Yeast and Filaments Have Specialized, Independent Activities in a Zebrafish Model of *Candida albicans* Infection

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ABSTRACT *Candida albicans* dimorphism is a crucial virulence factor during invasive candidiasis infections, which claim the lives of nearly one-half of those afflicted. It has long been believed that filaments drive tissue invasion and yeast mediates bloodstream dissemination, but observation of these activities during infection has been prevented by technical limitations. We used a transparent zebrafish infection model to analyze more comprehensively how *C. albicans* utilizes shape to disseminate and invade. This model facilitated the use of diverse, complementary strategies to manipulate shape, allowing us to monitor dissemination, invasion, and pathogenesis via intravital imaging of individual fungal cells throughout the host. To control fungal cell shape, we employed three different strategies: gene deletion (*efg1Δ/Δ cph1Δ/Δ*, *eed1Δ/Δ*), overexpression of master regulators (*NRG1* or *UME6*), and modulation of the infection temperature (21°C, 28°C, or 33°C). The effects of these orthogonal manipulations were consistent, support the proposed specialized roles of yeast in dissemination and filaments in tissue invasion and pathogenesis, and indicate conserved mechanisms in zebrafish. To test if either morphotype changes the effectiveness of the other, we infected fish with a known mixture of shape-locked strains. Surprisingly, mixed-strain infections were associated with additive, but not synergistic, filament invasion and yeast dissemination. These findings provide the most complete view of morphotype-function relationships for *C. albicans* to date, revealing independent roles of yeast and filaments during disseminated candidiasis.

KEYWORDS *Candida albicans*, zebrafish, yeast, hypha, dimorphism, dissemination, filamentation

Differentiation of shape during infection can affect how a pathogen evades the immune system and develops disease. To cause invasive infections, some pathogenic fungi transition between yeast and filament forms, while others cause infections as single morphotypes (1–7). *Candida albicans* is unusual among fungal pathogens in that it grows in both yeast and filamentous forms during infection of humans, mice, zebrafish, and invertebrate hosts (8–15). While both forms contribute to virulence, it is still unclear how this differentiation between shapes drives specific aspects of pathogenesis.

Candida is the agent of the most common hospital-acquired fungal infection in the United States, and the fourth most common bloodstream infection overall (16), with invasive infections causing as much as 50% mortality in those affected (17). *C. albicans* can transition between yeast and filament growth, and both morphotypes have long been hypothesized to possess specific roles for pathogenesis and disease spread in the host: yeast are presumed to spread infection, while filaments are implicated in tissue damage and destruction (18). Despite contributions from both murine and *in vitro* models to the understanding of fungal dimorphism during invasive infections, we are

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still held back by an inability to completely visualize morphotype-function relationships in the host.

C. albicans regulates growth between the yeast and hyphal forms by integrating many environmental cues, including temperature, pH, oxygen, carbon dioxide, and nutrients (19–21). Several genetic pathways transduce these shifts and other environmental signals to positively and negatively regulate morphological switching through filament-specific genes, such as *CPH1*, *EFG1*, *EED1*, and *UME6*, and yeast-promoting regulators, such as *TUP1* and *NRG1* (22). Overexpression of negative regulators, such as *NRG1*, confers a hypofilamentous phenotype, whereas overexpression of filament-specific genes, such as *UME6*, results in hyperfilamentous growth *in vivo* (23, 24).

The importance of *C. albicans* morphological transitions has made them a target for therapeutic exploration. Recent discoveries of small-molecule inhibitors have shown that filamentous growth can be blocked both *in vitro* and *in vivo*, with little to no detrimental effect on *in vitro* cell lines or animal models (12, 25, 26). Now that we have the prospect of regulating morphogenesis without killing the fungi, it is a critical time to determine the roles of each morphotype in driving both spread and invasion during infection.

Early in the study of *C. albicans* as a pathogen, deductive reasoning led to the hypotheses that filaments are more likely to be invasive and yeast has a smaller shape that can more readily spread throughout the host (10, 15). In fact, infections with hypofilamentous mutant strains of *C. albicans* lead to rapid clearance and low mortality but leave dissemination relatively intact (24, 27). Some hyperfilamentous strains also have lower virulence than the wild type, even though they can still cause tissue damage and some mortality (28–30). In work complementary to these studies, *in vitro* infections of epithelial and endothelial tissues have shown that filaments are required for epithelial invasion and damage (31, 32). These results are consistent with the original hypothesis that yeast is better suited for disease spread than for damaging invasion, whereas filaments are less involved in spread but are important for invasion, virulence, and infection progression. However, the attenuated virulence found in infections with both yeast-locked and filament-locked strains suggests that the combined activity of yeast and filaments is important for pathogenesis.

Limitations remain for both *in vitro* and murine models in testing the roles of shape in *C. albicans* pathogenesis. For instance, dissemination cannot be dissected *in vitro* or in invertebrate models due to the lack of vertebrate host anatomy (33–35). Moreover, although limited intravital imaging is possible and the technology is improving, the size and opacity of mice prohibits the tracking of individual fungal cells and morphotype differentiation throughout the host during infection progression (36–39). To investigate these hypotheses with intravital imaging, and to bridge the gaps between *in vitro* and murine work, we sought to adapt the larval zebrafish as a model with which to examine fungal and host determinants of pathogenesis.

The larval zebrafish offers a unique vertebrate model for studying bacterial, viral, and fungal infections (40–42). This transparent host allows the simultaneous longitudinal study of host and pathogen interactions intravitaly, enabling us to ask questions we cannot investigate *in vitro* or in murine models. Noninvasive imaging enables us to monitor the locations and shapes of all fungal cells during infection, which allows the measurement of specific morphotype-function relationships of yeast and filaments. Further, the environmental resiliency of zebrafish allows us to alter the infection temperature *in vivo*, augmenting our ability to manipulate fungal morphology in concert with conventional genetic manipulation.

