Fig. 1

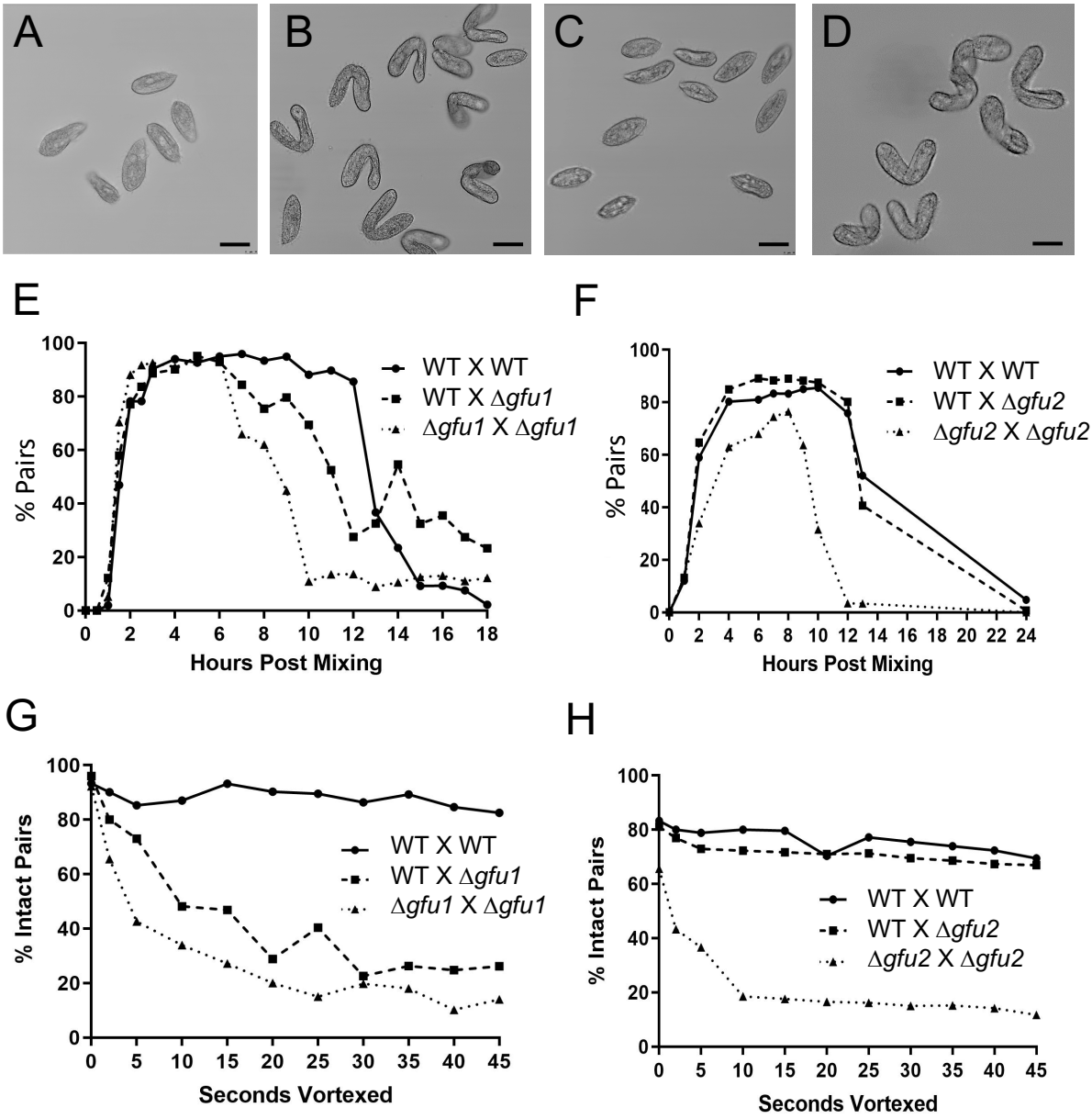


Figure 1. Characteristics of cell-cell pairing by *Δgfu1* and *Δgfu2* mutant cell lines. Wild type and deletion strains lacking either *GFU1* or *GFU2* were starved to induce mating competency and then mixed with equal numbers of cells of complementary mating type as indicated in each panel. Panels (A-D) are phase contrast images showing mixtures of *Δgfu1* (A,B) or *Δgfu2* (C,D) deletion strains fixed immediately after mixing (A,C) or 4 hr post-mixing (B,D) (bar = 20 μm). Note the heart-shaped mating pairs visible at the 4 hr time point in (B) and (D). Panels (E,F) show the kinetics of pair formation in undisturbed cultures. Following starvation, cells were mixed at ratios of 1:1, then fixed at the indicated time points and counted to determine the percentage of cells in pairs. Panels (G,H) show the relative stability of pairs from mating cultures of WT, *Δgfu1,* and *Δgfu2* strains following vortexing. Samples were collected from undisturbed cultures at 4 hr post-mixing, vortexed at a fixed speed for the times shown on the x-axis, then fixed and counted to determine the περχενταγε of cells in pairs.

