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1 **The two-component response regulator Skn7 belongs to a network of transcription**
2 **factors regulating morphogenesis in *Candida albicans* and independently limits**
3 **morphogenesis-induced ROS accumulation**

4

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28

29 **Running title:** Skn7 regulatory network in *Candida albicans*

30

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32 reactive oxygen species, chromatin immunoprecipitation, transcriptome

33 **SUMMARY**

34 Skn7 is a conserved fungal heat shock factor-type transcriptional regulator. It participates in
35 maintaining cell wall integrity and regulates the osmotic/oxidative stress response (OSR) in *S.*
36 *cerevisiae*, where it is part of a two-component signal transduction system. Here, we
37 comprehensively address the function of Skn7 in the human fungal pathogen *Candida*
38 *albicans*. We provide evidence reinforcing functional divergence, with loss of the cell
39 wall/osmotic stress-protective roles and acquisition of the ability to regulate morphogenesis
40 on solid medium. Mapping of the Skn7 transcriptional circuitry, through combination of
41 genome-wide expression and location technologies, pointed to a dual regulatory role
42 encompassing OSR and filamentous growth. Genetic interaction analyses revealed close
43 functional interactions between Skn7 and master regulators of morphogenesis, including
44 Efg1, Cph1 and Ume6. Intracellular biochemical assays revealed that Skn7 is crucial for
45 limiting the accumulation of reactive oxygen species (ROS) in filament-inducing conditions
46 on solid medium. Interestingly, functional domain mapping using site-directed mutagenesis
47 allowed decoupling of Skn7 function in morphogenesis from protection against intracellular
48 ROS. Our work identifies Skn7 as an integral part of the transcriptional circuitry controlling
49 *C. albicans* filamentous growth and illuminates how *C. albicans* relies on an evolutionarily-
50 conserved regulator to protect itself from intracellular ROS during morphological
51 development.

52

53 INTRODUCTION

54 *Candida albicans* is a diploid fungus, part of the normal human flora that colonizes the
55 digestive tract and the genital mucosa of most healthy individuals. However, it is also one of
56 the most prevalent fungal pathogens of humans, causing either superficial infections, such as
57 vaginal and oral thrush, or life-threatening bloodstream infections in immunocompromised
58 patients (Kullberg *et al.*, 2002; Perlroth *et al.*, 2007; Yapar, 2014). A distinctive feature of this
59 fungus is the ability to undergo a reversible morphogenetic transition between the unicellular
60 budding yeast and the filamentous pseudohyphal and hyphal forms (Sudbery, 2011).

61 The yeast-to-hyphal transition plays a central role in virulence. Indeed while yeast cells
62 contribute to the colonization of mucosa and dissemination into the bloodstream, hyphal
63 forms play important roles in crossing of barriers, invasion of tissues and formation of
64 biofilms (Dalle *et al.*, 2010; Khan *et al.*, 2010). A number of environmental cues trigger the
65 transition, such as temperature, pH or CO₂ levels (Berman, 2006; Shapiro *et al.*, 2011). Signal
66 transduction pathways have been identified linking these environmental cues to the activation
67 of so-called hyphal-specific genes (HSGs) that contribute to cell cycle deregulation, polarity
68 establishment and changes in the cell wall (reviewed in Sudbery, 2011; Huang, 2012).
69 Morphogenesis is under the control of an intricate network of transcriptional regulators that
70 act negatively or positively on the expression of HSGs (Shapiro *et al.*, 2011; Sudbery, 2011).
71 Negative regulation of HSGs is mediated by the transcriptional repressor Tup1, in association
72 with the DNA-binding proteins Nrg1, Rfg1 and Sfl1 and the co-repressor Ssn6 (Keleher *et al.*,
73 1992; Smith and Johnson, 2000; Conlan and Tzamarias, 2001; García-Sánchez *et al.*, 2005;
74 Kadosh and Johnson, 2005). Positive regulation of HSGs is mediated in almost all filament-
75 inducing conditions by the transcription factors (TFs) Efg1, Flo8 and Ndt80 (Stoldt *et al.*,
76 1997; Bockmühl and Ernst, 2001; Cao *et al.*, 2006; Sellam *et al.*, 2010) whereas other TFs,
77 such as Cph1, Czf1, Tec1, Sfl2 and Rim101, positively regulate HSGs in a condition-specific

78 manner (Liu *et al.*, 1994; Brown *et al.*, 1999; Ramon *et al.*, 1999; Schweizer *et al.*, 2000;
79 Baek *et al.*, 2006; Song *et al.*, 2011, Znaidi *et al.*, 2013). While these TFs act at the onset of
80 morphogenesis and are required for continued filamentation, another TF, namely Ume6,
81 shows a predominant role in the maintenance of hyphal growth (Banerjee *et al.*, 2008; Zeidler
82 *et al.*, 2009). Regulation of morphogenesis is also dependent on chromatin remodeling (Lu *et*
83 *al.*, 2008; Schaekel *et al.*, 2013). In this respect, it has been shown that Brg1, by binding to
84 promoters of HSGs, allows removing the repression exerted by the Nrg1-Tup1 complex and
85 promoting chromatin remodeling thus favoring the activation of HSGs (Lu *et al.*, 2012; 2013).
86 While a full understanding of the mechanisms by which these TFs regulate morphogenesis
87 from its onset is still lacking, it is increasingly clear that their interplay is central to this
88 regulation as many of them bind to the same promoter regions and are subject to reciprocal
89 regulation (Vinces *et al.*, 2006; Znaidi *et al.*, 2013).

90 Additional TFs appear to play a role in morphogenesis and yet their precise contribution
91 remains to be investigated. This is the case of the Skn7 response regulator that we
92 characterized here. Skn7 was shown to be required for hyphal morphogenesis on several solid
93 media (Singh *et al.*, 2004), but, as reported here, not in liquid media, suggesting a specific
94 role in surface-induced morphogenesis. In addition, over-expression of the *SKN7* gene was
95 shown to trigger hyphal formation regardless of the environment (Chauvel *et al.*, 2012). *SKN7*
96 encodes a TF specific of and highly conserved among ascomycetous and basidiomycetous
97 fungi (reviewed in Fassler and West, 2011). Skn7 proteins have been implicated in the control
98 of different cellular activities, such as the response to stress and cell cycle regulation. For
99 instance, in *Saccharomyces cerevisiae*, Skn7 acts as a response regulator in the Sln1 cell wall
100 integrity pathway, one of the best-studied eukaryotic examples of a « two-component » signal
101 transduction pathway (Fassler and West, 2011; Mulford and Fassler, 2011). These
102 transduction pathways, conserved in plants and microorganisms, rely on phosphotransfer

103 steps between histidine kinases, and eventually phosphorylation of a canonical Aspartate
104 residue on the response-regulator protein (Stock *et al.*, 2000; Chauhan, 2015). In the insect-
105 pathogenic fungus *Metarhizium robertsii*, Skn7 is required for cell wall integrity (Shang *et al.*,
106 2015), while in the human pathogen *Aspergillus fumigatus* it is involved in the cell wall stress
107 response (Lamarre *et al.*, 2007). *S. cerevisiae* Skn7 has also been linked to the response to
108 other types of stress: oxidative stress (Krems *et al.*, 1996; Morgan *et al.*, 1997; Charizanis *et*
109 *al.*, 1999; Lee *et al.*, 1999), osmotic stress (Li *et al.*, 2002), or nitrogen starvation-induced
110 diploid filamentous growth (Lorentz and Heitman, 1998). In *C. albicans* and in the other
111 fungal pathogens of humans *Cryptococcus neoformans*, *Candida glabrata* and *Candida*
112 *lusitaniae*, *SKN7* contributes to the oxidative stress response (Singh *et al.*, 2004; Wormley *et*
113 *al.*, 2005; Ruprich-Robert *et al.*, 2008; Saijo *et al.*, 2010). Indeed, *C. albicans* deletion
114 mutants for *SKN7* show increased sensitivity to oxidative stress mediated by hydrogen
115 peroxide (Singh *et al.*, 2004). Interestingly, Skn7 proteins possess a Heat Shock Factor
116 (HSF)-type DNA binding domain (DBD), and it has been reported that *S. cerevisiae* Skn7
117 interacts with Hsf1, suggesting that it is also involved in the heat shock-response in this
118 species (Raitt *et al.*, 2000). In *C. albicans*, two other transcription factors, namely Sfl1 and
119 Sfl2, also contain an HSF-type DBD (Li *et al.*, 2007; Spiering *et al.*, 2010). Interestingly, both
120 factors are regulators of filamentous growth, with antagonistic functions (Bauer and
121 Wendland, 2007; Song *et al.*, 2011; Znaidi *et al.*, 2013), and Sfl2 has been shown to be a
122 temperature-responsive regulator (Song *et al.*, 2011).

123 In this study, we have investigated the role of *C. albicans* Skn7 in morphogenesis and
124 oxidative stress response. By performing genome-wide transcriptomic and chromatin
125 immunoprecipitation analyses, we mapped the transcriptional circuitry that is directly
126 regulated by Skn7. We conducted epistasis analyses to further resolve Skn7 interplay with
127 other regulators of morphogenesis in *C. albicans*. Furthermore, we provided data unveiling a

128 role of Skn7 in preventing intracellular accumulation of reactive oxygen species (ROS) during
129 growth on a filamentation-inducing solid medium. Importantly, analysis of key residues in
130 different Skn7 domains allowed uncoupling Skn7 function in the regulation of morphogenesis
131 from protection against intracellular ROS.

132

133 **RESULTS**

134 **Knockout, overexpression and site-specific mutants support a role of *C. albicans* SKN7** 135 **in oxidative stress defense and morphogenesis, but not in cell-wall/osmotic stress.**

136 *C. albicans* SKN7 encodes a ~70 kDa protein whose structure is highly conserved with *S.*
137 *cerevisiae* Skn7: an N-terminal HSF-type DNA binding domain, a central coiled-coil domain,
138 which in *C. albicans* contains a leucine-zipper motif, and a C-terminal receiver domain (Fig.
139 1A). In *S. cerevisiae* both HSF- and receiver domains are as important for oxidative stress
140 response as for cell wall stress response (Raitt *et al.*, 2000; Fassler and West, 2011; Mulford
141 and Fassler, 2011). We sought to determine whether *C. albicans* Skn7 retained similar
142 functions to those in *S. cerevisiae*. We confirmed some of the results obtained by Singh *et al.*
143 (2004) *i.e.* that inactivation of SKN7 resulted in a filamentation defect of *C. albicans* on
144 filamentation-inducing solid media such as Spider, as well as an increased sensitivity to
145 oxidative stress (Fig. 1B, top left panel and Fig. 2A, respectively). Nevertheless, our *C.*
146 *albicans* $\Delta\Delta skn7$ null mutant (strain CEC4220; Table S1) did not exhibit a reduced growth on
147 YPD + 10% serum as observed by Singh *et al.* (2004); instead, it did not filament on this solid
148 medium (Fig. 1B, bottom left panels). By contrast, this mutant retained its ability to filament
149 in filament-inducing liquid media (Fig.1B, bottom right panels). Furthermore, the $\Delta\Delta skn7$
150 strain exhibited unaltered growth as compared to the wild-type strain on media inducing cell
151 wall and osmotic stress, such as those containing Congo Red, or NaCl, and slightly reduced
152 growth on Calcofluor White (Fig. 1C). We also confirmed results obtained by Chauvel *et al.*

153 (2012) *i.e.* that *C. albicans SKN7* overexpression led to uncontrolled filamentation in liquid
154 and solid filamentation-repressing media (Fig. 1D, right panel). However, *SKN7*
155 overexpression did not influence *C. albicans* sensitivity to oxidative, cell wall and osmotic
156 stress (not shown).