Here we describe a new zebrafish yolk infection model and leverage this infection route to study tissue-to-bloodstream dissemination of *C. albicans*. Multiple genetic and environmental perturbations were used to morphologically bias the population and to test the roles of each morphotype during disease development. Our results confirm the specialized functions of yeast in infection spread and filaments in tissue invasion. Further, our data suggest that each shape has an independent, additive effect on dissemination and mortality that is not enhanced by the presence of other morpho-

FIG 1 Wild-type *C. albicans* filaments invade and cause death, while yeast-locked mutants disseminate, in larval zebrafish. Larval AB zebrafish were raised at 33°C, infected with fungi at ~35 h postfertilization, screened for the inoculum, and kept at 28°C postinfection. Infected fish were screened and imaged on an Olympus confocal microscope at 24 or 30 hpi for dissemination and mortality. Shown are results of infections with hypofilamentous mutants, pooled from 9 independent experiments. (A) Single-strain yolk infections with wild-type SC5314-GFP (a and b) and hypofilamentous *efg1Δ/Δ cph1Δ/Δ*-dTomato (c and d) and *eed1Δ/Δ*-dTomato (e and f) mutants. (a, c, and e) Images of whole fish. The white arrowhead indicates yeast dissemination. Bars, 100 μ m. (b, d, and f) Insets for panels a, c, and e, respectively. Bars, 50 μ m. (B) Kaplan-Meier survival curves for fish during infection with mutant or wild-type fungi. Shown are pooled results for 196 individual uninfected fish and 194, 94, and 314 individual fish infected with the *eed1Δ/Δ* mutant, the *efg1Δ/Δ cph1Δ/Δ* mutant, and SC5314, respectively. (C) Dissemination frequencies in infections with mutant and wild-type strains. The number of live fish screened for each infection at each time point is shown. Statistical analysis was carried out by use of the Mantel-Cox log rank test with Bonferroni's correction (B) or Fisher's exact test with Bonferroni's correction (C). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

types. Thus, by using orthologous tools to manipulate the fungus, in combination with whole-animal imaging, we demonstrate the consequences of *C. albicans* morphological diversity during disease development in the host.

RESULTS

Yeast growth leads to dissemination, while filamentous growth leads to invasion and death. To visualize the functions of different *C. albicans* morphotypes during infection, we developed a zebrafish yolk infection route in which a localized tissue infection can spread to the bloodstream. At the normal temperature for zebrafish husbandry, 28°C, wild-type *C. albicans* yeast rapidly germinates in the zebrafish host unless contained by phagocytes (8, 43). Therefore, to test the roles of different morphological forms, we infected fish at this temperature with either the filament-dominant wild-type strain (green fluorescent protein-labeled SC5314 [SC5314-GFP]) (Fig. 1Aa and b) or hypofilamentous strains (the *efg1Δ/Δ cph1Δ/Δ*-dTomato or *eed1Δ/Δ*-dTomato mutants) (Fig. 1Ac through f). Although both the *efg1Δ/Δ cph1Δ/Δ* and *eed1Δ/Δ* strains were hypofilamentous during infections, pseudohyphae were still observed (see Fig. S1 in the supplemental material). The production of pseudohyphae by hypofilamentous strains *in vivo* is also seen in mammalian infection models (44, 45) but did complicate the interpretation of our results. The NRG1^{OEX} strain overexpresses the *NRG1* gene, which is necessary and sufficient for yeast growth (23). In contrast to the *efg1Δ/Δ cph1Δ/Δ* and *eed1Δ/Δ* mutants, the NRG1^{OEX}-dTomato strain was morphologically stable and yeast-locked *in vivo* and could be compared to its strongly filament producing control strain, TT21-dTomato (Fig. 2A).

FIG 2 Yeast disseminates, while filaments invade and cause death, during infections with overexpression strains. Larval AB zebrafish were raised at 33°C, infected with fungi at ~35 h postfertilization, screened for the inoculum, and kept at 28°C postinfection. Infected fish were screened and imaged on an Olympus confocal microscope at 24, 30, or 44 hpi for dissemination and mortality. Shown are results of infections with yeast-locked NRG1^{OEX}, pooled from 5 independent experiments. (A) (a and c) Yolk infected with the single strain TT21-dTomato (a) or NRG1^{OEX}-NEON (c) were imaged at 24 hpi. Bars, 100 μ m. (b and d) Insets for panels a and c, respectively. Bars, 50 μ m. (b) TT21 morphology/invasion in the yolk/heart. (d) NRG1^{OEX} yeast disseminating in the yolk. (B) Kaplan-Meier survival curves for fish during infection with a wild-type (TT21-dTomato) or yeast-dominant (NRG1^{OEX}-dTomato) strain. Shown are pooled results for 74 individual uninfected fish and for 142 and 168 individual fish infected with NRG1^{OEX} and TT21, respectively. (C) Comparisons of dissemination frequencies in infections with wild-type and yeast-dominant strains. The number of live fish screened for each infection at each time point is shown. Statistical analysis was carried out by use of the Mantel-Cox log rank test with Bonferroni's correction (B) and Fisher's exact text (C). ***, $P \leq 0.001$; ****, $P \leq 0.0001$; #, data not analyzed, because mortality was >90%.

Filaments are found in the infection foci of organs, and *in vitro* studies suggest that filaments invade and damage epithelial layers (46). To test if filaments are highly invasive in this zebrafish infection model and to observe the destructive power of filaments *in vivo*, we performed infections with filament-dominant strains SC5314 and TT21. Imaging infected zebrafish by longitudinal confocal microscopy, we determined that infections with the filament-dominant wild-type strains (SC5314 or TT21) result in very little dissemination but cause a high rate of mortality (Fig. 1B and C and 2B and C). Mortality is closely associated with filaments invading the host tissue surrounding the yolk, including the heart and nearby vasculature. These experiments show that filaments are invasive but also show that they are ineffective at spreading through the bloodstream in the zebrafish.

Yeast-dominant mutants have been found to disseminate at higher rates from the intestinal tract and show no loss in the ability to colonize organs in an intravenous route of infection, but they are hypovirulent in the murine model (24, 47). In agreement with these previous findings, yeast-dominant infections of zebrafish with hypofilamentous mutants and the yeast-locked NRG1^{OEX} strain resulted in the dissemination of yeast throughout the fish (Fig. 1C and 2C). Despite this dissemination, the yeast was less virulent, and survival was not significantly different from that for uninfected controls over the first 2 days of infection (Fig. 1B and 2B). Further, levels of both mortality/invasion from filaments and dissemination/colonization of yeast were robust over a range of infectious doses between 1 and 20 cells (Fig. 3A and B; see also Fig. S2 in the supplemental material). Quantification of filament invasion from longitudinal imaging also showed that high levels of tissue invasion at 18 h postinfection (hpi) correlate with early death of infected fish, establishing a link between invasion and virulence (Fig. 3C). Note that this time point was chosen carefully to allow for a significant period of infection progression while ensuring that a wide range of infected fish could be imaged before significant mortality occurred. These results support the long-hypothesized roles of yeast and filaments during infection, in addition validating the zebrafish as a model with which to study *C. albicans* morphology.

