**Title:** An opaque cell-specific expression program of secreted proteases and transporters allows cell-type cooperation in *Candida albicans*

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**File S1:** Supplemental Materials and Methods, Supplemental Results, and legends for all supplemental items.

**Supplemental Materials and Methods**

**White-Opaque Switching Assay and Variants**

*Standard White-Opaque Switching Assays*

The white-to-opaque and opaque-to-white switching assays evaluating the effect of *SAP* deletions on switching rates followed previously reported protocols (Miller and Johnson 2002; Zordan *et al.* 2007; Lohse *et al.* 2016). In brief, following seven days growth at 25°C after recovery from glycerol stocks, five colonies without visible sectors were resuspended in water, diluted, and plated on six SCD+aa+Uri plates. After seven days of growth, plates were scored for (A) colonies of the starting cell type, (B) colonies of the starting cell type with one or more sectors, and (C) colonies of the other cell type. We have calculated the switching frequency as (B+C) / (A+B+C). If no switching events were detected for a given strain, the switching frequency was treated as 1 / (total number of colonies counted on all of the plates for that strain). The switching frequency for each deletion strain was then normalized to the average switching frequency of wild type replicates, screened in parallel, from the same day.

*BSA Media Switching Rate Assays*

Evaluation of white-opaque switching when BSA is the sole nitrogen source is complicated by the white cell proliferation defect on SD+BSA+Uri media. Two changes were made to the standard switching assay in an attempt to circumvent this problem. The first variation on the switching assay measured switching rates when both BSA (1%) and ammonium sulfate (0.5%) were present (SD+AmS+BSA+Uri); the addition of ammonium sulfate allows for white cell growth while seeing if there is a change (relative to SD+AmS+Uri plates) in the presence of BSA. We note that the presence of ammonium sulfate might reduce or even eliminate effects due to the presence of BSA, as such we also pursued an alternative approach. The second variation on the standard switching assay involved plating a mixture of white and opaque cells on the same SD+BSA+uri plates so that the opaque cells facilitate white cell growth. White cells were diluted and plated for single colonies and, after one hour incubation at 25°C, 1 µl drops of a concentrated opaque cell solution (OD600 > 5) were spotted in a 2x3 grid in the center of the plates with 20 mm spacing between drops. For both variants of this assay, the plates were incubated at 25°C and scored after seven days (all plates) and after ten days for SD+AmS+BSA+Uri and SD+BSA+Uri plates. The SD+AmS+BSA+Uri and SD+BSA+Uri plates were scored at the second, later timepoint due to the slower growth observed on these plates, and there was not a noticeable change in switching rates between the two time points. The SD+BSA+Uri and SD+AmS+BSA+Uri plates for these assays contained BSA from a fresh 20% stock solution that was filtered on 0.45 µm PES filters (Thermo #725-2545) and added to the other media components after the autoclave step in order to minimize protein denaturation or degradation.

*37°C BSA opaque cell stability assay*

For the 37°C BSA opaque cell flow cytometry proliferation assay, we were interested in any changes in the cell type composition of the population during the course of the assay. To track this, we harvested cells immediately following inoculation into SD+BSA+Uri media as well as one, two, and seven days following the shift to 37°C. The harvested cells were diluted and plated on SCD+aa+Uri plates at 25°C, two plates for each culture at each timepoint. As we were interested in the cell type composition of the population when the cells were plated, rather than any switching that occurred as the cells proliferated on the plates, we scored only entirely white or opaque colonies and calculated the switching frequency as (white colonies) / (white colonies + opaque colonies) for this assay.