157 The HSF and receiver domains contain several well-conserved amino acids that play key roles
158 in mediating Skn7 function in *S. cerevisiae* (Fig. 1A). In the HSF domain, within the well-
159 conserved helix-turn-helix DNA binding motif, the F135 and L142 residues (equivalent to
160 F76 and L83 in the *C. albicans* protein, respectively) are both critical for Skn7 activity
161 (Fassler and West, 2011). In the receiver domain, the D427, T437 and T449 residues
162 (equivalent to D474, T484 and T496 in the *C. albicans* protein, respectively) have been
163 shown to be important for *S. cerevisiae* Sln1-dependent cell wall signaling, Skn7-Yap1
164 interaction upon oxidative stress and Skn7 DNA binding ability, respectively (He *et al.*,
165 2009). D427 (D474) corresponds to the conserved residue targeted by the histidine
166 phosphotransfer protein. We tested whether the HSF- and the receiver domains contribute to *C.*
167 *albicans* Skn7 activity in oxidative stress defense or morphogenesis. We generated a *C.*
168 *albicans* strain in which the F76 and L83 residues in the HSF domain are both mutated to
169 Alanine (CEC4277). We also generated *C. albicans* strains in which the D474, T484 or T496
170 residues in the receiver domain are individually mutated to Alanine (CEC4272, CEC4273 and
171 CEC4274, respectively). These strains are derivatives of the $\Delta\Delta skn7$ null mutant in which the
172 mutant versions of *SKN7* expressed from their own promoter have been integrated at the
173 *RPS1* locus. Results presented in Fig. 2 indicated that integration of a wild-type (WT) copy of
174 *SKN7* at the *RPS1* locus in the $\Delta\Delta skn7$ null mutant restored oxidative stress resistance and
175 morphogenesis to wild-type levels. Furthermore they showed that the F76/L83 residues in the
176 HSF domain were essential for *SKN7* function in oxidative stress defense and morphogenesis.
177 In contrast, the T484 or T496 residues in the receiver domain appeared essential for oxidative

178 stress defense and partly dispensable for morphogenesis while the D474 residue was
179 dispensable for both oxidative stress resistance and morphogenesis.

180 Taken together, these results indicate that *C. albicans* Skn7 plays important roles in oxidative
181 stress defense and morphogenesis, mediated by both the HSF and receiver domains. They also
182 show that Skn7 is not required for sustaining cell wall and osmotic stress in *C. albicans*,
183 contrasting with what has been observed in *S. cerevisiae* and other fungal species and
184 possibly reflecting a divergent function in *C. albicans*.

185

186 **Transcript profiling reflects the function of *SKN7* in oxidative stress defense and** 187 **morphogenesis**

188 As *SKN7* encodes a transcription factor, we sought to identify genes that are under its control
189 and whose differential expression may explain its contribution to morphogenesis and
190 oxidative stress adaptation. We performed a transcript profiling analysis of CEC4228 (P_{TET} -
191 *SKN7-HA₃*), a WT strain expressing an ectopic allele of *SKN7* fused to a triple-hemagglutinin
192 epitope (HA₃)-encoding sequence, and placed under the control of the tetracycline derivative-
193 inducible promoter (P_{TET} , Fig. 3A), and of CEC3785, with no gene fused to the P_{TET} promoter,
194 as a negative control. The expression and stability of Skn7-HA₃ were verified by Western
195 blotting (Fig. 3B), and we confirmed that the phenotype associated to the overexpression of
196 *SKN7-HA₃* was indistinguishable from that observed upon overexpression of the WT *SKN7*
197 allele (Fig. 1D).

198 We examined the global gene expression levels through the comparison of samples treated
199 with 3 $\mu\text{g}\cdot\text{mL}^{-1}$ anhydrotetracycline (ATc), for 2 h and 4 h and untreated samples grown in
200 parallel (Experimental Procedures). At this concentration, ATc achieves the same
201 overexpression levels as 50 $\mu\text{g}\cdot\text{mL}^{-1}$ doxycycline, with less inhibition of hyphal growth
202 (Chauvel *et al.*, 2012), and the cells do not exhibit any detectable phenotype (Fig. S1). We

203 found 76 upregulated and 36 downregulated genes after 2 h of induction, and 196 upregulated
204 and 280 downregulated genes after inducing for 4 h (fold change ≥ 1.5 , $P < 0.05$; Table S2).
205 Many of the genes highly upregulated at 2 h and/or 4 h are involved directly or indirectly in
206 oxidative stress adaptation (*CCPI*, *GPX2*, *TSAI* and *TSAIB* encoding peroxidases, *TRR1*
207 encoding a thioredoxin reductase) and filamentation (*CPHI*, *CZF1*, *SFL2*, *TEC1*, *UME6*
208 encoding TFs involved in hyphal morphogenesis, *EED1/DEF1* (further referred to as *EED1*)
209 encoding a regulator of *UME6* expression and consequently sustained hyphal growth, *ECE1*,
210 *HWP1*, *IHD1*, *RBT1* encoding hyphal-induced cell wall proteins, *PTP3* encoding a hypha-
211 induced tyrosine phosphatase; Fig. 3C and Table S2). Conversely, genes whose expression is
212 repressed under filament-inducing conditions were repressed upon *SKN7* overexpression (eg
213 *ALS2/4*, *ALD5*, *ASR1*, *ASR2*, *CHT3*, *MNN1*, *PIR1*, *RHD1/BMT2*, *RME1*, *YWP1*, *C1_05160C*,
214 *C2_07630C*, *C4_02930W*; Fig. 3C). Treatment of the negative control strain CEC3785 with
215 ATc had a mild impact on gene expression at 2h, since only 18 and 20 genes were
216 upregulated and downregulated with maximum fold-change values of 1.9 and -1.8,
217 respectively (Table S3), and only one overlapped with *Skn7* OE data. At 4 h, the number of
218 upregulated and downregulated genes was 44 and 27, respectively, with maximum fold-
219 change values of 2.5 and -1.9, and only 4 overlapped with *Skn7* OE data (Table S3). The genes
220 present in both analyses were not considered further.

221 We validated our data using RT-qPCR analyses on selected genes (*CPHI*, *CZF1*, *EED1*,
222 *SFL2* and *UME6* encoding regulators of morphogenesis; *HWP1* and *IHD1* that encode
223 hyphal-specific cell wall proteins; *TSAI* and *GPX2* encoding effectors of oxidative stress
224 response and *SKN7* as a control). We confirmed that these genes were significantly
225 upregulated upon *SKN7-HA₃* overexpression (Fig. 3D). We also confirmed that *EED1*, *UME6*
226 and *TSAI* were significantly upregulated upon untagged *SKN7* overexpression (not shown).
227 Notably, in this assay, *BRG1* that encodes another TF positively regulating morphogenesis

228 (Cleary *et al.*, 2012; Du *et al.*, 2012), showed elevated expression upon *SKN7-HA₃*
229 overexpression (Fig. 3D), a result that differed from those in the global gene expression
230 profiling which did not detect differential expression of *BRG1* (Table S2). In contrast, and as
231 predicted from the transcript profiling, *EFG1* expression was not altered upon *SKN7-HA₃*
232 overexpression (Fig. 3D).

233 Using gene-set enrichment analyses (Mootha *et al.*, 2003; Subramanian *et al.*, 2005; Lopes *et*
234 *al.*, 2010), we built a comprehensive map of the metabolic processes and pathways that were
235 significantly enriched among the upregulated (red spheres) and downregulated (blue spheres)
236 genes at time-points 2 h (Fig. 4A) and 4 h (Fig. 4B) following induction of *SKN7-HA₃*
237 expression. At time-point 2 h (Fig. 4A), there was a remarkable enrichment of processes
238 pertaining to antioxidant activity, response to oxidative shock and mitochondrial function
239 (energy generation, cellular respiration, mitochondrial components). Of particular interest was
240 the overrepresentation of gene sets that are downregulated in a $\Delta\Delta tsal$ mutant and those
241 upregulated upon *RASI/CYRI* deletion or *CDC5* depletion, suggesting alterations in signal
242 transduction pathways. Cell wall genes, filamentous growth and interaction with the host were
243 also significantly overrepresented among the upregulated genes, with a noticeable enrichment
244 of the glucose/hexose metabolic process, whereas genes involved in amino acid and
245 carboxylic acid metabolism were significantly enriched among the downregulated genes (Fig.
246 4A, blue network nodes). At time-point 4 h, processes pertaining to oxidative stress response,
247 mitochondrial function, filamentous growth, interaction with the host and signal transduction
248 activity were still enriched (Fig. 4B, upper right and left subnetworks), while genes involved
249 in ribosome biogenesis and assembly, chromatin integrity, cell-cycle control and DNA
250 damage response became overrepresented among the *SKN7*-induced genes (Fig. 4B, large
251 subnetworks). Among the downregulated genes, there was a significant enrichment of
252 processes linked to peroxisome function, lipid catabolism, protein degradation (proteasome),

253 and vacuolar transport/membrane biogenesis (Fig. 4B, blue subnetwork nodes).

254 Taken together, our transcript profiling data point to an unambiguous function of *C. albicans*
255 Skn7 in oxidative stress response and cellular morphogenesis. It also suggests an important
256 reprogramming of cellular metabolism and signal transduction activity upon *SKN7*
257 overexpression.

258

259 **Skn7 binds to the promoter of genes involved in oxidative stress adaptation and** 260 **morphogenesis**

261 To test if the genes that emerged from the transcript profiling analysis were directly regulated
262 by Skn7, a chromatin immunoprecipitation coupled with hybridization to genome-wide tiling
263 array (ChIP-chip) was performed. We compared binding of the HA₃-tagged Skn7 to untagged
264 control following 2 h of ATc induction (see Experimental Procedures). We used the
265 CisGenome peak finding algorithm to identify Skn7 binding sites (Ji *et al.*, 2008). In total, 74
266 binding peaks were found at the promoters for individual ORFs, and 38 peaks were found in
267 regions shared between two ORFs in opposite orientations (Table 1 and Table S4). Additional
268 *bona fide* binding peaks associate with 16 target genes were not detected by the peak-finding
269 algorithm and were added to our target list (Table S2). In some instances, e.g. long promoter
270 regions, more than one peak were detected, suggesting the presence of multiple binding sites
271 (for example at the *EFG1* promoter, Fig. 5A and Table S4). This analysis revealed that, as
272 previously shown for the hyphal growth regulators Sfl1 and Sfl2 (Znaidi *et al.*, 2013), most of
273 the regions bound by Skn7 exhibited binding sites for at least one other morphogenesis
274 regulator, including Efg1 (Lassak *et al.*, 2011), Ndt80 (Sellam *et al.*, 2009), Sfl1 and Sfl2
275 (Znaidi *et al.*, 2013), (Table 1 and Table S4).

276 We performed independent ChIP-qPCR experiments at the promoter regions of *EEDI*, *RBT4*
277 *SFL1* and *TSAI*, confirming the enrichment of these regions in the Skn7 immunoprecipitated

278 fraction, and comparing with regions that did not show any enrichment (*SFL2*, *TEC1*, *UME6*)
279 (Fig. 5B).

280 We also conducted motif-enrichment analyses of the DNA sequences encompassing 250 bp
281 around peak summits in Skn7 binding data, using three motif discovery algorithms: the RSA-
282 tools (RSAT) peak-motifs (<http://rsat.ulb.ac.be/rsat/>, Thomas-Chollier *et al.*, 2011; 2012), the
283 MEME suite (Bailey *et al.*, 2009) and SCOPE (genie.dartmouth.edu/scope/; Carlson *et al.*,
284 2007; see Experimental Procedures for details). The three algorithms identified the same three
285 motifs, CACAAA (Ndt80 binding site), TATGCA (Efg1 binding site), and TCTAGA (Fig.
286 5C), highlighting the high frequency of co-binding of regulators at the same promoter regions.
287 The latter sequence, similar to the *S. cerevisiae* transcription factor Abf2 binding motif
288 (involved in mitochondrial function; JASPAR database, <http://jaspar.genereg.net/>), lacks a
289 GAA triplet motif characteristic of heat-shock factor elements (Yamamoto *et al.*, 2005) as
290 observed for a subset of *S. cerevisiae* Skn7 binding motifs (www.yeasttract.com).

291 In order to get a global view of the Skn7 transcriptional network we combined our genome-
292 wide expression and location data (Fig. 5D). We intersected the genes regulated either at 2 h
293 or at 4 h with the targets bound by Skn7, and found an overlap between the genes bound and
294 regulated at the two different time points, with the exception of *ROBI*, which is bound and
295 regulated only at 2 h. Overall, 22 genes were bound and upregulated while 18 were bound and
296 downregulated (Fig. 5D) suggesting that Skn7 carries a dual, both positive and negative,
297 transcriptional regulatory activity. Among the upregulated targets, we found genes involved
298 in the oxidative stress response (*TSAI*, *TSAIB*, *SSU81*) and encoding cell wall proteins
299 (*MP65*, *IHD1*, *SIMI*, *PGA62*). Interestingly, only one gene encoding a regulator of hyphal
300 growth, namely *EED1*, was directly controlled by Skn7. *EED1* is a regulator of hyphal
301 maintenance, governing *UME6* and *NRG1* expression (Zakikhani *et al.*, 2007; Martin *et al.*,
302 2011). The strongly downregulated targets were represented mainly by genes encoding either

303 yeast-specific cell wall proteins (*ALS2*, *YWPI*) and cell wall remodeling enzymes (*CHT3*) or
304 proteins involved in galactose metabolism (*GALI0*, *GALI*).