Temperature modulation of fungal morphology confirms the morphotype-function relationship. While our results with hypofilamentous strains confirmed the long-suspected roles of yeast and filaments in the host, the genetic modifications in these strains can affect cellular functions other than morphology (21, 48–51). To enhance yeast growth without relying on genetic manipulation, we took advantage of

FIG 3 Inoculum size is not a determining factor in mortality during filament infections, but invasion determines death in filament-dominant infections. Larval AB zebrafish were raised at 33°C, infected with fungi at ~35 h postfertilization, screened for the inoculum, and kept at 28°C postinfection. Zebrafish infected with UME6^{OEX}-dTomato were screened for the exact inoculum and were individually placed in 48-well plates, with known inoculum sizes recorded for each fish. At 18 hpi, fish yolks were imaged for filament invasion, and imaged fish were monitored for mortality up to 24 and 30 hpi. (A, B, and C) Mortality and invasion in UME6^{OEX} infections with split inocula. Results were pooled from 4 independent experiments. (A) Kaplan-Meier survival curves for fish during infection with low (1 to 10 cells) or high (11 to 20 cells) UME6^{OEX} inocula. Shown are pooled results for 40 individual uninfected fish and for 195 or 85 individual fish infected with a low or high UME6^{OEX} inoculum, respectively. (B) Filament invasion, quantified by pixels in ImageJ, in fish with either low (1 to 10 cells) or high (11 to 20 cells) filament inocula (black circles, low inocula; blue squares, high inocula). (C) Mortality in fish with filament invasion at 18 hpi. Filament invasion was quantified in confocal images taken at 18 hpi, and mortality was scored at 24 and 30 hpi (black circles indicate early fish death, at 24 hpi; blue squares indicate late fish death, at 30 hpi). Statistical analysis was carried out by use of the Mantel-Cox log rank test with Bonferroni's correction (A) or the Mann-Whitney U test (B and C). Medians with interquartile ranges are displayed. **, $P \leq 0.01$; ***, $P \leq 0.001$; n.s. not significant.

the facts that yeast growth in wild-type *C. albicans* is enhanced at lower temperatures (20) while zebrafish can be grown at a wide range of temperatures (52–54). We infected fish with the wild-type TT21-dTomato strain and incubated fish at the yeast-inducing temperature of 21°C (Fig. 4A). We found that the wild-type strain produces a mix of morphologies at this temperature (21.7% $\pm$ 5.5% yeast at 30 hpi, 24.5% $\pm$ 9.0% yeast at 44 hpi, 67.5% $\pm$ 6.2% yeast at 68 hpi). As expected, the presence of moderate levels of yeast led to increased dissemination over time and prolonged survival up to 68 hpi, results similar to those obtained with the genetically modified yeast-dominant strains (Fig. 4B and C).

We then sought to ensure that these temperature manipulations by themselves did not affect the activities of shape-locked *C. albicans* strains or overall zebrafish immune function. We used morphology-locked strains to test whether temperature affects the ability of yeast to disseminate or of filaments to invade. After fish were raised at 33°C, they were infected with the filament-locked UME6^{OEX}-dTomato strain or with the yeast-locked NRG1^{OEX} strain labeled with near-infrared fluorescent protein (NRG1^{OEX}-iRFP). After fish were screened for the inoculum number, they were incubated at either the yeast-inducing temperature of 21°C or the filament-inducing temperature of 33°C. As we expected, we found the yeast-dominant NRG1^{OEX} strain continued to disseminate at both low and high temperatures (Fig. 4D and F). Further, the UME6^{OEX} strain did not disseminate at either temperature, and most fish died by the last time point during infection (Fig. 4D to G). These results indicate that the functions of yeast and filaments are conserved at temperatures higher and lower than 28°C in zebrafish.

To determine if downshifting the infection temperature would affect immune homeostasis or a sterile inflammatory response in zebrafish larvae, we assayed the immune system in two different ways. Zebrafish were raised at 33°C until the time of infection (as was done throughout all the experiments described above) and were then either kept at 33°C or shifted to 28°C or 21°C for 24 h to mimic the conditions of infection. At 24 h postshift (a developmental stage equivalent to 24 hpi), immune homeostasis was assayed by counting overall neutrophil numbers (see Fig. S3 in the supplemental material), and immune response was quantified by measuring neutrophil recruitment to a sterile tail wound (see Fig. S4 in the supplemental material). No differences were found at any temperature measured, suggesting that there are no significant effects of temperature downshift during the time scale of these experiments.

Morphotypes do not synergize to enhance infection outcome during mixed infections. The quantitative results from our experiments using shape-locked strains

