::TEF2pr-SPKar2-mCherry::NCW2	Weil et al. 2018
3xFLAG-NCW2	BY4741 *ncw2Δ NCW2*-FLAG	Present work

4.3.2 NCW2 - FLAG Tagging

A 3x FLAG tag was inserted in the 24th amino acid position of the Ncw2p, right after the signal peptide cleavage site. For this, an insert cassette of 3,413 base pairs was built by overlapping PCR containing *URA3* gene as selective marker and keeping the native promoter, signal peptide and 3'UTR regulatory regions of *NCW2* (Figure 1). The insert cassette has in its ends homologous sequences that allow the yeast cell to promote a homologous recombination with the *NCW2* gene flanking regions, with 862 base pairs

upstream the gene, containing the promoter, and 58 base pairs downstream at another end. The primers used here are presented below (table 2)

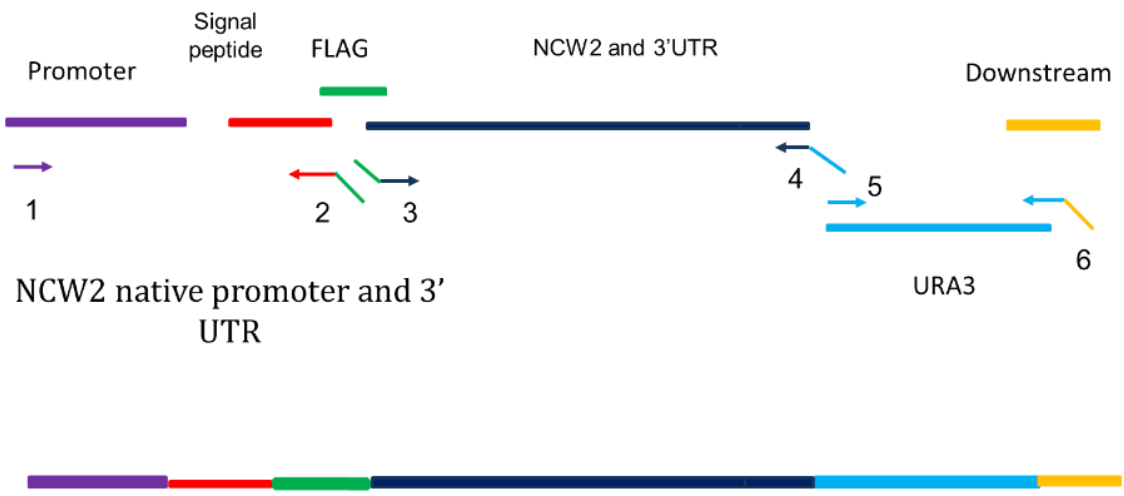

Figure 1: Scheme of the integration cassette with Ncw2p FLAG-tagged. The primers are identified by numbers as in the table 1.

These fragments were separately amplified and then the overlapping PCR was performed first with two fragments and subsequently with the third one, all the reactions were carried out by the Q5® high fidelity DNA polymerase (NEB). The last overlapping PCR reaction was loaded in a 1% agarose gel, and the band corresponding to the final cassette construct was cut out from the gel and submitted to purification by GeneJET Gel Extraction Kit (Thermo fisher). The DNA purified from that was introduced into ncw2Δ and a PMT mutant collection cells by transformation using Lithium Acetate method. The final overlapping PCR sequence obtained was checked by sequencing.

Table 2: Primers used to construct the insertion cassette Ncw2p tagged with FLAG 3X Primers 1 and 2 for amplifying a fragment containing native NCW2 promoter and native signal peptide (sequence for FLAG - 3X is already inserted in the reverse primer 5' end. At the very end of this cassette, corresponding to primer 6's 5' end, there is the 58 base pairs sequence complementary to the NCW2 gene downstream flanking sequence that will be used for homologous recombination and the cassette insertion into the genome, at the same NCW2 native locus.

	Primer	5' – 3'
1	Foward	TGTAGTCGAACAGCCCGATG
2	Reverse	AATCTATATCATGGTCTTTGTAGTCACCATCGTGGTCTTTGTAATCTAAGGAACCAGAGTCTTTTTGAGC
3	Foward	TGACTACAAAGACCATGATATAGATTACAAGGATGATGATGATAAAGATGGCCAGAACTCTGAAGATAGC
4	Reverse	CTGCTCTGATGCCGCATAGTTTTCAACAGCCTCGTTGTGC
5	Foward	ACTATGCGGCATCAGAGCAG
6	Reverse	GCCGTCTCAGAAGCCAGACAATACTTATTGAATATGATTACTCATCCATCTGTCATATAACTGGAACAACAC TCAACCCTATC

4.3.3 Protein Subcellular Fractioning and Western Blot Analysis

The transformed cells were grown in YPD with and without PHMB, in order to check the PHMB sensible phenotype rescue of the *ncw2*D mutant (Data not shown). Upon the same growth condition, these cells were also submitted to subcellular proteins fractionation following the protocol according to Mrsă et al. (1997), subsequently, around 200 µg of these samples were loaded onto a 9% SDS-PAGE, blotted on a nitrocellulose membrane, which was blocked with 5% milk solution, followed by incubation with anti-FLAG monoclonal antibody produced in mouse (Sigma-Aldrich), and after washing three times, the membranes were incubated in anti-mouse IgG peroxidase conjugate (Sigma-Aldrich), according to manufacturer's conditions. The membranes were visualized by enhanced chemiluminescence using ECL Prime Western Blotting. N-mannosylation and phosphorylation were tested by the digestion with EndoH (#P0702; New

England Biolabs; Frankfurt/Main, Germany) and Thermosensitive Alkaline Phosphatase FastAP (EF0654 ;Thermofisher). The reactions were carried out using the supplied buffers and according to manufacturer's instructions.

4.3.4 Fluorescence Microscopy

The fluorescence analysis was conducted using the strain TEF2prmCherryNcw2, kindly provided by Dr. Maya Schuldiner. This strain belongs to the SWAT library (Yofe et al. 2016; Weill et al. 2018; Meurer et al. 2018), and it harbors a mCherry-tagged *NCW2* gene after the predicted signal peptide. The regulators elements in TEF2prmCherryNcw2 are heterologous, with the strong promoter TEF2 and constitutive signal peptide Kar2 (Weill et al. 2018). The cells were grown as described above and visualized under the Olympus CellR microscope at ZMBH imaging facility (Zentrum für Molekulare Biologie der Universität Heidelberg).

