Title
Mucins suppress virulence traits of Candida albicans

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Candida albicans is an important opportunistic fungal pathogen in humans that can cause superficial infections, such as vaginitis in women or thrush in babies and HIV patients, and systemic, often fatal, disease in more advanced cases (1). C. albicans possesses a range of virulence traits, including adherence, filamentation, and secretion of proteases (2). At the heart of many infections is the formation of surface-associated C. albicans communities, also termed biofilms, which can form on mucus epithelial surfaces and on implanted medical devices, such as catheters and heart valves. Biofilms show increased resistance both to the immune system and to antifungal treatment (3). Despite the ability of C. albicans to cause disease, the healthy human body accommodates it as part of the microbiota (4–6). How the body tolerates the continued presence of potentially virulent C. albicans is largely unknown.

Mucus is the slimy coating found on all wet epithelia in the human body, including the eyes, airways, and the gastrointestinal and female genitourinary tracts, and many host-microbe interactions take place in this context. Its major gel-forming components, the mucin glycopolymers, are emerging as important regulators of microbial virulence. For example, the human cell-surface mucin MUC1 can inhibit surface adhesion of the gastric bacterium Helicobacter pylori (7). Moreover, the secreted human mucin Muc5AC can prevent Pseudomonas aeruginosa surface attachment and biofilm formation by promoting a dispersive state of bacteria (8). Other examples include mucus-mediated clearance of the bacterium Streptococcus pneumoniae (9) and modulation of HIV-1 (10) and influenza virus (11) infectivity by mucins. These observations indicate that mucin biopolymers help prevent bacterial and viral infections by regulating cellular processes related to virulence. Fungal pathogens are only distantly related to bacteria and viruses, and little is known about their interactions with mucins.

Here, we investigate the role of mucins as potential regulators of C. albicans virulence. Using a combination of gene expression analysis, mucus-secreting cell lines, and defined in vitro assays with natively purified mucins, we show that exposure to mucins induces a new oval-shaped morphological state in C. albicans in which various virulence traits are downregulated, including surface adhesion, the morphological transition to the filamentous
Mucins regulate *C. albicans* physiology. To determine the effects of mucins on *C. albicans*, the strain SC5314 was cultured in RPMI medium with and without pig gastric mucins (Muc5AC); RPMI favors growth in the filamentous state. Mucins were supplemented in the medium to create a 3-dimensional environment, as is found in the native mucus barrier (8). In the absence of mucins, the cells formed extensive hyphae which clumped together into flocs (Fig. 1A). In contrast, mucin-exposed cells predominantly formed short chains resembling pseudohyphae (Fig. 1A) or unicellular, ellipsoidal cells that are distinct from the round, yeast form cells. An analysis of the growth rate of *C. albicans* in mucins shows that cells continue to grow over time and even show an enhanced increase in optical density (OD) due to the homogenous suspension of individual cells as opposed to the filamentous flocs formed in RPMI medium alone (see Fig. S1 in the supplemental material).

Importantly, the effect of Muc5AC on *C. albicans* physiology is not limited to this type of mucin but was also observed for two other mucins: Muc2 from pig intestinal mucus and Muc5B from human saliva (Fig. 1B). This indicates that mucins have a general effect on *C. albicans* that likely extends across all mucosal surfaces. Due to the abundant availability of Muc5AC, the remainder of the experiments in this study were performed using this mucin.

At first sight, the mucin-induced morphology resembles opaque cells, which are the mating competent form of *C. albicans* (12) that have reduced virulence in systemic infection models (13). Since the ability of *C. albicans* to switch to the opaque state is controlled by genes at the mating type-like (MTL) locus, such that only *MTLa* or *MTLa* strains can undergo the transition to the opaque form, we first tested whether mucins would induce our starting *MTLa/α* heterozygous strains to become homozygous *MTLa* or *MTLa* mating-competent cells. Using PCR with primers specific to the MTL locus, we found that mucin-exposed cells remained heterozygous at the MTL locus (*n* = 100). To further probe their identity, the mucin-exposed cells were assayed for three features that are indicative of opaque cells: the abilities to form opaque colonies on agar plates (14), mate in the presence of the opposite mating type (15, 16), and form mating protrusions in the presence of mating pheromone (17). Our data show that the mucin-exposed cells were incapable of these three traits: they did not form opaque colonies, were not mating competent, and did not form mating protrusions in the presence of mating ρ-pheromone (see Table S1 in the supplemental material).