FIG 4 Morphology-specific infection phenotypes are conserved at high and low temperatures. Larval AB zebrafish were infected with fungi at ~35 h postfertilization, screened, and incubated at either 33°C or 21°C postinfection. Infected fish were screened for dissemination and mortality and were imaged on an Olympus confocal microscope at 18 to 68 hpi, as indicated. (A, B, and C) Low-temperature infections with TT21 or NRG1^{OEX}. Results were pooled from 4 independent experiments. (A) Representative image at 30 hpi of a zebrafish infected with the single strain TT21-dTomato (a) and insets (b and c). White arrowheads indicate yeast cells in the yolk. (B) Dissemination frequencies of TT21 and NRG1^{OEX} after infection at 21°C. The number of live fish screened for each infection at each time point is shown. (C) Survival of fish from the TT21-dTomato, NRG1^{OEX}-dTomato, and uninfected-control groups at 21°C. Shown are pooled results for 63 individual uninfected fish and for 100 and 140 individual fish infected with NRG1^{OEX} and TT21, respectively. (D and E) High-temperature infections with UME6^{OEX} and NRG1^{OEX}. Results are pooled from 4 independent experiments. (D) Dissemination frequencies for single infections with UME6^{OEX}-dTomato and NRG1^{OEX}-iRFP at 33°C. The number of live fish screened for each infection at each time point is shown. (E) Survival of fish during single infections with UME6^{OEX} and NRG1^{OEX} at 33°C. Shown are pooled results for 78 individual uninfected fish and for 49 and 67 individual fish infected with NRG1^{OEX} and UME6^{OEX}, respectively. (F and G) Low-temperature infections with UME6^{OEX} and NRG1^{OEX}. Results are pooled from 4 independent experiments. (F) Dissemination frequencies for single infections with UME6^{OEX} and NRG1^{OEX} at 21°C. The number of live fish screened for each infection at each time point is shown. (G) Survival of fish during single infections with UME6^{OEX} and NRG1^{OEX} at 21°C. Shown are pooled results for 74 individual uninfected fish and for 40 and 59 individual fish infected with NRG1^{OEX} and UME6^{OEX}, respectively. Statistical analysis was carried out by use of Fisher's exact test (B, D, and F) or the Mantel-Cox log rank test with Bonferroni's correction (C, E, and G). **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; n.s., not significant; #, data not analyzed, because mortality was >90%.

suggested the possibility that invasion and dissemination are driven by the presence of a specific fungal shape rather than by the process of dimorphic transition alone. To test if a mix of shapes is sufficient to make up for the inability to change shape, we infected fish with a mixture of filament-locked (UME6^{OEX}) and yeast-locked (NRG1^{OEX}) strains and compared dissemination and lethality between these mixed- and single-strain

FIG 5 Morphotypes do not affect each other's abilities to disseminate or invade in mixed infections. Larval AB zebrafish were infected at ~35 h postfertilization with UME6^{OEEX}-dTomato and NRG1^{OEEX}-NEON at a 1:1 ratio, screened for the inoculum, and incubated at 28°C. Infected fish were screened for dissemination and mortality and were imaged on an Olympus confocal microscope at 18, 24, or 30 hpi. (A) (a) Representative image of a fish with a mixed infection with NRG1^{OEEX}-NEON and UME6^{OEEX}-dTomato at 24 hpi. Bar, 100 μ m. (b and c) Insets showing yeast and filaments in yolk (b) and disseminated yeast (c). Bars, 50 μ m. (B and C) Single- and mixed-strain infections with NRG1^{OEEX} and UME6^{OEEX}. Results are pooled from 4 independent experiments. (B) Comparison of dissemination frequencies for single- and mixed-strain infections with NRG1^{OEEX} and UME6^{OEEX}. The number of live fish screened for each infection at each time point is shown. (C) Survival of fish during NRG1^{OEEX}, UME6^{OEEX}, and mixed-morphology fungal infections. Shown are pooled results for 65 individual uninfected fish and for 85, 113, and 180 individual fish infected with NRG1^{OEEX} alone, UME6^{OEEX} alone, and a mixture of the two, respectively. (D, E, and F) Dissemination and yeast levels in mixed-strain infections with different filament inocula. Results are pooled from 7 independent experiments. (D) Percentage of dissemination for mixed-strain infections with low (1 to 10 cells) or high (11 to 20 cells) filament inocula. The number of live fish screened for each inoculum is shown. (E) Yeast levels, quantified by pixels in ImageJ, in fish with either low (1 to 10 cells) or high (11 to 20 cells) filament inocula (black circles, low inocula; blue squares, high inocula). (F) Levels of invasion at 18 hpi in fish that go on to have disseminated infection at 24 hpi (black circles) or 30 hpi (blue squares), compared to those in fish that remain without dissemination by 30 hpi (gray triangles). Filament invasion was quantified in confocal images taken at 18 hpi, and dissemination was scored at 24 and 30 hpi. (G and H) Invasion and mortality in mixed-strain infections with different yeast inocula. Results are pooled from 4 to 7 independent experiments. (G) Survival of fish during infection with low (1 to 10 cells) or high (11 to 20 cells) NRG1^{OEEX} inocula. Shown are pooled results for 115 individual uninfected fish and for 517 and 257 fish infected with mixed-strain, low-NRG1^{OEEX} inocula and with mixed-strain, high-NRG1^{OEEX} inocula, respectively. (H) Filament invasion at 18 hpi, expressed in pixels as quantified by ImageJ (Fiji), during infections with low (blue circles) or high (gray squares) yeast inocula. Statistical analysis was carried out by use of Fisher's exact test with Bonferroni's correction (B and D), the Mantel-Cox log rank test with Bonferroni's correction (C and G), the Mann-Whitney U test (E and H), or the Kruskal-Wallis test with Bonferroni's correction (F). Medians with interquartile ranges are displayed. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; n.s., not significant.

infections (Fig. 5A). These experiments were enabled by our earlier finding that a 2-fold change in the infectious dose of either strain did not quantitatively affect the efficiency of yeast dissemination or filament invasion during single-strain infections (Fig. 3; also Fig. S2 in the supplemental material). Surprisingly, we now found similar dissemination

frequencies for mixed-strain (UME6^{OEX} plus NRG1^{OEX}) and single-strain (NRG1^{OEX}) infections (Fig. 5B). In accord with these findings, we also measured similar dissemination frequencies in infections with SC5314 and hypofilamentous mutants at all temperatures tested (see Fig. S5A and B and Fig. S6A and C in the supplemental material). These results suggest that filaments do not affect the ability of yeast to disseminate. Interestingly, we found less mortality in mixed-strain infections than in infections with filament-dominant strains, suggesting that yeast does somehow affect the mortality associated with filament invasion (Fig. 5C; also Fig. S5C and S6B and D). Overall, these results suggest that morphotypes do not synergize to enhance each other's activities.