4.3.5 Growth and Lysis Experiments

Yeast cells were incubated as described above in YPD and YPD containing PHMB 0,0005%. The absorbances 600nm were measure in intervals of 1 hour for 24 hours as described by (Elsztein et al. 2016). For the lysis assay, the cells were pre-cultivated, as detailed above, until exponential phase (0,5 A600nm) when they were centrifuged and incubated with and without PHMB 0,0005% for 3 hours. Subsequently, the cells were washed and submitted to lyticase enzyme following protocol described by Queiroz et al (2020). The growth experiment and the Lysis assay were carried out in the Synergy HT Multidetection reader (BioTek, Luzern, Switzerland).

4.4 RESULTS AND DISCUSSION

4.4.1 Compartment localization of Ncw2p

There have been published some evidences pointing that Ncw2p is located at the yeast cell wall in the surrounding area of the bud scars taking the advantage of high throughput analyses available at SGD and Yeast RGB databases (Terashima et al. 2000; Yofe et al. 2016; Weill et al. 2018). Recently, we provided more evidences on this localization using Ncw2p-GFP fusion protein when the yeast cells were treated with PHMB (Queiroz et al. 2020). However, that construct had that limitation that Ncw2p is thought to be processed at its carboxi terminal in the omega site for its attachment to a GPI anchor.

To solve this problem, we used in the present work a strain that express Ncw2p target with mCherry at its N terminal after the signal peptide. It means that the marker is kept in the processed protein after the trafficking along the Golgi complex and attachment to the outer cell membrane. Microscopic inspection revealed the accumulation of fluorescent dots of mCherry-Ncw2p that coincided with the yeast bud scars in exponentially growing cells in YPD medium (Figure 2). Hence, this result confirms that Ncw2p is located to the cell bud scars during the growth of *S. cerevisiae* cells. Ncw2p might have auxiliary role in the correct chitin deposition by its link to the cell wall glucan network (Queiroz et al. 2020). This association has been demonstrated by a series of cell wall phenotypes and physiological aspects due to the absence of Ncw2p, as for example increased chitin deposition and resistance to lysis by glucanases, which is even stronger in this mutant after treatment with PHMB (Elsztein et al. 2016; Queiroz et al. 2020).

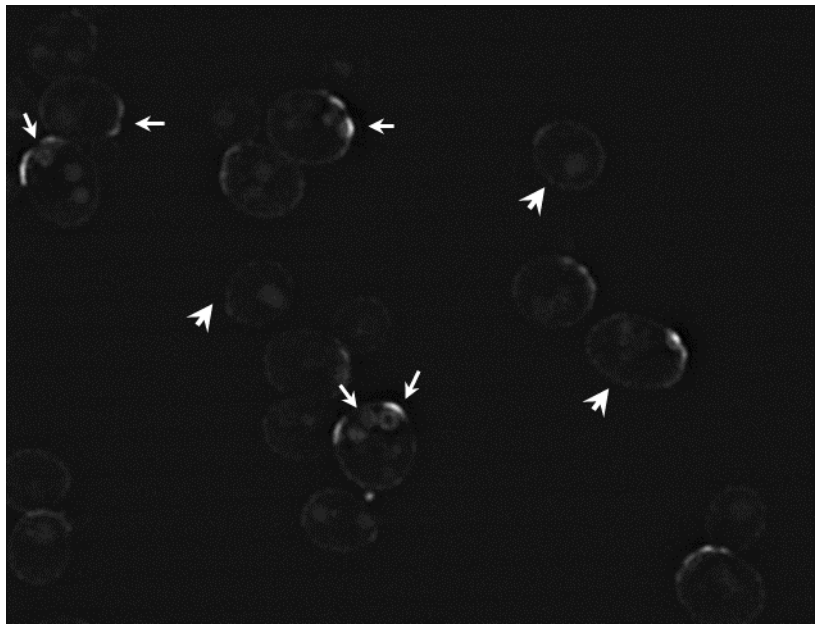

Figure 2: Subcellular compartment of Ncw2p identified by fluorescence microscopy analysis of mCherry-Ncw2pfusion, in Saccharomyces cerevisiae cells during exponential growth.Ncw2p is localized in plasma membrane, distributed in a punctate pattern (arrow heads), and in the bud scars (arrows).

During the cell division, the cytokinesis process promotes a constriction between the mother and daughter cell, due to the contractile actomyosin ring (Lippincott and Li 1998; Cabib et al. 2001). In budding yeast cells, the plasma membrane invagination generates the bud neck structure that is surrounded by a chitin ring, which is secreted from membrane to form the primary septum. Afterwards a secondary septum is formed together with the digestion of the primary septum by chitinase, which indicates the ending of cytokinesis the ultimate separation of the two cells (Kuranda et al. 1991). This structure remains in the surface of the mother cell as a bud scars entirely composed by chitin (Cabib et al. 2001; Cabib and Arroyo 2013).

Predominantly, chitin in the lateral wall is bound to β-1,6-glucan where proteins involved in the cell wall remodeling and growth of glucan are

attached (Kóllar et al. 1995; Kapteyn et al. 1995; Kóllar et al. 1997). Once in the bud neck, chitin plays a role in preventing growth and cell wall expansion of this region due to its linkage to β-1,3-glucan instead of b-1,6-glucan (Cabib and Duran 2005). It prevents the action of proteins that work on the remodeling of the glucan net (Cabib and Arroyo 2013). Chitin in the septum structure located inside the bud neck is synthesized by the chitin synthase Chs2p while chitin ring around the bud neck is synthesized by the chitin synthase Chs3p (Shaw et al. 1991).

In addition to differences of chitin formation, the constitution of the glucan structure also changes. For example, β-1,3-glucan in the lateral wall presents low relative molecular mass that is different from β-1,3-glucan linked directly to chitin in the bud neck that presents high relative molecular mass fraction (Cabib et al. 2012). This last is called static network because it is not submitted to remodeling process (Cabib et al. 2012). It means that the chemistry behind the chitin-glucans linkages is more important to the bud neck function than a mechanic function that is used to be proposed (Cabib and Arroyo 2013).

In this scenario, Ncw2p might play a role in the cell wall organization by acting on the chitin linkages (Queiroz et al, 2020). Hence, its function seems especially important for those different chitin-glucan links in the bud neck, during the whole budding process, once it can be found in the remains of the bud scar.

In addition to chitin, there is a small group of proteins found in the bud scars and chitin-rich structures that belongs to the Pir protein family, a GPI-independent alkali-sensitive cell wall attached proteins bound to β-1,3-glucan net (Kapteyn et al. 1999; Groot et al. 2005). Pir1p was the first protein detected in the structure of the chitin ring in the bud scars after cell separation, even though it is not found at the bud neck during the budding process, while

the Pir2 is located both in the bud scar and the lateral wall (Sumita et al. 2005). On the other hand, Pir3p and Pir4p are uniformly found in the lateral cell wall, but not in the bud scar (Sumita et al. 2005).