Moreover, exposure of *wor1Δ/Δ* cells (which cannot form opaque cells) to mucins also induced the oval-shaped morphology (Fig. S2), suggesting that the mucin-dependent morphology can develop independently of the master regulator of opaque status. As one further control, we repeated the white-opaque switching, quantitative mating, and pheromone response assays using mating competent *MTLa* or *MTLa* cells and found that mucins do not affect these opaque processes in mating competent strains (Table S1). Taken together, these results indicate that exposure to mucins suppresses the formation of hyphae while inducing the formation of a novel phenotype that superficially resembles the opaque cell type but is distinct in its physiological responses.

**Mucins downregulate virulence-associated genes in *C. albicans*. To better characterize the mucin-induced morphological change, we carried out transcriptional profiling experiments. A wild-type *C. albicans* strain (SC5314) was grown for 8 h in RPMI at 37°C in the presence and absence of 0.5% mucins. We performed quantitative PCR (qPCR) (Fig. 1C) to measure the expression levels of selected virulence genes (listed in Table S2 in the supplemental material), including those associated with adhesion (18), biofilm formation (19), and secreted proteinases (20). TAF145, a general transcription factor TFIID subunit (21), did not change expression in the presence of mucins and was used as a reference for calibration. The results of this experiment show that 7 of the 16 genes tested (Fig. 1C, P values indicated by asterisks) were downregulated by more than 1.5-fold in the presence of mucins (*P* < 0.05) as determined by a two-tailed, unpaired *t* test comparing the cycle threshold (ΔCt) values of samples with and without mucins.

**Mucins suppress *C. albicans* transition to the filamentous state.** To understand in more detail the effect of mucins on *C. albicans* physiology, we monitored the *C. albicans* strain HGFP3,
FIG 2 Mucins suppress hyphal growth from both yeast and hyphal cells. Fluorescence microscopy images overlaid on phase-contrast images (A) and quantitative PCR of the expression of hyphal-specific genes from yeast (B) or hyphae (C) after incubation for 8 h in RPMI alone or with 0.5% methylcellulose, 0.5% native mucins, or 0.5% industrially purified mucins. The strain used, HGFP3, expresses GFP only when cells are true hyphae. RNA was extracted from 6 independent biological replicates. Error bars represent SEM.

which expresses green fluorescent protein (GFP) when the hyphal-specific gene HWP1 is transcribed, during growth in RPMI with or without 0.5% natively purified mucins. To test whether the effects are simply a response to an increased viscosity or osmotic stress, we also subjected the cells to 0.5% industrially purified mucins (Sigma-Aldrich), 0.5% methylcellulose, or 1 M sorbitol. Industrially purified mucins are proteolytically processed and, as a result, have lost the gel-forming capacity characteristic of native mucins (22, 23). The polysaccharide methylcellulose is commonly used to mimic the viscosity of a mucus environment (24, 25). Sorbitol at high concentrations can induce osmotic stress; testing this condition is informative because the osmotic stress pathway is linked to hyphal formation (26). Our data show that in the no-polymer control, as well as in the presence of methylcellulose or 1 M sorbitol, the majority of cells formed hyphae, as indicated both by the elongated structures and the presence of GFP fluorescence (Fig. 2A, right). In comparison, hyphae in the absence of mucins continued to produce hyphae. Hyphal cells inoculated into medium containing methylcellulose also continued to produce more hyphal cells. These results were verified by qPCR, which showed that native mucins have the strongest capacity to suppress hyphal formation (Fig. 2C). We note that the levels of expression of the gene HWP1 showed a lower degree of suppression (yet still significant; \( P < 0.001 \)) when hyphae were added as the starting culture than when yeast cells were added (−1.3-fold change in expression with hyphal inoculum versus −15.9-fold change for yeast). Thus, in addition to inhibiting the growth of hyphae in yeast form cells, we can also conclude that the hypha-suppressive capacity of mucins is strong enough to downregulate the responsible pathway in existing hyphae.

