Hexokinase and glucokinases are essential for fitness and virulence in the pathogenic yeast *Candida albicans*

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Abstract

Metabolic flexibility promotes infection and commensal colonization by the opportunistic pathogen *Candida albicans*. Yeast cell survival depends upon assimilation of fermentable and non-fermentable locally available carbon sources. Physiologically relevant sugars like glucose and fructose are present at low level in host niches. However, because glucose is the preferred substrate for energy and biosynthesis of structural components, its efficient metabolization is fundamental for the metabolic adaptation of the pathogen. We explored and characterized the *C. albicans* hexose kinase system composed of one hexokinase (*Ca*Hxk2) and two glucokinases (*Ca*Glk1 and *Ca*Glk4). Using a set of mutant strains, we found that hexose phosphorylation is mostly assured by *Ca*Hxk2, which sustains growth on hexoses. Our data on hexokinase and glucokinase expression point out an absence of cross regulation mechanisms at the transcription level and different regulatory pathways. In the presence of glucose, *Ca*Hxk2 migrates in the nucleus and contributes to the glucose repression signaling pathway. In addition, *Ca*Hxk2 participates to oxidative, osmotic and cell wall stress responses, while glucokinases are overexpressed under hypoxia. Hexose phosphorylation is a key step necessary for filamentation, that is affected in the hexokinase mutant. Virulence of this mutant is clearly impacted in the *Galleria mellonella* and macrophage models. Filamentation, glucose phosphorylation and stress response defects of the hexokinase mutant prevent host killing by *C. albicans*. By contributing to metabolic flexibility, stress answer response and morphogenesis, hexose kinase enzymes play an essential role in the virulence of *C. albicans*.

Author summary

The pathogenic yeast *C. albicans* is both a powerful commensal and pathogen of humans that can infect wide range of organs and body sites. To grow in its host and establish an infection, the pathogen must assimilate carbon from these heterogenous environments. *C. albicans* regulates central carbon metabolism in a niche-specific manner, activating alternatively gluconeogenesis, glyoxylate cycle and the glycolytic metabolism. For yeast and other microorganisms, glucose is the preferred carbon and energy source and its accurate detection and metabolism is essential. However, the glycolytic hexose kinase system has not been investigated yet in *C. albicans*. In this report, we showed that hexokinase and glucokinases contribute to the fitness and virulence of *C. albicans.* We revealed the main metabolic role of the hexokinase *Ca*Hxk2 which impacts on growth, glucose signalling, morphological transition and virulence. However, glucokinases contribute to the anoxic response and their implication in regulation processes is suggested.

Introduction

C. albicans is an opportunistic pathogen which exists in a relatively harmless state in the microbial flora of healthy individuals. It is notably present on the mucosal surfaces composing the digestive tract [1,2]. Perturbations of the normal microbiota, use of medical implants, or predisposing factors like diabetes can trigger *C. albicans* infection. *C. albicans* is the most common cause of fungal nosocomial infections associated with high mortality rates in immunocompromised patients [3-5].

C. albicans colonizes diverse host microenvironments such as skin, mucosa, blood, organs, [1]. Among the wide range of virulence traits, survival at 37°C, pH and osmolarity adaptation, secretion of lytic enzymes, alteration of the immune response, morphological

47 changes such as a transition between yeast and hyphae, occur during infection and promote
48 host invasion [6]. Another crucial factor is the metabolic capacity to assimilate host
49 nutrients. The importance of metabolic flexibility to promote systemic infection and
50 commensal colonization has been clearly emphasized during the past years. Genomic tools
51 revealed that rapid transcriptional responses take place to set up a niche-specific carbon
52 metabolism [7-11]. Utilization of alternative non-fermentable sources through the glyoxylate
53 and gluconeogenesis pathways is essential to support *C. albicans* proliferation *in vivo*
54 [12,13]. However, physiologically relevant hexose sugars like glucose, galactose, and fructose
55 are transiently available at low level in the gastrointestinal tract and only glucose (0.05 to
56 0.1%) is present in the bloodstream [9,13,14]. During colonization of plasma, kidneys and
57 liver by *C. albicans* [14-16], expression of infection-associated genes involved in glycolysis
58 has been reported. Complete glycolytic activation by the two key transcriptional regulators
59 Gal4p and Tye7p is required for full virulence in *Galleria mellonella* and mice [17]. Glucose is
60 the preferred substrate for ATP generation, metabolic precursors synthesis and maintenance
61 of a reductive potential in eukaryotes [18,19]. Hence, accurate and efficient glucose
62 detection and metabolization pathways constitute a fundamental basis for metabolic
63 adaptation of the pathogen. In *C. albicans*, there are 20 predicted glucose transporters, one
64 of them, *Ca*Hgt4, a high affinity sensor of the SRR pathway (Sugar Receptor Repressor), is
65 essential for low glucose level induction of six of the *C. albicans* transporters. In addition,
66 *Ca*Hgt4 is also required for filamentation and contributes to virulence in mice [20,21].
67 Once detected, the initial step in glucose utilization is its transport and following
68 activation to a sugar phosphate. Most fungi contain at least two active hexose kinases:
69 glucokinase and hexokinase. In *S. cerevisiae*, the hexokinase Hxk2 is the predominant
70 glucose kinase in cells growing in high glucose conditions [22]. Both enzymes can support

71 growth on glucose but hexokinases and glucokinases can also phosphorylate other hexoses
72 like fructose and mannose [23]. The enzymatic equipment for hexose phosphorylation varies
73 among different yeasts, although no physiological explanation for the differences has been
74 found. A search in the genome of *C. albicans* revealed the presence of two hexokinases
75 (*CaHXK1* and *CaHXK2)* and two glucokinase genes (*CaGLK1* and *CaGLK4*). The hexokinase
76 *Ca*Hxk1 does not phosphorylate glucose but GlcNAc, an extracellular carbon source present
77 in the mucous membranes, triggering the transition between yeast and hyphal form [24,25].
78 However, the hexokinase *Ca*Hxk2 and the glucokinases *Ca*Glk1 and *Ca*Glk4 have not been
79 characterized so far and their respective role in *C. albicans* fitness and virulence have not
80 been investigated yet. Moreover, nothing is known on the enzymatic functions of *Ca*Hxk2,
81 *Ca*Glk1 and *Ca*Glk4 and their putative dual regulatory role in glucose repression. The
82 preferential use of glucose by yeasts results from glucose-induced transcriptional repression
83 via the *Ca*Snf1, essential AMP-kinase, which phosphorylates the transcription factor *Ca*Mig1
84 [26,27]. Based on the *S. cerevisiae* model, *Ca*Mig1 should form a necessary complex with the
85 hexokinase *Ca*Hxk2 to shuttle in the nucleus and generate the glucose repression signal [28],
86 but this particular step has not been described yet in *C. albicans*.
87 In this study, we evaluated the contribution of *Ca*Hxk2, *Ca*Glk1 and *Ca*Glk4 to the
88 phosphorylation of hexoses and to the glucose repression process. Substantial insights in the
89 functional consequences of hexokinase and/or glucokinases deficiency for *C. albicans*
90 growth, various stress responses, morphological transition and virulence are also proposed.
91

92 ## Results

93 ### Deciphering the hexose kinase activity in *C. albicans*

One hypothetical hexokinase (*CaHXK2*, GenBank XM_712312) and two hypothetical glucokinase genes (*CaGlK1*, GenBank XM_705084 and *CaGLK4*, GenBank XM_707231) were found in the genomic sequence of *C. albicans* (http://www.candidagenome.org/). Analysis of the *C. albicans HXK2*, *GLK1* and *GLK4* sequences revealed the presence of a classical hexose kinase conserved domain organized in two regions (http://prosite.expasy.org/): a small and a large subdomain. The small subdomain contains the sugar-binding site of typical hexose kinases: -LGFTFSF/YP- [29]. Protein function prediction (http://bioinf.cs.ucl.ac.uk) revealed that *Ca*Hxk2, *Ca*Glk1 and *Ca*Glk4 are involved in phosphate-containing compound metabolic processes, transferase activity and ATP-binding. The localization of these conserved domains is provided in S1 Table [30,31]. Two nuclear localization sequences have been identified in the hexokinase sequence: -PAQKRKGTFT- (8-17) and -QKRGYKTAH- (405-413). These sequences were not found in the glucokinase sequences. Both glucokinase and hexokinase genes are located on chromosome R (Fig. 1A), spaced by 70 Kbp and oriented in opposite directions. The *CaGLK1* and *CaGLK4* sequences share 98.6% and 99.2% identity at the nucleotide and amino acid level, respectively. Alignment of the *CaGLK1* and *CaGLK4* genomic regions, showed that a high level of identity (98%) spanned from 1500 bp before and after the coding sequences. Within these conserved regions, separated by a few hundred of base pairs, both glucokinase genes are framed by two uncharacterized coding sequences. Alignment of these sequences, spanning the 5' and 3' regions of both glucokinases, revealed a level of 95.1% and 98.8% identity, respectively. This strongly indicates that the whole conserved region containing the *CaGLK1* and *CaGLK4* genes has been duplicated and conserved.

To identify the function of *CaHXK2*, *CaGLK1* and *CaGLK4,* a set of gene disruption strains was constructed into the *C. albicans* wild type strain SC5314. Single homozygous null

Cahxk2 (*Cahxk2Δ/Δ*) and *Caglk1* (*Caglk1Δ/Δ*) mutants, double homozygous null *CaHXK2 CaGLK1* (*Cahxk2glk1Δ/Δ*) and *CaGLK1 CaGLK4* (*Caglk1glk4Δ/Δ*) mutants were constructed by replacing both wild type alleles using the excisable *CaSAT1* flipper cassette [32]. A *CaHXK2* complemented strain (*Cahxk2Δ/Δ c/c*) was also constructed by reintegrating the wild type coding sequence at the *HXK2* locus, using the same strategy (S1 Appendix).

To investigate the contribution of *Ca*Hxk2*, Ca*Glk1 and *Ca*Glk4 to the phosphorylation of hexoses in *C. albicans,* we measured the hexose kinase activity displayed by the wild type strain (SC5314) and the generated mutant strains (Fig 1B). Data obtained with *Cahxk2Δ/Δ* cell extracts revealed that glucose kinase and mannose kinase activities decreased by 65 % and 75 %, respectively, while the phosphorylation of fructose was totally abolished. This suggests that other enzymes, like glucokinases, could phosphorylate glucose and mannose, while fructose is phosphorylated by *Ca*Hxk2 only. Values obtained with the complemented strain *Cahxk2Δ/Δ c/c*, statistically comparable to the data from the wild type strain, indicated that the lack of fructose phosphorylation was due to the deletion of the gene. Deletion of one or both glucokinase genes (*CaGLK1*, *CaGLK4*) had no apparent consequence on the level of hexose phosphorylation, suggesting that glucokinase activity could be compensated by the hexokinase activity. Hexose kinase activity measured in the *Cahxk2Δ/Δ* strain corresponds to the sum of the activities of *Ca*Glk1 and *Ca*glk4 (33% of the total activity). Glucose kinase activity, measured in the double mutant strain *Cahxk2glk1Δ/Δ,* which corresponds to the activity of *Ca*Glk4, was drastically reduced compared to the *Cahxk2Δ/Δ* mutant. This activity corresponds to 6% of the total glucose kinase activity. Taken together these results suggest that glucokinases enzymes contribute unevenly and seem to play a minor role in glucose and mannose phosphorylation.