**Optical Density Proliferation Assays**

The optical density proliferation assays modified our previously reported protocol (Lohse *et al.* 2016) to account for the increased delay before proliferation when BSA was the sole nitrogen source. In brief, SCD+aa+Uri overnight cultures (25°C) were started from white or opaque colonies. The following morning, overnight cultures were diluted to OD600 = 0.1 in SD+Uri and these 10x working stocks were then diluted 1:10 into 90 µL of the indicated media (generally SD+BSA+Uri or SD+AmS+Uri). Optical density proliferation assays were performed in 96-well plates (Falcon 351172) sealed with Breathe-Easy membranes (Diversified Biotech BEM-1) on a Tecan Infinite M1000 Pro or a Tecan Spark 10M at 25°C. Absorbance was measured every 15 minutes for 67 hours (270 cycles) with continuous shaking between reads. Absorbance values represent the average of five reads per well (Tecan Infinite M1000 Pro) or a single read per well (Tecan Spark 10M). Three replicate wells were started from the same overnight culture and working stock for each strain on a given day. Wild type samples and uninoculated wells were included on each plate to allow for comparisons between experiments and for background subtraction. When indicated, Pepstatin A (Sigma P5318) was diluted from 5 mM (in DMSO) to 1 mM (in DMSO) before being added to media at a final concentration of 1 or 10 µM as indicated. Equivalent volumes of DMSO were used as loading controls for these assays.

Maximum proliferation rates were estimated using the plyr package in R; in brief the script determined the four hour (17 time point) window with the maximum slope that satisfied other criteria (e.g. r2 greater than 0.9, no blank subtracted absorbance values below 0.05). All 270 points were used for SD+AmS+Uri curves and points 81-270 (20-67.25 hours) were used for SD+BSA+Uri curves. The initial point from the four hour window of maximum proliferation was defined as the time to maximum proliferation, used for comparing delays before active proliferation. The maximum proliferation rate and time to maximum proliferation for each well were normalized to the average values from the wild type control(s) from the same day in order to allow for comparisons between assays. The three wells for each strain were analyzed separately and only averaged after normalization.

The initial opaque transcriptional regulator knockout library BSA proliferation screen involved one set of three wells for each of the 188 strains. Candidates identified during the first screen of the library did not proliferate or had either a 25% reduction in maximum proliferation rate (“slow” proliferation phenotype) or a 40% increase in the time to maximum proliferation (“late” proliferation phenotype). The 52 candidates that met one or more of these criteria were then screened a second time on SD+BSA+Uri as well as on SD+AmS+Uri (on the same 96-well plate) and evaluated using the same thresholds as the initial screen. We considered strains with a reproducible slow and/or late SD+BSA+Uri phenotype, but not slow phenotypes on SD+AmS+Uri or in our previous SCD+aa+Uri proliferation screen (Lohse *et al.* 2016), to be BSA-specific as opposed to general proliferation defects. Three of the deletion strains (*brg1*, *zcf16*, *swi4*) were tested three, rather than two, times; for these strains we required a phenotype to repeat in all three experiments. We could not reliably calculate maximum proliferation rates or times to maximum proliferation for three strains due to opaque filamentaion (*czf1*, *bcr1*) or high rates of opaque-to-white switching (*fgr15*).

A breakdown of the ten slow-, twelve late-, and two no-proliferation defects (BSA-specific or general) is included in the Supplemental Results and the complete list of maximum proliferation rates and times to maximum proliferation for this screen are provided in File S7. We note that we could not calculate maximum proliferation rate or time to maximum proliferation for the two strains that did not proliferate on SD+BSA+Uri media after 67 hours (*stp1*, *adr1*). These two strains were evaluated during the second round of screening in order to distinguish between those with BSA-specific proliferation defects that could grow on SD+AmS+Uri (*stp1*) and those with general proliferation defects that could not (*adr1*).

**Supplemental Results**

**Proliferation on BSA does not increase white-opaque switching or stabilize opaque cells at 37°C**

We observed little opaque-to-white and no white-to-opaque switching when cells proliferated on either SD+BSA+Uri or SD+AmS+BSA+Uri (where ammonium sulfate was added to help white cell proliferation) plates (Table S2). As such, proliferation on BSA does not appear to trigger *en masse* switching in either direction. Given the decrease in switching seen on SD+AmS+Uri plates relative to the normal switching assay plates (SCD+aa+Uri, containing 0.5% ammonium sulfate and 0.2% amino acids), we cannot determine whether proliferation on BSA reduces either switching rate.