Mucins suppress surface adhesion of *C. albicans* A critical, early step of infection by *C. albicans* is its attachment to a solid surface. From the results shown in Fig. 1C, we observed that genes involved in cell adhesion (*ALS1* and *ALS3*) were downregulated in the presence of mucins. Moreover, we know that mucins may physically trap certain particles and cells, thereby preventing their association with an underlying surface. Hence, we tested whether medium containing gel-forming mucins also decreases the surface attachment of *C. albicans*. We analyzed the ability of *C. albicans* to colonize two different surfaces in the presence of mucins: abiotic polystyrene, which is often used in the context of biofilm formation assays, and human epithelial mucus-secreting cells. For adhesion to polystyrene, a suspension of yeast cells was inoculated into polystyrene 96-well plates containing RPMI without or with 0.5% native mucins, industrially purified mucins, or methylcellulose. The phase-contrast microscopy images and quantification results in Fig. 3A and B show that native mucins significantly reduced cell attachment to polystyrene. This effect was detectable as early as 30 min and became stronger over the course of the hour (unpaired two-tailed \( t \) test; \( P < 0.05 \)). Methylcellulose was similarly effective in suppressing cell surface attachment (Fig. 3A). For comparison, industrially purified mucins provided no significant protection from surface attachment. Native mucins and methylcellulose both increase the viscosity of the medium, and hence, this parameter could be responsible for the observed antiahesive effect. To test this, we subjected the yeast to medium containing 0.5% polyethylene glycol (PEG), which is often used as an anti-fouling coating (27). Our data show that PEG was not able to decrease surface attachment to the same degree as mucins and methylcellulose (see Fig. S3B in the supplemental material), sug-
Mucins reduce attachment of \textit{C. albicans} to polystyrene and mucus-secreting human cells. (A) Fluorescence microscopy images of 96-well polystyrene plates after incubation with \textit{C. albicans} under different medium conditions (RPMI alone or with 0.5% methylcellulose, 0.5% industrially purified mucin, or 0.5% native mucin). Images were obtained every 15 min, after removal of nonadherent cells. (B) Quantification of attachment to the polystyrene plates. Error bars represent standard deviations of the results from 3 replicates. (C) Diagram illustrating the experimental setup of the infection assay, and fluorescence and phase-contrast microscopy images of \textit{C. albicans} SC5314 stained with calcofluor white after 2 h of incubation with HT29-MTX human mucus-secreting cells. (D) Quantification of \textit{C. albicans} attachment to HT29-MTX cells. Error bars represent standard deviations of the results from 3 replicates.

Mucins decrease \textit{C. albicans} biofilm formation. \textit{C. albicans} readily forms surface-attached biofilms on both abiotic and biotic surfaces. Because biofilm formation relies on attachment, filamentation, and cell-cell interactions (30, 31), we tested whether mucins inhibit biofilm formation by \textit{C. albicans}. We used a standard biofilm assay in which a polystyrene surface is immersed in a yeast culture (in RPMI) to allow attachment of the cells and, after 90 min, washed to remove nonadherent cells. Then, the surface is submerged in fresh, cell-free medium to follow the development of the initially attached cells into a biofilm over 8, 24, and 48 h. We performed this assay in the presence of native mucins and, for comparison, with methylcellulose and industrial mucins.

To evaluate the effect of the individual polymers on \textit{C. albicans} surface attachment, we removed the supernatant after the time points indicated above (8, 24, and 48 h) and analyzed the resulting biofilms (Fig. 4). Surface attachment was visibly reduced in the presence of native mucins and even more effectively prevented in the presence of methylcellulose (Fig. 4A). Confocal zy and xz sections of the biofilms show that both the surface coverage (zy images) and thickness (xz scan) of the biofilm are reduced in the presence of mucins (Fig. 4C). Moreover, a smaller percentage of cells in the mucin-treated biofilms form hyphae within the thin biofilms, confirming the previous observation from the experiment whose results are shown in Fig. 2. Finally, quantification of the biofilm biomass shows a significant reduction in the presence of mucins (Fig. 4D). These data suggest that both mucins and methylcellulose have the capacity to suppress, or destabilize, the attachment of \textit{C. albicans} to an underlying polystyrene surface over a relatively long period of time. In an experiment whose results are shown in Fig. S1 in the supplemental material, we showed that the cells continued to grow strongly in the presence of both mucins and methylcellulose, indicating that the reduced number of attached cells is not due to toxicity of the polymers.