141 To further investigate the specificity of *Ca*Hxk2, *Ca*Glk1 and *Ca*Glk4, we determined
142 their apparent Michaelis constant for glucose (Fig 1C). Apparent K_m of *Ca*Hxk2 was measured
143 in the *Caglk1glk4Δ/Δ* strain, while the K_m of Glk4 was determined in *Cahxk2glk1Δ/Δ* strain.
144 The apparent K_m of Glk1 was estimated in the *Cahxk2Δ/Δ* by subtracting the effect of
145 *Ca*Glk4. Data revealed that hexokinase 2 and glucokinase 1 have much lower K_m values (K_m
146 104.87 ± 7.05 µM and K_m 84.86 ± 6.23 µM, respectively) than glucokinase 4 (K_m 3900 ± 400
147 µM), using glucose as a substrate. The low glucose affinity of *Ca*Glk4 could partially explain
148 the poor contribution of this protein to glucose and mannose phosphorylation.
149

150 ## *Ca*Hxk2 mostly sustains growth in the presence of hexoses

151 Impact of hexokinase and glucokinase gene deletion on growth in the presence of hexoses
152 was evaluated (Fig 2). Delayed growth of the hexokinase mutant *Cahxk2Δ/Δ* on glucose and
153 mannose and severely impaired growth on fructose, confirmed the absence of a functional
154 hexokinase. Slow growth on glucose and mannose was consistent with the presence of an
155 additional glucokinase activity. The strong growth defect observed on fructose for this
156 mutant confirmed the fact that fructose is phosphorylated by *Ca*Hxk2 only. The residual
157 growth observed on fructose could be due to the metabolism of the alternative carbon
158 sources present in YPG. Growth of the mutant *Cahxk2Δ/Δ* was not affected in the presence
159 of glycerol or galactose, substrates that are not phosphorylated by *Ca*Hxk2. This indicates
160 that growth defects are linked to an impaired phosphorylation of hexoses. Moreover,
161 growth of the complemented strain was comparable to the wild type. Altogether, these data
162 clearly show that the hexokinase *Ca*Hxk2 is necessary for proper growth in *C. albicans*.
163 Deletion of *CaGLK1* or both *CaGLK1* and *CaGKLK4* did not affect growth. Growth of
164 the double mutant *Cahxk2glk1Δ/Δ* was drastically affected in the presence of glucose,

fructose and mannose. Growth failure was also observed, but less pronounced, in the presence of carbon sources that are not phosphorylated by hexokinase or glucokinase (galactose, lactate, glycerol). This strongly suggests that the presence of *Ca*Glk4 alone is not sufficient to sustain growth in the presence of hexoses and that the lack of both *Ca*Hxk2 and *Ca*Glk1 could affect general physiological properties, beyond hexose phosphorylation in *C. albicans.*

Glucokinases and hexokinase do not compensate at the transcriptional level, but exhibit functional redundancy at the protein level

To highlight the respective role of hexokinase and glucokinases, *CaHXK2*, *CaGLK1* and *CaGLK4* expression was analyzed (Fig 3A). Due to the high level of homology of their coding sequences (98.6 % identity) it has not been possible to amplify *CaGLK1* transcripts alone. Therefore, the transcription level corresponded to the sum of *CaGLK1* and *CaGLK4* transcripts (indicated as *CaGLK1/4*). In the presence of glucose, *CaHXK2* was 3 times more expressed than *CaGLK1*/4 (Fig 3A). Transcription of hexokinase and glucokinase genes was strongly induced by glucose (0.1% and 2%). In the presence of glucose, *CaHXK2* was 3 to 5 times more expressed that *CaGLK1/4* (Fig 3A, B) . Contrary to glucokinase genes, the level of *CaHXK2* transcripts was dependent on the glucose concentration. Transcription of hexose kinase genes was also strongly induced by mannose and fructose. Surprisingly, the transcription of glucokinase genes was induced by fructose and glycerol which are not substrates for glucokinases (Fig 3B).

To better elucidate hexose kinase gene regulation, we examined their transcription after growth on 2% glucose in the different mutant strains (Fig 3C). Expression data confirmed an absence of transcripts in the corresponding gene-deleted strains and revealed

189 a complete restoration of the hexokinase transcription level after re-introduction of both
190 wild type alleles. *CaHXK2* expression was not increased in the glucokinase mutants
191 (*Caglk1Δ/Δ, Caglk1glk4Δ/Δ*). Likewise, *CaGLK1/4* gene expression was not increased in
192 *Cahxk2Δ/Δ.* This suggests that unlike what happens in *S. cerevisiae* [33], no compensation
193 mechanisms interfere to regulate glucokinases and hexokinase at the transcriptional level in
194 the absence of one or the other gene. This points out different regulation pathways.
195 However, this compensation could occur at the protein level since the double glucokinase
196 mutant shows no hexose phosphorylation deficiency. Moreover, the fact that the level of
197 *CaGLK1/4* transcripts was unchanged in the absence of the hexokinase (*Cahxk2Δ/Δ*) revealed
198 that glucokinases genes are not subjected to glucose repression (Fig 3C). Interestingly, the
199 level of *CaGLK4* expression, detected in *Caglk1Δ/Δ* and *Cahxk2glk1Δ/Δ* was very low, just
200 above the detection threshold. Considering that the glucokinase gene expression level
201 detected in the mutant *Cahxk2Δ/Δ* is the sum of *GLK1* and *GLK4* transcripts, we can again
202 assume that *CaGLK1* and *CaGLK4* are not equally expressed.
203 To investigate the expression of the enzymes, GFP-tagged CaHxk2 and *Ca*Glk1 were
204 detected in cell extracts by immunoblotting, after growth in the presence of glucose.
205 *Ca*Hxk2-GFP and *Ca*Glk1-GFP were detected whatever the glucose concentration. However,
206 *Ca*Hxk2-GFP was much more abundant than *Ca*Glk1-GFP (Fig 3D and 3E). This is consistent
207 with the higher transcription level observed for *CaHXK2* but could also reflect a faster
208 turnover for glucokinases. *Ca*Hxk2-GFP was equally detected in the presence of various
209 carbon sources that are both inducers of its transcription and substrates of the enzyme, but
210 also and in the presence of glycerol and lactate that do not induce *CaHXK2* transcription (Fig
211 3B). This could be explained by the long half-life of *Ca*Hxk2. In addition to the lowest
212 abundance of *Ca*Glk1-GFP, the main difference between *Ca*Hxk2-GFP and *Ca*Glk1-GFP

protein content was that *Ca*Glk1 was barely detectable in cell extracts after growth on lactate. This may again reflect different regulation processes for *Ca*Hxk2 and *Ca*Glk1/4.

Hexokinase mediates glucose repression but not glucokinases

To highlight the regulatory functions of *Ca*Hxk2, we constructed a *HXK2::GFP* strain (*CaHXK2-GFP*) expressing a functional *CaHXK2-GFP* from its own promoter, to examine the localization of *Ca*Hxk2 in living cells exposed to glucose (Fig 4A). Upon growth in glucose (2%) the GFP signal was distributed in all the cell (except in the vacuole) with a strong accumulation into a structure that colocalize with the nucleus. This nuclear GFP signal was less important in cells grown in 0.1% glucose and nearly absent in cells grown without glucose (2% lactate) or at very low glucose concentration (0.05%). This indicates that, in *C. albicans*, *Ca*Hxk2 is able to shuttle from the cytoplasm to the nucleus in presence of high glucose (0,1% and more). This observation is similar to what observed in *S. cerevisiae* grown in 2% glucose where Hxk2 is known to accumulate into the nucleus where it exerts a transcriptional regulatory function necessary for glucose repression independent of its hexokinase activity [34,35].

To ascertain the impact of *Ca*Hxk2, *Ca*Glk1 and *Ca*Glk4 on glucose repression, we analyzed the expression of high affinity hexose transporter genes that are known to be controlled by the central repressor of the glucose repression pathway, *Ca*Mig1, in response to glucose [27,36] (Fig 4B). These transporter genes (*CaHGT7*, *CaHGT12*, *CaHXT10*) are also regulated by another main glucose sensing pathway, the SRR pathway, except *CaHGT8* which is not [20]. Hexose transporter gene expression was drastically enhanced (up to 10 times for *CaHXT10*) in the hexokinase mutant after transfer on 2% glucose medium. On the contrary, *CaHGT7*, *CaHGT12* and *CaHXT10* expression level was either lowered in the double

glucokinase mutant or unaffected in the case of *CaHGT8*. These data suggest that *CaHxk2* but not glucokinases, could have a repressor function on hexose transporter gene expression. Moreover, SRR-dependent transporter genes expression in the glucokinase mutant may suggest an unexpected regulatory role for glucokinase in transporter gene expression.

Hexose kinase enzymes mediate protection during harmful environmental challenges: glucokinase contributes to the hypoxic response

In *C. albicans* and a number of yeasts, one strategy to counteract oxidative and osmotic stresses is the rapid endogenous synthesis of compatible solutes or, under exposure to cell wall stresses, cell wall biogenesis [37,38]. These stress responses which are directly or indirectly linked to glucose metabolism, could have been affected in the hexose kinase mutants. For that purpose, wild type and mutant strains were grown in the presence of 2% glucose (YPG) supplemented with 1.2 M KCl (osmotic stress), 5 mM H_2O_2 (oxidative stress), 0.05% SDS and 5 mM caffeine (cell wall stresses). Data presented Fig. 5A revealed that all stresses had an impact by decreasing growth of the hexokinase mutants (*Cahxk2Δ/Δ, Cahxk2glk1Δ/Δ*). On the opposite, single and double glucokinase mutant strains were not significantly susceptible to the applied stresses. This suggests that *Cahxk2* is involved in stress responses through its central metabolic position.

