

# Membrane fusion triggers rapid degradation of two gamete-specific, fusion-essential proteins in a membrane block to polygamy in *Chlamydomonas*

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## SUMMARY

The plasma membranes of gametes are specialized for fusion, yet, once fusion occurs, in many organisms the new zygote becomes incapable of further membrane fusion reactions. The molecular mechanisms that underlie this loss of fusion capacity (block to polygamy) remain unknown. During fertilization in the green alga *Chlamydomonas*, the *plus* gamete-specific membrane protein FUS1 is required for adhesion between the apically localized sites on the plasma membranes of *plus* and *minus* gametes that are specialized for fusion, and the *minus*-specific membrane protein HAP2 is essential for completion of the membrane fusion reaction. HAP2 (GCS1) family members are also required for fertilization in *Arabidopsis*, and for the membrane fusion reaction in the malaria organism *Plasmodium berghei*. Here, we tested whether *Chlamydomonas* gamete fusion triggers alterations in FUS1 and HAP2 and renders the plasma membranes of the cells incapable of subsequent fusion. We find that, even though the fusogenic sites support multi-cell adhesions, triploid zygotes are rare, indicating a fusion-triggered block to the membrane fusion reaction. Consistent with the extinction of fusogenic capacity, both FUS1 and HAP2 are degraded upon fusion. The rapid, fusion-triggered cleavage of HAP2 in zygotes is distinct from degradation occurring during constitutive turnover in gametes. Thus, gamete fusion triggers specific degradation of fusion-essential proteins and renders the zygote incapable of fusion. Our results provide the first molecular explanation for a membrane block to polygamy in any organism.

**KEY WORDS:** *Chlamydomonas*, FUS1, HAP2, Gamete fusion, Polygamy

## INTRODUCTION

In many organisms, a key requirement for a newly formed zygote is that it render itself refractory to interactions with non-fused gametes and thus ensure formation of a diploid cell. In multicellular animals, blocks to fusion of multiple gametes (i.e. blocks to polygamy) occur both at the site of initial recognition between gametes, the egg coat, and at the fusion-competent plasma membrane of the newly fertilized oocyte (zygote). Fusion triggers rapid changes within the fertilized egg that in many organisms activate two blocks to polygamy – a block at the egg coat and a block at the level of the egg plasma membrane. Proteases, glycosidases and other enzymatic and non-enzymatic proteins released from the oocyte upon fusion chemically and physically convert the egg coat to a form that cannot support sperm binding (Hedrick, 2008; Gardner and Evans, 2006; Wong and Wessel, 2006). And, recent findings show that oviduct secretions influence changes in the egg coat contributing to the block to polygamy (Coy et al., 2008).

The membrane block is poorly understood. In some animals (sea urchins and frogs being the best studied), the block at the membrane is accomplished by a fusion-induced depolarization of the egg plasma membrane that through unknown mechanisms disallows penetration of the egg by additional sperm (Jaffe et al., 1982). In mammals, the membrane block to polyspermy is slower (30-60 minutes) and requires sperm membrane fusion with the egg, but otherwise is uncharacterized (Wortzman-Show et al., 2007). For

example, whether the egg membrane proteins CD9 and CD81, which participate in fusion (Kaji et al., 2000; Rubinstein et al., 2006), are modified after fusion is unknown. In angiosperms, polygamy also is rare and its molecular basis is unknown, although in vitro studies in *Nicotiana* have documented a temporary membrane block accompanied by changes in the amounts and distribution of lectin binding molecules on the egg surface (Sun et al., 2000; Fang et al., 2008).

A two-step process for fertilization – an initial recognition/adhesion interaction that triggers gamete activation, followed by adhesion and fusion of the gamete plasma membranes – is an ancient invention and holds true for the mating reaction between *plus* and *minus* gametes in the unicellular bi-flagellated green alga *Chlamydomonas*. In this organism, initial recognition and signaling are accomplished by mating type-specific adhesion molecules, agglutinins, on the flagella of mating-type *plus* and mating-type *minus* gametes. When the gametes are mixed together, the cells rapidly adhere to each other by their flagella, forming large, multi-cell aggregates composed of as many as 10-30 cells. A signaling pathway triggered by flagellar adhesion activates the gametes to prepare for fusion and induces them to form membrane protrusions (the *plus* and *minus* mating structures) between the two sets of flagella. The mating structures are the sites for the second step in fertilization, adhesion and fusion of the cell body plasma membranes. The motility of the flagella causes the apical ends of the cell bodies to be flung against each other and consequent interactions between the activated *plus* and *minus* mating structures lead to tight adhesion between the organelles (Goodenough et al., 1982; Liu et al., 2008). Mating structure adhesion is followed quickly by fusion of the tips of the organelles. And, almost immediately, the tube-like fusion pore connecting the two gametes shortens and expands and the two cells coalesce into a quadri-

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flagellated zygote. Fertilization is rapid; zygotes can be detected within minutes after gametes are mixed together and, by ~30 minutes, most gametes have fused. Previously, we showed that soon after fusion, the flagella of the zygote become non-adhesive (Hunnicut and Snell, 1991), thereby providing one element to what is likely to be a complex mechanism for blocking formation of triploid zygotes.