305 Taken together, these data suggest that Skn7 acts as both activator and repressor of gene
306 expression and directly controls the expression of a specific set of genes involved in oxidative
307 stress response and morphogenesis.

308

309 **Skn7 is required for the expression of morphogenesis regulators and oxidative stress**
310 **defense genes during hyphal development on solid media**

311 Results presented above suggested that Skn7 might be directly and indirectly involved in the
312 positive control of genes involved in morphogenesis, and that lack of regulation of these
313 genes might explain the morphogenetic defect of the $\Delta\Delta skn7$ mutant on filamentation-
314 inducing solid media. Therefore, we measured the expression levels of genes encoding
315 regulators of morphogenesis, namely *EED1*, *CPHI* and *UME6* in the $\Delta\Delta skn7$ mutant
316 (CEC4220) and $\Delta\Delta skn7+SKN7$ complemented strain (CEC4682) grown on Spider solid
317 medium where the $\Delta\Delta skn7$ mutant shows defective filamentation (Fig. 1C). We also measured
318 the expression levels of hypha-specific genes (*HWPI*, *IHDI*) and oxidative stress defense
319 genes (*TSAI*, *GPX2*) that our results above had shown to be directly or indirectly regulated by
320 Skn7. The Spider medium-dependent induction was significantly decreased for all tested
321 genes in the $\Delta\Delta skn7$ mutant when compared with the complemented strain (Fig. S2). Notably,
322 the expression level of *HWPI* remained very high in the $\Delta\Delta skn7$ mutant, suggesting that Skn7
323 is partly dispensable for the upregulation of this gene in hypha-inducing solid medium.

324 These results confirm that the presence of *SKN7* is required for full upregulation of several
325 positive regulators of morphogenesis and hypha-specific genes on hypha-inducing solid
326 medium, which may explain the filamentation defect of the $\Delta\Delta skn7$ mutant. Strikingly, Skn7
327 also appears to contribute to the upregulation of genes encoding enzymes involved in

328 oxidative stress defence during growth on a hypha-inducing solid medium suggesting that it
329 may provide a link between morphogenesis and protection against oxidative stress.

330

331 ***SKN7*-dependent filamentation on solid media requires the function of several**
332 **transcription factors**

333 Results presented above imply that the filamentation phenotype associated with the
334 overexpression of *SKN7* and *SKN7*-HA₃ might be dependent on the function of transcription
335 factors whose genes are transcriptionally activated by Skn7 in a direct or indirect manner. In
336 order to test these hypotheses, we overexpressed *SKN7* in strains harboring null mutations for
337 *EEDI* (directly regulated by Skn7), *CPHI*, *CZF1*, *SFL2*, *TEC1* and *UME6* (all indirectly
338 regulated by Skn7) as well as *EFG1* and *SFL1* (both bound but not regulated by Skn7).
339 Growing on ATc had no effect on the knockout strains colony phenotypes (Fig. S3).

340 As stated previously, overexpressing *SKN7* in a WT strain altered the morphology of the
341 colonies that appeared wrinkled and contained filamentous cells (Chauvel *et al.*, 2012; Fig. 1E
342 and Fig. 6A). Conversely, *SKN7* overexpression in the $\Delta\Delta eed1$, $\Delta\Delta efg1$ or $\Delta\Delta cph1$ strains did
343 not yield wrinkled colonies, and only yeast cells were found within these colonies (Fig. 6A),
344 suggesting that these three genes are essential for Skn7 function in morphogenesis. Ectopic
345 overexpression of *SKN7* in the $\Delta\Delta ume6$ strain yielded slightly wrinkled colonies with a
346 mixture of yeast cells and pseudohyphae (Fig. 6A), suggesting that *SKN7* involvement in
347 morphogenesis only partially relies on Ume6. In contrast, *SKN7* overexpression in the $\Delta\Delta sfl1$,
348 $\Delta\Delta sfl2$, $\Delta\Delta tec1$ and $\Delta\Delta czf1$ strains was still able to activate the morphogenetic switch (Fig.
349 6A), suggesting that Skn7 either functions downstream of these four transcription factors, or
350 bypasses their unrelated contribution to morphogenesis. These results indicated that the
351 functionality of *CPHI*, *EEDI*, *EFG1*, and to some extent *UME6*, is required for Skn7 to exert
352 its function.

353 To further explore the genetic interaction between *SKN7* and other genes encoding
354 transcriptional activators of morphogenesis, we examined the phenotype of the ectopic
355 overexpression of the morphogenesis regulators *EED1*, *UME6*, *CPH1*, *SFL2* and *TEC1* in the
356 $\Delta\Delta skn7$ background (strains CEC4813, CEC4650, CEC4474 and CEC4649, respectively; Fig.
357 6B). These transcription factors are known to activate HSGs and hence to promote
358 filamentation (Liu *et al.*, 1994; Schweizer *et al.*, 2000; Lane *et al.*, 2001; Giusani *et al.*, 2002;
359 Banerjee *et al.*, 2008; Martin *et al.*, 2011; Song *et al.*, 2011) and their overexpression in a WT
360 background resulted in wrinkled colonies (Chauvel *et al.*, 2012 and Fig. 6B, upper panel).
361 Strikingly, we found that overexpression of *UME6*, *CPH1* or *TEC1* in the $\Delta\Delta skn7$ strain did
362 not promote colony wrinkling, indicating that *SKN7* is required for the function of these genes
363 in activating the morphogenetic switch on solid medium. The absence of phenotype linked to
364 *UME6* overexpression in the *skn7* mutant is highly surprising, since it has been shown that it
365 is able to bypass the *efg1* (Zeidler *et al.*, 2009) or *eed1* (Martin *et al.*, 2011) knockout
366 mutants. In contrast, overexpression of either *EED1* or *SFL2* resulted in the same wrinkled
367 colony morphology reported in the WT background, although overexpression of *SFL2* in
368 $\Delta\Delta skn7$ led to a more invasive phenotype (Fig. 6B, lower panel). These latter results indicated
369 that Eed1 and Sfl2 act downstream of Skn7 in the regulation of morphogenesis.

370 Among the genes upregulated upon *SKN7-HA₃* overexpression we found *CZFI* that encodes a
371 positive regulator of morphogenesis when *C. albicans* grows in embedded conditions (Brown
372 *et al.*, 1999). Therefore, we tested the behaviour of the $\Delta\Delta skn7$ strain (CEC4220) when grown
373 in YPS agar embedded conditions at room temperature and observed defective filamentous
374 colony formation (Fig. 6C), as reported for $\Delta\Delta czf1$ (Brown *et al.*, 1999). Furthermore, ectopic
375 overexpression of either *SKN7* or *CZFI* in the $\Delta\Delta skn7$ background restored vigorous
376 filamentation in embedded conditions (Brown *et al.*, 1999; Fig.6C). We showed that ATc had
377 no effect on filamentation in embedded conditions on SC5314 and that overexpressing *SKN7*

378 in a WT background did not extensively modify embedded filamentation (Fig. S4). To test the
379 connection between *SKN7* and *CZF1*, we overexpressed either *SKN7* in $\Delta\Delta czf1$ (CEC4815) or
380 *CZF1* in $\Delta\Delta skn7$ (CEC5069). As reported in Fig. 6C, *SKN7* overexpression in $\Delta\Delta czf1$ results
381 in hyper-filamentous colonies as observed in the WT background. Similarly, *CZF1*
382 overexpression conveys a hyper-filamentous phenotype in $\Delta\Delta skn7$ background. These results
383 suggest that overexpression of *SKN7* can bypass the requirement of *CZF1* for embedded
384 filamentous growth, and vice versa.

385 Taken together, our epistasis analyses point to a complex interplay between *SKN7* and genes
386 encoding important regulators of *C. albicans* morphogenesis. *SkN7* requires the function of
387 *Cph1*, *Eed1*, *Efg1* and *Ume6* to induce morphogenesis, whereas *Cph1*, *Tec1* and *Ume6*
388 require a functional *SkN7*. Moreover, our results show that *SKN7* overexpression can bypass
389 the requirement for *Tec1*, *Sfl2* and *Czf1* for filamentation.

390

391 **SkN7 is required to limit the accumulation of reactive oxygen species (ROS) content**
392 **associated to filamentation on solid medium**

393 Results presented above indicate that *SkN7* plays a direct and important role in regulating
394 expression of genes involved in morphogenesis and oxidative stress response on solid
395 medium, possibly explaining the filamentation defect and oxidative stress sensitivity of the
396 $\Delta\Delta skn7$ mutant on solid medium. Schröter *et al.* (2000) have shown that the yeast-to-hyphal
397 transition is associated with the accumulation of Reactive Oxygen Species (ROS). Therefore,
398 we questioned whether *SkN7* might be involved in controlling the accumulation of ROS
399 during the yeast-to-hyphal transition.

400 We measured the ROS content in the WT SC5314, $\Delta\Delta skn7$ (CEC4220) and $\Delta\Delta skn7+SKN7$
401 (CEC4682) strains grown on different solid media inducing filamentation or promoting yeast
402 growth (solid Spider, YPD + serum and Lee's medium at 37°C or YPD agar and Lee's

403 medium at 25°C, respectively) for 4 h. We used the cell permeable non fluorescent probe
404 2',7'-dichlorofluorescein diacetate (DCFH-DA) which, upon intracellular de-esterification
405 and oxidation by ROS, is converted to highly fluorescent 2',7'-dichlorofluorescein. A
406 significant increase of intracellular ROS species was observed in the knockout strain as
407 compared either to the complemented strain or to the WT reference strain when grown on all
408 tested media (Fig. 7A). Conversely, no difference was detected between the 3 strains grown
409 on YPD or Lee's medium at 25°C (Fig. 7A). We also measured the ROS content when these
410 strains were exposed to oxidative stress (5 mM H₂O₂ for 4 h) or in liquid medium inducing
411 filamentation or promoting yeast growth (Lee's medium at 37°C or 25°C for 4 h,
412 respectively). Results in Fig. 7B show that *SKN7* was required to limit the intracellular
413 accumulation of ROS upon oxidative stress. In contrast, while ROS accumulated upon
414 filamentation in liquid Lee's medium, this was not affected by Skn7 functionality.
415 Finally, we tested whether ROS accumulation is systematically associated with defective
416 filamentous growth on solid medium. Results presented in Fig. 7D showed that the ROS
417 content in several mutants defective for filamentation, such as $\Delta\Delta eed1$, $\Delta\Delta ume6$ and $\Delta\Delta cph1$
418 (or only partially defective, e.g. $\Delta\Delta tec1$) (Fig. 7C), did not increase, indicating that ROS
419 detoxification upon filamentation on solid media is a Skn7-specific function (Fig. 7D).
420 Taken together, these results indicate that *SKN7* is required to limit the accumulation of ROS
421 when *C. albicans* is exposed to conditions that promote the yeast-to-hypha transition on solid
422 medium.

423

424 **Skn7 function in morphogenesis is uncoupled from its role in limiting intracellular ROS** 425 **accumulation**

426 To investigate if Skn7 functions in morphogenesis and ROS quenching were decoupled, we
427 analyzed the accumulation of ROS in the *SKN7* site-specific mutants. Results presented in

428 Fig. 8 showed that the HSF domain mutant (F76A,L83A) and the two strains with mutations
429 in the receiver domain affecting filamentation (T484A and T496A) were still able to limit
430 ROS accumulation. In contrast, the strain with a mutation in the receiver domain that did not
431 affect filamentation (D474A) accumulated ROS to the same extent as the $\Delta\Delta skn7$ mutant (Fig.
432 8). These data supported our hypothesis that Skn7 has a role in morphogenesis that is
433 decoupled from its role in quenching intracellular ROS accumulation upon solid-media
434 induced morphogenesis. Indeed these two roles could be linked to different residues in the
435 protein. While the F76 and L83 residues in the HSF DNA-binding domain and the T484 and
436 T496 in the receiver domain are critical for hyphal growth, the D474 residue in the receiver
437 domain is important for decreasing the intracellular ROS content, but has no role in
438 filamentous growth.