Fig. 2

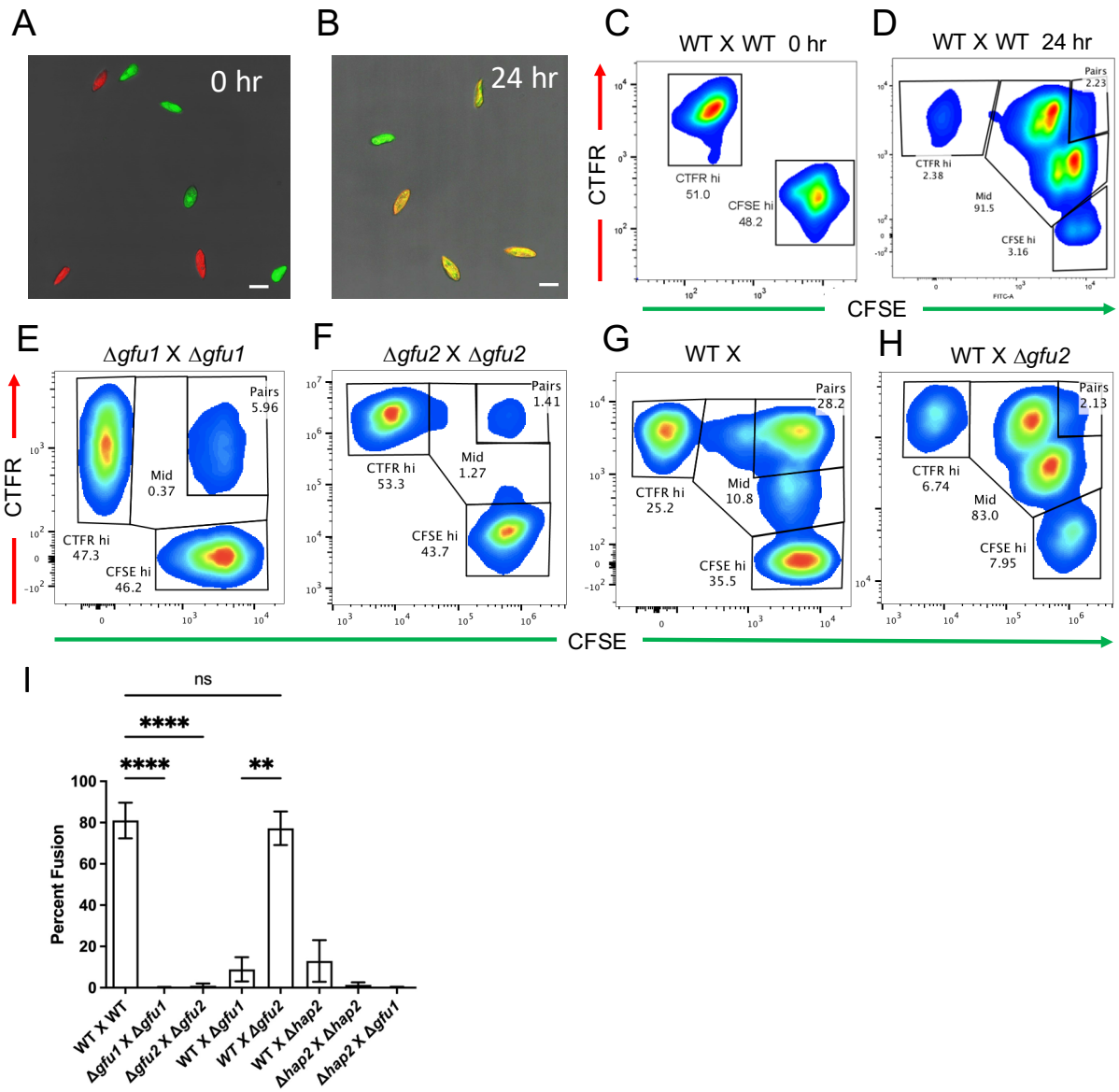
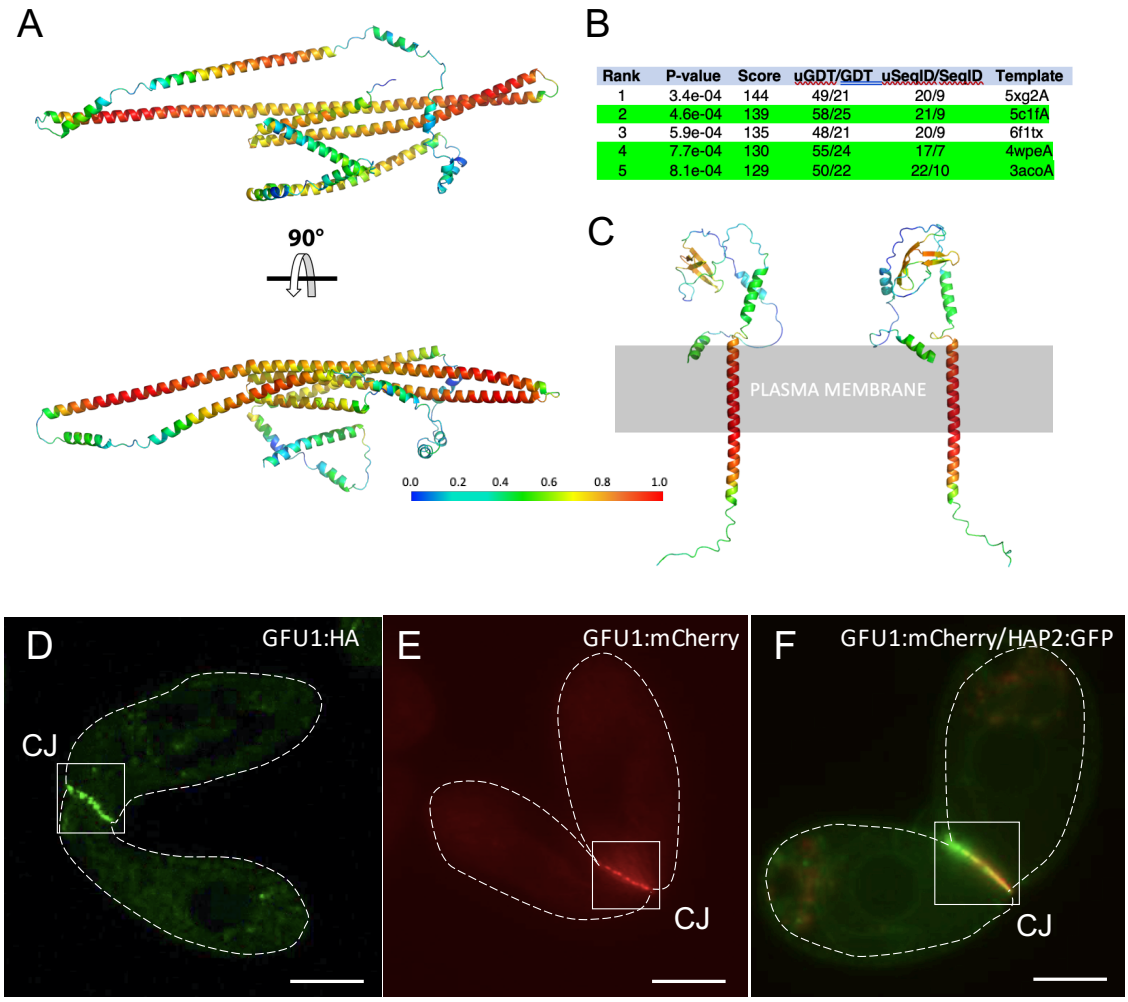