During host infection, *C. albicans* colonizes multiple niches that greatly differ in oxygen content, meaning that it is adapted to hypoxic environments. Growth of the wild type and mutant strains under hypoxic conditions revealed the impact of *CaHXK2* deletion (Fig 5B). Growth of *Cahxk2Δ/Δ* and *Cahxk2glk1Δ/Δ* was affected by 50% after 24 h, as compared to normoxia while the deletion of one or two glucokinases had minor or no

261 effects. The transcriptional response to hypoxia, elucidated in *C. albicans*, revealed a global
262 upregulation of glycolytic genes [39,40]. This prompted us to investigate the expression of
263 hexokinase and glucokinase in response to hypoxia (Fig 5C). After one hour of exposure,
264 *CaGLK1/4* transcript level increased by a factor of 25, while *CaHXK2* upregulation was much
265 less detectable. This shows that glucokinases and hexokinase transcription is differently
266 regulated by hypoxic conditions. This was confirmed at the protein level. GFP-tagged
267 hexokinase was equivalently detected in normoxia and hypoxia. In contrast, immunoblot of
268 *Ca*Glk1-GFP revealed a constant amount of protein in response to hypoxia that persisted
269 along the growth, while in normoxia, the amount of *Ca*Glk1-GFP detected clearly decreased
270 (Fig 5D).
271

272 **Hexose phosphorylation by *Ca*Hxk2 is necessary to filamentation**

273 As glucose is one of the several stimuli that can trigger yeast-to-hypha development in *C.*
274 *albicans* [41,42], we checked the ability of hexokinase and glucokinase mutants to undergo a
275 yeast-to-hyphae morphological transition. To evaluate the impact of the hexose
276 phosphorylation step on filamentation, hyphal formation was induced by growth on
277 different media containing known inducing carbon sources, requiring or not hexose kinase
278 enzymes for further metabolization. Spider and serum media, contain respectively mannitol
279 and glucose that depends upon the hexose kinase step to be metabolized. The third medium
280 contains *N*-acetylglucosamine, that do not require *Ca*hxk2, *Ca*Glk1 or *Ca*Glk4 to be
281 metabolized, but require *Ca*Hxk1 [24,25]. After two days of growth at 37°C on serum and
282 spider media, the wild type, *CaHXK2* complemented strain and glucokinase mutants showed
283 abundant filaments at the periphery of the colony, while the hexokinase mutants
284 (*Cahxk2Δ/Δ*, *Cahxk2glk1Δ/Δ*) produced hyphae-deficient colonies (Fig 6A). Morphological

285 changes were also observed at the cell level. Microscopic observations revealed a drastically
286 decreased proportion of filamentous structures for the hexose kinase mutant cells,
287 suggesting that the hexokinase *CaHXK2* is necessary to the yeast-to-hyphae transition. By
288 contrast, filamentation of the *Cahxk2Δ/Δ* hexokinase mutant was not affected during growth
289 in the presence of *N*-acetylglucosamine. All strains behave similarly except the double
290 mutant *Cahxk2glk1Δ/Δ*, which appeared hypofilamentous. This suggests that hexose
291 phosphorylation by *Ca*Hxk2 could be an essential step for filamentation. Moreover,
292 filamentation defect of the double mutant *Cahxk2glk1Δ/Δ* grown on *N*-acetylglucosamine
293 could be the consequence of severe physiological disturbances.
294 To eventually highlight a specific role in pathogenic behaviour for *C. albicans* hexose
295 kinases, we compared hexokinase and glucokinase gene expression levels during the early
296 steps of the morphological switch. Our data did not reveal any particular transcriptional
297 response of one gene or another (Fig 6B). Both profiles revealed a two-time increase of
298 transcripts 30 or 60 min after the initiation of the filamentation by serum and a shift at 37°C.
299 However, after one hour of growth, glucokinases expression continues to increase while
300 *CaHXK2* transcription level decreases after 30 min.
301

302 ## *Ca*hxk2 mutant is hypovirulent in *Galleria mellonella* and macrophage models

303 To explore the impact of altering hexokinase and glucokinases on *C. albicans* virulence, we
304 examined first the survival rate of the host model *G. mellonella* following infection with the
305 wild type, mutant and complemented strains (Fig 7A). *G. mellonella* survival data indicated
306 that there was a statistically significant difference between the survival rate of larvae
307 infected by the mutants and the wild type strains, except for the *Caglk1Δ/Δ* single mutant.
308 Seven days post infection, 100% of the larvae were killed by the wild type strain while the

309 survival of the larvae infected by *Cahxk2Δ/Δ* and *Cahxk2glk1Δ/Δ* was still 70% and 85%,
310 respectively. The double glucokinase mutant was also significantly less virulent than the wild
311 type strain, with an intermediary survival rate of 50%. The *Cahxk2Δ/Δ c/c* complemented
312 strain revealed a partially restored virulence.
313 Secondly, we analysed the ability of the mutant to kill macrophages at different
314 interaction times using an *in vitro* model assay (Fig 7B). Hexokinase and glucokinase gene
315 deletions did not modify macrophages association with yeast for any strains, except for the
316 *Cahxk2glk1Δ/Δ* double mutant which shows a slightly decreased number of recruited
317 macrophages at the early time of infection (60% compared to approximately 80% for the
318 other strains). This suggests that the absence of hexokinase or glucokinase has no impact on
319 the recognition step. Survival of macrophages was severely enhanced when *Cahxk2Δ/Δ* and
320 *Cahxk2glk1Δ/Δ* were tested. After 4 hours in the presence of the hexokinase mutant
321 (*Cahxk2Δ/Δ*) the number of alive macrophages was nearly twice as high as in the presence of
322 the wild type strain. Moreover, 90% of the macrophages infected by *Cahxk2glk1Δ/Δ* were
323 still alive after 4 h, while 34% survived with the wild type strain. After 24 hours, 39% of the
324 macrophages infected with *Cahxk2glk1Δ/Δ* survived, compared to only 2% with the wild
325 type strain and 1% for the other strains. This underlines again the very affected virulence
326 capacities of this double mutant. Reintegration of the wild type *CaHXK2* gene restored the
327 killing capacities, suggesting that the virulence defect was linked to the absence of *CaHXK2*.
328 As compared to the wild type strain, interactions performed with glucokinase mutants
329 (*Caglk1Δ/Δ, Caglk1glk4Δ/Δ*) and macrophages did not reveal significant differences. Because
330 the process of macrophage killing relies on the formation that pierce the phagocytic
331 membrane, the morphogenesis of the strains was analysed at 4 and 24 h post infection (Fig
332 7C). Our data clearly reveal that *Cahxk2Δ/Δ and Caglk1glk4Δ/Δ* did not develop hyphae

during macrophage infection. In order to make sure that the growth defect of the hexokinase mutant strains was not the main cause for avirulence, *C. albicans* cells were released from macrophage after 4 hours of phagocytosis by cell lysis and counted (S1 Fig). As compared to the wild type, there was no significant differences in the capacity of the mutant strains to divide inside the macrophage.

Altogether, these data suggest that the virulence defect associated to the deletion of *Cahxk2* could concern the fungal escape phase rather than the recognition and initial phagocytosis steps.

Discussion

In this study, we sought to assign functions to the hexokinase and glucokinases that could potentially contribute to the fitness and virulence of *C. albicans*. We showed that hexose phosphorylation is mostly assured by *Ca*Hxk2, which mainly sustains *in vitro* growth in the presence of hexoses. Hexokinase expression is induced by glucose and higher than glucokinase expression. But proteins are both detectable even in the absence of any phosphorylable hexose. As shown for *S. cerevisiae* glycolytic enzymes, regulation is the result of a complex mixture of gene expression and metabolic effects, in order to optimize simultaneously fluxes, protein and metabolite concentrations [43]. *C. albicans* inhabits niches containing contrasting carbon sources. Metabolic flexibility implies that alternative carbon sources and glucose are assimilated simultaneously [9]. The discrepancy between *C. albicans* transcriptome and proteome has been already clearly highlighted [44]. Upon glucose exposure, *Ca*Icl1 and *Ca*Pck1, enzymes involved in the assimilation of alternative carbon sources are not degraded, while their transcripts are subjected to glucose repression. We can assume that a persistent level of *Ca*Hxk2, *Ca*Glk1 and *Ca*Glk4 could promote

357 metabolic flexibility and stress response to cope with changing microenvironments reached
358 by the pathogen in the various host niches.

359 The affected growth profiles of *Cahxk2Δ/Δ* and *Cahxk2glk1Δ/Δ* indicates the limited
360 ability of *Ca*Glk1 and *Ca*Glk4 to allow glucose utilization in the absence of *Ca*Hxk2, while
361 normal growth was observed in the absence of *Ca*Glk1 and/*Ca*Glk4. One possible hypothesis
362 would be a limited glucose uptake caused by the absence of hexokinase. Hence, in *S.*
363 *cerevisiae* and *K. lactis* glycolytic mutants, glycolysis controls glucose signalling via the SRR
364 pathway. The expression of several glucose-regulated genes, like hexose transporter genes,
365 depends on a functional glycolysis, limiting therefore glucose import [45]. However, this
366 control does not seem to exist in *C. albicans* (Fig. 3C). On the contrary, expression of
367 transporter genes controlled by the SRR pathway (*HGH12, HGT7, HXT10*) was enhanced in
368 the hexokinase mutant *Cahxk2Δ/Δ*. Therefore, the poor expression of glucokinase genes, the
369 low intracellular concentration of *Ca*Glk1 and *Ca*Glk4 and their low participation in hexose
370 kinase activity, could mainly explain the growth defect in the absence of hexokinase.
371 Moreover, glycolysis constitutes an interface between metabolism and gene transcription.
372 For instance, glycolysis yields pyruvate which can be oxidized into acetyl-CoA, directly
373 implicated in histone acetylation and gene expression. In stationary yeast cells, increase
374 glucose availability leads to higher levels of acetyl-CoA synthesis, global histone acetylation,
375 accompanied by the induction of a thousand of growth-related genes [46]. The reduced
376 glycolytic flux of the hexokinase mutant could therefore lead to transcription defects and
377 slower growth. In all the tested conditions, the double mutant *Cahxk2glk1Δ/Δ* presented an
378 altered phenotype. In this context, *Ca*Glk4 is the only hexose kinase enzyme present.
379 Because of the low affinity of *Ca*Glk4 for glucose and its very low expression, growth of
380 *Cahxk2glk1Δ/Δ* is drastically affected in the presence of hexoses. This could be explained by

the lack of efficient hexose kinase enzymes. Moreover, hexokinase and glucokinase gene deletions could lead to several drastic intracellular changes. In *S. cerevisiae*, the *hxk2* mutant has a higher H^+-ATPase activity and a lower pyruvate decarboxylase activity which coincided with an intracellular accumulation of pyruvate [47]. Absence of glucose repression, could also contribute to redirect carbon flux. In *K lactis*, the identification of hexokinase-dependent proteins related to chromatin remodeling, amino acids and protein metabolism, redox maintenance and stress response reinforces the idea that glucose kinase enzymes exert broader functions than hexose phosphorylation and glucose repression [48].