Our results suggesting that yeast and filaments do not enhance each other during infection were unexpected and drove us to further investigate this lack of synergy. We postulated that the relative number of filament- or yeast-locked cells in the mixed infectious dose may affect dissemination or invasion/mortality. To test this idea, we split mixed infections between low (1 to 10 cells) and high (11 to 20 cells) filament or yeast inocula and analyzed the impact of these dosages. As noted above, we found no significant differences in these two different infection doses in single-strain infections (Fig. 3; also Fig. S2 in the supplemental material). In the context of yeast function, we found that the filament inoculum size had no impact on the ability of yeast to disseminate or proliferate in the yolk (Fig. 5D and E). Filament invasion also did not impact the ability to disseminate (Fig. 5F). In the context of filament function, we found that the yeast inoculum size does not affect survival or impact the ability of filaments to invade tissue (Fig. 5G and H). These results are consistent with the idea that the intrinsic abilities of each shape to disseminate or invade tissue are insensitive to the presence of both morphotypes. While comparison of mixed-strain infections and monoinfections (Fig. 5C; also Fig. S5C and S6B and D in the supplemental material) suggests that the presence of yeast limits filament-driven mortality, these experiments with different levels of yeast suggest that a more subtle alteration in the yeast dose (either 1 to 10 cells or 11 to 20 cells) does not significantly impact survival.

The lower mortality associated with mixed-morphology (yeast-plus-filament) infections than with filament-only infections suggested the possibility that yeast might reduce mortality by signaling to either the host or the filamentous cells early during the infection. To further investigate the role of yeast in reducing mortality, we took advantage of the potential to shut off expression of the extra copy of the yeast-inducing *NRG1* gene in the NRG1^{OEX} strain. Because the extra copy of *NRG1* is under the control of the TET repressor, doxycycline (DOX) blocks expression both *in vitro* and in murine infections (23, 24). Although initial experiments with the addition of DOX to the water were ineffective in reversing the yeast-locked phenotype of the NRG1^{OEX} strain *in vivo*, we found that coinjection of DOX at the time of infection was highly effective in allowing filamentous growth of this strain *in vivo* (see Fig. S7 in the supplemental material). Lower concentrations of DOX were less effective, and higher concentrations were slightly toxic to fish (data not shown). At several times postinfection, cohorts of fish were euthanized, flattened, and imaged by epifluorescence microscopy. Images were then quantified as described in Materials and Methods. For infections conducted at 28°C and 33°C, this resulted in a nearly complete switch of morphology from almost 100% yeast to almost 100% hyphae, in line with what is seen for the wild-type TT21 strain. For infections at the low temperature of 21°C, this resulted in a phenocopy of the wild-type morphology mix of approximately 25% yeast and 75% filaments. These experiments are the first to demonstrate TET-regulated expression of fungal genes during infection of zebrafish. However, there appeared to be slightly more yeast growth in the wild-type TT21 strain when it was coinjected with DOX during infections at 21°C and 28°C.

This inhibition by DOX is consistent with previous work suggesting that the iron-chelating ability of DOX leads to inhibition of *C. albicans* growth (55). We confirmed that DOX induces filamentous growth of the NRG1^{OEX} strain but also found that higher levels of DOX inhibited the growth of both the NRG1^{OEX} strain (see Fig. S8 in the supplemental material) and the wild-type CAF2 strain (data not shown). However, as

expected on the basis of previously published work (55), the addition of approximately equimolar levels of ferric chloride eliminated this inhibition without blocking the ability of DOX to allow the NRG1^{OEX} strain to make filaments. These experiments confirm the previous work and extend it to the NRG1^{OEX} strain, suggesting that coadministration of DOX with equimolar levels of ferric chloride is an effective way to eliminate this potential adverse effect of drug addition.

Our successful demonstration of conditions for DOX administration for altering morphology *in vivo* put us in a position to test if switching morphology midway through the infection could alter the survival of coinfecting fish. We first coinfecting fish, then injected either a vehicle (saline) or DOX at 18 hpi, and monitored survival for an additional 24 h. We performed all infections at 28°C, a temperature at which wild-type strains grow almost universally as filaments, so that the addition of DOX would switch morphology completely. We found that, in line with previous experiments, coinfecting fish injected with a vehicle at 18 hpi had lower mortality than monoinfecting fish (see Fig. S9A in the supplemental material). Interestingly, there was no difference in survival between monoinfections and coinfections with the injection of DOX at 18 hpi. To confirm that the later injection of DOX led to a change in the morphology of the NRG1^{OEX} strain, we imaged live fish and found high levels of filamentous growth at 30 hpi by live confocal microscopy (Fig. S9B) and at 44 hpi after euthanizing the fish, flattening the samples, and imaging by epifluorescence microscopy (Fig. S9C). These experiments suggest that switching the morphology of NRG1^{OEX} *C. albicans* from yeast to filaments midway through infection eliminates the reduced mortality associated with mixed-strain infection. Thus, the presence of yeast throughout the infection is important in limiting mortality during coinfection.

DISCUSSION

Invasive, disseminated *C. albicans* infections claim as many as 30 to 50% of those diagnosed (16, 17). *C. albicans* has a pleomorphic ability to switch between yeast and filament growth that is a crucial virulence factor during these deadly infections. It has long been hypothesized that yeast drives dissemination while filaments are primarily invasive, but technical limitations have prevented the visualization of these processes during infection. We used a simplified, transparent vertebrate infection model to probe the individual and combined abilities of both the yeast and filament forms of *C. albicans*. Our findings with the larval zebrafish suggest that yeast and filaments have specialized, independent roles during tissue-to-bloodstream spread in candidiasis. Infections with genetically modified strains and infections with wild-type strains during temperature shifts all show that yeast disseminates, in contrast to filaments, which invade tissue and ultimately kill the host. Mixed-strain infections provide further evidence of each of these roles and, surprisingly, demonstrate that the two morphotypes, with defined numbers of morphology-locked strains, do not synergize to enhance infection progression. Overall, our study confirms the long-suspected roles of each morphotype during infection and demonstrates that there is little influence of one morphotype on the other during disease development.

Zebrafish provide a platform with which to answer questions that cannot be investigated in murine or *in vitro* models during host-*C. albicans* interactions. Transparency, a unique trait of this vertebrate, allowed us to visualize all fungal cells throughout the host at high resolution. This enabled noninvasive quantification of morphology, invasion, yeast proliferation, and dissemination frequency; of these, only invasion has been quantified *in vitro* previously, through damage assays (56). Longitudinal imaging of individual fish enabled us to link invasion directly with mortality and to test connections between the inoculum size and pathogenesis, consistent with results from simpler inoculum size-mortality correlations that have been analyzed in mice (29). Another advantage of the zebrafish is the cost-effectiveness of husbandry (57), which permitted the testing of multiple fungal strains and environmental conditions using the same infection protocol. Furthermore, we could take advantage of the flexibility of zebrafish for growth at different temperatures to manipulate *C. albicans*

morphology, which has so far been analyzed only *in vitro* (19). In the future, we can take advantage of multiple transgenic lines (58, 59) to examine the role of immune response in yeast dissemination, the prevention of invasion, and the unexpected contributions of yeast to reduced mortality during coinfections.