Figure 2. Quantitation of cell-cell fusion by flow cytometry. Panels (A,B) are fluorescence confocal images of a mating culture of wild type *T. thermophila* containing equal numbers of cells of complementary mating type that had been labeled with CFSE (green) or CTFR (red) prior to mixing. Panel (A) shows cells immediately after mixing (0 hr), while panel (B) shows cells 24 hr after mixing when sexual development was complete, and pairs had come apart as new progeny. Note that cells in (A) are either red or green, while in (B), four of five cells show both colors and appear yellow (bar = 30 μm). Panels (C) and (D) are representative smoothed display pseudocolor flow cytometry plots of the same cultures in (A) and (B) either before (C) or after completion of mating (D). Initially (0 hr), only two populations were present labeled CTRF^{hi} and CFSE^{hi} (panel (C)). After mating (24 hr), the original single-labeled populations were diminished, and >90% of cells were present in two new populations labeled with both dyes, one being more red than green, and the other more green than red (labeled "Mid" gate in (panel (D)). An additional small population was also present at the 24 hr time point labeled "Pairs". As shown previously, this gate represents mating pairs that either failed to come apart or continued to attempt to pair after normal mating was complete[3]. Panels (E-H) are representative flow cytometry plots of mating cultures of *Δgfu1* or *Δgfu2* deletion strains crossed to complementary mating types carrying the *Δgfu1* or *Δgfu2* deletions (panels E,F) or to WT cells (panels G,H) 18-24 hr post-mixing. Panel (I) is a bar graph