In *Chlamydomonas*, two proteins are known by gene disruption to be essential for the membrane fusion reaction. *Plus* gametes express a species-specific, single-pass transmembrane protein, FUS1, on the surface of the actin-filled, microvillus-like *plus* mating structure (Ferris et al., 1996; Misamore et al., 2003). FUS1, which has domains related to the Ig-like domains of prokaryotic invasins and adhesins, is essential for adhesion of the *plus* mating structure to an as yet unidentified receptor on the shorter, more bulbous mating structure present between the flagella of *minus* gametes (Misamore et al., 2003).

The second identified protein required for the membrane fusion reaction, HAP2, is expressed on the surface of the *minus* mating structure (Liu et al., 2008). *Chlamydomonas* HAP2 is a member of a broadly conserved protein family whose founding member was identified in *Arabidopsis* in a screen for male sterile mutants (Johnson et al., 2004). [HAP2 more recently has also been termed GCS1 (Mori et al., 2006).] Previously, using gene disruption methods, we showed that in both *Chlamydomonas* and the rodent malaria organism *Plasmodium berghei*, HAP2 is required at a step in the membrane fusion reaction after initial, species-specific adhesion between gamete membranes (Liu et al., 2008). Although they did not identify the step that was blocked, Hirai et al. (Hirai et al., 2008) also showed that *P. berghei* zygote formation requires HAP2. HAP2 family members are present in most higher plants (Liu et al., 2008). In *Arabidopsis*, the protein is present on sperm and required both for pollen tube guidance (Johnson et al., 2004; von Besser et al., 2006) and at an as yet uncharacterized step in sperm-egg interactions in this higher plant (Mori et al., 2006).

Here, we report that during *Chlamydomonas* fertilization, gamete fusion triggers rapid degradation of FUS1 and HAP2 and renders the zygote incapable of subsequent fusion. Moreover, although the proteins undergo constitutive loss and replacement in non-activated and activated gametes, fusion is required for their rapid cleavage; fusion-triggered HAP2 degradation products are unique to zygotes.

## MATERIALS AND METHODS

### Cells and cell culture

*Chlamydomonas reinhardtii* wild type strains *21gr* (mating type *plus*; mt+; CC-1690) and *6145C* (mating type *minus*; mt-; CC-1691) are available from the *Chlamydomonas* Culture Collection. The fusion-defective, *HAP2 minus* mutant (*hap2*) and *hap2* rescued for gamete fusion by transformation with a *HAP2-HA* construct have been previously described (Liu et al., 2008). The fusion-defective *fus1* mutant (*fus1-1*; CC-1158) *plus* strain (Goodenough et al., 1976) was rescued for fusion with a *FUS1-HA* plasmid (see Fig. S1 in the supplementary material) by co-transformation with the pSI103 plasmid (Silflow et al., 2001; Schroda et al., 2000; Kindle, 1990; Sizova et al., 2001). The *gam-10 minus* strain (CC-4164), which has a temperature-sensitive, fusion-defective phenotype (Goodenough et al., 1976; Forest, 1983), recently became available and was obtained from Dr Charlene Forest (Brooklyn, NY, USA). The *HAP2-HA* plasmid was introduced into the *gam-10* strain by co-transformation with the pSI103 plasmid.

Gametogenesis was induced as previously described (Liu et al., 2008). Gamete mixing experiments were performed at 23°C, including those using *gam-10* cells that had undergone gametogenesis at 32°C. Trypsin treatment of gametes ( $5 \times 10^7$  cells/ml) was described previously (Misamore et al., 2003) with the modification that 0.01% chicken egg white trypsin inhibitor

(Sigma) was used in the wash solutions. For some experiments, gametes ( $1.5 \times 10^7$  cells/ml) were incubated with 4 µg/ml of tunicamycin (Sigma) or 10 µg/ml cycloheximide (Sigma).

### Quantifying formation of diploid zygotes, triploid zygotes and tri-cell adhesions

The percent of cells forming diploid zygotes was calculated using the following equation:  $(2 \times \text{number of zygotes}) / [(2 \times \text{number of zygotes}) + \text{number of unfused gametes}] \times 100$ . At least 100 randomly selected cells were counted. To detect formation of triploid zygotes, wild-type *plus* and *minus* gametes ( $5 \times 10^7$  cells/ml) were mixed together for 20-30 minutes at *plus:minus* ratios of 1:1, 1:3 and 3:1 and fixed with glutaraldehyde (2.5%). Diploid zygotes (cells with four flagella) and triploid zygotes (cells with six flagella) were distinguished from non-fused gametes by phase contrast microscopy. To detect tri-cell adhesions, wild-type *plus* or *fus1-1 plus* gametes were mixed with *hap2 minus* gametes ( $1.5 \times 10^7$  cells/ml) for 7 minutes at the *plus:minus* ratios indicated above, fixed and the numbers of cells that were single, in pairs or in tri-cell adhesions were counted. Tri-cell adhesions were defined as clusters of three cells that were interacting with each other at their apical ends. Cells in larger clumps that were occasionally observed (5-10 cells/clump) were excluded from the analysis because of the difficulty of visualizing the individual cells and their interactions. More than 300 randomly selected cells were counted for each sample.