If cell proliferation is not suppressed by the polymers and, yet, a reduction of biomass is observed on the surface, one would expect that a significant proportion of the population has been shifted to the supernatant. This could occur if cells are released from the biofilm and continue to propagate in the planktonic phase. Indeed, the results in Fig. 4E show that the supernatant from biofilms in the presence of mucins or methylcellulose contained higher numbers of cells than the supernatants from biofilms growing without mucins. This experiment also reveals another important point: in the presence of mucins, the nonattached cells are largely devoid of filaments and, therefore, presumably are in the less invasive form (Fig. 4B). In contrast, the cells in the supernatant of methylcellulose cultures were all highly filamentous. Together, these data suggest that both mucins and methylcellulose can suppress the formation and maturation of biofilms by reducing attachment of the cells to an underlying surface, possibly by preventing stable maintenance of cells within the biofilm. However, mucins have a distinct effect: they render nearly the entire population devoid of filaments.

**DISCUSSION**

This work shows that the mucin Muc5AC, which is expressed in the stomach and in the lungs, can induce the downregulation of several virulence traits in \textit{C. albicans}, both at the level of gene expression and phenotype. These include the suppression of both filamentous growth and the formation of surface-attached biofilms. Studies with other types of mucins (Fig. 1B) (32, 33) suggest that the ability of mucins to manage microbial virulence may be a general mechanism that is present on all mucosal surfaces as part of the innate mucosal immune system.
How might mucins prevent the transition to the hyphal form? Mucins, but not the other tested polymers, were capable of blocking this transition, suggesting that specific, mucin-associated glycans might be involved in this process. Consistent with this idea, glucose, maltose, and galactose in solution can all influence the formation of hyphae in *C. albicans* (34). The identity of the mucin glycan moieties that are recognized by *C. albicans*, as well as the receptors and pathways in the yeast that are affected by these sugars, are currently unknown; their identification may suggest new valuable strategies for preventing or recovering *C. albicans* infections of mucosal surfaces.

Preventing biofilm formation on materials exposed to living organisms presents a vexing engineering challenge; the results obtained for mucus hydrogels may provide some interesting new strategies. Our experiments show that mucins and methylcellulose are both effective in suppressing *C. albicans* surface attachment. Both polymers appear to reduce the ability for initial surface attachment; moreover, they render newly formed cells less capable

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**FIG 4** Mucins reduce biofilm formation of *C. albicans*. Biofilms were grown using strain HGFP3 that produces GFP upon transcription from a hyphal-specific promoter. (A) Macroscopic view of biofilms grown in the presence or absence of mucins or methylcellulose. (B) Fluorescence microscopy images overlaid on phase-contrast images of *C. albicans* found in biofilm supernatants. (C) Confocal images of biofilms grown in the presence or absence of mucins. (D and E) Quantification of biofilm biomass (D) and of *C. albicans* found in biofilm supernatants (E). RFU, relative fluorescence units. Error bars show SD. P values indicated by asterisks are <0.05.
of stably integrating into an emerging biofilm. How these polymers work to suppress surface attachment and even whether they function by the same mechanisms are open questions. Despite their superficial resemblance in surface protection, mucins and methylcellulose have different effects on the surrounding *C. albicans* cell population. With methylcellulose, the vast majority of cells remain in the filamentous form, while a cell population exposed to mucins remains largely devoid of filaments. There is good experimental evidence that the ability to form filaments is required for *C. albicans* virulence (35). Therefore, we have shown that the native mucins in the body are capable of providing a dual mechanism for virulence control, suppressing both the yeast-hyphal transition and surface attachment.

The ability of mucins to suppress virulence traits is not specific for *C. albicans* but appears to apply also to a range of other microorganisms, including the bacteria *Helicobacter pylori* and *Pseudomonas aeruginosa* (7, 8) and, also, certain viruses, such as HIV and influenza virus (10, 11). Understanding the mechanism of the mucin- *Candida* host-pathogen interaction could direct treatment strategies for regulating the healthy microbiota and also shed light on the molecular origins of increased susceptibility to microbial disease.