Besides in the bud scar structure, mCherry-Ncw2p fluorescence signal was also visible as a punctate pattern on the cell surface (Figure 2). This is a similar feature of that displayed by the b-1,3-glucanosyltransferase Gas1p found in the bud neck, in the bud scars as well in punctate structures over the plasma membrane (Rolli et al. 2009). Like Ncw2p, Gas1p has its C-terminal domain cleaved for binding to a GPI moiety (Yin et al. 2005; Rolli et al. 2009). Another similarity is the fact that both *gas1* and *ncw2* mutant cells accumulate chitin in their surface and are more resistant to lytic enzymes (Elstein et al. 2016). All these coincidences suggest that these proteins work together for the correct structuring of cell wall, with Ncw2p acting as auxiliary player in this biological process.

Despite this indicative of cooperation, there are no evidences that Ncw2p and Gas1p physically interact. The only evidence of Ncw2p interaction come from a high throughput study using the two-hybrid technology to show its binding with the 270-aa protein encoded by the open reading frame YNL050c (Krugan et al. 2006). To date, there is no information about the function of this protein. Afterwards, Qui and Noble (2008) used a Karnel-based computation modelling to predict co-complexed protein pairs (CCPP) and identified putative functional interaction of Ncw2p with four GPI-anchored proteins Dcw1p and Dfg5p (both putative mannosidases required for cell wall biogenesis and bud formation), Sed1p (associated to entry to stationary phase and to response to cell wall damage) and Yps3p (aspartic protease), as well as with Kre1p that is a cell wall glycoprotein involved in beta-glucan assembly.

Regarding to its predicted structure, theoretical analysis available at the Yeast Resource Center Public Data Repository (YRC-PDR) protein database predicted two major regions of primary Ncw2p composed by a signal peptide (1-22 aa) and a non-cytoplasmic (extracellular) mature protein (23-254 aa). This second region contains a series of b-sheet structures, disordered domains and an a-helix at the C terminus, as computationally predicted (Malmström et al. 2007). That a-helix domain might be lost during the GPI-anchoring to position Ncw2p at cell surface. That non-cytoplasmic region seems to form β-barrel structure that resembles that observed in intimin, an autotransporter-family protein involved in cell adhesion in bacteria (Leo et al. 2015). All those evidences, both experimental and computation-predicted, confirmed the localization of Ncw2p at the cell surface and functioning in the correct assembly of chitin-glucan structure during cell growth.

4.4.2 Subcellular localization of Ncw2p

The Ncw2p function on specific sites of the cell wall and its close involvement with specific chitin-glucan linkages raises the question about how Ncw2p interacts with those elements. For that, it is important to identify which subcellular fraction Ncw2p is found. For that purpose, Ncw2p was fused with a 3xFLAG tag at its N terminal after signal peptide to allow protein identification using anti-FLAG IgG. Yeast components were fractionated (Mrsa et al. 1997). The results showed that Ncw2p was detected in the membrane fraction of exponentially growing cells at three different molecular weight bands of 35, 45 and 70 kDa (Figure 3, lanes A and B).

According to the SGD database, the predicted size of Ncw2p would be 25 kDa based on the primary protein sequence, indicating that this protein

was targeted to post-translational modifications at different levels to reach cell surface. This protein was not found in the cell wall fraction (not shown), indicating its linkage to this cell component probably in GPI anchors as predicted by the presence of an omega site near to its C terminus (Terashima et al. 2000).

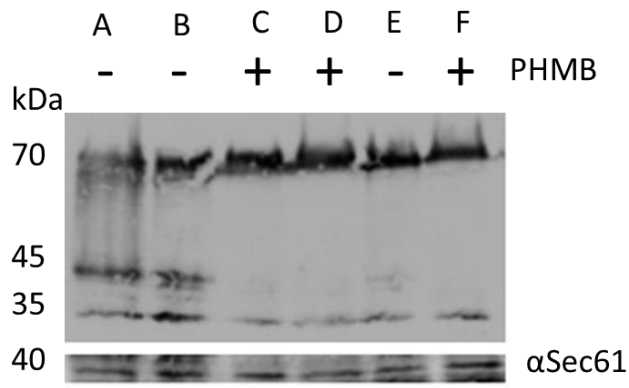

Figure 3: Membrane-bound Ncw2pin exponential (A to D) and stationary phase (E and F). Western blot analysis of a 3xFLAG-tagged Ncw2p strain, using anti-FLAG, and anti-Sec61 as loading control. Subcellular protein fractioning; Three independent experiments: two from cells collected in the exponential growth (A/C and B/D); one from cells collected in the stationary phase (E/F).

The GPI anchor is a glycolipid structure that is synthesized and attached to proteins in the endoplasmic reticulum (ER). Afterwards, through the Golgi apparatus vesicles, the GPI- anchored protein reaches the cell surface where the inositol-containing lipid moiety can be cleaved or not, which makes the GPI-anchored protein be covalently linked to the β-1,6-glucan in the cell wall or retained in the plasma membrane, respectively, by the GPI structure. Most of the yeast GPI-anchored proteins are covalently linked to the cell wall, and consequently detected in the cell wall fraction, from which they can be released using a glucanase enzyme, or an exochitinase if it is linked to the chitin. In the case of Gas1p, approximately 80% of this protein

is retained in the cell membrane, found in the lipid raft fraction (Bagnat et al. 2000; Rolli et al. 2009). Interestingly, the remaining 20% is linked to β-1,6-glucan, which can be either linked to β-1,3-glucan or to the chitin structure (Rolli et al. 2009).

The present work showed that Ncw2p remains retained in the membrane through its GPI anchor, and plays a role on the chitin-glucan linkages without being covalently attached to β-1,6-glucan, and consequently to the cell wall. It is noteworthy that exponentially yeast cells growing in the presence of PHMB lacked the 45 kDa form of Ncw2p, with reduction of 35 kDa form and increasing the 70 kDa form (Figure 3, lanes C and D). This pattern was similar to that observed for cells in stationary phase, irrespective the presence of PHMB (Figure 3, lanes E and F).

The observation of intermediate modified forms means that Ncw2p is actively produced during the exponential phase. Hence, in exponentially growing cell, Ncw2p is required for the correct chitin-glucan linkage during the cell wall expansion, especially in the emergent bud area. The cell wall must be in constant remodelling to allow its expansion during the cell division (Cabib et al. 2001). Whereas the stationary phase approaches, the cell wall should turn to a more rigid stage, in such a way, its structure does not promote cell wall growth and expansion. Besides, at this phase, the cells had many rounds of cell divisions, after every each, they accumulate more bud scars where Ncw2p remains localized. There are evidences that links some cell wall properties to replicative age, also including the budding process (Cabib et al. 1997). It is not clear yet how cell surface proteins play in the lifespan, but the deletion of some proteins can either increase or decrease lifespan, as for example, Knr4 and Mnn9, respectively (Molon et al. 2017).