### Protein and glycoprotein analysis

For immunoprecipitation of HAP2-HA, gametes ( $4 \times 10^8$  cells) were suspended in 1 ml RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail, Roche Applied Science) and sonicated (three times, 10 seconds each) on ice. After 30 minutes on ice, the sample was centrifuged at 12,000 g for 30 minutes and the supernatant was incubated with an anti-HA monoclonal antibody (Santa Cruz) and protein A agarose beads (Progena Biotech, San Diego, CA, USA) for 4 hours or overnight. The agarose beads were washed with RIPA buffer four times, boiled in  $2 \times$  SDS-PAGE sample buffer (Misamore et al., 2003) and subjected to SDS-PAGE followed by immunoblotting or silver staining (Pierce). Gels were stained for glycoproteins using a Pro-Q Emerald 300 glycoprotein staining kit (Molecular Probes) and viewed in a UV transilluminator (Alpha imager from Alpha Innotech).

To assess protein glycosylation, immunoprecipitated HAP2-HA protein bound to protein A beads was denatured and incubated with PNGase F (NEB Biolabs) or O-glycosidase (Roche Applied Science) according to the manufacturer's instructions.

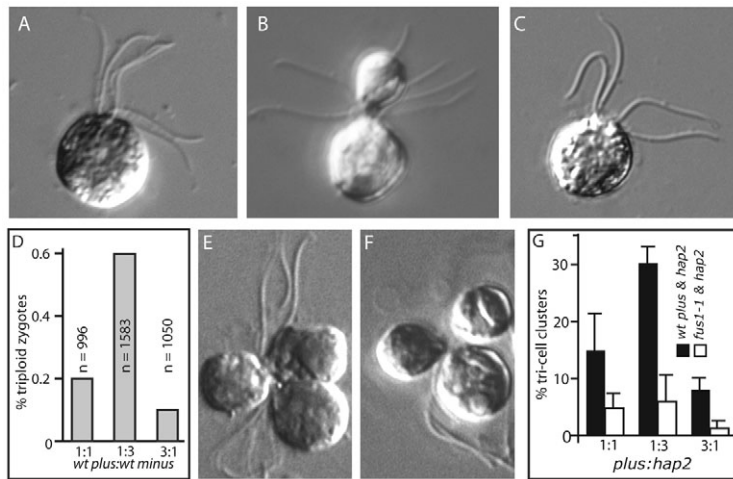
### Immunoblotting and immunofluorescence microscopy

Gametes ( $1 \times 10^7$  cells) suspended in 25 µl HMDEK buffer (20 mM HEPES pH 7.2, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM EDTA, 25 mM KCl) containing a protease inhibitor cocktail (Roche Applied Science) were mixed with an equal volume of  $2 \times$  SDS-PAGE sample buffer, boiled for 5 minutes, and proteins were analyzed by SDS-PAGE and immunoblotting as described previously (Misamore et al., 2003). Methods for DIC, immunofluorescence and for use of PKH26 to detect membrane fusion were previously described (Liu et al., 2008; Misamore et al., 2003). The DIC images of triploid zygotes are from serial z-stacks prepared using Image J (NIH).

## RESULTS

### Gamete fusogenic sites can support multi-cell adhesions, but multi-cell fusions are rare

We found that ~0.2-0.6% of the *Chlamydomonas* gametes in mixtures of wild-type *plus* and *minus* gametes formed triploid zygotes as evidenced by the presence of cells that possessed six flagella (Fig. 1A,B) rather than the four that characterize diploid zygotes (Fig. 1C). Gametes that had been mixed at a *plus* gamete:*minus* gamete ratio of 1:3 formed more triploid zygotes than did gametes mixed in ratios of 3:1 or 1:1 (Fig. 1D). In most of the triploid zygotes, the cell bodies of all three of the gametes had fully



**Fig. 1. Triploid zygotes and tri-cell clusters.** (A) Fully coalesced triploid zygote with six flagella emerging from the apical end of the cell. (B) Partially coalesced triploid zygote. (C) Typical diploid zygote with four flagella emerging from the apical end of the cell. (D) Triploid zygotes in mixtures of wild-type *plus* and *minus* gametes. Wild-type *plus* and *minus* gametes were mixed at the indicated ratios and the percent of zygotes that were triploid was determined by phase contrast microscopy.  $n$ =total number of zygotes counted. (E,F) Tri-cell clusters formed by adhesions between mating structures of wild-type *plus* and *hap2 minus* gametes mixed at a ratio of 1:3. (G) Wild-type *plus* or *fus1-1 plus* gametes were mixed with *hap2 minus* gametes at the indicated ratios and the percent of cells in tri-cell clusters was assessed.

coalesced (Fig. 1A), whereas in others, coalescence was only partial (Fig. 1B). Thus, although it is an infrequent event, *plus* and *minus* gametes are capable of forming triploid zygotes during a normal mating reaction.