Our findings reveal that the well-established bifunctional functions of Hxk2 in *S. cerevisiae* [22,49] also exists in *C. albicans*, while glucokinases do not seem to play a role in glucose repression. We detected *Ca*Hxk2-GFP in the nuclei in 0.1% glucose-grown cells (5 mM), which corresponds to the glucose level maintained in the bloodstream and in vaginal secretions [7,9]. Glucose repression pathway via *Ca*Hxk2, could thereby promote metabolic adaptation to favor the fitness of the pathogen, even in glucose-limited host niches. In response to glucose and according to the *S. cerevisiae* model, CaHxk2 should act as a transcriptional carbon catabolite corepressor binding to *Ca*Mig1 [50]. *C. albicans* has two orthologs of *Sc*Mig1, *Ca*Mig1 and *Ca*Mig2 but, to our knowledge, no functions have been assigned yet to *Ca*Mig2.Transcriptional studies realized on *C*aMig1 revealed that it regulates a unique set of genes, annotated as carbohydrate uptake and catabolism factors [51]. However, works conducted on *Ca*Mig1 revealed that it has no phosphorylation sequence for the kinase *Ca*Snf1, essential for the removal of glucose repression [27,52]. Deletion of *Ca*Mig1 has no effect on the expression of *CaGAL1*, a glucose repressed gene [27] but impacts the transcription of hexose transporter genes [53]. Moreover, CaMig1 has been recently implicated in the resistance to weak organic acids, a novel function [54]. All these

405 evidences show that some of the molecular mechanisms involved in glucose repression in *C.*
406 *albicans* remain to be elucidated, in particular concerning the direct partners of *Ca*Hxk2.
407 Moreover, contrary to *S. cerevisiae* [33] *C. albicans* glucokinases are not subjected to glucose
408 repression. This suggests that glucokinases are not involved into the control of glucose
409 phosphorylation in *C. albicans* and underline their minor role in glucose metabolism.
410 Contrary to glucokinases, hexokinase gene deletion had an impact on various *in vitro*
411 stress responses. Glucose has been shown to promote stress resistance and to induce some
412 stress genes in *C. albicans* [55]. Our data support this finding, but furthermore indicate a role
413 for glucose phosphorylation in stress resistance. Osmotic and oxidative stresses induce
414 storage of trehalose, glycerol and arabitol [38]. Biosynthesis of such osmolyte sugars and
415 polyols, directly connected to the upper part of the glycolytic pathway, depends on glucose-
416 6-phosphate availability. This was confirmed in *S. cerevisiae* where the analysis of metabolic
417 fluxes in a *Δhxk2* mutant revealed a synthesis of glycerol reduced by a factor of 4.5 [56].
418 Moreover, the carbon source modulates cell wall architecture and strongly influences the
419 resistance of *C. albicans* to osmotic and cell wall stresses. Glucose and lactate-grown cells
420 display significant differences in cell wall elasticity and ultrastructure [8]. β-glucans are
421 major constituents of *C. albicans* cell wall. Glucan synthases assemble UDP-glucose residues
422 produced from phosphorylated glucose [57]. Thus, any hexose phosphorylation defect
423 would affect the cell wall and render it more sensitive to stress.
424 Glycolytic gene expression has been associated to the global response of *C. albicans*
425 to hypoxia [58]. Several transcription factors are involved in hypoxia-responsive expression
426 of glycolytic genes. Among them, the key filamentation regulator Efg1 and the transcription
427 factors Tye7 and Gal4 contribute to the early hypoxic response [11,17,39,40]. Contrary to *S.*
428 *cerevisiae* which ferments sugars even under aerobic conditions, *C. albicans*, a Crabtree-

429 negative yeast, ferments carbohydrates under hypoxic conditions [59]. Our findings specify a
430 clear and drastic effect of hypoxia on glucokinase expression at the mRNA and protein levels.
431 Glucokinase enzymes could be part of the global early hypoxic response, as a spare wheel, to
432 maintain a necessary glycolytic flux during fermentation conditions in oxygen-poor niches.
433 Moreover, this data confirms the fact that hexokinase and glucokinases are not targeted by
434 the same regulatory pathways.

435 Our results show that the hexokinase mutant retains the filamentation capacity when
436 the carbon source does not require *Ca*Hxk2 to be assimilated. Thus, the filamentation-
437 defective phenotype of the hexokinase mutant could be linked to a phosphorylation defect.
438 The absence of one or both glucokinases has no impact on filamentation. This could be
439 related to their low contribution to hexose phosphorylation and, concerning the spider
440 medium, because glucokinases do not phosphorylate fructose. The activities of several major
441 glycolytic enzymes are known to differ in yeast and hyphal forms [60]. We have shown that
442 induction of the filamentation requires upregulation of hexose kinase genes. Morphological
443 switch to filamentous growth requires energy and carbon source, notably to build
444 membranes and cell walls. Thereby, several links between morphogenesis and expression of
445 metabolic genes are established in *C. albicans.* The transcription factor Efg1, part of a Ras-
446 cAMP-PKA signaling network and involved in morphogenesis in *C. albicans,* strongly induces
447 glycolytic and fermentation genes [61]. Moreover, mutants of the *Ca*Hgt4/*Ca*Rgt1 pathway
448 (SRR pathway) involved in the control of gene expression in the absence of glucose display
449 affected filamentation phenotypes [20,36]. Thus, nested pathways control simultaneously
450 morphogenesis, glucose signalization and metabolism and by this way *Ca*Hxk2, which
451 directly impacts on filamentation through its kinase activity.

The hypovirulence of the hexokinase mutant suggests a central function for *Ca*Hxk2. We have shown that the glucose phosphorylation step controls filamentation. Morphological switch is a determining factor of virulence in both *Galleria* and macrophage models. Hyphae are observed in the *G. mellonella* infected tissues [62]. Histological investigations of infected larvae revealed that the SC5314 wild type strain shows a high propensity to filament, leading to gut invasion [63]. Time-lapse microscopy studies have shown a strong correlation between intra-phagocytic hyphal growth and macrophage lysis [64]. Numerous experimental data support a model by which *C. albicans* hyphae enable escape from phagocytes by growing and consequently lysing the cell [65]. *Ca*Hxk2 could then be required to sustain hyphal growth within the host cell. Moreover, the impact could be situated at the metabolic level. *C. albicans* hexokinase mutant could degrade trehalose, major hemolymph sugar in *G. mellonella* larvae [66] to recover glucose, but remain unable to phosphorylate it. Recently, Tucey et al., [67] revealed concomitant up-regulation of host and pathogen glycolysis, setting up glucose competition by depleting glucose. *C. albicans*-activated macrophages shift to Warburg metabolism and become dependent on glucose for survival. During macrophage infection, both *C. albicans* free cells and escaped from macrophage could trigger rapid death to the phagocytes by depleting glucose levels. The hexokinase mutant could not compete efficiently for glucose and then turn out to be hypovirulent.

Our data decipher the role of glucose kinase enzymes, not only as a central point of metabolism, but also as actors in regulation, stress response and morphogenesis. Altogether, those different interconnected functions influence the virulence of the yeast. Surprisingly, while the lack of glucokinases did not impact on the phenotype of the mutants, *Ca*Glk1 clearly appeared implicated in the hypoxic response. Moreover, the fact that hexose transporter gene expression level is affected in *Caglk1glk4Δ/Δ* suggests that glucokinases

476 could be implicated in regulation processes that remain to be elucidated. Future research
477 might provide further insights in this challenging area.
478

479 ## Methods

480 ### Strains and media

481 *C. albicans* strains used in this study are listed in S2 Table. Strains were grown at 30°C or
482 37°C on YPG medium (1% yeast extract, 2% peptone, 2% glucose). When necessary glucose
483 was added at various concentrations (from 0.01 to 2%) or replaced by other carbon sources
484 like 2% glycerol or 2% lactate. For filamentation assays, *C. albicans* cells were grown for 48 h
485 at 37°C on Spider medium (1% Nutrient Broth, 1% mannitol, 0.2% KH_2PO_4, 2% agar) or 96 h
486 on YP medium (1% yeast extract, 2% peptone) supplemented with 2.5 mM N-acetyl-
487 glucosamine. Five percent calf serum was also used to induce the morphological switch at
488 37°C after a 2 to 5 days incubation period. The utilization of different carbon sources and
489 sensitivity to different compounds (5 mM H_2O_2, 1.2 M KCl, 0.05% SDS, 5 mM caffeine) was
490 monitored in liquid YPG at 30°C by spectrophotometry (Tecan Infinity 200 Pro Serie). Five ml
491 of YPG inoculated with stationary phase cells were cultivated to an optical density at 600
492 (OD_{600}) = 0.6. Ten µl of culture were used to inoculate 180 µl of YP medium containing
493 different carbon sources or the required additives distributed in the wells of a plate. Controls
494 lacking carbon source or specific compounds were performed. Plates were sealed with gas-
495 permeable plastic film. OD_{600} was measured every 30 min during 48 h, with shaking at 380
496 rpm. Growth data are based on three independent experiments, each of which consisted of
497 assays performed in triplicate. For growth under hypoxic conditions, aerated flasks were
498 inoculated with an overnight culture of *C. albicans*, to OD_{600} of 0.2. Cells were grown until
499 the beginning of the exponential phase ($OD_{600} \approx 1.8$). Hypoxic conditions were created by

collecting and transferring cells suspension in hermetic and filled tubes. Different time points following the shift from normoxic to hypoxic growth conditions were considered (30, 60, 90 and 120 min). After appropriate time, cells were collected by centrifugation at 3,000 rpm for 5 min, washed twice with sterile water and rapidly frozen at -80°C. For each time point, three biological replicates were performed. For growth in 96-well plates, anoxic condition were generated by adding 50 µl of mineral oil in each well.

Construction of mutant strains

Mutant strains used in this study are listed in S2 Table. Mutant strains were constructed using the *SAT1* flipper selection cassette kindly provided by J. Morschhäuser [32]. *CaHXK2* homozygous mutant strain and the complemented strain *Cahxk2Δ/Δc/c* were generated according to methods described by Reuβ et al., [32]. The *Caglk1Δ/Δ* and *Caglk1glk4Δ/Δ* homozygous null mutant strains were constructed by one step cloning-free fusion PCR-based strategy. The *Cahxk2glk1Δ/Δ* mutant strain was constructed by deleting successively both *CaHXK2* alleles of the *Caglk1Δ/Δ* mutant using the *CaHXK2* deletion cassette (S1 Appendix). *Ca*Hxk2 and *Ca*Glk1 GFP epitope tagging was performed using a PCR-based strategy using pGFP-NAT1 as a template (kindly provided by S. Bates) [68]. The appropriate mutants were identified by PCR analysis using a combination of primers outside the sites of cassette integration and internal primers.

Yeast transformation

C. albicans transformation was performed using the PEG Lithium technique [69]. After transformation, mixtures were incubated in YPG for 4 h at 30°C and then plated on YPG + nourseothricin 250 µg/ml (Werner BioAgent, Jena, Germany). Nourseothricin-sensitive cells

were obtained according to Reuβ et al., [32]. Transformants were grown overnight in YPG medium without selective pressure. Cells were plated on YPG containing nourseothricin (25 µg/ml). Small colonies containing nourseothricin-sensitive cells were selected after 2 days of growth at 30°C. Both alleles were disrupted or complemented in a similar manner after elimination of the *SAT1* flipper cassette. In the case of *in vivo* epitope tagging using pGFP-NAT1 (S3 Table) it was not possible to eliminate the *NAT1* marker, one allele was modified by transformation, only.