We conducted the bulk of our experiments at 25°C, the temperature most conductive to the stability of the opaque cell type, since opaque cells switch *en masse* to white cells at 37°C on standard lab media (2% glucose, 0.5% ammonium sulfate, 0.2% free amino acids) (Slutsky *et al.* 1987; Rikkerink *et al.* 1988). Certain conditions, such as media lacking glucose, however, can reduce or even block this temperature induced switching (Huang *et al.* 2009, 2010; Lohse *et al.* 2013). In order to determine whether proliferation with a protein as the sole nitrogen source affected opaque cell stability at 37°C, we modified our standard flow cytometry based proliferation assay by shifting the incubation temperature to 37°C and collecting cells for plate based switching assays after one, two, and seven days. We found that, for a population that started as predominately opaque, roughly one-third of cells were white after two days and nearly all cells (>90%) where white after seven days proliferation on BSA at 37°C (Figure S2B). The slight delay this represents relative to opaque cells proliferating on ammonium sulfate presumably results from the later and slower proliferation of opaque cells on BSA.

Although the populations that started as opaque cells divided seven or eight times during the course of this experiment, the largely white nature of the final population (>90%) obscures whether the bulk of the observed proliferation was due to white or opaque cells (Figures S2A and S2B). To address this question, we consider the same population after two days proliferation on BSA at 37°C. By this point, the starting cells had divided approximately four times (going from roughly 500 to 10,000 cells/µL) and, based on the plating assays, roughly one-third of the population were white cells (Figures S2A and S2B). As the white cell subpopulation does not account for the entire increase in cell numbers, we conclude that opaque cells are capable of proliferation on BSA at 37°C. While the opaque cells can still proliferate, the 37°C temperature signal still pushes them to switch to the white cell type. We also note that white cell populations exposed to the same conditions (seven days proliferation on BSA at 37°C) divided seven or eight times during the course of this experiment (Figure S2A).

**Deletion of SAP genes does not affect white-opaque switching rates**

Given the differential expression of a number of the *SAP*s between white and opaque cells, we examined whether the opaque-expressed *SAP*s affect cell type switching or stability. We considered strains with different combinations of four or five of the opaque-expressed SAPs (*SAP1*, *SAP2*, *SAP3*, *SAP8*, and *SAP99*) deleted but did not observe large (at least five-fold) changes in either white-to-opaque or opaque-to-white switching rates in any of these strains on standard lab media (2% Glucose, 0.5% ammonium sulfate, 0.2% amino acids) at 25°C (Table S3). As none of these *SAP* deletions prevented white-to-opaque switching and the effects were small compared to those observed for many transcriptional regulators (Lohse *et al.* 2016), we conclude that the opaque-expressed *SAP*s do not contribute to cell type switching and/or stability under standard laboratory conditions.

**Transcriptional Regulator Knockout Library Proliferation Screen**

We looked for three phenotypes in the BSA proliferation assay screen: (1) strains that were unable to proliferate, (2) strains that had a reduced maximum proliferation rate (slow proliferation), and (3) strains that took longer to reach their maximum proliferation rate (late proliferation); the latter two phenotypes are not mutually exclusive. Approximately 50 transcriptional regulator mutants exhibited one or more of these phenotypes in our initial screen and were thus tested a second time (File S7). Furthermore, we screened these candidates on media with ammonium sulfate as the sole nitrogen source (and looked at our previously published data for proliferation on media containing ammonium sulfate and amino acids) in order to eliminate general, as opposed to BSA-specific, proliferation defects.