Because the presence of triploid zygotes implied that multiple gametes adhered to each other by their mating structures, we tested the capacity of cells to form multi-cell adhesions. In wild-type gametes, fusion occurs almost immediately after mating structure adhesion, making it difficult to detect even pairs of cells adhering at their mating structures (Liu et al., 2008). To optimize detection of multiple mating structure adhesions, we mixed wild-type *plus* gametes and fusion-defective *hap2 minus* gametes in the same ratios as above, fixed with glutaraldehyde (Forest, 1983), and determined the numbers of cells in clusters of three adhering to each other by their mating structures.

We found that in addition to many cells being present as pairs (20-40%; not shown), up to 30% of the cells assessed were present in tri-cell clusters (Fig. 1E,F), with the highest proportion of tri-cell clusters found in the samples with a *plus:minus* ratio of 1:3 (Fig. 1G). In control experiments carried out with *fus1* gametes, which are incapable of mating structure adhesion, ~5% of cells assessed were in tri-cell aggregates, indicating that mating structure adhesion was required for formation of the aggregates and confirming the validity of the assay (Fig. 1G). Our results are similar to those with another *minus* fusion-defective mutant, *gam-10* (Forest, 1987). The gene mutated in *gam-10* is unknown (see below). The results that gametes are capable of forming tri-cell mating structure interactions, yet triploid zygotes are rare, suggest that fusion between two gametes activates a mechanism that blocks subsequent membrane adhesions and fusions.

### FUS1 is degraded rapidly after gamete fusion

Because genetic methods have shown that the membrane fusion reaction is blocked in mutant gametes that fail to express FUS1 or HAP2, we tested whether FUS1 and HAP2 were present after fusion. In samples of wild type *plus* and *minus* gametes that had been mixed for 5 minutes, FUS1 [detected by an antibody that recognizes the endogenous FUS1 protein (Misamore et al., 2003)] was present at the *plus* mating structure (arrow) between the apical ends of interacting gametes (Fig. 2A, upper panels; the pair of gametes shown were about to fuse or had just fused). In samples examined 20 minutes after mixing, FUS1 was undetectable in zygotes, even in those fixed before complete coalescence of the two cytoplasms had occurred (Fig. 2A, lower panels; arrowheads show

the bases of the flagella in the zygotes, the sites of the gamete mating structures). As expected, non-fused *plus* gametes in the mixture retained FUS1 at their mating structures (Fig. 2A, lower panels, arrows).

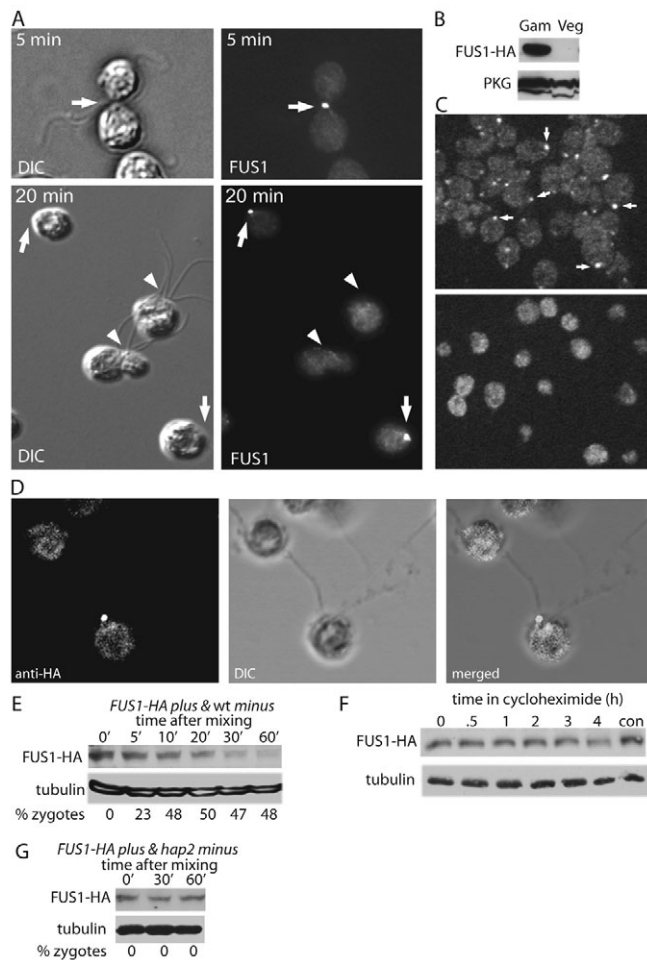
To learn more about FUS1 loss, we studied the properties of the protein in a *FUS1* sterile mutant strain, *fus1-1* (Goodenough et al., 1976), that we had rescued for fusion with a DNA construct encoding *FUS1* bearing an HA epitope. Immunoblotting with an HA antibody indicated that the 105-kDa FUS1 protein, whose expression was driven by a constitutively expressed heat shock-RUBISCO promoter, was stably expressed in *plus* gametes (Fig. 2B). The absence of FUS1-HA expression in *plus* vegetative cells suggested that the protein was stable only in gametes (Fig. 2B). As expected, FUS1-HA was localized at the *plus* mating structure on the apical ends of the gametes (Fig. 2C,D). And, consistent with the results above, when zygotes formed in a mixture of *fus1-1-FUS1-HA* gametes and *minus* gametes, after a short lag, the levels of the protein detected by immunoblotting were substantially reduced (Fig. 2E).