Yeast cell extract and immunoblotting

To prepare proteins extracts, cells were centrifuged and suspended in 500 µl of 0.1 M Tris-HCl buffer supplemented with 10% PMSF (Phenylmethylsulfonyl fluoride). 1.5 ml of glass beads were added and proteins were extracted using FastPrep®-24 (MP Biomedicals). A succession of five grinding (6.5 m/s for 30 sec) was performed. Following this lysis step, cell extracts were centrifuged at 1,500 rpm for 10 min at 4°C. Proteins from the supernatant were quantified using Nanodrop 2000®.

Immunodetection conditions were as described by Rolland *et al.,* [70]. The α-GFP antibody (monoclonal anti-mouse, Roche) and secondary antibody (mouse antibody, HRP conjugated, Bethyl Laboratories) mouse HRP were used at 1/5000^{e} and 1/10000^{e} final concentration respectively.

Determination of hexose kinase activity

Either glucose, fructose or mannose were used as substrates. The hexokinase II activity was measured spectrophotometrically through $NADP^{+}$ reduction in a glucose-6-phosphate dehydrogenase-coupled reaction. Each reaction was performed in 1 mL spectrophotometer

548 cuvette at room temperature. The final assay mixture consisted of 100 µl of 25 mM HEPES
549 buffer pH 7.5, 100 µl of 10 mM $MgCl_2$, 100 µl of 1 mM ß-NADP, 500 µg of crude proteins
550 extract, 2 units of Glucose-6-phosphate dehydrogenase and (i) 100 µl of 10 mM D-Glucose
551 for glucose kinase activity, (ii) 2 units of phosphoglucose isomerase and 100 µl of 10 mM D-
552 Fructose for fructokinase activity, (iii) 2 units phosphomannose isomerase, 2 units of
553 phosphoglucose isomerase, and 100 µl of 10 mM D-Mannose for mannokinase activity.
554 Reactions were started with the addition of 100 µl of 5 mM ATP. Absorbance was
555 continuously recorded at 340 nm, for 10- or 15-min. Activities are obtained from the mean
556 of three independent experiments and expressed as a percentage of the activity obtained
557 with wild type crude protein extract. The apparent *K*m of crude extracts of the glucose
558 kinases were determined with a final ATP concentration of 5 mM, a final concentration of
559 glucose ranging from 1 µM to 100 mM. The NADPH apparition at 340 nm was measured
560 using a Tecan Infinite M200 (Salzburg, Austria) microtiter plate reader at 30°C. A single well
561 is composed of 10 µl Glucose-6-P dehydrogenase (0.2 U/mL), 10 µl of 1 mM $NADP^+$, 10 µl of
562 10 mM $MgCl_2$, 10 µl of HEPES buffer (25 mM, pH 7.6) and 50 µg of crude extracts. The
563 reaction was initiated by the addition of 10 µL of ATP (5 mM in potassium HEPES buffer). The
564 activity was determined using a calibration curve of NADH in the range of 0-500 µM to
565 consider the variability of the optical pathway. The parameters were obtained using Dynafit
566 software and the rapid equilibrium approximation of the Michaelis-Menten equation [71].
567
568 **GFP detection by Microscopy**
569 Yeast strain expressing the *Ca*Hxk2::GFP and *Ca*Glk1::GFP fusion proteins were grown to
570 exponential phase (OD_{600} ≈ 0.8) in YP containing 0.05, 0.1 or 2% glucose or 2% lactate. Nuclei
571 were stained by addition of DAPI to 10 µg/ml to the cultures and incubated at 28°C, 180 rpm

for 60 min. Cells were washed twice with phosphate buffer saline (PBS) (10 mM Na_2HPO4, 1.76 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl), collected by centrifugation and resuspended in 20 µl of PBS. GFP and DAPI localization were monitored in live cells cultures using a Zeiss Axioskop 2 Plus fluorescence microscope. Images were taken with a Zeiss AxioCam MR camera using AxioVision software and processed using LiveQuartz Images Editor.

RNA extraction and RT-q-PCR analysis

Total RNA was extracted from cells grown to OD_{600}≈ 1.5 by the acid phenol method [72]. For reverse transcription-quantitative PCR (RT-qPCR) experiments, 10 µg of total RNA extract were treated with DNase I (Ambion). Then, ReVertAid H Minus reverse transcriptase (Thermo Scientific), was used as described by the manufacturer, to generate cDNAs. RT-qPCR experiments were performed with the CFX 96 Bio-Rad light cycler using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Relative quantification was based on the 2Δ*CT* method using *CaACT1* (actin) as calibrator. The amplification reaction conditions were as follows: 95°C for 1 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and the final step 95°C for 10 s. A melting curve was generated at 95° for 10 s, 65°C for 5 sec with an increment of 0.5 °C until 95°C at the end of each PCR cycle, to verify that a specific product was amplified.

Infection of *G. mellonella* larvae

For *G. mellonella* infection, overnight cultures of WT (SC5314), mutant or complemented strains of *C. albicans* were grown to stationary phase (OD_{600} = 5) in 2% YPG medium. Cells were centrifuged and washed three times with 0.9% NaCl. Larvae were infected with 10 µl of suspension (2.5 x 10^5 cells) injected using a Hamilton syringe, between the third pair of

596 prothoracic legs. Three replicates, each consisting of 10 insects, were carried out with
597 survival rates measured daily for a period of 8 days. Infected larvae were incubated at 37°C
598 in the dark. A control group injected with 10 µl of 0.9% NaCl was included. Death was
599 determined based on the lack of motility and melanisation. Survival curves were plotted and
600 their statistical significance were determined by Kaplan-Meier analysis using the GraphPad
601 Prism 6.0 program. *P* values were estimated using Log rank tests.
602
603 **Infection of phagocytes with yeasts**
604 Macrophages from the J774A.1 (ATCC TIB-67) murine cell line were infected as previously
605 described [73] in cRPMI medium (RPMI-1640 without phenol red and supplemented with
606 10% heat-inactivated fetal bovin serum, 1 mM sodium pyruvate and 2 g/L sodium
607 bicarbonate) at 37°C under 5% CO_2. Briefly, 2 x 10^5 macrophages per well were adhered
608 overnight in 96-well plates, and infected with 1 x 10^6 Calcofluor White (CFW)-labeled yeast
609 cells in stationary phase in cRPMI medium supplemented with 5 µg/mL CFW. Interaction was
610 followed over a 24-hour time course experiment. To count yeasts after phagocytosis (S1 Fig),
611 macrophages were infected with *C. albicans* strains as described in Materials and Methods,
612 using 10 macrophages to 1 yeast Multiplicity Of Infection (MOI). After 4 hours of interaction,
613 infected macrophages were collected after trypsin treatment, centrifuged for 10 min at
614 10000 X g, and lysed in 1 ml of 0.2% ice-cold Triton X-100. Released yeast cells were
615 resuspended in YPD and counted using Kova slides (Kova International). Triplicates were
616 done for each experiment. The results are shown as the average of five
617 independent experiments +- standard errors.
618
619 **Flow Cytometry analysis**

Flow Cytometry assays were conducted as previously described [73] using a FACSCantoII (Becton Dickinson). Macrophage viability and the ratio of macrophages engaged in phagocytosis were determined after 30 min, 4 h and 24 h of infection with CFW-labeled yeasts. Quintuplets of each condition were done for each experiment. After trypsin treatment, macrophages were labeled with 0.2 µg/mL anti-mouse CD16-APC (a membrane stain) and 0.2 µM calcein-AM (Sigma) (a marker of active metabolism). The percentage of macrophage viability was calculated using the number of macrophages positive for both fluorescence (anti-CD16-APC and calcein-AM) when infected with yeasts compared to the control uninfected macrophages. Phagocytosing macrophages were quantified as the percentage of the double-stained macrophages also positive for CFW fluorescence. *t*-test was used to establish statistical significance with a significance level set at $P<0.05$.

Statistical analysis

Experiments were performed at least three times independently. All statistical data were calculated with GraphPad Prism 7 software. For comparisons of multiple groups one-way ANOVA method was used. Significance of mean comparison is annotated as follow: ns, not significant; *P=0.033; **P=0.002; ***P=0.0002; ****P<0,0001.

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839

840 ## Figure Captions

841 **Fig 1. The hexose kinase system in *C. albicans.*** (A) The hexose kinase genes are located on
842 *C. albicans* chromosome RA (Ca22chrRA_C_albicans_SC5314:994,376..997,830). Glucokinase
843 genes are oriented in opposite directions and bordered by highly homologous regions (light
844 green). (B) Hexose phosphorylation rates in *C. albicans* wild type and hexose kinase mutant
845 cell extracts. For each strain, the amount of glucose-6-phosphate produced was measured
846 and expressed as a percentage of the wild type strain. Data are presented as a mean (+
847 standard deviation) of 3 independent experiments performed in triplicates (n = 9); **** P <
848 0.0001; one-way ANOVA using Dunnett's method. (C) Kinetic constant of hexose kinases in *C.*
849 *albicans* in the presence of glucose. The experiment was performed in triplicate.
850 Representative data are presented here.

Fig 2. Hexokinase 2 is necessary to sustain *C. albicans* growth. Wild type and mutant strains were grown to the mid-log phase on medium containing 2% of various carbon sources (glucose, fructose, mannose, galactose, glycerol or lactate). A 96-well plate containing appropriate medium was inoculated with each strain at starting OD_{600nm}= 0.2. Cells growth was performed at 30°C and recorded during 48 h using microplates reader (TECAN infinite pro200). Data are presented as a mean (± standard deviation) of 3 independent experiments performed in triplicates (n = 9).