We identified ten strains with consistent slow proliferation phenotypes on BSA (*efg1*, *hap2*, *hap31*, *hap41*, *hap42*, *hap5*, *hcm1*, *mac1*, *rfg1*, *zcf21*), however all ten of these strains also grew slowly on ammonium sulfate (all but *mac1*) and/or standard media (SCD+aa+Uri; all but *efg1*, *rfg1*), indicating general, as opposed to BSA-specific, proliferation defects (File S7). We identified twelve strains with consistent late proliferation phenotypes on BSA (File S7). Eight of these strains (*csr1*, *efg1*, *grf10*, *hap2*, *hap42*, *mac1*, *ssn6*, *zcf8*) were either slow proliferators on BSA (two repeats for *efg1*, *hap2*, *hap42*, *mac1*; one of two repeats for *csr1*, *ssn6*), slow proliferators on ammonium sulfate (*efg1*, *grf10*, *hap2*, *hap42*, *ssn6*, *zcf8*), slow proliferators on standard media (*hap2*, *hap42*, *mac1*, *ssn6*), or late proliferators on ammonium sulfate (*grf10*, *hap2*) (File S7). The remaining four strains (*ahr1*, *cap1*, *gat1*, *ndt80*), however, had late BSA proliferation phenotypes but not other proliferation defects (File S7). We identified two strains that did not proliferate at all on SD+BSA+Uri media (*stp1*, *adr1*), of which one could not proliferate on SD+AmS+Uri (*adr1*), indicating a general proliferation defect (File S7). The remaining no proliferation strain (*stp1*) could proliferate on SD+AmS+Uri, indicating a BSA-specific proliferation defect (File S7). One additional strain (*isw2*) had an inconsistent general proliferation defect (File S7). A complete list of maximum proliferation rates and times to maximum proliferation for the BSA proliferation screen are provided in File S7.

**Supplemental Figure Legends**

Figure S1: Opaque cells secrete more proteolytic activity than white cells. (A) Quantification of the unique 8-mer cleavages common to both cell types or specific to one cell type at either pH 5.5 or 7.4 (60 minutes). (B) Wild type opaque cell conditioned media cleaves more BSA than wild type white cell conditioned media. Conditioned media was incubated with 0.5% BSA, aliquots were harvested at the indicated time points, and samples run on a 12.5% SDS-PAGE gel. The location of the full length BSA band as well as the 20kDa and 50kDa molecular weight markers are indicated to the left of the gel. These experiments were performed on conditioned media from cells grown in media containing ammonium sulfate; as such, these results reflect basal, rather than protein induced, proteolytic activity levels

Figure S2: Proliferation on media with BSA as the sole nitrogen source does not block temperature induced *en masse* opaque-to-white switching. (A) Proliferation of white (red) and opaque (blue) cells from the SC5314 background at 25°C (circles) or 37°C (squares) when BSA is the sole nitrogen source. (B) Percentage of white cells in populations proliferating at 37°C harvested during the proliferation assay in panel A, as determined by plating. Opaque starting populations are in blue, white starting populations are in red. (C) Proliferation of white (red) and opaque (blue) cells from the SC5314 background when BSA is the only potential carbon source in the presence (upward facing triangles) or absence (downward facing triangles) of ammonium sulfate as a nitrogen source. Cell counts in panels A and C were determined by flow cytometry.

Figure S3: Volcano plots depicting the fold change in gene expression between proliferation on ammonium sulfate and BSA as the sole nitrogen source. Opaque (top) and white (bottom) cells undergoing logarithmic proliferation (right) or after two hours (left) are shown. Vertical red lines indicate a five-fold change in expression between ammonium sulfate and BSA as the sole nitrogen source. The horizonal red lines indicate a five percent false discovery rate.

Figure S4: Overlap between the transcriptional responses to different proteins as the sole nitrogen source and between the responses to BSA and to nitrogen starvation. (A) Number of genes up-regulated at least five-fold in opaque cells logarithmically proliferating when BSA, myoglobin, or hemoglobin are the sole nitrogen sources. (B) Number of genes up-regulated (left) or down-regulated (right) at least five-fold in white cells logarithmically proliferating when BSA, myoglobin, or hemoglobin are the sole nitrogen sources. (C) Number of genes up-regulated (left) or down-regulated (right) at least five-fold in white or opaque cells after two hours exposure to media containing BSA as the sole nitrogen source or no nitrogen source.

Figure S5: White and opaque cells express different *SAP*s and different peptide transporters in response to two hours growth on different nitrogen sources. (A) Enrichment of selected genes in opaque cells (y-axis) plotted relative to enrichment in white cells (x-axis) after two hours proliferation when ammonium sulfate (left chart) or BSA (right chart) is the sole nitrogen source. Names are indicated for genes differentially regulated at least 5-fold between cell types, the line (y = x) indicates equal expression in both cell types. (B) Enrichment of selected genes after two hours proliferation when BSA is the sole nitrogen source (y-axis) plotted relative to two hours proliferation when ammonium sulfate is the sole nitrogen source (x-axis). Names are indicated for genes differentially regulated at least 5-fold between the two nitrogen sources in white (red) and opaque (blue) cells, the line (y = x) indicates equal expression on both nitrogen sources.