showing the compiled data from multiple crosses between WT, *Δgfu1*, *Δgfu2,* and *Δhap2/gcs1* deletion strains as indicated on the x-axis (n = 12-78 biological replicates/cross). Bars show the mean percentage of cells that had undergone fusion (calculated from the "Mid" gate) and the bracketed lines, the standard deviations (+/-). Asterisks represent the level of statistical significance between indicated crosses based on one-sided Kruskal-Wallis tests and Dunn's post-tests (**** = $p<0.0001$; ** = $p<0.01$; ns = no significance).

Fig. 3.



Rank	P-value	Score	uGDT/GDT	uSeqID/SeqID	Template
1	3.4e-04	144	49/21	20/9	5xg2A
2	4.6e-04	139	58/25	21/9	5c1fA
3	5.9e-04	135	48/21	20/9	6f1tx
4	7.7e-04	130	55/24	17/7	4wpeA
5	8.1e-04	129	50/22	22/10	3acoA

Figure 3. Localization and predicted structural features of GFU1 and GFU2. AlphaFold2 was used to generate predicted 3-dimensional protein structures of full-length GFU1 (panel (A)) and the mature GFU2 protein minus the signal peptide (panel (C)) with color coding based on prediction reliability scores. Blue (0.0) represents the lowest and red (1.0) the highest confidence scores. Note the curvature along the long axes of the extended helices (visible in the orthogonal view at the bottom of the panel), a common feature of a-helical BAR domains[33]. Panel (B) shows the results of a structure homology search comparing GFU1 to known structures in the PDB Protein Data Bank (https://www.rcsb.org/) using the search algorithm, RaptorX[58]. Three of the five top hits are to F-BAR domain-containing proteins[59–61] (highlighted in green) with P-value and uGDT scores reflecting high quality predictions in each case. Orthogonal views of the AlphaFold2 prediction of mature GFU2 (residues 18-228) are shown in panel (C) as they would appear in the plasma membrane (gray bar) with the N-terminal extracellular domain on top. Note that the a-helix at the proximal N-terminus is aliphatic and probably lies flat on the membrane rather than penetrating it as shown. Panels (D-F) are conventional fluorescence light microscopic images of mating pairs formed by WT cells and transgenic cell lines expressing C-terminal HA- (D) or mCherry-tagged (E,F) versions of GFU1 from chimeric genes introduced at the endogenous *GFU1* locus. In panel (D), the HA-epitope tag was detected with a fluorescein-tagged secondary antibody and appears green. In panel (F), the transgenic cell is co-expressing an inducible, GFP-tagged version of HAP2/GCS1 (green) from the β-tubulin 1 locus. Areas where the two proteins co-localize appear yellow. White boxes indicate the conjugation junction (CJ) where complementary mating types adhere and dashed white lines demarcate the borders of mating cells (bars = 10 μm).