In the present work, exponential-phase cells treated with PHMB behaved like untreated stationary-phase cells regarding Ncw2p processing.

Based on this criterion, it seems that the PHMB treatment induces changes in the cell wall that mimics or anticipate the stationary phase. It explains why stationary-phase cells are less sensitive to PHMB than exponential-phase cells (Elsztein et al. 2008). Besides, PHMB-induced early hardening of the cell surface very well explains the fact that these cells are more resistant to lysis by lyticase (Queiroz et al. 2020). This mechanism impairs the cell wall expansion and, consequently cell growth, increasing the Ncw2p production (Queiroz et al. 2020; Elsztein et al. 2016) and accelerating its sorting to the plasma membrane mostly in the 70 kDa isoform In this case the disappearance of the 45 kDa isoform might reflect the entrance of the yeast cells to stationary growth phase or the early stiffness of cell surface caused by stressful environmental conditions.

4.4.3 Post-translational modifications of Ncw2p

As soon as the protein is translated, the signal peptide targets it to be translocated into the endoplasmic reticulum (ER) lumen, where the omega site is cleaved, and a GPI moiety is attached to it. In this moment, the protein is associated to the ER membrane through the GPI anchor. Subsequently, it is transported to the Golgi apparatus, from where it is sorted in vesicles to the cell surface (reviewed by Mayor et al. 2004).

The results in Figure 3 showed that the isoforms of Ncw2p might represent different levels of protein modification that, given its localization at cell surface, be the result of glycosylation process occurring to asparagine residues in N-glycosylation type or to serine or threonine residues in O-glycosylation type (Spiro 2002; Li et al. 2019). This can be discriminated by exposing protein preparation to endoH digestion. When membrane preparation of exponentially growing cells was submitted to this treatment it

was observed the appearance of two gross bands of 100 kDa and 130 kDa (Figure 4, lane A), with disappearance of the 70 kDa band (data not shown).

These bands might represent the concentration of partially glycosylated proteins produced by the hydrolysis of N-hyperglycosylated forms represented in the high-weight smear in the gels. Cells treated with PHMB showed higher content of N - hyperglycosylated Ncw2p as indicated by thickness of the 130 kDa band (Figure 3, lane C) when compared to untreated cells (Figure 3, lane A). In this case, the high-weight smear was more evident when the extratact was not digested with endo H (Figure 4, lane D). Therefore, in the course of its export Ncw2p is N-glycosylated at different levels that can vary depending on the growth phase or the exposure to cell wall damaging agent.

Besides the membrane localization, Ncw2p was unexpectedly detected even at low concentration in the soluble fraction of cell extract as the 70 kDa isoform, which corresponds to this protein in the cytosol of PHMB-untreated cells (Figure 5A). When the cells were exposed to PHMB, the band of 70 kDa still present and the band of 100 kDa was detected (Figure 5A).

The orthologous of Gas1p in the methylotrophic yeast *Ogataea minuta* was also detected in soluble fractions most likely as the result of its transported from the membrane or Golgi apparatus to vacuoles for degradation (Rolli et al. 2009; Xu et al. 2017).

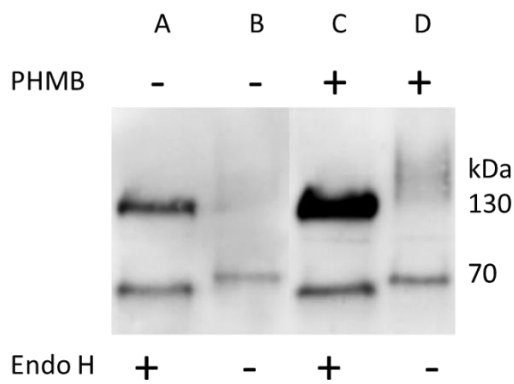

Figure 4: EndoH digestion of the membrane fraction. Western blot analysis of a 3xFLAG-tagged Ncw2p strain, using anti-FLAG. Lines B and D: membrane fraction sample; controls with no enzyme.

This protein is intensively produced during the exponential growth phase, which also increases its turnover and vacuolar degradation (Xu et al. 2017). Considering that cell exposure to PHMB increases *NCW2* expression (Elsztein et al. 2011; Elsztein et al. 2016) and Ncw2p production (Queiroz et al. 2020), it can also be considered that this agent also induces higher turnover to such level that Ncw2p isoforms can be found in cytosolic fractions. Following the post-translation modification analyses, cytosolic fraction was digested with endo H for the decreasing of 100 kDa isoform and appearance of 130 kDa isoform (Figure 5B).

It means that most of the cytosolic protein was indeed hyper N-glycosylated and did not migrated in the gel until the level of glycosylation diminish to produce that 130 kDa isoform. That soluble fraction was also submitted to digestion with FastAP alkaline phosphatase and the absence of isoforms other than 100 kDa band revealed that Ncw2p is not phosphorylated (Figure 5B).

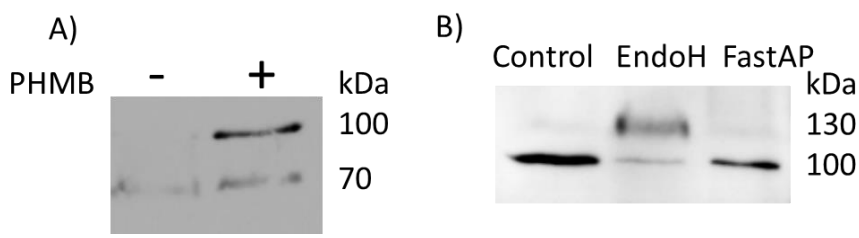

Figure 5: Detection of Ncw2p in the soluble fraction from PHMB-treated cells. Western blot analysis of A) 3xFLAG-Ncw2p in the soluble fraction; B) Post- translational identification of Ncw2p soluble form; EndoH for N-mannosylation; FastAP for phosphorylation.

N-glycosylation is one of the most common PTM and acts on proper folding of newly synthesized protein in the ER (Skropeta et al. 2009). A misfolded protein is degraded by ubiquitination, then the correct folding allows the protein to leave the ER and to go to the Golgi apparatus and to be secreted to the cell surface, also because the N-glycans binds specifically to cargo receptors responsible for the transport from ER to Golgi (Han et al. 2015). Studies about heterologous protein production have shown that the introduction of N-glycosylation sites in recombinant proteins can enhance their secretion, thermostability, their production in general (Ge et al. 2018; Yang et al. 2015; Han et al. 2015). Hence, the overproduction of Ncw2p induced by PHMB can also be accompanied by hyper N-glycosylation, misfolding and, consequently, migration from Golgi complex to cytosol for degradation.