Figure S6: Opaque cells’ basal proteolytic activity and ability to proliferate when proteins are the sole nitrogen source are dependent on the Sap family of proteins. (A) Cleavage of BSA by wild type opaque cell conditioned media in the presence of Pepstatin A or DMSO loading control. Conditioned media was incubated with 0.5% BSA, aliquots were harvested at the indicated time points, and samples run on a 12.5% SDS-PAGE gel. The location of the full length BSA band as well as the 20kDa and 50kDa molecular weight markers are indicated to the left of the gel. (B) Proliferation of wild type opaque cells on SD+AmS+Uri and SD+BSA+Uri media in the presence of Pepstatin A or DMSO loading control. Data represent the blank-subtracted average of three wells per condition from the same assay. (C) Cleavage of BSA by opaque cell conditioned media from strains with four or five of the five opaque-expressed *SAP*s (*SAP1*, *SAP2*, *SAP3*, *SAP8*, and *SAP99*) deleted. Conditioned media was incubated with 0.5% BSA, aliquots were harvested at the indicated time points, and samples run on a 12.5% SDS-PAGE gel. The location of the full length BSA band as well as the 20kDa and 50kDa molecular weight markers are indicated to the left of each gel.

Figure S7: Deletion of the five opaque-expressed *SAP*s (*SAP1*, *SAP2*, *SAP3*, *SAP8*, and *SAP99*) has minimal effects on expression of *SAP*s other than *SAP98*. Enrichment of the *SAP* genes in quintuple *SAP* deletion opaque cells undergoing logarithmic proliferation on media where ammonium sulfate is the sole nitrogen source (y-axis) plotted relative to enrichment in wild type opaque cells proliferating under the same conditions (x-axis).

Figure S8: Identification of regulators controlling basal proteolytic activity and proliferation when proteins are the sole nitrogen source. (A) Cleavage of BSA by wild type (24 hours, left; 44 hours, right), *stp1* (24 hours), *csr1* (24 hours), *efg1* (44 hours), and *wor3* (44 hours) opaque cell conditioned media. Conditioned media was incubated with 0.5% BSA, aliquots were harvested at the indicated time points, and samples run on a 12.5% SDS-PAGE gel. The location of the full length BSA band as well as the 20kDa and 50kDa molecular weight markers are indicated to the left of each gel. (B) Proliferation of opaque (top) and white (bottom) wild type, *stp1*, *efg1* (opaque only), *wor3*, *csr1*, and *ssy1* strains when BSA is the sole nitrogen source. Cell counts were determined by flow cytometry. (C) Proliferation of wild type and constitutively active Stp1 (Δ2-61) white and opaque strains when BSA is the sole nitrogen source. Data represent the blank-subtracted average of three wells per condition from the same assay.

Figure S9: Characterization of the role of Stp1 in regulating the opaque cell response after two hours exposure to proteins as the sole nitrogen source or nitrogen starvation. Deletion of *STP1* prevents the normal opaque cell responses after two hours exposure to protein as the sole nitrogen source or nitrogen depletion but does not affect other aspects of the nitrogen starvation response like up-regulation of *UGA4*. Red stars indicate that a given gene has been deleted in that condition.

Figure S10: Co-culture with wild type opaque cells rescues opaque quintuple *SAP* deletion strain proliferation but has a more limited effect on opaque *stp1* deletion strain proliferation when BSA is the sole nitrogen source. Proliferation of the wild type white strain, opaque quintuple *SAP* deletion strain, and opaque *stp1* deletion strain, grown by themselves or co-cultured with wild type opaque cells at different ratios, when BSA is the sole nitrogen source. Proliferation of the wild type white strain when co-cultured with the opaque quintuple *SAP* deletion strain at different ratios, when BSA is the sole nitrogen source, is also shown. The fluorescent strain in each co-culture pair is indicated. Cell counts and GFP fluorescence to assign cell identity were determined by flow cytometry.