Fig. 4

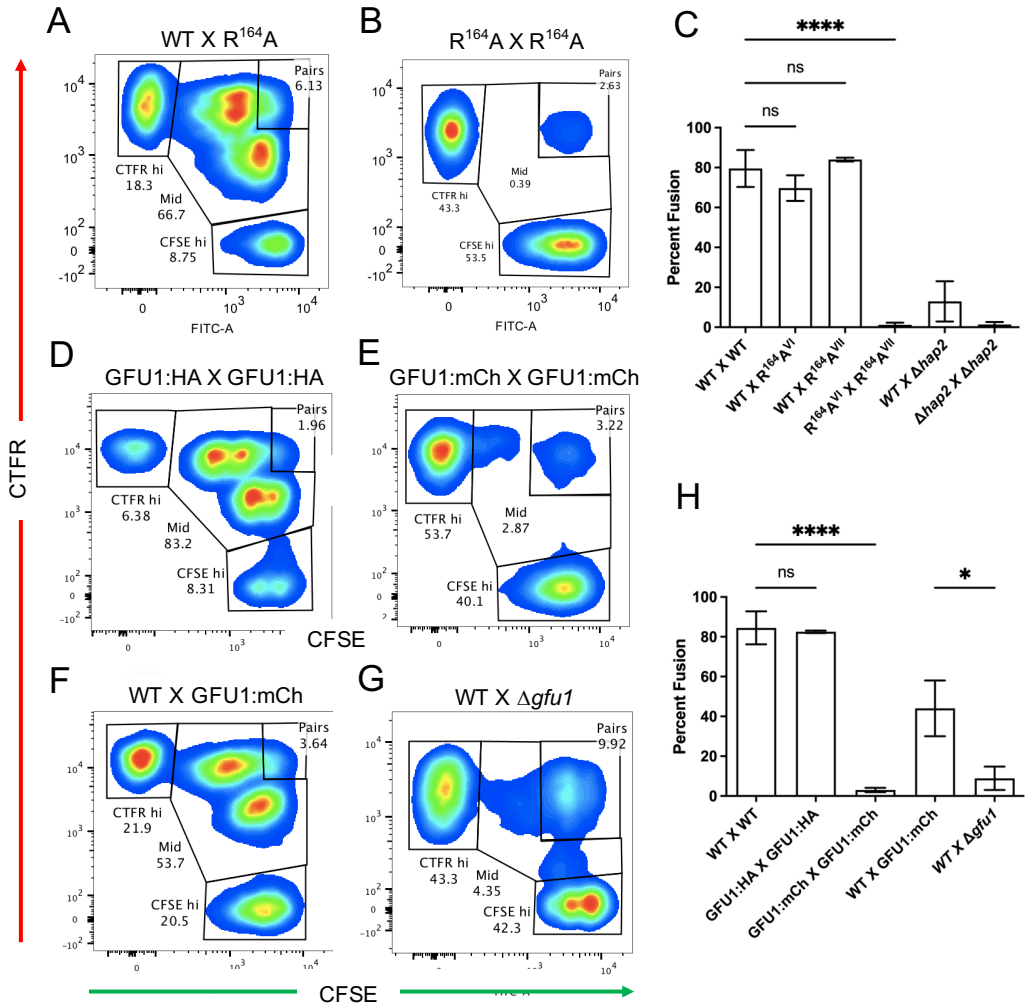


Figure 4. Cooperativity between the fusion machinery on apposed membranes. Panels (A,B) are representative flow cytometry plots of CFSE and CTFR-labeled cells expressing the *R164A* mutant allele of *HAP2/GCS1* mated to either WT cells (panel A) or a complementary mating type carrying the identical *R164A* mutant allele (panel B) fixed at 24 hr post-mixing when mating was complete. Panel (C) is a bar graph showing the compiled data of percent fusion in crosses between WT, *R164A* mutant, and *Δhap2/gcs1* deletion strains as indicated on the x-axis (n = 6-78 biological replicates/cross). The mating types (VI and VII) of strains carrying the *R164A* mutant allele are shown in superscript. Panels (D-E) are representative flow cytometry plots of crosses between CFSE and CTFR-labeled cells expressing either C-terminal HA- (D) or mCherry-tagged (E) versions of GFU1 fixed at 24 hr post-mixing. Fluorescence from the endogenously expressed mCherry construct is negligible in fixed cells and does not contribute to the recorded signal. In panels (F,G), WT cells were mixed with either GFU1:mCherry expressing cells (F) or with a *Δgfu1* deletion strain lacking the gene (G), and fixed 24 hr post-mixing. Note the substantially larger population of cells in the "Mid" gate in (F) compared to (G). Panel (H) is a bar graph showing the compiled data for percent fusion from all experiments with tagged *GFU1* constructs (n = 3-18 biological replicates/cross). Bars in panels (C,H) represent the mean and bracketed lines the standard deviations (+/-). Asterisks represent the level of statistical significance between indicated crosses based on one-sided Kruskal-Wallis tests and Dunn's post-tests (**** = p<0.0001; * = p<0.05; ns = no significance).