439

440 **DISCUSSION**

441 *Conserved and divergent functions of Skn7*

442 Two-component signal transduction pathways play important roles in the biology of
443 prokaryotic and eukaryotic microorganisms (Wuichet *et al.*, 2010; Capra and Laub, 2012;
444 Hérivaux *et al.*, 2016). Response-regulator systems occur in most fungi (Schaller *et al.*, 2011),
445 and compelling evidence indicates that they originate from multiple independent horizontal
446 gene transfer events from bacteria to eukaryotes, that occurred after the divergence of
447 Archaea, Eubacteria and Eukarya (Wuichet *et al.*, 2010, Schaller *et al.*, 2011). It is tempting
448 to speculate that in fungi the response-regulator systems, which originally relied on a unique
449 regulatory site (phosphorylatable aspartate) to efficiently respond to a wide variety of
450 environmental stresses, have progressively evolved to regulate additional niche-specific
451 biological processes through the selection of supplementary functional domains (*e.g.* HSF
452 domain) and regulatory amino acid residues within the regulatory receiver domain.

453 In *C. albicans*, three histidine kinases (Sln1, Chk1 and Nik1), a histidine phosphotransfer
454 protein (Ypd1) and three response regulators (Ssk1, Skn7 and Srr1) have been identified
455 (Nagahashi *et al.*, 1998; Calera *et al.*, 1999, 2000; Singh *et al.*, 2004; Bruce *et al.*, 2011), all
456 involved in the oxidative stress response (Bruce *et al.*, 2011). Ssk1 and Srr1 are localized in
457 the cytosol and in mitochondria, respectively, and are required for stress adaptation, cell wall
458 biosynthesis, morphogenesis and virulence (Chauhan *et al.*, 2003; Bruce *et al.*, 2011; Desai *et*
459 *al.*, 2011; Mavrianos *et al.*, 2013). Skn7 is localized in the nucleus (our unpublished results),
460 and is thought to act downstream of Sln1 and Ypd1 (Ketela *et al.*, 1998; Li *et al.*, 1998; Li *et*
461 *al.*, 2002). Our results combined with those of Singh *et al.* (2004) show that Skn7 is necessary
462 for *C. albicans* tolerance to H₂O₂-induced oxidative stress while being dispensable for
463 adaptation to cell wall stress (mediated by the chitin synthase inhibitor nikkomycin Z or the
464 cell wall stressors Congo Red and Calcofluor White), osmotic stress and membrane stress
465 (mediated by amphotericin B or miconazole). Importantly, Skn7 also appears to be required
466 for *C. albicans* filamentation on solid medium (Singh *et al.*, 2004 and this study) or under
467 embedded growth conditions (this study) while it is dispensable for morphogenesis in liquid
468 medium (this study). Our characterization of site-specific mutants in the *SKN7* gene shows
469 that the Skn7 DBD is essential for both oxidative stress tolerance and filamentation. This is
470 reminiscent of the role of the Skn7 DBD in oxidative stress tolerance in *S. cerevisiae* (Raitt *et*
471 *al.*, 2000) and is consistent with Skn7 functions in oxidative stress tolerance and filamentation
472 being primarily exerted through transcriptional regulation (see below). A typical feature of
473 Skn7 is the occurrence in its C-terminal moiety of a receiver domain characteristic of
474 response regulator proteins (Bourret, 2010). In *S. cerevisiae*, three key residues have been
475 identified in this domain that play important roles in Skn7 function: D427, the Aspartate
476 targeted by the histidine kinase (D474 in *CaSkn7*), is important for Sln1/Ypd1-mediated
477 adaptation to cell wall/osmotic stress (Morgan *et al.*, 1997; Li *et al.*, 2002) while T437 and

478 T449 (T484 and T496 in *CaSkn7*) are important for *S. cerevisiae* tolerance to oxidative stress
479 (He *et al.*, 2009). The D474 residue in *C. albicans* Skn7 does not participate in H₂O₂-induced
480 oxidative stress tolerance, (as reported for *S. cerevisiae* D427, Morgan *et al.*, 1997), nor
481 morphogenesis. The observation that Skn7 is not necessary for *C. albicans* adaptation to cell
482 wall/osmotic stress suggests that in *C. albicans* Skn7 is - at least for functions related to
483 filamentation, oxidative stress and osmotic stress tolerance - disconnected from the
484 Sln1/Ypd1 pathway, contrary to what has been observed in *S. cerevisiae*. This is supported by
485 distinct phenotypes of the *C. albicans* *SLN1*, *YPD1* and *SKN7* null mutants when exposed to
486 osmotic, cell wall or oxidative stress or induced to undergo the yeast-to-hypha transition
487 (Nagahashi *et al.*, 1998; Yamada-Okabe *et al.*, 1999; Kruppa *et al.*, 2004; Singh *et al.*, 2004;
488 Mavrianos *et al.*, 2014). Mutations of the T484 and T496 residues in the *C. albicans* Skn7
489 receiver domain affect both oxidative stress tolerance and morphogenesis. In *S. cerevisiae*
490 these residues are phosphorylated and the fact that converting them into alanine alters *C.*
491 *albicans* Skn7 functions in oxidative stress tolerance and morphogenesis suggests that they
492 are also subject to phosphorylation in this species. The nature of the protein kinases that are
493 responsible for such phosphorylation is unknown. Protein kinase A (PKA) plays a central role
494 in the regulation of morphogenesis and it is noteworthy that null mutants for the *TPK1*-
495 encoded PKA catalytic subunit are defective for morphogenesis predominantly on solid
496 media, such as the $\Delta\Delta skn7$ mutant, whereas null mutants for the *TPK2*-encoded PKA catalytic
497 subunit show filamentation defects primarily in liquid media (Sonneborn *et al.*, 2000;
498 Bockmühl *et al.*, 2001; Biswas *et al.*, 2007). This suggests that Tpk1 might be one of the
499 protein kinases responsible for activation of Skn7 under solid filamentation-inducing
500 conditions and possibly, oxidative stress as inactivation of *TPK1* leads to oxidative stress
501 sensitivity (Giacometti *et al.*, 2009). Similarly, *chk1* KO mutants exhibit some peroxide
502 sensitivity (Li *et al.*, 2004) as well as a hyphal growth defect on serum agar (Yamada-Okabe

503 *et al.*, 1999), suggesting that Chk1 could also participate in Skn7 regulation under these
504 specific conditions.

505 Skn7 function in oxidative stress tolerance appears highly conserved as *SKN7* null mutants in
506 almost all fungal species tested show sensitivity to externally applied oxidative stress
507 (Charizanis *et al.*, 1999; Lee *et al.*, 1999; Singh *et al.*, 2004; Wormley *et al.*, 2005; Ruprich-
508 Robert *et al.*, 2008; Saijo *et al.*, 2010; Zhang *et al.*, 2016). Our results in *C. albicans* show
509 that Skn7 regulates the expression of a number of genes encoding ROS-detoxifying enzymes
510 in a direct or indirect manner. Yet, as our main focus was to understand the contribution of
511 *SKN7* to the yeast-to-hypha transition, we have limited our study to evaluating the impact of
512 overexpressing *SKN7* on the expression of these genes and have not assessed the extent to
513 which a *SKN7* knockout influences their expression when *C. albicans* is exposed to oxidative
514 stress. *C. albicans* adaptation to oxidative stress is under the control of the Cap1 TF (Zhang *et*
515 *al.*, 2000; Wang *et al.*, 2006). In this respect, it has been shown that *C. albicans* *TSA1*
516 transcriptional regulation upon H₂O₂-induced oxidative stress depends only on Cap1, and is
517 independent of Skn7 (Urban *et al.*, 2005). Moreover, our search for Skn7 targets did not
518 reveal co-occurrence with Cap1 targets (Znaidi *et al.*, 2009), suggesting that these two TFs do
519 not necessarily cooperate in the response of *C. albicans* to oxidative stress. Further work will
520 be needed to understand how Skn7 contributes to oxidative stress adaptation in *C. albicans*,
521 and more generally, the mechanisms that lead to Skn7 activation upon fungal cell exposure to
522 external oxidative stress. As mentioned above this probably involves phosphorylation of
523 threonine residues in the receiver domain but may also rely on the production of an oxidized
524 form (da Silva Dantas *et al.*, 2010).

525 *Skn7 and other regulators of morphogenesis*

526 Skn7 involvement in hyphal morphogenesis is reminiscent of previous observation in *S.*
527 *cerevisiae* showing that inactivation of *SKN7* impairs pseudohyphal growth while

528 overexpression of *SKN7* or its paralog, *HMS2*, triggers more vigorous pseudohyphal growth
529 under certain conditions (Lorenz and Heitman, 1998). Yet, little is known about the
530 mechanisms by which *SKN7* impacts pseudohyphal growth in this species. Here, we have
531 used a combination of transcript profiling, chromatin immunoprecipitation and epistasis tests
532 with overexpression and knockout mutants in order to decipher some of the mechanisms by
533 which Skn7 positively regulates hyphal morphogenesis in *C. albicans*. Taken together, our
534 results show that *SKN7* is highly interconnected with other regulators of morphogenesis in *C.*
535 *albicans*. Skn7 positively regulates the expression of Brg1, Cph1, Czf1, Eed1, Sfl2, Tec1 and
536 Ume6 that are all positive regulators of morphogenesis. Moreover, while Cph1, Eed1, Efg1
537 and Ume6 appear required for Skn7 function in morphogenesis, Skn7 appears required for the
538 functions of Cph1, Tec1 and Ume6 in this process. We demonstrated that Skn7 is one of the
539 components of the network of TFs that controls morphogenesis in *C. albicans*, although we
540 are aware of the limitations of our overexpression-based approach, particularly when
541 searching for Skn7-regulated or -bound genes. Characterization of biological processes such
542 as the switch between the white and the opaque forms of yeast growth, biofilm formation or
543 commensal vs. pathogenic growth has led to the identification of highly interwoven TF
544 networks allowing the orchestration of gene expression programs according to specific *C.*
545 *albicans* lifestyle within the host environment (Linde *et al.*, 2010; Nobile *et al.*, 2012;
546 Hernday *et al.*, 2013; Pérez *et al.*, 2013; Fox *et al.*, 2015). For instance, Bcr1, Brg1, Efg1,
547 Flo8, Gal4, Ndt80, Rfx2, Rob1 and Tec1 form a TF network regulating biofilm growth
548 (Nobile *et al.*, 2012; Fox *et al.*, 2015). This network (or part of this network) might also be
549 associated to hyphal differentiation as biofilm formation is intimately linked to hyphal growth
550 and many of the TFs in this network are regulators of hyphal growth, including Brg1, Efg1,
551 Flo8, Ndt80, Rfx2, Rob1 and Tec1 (Stoldt *et al.*, 1997; Schweizer *et al.*, 2000; Cao *et al.*,
552 2006; Hao *et al.*, 2009; Sellam *et al.*, 2010; Vandeputte *et al.*, 2011; Du *et al.*, 2012).

553 Strikingly, when assessing whether Skn7 was binding to Cph1-, Efg1-, Ndt80-, Rob1-, Sfl2-
554 or Tec1 targets (binding data for Flo8 and Rfx2 are lacking), we found 2.2 % of the 91 Skn7
555 binding regions to be shared by six of these TFs, 11% by five, 19.8 % by four , 20.9 % by
556 three, 23 % by two and 9.9 % by one (Table S4). Only 13.2 % of the Skn7 binding regions
557 were unique to Skn7 including those upstream of *TSA1* and *TSA1B* (Table S4). This is
558 illustrated in the network displayed in Fig. 9 and supports the contribution of Skn7 to an
559 interwoven network of transcription factors regulating morphogenesis in *C. albicans*. In
560 agreement with these observations, a search for motifs enriched in the Skn7-bound regions
561 retrieved consensus binding sites for Efg1 and Ndt80, suggesting that Skn7 may cooperate
562 with at least these two TFs to regulate morphogenesis. A similar observation was made for
563 Sfl1 and Sfl2 and concomitant binding of these TFs and Efg1 to DNA in a condition-
564 dependent manner was demonstrated (Znaidi *et al.*, 2013). Despite these observations, it is
565 interesting to note that, among known regulators of morphogenesis, only *EED1* was both
566 bound and regulated by Skn7. Eed1 is unique to *C. albicans* and has been shown to regulate
567 morphogenesis through Ume6, allowing maintenance of hyphal growth (Martin *et al.*, 2011).
568 Our results suggest that Skn7 activation by overexpression or growth on solid filament-
569 inducing media or under embedded growth leads to *EED1* direct activation, which is required
570 for Skn7-dependent filamentation to proceed. Whether Eed1 is the sole mediator of Skn7
571 positive regulation of morphogenesis remains to be further evaluated.