Figure S11: White cells respond more rapidly to BSA as the sole nitrogen source when co-cultured with opaque cells or when BSA is pretreated with Proteinase K. (A) Expression of a mCherry reporter driven by the *OPT1* (left) or *OPT2* (right) promoter in white cells, grown by themselves (red) or with an equal number of opaque cells (orange), when BSA is the sole nitrogen source. (B) Expression of a mCherry reporter driven by the *OPT1*, *OPT2*, *SAP2*, or *UGA4* promoter in white cells when BSA has (dashed lines and boxes) or has not (solid lines and boxes) been pretreated with Proteinase K. Boxes indicate the 25th to 75th percentiles of the data and the whiskers indicate the 5th to 95th percentiles of the data for each sample at each time point. mCherry fluorescence was determined by flow cytometry and normalized by side scatter.

Figure S12: *C. dubliniensis*, *C. tropicalis*, and *C. parapsilosis* proliferate better in the presence of *C. albicans* opaque cells when BSA is the only nitrogen source. Proliferation of *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, a cell type switching capable white cell *C. tropicalis* isolate, and a cell type switching capable opaque cell *C. tropicalis* isolate, grown by themselves (circles) or co-cultured with an equal number of opaque (square) or white (diamond) *C. albicans* cells, when BSA is the sole nitrogen source. Cell counts and GFP fluorescence to assign cell identity were determined by flow cytometry.

**Supplemental Table Legends**

Table S1: Average basal Sap abundance. The total abundance of all Saps detected in white and/or opaque cells, broken down by cell type and strain background. Sap4, Sap5, and Sap6, which are normally associated with biofilms, were not detected in any sample of either cell type. These experiments were performed on conditioned media from cells grown in media containing ammonium sulfate; as such, these results reflect basal, rather than protein induced, levels of these proteins.

Table S2: Growth on media containing BSA as a nitrogen source does not induce white-opaque switching. White-to-opaque (top) and opaque-to-white (bottom) switching rates for the wild type strain on the indicated plate types.

Table S3: Deletion of opaque enriched *SAP*s does not induce white-opaque switching. White-to-opaque and opaque-to-white switching rates for strains with combinations of four or five of the five opaque-expressed *SAP*s (*SAP1*, *SAP2*, *SAP3*, *SAP8*, and *SAP99*) deleted. Switching rates are normalized to the rate of the equivalent wild type strain on the same day.

**Supplemental File Legends**

File S1: Supplemental Materials and Methods, Supplemental Results, and legends for all supplemental items.

File S2: Lists of media, oligonucleotides, plasmids, and strains used in this study.

File S3: A summary and compilation of all 8mer cleavages detected in at least one MSP-MS experiment as well as a breakdown of the number of times, if any, a given cleavage was detected at each time point.

File S4: A summary of peptide counts and a compilation of all unique proteins detected in the proteomic analysis of trypsin digested conditioned media from white and opaque cells. The data summaries for the three replicates as well as the Average Peptide Count and Percent of Total Counts calculations for detected proteins for each cell type-strain background pairing are also included.

File S5: Growth rates, lag times, and doubling times for stand alone and co-culture flow cytometry proliferation experiments. Data reflect the mean of all replicates performed of a given condition (e.g. WT Opaque on BSA), Strains with NaN and N/A values did not proliferate during the course of an experiment and as such growth rates could not be calculated.

File S6: Compiled data for NanoString transcriptional profiling experiments. File includes lists of 59 genes targeted, probe sequences, raw data for individual experiments, averaged normalized data for specific experiments (e.g. Wild Type Opaque cells proliferating when BSA was the sole nitrogen source), and selected comparisons between conditions (e.g. Wild Type Opaque cells proliferating when BSA or ammonium sulfate are the sole nitrogen sources).

File S7: Compiled data for the opaque transcriptional regulator knockout library BSA proliferation screen, including lists of regulators with Slow and/or Late Phenotypes as well as whether they exhibited general, as opposed to BSA specific, growth defects.

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