**MATERIALS AND METHODS**

*C. albicans* strains and media. Strains were maintained on YPD agar (2% Bacto peptone, 2% glucose, 1% yeast extract, 2% agar) and grown at 30°C. Single colonies were inoculated into YPD broth and grown with shaking overnight at 30°C prior to each experiment.

The experiments were performed using either Gibco RPMI 1640 medium (catalog number 31800-089; Life Technologies) buffered with 165 mM morpholinepropanesulfonic acid (MOPS) and supplemented with 0.2% NaHCO₃ and 2% glucose or YPD medium with 10% fetal bovine serum (FBS). Medium with 0.5% methylcellulose (15 centipoise [cP]; Sigma) was prepared from a 5% stock solution by dilution in RPMI. Type II mucin from porcine stomach (Sigma) was dialyzed in a Spectra/ Por Float-A-Lyzer G2 dialysis tube with a 100-kDa molecular mass cutoff, followed by lyophilization. Industrial PGM and native PGM were dissolved in RPMI and gently vortexed at 4°C overnight.

The *C. albicans* yeast strains used in this study were SC5314 and HGFP3. Strain HGFP3 (36) was constructed by inserting the GFP gene next to the promoter of *HWP1*, a gene encoding a hyphal cell wall protein, and was provided by E. Mylonakis (Massachusetts General Hospital, Boston, MA) with the permission of P. Sundstrom.

**Mucin purification.** The mucins were natively purified to preserve their properties, as opposed to industrially purified mucins which do not form gels in solution (23, 22). Porcine gastric mucins (PGM) were purified from fresh pig stomachs as previously described (37). Briefly, the mucin layer was isolated from pig stomachs and solubilized in sodium chloride buffer containing protease inhibitors to prevent mucin degradation and sodium azide to prevent bacterial proliferation. Insoluble components were removed via centrifugation, and the mucins were isolated using gel filtration chromatography on a Sepharose column (CL2B). The mucins were then concentrated and lyophilized. As a control to ensure that there were no contaminants in the mucin preparation, mucins prepared by CaCl₂ gradient centrifugation (as described in references 38 and 39) were compared to those prepared without this step.

**Extraction of RNA and cDNA synthesis.** One milliliter of RPMI or 0.5% PGM in RPMI in a culture tube was inoculated with 10 µl of an overnight culture of strain SC5314 and incubated at 37°C and 180 rpm for 8 h. RNA was extracted using the Epicentre MasterPure yeast RNA purification kit and treated with Sigma-Aldrich AMPD1 amplification-grade DNase I. Five hundred nanograms of RNA per sample was used to generate cDNA using the Invitrogen SuperScript III first-strand synthesis system. cDNA samples were stored at −80°C until use.

**Quantitative PCR.** All primers were obtained from Sigma and analyzed for efficiency before use in experiments. Efficiency was calculated by performing qPCR with serial 1:10 dilutions of genomic DNA. Bio-Rad iQ SYBR green supermix was used for qPCRs. Experiments were performed in a Roche LightCycler 480 II machine with the following run protocol: 95°C for 3 min and then 40 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s. Cycle threshold (Cₜ) values were obtained and used for analysis. Fold changes were calculated using the ΔΔCₜ method in comparison to the results for the reference gene TAF145.

**Filamentation assay.** One hundred microliters each of RPMI, 0.5% methylcellulose medium, 0.5% industrial PGM medium, and 0.5% native PGM medium were inoculated with the strain HGFP3 as yeast form cells or hyphae in a 96-well plate. One microliter of an overnight culture was used as the source for yeast form cells. For hyphae, an overnight culture was diluted 1:100 into YPD plus 10% FBS, which stimulates the transition to hyphae, and grown to an optical density at 600 nm (OD₆₀₀) of 0.5. The hyphae were centrifuged and then resuspended to an OD₆₀₀ of 5, and 10-µl amounts were added to media under the aforementioned conditions. The cells were incubated at 37°C with shaking at 180 rpm for 8 h. Adherent cells were scraped off the surface, and samples were pipetted vigorously to break up aggregates. A 15-µl amount of each sample was placed on a microscope slide for visualization. Slides were imaged with a Zeiss Observer Z1 inverted fluorescence microscope with a Zeiss Plan-Apochromat 20× objective lens using phase-contrast and fluorescein isothiocyanate (FITC) (excitation at 475 nm and emission at 530 nm).