572

573 *Skn7 and the regulation of intracellular ROS accumulation*

574 There is a striking overlap between Skn7 involvement in morphogenesis on solid media and
575 adaptation to H₂O₂-induced oxidative stress. Indeed, Skn7 is required for both processes, with
576 mutations in the HSF and receiver domains affecting them in similar ways. Here, we have
577 observed that a distinctive feature of Skn7 among *C. albicans* regulators of morphogenesis is

578 to limit the intracellular accumulation of ROS when *C. albicans* is induced to undergo the
579 yeast-to-hypha transition on solid media. Although this would suggest a link between Skn7
580 functions in morphogenesis and prevention of intracellular ROS accumulation, it does not
581 appear to be the case. Indeed, while site-specific mutations in Skn7 affecting morphogenesis
582 do not result in increased ROS accumulation, mutating Asp474 into Ala has no effect on
583 morphogenesis but leads to elevated levels of intracellular ROS. Strikingly, mutants of the
584 DNA binding domain, although not able to bind target DNAs (not shown) did not accumulate
585 ROS, suggesting an interaction between Skn7 and a cofactor, through Asp474
586 phosphorylation. Therefore, the functions of Skn7 in morphogenesis and prevention of
587 intracellular ROS accumulation are decoupled. More surprisingly, the functions of Skn7 in
588 adaptation to H₂O₂-induced oxidative stress and prevention of intracellular ROS accumulation
589 appear similarly decoupled. This may reflect different mechanisms in handling ROS
590 according to whether they are of extracellular or intracellular origin, the latter possibly
591 involving the Sln1/Ypd1 pathway as it is dependent upon Asp474 which, in *S. cerevisiae*, is
592 proposed to be subject to phosphotransfer from Ypd1 (Ketela *et al.*, 1998).

593

594 In summary, *C. albicans* Skn7 appears to play important roles in morphogenesis and
595 adaptation to intracellular and extracellular oxidative stress, regulating the expression of
596 numerous genes encoding morphogenetic regulators, hypha-specific proteins and ROS
597 detoxifying enzymes, while having lost its contribution to the adaptation to cell wall/osmotic
598 stress. It is striking that despite such roles, the lack of *SKN7* does not impact virulence of *C.*
599 *albicans* in animal models of systemic candidiasis (Singh *et al.*, 2004; our unpublished
600 results) or oropharyngeal candidiasis (not shown). This suggests redundancy in the regulators
601 of morphogenesis and adaptation to oxidative stress, allowing a bypass of the requirement for
602 Skn7 that is observed *in vitro*. Further work will be needed to understand such bypass and to

603 decipher the mechanisms by which Skn7 is activated in response to morphogenetic cues and
604 to extracellular and intracellular ROS and the precise interplay between them.

605

606 **Experimental Procedures**

607 **Strains and growth media**

608 Strains used in this study are listed in Table S1. *C. albicans* strains were grown in YPD (1%
609 yeast extract, 2% peptone, 1% dextrose) or SD minimal medium (0.67% yeast nitrogen base
610 (YNB, Difco) with 0.4 or 2% glucose) (Sherman, 1991) supplemented if necessary with
611 arginine, histidine and uridine at 20 mg.L⁻¹ and 2% agar for solid medium. Filamentation was
612 assayed either with YPD supplemented with 10% Fetal Bovine Serum (FBS), or Spider
613 medium (1% nutrient broth, 1% mannitol, 0.2% K₂HPO₄) (Liu *et al.*, 1994), or modified
614 Lee's medium (O'Connor *et al.*, 2010).

615 To test the oxidative stress response, 3 mM H₂O₂ (Fisher Bioblock Scientific) were added to
616 YPD 2% agar plates. For the osmotic and cell wall stress, 1.5 M NaCl, 200 µg.mL⁻¹ and 300
617 µg.mL⁻¹ CongoRed, and 20 µg.mL⁻¹ and 30 µg.mL⁻¹ CalcoFluor White were added to YPD
618 2% agar plates.

619 P_{TET}-driven over-expression was induced by addition of 3 µg.mL⁻¹ anhydrotetracycline (ATc-
620 Fisher Bioblock Scientific) in YPD medium, and incubation at 30°C in the dark (Chauvel *et*
621 *al.*, 2012).

622 For embedded conditions, YPS medium (1% yeast extract, 2% bacto peptone, 2% sucrose)
623 supplemented with uridine 20 mg.L⁻¹, with 2% or 1% agar (molten) was prepared. The strains
624 grown overnight were diluted and mixed to a molten agar layer poured over a YPS 2% agar
625 layer. A second slab of YPS 2% agar was poured over the cells. For overexpression, 3 µg.mL⁻¹
626 ¹ATc was added to each layer. Plates were incubated in the dark at room temperature.

627 **Plasmid construction and generation of mutant strains**

628 Sequences of the primers used for strains and plasmids constructions are listed in Table S6.
629 DNA fragments containing mutations into Alanine of chosen residues (D474, T484, T496)
630 were amplified by PCR and cloned into TOPO-BLUNT cloning vector (Invitrogen). The
631 resulting plasmids, carrying a mutated fragment of *SKN7* were digested by selected restriction
632 enzymes and ligated in pENTRY-*SKN7* (Chauvel *et al.*, 2012) carrying the wild-type gene,
633 cut similarly. For the double mutant F86A,L83A, two overlapping PCR fragments were
634 generated, and fused by PCR. All the resulting ENTRY plasmids were checked by Sanger
635 sequencing.

636 The GatewayTM system allowed to construct a series of plasmids in which the *skn7* mutants
637 were placed either under the control of the inducible P_{TET} promoter or the native P_{SKN7}
638 promoter, and their 3' end fused to a HA₃ tag, by recombining either the Cip10- P_{TET} -GTW-
639 HA₃ vector or the Cip10- P_{SKN7} -GTW-HA₃ vector with the pENTRY vectors obtained
640 previously. To build Cip10- P_{SKN7} -GTW-HA₃, the promoter region of *SKN7* was PCR
641 amplified with SB095 and SB096, and the fragment used to replace P_{TET} from Cip10- P_{TET} -
642 GTW-HA₃. *E. coli* TOP10 (Invitrogen) or DH5 cells were used for cloning and maintaining
643 the plasmid constructs (Cabral *et al.*, 2012).

644 The Cip10- P_{TET} or - P_{SKN7} derivatives were integrated at the *RPS1* locus by transforming *C.*
645 *albicans* strains CEC2907 and CEC4617, respectively (Table S1) using the lithium-acetate
646 transformation protocol (Walther and Wendland, 2003). Transformants were selected on SD
647 medium. The integrations of the constructs were verified by colony-PCR with CipUL and
648 CipUR primers (Table S6) and the protein overexpression was checked by Western Blot as
649 described in Chauvel *et al.*, 2012 (see below).

650 A *SKN7* heterozygous mutant was generated as described in Noble and Johnson (2005).
651 Briefly, transformation with a linear DNA fragment bearing *HIS1* as a selectable marker
652 amplified from pSN52 (Noble and Johnson, 2005) and a short region of homology to the

653 sequences that flank the *SKN7* open reading frame were performed using the *C. albicans*
654 SN76 strain using SKN7KO_F and SKN7KO_R primers (Table S6). To delete the second
655 allele, the hererozygous strain was transformed by a linearized plasmid carrying the selectable
656 marker *ARG4* flanked by *SKN7* upstream and downstream regions.

657 The correct replacement of the alleles and deletion of the gene were checked by Southern
658 blotting using a DIG labeled probe as recommended by the supplier (Roche).

659 The *CZF1* overexpression plasmid (Chauvel *et al.*, 2012) was modified as follow: a fragment
660 encompassing *CZF1* 3' end was PCR amplified with primers SB204 and SB205 on SC5314
661 DNA, cut with *PstI* and *BstZ17I* and inserted in the same sites of Clp-*P_{TET}-CZF1*, to generate
662 Clp-*P_{TET}-CZF1*ΔattB2.

663 **Total protein extraction and Western Blotting**

664 Overnight cultures were diluted to OD₆₀₀ = 0.2 in 10 mL of either YPD or YPD + 3 μg.mL⁻¹
665 ATc. After 2 h of growth at 30°C cells were collected by centrifugation at 1,000 x g for 5
666 min. Pellets were resuspended in 150 μL of ice-cold TE buffer (10 mM Tris.HCl pH 7.5, 1.5
667 mM EDTA) supplemented with a protease inhibitor cocktail (cOmplete™ Roche). 100 μL of
668 ice-cold glass beads were added and the tubes were vortexed 4 times for 30 seconds with 30
669 seconds incubation on ice in-between. The extracts were centrifuged for 5 minutes at 1,000 x
670 g and the supernatants were transferred in a new tube.

671 Loading buffer (0.25 M Tris.HCl pH 6.8, 50% glycerol, 0.05% bromophenol blue) was added
672 and the samples were heated at 70°C for 10 minutes.

673 Proteins were separated on an Invitrogen 8% NuPage Bis-Tris gel and transferred to
674 nitrocellulose membrane by iBlot® using the standard P3 program. Detection of the HA₃-
675 tagged proteins was performed by Western blotting using monoclonal Anti-HA peroxidase
676 conjugate-High Affinity (clone 3F10 Roche) at a 1:2000 dilution. For the internal control an
677 anti α-tubulin antibody (AbD Serotec, diluted 1:1000) was used. Briefly, membranes were

678 stripped with a buffer containing 2% SDS, 0.7% β -mercaptoethanol and 125 mM Tris.HCl pH
679 6.8 for 30 min at 50°C and re-probed with the primary rat anti- α -tubulin antibody and a
680 secondary goat anti-rat HRP-conjugated antibody (1:2000, Santa Cruz). Membranes were
681 revealed with an ECL kit (GE Healthcare).

682 **Chromatin Immunoprecipitation (ChIP) and ChIP-on-chip**

683 Two independent cultures of CEC4228 (P_{TET} - $SKN7$ - HA_3) and CEC4848 (P_{TET} - $SKN7$,
684 untagged control strain were grown overnight at 30°C in 2 mL of YPD. Strains were diluted
685 at $OD_{600} = 0.2$ and incubated in YPD for 1 h at 30°C, then 3 $\mu\text{g.mL}^{-1}$ ATc was added
686 followed by growth during 2 additional hours.

687 The ChIP procedure was performed as described in Znaidi *et al.* (2013). Immunoprecipitation
688 of soluble chromatin was performed by incubating clarified sonicated extracts (500 μL out of
689 ~ 700 μL) overnight at 4°C with a mouse monoclonal anti-HA antibody (Santa Cruz Biotech)
690 coupled with magnetic beads (Pan-mouse immunoglobulin G Dynabeads; Dynal Biotech,
691 Brown Deer, WI). The concentration of the purified immunoprecipitated DNA was measured
692 by Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies). DNA was labeled as
693 described by Drouin and Robert
694 ([http://www.ircm.qc.ca/LARECHERCHE/axes/Biologie/Chromatine/Documents/ProtocoleIR](http://www.ircm.qc.ca/LARECHERCHE/axes/Biologie/Chromatine/Documents/ProtocoleIRCM_LevureYeast1.pdf)
695 [CM_LevureYeast1.pdf](http://www.ircm.qc.ca/LARECHERCHE/axes/Biologie/Chromatine/Documents/ProtocoleIRCM_LevureYeast1.pdf)). Briefly, the IP DNA (40 μL out of 50 μL) fragments were blunted
696 with T4 DNA polymerase and ligated to unidirectional linkers. The DNA was amplified by
697 ligation-mediated PCR in the presence of aminoallyl-modified dUTP. Labeling was carried
698 out post-PCR using monoreactive Cy dye N-hydroxysuccinimide esters (Cy5/Cy3
699 monoreactive dye packs; Amersham Biosciences) that react specifically with the aminoallyl-
700 modified dUTP [5-(3-aminoallyl)-2' deoxyuridine-5'-triphosphate; Sigma-Aldrich]. Labeled
701 IP DNA from the tagged (Cy5) and untagged control strains were mixed and hybridized to
702 previously described *C. albicans* whole-genome tiled DNA microarrays from Assembly 20

703 (Tuch *et al.*, 2008; GEO platform accession # GPL13696). Images of Cy5 and Cy3
704 fluorescence intensities were generated by scanning arrays using an Axon Autoloader
705 4200AL scanner and analyzed with the GenePix software version 7.0 (Molecular Devices).
706 Data normalization (Quantile) and peak finding were conducted using CisGenome (Ji and
707 Wong, 2005; Ji *et al.*, 2008). The Integrated Genomics Viewer (IGV) software was used for
708 visualization of the ChIP-chip results (Robinson *et al.*, 2011; Thorvaldsdóttir *et al.*, 2013)
709 (Fig. 5A). The complete Skn7 binding datasets are available in Table S4. ChIP-on-chip raw
710 data were deposited at GEO under accession number GSE85275, and transcriptomics data
711 under the accession number GSE85276.