4.4.4 O-mannosyl transferases are important to PHMB tolerance

Like N-glycosylation, the O-glycosylation type is also initiated in the ER, with an overlapping role in proper protein folding that impacts in the ER homeostasis, in such a way N-glycans and O-glycans can partially compensate each other (Loibl and Strahl 2013; Arroyo et al. 2011). In S. cerevisiae there are seven different proteins with O-mannosyltransferases (PMT) activity and they can be subdivided in the subfamilies PMT1 (Pmt1p and Pmt5p), PMT2 (Pmt2p, Pmt3p and Pmt6p) and PMT4 (Pmt4p). They work by transferring mannose from dolichyl phosphate-D-mannose to the serine/threonine residues of the target proteins (Goto 2007; Loibl et al. 2014). These PTMs have specificity for their protein substrate, acting in different sites (Ecker et al. 2003; Gentzsch and Tanner 1997), although the heterodimer Pmt1p-Pmt2p is the responsible for the majority of O-

mannosylation in yeast, with the heterodimer Pmt5p-Tmp3p playing a secondary action on this activity (Lommel and Strahl 2009). Other combinations are also possible, in which Pmt1p interact with Pmt3, and Pmt2p with Pmt5p (Girrbach et al. 2003). It means that under physiological situation heterodimers are formed with one member of PMT1 and one member of PMT2 protein subfamilies. On the other hand, Pmt4p acts as a homodimer or forming a heterodimer with Pmt6p (Gentzsch and Tanner 1997).

Ncw2p was predicted to be O-mannosylated at the amino acid positions 54, 57, 60 and 61 (Neubert et al. 2016) and, therefore, it is important to define which complex was involved in this post-translation modification. For this purpose, the construct 3xFLAG-Ncw2p fusion was transferred to different PMT mutants. The results showed that O-mannosylation is an important modification of Ncw2p responsible for the 70 kDa protein band identified. In addition, no modification in Ncw2p mobility profile was observed in the protein extract of yeast cells with single deletion of *PMT1* or *PMT2* genes (data not shown). It indicated that neither Pmt1 nor Pmt2 were essential for O-mannosylation modification of Ncw2p. Similar situation was observed for the single mutant *pmt4*Δ (Figure 6, lane A) and the quadruple mutant *pmt3,4,5,6*Δ (Figure 6, lane G). On the other hand, the simultaneous absence of Pmt proteins 3, 5 and 6 in triple mutant (Figure 6, lane C) and 1, 3, 5 and 6 in quadruple mutant (Figure 6, lane E) mutants significantly reduced the presence of 70 kDa form of Ncw2p in exponentially growing cells. In the case of *pmt1,3,5,6*Δ mutant the amount of the 35 kDa isoform of Ncw2p was higher than the other mutants.

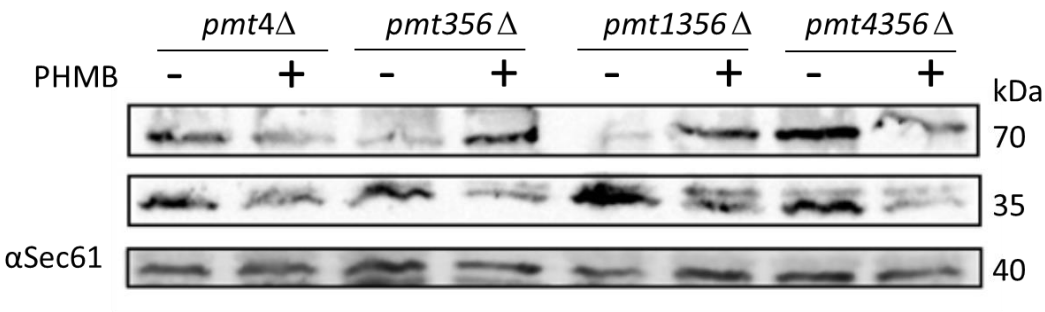

Figure 6 : Identification of O-mannosylation in the 70 kDa-sized Ncw2p form, and differential Ncw2p processing under PHMB treatment.Western blot analysis of 3xFLAG-tagged Ncw2p inserted in the genome of PMT mutants. Anti-Sec61 as loading control.

These results indicated that Ncw2p is constitutively O-mannosylated to 70kDa form by Pmt3/5 complex. When the cells were exposed to PHMB, the presence of Pmt4p was paramount for the accumulation of 70 kDa Ncw2p isoform since it was very reduced in *pmt4*Δ (Figure 6, lane B) and the quadruple mutant *pmt3,4,5,6*Δ (Figure 6, lane H) and present in the other two mutants (Figure 6, lanes D and F). Therefore, *PMT4* gene seems to encode an O-mannosyltransferase that works in the repair mechanism that keep cell integrity upon exposure to cell wall damaging agents.

All these results showed that Ncw2p can be O-mannosylated by at least three PMT complexes. It seems plausible to suppose that each complex can act specifically on one of the four putative O-mannosylation sites mapped in this protein (Neubert et al. 2016). During the vegetative growth, the complex pmt3/pmt5 seems to be relevant for the ultimate Ncw2p processing, and its absence produces accumulation of Ncw2p in the ER. However, the damages caused by PHMB on cell wall trigger the CWI cell response that induces a specific set of proteins involved in the cell wall remodelling (Elsztein et al. 2016). Since *PMT4* gene was sought to be important for activation and action of Ncw2p, this gene could also be included in the list of PHMB-tolerance CWI genes.

4.4.5 Cell growth and resistance to lysis in the absence of Pmt proteins

After defining the importance of Pmt proteins in the O-mannosylation of Ncw2p, we tested the relevance of these proteins for the tolerance to PHMB. Deletion of *NCW2* gene decreases the yeast growth rate when PHMB was in the medium relative to parental cells, leading us to suppose that Ncw2p is involved to tolerance to PHMB (Elsztein et al 2016). However, the recent data showed that this reduction in growth rate might be the result of early stiffening of the cell wall caused by the additive combination of *NCW2* deletion and PHMB treatment caused by the accumulation of chitin (Queiroz et al 2020).

In the present work, we tested the effect of PHMB on different *PMT* mutants (Figure 7 and 8). First, *pmt1*Δ and *pmt4*Δ mutants showed the same growth pattern of the parental BY4741 strain in absence or presence of PHMB (Figure 7, panels A and B). The triple mutant *pmt3,5,6*Δ showed a slight reduction in growth compared to parental strain when PHMB was present in the medium (Figure 7, panel C), while the mutants *pmt2*D, *pmt1,3,5,6*Δ and *pmt3,4,5,6*Δ showed significant growth reduction in PHMB-containing medium relative to BY4741 (Figure 8, panels A-C). These last four mutants had in common the fact that their cultures reached higher biomass than the parental strain in medium without PHMB (Figure 7C and Figure 8 A-C). Interestingly, the absence of Pmt1 alone does not have any effect under PHMB treatment, while PMT2 deletion causes a more sensitive phenotype in this condition.