**Polystyrene attachment assay.** Polystyrene 96-well plates were inoculated with 100 µl of RPMI, 0.5% methylcellulose medium, 0.5% industrial PGM medium, and 0.5% native PGM medium containing yeast form cells from the strain SC5314. The plates were incubated statically at 37°C. Every 15 min, a time point sample was taken by washing the wells with 200 µl of phosphate-buffered saline (PBS) twice, followed by the addition of 100 µl of PBS. After 1 h, 1 µl of 1 mg/ml calcofluor white solution was added to each well. The samples were imaged similarly to the description above using a 10× objective (excitation at 365 nm and emission at 445 nm). The experiment was performed in triplicate with 5 pictures taken of each well. The images were analyzed in ImageJ as follows: each image was converted to 8-bit and the contrast was enhanced (0.4% saturated pixels). The image was then thresholded to create a binary image. The image was then analyzed using the Analyze Particles tool to measure the surface area covered by cells. The surface area measurements of the 15 images for each condition and time point were averaged.

**Attachment to human mucus-secreting colorectal cells (HT29-MTX).** HT29-MTX cells mucus-secreting cells reliably secrete a thick, homogeneous layer of mucus as soon as 7 days postconfluence. The cells were grown in a 24-well plate. At 2 weeks postconfluence, the cells were treated with 10 mM N-acetylcysteine, which cleaves disulfide bonds between mucins (40), for 30 min to remove the adherent mucus layer or with PBS as a control. For infection, *C. albicans* strain SC5314 was diluted from an overnight culture into Dulbecco modified Eagle medium to an OD₆₀₀ of 0.5. Five hundred microliters of *C. albicans* was added on top of the HT29-MTX cells and incubated statically at 37°C for 2 h. After 2 h, the medium was removed from the wells, which were subsequently washed twice with 500 µl of PBS. The remaining *C. albicans* cells were stained with calcofluor white and analyzed using a Zeiss Observer Z1 inverted fluorescence microscope and a plate reader (excitation at 355 nm and emission at 460 nm).

The HT29-MTX cells were derived from HT-29 cells (ATCC HTB-38) as described previously (29). HT-29 cells were derived from an anonymous donor.

**Biofilm formation assay and visualization.** Biofilms were grown on either of two surfaces: in a 96-well plate (for macroscopic views and quantification) or on 8-mm-diameter silicone circles (for confocal imaging). Before the experiment, the silicone circles were washed with water and
autoclaved. The surfaces were incubated in adult bovine serum overnight with shaking at 37°C. The next day, the surfaces were washed in PBS and submerged in a C. albicans cell suspension with an OD600 of 0.5 in RPMI with or without 0.5% mucins. The samples were incubated at 37°C with shaking at 180 rpm for 90 min to facilitate attachment of yeast cells to the surface. Nonadherent cells were washed away with PBS, and the samples were subsequently submerged in fresh RPMI with or without 0.5% mucins, 0.5% industrial mucins, or 0.5% methylcellulose. The biofilms were allowed to grow with shaking (180 rpm) at 37°C for the times indicated in Fig. 4. For biomass quantification, the biofilms were stained with 20 μg/ml calcofluor white in PBS for 10 min and analyzed in a SpectraMax M3 plate reader (excitation at 355 nm and emission at 460 nm). Biofilm supernatant quantification was performed in the same plate reader (absorbance at 600 nm). For confocal imaging, the biofilms were submerged in 6 ml of 20 μg/ml calcofluor white and stained for 10 min. The biofilms were imaged using a photo scanner or a Zeiss LSM 700 upright confocal microscope. Planktonic cells were placed on microscope slides and imaged using a Zeiss wide-field fluorescence microscope.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01911-14/-/DSupplemental.

SUPPLEMENTAL MATERIAL

Text S1, DOCX file, 0.02 MB.
Figure S1, TIF file, 0.3 MB.
Figure S2, TIF file, 3.3 MB.
Figure S3, TIF file, 2.1 MB.
Figure S4, TIF file, 0.7 MB.
Figure S5, TIF file, 1.5 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

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