In experiments not shown, we searched for FUS1-HA and possible degradation products in zygotes and in the fertilization medium. Using immunoprecipitation and immunoblotting with the HA antibody (including using longer exposure times for the immunoblots), however, we failed to detect intact FUS1 in the fertilization medium or any FUS1 degradation products in zygotes or in the fertilization medium (data not shown). The failure to detect intact FUS1 in the medium or in any form in cells after fusion indicates that the protein was modified upon fusion, presumably by extensive proteolytic cleavage.

### Fusion is required for rapid degradation of FUS1 and for loss of fusion capacity

To investigate the stability of FUS1 protein in non-mixed gametes, we added the protein synthesis inhibitor cycloheximide (CH) to *fus1-1-FUS1-HA plus* gametes and, at various times after addition, we determined the amounts of FUS1-HA remaining using immunoblotting. As shown in Fig. 2F, FUS1-HA levels changed little during the 4 hour incubation with CH. Thus, constitutive loss of FUS1 failed to explain the rapid loss of the protein that occurs during fertilization, indicating that FUS1 degradation was activated during fertilization.

Although gamete fusion was one possible trigger for the degradation of FUS1 and for loss of fusion competence, gamete activation or FUS1-dependent mating structure adhesion alone



**Fig. 2. FUS1 becomes undetectable after gamete fusion.**

(A) Samples of mixed *plus* and *minus* gametes were fixed at 5 or 20 minutes after mixing and stained with anti-FUS1 antibody. FUS1 was visible on the *plus* mating structures (arrow) at 5 minutes but was undetectable in zygotes at 20 minutes (arrowheads). The arrows at 20 minutes point to unactivated *plus* gametes whose mating structures stained for FUS1. (B) *FUS1-HA*-transformed *fus1-1* gametes (Gam), but not vegetative cells (Veg), express FUS1-HA. The loading control in the lower panel is CrPKG (Wang and Snell, 2003). (C) FUS1-HA is localized at the mating structure of *plus* gametes (arrows). Upper panel, *FUS1-HA*-transformed *fus1-1* strain stained with HA antibody; lower panel, *fus1* strain with HA antibody. (D) Anti-HA immunostaining combined with DIC microscopy of *FUS1-HA* gametes demonstrates that FUS1-HA is localized at the site of the *plus* mating structure. (E) FUS1-HA is lost after fusion. At various times after mixing, *fus1-1-FUS1-HA plus* and wild-type *minus* gamete samples were assessed for zygote formation and the presence of FUS1-HA. The lower panel is a loading control showing tubulin staining. (F) *fus1-1-FUS1-HA* gametes incubated for the indicated times in cycloheximide (CH) were analyzed by immunoblotting. The lane at the right shows cells incubated for 4 hours without CH. The lower panel is a tubulin loading control. (G) *fus1-1-FUS1-HA* gametes mixed with *hap2* gametes failed to fuse and also retained constant levels of FUS1-HA.

could have triggered loss. To test whether activation of *plus* gametes induced more rapid turnover of FUS1, we used immunoblotting to examine the stability of FUS1-HA in *fus1-1-FUS1-HA plus* gametes that were mixed with *minus* gametes of the fusion-defective *hap2* mutant and underwent gamete activation. As shown in Fig. 2G, FUS1-HA levels were unchanged in the *plus* gametes that had been

induced to undergo gamete activation. Thus, gamete activation was insufficient to trigger loss. Moreover, because the interacting cells in such samples would have undergone adhesion of their mating structures (Liu et al., 2008), mating structure adhesion also failed to activate loss.

Even though FUS1 was retained in the activated *plus* gametes in these experiments, it was possible that the fusion capacity of the *plus* gametes had been lost. We tested the fusion competence of *plus* gametes that had been mixed with *hap2 minus* gametes by subsequently adding wild-type *minus* gametes to the mixture and assaying for zygote formation. We found that *plus* gametes that had failed to fuse with *hap2 minus* gametes after 30 minutes were still capable of forming zygotes when subsequently mixed with wild-type *minus* gametes (see Fig. S2 in the supplementary material). Taken together, our results indicated that the fusion-triggered degradation of FUS1, a species-specific protein essential for membrane adhesion during the gamete membrane fusion reaction, was accompanied by loss of fusion capacity. Moreover, fusion was required for loss of the protein and for extinction of the ability of gametes to fuse.