Balancing these unique advantages, a few differences between our model and murine infections should be noted. Due to the small size of the fish, *C. albicans* filaments can damage a significant percentage of the whole fish quite rapidly. Thus, only a few filament cells are sufficient to kill a larval fish, while a much larger dose is necessary in mice (29). Furthermore, since the infection spreads over much shorter distances in the fish, extensive invasion of the whole host does not require dissemination. Therefore, movement into the bloodstream and spread to other organs likely play a much less important role in overall virulence in larval fish than in larger animals, such as mice and humans. Similar results have also been found in a zebrafish model of mycobacterial infection, where comparably extensive mycobacterial growth and pathogenesis occur in both localized hindbrain and bloodstream infections during macrophage ablation (60). Due to these differences, we focused on aspects of infection that are most likely to be conserved among small and large vertebrate hosts: basic behaviors of yeast and filaments, and the interplay between morphological forms. These behaviors are likely similar across species, in part because of the conservation of host factors relevant for *C. albicans*–host interaction, such as immune cell types and receptors on epithelial and endothelial cells that mediate endocytosis (57, 61–63). Furthermore, the applicability of our study to mammalian infection is supported by the fact that our findings are consistent with the morphotype-function relationships originally suggested by experiments with mice. Specifically, intravenous murine infections with yeast- or filament-locked strains are each associated with reduced overall virulence, although filament-dominant infections are more virulent than infections with yeast-locked strains (24, 28). In the murine model, it has not been feasible to quantify dissemination and invasion in these infections, but such quantification has been made possible here by the development of a localized infection model in zebrafish. In this zebrafish model, we found similar overall infection dynamics with morphology-locked strains, where filaments are associated with invasion and mortality but are not able to disseminate, while yeast is able to disseminate but causes less invasion and delayed mortality. These similarities between zebrafish and murine infections, combined with the conservation of basic anatomy and cellular architecture between the two vertebrate hosts, suggest that our findings and our model are relevant for understanding mammalian infection. It will be interesting to examine antagonism and synergy between yeast and filaments in more-localized models of murine infection, such as intraperitoneal infection (64).

Experiments with genetic manipulation of five separate *C. albicans* morphology regulators provide strong evidence for a filament–invasion and yeast–dissemination dichotomy. It was important to use several different genetic manipulations, since each individual change could produce non-morphotype-specific artifacts during infection, potentially altering other cellular functions, such as metabolism and cell wall structural integrity (21, 48–51). Both wild-type-strain and filament-dominant UME6^{OEX} infections produced rapid invasion and mortality but did not spread through the blood, findings similar to those for other zebrafish and mouse infection models (8, 28, 29). In contrast, two hypofilamentous mutants (the *efg1Δ/Δ cph1Δ/Δ* and *eed1Δ/Δ* mutants) and a yeast-locked overexpression strain (NRG1^{OEX}) caused disseminated infection with low mortality, results similar to those for murine infection models (24, 27). Both hypofilamentous mutants grew pseudohyphae at times, in line with mouse studies where filamentous growth of the *efg1Δ/Δ cph1Δ/Δ* mutant was observed in kidneys (44, 45). However, similar rates of dissemination and mortality were seen for infections with the morphologically stable NRG1^{OEX} strain, suggesting that pseudohyphae may not play the same role as hyphae. Overall, the use of these genetic manipulations to alter morphology and monitor the shape/location of all fungal cells in this model provided

us with a more thorough understanding of how *C. albicans* utilizes shape to invade tissue and disseminate infection throughout the host.

Temperature manipulations of *C. albicans* morphogenesis in the host provide further evidence for the independent and specialized roles of each morphotype, complementing our results with hypofilamentous strains. These are, to our knowledge, the first experiments using environmental manipulation *in vivo* to perturb and study both yeast and filament contributions to dissemination and invasion in candidiasis. However, temperature alterations were recently used to probe the virulence of the fungal dimorph *Talaromyces marneffe* (65). Temperature shifts can alter gene expression and physiology in zebrafish, including responses to oxidative stress and motor activities, and immune responses to infection and wounding can be temporarily altered (66–68). Nonetheless, our control experiments monitoring immune homeostasis and wounding responses provide support for the immune stability of zebrafish over short-term infections at temperatures higher and lower than the usual 28°C. Extensive testing was also done as an adjunct to the investigations of *T. marneffe* in zebrafish larvae (65). While it is possible that other physiological changes at different temperatures may occur, the conservation of morphotype-specific activities throughout the range of temperatures (Fig. S6 in the supplemental material) suggests that any other physiological changes do not change the specialized activities of yeast and filaments. Taken together with the consistent results in control experiments with shape-locked strains at multiple temperatures, these data suggest that temperature shifts can be used over these time scales to test the functions of *C. albicans* pathogenesis in zebrafish without gross consequences for immune function.

In human patients, both yeast and filaments of *C. albicans* are found at the infection site, suggesting that the presence of both morphotypes contributes to systemic candidiasis (69). Our results show that yeast and filaments have an additive effect on dissemination and mortality during coinfections in zebrafish, providing the first evidence to date for the separability of their functions *in vivo*. Murine infection studies indicate that both yeast- and filament-locked strains have lower levels of virulence than a wild-type strain that can switch forms (21, 24, 49, 70, 71). This suggests that either the presence of both forms or a dimorphic transition is required for virulence. However, there are no studies in mice focused on defined coinfections with yeast- and filament-locked strains, and results from pooled mixed-strain infections suggest that *in vitro* morphology and relative infection progression *in vivo* are not perfectly correlated (72). The additive effect of yeast and filaments on dissemination and mortality that we find in our mixed-strain infections supports the idea that the simultaneous presence of both forms is a key factor in promoting full pathogenesis. Thus, while each form contributes an important component to successful infection, the simple presence of both morphology-locked forms allows for the distinct functions of dissemination and invasion, even if the strains cannot switch back and forth between shapes.