Fig. 5

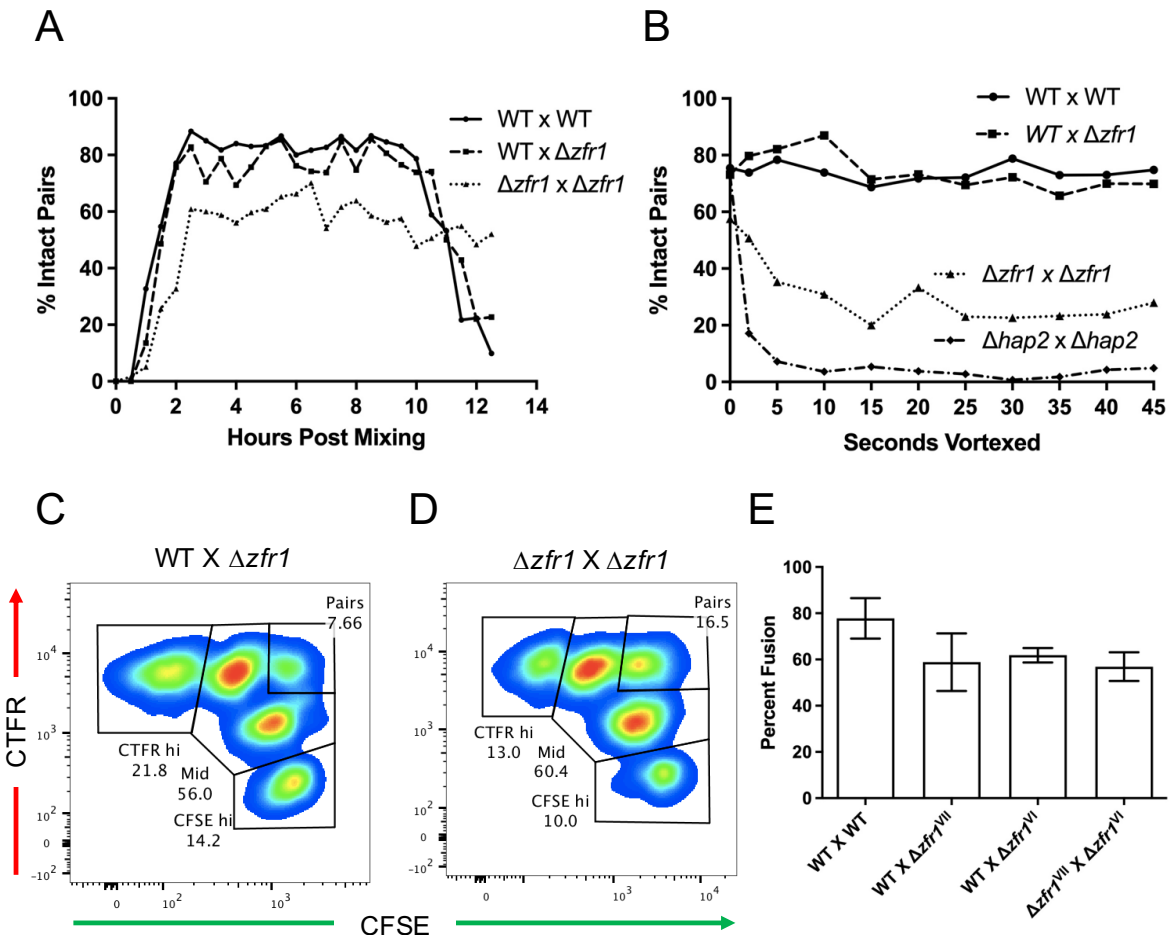


Figure 5. Stability and cell-cell fusion of *Δzfr1* mating pairs. Panels (A) and (B) show the kinetics of pairing (A) and mechanical stability (B) of pairs formed by *Δzfr1* deletions strains. Note that *Δzfr1* X *Δzfr1* pairs were comparable to WT X WT pairs in their overall kinetics of pairing through roughly 10 hr post-mixing, but their mechanical stability was considerably lower and approached that of *Δhap2/gcs1* X *Δhap2/gcs1* pairs which are unable to form pores[3,24]. Panels (C,D) show representative flow cytometry plots, and panel (E) a bar graph of compiled data from experiments involving multiple crosses between WT and *Δzfr1* deletion strains constructed in different mating type backgrounds (VI and VII) as shown on the x-axis (n = 6-42 biological replicates/cross). In all cases, cells were labeled with CFSE or CTFR and fixed 16-20 hr post-mixing. Note that the percentage of cells that formed pairs in *Δzfr1* X *Δzfr1* crosses (panel (A)) was essentially the same as the percentage that underwent fusion (panel (E)). Bars represent the means and bracketed lines the standard deviations in each case.