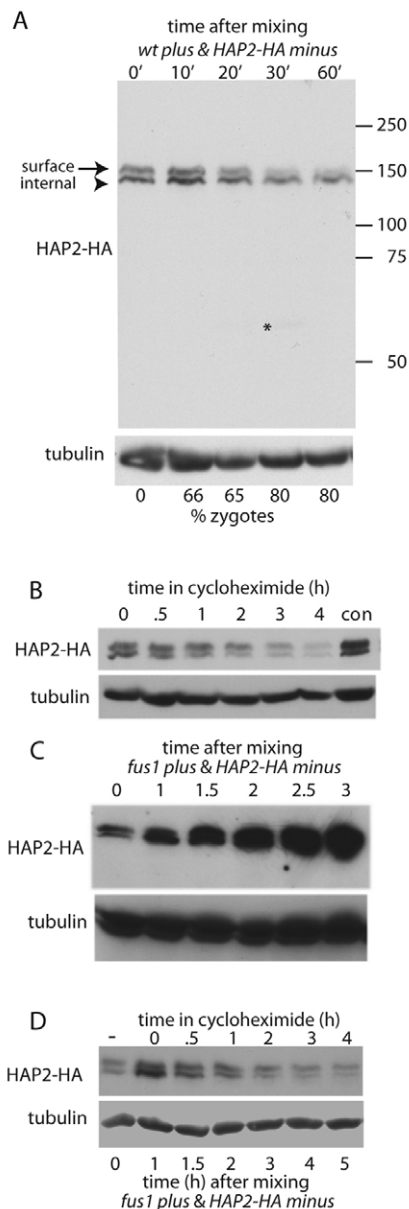
### The surface form of HAP2, the *minus* gamete protein essential in the membrane fusion reaction, also becomes undetectable during fertilization

We used methods similar to those described above to test for degradation of the *minus*-specific protein HAP2 during zygote formation. Previously, we showed that *hap2* mutant cells, transformed with an HA-tagged form of HAP2 driven by the endogenous *HAP2* promoter, were rescued for fusion. As described earlier (Liu et al., 2008), HAP2-HA was present as two closely migrating isoforms of ~150 kDa (the upper form) and 140 kDa (the lower form) in immunoblots of *hap2-HAP2-HA minus* gametes (Fig. 3A, 0 minutes). We also showed previously that only the upper form (HAP2-HA-150) was present on the cell surface and sensitive to trypsin treatment of live cells (Liu et al., 2008). When *hap2-HAP2-HA minus* gametes were mixed with wild-type *plus* gametes, they fused to form zygotes (80% fusion). And, whereas the lower form of HAP2 remained at relatively constant levels, the upper form of HAP2 almost completely disappeared in the zygotes (Fig. 3A). Thus, the second protein known to be essential for the membrane fusion reaction, HAP2, also became undetectable almost immediately after gamete fusion.

### The fertilization-related change in HAP2 detectability requires gamete membrane fusion

To determine whether a constitutive mechanism of turnover of HAP2 could explain the fusion-related change in detectability of HAP2-HA-150, we assessed HAP2 turnover in non-mixed gametes. As shown in Fig. 3B, HAP2 was relatively stable in non-mixed gametes placed in CH and both isoforms turned over with a half-life of 1.5–2.0 hours. Thus, constitutive loss of HAP2-HA-150 (Fig. 3B) was different from the fusion-related loss. Moreover, only HAP2-HA-150 was lost upon fusion, whereas both forms were lost during constitutive turnover; fusion-triggered loss was nearly complete by 30 minutes after *plus* and *minus* gametes were mixed together to form zygotes (Fig. 3A), whereas constitutive loss was much slower.

We then tested whether gamete activation alone led to the loss of detectability of HAP2-HA-150 by mixing *hap2-HAP2-HA minus* gametes with *fus1-1 plus* gametes. The mixed gametes underwent gamete activation as assessed by cell wall loss, but failed to fuse. And, as shown in Fig. 3C, activation alone failed to trigger HAP2 loss.



**Fig. 3. HAP2 changes after gamete fusion.** (A) The upper form of HAP2 becomes undetectable after gamete fusion. HAP2-HA at the indicated times after mixing *hap2-HAP2-HA minus* and wild type *plus* gametes was analyzed by immunoblotting. The percent of cells forming zygotes is shown below the blot. The asterisk indicates the location of a faintly staining band that appeared in zygotes. (B) Constitutive turnover of HAP2-HA is relatively slow. *HAP2-HA minus* gametes incubated with the protein synthesis inhibitor cycloheximide were analyzed by immunoblotting. The lane on the right shows control gametes incubated for 4 hours without cycloheximide. (C) Upregulation of HAP2-HA during gamete activation without fusion. *HAP2-HA minus* gametes mixed with fusion-defective *fus1-1 plus* gametes were analyzed by immunoblotting (upper panel). (D) Gamete activation alone does not trigger rapid loss of HAP2-HA. *HAP2-HA minus* gametes were mixed with *fus1-1* gametes for 1 hour, cycloheximide was added to the mixture and samples were analyzed by immunoblotting. Lower panels in A-D are tubulin loading controls.