712 **ChIP-qPCR**

713 ChIP-qPCR was used to validate the ChIP-on-chip experiment. The primers used to assess
714 binding enrichment at the observed ChIP peaks were designed with Primer3 and their
715 sequences are listed in Table S7. ChIP samples were diluted to a concentration of $2.5 \text{ pg} \cdot \mu\text{L}^{-1}$
716 in $100 \mu\text{L}$ of water. $2 \mu\text{L}$ of these dilutions were mixed with $4 \mu\text{L}$ of a primer mix at $10 \mu\text{M}$
717 each and $10 \mu\text{L}$ of 2x Takyon™ Rox SYBR MasterMix dTTP Blue (Eurogentec) in a total
718 volume of $20 \mu\text{L}$. Reactions were processed in a MicroAmp Optical 96-Well Reaction Plate
719 (Applied Biosystems) using an Eppendorf *realplex*⁴ Mastercycler real-time PCR instrument
720 (Eppendorf) with 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min and 50 cycles at 95°C
721 for 15 sec and at 58°C for 1 min, followed by melting-curve generation to rule out
722 amplification of unspecific products. Data were analyzed using the Realplex software version
723 2.2 (Eppendorf). For each experiment, threshold cycle (C_T) values were determined using the
724 *realplex* software. Skn7 binding enrichment was calculated using relative quantification
725 according to the $2^{-\Delta\Delta\text{CT}}$ method, as follows: $\Delta C_T = C_T(\text{target}) - C_T(\text{TEF3 reference})$ calculated
726 in both tagged (Skn7-HA₃) and untagged samples, and $\Delta\Delta C_T = \Delta C_T(\text{tagged}) -$
727 $\Delta C_T(\text{untagged})$, where $C_T(\text{TEF3 reference})$ is the C_T for the *TEF3* amplicon (primers TEF3-

728 F/R, Table S7). The qPCR assays were performed three times independently using two or
729 three biological replicates each time. A two-tailed Student's *t*-test was applied by comparing,
730 for a given ChIP target, the n-fold enrichment values of the selected target gene to those of the
731 corresponding *ACT1* control, followed by false discovery rate according to Benjamini and
732 Hochberg (1995). Statistical significance threshold was $P < 0.05$.

733 **RNA isolation, first-strand cDNA synthesis and microarray hybridization.**

734 Overnight cultures of CEC4228 and CEC3785 (P_{TET} -Gtw) were diluted at $OD_{600} = 0.2$ in 50
735 mL of YPD or YPD + ATc $3 \mu\text{g}\cdot\text{mL}^{-1}$ and grown for 0 h, 2 h and 4 h at 30°C . Total RNA was
736 extracted using the hot phenol method (Wodicka *et al.*, 1997) as described in Znaidi *et al.*
737 (2013). First-strand cDNA synthesis was performed using the SuperScript III indirect cDNA
738 labeling system (Invitrogen) from $20 \mu\text{g}$ of total RNA. Labeling was performed as described
739 above. The Qiagen-PCR Purification kit was used to remove the unincorporated aminoallyl-
740 dUTP. A phosphate wash buffer (5 mM Phosphate buffer ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 8.5), 80%
741 ethanol) and a phosphate elution buffer (4 mM Phosphate buffer $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 8.5)
742 were used instead of the kit supplied buffers. The Cy3- and Cy5-labeled pellets were
743 resuspended in $10 \mu\text{L}$ of DNase-free water. $2.5 \mu\text{L}$ of 10X blocking agent and $12.5 \mu\text{L}$ of 2X
744 hybridization buffer (Agilent Technologies) were added to each sample. A 3-minute
745 incubation at 95°C was followed by a snap cool on ice during 1 minute and the samples were
746 hybridized to a *C. albicans* expression array (Agilent Technologies) designed such that two
747 non-overlapping probe sets target each of 6,105 *C. albicans* ORFs from Assembly 19 for a
748 total of 15,744 probes (GEO acc# GPL19932, Znaidi *et al.*, 2013). Hybridization was
749 performed by an overnight incubation at 65°C .

750 **Gene expression microarray data analyses**

751 The microarrays were scanned using the Axon Autoloader 4200AL scanner (Molecular
752 Devices, Downingtown, PA). The images were analyzed with GenePix Pro 6.1.0.2 software

753 (Molecular Devices, Downington, PA). The GenePix Results (GPR) files were imported into
754 the Arraypipe 2.0 software (Hokamp *et al.*, 2004). Following spot filtering and bad spot
755 flagging, global signal intensities were normalized using Loess normalization and replicates
756 slides (n=3) were combined and P-values calculated using standard Student's *t*-test.
757 Microarray data were deposited at GEO under accession number GSE85276.

758 **RNA extraction and quantitative RT-PCR analyses**

759 For the OE experiments, the strain CEC4228 was treated for 4 h with 3 $\mu\text{g}\cdot\text{mL}^{-1}$ ATc, Three
760 biological replicates were processed. Cells were collected by centrifugation at 1,000 x g,
761 washed with RNAlater[®] RNA Stabilization Solution (Ambion) and mechanically disrupted
762 with glass beads using a Fastprep (MP Biomedicals). Total RNA was extracted either using
763 the hot-phenol method (see above) or using the RNeasy Mini Kit (Qiagen) according to the
764 manufacturer's instructions. RNA was resuspended in 50 μL of DEPC-treated water, and the
765 concentration of RNA samples was determined by NanoVue Plus (GE Healthcare) readings,
766 while the RNA integrity was determined by electrophoresis on 1.5% agarose gel.

767 For comparison between the *skn7* Δ/Δ (CEC4220) and *skn7* Δ/Δ + *SKN7* (CEC4682) strains
768 grown on solid media (Spider and YPD) the following procedure was used. 100 μL of o/n
769 cultures were plated on solid media covered with a sterile cellophane sheet and incubated at
770 30°C for 3 (YPD) or 8 (Spider) days. Cellophane sheets were transferred in 50 mL tubes,
771 washed with 10 mL of RNase-free H₂O and cells resuspended through vigorous vortexing.
772 Cells were harvested and stored at -80°C until the RNA extraction (hot-phenol method).
773 Three independent biological replicates were used.

774 Five μg of RNA were used to synthesize the cDNA using SuperScript III (Invitrogen);
775 alternatively, 500 ng of RNA were used with SuperScript IV (Invitrogen). Reactions were
776 done according to the supplier's instructions.

777 Primers were designed using Primer3 and the sequences are listed in Table S7. Quantitative

778 PCR was performed using 1 μ L of a 1/100 dilution of cDNA, mixed with 7.5 μ L of Takyon™
779 mastermix and 2 μ L of each primer at 10 μ M, in a final volume of 15 μ L. The PCR program
780 was as described above. Three technical replicates of each sample were processed. *TEF3* and
781 *RPL18 C. albicans* genes were used as calibrators.

782 **Bioinformatic analyses**

783 Gene Set Enrichment analyses were conducted as described previously (Sellam, 2014).
784 Briefly, the GSEA PreRanked tool (<http://www.broadinstitute.org/gsea/>) (Subramanian, 2005)
785 was used (default parameters) to determine if the lists of ranked genes (CEC4848 data at 2 h
786 and 4 h sorted from the highest upregulated genes to the highest downregulated ones)
787 displayed a significant bias with any gene sets from a compendium of published microarray
788 data and GO term categories/pathways (Sellam, *et al.*, 2014; Chatr-Aryamontri, 2015).
789 Enrichment scores for the GSEA analyses are available in Table S5. The Cytoscape
790 EnrichmentMap plugin (Merico, 2010) was used to further visualize the GSEA network using
791 the default parameters.

792 **ROS measurement**

793 To measure the intracellular ROS levels, 100 μ L of overnight cultures were plated on a sterile
794 cellophane layer (Dominique Dutscher) placed on solid Spider or YPD plates and incubated at
795 30°C for 4 h. Cells were collected by washing the cellophane with 1% phosphate-buffered-
796 saline (PBS) buffer and recovered in fresh YPD medium with 20 μ g.mL⁻¹ of 2',7'-
797 dichlorodihydro-fluorescein diacetate (DCFH-DA, Sigma-Aldrich) for 30 min at 30°C. Cells
798 were harvested and washed 3 times with 1% PBS buffer, 100 μ L were transferred in Nunc™
799 flat black MicroWell plates (Thermo Scientific), ROS levels were quantified by measuring
800 the fluorescence in a Tecan Infinite M200 microplate reader, using an excitation filter of
801 486±9 nm and an emission filter of 535±20 nm. The results were normalized to cell
802 concentration by measuring the optical density (OD₆₀₀).

803 **Microscopy and Image analyses**

804 Colony morphology was imaged with a Leica M80 Stereomicroscope at a x25 magnification.

805 Images were captured with a DMC 2900 camera, using the Leica Application Suite (LAS)

806 imaging software.

807 Cells phenotypes were observed with a Leica DM RXA microscope (Leica Microsystems).

808 Images were captured with a Hamamatsu ORCA II-ER cooled CCD camera, using Openlab

809 software version 3.5.1 (Improvision Inc.).

810 Images were then processed with Adobe Photoshop CS6.

811

812

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831

832 **Authors Contributions**

833 Conceived the experiments: CdE, SBB, SLL, SZ, VB. Performed the experiments: FS, SBB,
834 SZ, VB, VC, VL. Analyzed the data: CdE, FS, SBB, SLL, SZ, VB. Wrote the paper: CdE,
835 SBB, SZ, VB.

836

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Table 1. List of Skn7 targets