Surprisingly, *C. albicans* morphotypes do not synergize to enhance yeast spread or filament invasion in zebrafish. Interestingly, comparison of mono-infection (filament only) to mixed-morphology infection (filaments plus yeast) suggests that the presence of yeast during infection results in higher survival (Fig. 5C; also Fig. S5C and S6B and D in the supplemental material), although there was no difference in overall mortality between low and high yeast inocula in mixed-strain infections (Fig. 5G). This suggests that the presence of yeast makes a subtle but consistent difference in reducing mortality, but that this difference is associated only with the presence or absence of yeast rather than with the overall level of the yeast inoculum. The DOX-mediated morphology switching results further suggest that the reduction in mortality requires the continuous presence of yeast, although this is a tentative conclusion that awaits more experiments with varied times of DOX-mediated switching. We do not yet understand how the presence or absence of yeast affects the lethality of the filaments, but this influence could be dependent on other fungal or host factors. For instance, several quorum-sensing factors are known to mediate intercellular communication among *C. albicans* cells (73, 74). Alternatively, yeast presence could result in ongoing

immune modulation due to differential immune responses to yeast and filaments (75, 76). Future studies concurrently analyzing immune responses in mixed-morphotype infections may provide us with a better understanding of how yeast affects the ability of filaments to kill fish but not to invade tissue.

Overall, the unique advantages of the zebrafish model described in this study offer a new outlook on host-*C. albicans* interactions in the context of morphological effects on pathogenesis. Taken together, these results provide detailed evidence confirming the previously suspected links between shape and pathogenesis, revealing independent roles for each morphotype during infection. Characterizing the roles of each morphotype during infection has the potential to inform the development and use of promising new morphotype-specific interventions against *C. albicans* (12, 25, 26).

MATERIALS AND METHODS

Ethics statement. All zebrafish protocols were conducted in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (77). All animals were treated in a humane manner according to the guidelines of the University of Maine Institutional Animal Care and Use Committee (IACUC), protocols A2012-11-03 and A2015-11-03.

Zebrafish care and maintenance. Adult zebrafish were kept at 28°C in recirculating systems (Aquatic Habitats, Apopka, FL) at the University of Maine Zebrafish Facility. Zebrafish embryos were collected and grown at 33°C at a density of 150 eggs/dish in 150-mm petri dishes containing 150 ml egg water (Nanopure water with 20 g/liter [final concentration, 60 mg/liter] Instant Ocean salts [Aquarium Systems, Mentor, OH]). Egg water was supplemented with 0.1% methylene blue (final concentration, 0.00003%) (Alfa Aesar, Haverhill, MA) for 24 h to prevent microbial growth. Water was changed once a day to keep larvae clean. For temperature experiments, larvae were placed at either 33°C, 28°C, or 21°C after microinjection. The wild-type AB fish line from the Zebrafish International Resource Center and the Tg(BACmpo:gfp) fish line (for neutrophil flow cytometry data) described previously (59) were used for experiments. All zebrafish husbandry was performed as described previously (78, 79).

Fungal strains and growth conditions. *Candida albicans* strains are described in Table S1 in the supplemental material. The nonfluorescent overexpression strains have been described previously (23). *C. albicans* strains were grown on YPD (yeast-peptone-dextrose) agar (20 g/liter Bacto peptone, 10 g/liter Bacto yeast extract, 20 g/liter Bacto agar, 20 g/liter dextrose; BD/Fisher Chemical) at 37°C. Liquid cultures were grown overnight in YPD medium at 30°C for infections. For UME6^{OE}, the strain was grown on YPD agar or in liquid supplemented with 50 µg/ml or 25 µg/ml doxycycline hydrochloride (DOX; Calbiochem, EMD Millipore, Billerica, MA), respectively, to produce nonfilamentous colonies. Overnight cultures were resuspended and diluted in phosphate-buffered saline (PBS) and were counted on a hemocytometer for a concentration of 1×10^7 cells/ml or 5×10^6 cells/ml for injection. For mixed-strain infections, concentrations of each fungal strain (1×10^7 cells/ml) were made separately before being mixed together in a 1:1 ratio for injection (final concentration for each strain, 5×10^6 cells/ml). For infections involving UME6^{OE}-dTomato, yeast cells were stained for 5 min with 750 µg/ml calcofluor white (CFW) before being washed in PBS once or twice (Sigma-Aldrich, St. Louis, MO) for better visualization during postinfection screening.

Engineering of NEON-expressing *C. albicans* strains. The NRG1^{OE}-NEON-NAT^R *C. albicans* strain (NRG1^{OE}-NEON) was constructed by transforming the NRG1^{OE} strain (Table S1 in the supplemental material) (23) with the pENO1-NEON-NAT^R plasmid, engineered in our lab. This plasmid contains a codon-optimized version of the NEON gene under the control of the constitutive *ENO1* promoter, with a nourseothricin resistance selection marker (NAT^R) (plasmids themselves are on a pUC57 backbone). The plasmid was cloned and ligated into the *Escherichia coli* competent strain NEB5α (New England BioLabs, Ipswich, MA). Transformed cells were prepared (QIAprep Spin Miniprep kit; Qiagen, Valencia, CA), and DNA was digested with restriction enzymes flanking the beginning and end of the NEON insert (NcoI and PacI; New England BioLabs, Ipswich, MA) to identify correct transformants (the backbone is ~5 kb; the NEON insert is 715 bp). *C. albicans* was transformed using the lithium acetate protocol published previously (80), with nourseothricin resistance as a selection marker (100 µg/ml NAT; Werner Bioagents, Jena, Germany). At least 3 to 20 colonies were selected and screened for fluorescence via epifluorescence microscopy (Carl Zeiss Microscopy LLC, Thornwood, NY) and flow cytometry (for NEON, a 488-nm laser with a fluorescein isothiocyanate [FITC] filter; LSRII; Becton, Dickinson and Company, Franklin Lakes, NJ). PCR was used to check for correct integration of the NEON plasmid in the *ENO1* locus using primers P_{ENO1} Fw and NEON Rv (~1.2 kb). All primer sets can be found in Table S2 in the supplemental material. Strains containing dTomato or iRFP were transformed with pENO1-dTomato-NAT^R (68) or pENO1-iRFP-NAT^R (70), respectively, according to this protocol. PCR was used to check for correct integration of the dTomato plasmid in the *ENO1* locus using primers Sp6 and dTomato Rv (~680 bp) or P_{ENO1} Fw and dTomato Rv (~1.2 kb). PCR was used to check for the correct integration of the iRFP plasmid using primers P_{ENO1} Fw and iRFP Rv1 (~1.2 kb).