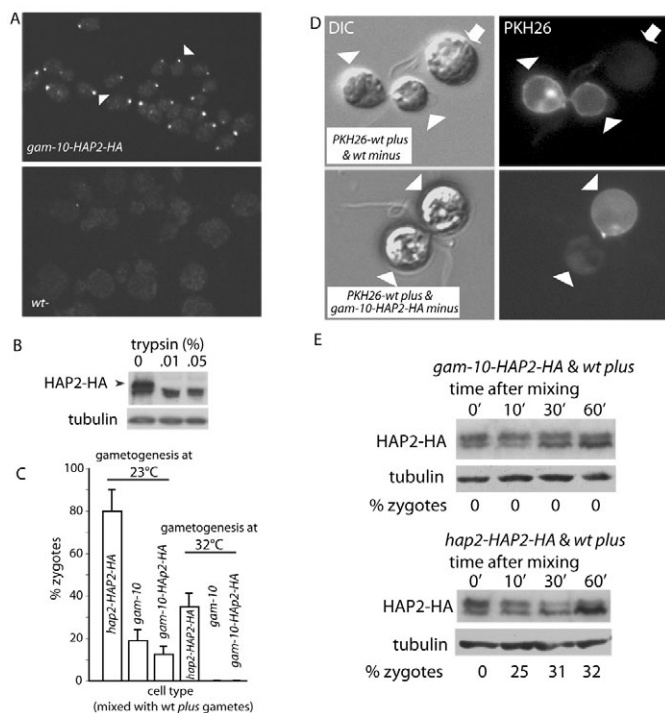
Rather, activation led to a substantial increase in HAP2 levels, with the amount of HAP2 increasing ~5- to 10-fold by 2 hours. Furthermore, addition of CH to activated gametes brought about

gradual loss of both forms of HAP2 (Fig. 3D) and the rate of loss (half-life of ~1.5-2.0 h) was similar to that seen when non-mixed gametes were incubated in CH (Fig. 3B). Thus, like FUS1, HAP2-HA-150 became undetectable during fertilization and the disappearance occurred at a step in fertilization downstream of gamete activation.

We also tested whether FUS1-dependent adhesion alone of the *plus* and *minus* mating structures was sufficient to activate the loss of HAP2-HA-150. To do this, we generated a *minus* gamete strain that expressed a wild-type, tagged form of HAP2 and was capable of mating structure adhesion, but was blocked at a separate step in the membrane fusion reaction. As described above, *gam-10* gametes had been reported to be defective in fusion at the non-permissive temperature (32°C), but competent for mating structure adhesion as evidenced by the ability of the *gam-10 minus* gametes to adhere to *plus* gametes at their mating structures (Forest, 1983). During our earlier studies on mating structure adhesion (Misamore et al., 2003; Liu et al., 2008) we were unable to confirm whether *gam-10* gametes were capable of mating structure adhesion or to determine if *gam-10* cells possessed a mutant allele of *HAP2* because the strain was unavailable to us. Fortunately, during the course of our current studies, the *gam-10* strain became available and was deposited in the *Chlamydomonas* Culture Collection.

*gam-10* cells transformed with *HAP2-HA* expressed HAP2-HA on their mating structures (Fig. 4A) but the transformation failed to rescue fusion under non-permissive conditions. Moreover, as in *HAP2-HA*-transformed *hap2* cells, the upper form of HAP2-HA was on the surface of the *HAP2-HA*-transformed *gam-10* gametes, as shown by its sensitivity to trypsin (Fig. 4B); and, although fusion was blocked, the *gam-10* gametes, under non-permissive conditions, adhered to wild-type *plus* gametes by their mating structures, consistent with the original report (Forest, 1987). At 32°C, over 20% of *hap2*, *gam-10* and *HAP2-HA*-transformed *gam-10* gametes showed mating structure adhesion with wild-type *plus* gametes 20 minutes after mixing, compared with 3% of *fus1 plus* and wild-type *minus* gametes. Control *hap2-HAP2-HA minus* gametes generated at 32°C showed reduced fusion compared with those that underwent gametogenesis at 23°C (Fig. 4C), but fusion was not abrogated. Moreover, even when cultured at 23°C, expression of HAP2-HA in *gam-10* cells failed to increase their fusion competence compared with non-transformed *gam-10* cells (Fig. 4C). The failure of the HAP2-HA-expressing *gam-10* cells to fuse in the non-permissive conditions indicated that the gene disrupted in the mutant is unlikely to be *HAP2*.

It is possible that, even though the *HAP2-HA*-transformed *gam-10* gametes failed to undergo complete coalescence, their membranes had begun the fusion process. The membrane fusion reaction in viral fusion and other cell-cell fusion events proceeds through a hemi-fusion intermediate (Top et al., 2005; Podbilewicz et al., 2006) in which lipids of the outer layers of the interacting membranes become freely exchangeable. Previously, we showed that *hap2* gametes fail to undergo hemi-fusion (Liu et al., 2008). To test for lipid mixing in the experiments with the *gam-10-HAP2-HA* cells, *plus* gametes were incubated with the fluorescent membrane dye PKH26, mixed at 23°C with *gam-10-HAP2-HA* gametes (both sets of cells had been cultured at 32°C) and pairs were examined for lipid mixing. As expected, in wild-type control gametes, the PKH26 diffused rapidly after fusion to cover the entire surface of the just-formed zygote (Fig. 4D, upper panels) but we never detected dye redistribution in mating structure-adhering pairs composed of *gam-10-HAP2-HA minus* gametes and PKH26-labeled *plus* gametes generated at the non-permissive temperature (over 60 pairs examined; Fig. 4D, lower panels).