Fig 3. Hexokinase and glucokinases expression. (A) Relative expression of *CaHXK2* and *CaGLK1*/4 in wild type strain in presence of 2% lactate, 0.1% and 2% glucose. (B) Relative expression of *CaHXK2* and *CaGLK1*/4 in wild type strain in presence of various carbon sources (2% lactate, glycerol, glucose, fructose or mannose). For the panels A and B the results were normalized to the expression of *CaACT1*. The level of *CaHXK2* and *CaGLK1/4* mRNAs was expressed relatively to their abundance in 2% lactate, which was set to 1. (C) Relative expression of *CaHXK2* and *CaGLK1*/4 in wild type and hexose kinase mutant strains after growth in 2% YPG. The expression was normalized to the level of the *CaACT1* mRNA internal control. mRNA levels were expressed relatively to their abundance in the wild type strain, which was set to 1. Results represent a mean (+ Standard Deviation) of 3 independent experiments performed in duplicate (n=6); ns, non-significant; ** P = 0.002; **** P < 0.0001. Pvalues were calculated by one-way ANOVA using Tukey's method. (D) Strains of *C. albicans* expressing C-terminally GFP-tagged CaHxk2 or CaGlk1 were cultivated in presence of 0.1, 0.5 or 2% glucose. Whole cell lysates were analyzed for CaHxk2-GFP and CaGlk1-GFP by Western blotting, using α-GFP antibody. Detection of total proteins by in-gel Coomassie staining was

875 used as a loading control (total extract). Western blots were performed 3 times.
876 Representative data are presented here. (E) and (F) *C. albicans* expressing C-terminally GFP-
877 tagged CaHxk2 or CaGlk1 were cultivated in presence of various carbon sources (2%). Whole
878 cell lysates were analyzed for CaHxk2-GFP and CaGlk1-GFP by Western blotting, using α-GFP
879 antibody. Detection of total proteins by in-gel Coomassie staining was used as a loading
880 control (total extract). Western blots were performed 3 times. Representative data are
881 presented here.
882
883 **Fig 4. Hexokinase 2, but not glucokinases, participates to glucose repression.** (A)
884 Subcellular localization of CaHxk2-GFP was followed using fluorescence microscopy. Direct
885 visualization of CaHxk2-GFP in live cells of *C. albicans* was performed as described in the
886 methods section. Nuclei were identified using DAPI staining. Transformants expressing
887 CaHxk2-GFP were grown on medium containing 2%, 0.1% or 0.05% glucose or 2% lactate as
888 carbon source. GFP and DAPI localization was monitored in live cell cultures using a Zeiss
889 Axioskop 2 Plus fluorescence microscope. Images were taken with a Zeiss AxioCam MR
890 camera using AxioVision software. (B) Expression of glucose permeases, controlled by the
891 Sugar Receptor Repressor pathway (*CaHGT12*, *CaHXT10* and *CaHGT7*) or not (*CaHGT8*) was
892 measured by qPCR in each strain. Cells were cultivated in the presence of 2% lactate and
893 then transferred for 1 h in 2% glucose before RNA was extracted. Expression levels were
894 normalized to the expression of *CaACT1*. For each permease, the mRNA levels were
895 expressed relatively to their abundance in the wild type strain, which was set at 1. Results
896 represent a mean (+ Standard Deviation) of 3 independent experiments performed in
897 duplicate; (n=6), ** P = 0.002; **** P < 0.0001. Pvalue were calculated by one-way ANOVA
898 using Tukey's method.

899

900 **Fig 5. Hexose kinase enzymes mediate protection during harmful environmental**
901 **challenges.** (A) Growth of the wild type and hexose kinase mutant strains in YPG and
902 exposed to various stresses was expressed as a percentage of growth in absence of stress
903 which was set to 100% (black line). (B) Growth of the wild type and hexose kinase mutant
904 strains under hypoxic conditions in 2% glucose YPG was expressed as a percentage of growth
905 in normoxia which was set to 100% (black line). (C) Relative expression of *CaHXK2* or
906 *CaGLK1*/4 measured in normoxia (N) or hypoxia (H) during growth in YPG 2% glucose.
907 Transcript level was analyzed by qPCR at 0, 30 and 60 min. Results were normalized to the
908 *CaACT1* transcript level. The level of *CaHXK2* and *CaGLK1/4* mRNAs was expressed relatively
909 to their abundance at time zero, which was set to 1. Histograms represents a mean of 3
910 independent experiments performed in triplicate (n=9); ns, non-significant; *** P = 0.0002;
911 **** P < 0.0001. Pvalue were calculated by one-way ANOVA using Tukey's method. (D)
912 Strains of *C. albicans* expressing CaHxk2-GFP or CaGlk1-GFP were grown in 2% glucose YPG
913 to the mid log phase. Cells were transferred into the new medium containing 2% glucose
914 and exposed (N= Normoxia) or not (H= Hypoxia) to oxygen. Following this shift, cells were
915 collected at 0, 20, 60, 90 and 120 min and the detection of CaHxk2-GFP or CaGlk1-GFP was
916 performed by Western Blot using α-GFP antibody. Detection of total proteins by in-gel
917 Coomassie staining was used as a loading control (total extract).

918

919 **Fig 6. Glycolytic flux is required to sustain hyphal growth of *C. albicans*.** (A) *C. albicans* wild
920 type and mutant strains were grown during 3 days at 37°C on spider, serum or N-acetyl-
921 glucosamine medium. For each medium, the upper and middle panels show photographs of
922 macroscopic appearance of the colonies. Photographs present in the lower panel were

obtained using Zeiss Axioskop 2 Plus microscope with dark field and show the microscopic aspect. (B) Relative expression of *CaHXK2* and *CaGLK1/4* in the wild type strain during filamentation after transfer from 0.5% YPG to 5% serum liquid medium. Expression level of *CaHXK2* and *CaGLK1/4* was measured by qPCR, at different time points (0, 5, 20, 30 and 60 minutes), and normalized to the level of the *CaACT1* mRNA internal control. mRNAs level was expressed relatively to their abundance at time zero, which was set to 1. Results represent a mean (+ Standard Deviation) of 3 independent experiments performed in duplicate (n=6); ns, non-significant; **** $P < 0.0001$. Pvalue were calculated by one-way ANOVA using Tukey's method.

Fig 7. Hexokinase 2 is required for full virulence of *C. albicans*. (A) *Galleria mellonella* model of systemic infection. 2.5 x 10^5 cells of wild type (*SC5314*), complemented (*Cahxk2Δ/Δc/c*) or hexose kinase mutant strains were injected into the hemocoel at the last left-pro leg of 30 *Galleria* larvae. Sterile NaCl (0,9%) was injected into control larvae. Survival was monitored for 8 days at 37°C and presented in a Kaplan-Meier plot. Statistical analysis was performed using log rank tests; ns, non-significant; *** $P = 0.0002$; **** $P < 0.0001$. *Cahxk2Δ/Δ*, *Cahxk2glk1Δ/Δ* and *Caglk1glk4Δ/Δ* mutant strains are significantly less virulent compared to the wild type strain ($P_{value} \leq 0.0001$). The difference observed between *Caglk1Δ/Δ* mutant and the wild type strain is not significant ($P_{value} > 0.05$). The complemented strain exhibits higher virulence than hexokinase mutants but lesser than the wild type ($P_{value} = 0.002$). (B) Analysis of mouse macrophage interaction with live *C. albicans* cells in stationary phase at MOI 1:5 (1 macrophage for 5 yeasts) over a 24-hour time course experiment. The horizontal bars represent the macrophage survival, indicated as a percentage on the left side of the bar. The white part of the bars represents the percentage of non-phagocytosing

947 macrophages. The shades tones part represents the percentage of phagocytosing
948 macrophages. (C) Representative pictures of the J774 macrophages after 24 h of infection
949 with wild type and hexose kinase mutant strains in culture flasks at MOI 1 :5. The scale bars
950 represent 100 µm.

951 ## Supporting information

952 **S1 Table.** Glucose and ATP binding sites and structural domains of yeast hexokinases and
953 glucokinases.

954 **S2 Table.** *C. albicans* strains used in this study.

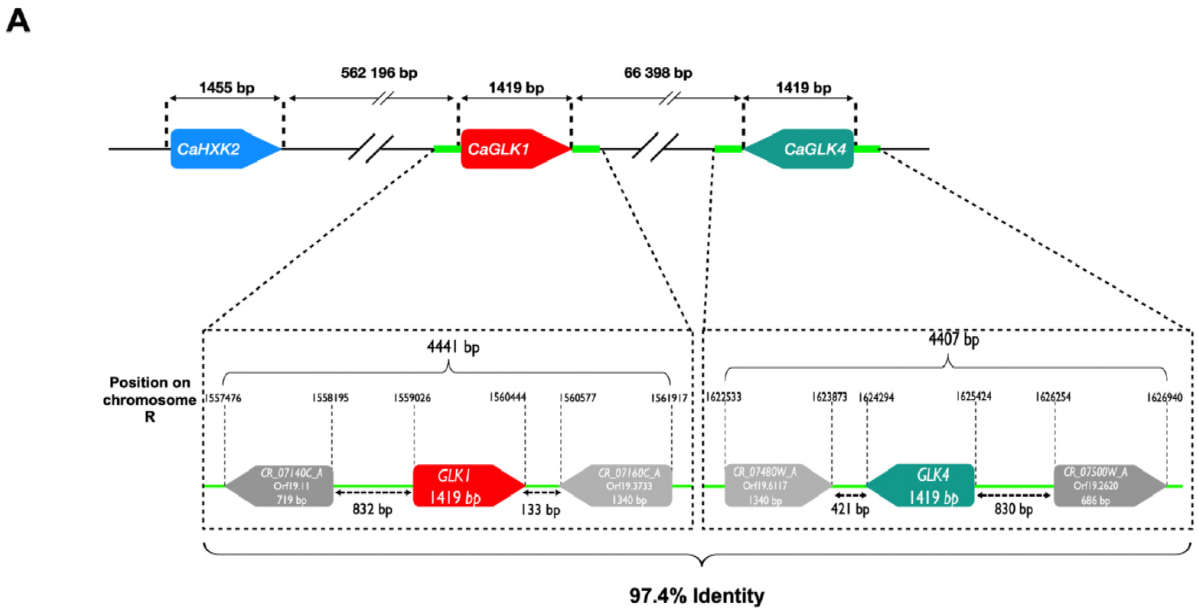
955 **S3 Table.** Plasmids used in this study.

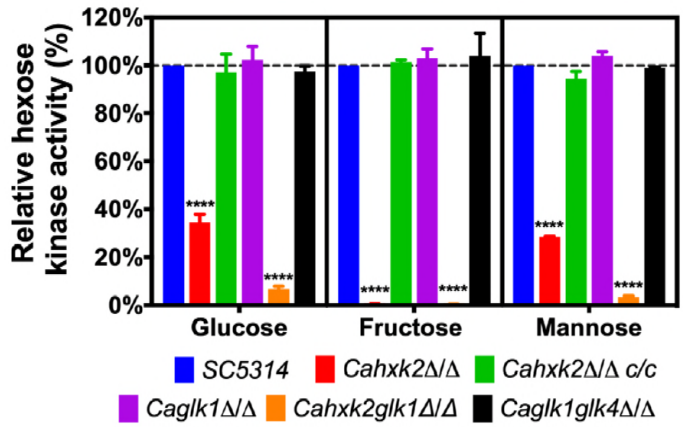
956 **S4 Table.** Primers used in this study.

957 **S1 Appendix.** Construction of *CaHXK2* deletion and complementation cassettes.

958 **S1 Fig**. *C. albicans* wild type and mutant strains divide equally during macrophage
959 phagocytosis.