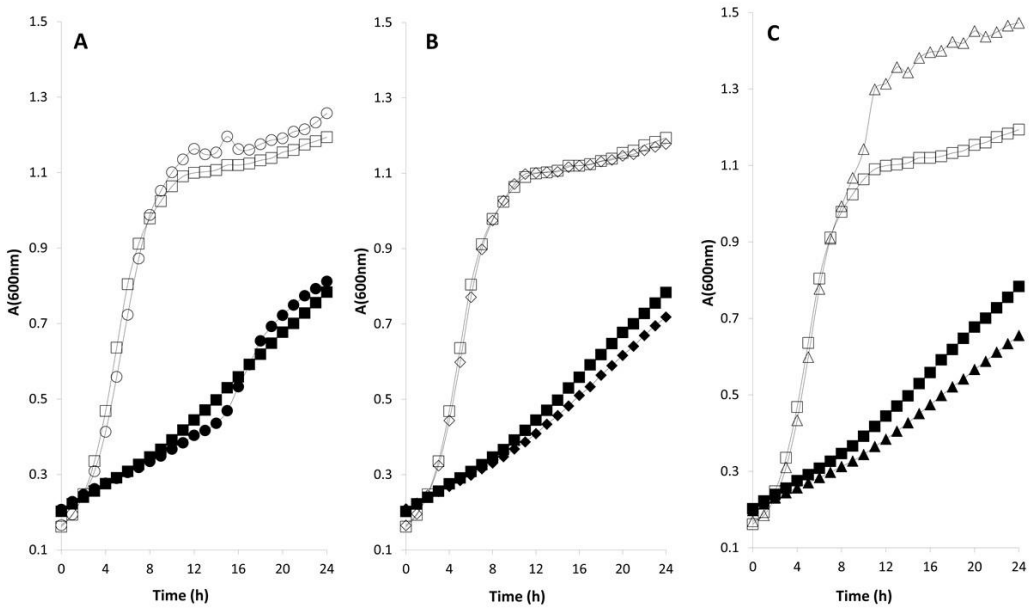

Figure 7: Growth curves of the pmt1Δ (A), pmt4Δ (B) and pmt356Δ, in YPD medium with (closed symbols) and without (open symbols) PHMB, compared to Saccharomyces cerevisiae parental strain BY4741 (square symbol).

The importance of Pmt2 over Pmt1 for PHMB cell response is clear once the double mutant *pmt*1Δ*pmt*2Δ has the same phenotype of *pmt*2Δ (Data not shown). Yet, Pmt1 alone does not play the major a role in the PHMB stress, and the same seem to be true for Pmt4. However, the *pmt*2Δ seems to be very important in this response, since the PMT2 deletion alone is enough to cause the same sensitive phenotype of the simultaneous deletion in PMT1, 3, 5, 6 and PMT 3, 4, 5 and 6 genes. It has been revealed that deletion of *NCW2* or treatment with PHMB increased chitin deposition in the cell wall, which turn the cells more resistant to lysis by lyticase (Elsztein et al., 2016; Queiroz et al., 2020).

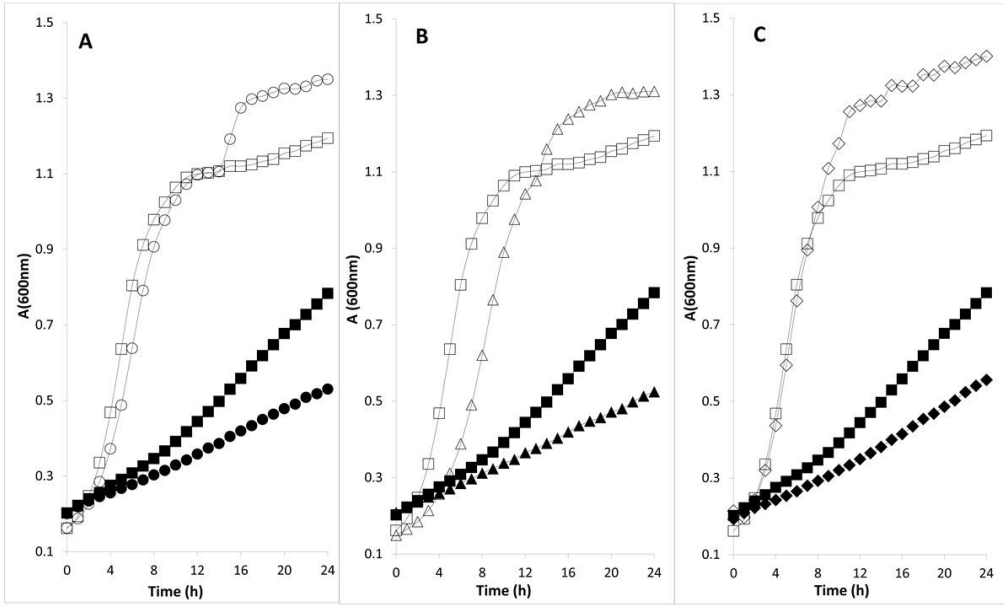

Figure 8: Growth curves of the pmt2Δ (A), pmt1356Δ (B) and pmt4356Δ, in YPD medium with (closed symbols) and without (open symbols) PHMB, compared to Saccharomyces cerevisiae parental strain BY4741 (square symbol).

Hence, both conditions might signal for the entrance to the stationary phase by stiffening cell surface. Exponentially growing cells of different mutant strains were tested for lysis by lyticase in the absence or presence of PHMB and the results were variable (Figure 9 and Figure 10). *pmt1*Δ cells showed a striking different lysis pattern, where cells exponentially growing are very susceptible to glucanases (Figure 9A).

These cells when treated with PHMB had a high increase in resistance to lysis compared to untreated cells, however, PHMB-treated *pmt1*Δ cells have the same lysis phenotype as untreated parental cells (Figure 9A). The deletion of PTM1 alone generates a remodelling inducing the susceptibility to glucan digestion.

It would be expected the same behaviour for *pmt2*Δ cells, once Pmt1 function as a heterodimer with Pmt2 to O-mannosylate proteins. Nevertheless. there was no difference between untreated parental cells and

treated and untreated *pmt*2Δ cells (Figure 10A), what means that the *PMT2* absence does not affect the glucan digestion by Lyticase in the cell wall. Considering only the *PMT4* deletion, amongst the other mutants, *pmt4*Δ seems to have the most similar phenotype to the parental strain (Figure 9B). Although, the lysis curve of untreated *pmt4*Δ cells starts very similar to the parental, but after four hours the *pmt*4Δ curve achieves a stationary state, while the parental continues a linear digestion.