		Target 1 ¹				Target 2 ²					# of TF binding ⁹	
H ³	Ox ⁴	orf19.XX ⁵	Gene ID ⁶	Gene ⁷	FC ⁸	H	Ox	orf19.XX	Gene ID	Gene		FC
		orf19.3337 ¹⁰	C1_01510W		2.3							4
		orf19.3338 ¹⁰	C1_01510W		4.8							4
		orf19.4527	C1_01980W	<i>HGT1</i>	-2.1							4
		orf19.3669	C1_02120C	<i>SHA3</i>	-1.5							3
		orf19.3670	C1_02130C	<i>GAL1</i>	-2.0			orf19.3672	C1_02150W	<i>GAL10</i>	-1.6	3
		orf19.3674	C1_02170C	<i>GAL102</i>	NS			orf19.3675	C1_02180W	<i>GAL7</i>	NS	3
		orf19.4459	C1_03870C		3.4							3
		orf19.1065	C1_04300C	<i>SSA2</i>	NS							1
		orf19.53	C1_04990C		-1.6			orf19.54	C1_05000W	<i>RHD1</i>	-4.1	3
		orf19.450	C1_05150C	<i>PPM2</i>	NS							6
		orf19.6202	C1_07030C	<i>RBT4</i>	7.3							4
		orf19.5070	C1_07990C		NA			orf19.5071	C1_08000W	<i>NRP1</i>	NS	0
		orf19.5105	C1_08270C	<i>MED15</i>	NS							2
		orf19.4772	C1_09140C	<i>SSU81</i>	2.0			orf19.4773	C1_09150W	<i>AOX2</i>	2.4	1
		orf19.4792	C1_09320C		1.5			orf19.4793	C1_09330W		NS	3
		orf19.4818	C1_09540W		NS							2
		orf19.1835	C1_10640C	<i>PEA2</i>	1.5							0
		orf19.2333	C1_10840C		NS			orf19.2332	C1_10850W		NS	3
		orf19.656	C1_11470C	<i>DPP1</i>	NS			orf19.655	C1_11480W	<i>PHO84</i>	-1.6	3
		orf19.4941	C1_13140C	<i>TYE7</i>	NS							2
		orf19.4991	C1_13570W	<i>MPT5</i>	-1.7							0
		orf19.4997	C1_13610C	<i>KIS2</i>	NS			orf19.4998	C1_13620W	<i>ROB1</i>	-1.8	5
		orf19.5032	C1_13940W	<i>SIM1</i>	2.4							4
		orf19.2026	C2_00970C	<i>UBP13</i>	NS			orf19.2023	C2_01000W	<i>HGT7</i>	NS	2
		orf19.1486	C2_01750C		-1.6							4
		orf19.1490	C2_01780W	<i>MSB2</i>	NS							3
		orf19.1505	C2_01930C		-1.6							1
		orf19.909	C2_03220C	<i>STP4</i>	NS							2
		orf19.868	C2_03520C	<i>ADAEC</i>	-1.8							2
		orf19.851	C2_03690C	<i>MNN42</i>	NS			orf19.850	C2_03700W	<i>NTAI</i>	NS	2
		orf19.829	C2_03940C	<i>SCH9</i>	NS			orf19.828	C2_03950W		1.9	3
		orf19.6869	C2_05570C		NS			orf19.6871	C2_05580W		2.1	5
		orf19.6874	C2_05640W	<i>BAS1</i>	NS							5
		orf19.3618	C2_08590W	<i>YWPI</i>	-2.4							5
		orf19.1779	C2_10030C	<i>MP65</i>	1.7							2
		orf19.5437	C3_00320W	<i>RHR2</i>	NS							1
		orf19.5431	C3_00360W		1.8							2
		orf19.1690	C3_01550C	<i>TOS1</i>	NS			orf19.1687	C3_01560W	<i>PRP43</i>	NS	2
		orf19.6937	C3_03800W	<i>PTR22</i>	-1.6							2
		orf19.5902	C3_04480C	<i>RAS2</i>	NS			orf19.5903	C3_04490W	<i>RAX1</i>	NS	2
		orf19.5992	C3_05170W	<i>WOR2</i>	NS							4
		orf19.6000	C3_05220W	<i>CDR1</i>	NS							1
		orf19.6983	C3_05450C		-1.6			orf19.6984	C3_05460W		NS	4
		orf19.7381	C3_06000W	<i>AHR1</i>	NS			orf19.7380	C3_05990C		NS	4
		orf19.7417	C3_06180C	<i>TSA1</i>	2.8							0
		orf19.7398.1	C3_06330W	<i>TSA1B</i>	2.7							0
		orf19.7436	C3_06470W	<i>AAF1</i>	NS							3
		orf19.7455	C3_06660C		NS							0
		orf19.6816	C3_06860C		-2.7							0
		orf19.6798	C3_07020W	<i>SSN6</i>	1.5							4
		orf19.6736	C3_07550C		1.7			orf19.6734	C3_07540C	<i>TCC1</i>	NS	4
		orf19.4167	C4_00750C		NS			orf19.4166	C4_00760W	<i>ZCF21</i>	NS	4
		orf19.2765	C4_02390W	<i>PGA62</i>	1.7							3
		orf19.2726	C4_02720C		-1.5			orf19.2724	C4_02740W		NS	5
		orf19.2723	C4_02750W	<i>HIT1</i>	NS							4
		orf19.5311	C4_04010W		NS							2
		orf19.5292	C4_04170C	<i>AXL2</i>	NS							1
		orf19.1415	C4_04320W	<i>FRE10</i>	1.8							2
		orf19.740	C4_05110C	<i>HAP41</i>	NS							3
		orf19.2892	C4_06420W		NS							4
		orf19.3134	C4_06750C		NS			orf19.3133	C4_06760W	<i>GUT2</i>	NS	2
		orf19.920	C5_00680W	<i>RMT2</i>	NS			orf19.921	C5_00670C	<i>HMS1</i>	1.5	3

	orf19.4145	C5_01500C	ZCF20	NS		orf19.4146	C5_01510W	SMD3	NS	0
	orf19.1106	C5_03880C		NS						3
	orf19.1285	C5_04050W		-2.2						3
	orf19.3997	C5_05050W	ADH1	NS						0
	orf19.4211	C6_00480C	FET31	2.2		orf19.4210	C6_00490W		NS	3
	orf19.85	C6_00840W	GPX2	4.4						3
	orf19.691	C6_02010C	GPD2	NS						2
	orf19.5536	C6_02660C		NS						2
	orf19.5619	C6_03310W		2.2						0
	orf19.5741	C6_03700W	ALS1	2.1						4
	orf19.5760	C6_03850C	IHD1	2.9						4
	orf19.7094	C7_00280W	HGT12	NS						1
	orf19.7053	C7_00660W	GAC1	-1.8		orf19.7056	C7_00630C	DIP53	NS	5
	orf19.6586	C7_01430C		-1.7						2
	orf19.6514	C7_02040C	CUP9	NS						5
	orf19.5170	C7_02910W	ENA21	NS		orf19.5171	C7_02890C	PMT1	NS	1
	orf19.7152	C7_04210C		NS		orf19.7150	C7_04230W	NRG1	NS	5
	orf19.2823	CR_02640W	RFG1	NS		orf19.2822	CR_02630C		NS	3
	orf19.2842	CR_02850C	GZF3	NS		orf19.2843	CR_02860W	RHO1	NS	2
	orf19.473	CR_03920C	TPO4	2.9						2
	orf19.6313	CR_04800W	MNT4	2.1						0
	orf19.454	CR_05990C	SFL1	NS		orf19.451	CR_06000W	SOK1	NS	4
	orf19.3869	CR_06140W		-1.7						5
	orf19.699	CR_06650C		NA		orf19.700	CR_06660W	SEO1	NS	0
	orf19.2356	CR_07060C	CRZ2	NS						5
	orf19.610	CR_07890W	EFG1	NS						6
	orf19.7550	CR_09780C	IFA14	1.8						2
	orf19.7561	CR_09880W	EED1	3.3						4
	orf19.7585	CR_10100C	INO1	NS		orf19.7586	CR_10110W	CHT3	-2.9	1
	orf19.7592	CR_10160W	FAA4	NS						4

1168

¹For bound promoter regions shared by two ORFs, name of the upstream gene

1169

²For bound promoter regions shared by two ORFs, name of the downstream gene

1170

³H: green colour indicates involvement or inferred role in hyphal growth

1171

⁴Ox: blue colour indicates involvement or inferred role in oxidative stress response

1172

⁵orf19 nomenclature at the Candida albicans genome database (CGD)

1173

⁶systematic name according to CGD

1174

⁷gene name according to CGD

1175

⁸FC: Fold change (Table S2) NS: not significant. NA: not on the microarray. Highest values are reported, regardless of the

1176

time point.

1177

⁹Number of transcription factors found to bind the same promoter region as Skn7 (Cph1: Borneman *et al.*, 2007; Efg1,

1178

Ndt80, Rob1, Tec1: Nobile *et al.*, 2012; Sfl2: Znaidi *et al.*, 2013). Detailed information is found in Table S4 – Co-binding)

1179

¹⁰orf19.3337 and orf19.3338 have been fused in CGD Assembly 22

1180 **FIGURE LEGENDS**

1181 **Fig. 1. Characterization of *SKN7* null and P_{TET} -driven overexpression mutants.**

1182 **(A) Schematic representation of Skn7 in *S. cerevisiae* and *C. albicans*.** The conserved key
1183 residues are shown in the HSF-like DNA-binding domain (black; HSF) and in the receiver
1184 domain (dark grey). The coiled coil domain is also represented (light grey).

1185 **(B) Phenotypic analysis of *skn7* deletion mutant on filament-inducing media.** *C. albicans*
1186 reference strain SC5314, $\Delta\Delta skn7$ (CEC4220) and $\Delta\Delta skn7+SKN7$ (CEC4682) strains were
1187 tested for their ability to filament on Spider and YPD+10% serum media either solid (left
1188 panel) or liquid (right panel). Pictures were taken after 8 days at 30°C (solid) or 18 h at 37°C
1189 (liquid).

1190 **(C) Phenotypic analysis of *skn7* deletion mutant upon exposure to cell wall or osmotic**
1191 **stress.** Serial dilutions of *C. albicans* SC5314, $\Delta\Delta skn7$ and $\Delta\Delta skn7+SKN7$ strains were
1192 spotted on YPD supplemented or not with different stress agents: 200 $\mu\text{g.mL}^{-1}$ and 300
1193 $\mu\text{g.mL}^{-1}$ Congo Red, 20 $\mu\text{g.mL}^{-1}$ and 30 $\mu\text{g.mL}^{-1}$ CalcoFluor White (cell-wall stress) or 1.5 M
1194 NaCl (osmotic stress). The $\Delta\Delta hog1$ mutant (CEC2293), sensitive to osmotic stress, and the
1195 $\Delta\Delta pga59, pga62$ mutant (CEC377), sensitive to cell wall perturbing agents, were used as
1196 controls. Phenotypes were observed after 3-4 days of growth at 30°C.

1197 **(D) Comparison between Skn7-HA₃ and Skn7 un-tagged strains.** *C. albicans* strains
1198 overexpressing either *SKN7* untagged or HA₃-tagged (CEC4848 and CEC4228, respectively)
1199 were either spotted on YPD agar- or grown in liquid YPD-supplemented or not with 3 $\mu\text{g.mL}^{-1}$
1200 ¹ ATc. The SC5314 strain was used as a control. Pictures of colonies were taken after 3 days
1201 of incubation at 30°C, whereas liquid cultures were induced o/n. The phenotypes observed for
1202 the untagged (middle panel) or HA₃-tagged (right panel) strains were similar, indicating the
1203 HA₃-tag did not interfere with protein function.

1204

1205 **Fig. 2. Specific residues are responsible for Skn7 functions**

1206 **(A) Phenotypic analysis of Skn7 mutants upon H₂O₂-induced oxidative stress.** Reference
1207 strain SC5314, $\Delta\Delta skn7$ (CEC4220) and $\Delta\Delta skn7+SKN7$ (CEC4682) strains and strains
1208 harboring *skn7* mutant alleles under the control of the P_{SKN7} promoter in the $\Delta\Delta skn7$
1209 background (D474A: CEC4272; T484A: CEC4273; T496A: CEC4274; F76A,L83A:
1210 CEC4277), were spotted on YPD supplemented or not with 3 mM H₂O₂ and incubated at
1211 30°C for 2 days.

1212 **(B) Phenotypic analysis of Skn7 mutants grown on filament-inducing medium.** *C.*
1213 *albicans* $\Delta\Delta skn7$ and $\Delta\Delta skn7+SKN7$ strains and strains harboring the site-specific *skn7*
1214 mutant alleles under the control of P_{SKN7} in the $\Delta\Delta skn7$ background were spotted on Spider
1215 medium and incubated at 30°C for 10 days.

1216

1217 **Fig. 3. Skn7 transcript profiling**

1218 **(A) Schematic representation of the SKN7-HA₃ tagging cassette that allowed the**
1219 **expression of the fusion protein.** A triple HA-tag was inserted in frame with the *SKN7*
1220 coding sequence. The tagged allele was placed under the control of P_{TET}, induced in the
1221 presence of ATc.

1222 **(B) Induced OE of SKN7-HA₃.** *SKN7-HA₃* overexpression strain (CEC4228) was grown in
1223 YPD supplemented or not with 3 $\mu\text{g}\cdot\text{mL}^{-1}$ ATc. Whole cell extracts were collected after 2 h at
1224 30°C and were separated by SDS-PAGE and probed with peroxidase-coupled antibody
1225 allowing the detection of HA₃-tagged proteins. Tub1 was used as an internal control.

1226 **(C) Heat maps of the 30 highest log₂-transformed relative gene expression levels in the**
1227 **P_{TET}-SKN7-HA₃ induced versus non-induced transcriptomics data.** The most upregulated
1228 (YELLOW) and downregulated (BLUE) genes at the three time points (0 h, 2 h and 4 h) are
1229 indicated with their name or the CGD Assembly 22 nomenclature. * the microarray bore

1230 probes for orf19.3337 and orf19.3338, which have been fused in CGD release 22 under the
1231 name C1_01510W. Red dots indicate known regulators of the yeast-to-hypha transition.
1232 Green dots indicate hypha-specific genes and genes that have been shown to be upregulated
1233 upon the yeast-to-hypha switch. Green circle indicate gene with an inferred link to
1234 morphogenesis. Blue dots indicate genes linked to the response to oxidative stress. Dots with
1235 two colors are the sign of a dual function. Heat maps were obtained using Java TreeView
1236 1.1.6r4.