960





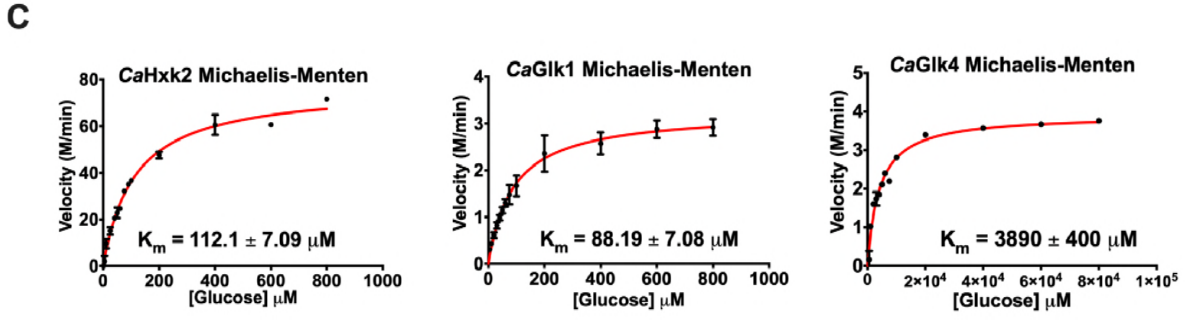


Figure 1

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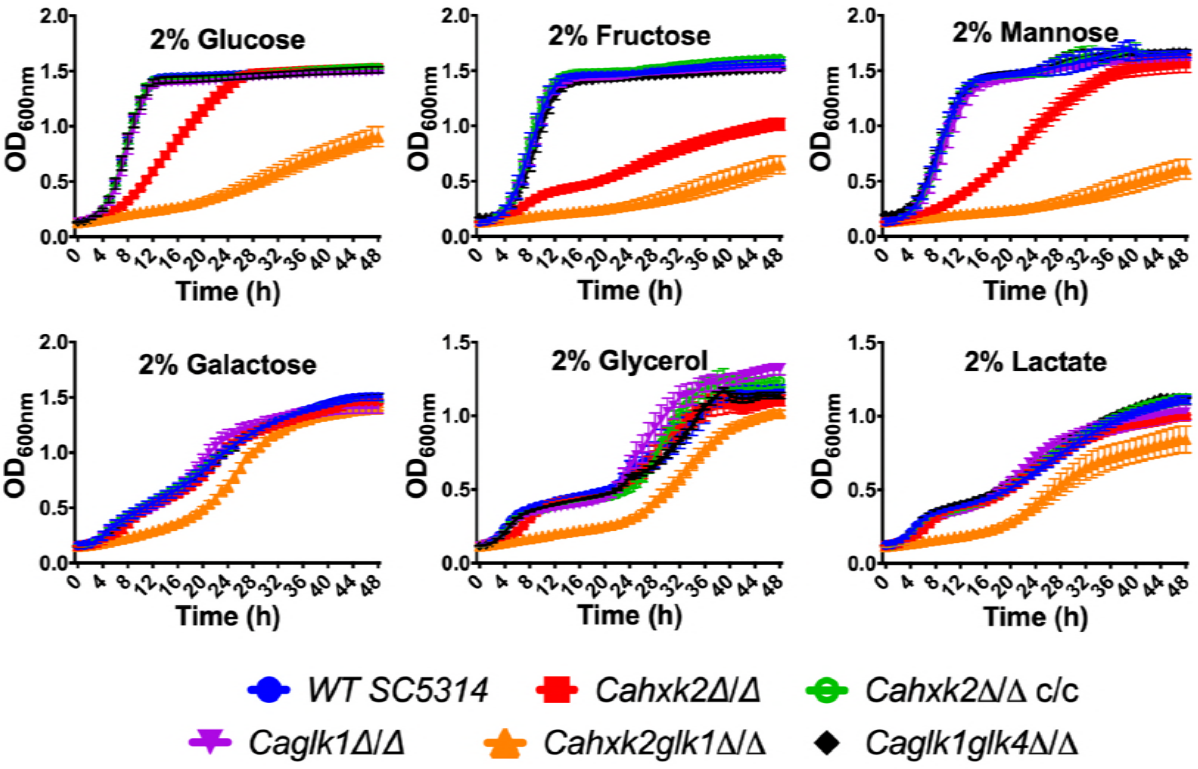

Figure 2

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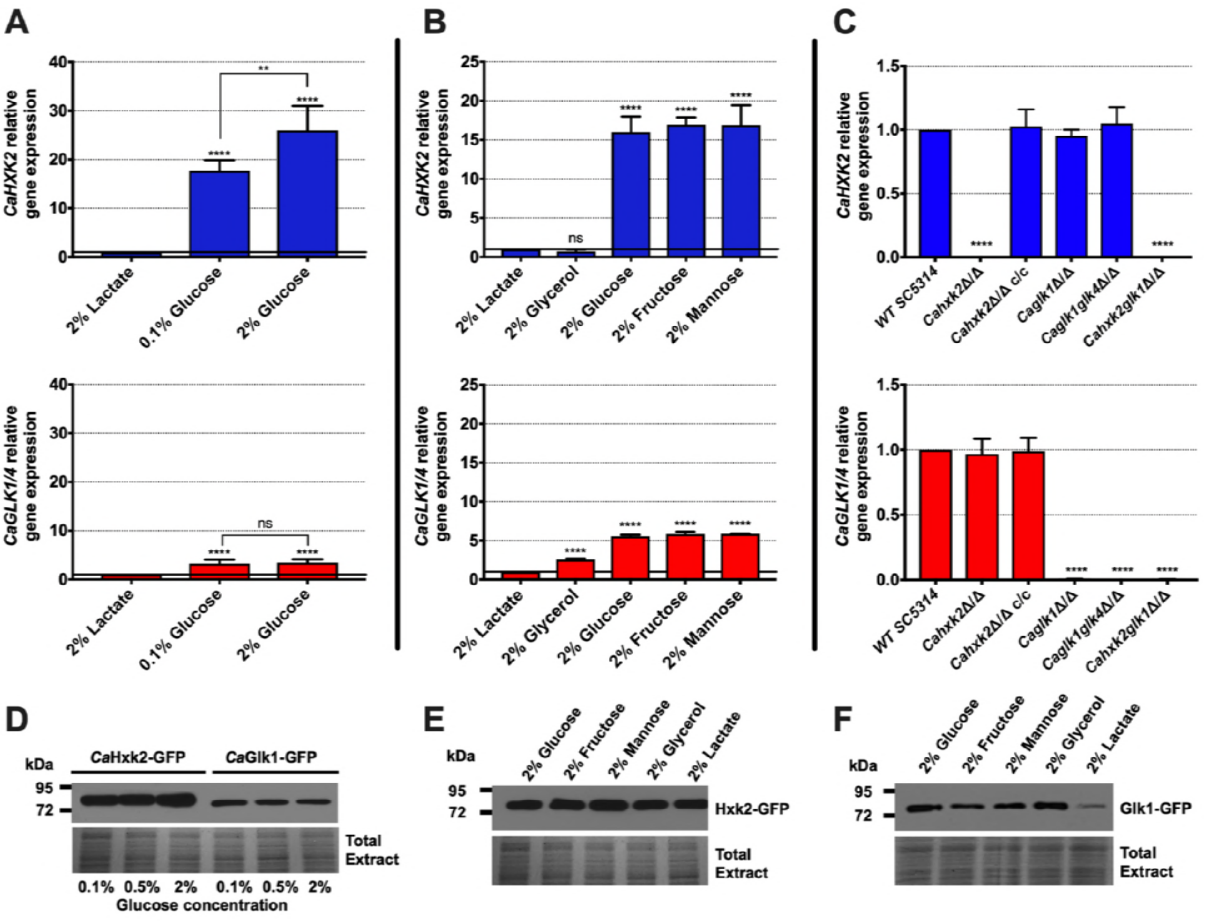

Figure 3

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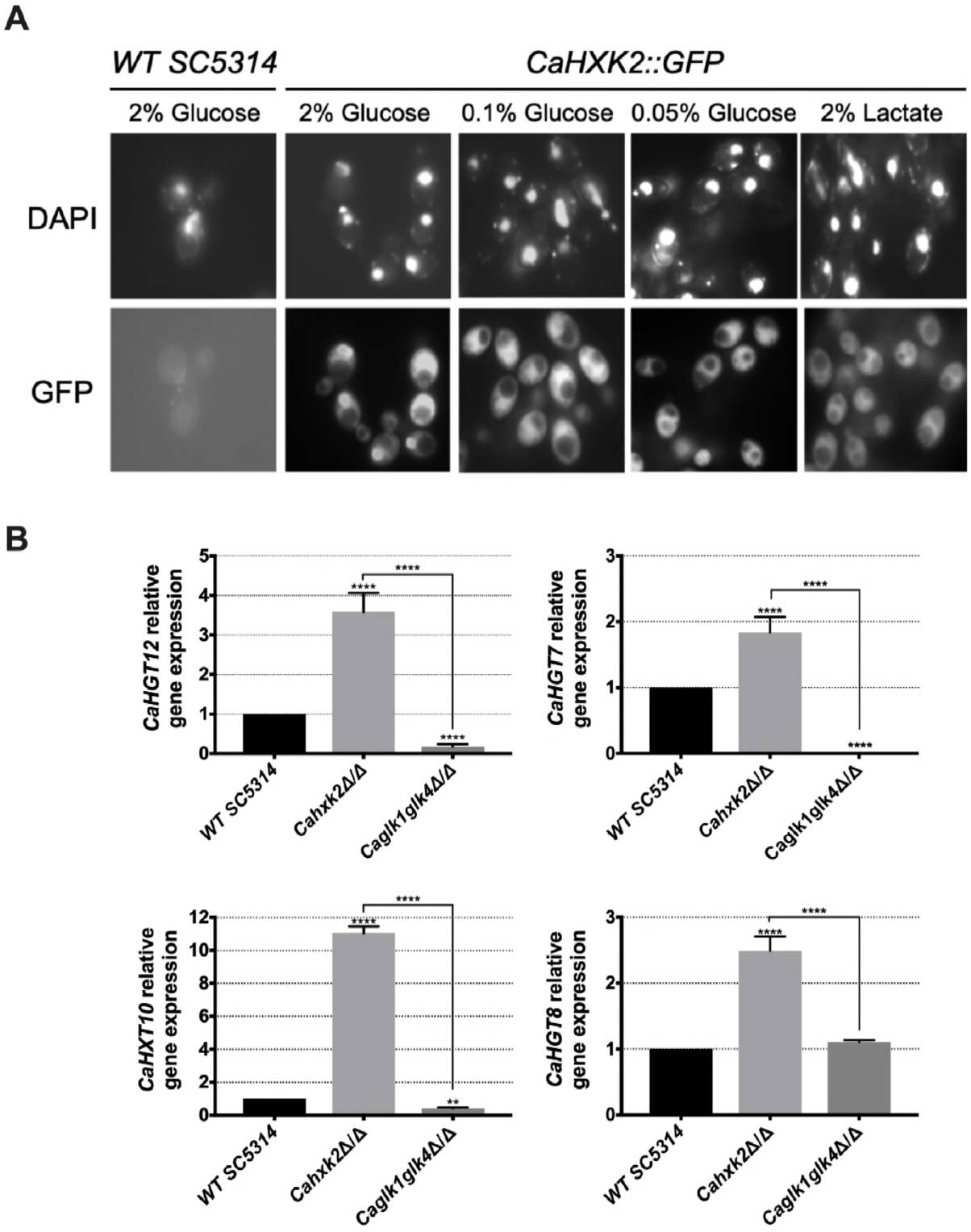