Microinjection. Zebrafish at the roughly prim-22 stage were physically dechorionated and anesthetized in 4 mg/ml Tris-buffered tricine methanesulfonate (final concentration, 160 µg/ml in 50 ml egg water) (Western Chemical, Ferndale, WA). For injection, 2 to 4 nl of PBS or *C. albicans* suspended at 1×10^7 cells/ml or 5×10^6 cells/ml in PBS was microinjected with borosilicate glass capillary needles (Sutter Instrument, Novato, CA) through the back of the yolk near the junction of

the yolk extension for a dose between 5 and 20 CFU. In experiments that needed exact known inoculum sizes, fish were screened, and the inoculum sizes for all strains were counted. Fish were then plated in individual wells until imaging and infection scoring. A small portion of fish mock infected with PBS do die due to natural causes in the first 4 days postinjection, and this proportion can differ from batch to batch of fish. Therefore, to make sure that these variations do not affect any comparisons among cohorts, all cohorts are included in experiments with a given batch of fish, and a full set of independent experiments is pooled.

Fluorescence microscopy. An FV-1000 laser-scanning confocal system on an Olympus IX-81 inverted microscope was used for confocal imaging (Olympus, Waltham, MA). Objective lenses with powers of 10 $\times$ (numerical aperture [NA], 0.40) and 20 $\times$ (NA, 0.70) were used. Live fish were prepared for imaging by anesthetizing and immobilizing them in 0.4% low-melting-point agarose (Lonza, Rockland, ME) with tricaine. Intact fish images are overlays of differential interference contrast (DIC) and fluorescence images or fluorescence image panels (e.g., red, red/green). Images consist of 18 to 25 slices stacked at a maximum z-projection. dTomato, enhanced green fluorescent protein (EGFP), NEON, and iRFP were detected by optical lasers/filters with a 10 $\times$ (NA, 0.4) or 20 $\times$ (NA, 0.7) lens objective for excitation/emission, respectively, at 546 nm/560 to 660 nm (dTomato), 488 nm/505 to 525 nm (both EGFP and NEON), and 647 nm/655 to 755 nm (iRFP). For 2-dimensional (2D) imaging of yeast and filaments, fish were euthanized by an overdose of tricaine (300 μ g/ml for 10 min) and were individually placed on a glass slide (25 by 75 mm; Corning, Corning, NY). A glass coverslip (12 by 12 mm; Corning, Corning, NY) was placed on top of the fish and was pressed down using the bulb of a plastic Pasteur pipette to gently flatten the fish. Images were acquired on a Zeiss AxioVision epifluorescence microscope (Carl Zeiss Microscopy LLC, Thornwood, NY) using a 40 $\times$ objective (NA, 0.75) and were processed using Photoshop (version CS5 12.1, 64 bit; Adobe Systems Incorporated) and Fiji (www.fiji.sc) software.

Imaging and quantification of filament invasion and yeast colonization in intact fish. To image fungal invasion and colonization in intact fish, fish infected with known yeast and/or filament inocula were plated into individual wells in either 24- or 96-well glass-bottom plates, anesthetized with tricaine, and stabilized in 0.4% low-melting-point agarose as described above. Each fish was placed on its left side, with the head facing the right side of the well. Random, nondisseminated fish were imaged at 18 hpi. This time point was chosen to allow for a significant period of infection progression while making sure to image before significant mortality occurred. Yolk sacs were imaged on an Olympus IX-81 inverted confocal microscope with consistent photomultiplier tube (PMT) voltage (high voltage [HV]; for the Alexa Fluor 488 laser, 660 V; for the Alexa Fluor 546 laser, 700 V). Images consist of 18 to 20 slices stacked at a maximum z-projection. Images were then transferred to ImageJ (Fiji) and were thresholded for either bright filaments (indicating invasion outside yolk) or whole yeast-colonized areas, and each area was quantified in pixels. If fish had infections on the far side of the yolk and thresholding could not detect fluorescence, these fish were excluded from data analysis. The yeast level was calculated as a percentage of the total area covered by fungi. Care was taken to ensure that images for quantification were taken with identical settings and were processed identically.

Longitudinal analyses of survival, dissemination, filament invasion, and yeast colonization level. To quantify the percentage of survival of infected fish for Kaplan-Meier curves, all fish were pooled at each time point and censored for either survival (score, 0) or death (score, 1) for each group (uninfected, infected). To quantify the percentage of dissemination in experiments with pooled infection groups that were not followed longitudinally (Fig. 1C, 2C, 3B, D, and F, and 4B; also Fig. S5B and S6A and C in the supplemental material), all scored, live fish were pooled from each experiment at each time point. For longitudinal imaging experiments (Fig. 2E and F and Fig. 4D to F and H; also Fig. S2), fish were kept in individual wells starting immediately postinfection (<1 hpi, when the inoculum size was determined), screened for lack of dissemination at 18 hpi, imaged to determine the yeast colonization level and filament invasion level at 18 hpi, and then followed and scored at 24 and 30 hpi for dissemination and mortality. Results for fish from multiple independent experiments were pooled for analysis as indicated in the figure legends.

Statistical analysis. All statistical analyses were performed in GraphPad Prism software (version 6; GraphPad, La Jolla, CA). Cohorts of infected fish start at similar numbers during longitudinal analysis, but some fish in infected groups succumb to infection over time, leading to a lower number of live fish at a given time point (depicted on dissemination graphs). All data were tested with nonparametric tests, as indicated in the corresponding figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00415-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 1.0 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 5, PDF file, 1.8 MB.

SUPPLEMENTAL FILE 6, PDF file, 3.4 MB.

SUPPLEMENTAL FILE 7, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 8, PDF file, 0.5 MB.

SUPPLEMENTAL FILE 9, PDF file, 3.0 MB.

SUPPLEMENTAL FILE 10, PDF file, 7.8 MB.

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