The following results show a strong genetic interaction between the PMT genes for lysis phenotype. The simultaneous deletion of *PMT3*, *PMT5* and *PMT6* induces an increased lysis resistance in untreated cells (Figure 9C), similarly to the deletion of *NCW2* (Elsztein et al., 2016). Although, the results with PHMB-treated *pmt356*D cells show no difference compared to its untreated cells and PHMB-treated parental cells. Besides, the simultaneous deletion of *PMT1*, in the *pmt1356*Δ, does not change the phenotype compared to PHMB-treated or untreated *pmt356*Δ (Figure 10B). It means that the deletion of these three genes suppresses the deletion of PMT1, as a positive genetic interaction, however, *PMT1* seem to be indifferent for lysis phenotype in this genetic background.

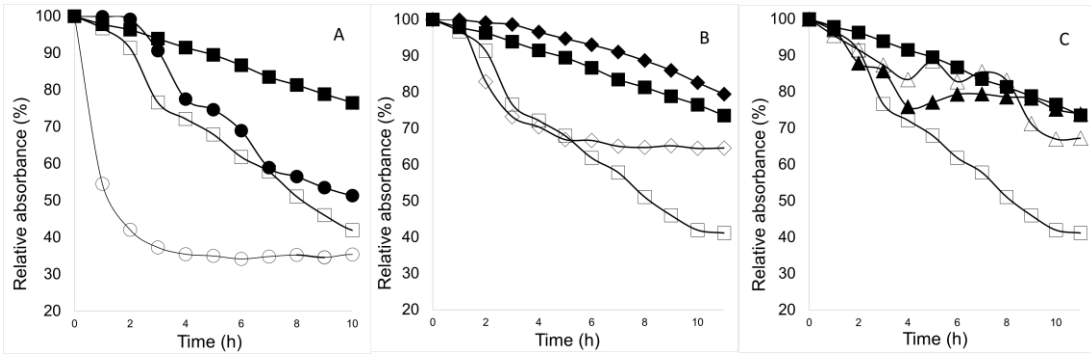

Figure 9: Cell wall digestion curves by Lyticase in Saccharomyces cerevisiae parental strain BY4741 (square) and mutants pmt1Δ (A), pmt4Δ (B), and pmt356Δ (C) cells previously incubated in YPD medium with (closed symbols) and without (open symbols) PHMB.

Then, the simultaneous deletion of *PMT4* in the *pmt356*Δ background does not affect the lysis curve (Figure 10D) compared to *pmt4*Δ cells (Figure 9B). In contrast to all phenotypic results obtained so far, PHMB-treated *pmt4356*Δ cells display a high susceptibility to lysis by Lyticase, which in six hours, around 70% of cells were lised (Figure 10D).

The impairment of mannosylation strongly affects the cell wall integrity pathway and GPI biosynthesis, which can affect the ER homeostasis (Zaratorska et al., 2017; Arroyo et al., 2011). Then, the absence of O-mannosylation increases the accumulation of misfolded protein in the ER, triggering a protective response that leads to ER-associated protein degradation (ERAD). However, it also affects the cell wall mannoproteins production, and consequently a cell wall stress response that is impaired due to lack of O-mannosylation machinery (Ron and Walter, 2007; Smith et al., 2011).

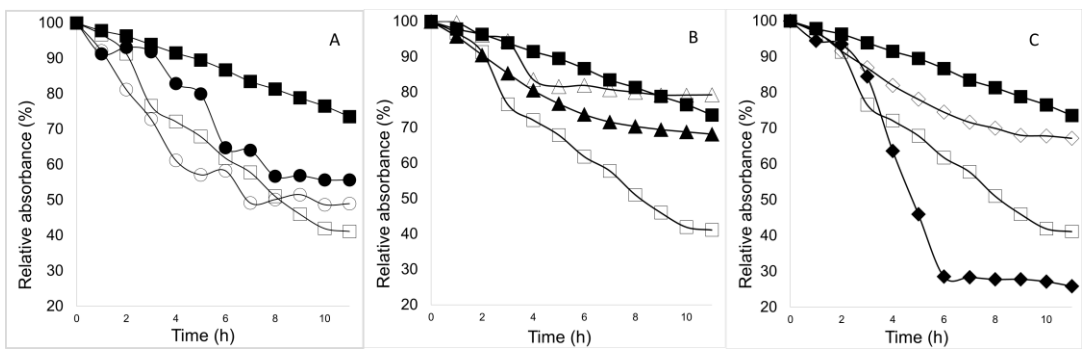

Figure 10: Cell wall digestion curves by Lyticase in Saccharomyces cerevisiae parental strain BY4741 (square) and mutants pmt2Δ (A), pmt1356Δ (B), and pmt4356Δ (C) cells previously incubated in YPD medium with (closed symbols) and without (open symbols) PHMB.

The consequence is a proteotoxic effect due to aberrant mannoproteins accumulating in the secretory pathway, then provoking cell

death (Loibl and Strahl, 2013). Besides, it has been demonstrated that D*pmt*2 is sensitive to Calcofluor White and Congo red, two cell wall stress agents (Zatorska et al., 2017). Hence, it is reasonable that *pmt2*D is also sensitive to PHMB, as well as many cell wall-related mutants, including *ncw*2D (Elsztein et al., 2011).

Even though, Pmt1p and Pmt2p have a similar structure (Bai et al., 2019), they are differently processed, for example Pmt2p is N-mannosylated by Ost3 enzyme, but not Pmt1p (Zatorska et al., 2017). It is possible that in the PHMB response, Pmt2p can also efficiently acts with Pmt5p, which seemed to be relevant to the PHMB cell response and together with Pmt3p is essential to mannosylate Ncw2p. The complex Pmt1/Pmt2 and Pmt4/ Pmt4 are responsible for the majority of O-mannosylation in yeast (Lommel et al., 2011). These different Pmt complexes have different set of substrates, normally, Pmt1/Pmt2 acts on both membrane-associated or soluble proteins, while Pmt4 homodimer mannosylates only membrane-associated proteins, transmembrane or GPI anchored, as for example Gas1p (Hutzler et al, 2007).

Hence, the phenotypes found here, especially about the lysis, reflect the impact of mannoproteins absence or its misfolded forms in the cell wall. Even Pmt1p and Pmt2p act as a heterodimer in the majority of the O-mannosylation in the cell, their absence causes different lysis phenotypes. On the other, when they are the only active machinery, as in the *pmt4356*D cells, the cell wall remodelling changes in a way that the typical resistance to lysis induced by PHMB treatment does not occur in those cells. The explanation for that can be the fact that in this mutant, the O-mannosylation level is strikingly reduced, because many proteins are specific targets of Pmt4 (Hutzler et al., 2007). This specificity occurs for Ncw2p only when there is no stress condition, where it is O-mannosylated by Pmt3/Pmt5. Nevertheless, as PHMB cell response, it is possible that ncw2p is O-mannosylated for more

two different Pmt complex in this condition, one in the amino acid positions 54 and 57, and the other in the 60 and 61. It is necessary to verify in which Pmt mutant Ncw2p is detected from the soluble fraction. Then, it will be possible to identify the differential mechanism involved in Ncw2p processing as a cell response to PHMB-induced stress.