Figure 4

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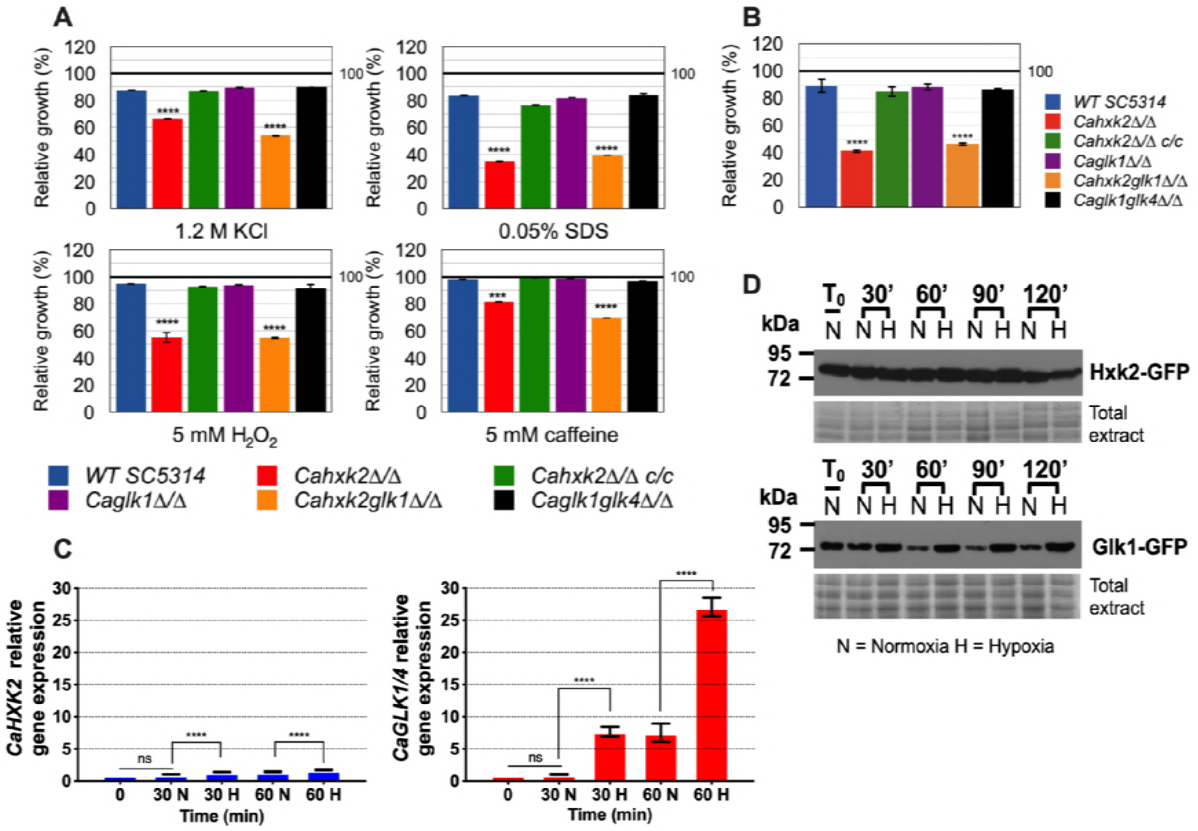

Figure 5

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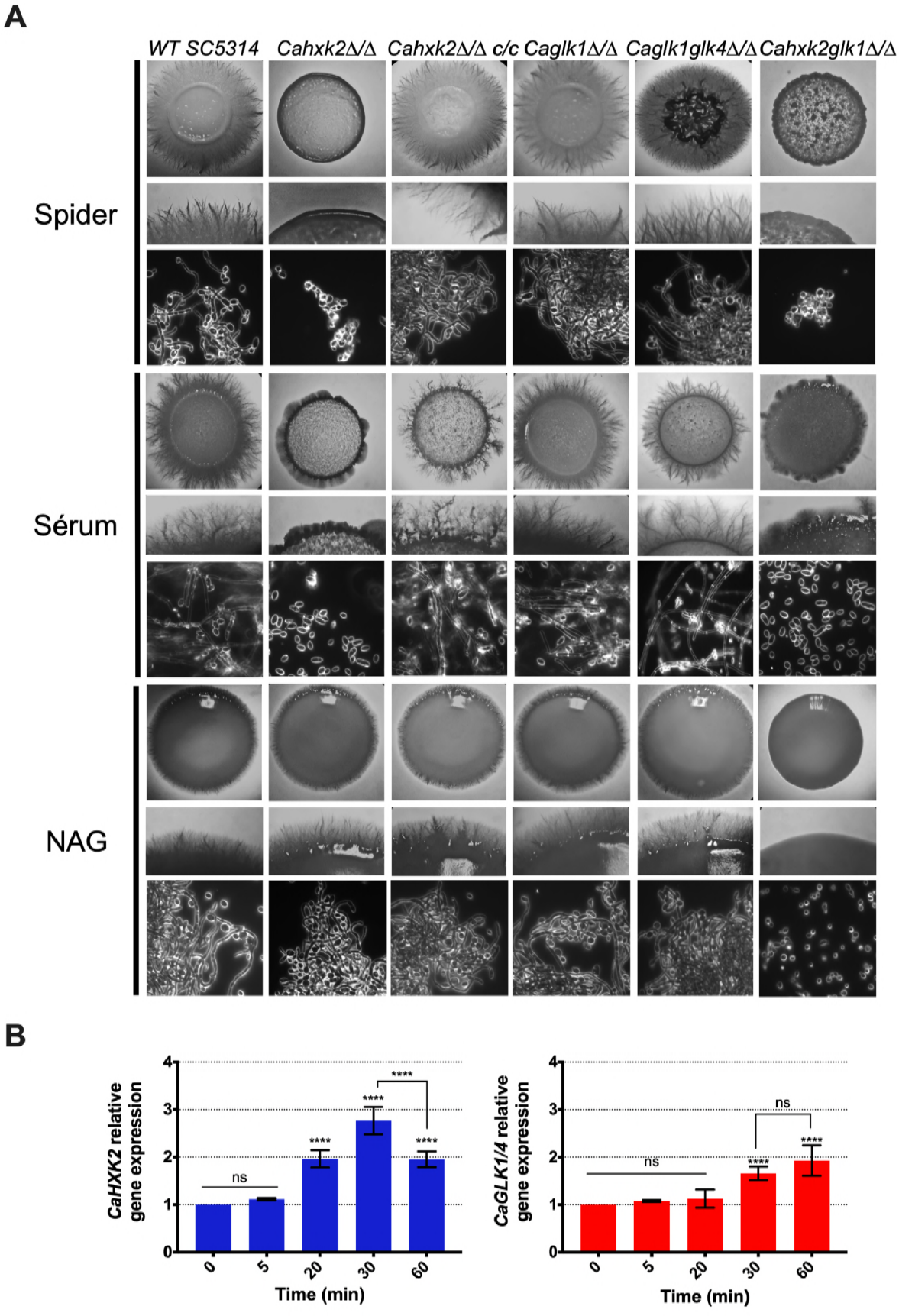

Figure 6

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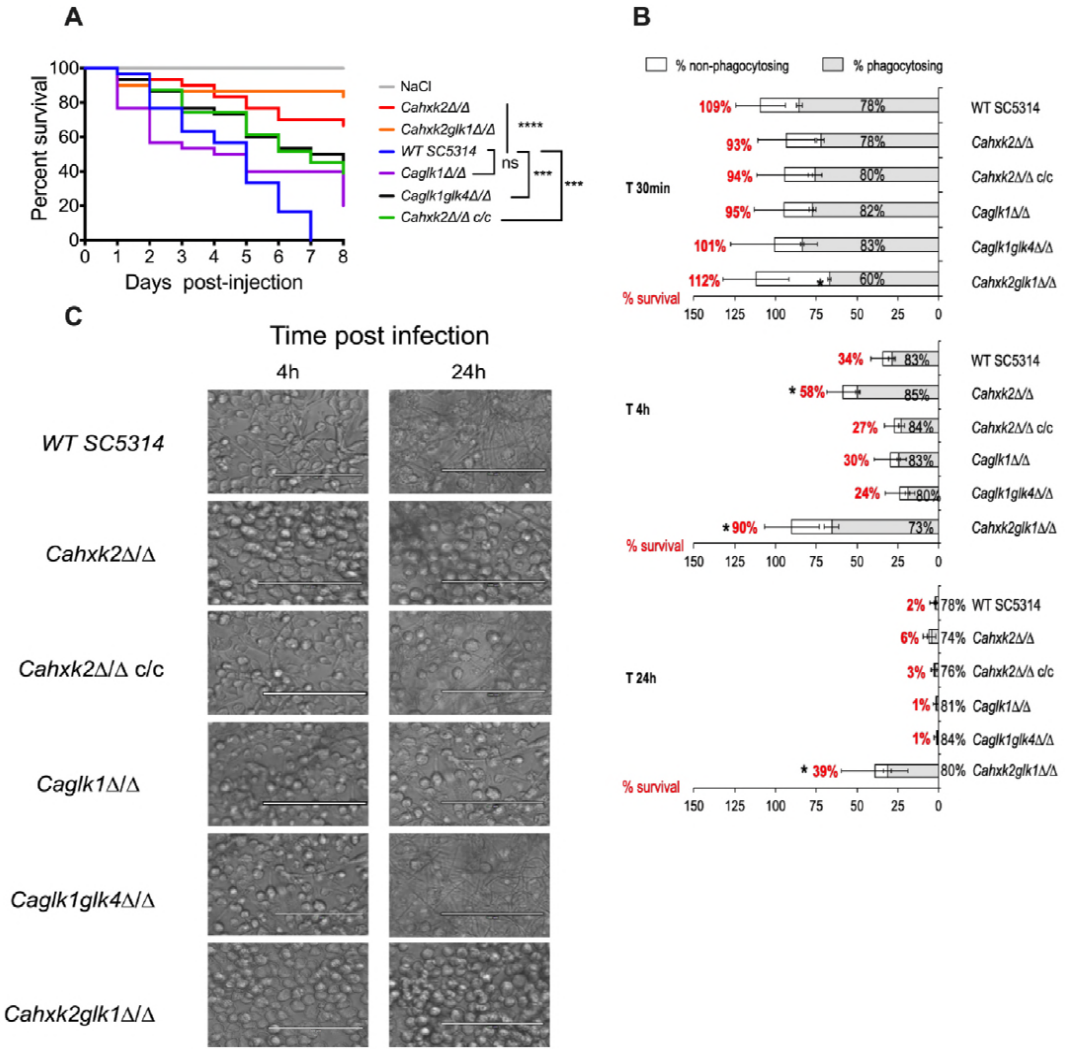

Figure 7