1237 **(D) Expression analysis of genes selected from *SKN7* transcriptomics data.** Expression of
1238 *SKN7*, *BRG1*, *CPH1*, *CZF1*, *EED1*, *SFL2*, *UME6*, *HWPI*, *IHD1*, *TSA1* and *GPX2* genes was
1239 quantified by RT-qPCR using specific primers for the selected regions and RNA from
1240 CEC4228 grown in the presence or absence of 3 $\mu\text{g.mL}^{-1}$ ATc. *ACT1* and two genes (*EFG1*
1241 and *SFL1*) not upregulated upon Skn7-HA₃ OE are used as controls. The experiments were
1242 performed on 3 biological replicates and error bars show the SD. Statistical significance was
1243 assigned by performing 2-tailed Student's *t*-tests comparing the same strain in inducing and
1244 non-inducing conditions, followed by false discovery rate according to Benjamini and
1245 Hochberg (1995). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

1246

1247 **Fig. 4. Gene-set enrichment among *Skn7* transcriptionally-modulated genes.** Gene set
1248 enrichment analysis maps at time points 2 h (A) and 4 h (B) post-induction of *SKN7* gene
1249 expression. Functional enrichment among the upregulated and downregulated genes is
1250 depicted with red and blue spheres, respectively. Sphere size is proportional to the number of
1251 genes. Network motif thickness is proportional to the extent of overlap in the list of genes
1252 between each node (blue/red spheres). Selected functional categories are indicated with
1253 dashed circles. Networks were constructed using Cytoscape version 3.4.0.

1254

1255 **Fig. 5. Genome-wide location of *Candida albicans* Skn7 *in vivo***

1256 **(A) Skn7 binding at selected *C. albicans* genomic regions *in vivo*.** Skn7 occupancies along
1257 selected areas from the *C. albicans* genome (Assembly 20, the corresponding target is
1258 indicated at the left of each panel). Plotted are the relative signal intensities of the 60-bp
1259 probes covering the whole *C. albicans* genome following enrichment of the HA₃-tagged
1260 Skn7-coimmunoprecipitated DNA relative to DNA from a mock immunoprecipitation (*i.e.* in
1261 an untagged-strain background). Some binding-enrichment signals extend beyond the
1262 maximum graduations (*y*-axis). The orientation of each ORF is depicted by the arrowed black
1263 rectangle. Binding maps were generated using the IGV genome browser (Thorvaldsdóttir *et*
1264 *al.*, 2012).

1265 **(B) ChIP-qPCR analysis of selected genes from *SKN7* transcriptomics data.** Relative
1266 DNA enrichment following immunoprecipitation of HA₃-tagged Skn7 was assessed at the
1267 promoters of *EED1*, *RBT4*, *SFL1* and *TSI1* by qPCR assays using specific primers for the
1268 selected regions. Promoter regions of *SFL2*, *TEC1* and *UME6* that did not show enrichment
1269 following immunoprecipitation of HA₃-tagged Skn7, were used as negative controls. The
1270 experiments were performed on 3 biological replicates of the HA₃-tagged (CEC4228) and
1271 untagged (CEC4848) strains grown in YPD supplemented with 3 $\mu\text{g.mL}^{-1}$ ATc for 2 h. Error
1272 bars show the SD. Statistical significance was assigned by performing 2-tailed Student's *t*-
1273 tests that compares binding enrichment values at the indicated loci to those at the *RPL18*
1274 locus (** $p \leq 0.01$, *** $p \leq 0.001$; negative control: $P < 0.05$).

1275 **(C) Discovery of Skn7 binding motif and Skn7 transcriptional module.** Motif-enrichment
1276 analyses of the DNA sequences encompassing 250 bp around peak summits in Skn7 binding
1277 data using three algorithms (RSAT, MEME and SCOPE, see Experimental procedures). The
1278 motifs identified are CACAAA (Ndt80 binding site), TATGCA (Efg1 binding site), and
1279 TCTAGA.

1280 **(D) Intersection of transcript profiling and ChIP-on-chip data.** Venn diagrams of the
1281 overlap between genes modulated (red circles, upregulated; green circles, downregulated;
1282 gene expression fold-change cut-off ≥ 1.5 ; P-value cut-off ≤ 0.05) and bound (blue circle) by
1283 Skn7 at 4 h. The name of the genes or their Assembly 22 nomenclature and the functional
1284 categories are shown in the boxes.

1285

1286 **Fig. 6. Genetic interactions between *SKN7* and other genes encoding regulators of**
1287 **morphogenesis.**

1288 **(A) Phenotypic analysis of *SKN7* ectopic OE in different KO backgrounds. Upper panel:**

1289 Mutants strains with an inducible overexpression of *SKN7* in $\Delta\Delta eed1$ (CEC4653), $\Delta\Delta ume6$
1290 (CEC4458), $\Delta\Delta cph1$ (CEC4462), $\Delta\Delta sfl1$ (CEC3836), $\Delta\Delta sfl2$ (CEC3424), $\Delta\Delta tec1$
1291 (CEC4464), $\Delta\Delta czf1$ (CEC4815), or $\Delta\Delta efg1$ (CEC3420) were spotted on YPD (-ATc) or YPD
1292 + 3 $\mu\text{g.mL}^{-1}$ ATc (+ATc) and incubated at 30°C for 3 days. One out of several clones tested for
1293 each strain is shown. **Lower panel:** Cells from the colonies shown in the upper panel were
1294 recovered and observed under the microscope; smooth colonies are made of yeast cells, while
1295 wrinkled colony morphology is linked to the presence of hyphal cells. Finally the colony
1296 morphology observed for *SKN7* OE in the $\Delta\Delta ume6$ (CEC4458) background conveyed in a
1297 mix of yeast and pseudohyphal cells.

1298 **(B) Phenotypic analysis of ectopic OE of different morphogenesis regulators in WT and**

1299 **$\Delta\Delta skn7$ backgrounds.** Strains with an inducible overexpression of *EED1* (CEC4812), *UME6*
1300 (CEC2994), *CPH1* (CEC1084), *SFL2* (CEC4393) or *TEC1* (CEC1085) in WT background
1301 (upper panel) were spotted on YPD (-ATc) or YPD + 3 $\mu\text{g.mL}^{-1}$ ATc (+ATc) and incubated at
1302 30°C for 3 days. The OE of *EED1* (CEC4813), *UME6* (CEC4650), *CPH1* (CEC4656), *SFL2*
1303 (CEC4474) or *TEC1* (CEC4649) in $\Delta\Delta skn7$ background was also assessed (lower panel)
1304 using the same conditions as above.

1305 **(C) Skn7 is required for filamentation under embedded conditions independently of**
1306 **Czf1.**

1307 *C. albicans* wild type (SC5314), $\Delta\Delta skn7$ (CEC4220) and $\Delta\Delta czf1$ (CEC4829) strains were
1308 grown in embedded conditions at room temperature for 5 days. For Skn7 OE in $\Delta\Delta czf1$
1309 background (CEC4815), and OE of Czf1 in $\Delta\Delta skn7$ background (CEC5069), 3 $\mu\text{g}\cdot\text{mL}^{-1}$ ATc
1310 was added and plates were incubated in the dark at room temperature for 5 days. Pictures
1311 were acquired on a Leica M80 stereomicroscope equipped with a DMC2900 colour camera, at
1312 a 16x magnification. Several fields of representative cells have been merged.

1313

1314 **Fig. 7. Skn7 is required to limit the accumulation of intracellular ROS upon**
1315 **filamentation on solid media.**

1316 **(A) The $\Delta\Delta skn7$ strain accumulates intracellular ROS in filamentous-inducing**
1317 **conditions on solid medium.** DCFDA fluorescence was measured in the WT strain SC5314,
1318 in the deletion mutant $\Delta\Delta skn7$ (CEC4220) as well as in $\Delta\Delta skn7+SKN7$ (CEC4682) grown on
1319 solid YPD, Spider or YPD+10% serum media for 4 h at 30°C, or grown on solid Lee's
1320 medium for 4 h at either 25°C or 37°C. The $\Delta\Delta skn7$ (CEC4220) shows a significant increase
1321 of intracellular ROS species as compared to SC5314 and $\Delta\Delta skn7+SKN7$ (CEC4682). Error
1322 bars show the SD of assays performed on three biological replicates. Statistical significance
1323 was assigned by performing 2-tailed Student's *t*-tests, comparing the knockout to either the
1324 WT or the reintegrant. The experiments were performed 4 times (** $p\leq 0.01$, *** $p\leq 0.001$). *y*-
1325 axis: ROS indicates DCFDA Fluorescence/ OD_{600} .

1326 **(B) The $\Delta\Delta skn7$ strain accumulates intracellular ROS only in the presence of H_2O_2 in**
1327 **liquid medium.** DCFDA fluorescence was measured in the 3 strains grown for 4 h in Lee's
1328 medium at either 25°C or 37°C to assess filamentation, and in YPD supplemented or not with
1329 5 mM H_2O_2 after 4 h of incubation at 30°C. Statistical significance was assigned by

1330 performing 2-tailed Student's *t*-tests comparing either the 3 strains grown in Lee's medium at
1331 37°C and 25°C or the deletion mutant $\Delta\Delta skn7$ with either $\Delta\Delta skn7+SKN7$ or SC5314 in
1332 presence of H₂O₂ 5mM. The experiments were performed 3 times (** $p\leq 0.01$, *** $p\leq 0.001$).

1333 **(C) Filamentation defect of selected knockout mutants in regulators of morphogenesis.**

1334 *C. albicans* SC5314, $\Delta\Delta skn7+SKN7$ (CEC4682) and deletion mutants $\Delta\Delta skn7$ (CEC4220),
1335 $\Delta\Delta eed1$ (CEC4637), $\Delta\Delta ume6$ (CEC2664), $\Delta\Delta cph1$ (CEC2297) and $\Delta\Delta tec1$ (CEC4831) were
1336 spotted on Spider solid medium and observed after 8 days of incubation at 30°C.

1337 **(D) Accumulation of intracellular ROS is not a mere consequence of filamentation**

1338 **defects.** DCFDA fluorescence was measured in SC5314, $\Delta\Delta skn7+SKN7$ (CEC4682), $\Delta\Delta skn7$
1339 (CEC4220), $\Delta\Delta eed1$ (CEC4637), $\Delta\Delta ume6$ (CEC2664), $\Delta\Delta cph1$ (CEC2297) and $\Delta\Delta tec1$
1340 (CEC4831) strains. Error bars show the SD of assays performed on three biological replicates.

1341 The ROS levels of all the mutants but $\Delta\Delta skn7$ are similar to SC5314 and $\Delta\Delta skn7+SKN7$
1342 (***) $p\leq 0.001$.

1343

1344 **Fig. 8. The D474 residue in the Skn7 response-regulator domain is critical for reducing**

1345 **intracellular ROS in filamentation-inducing conditions.** DCFDA fluorescence was

1346 measured in $\Delta\Delta skn7$ (CEC4220), and in strains with integrated CIP10-*P*_{SKN7}-GTW-HA₃ tag

1347 derivatives harboring *SKN7* (CEC4228) or the different *skn7* mutants. The mutants tested

1348 carry point mutations of the F76,L83 residues in the HSF domain (CEC4277), or of D474

1349 (CEC4221), T484 (CEC4273) or T496 (CEC4274) residues in the response-regulator domain.

1350 Error bars show the SD of assays performed on three biological replicates. Statistical

1351 significance was assigned by performing 2-tailed Student's *t*-tests (***) $p\leq 0.001$ comparing

1352 the mutant strains to the $\Delta\Delta skn7+SKN7$ strain. The experiments were performed 3 times.

1353 ROS: DCFDA fluorescence/OD₆₀₀.

1354

1355 **Fig. 9. Skn7 is a member of an interwoven network of morphogenetic regulators in *C.***
1356 ***albicans*.** The direct targets (yellow) of Skn7 (green), as revealed by ChIP-chip, are part of an
1357 intricate network of transcription factors that regulate morphogenesis in *C. albicans* (Ndt80,
1358 Efg1, Rob1, Tec1, Sfl2, Cph1, in blue). Binding data were taken from Nobile *et al.* (2012;
1359 Efg1, Ndt80, Rob1, Tec1), Borneman *et al.* (2007; Cph1) and Znaidi *et al.* (2013; Sfl2).
1360 Transcription factors are drawn as spheres. The transcriptional interactions of Skn7 with its
1361 direct targets are depicted by coloured arrows stemming from Skn7: red arrows, directly
1362 upregulated; green arrows, directly downregulated, black arrows, no change in gene
1363 expression. Transcriptional network was constructed using Cytoscape version 3.4.0.