4.5 CONCLUSION

The results presented herein showed molecular features of Ncw2p, and how they change along the cell growth and under cell wall stress caused by PHMB. The Ncw2p role in chitin-glucan linkages are relevant for the proper cell division at budding sites. However, this process is impaired after exposure to PHMB that anticipates a “stationary phase” state, which increases the secretion of Ncw2p to the membrane. This mechanism involves increasing in N-mannosylation, and possibly a different set of PMT complex responsible for O-mannosylation, in Ncw2p. Neverhteless, this cell response also involves a fine control of protein modifications, unrevealed genetic interaction network, besides increasing the CWI genes expression.

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5 DISCUSSÃO GERAL

A identificação de NCW2 como gene funcional e pertencente à via de integridade da parede celular (CWI) só foi possível devido à sua responsividade ao PHMB, o qual ainda era um dos poucos processos biológicos associados a este gene (Elsztein et al. 2016). O que levantou questões acerca de quais aspectos da parede celular em leveduras ainda não estão esclarecidos, e qual o papel de uma ORF, até aquele momento não bem conhecida, teria sobre tal. O presente trabalho mostra que o gene NCW2 codifica uma proteína cuja expressão, de fato, responde à um agente estressor de parede, e que seu papel está intimamente relacionado ao mecanismo de resposta ao PHMB, bem como ao efeito que este causa na parede.

Contudo, também observamos que Ncw2p também pode estar envolvida na resposta celular de outros tipos de estresse, tais como etanol em concentrações mais elevadas. Isso mostra que ainda há muito mais aspectos biológicos a serem revelados sobre esta proteína, como também aos mecanismos de resposta ao estresse de um modo geral, e a elucidação deles podem permitir novas ferramentas de estudo. Isso tem sido mostrado por estudos utilizando *S. cerevisiae* como modelo eucarioto e seu uso como ferramenta para tecnologias como "*cell surface protein display*", o qual utiliza a parede celular como esqueleto para imobilização e expressão heteróloga de proteínas de interesse farmacêutico e industrial (Urbar-Ulloa et al, 2019; Hossain et al., 2020).

Além disso, análises da proteína Pkc1, envolvida na via CWI em *S. cerevisiae*, têm sido aplicadas no estudo de isoformas específicas de humano, as quais estão envolvidas em doenças do tipo Alzheimer, diabetes e alguns tipos de câncer (Heinisch and Rodicio, 2018). Tal aspecto foi

revelado também pela sinergia entre estresse osmótico e por PHMB, o qual havia sido previamente relacionado a um aumento na tolerância a Calcofluor White ao passo que diminui a via HOG (Garcia-Rodrigues et al., 2000). Essa sinergia parece ser muito mais complexa, uma vez que ainda é necessário entender quais vias estão ativas em condições de estresse por PHMB, o qual interfere em diferentes vias e modificações proteicas ainda a serem elucidadas.

Isso mostrou que a ação do PHMB na célula era diferente dos demais agentes estressores de parede, fato este que ficava mais evidente na ausência de *NCW2*. E devido a análises fenotípicas de deposição de quitina e resistência a lise em células com ausência do gene *NCW2* e tratadas com PHMB, ficou evidente que os efeitos causados por essas duas variáveis pareciam se sobrepor. Portanto, havia a necessidade de identificar qual o efeito molecular do PHMB em Ncw2p. Com isso, foi verificado que Ncw2p é regulada em vários níveis, desde sua expressão gênica como também em nível pós-traducional em resposta ao PHMB, que parece induzir a aceleração do tráfego dessa proteína à membrana, onde auxilia na contenção da expansão da parede celular pela deposição correta da quitina na parede celular, especialmente na ligação deste polissacarídeo com os demais na estrutura da parede. Essa resposta ao PHMB altera as modificações sofridas por Ncw2p aumentando o grau de N-manosilação. No entanto, a O-manosilação de Ncw2p parece ser realizada de maneira diferencial em resposta ao PHMB, o que demonstra mais um novo aspecto sobre a ação deste biocida, o qual mostra haver uma complexa rede de interações genéticas da família PMT, cuja função é de extrema importância para biossíntese e remodelagem da parede celular (Willer et al., 2005).

Além disso, a regulação de Ncw2p também é relevante ao crescimento e divisão celular, independente da condição de estresse. Há uma clara

diferença na expressão da proteína Ncw2 durante o crescimento exponencial e em fase estacionária. Isso mostra uma interconectividade do crescimento com a expansão da parede, que parece ocorrer na cinética de modificação das proteínas envolvidas na remodelagem da parede, possivelmente em sincronia com o ciclo celular e os pontos de *checkpoint* (Suzuki et al., 2004). Esse *chekpoint* possivelmente regula também a produção de Ncw2p durante o ciclo celular, uma vez que a via CWI está coordenada com a formação do fuso mitótico (Negishi and Ohya 2010), e a deleção de *NCW2* induz a constitutiva expressão de parte dos genes da via CWI.

6 CONCLUSÃO GERAL

Dados os resultados e análises aqui apresentados, foram demonstrados novos aspectos acerca da remodelagem da parede celular sob condições estresse, por meio da caracterização de Ncw2p, bem como sobre o efeito que o PHMB tem sobre a parede celular. Essa proteína é significativamente mais expressa sob tratamento com PHMB e a ausência de NCW2 induz um estado de estresse de parede celular, o que definitivamente demonstra que Ncw2p é uma proteína que faz parte da via CWI. Seu papel principal está relacionado com a correta deposição de quitina, auxiliando na ligação diferencial desta com os glucanos β-1,3 e β-1,6 da parede celular. Tal mecanismo também envolve o efeito que o PHMB induz na célula, disparando um mecanismo compensatório de aumento de quitina, aumentando a resistência da parede à ação de glucanases.

Para uma resposta celular eficiente ao PHMB, Ncw2p não só é mais produzida, como também ocorre uma aceleração na via secretória que leva esta proteína para a membrana plasmática, onde está ancorada por GPI, para isto Ncw2p é altamente N-manosilada. Nessa condição, Ncw2p auxilia na remodelagem da parede, de modo a diminuir sua expansão da parede e controlar o crescimento celular. Além de N-manosilada, Ncw2p também é O-manosilada, em condições de crescimento vegetativo, pelo complexo Pmt3/Pmt5. Contudo, sob tratamento com PHMB, essa modificação parece não depender mais deste heterodímero, provavelmente por mais de um complexo PMT, o que possivelmente está relacionado com uma forma Ncw2p diferente encontrada na fração solúvel e mais modificada que a fração de membrana.

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