**Fig. 4. Gamete fusion is required for rapid loss of the upper form of HAP2-HA.** (A) Gametes of the *gam-10* fusion-defective *minus* strain transformed with the *HAP2-HA* construct express HAP2-HA at their mating structures (upper panel, arrowheads). Control wild-type *minus* gametes lack the HA antigen (lower panel). (B) The upper isoform of HAP2-HA is on the cell surface. *gam-10-HAP2-HA* gametes treated with trypsin were analyzed by immunoblotting. The upper form of HAP2-HA (arrowhead) was lost after trypsin treatment (upper panel); the lower panel is a loading control. (C) HAP2-HA failed to rescue fusion in *gam-10* cells. *Minus* gametes of the indicated cell types that had undergone gametogenesis at 23°C or 32°C were mixed with wild-type *plus* gametes at 23°C for 30 minutes and the number of cells forming zygotes was determined. (D) Lipid mixing failed to occur in pairs of *gam-10-HAP2-HA minus* gametes with wild-type *plus* gametes. *gam-10-HAP2-HA minus* gametes (upper panels) or wild-type *minus* gametes (upper panels) mixed at 23°C with wild-type *plus* gametes labeled with the PKH26 membrane dye were analyzed for redistribution of the dye by use of fluorescence microscopy. In wild-type mixtures (upper panels), an unstained *minus* gamete (arrow) and stained *plus* and *minus* gametes that just fused (arrowheads) are visible. In pairs of wild-type *plus* gametes (lower panels, upper arrowheads) with *gam10-HAP2-HA minus* gametes (lower panels, lower arrowheads), dye redistribution failed to occur. (E) HAP2-HA is retained in *gam-10-HAP2-HA minus* gametes generated at 32°C that interacted with, but failed to fuse with, wild-type *plus* gametes generated at 32°C (upper panels), whereas, HAP2-HA was lost from *hap2-HAP2-HA minus* gametes that fused with wild-type *plus* gametes (lower panels).

Finally, immunoblotting showed that when *gam-10-HAP2-HA minus* gametes grown at the non-permissive temperature were mixed with wild-type *plus* gametes, the upper form of HAP2-HA failed to decrease (Fig. 4E, upper panel). Conversely, in experiments with *hap2-HAP2-HA* cells grown at 32°C, the upper form of HAP2-HA had substantially decreased at 30 minutes (Fig. 4E, lower panel). At 60 minutes, the levels of both forms of HAP2 began to increase in both sets of cell mixtures, presumably because the non-fused gametes in the sample were activated and HAP2 expression was upregulated as described above.

### The fusion-triggered loss in detectability of HAP2 is due to its cleavage

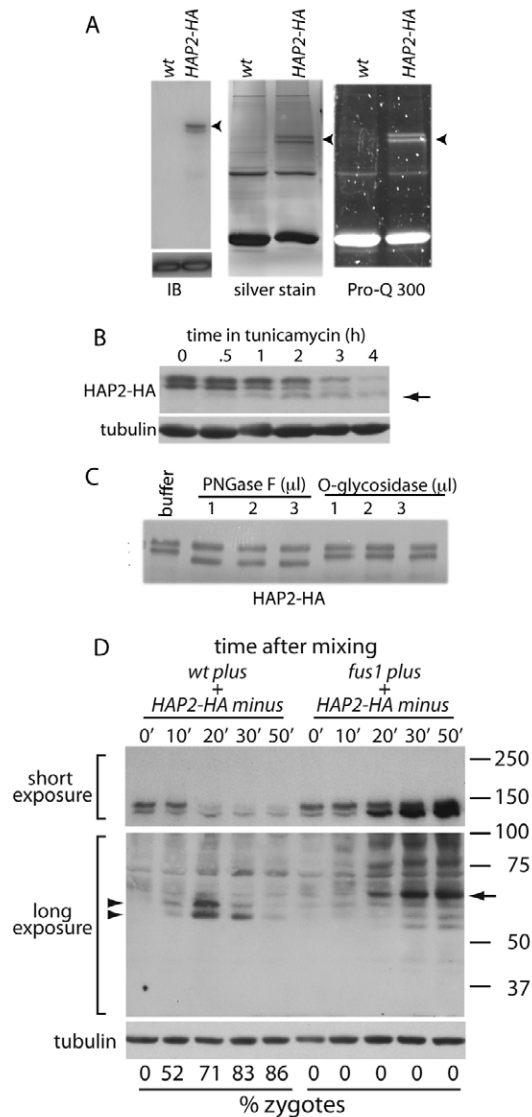
Having determined that fusion was required for the disappearance of the upper form of HAP2, we examined its fate after fusion. Because only the upper form became undetectable after fusion (Fig. 3A and see below), with no concomitant increase in the lower form in fertilization mixtures in which high percentages of the gametes fused to form zygotes, it was unlikely that the upper form was being converted to the lower form. Furthermore, even during constitutive turnover, we found no evidence for interconversion of the two forms and both were lost essentially simultaneously in gametes incubated for several hours in cycloheximide (Fig. 3B).

We looked for other possible relationships between the two that could provide insights into the fusion-triggered loss of the upper form. We found no evidence that either of the two isoforms of HAP2-HA were phosphorylated, ubiquitinated or SUMOylated [using methods described in Pan et al. (Pan et al., 2004) and Wang et al. (Wang et al., 2008)] (see Fig. S3 and Table S1 in the supplementary material). SDS-PAGE-based methods for detecting glycosylation, however, indicated that both isoforms were glycoproteins (Fig. 5A) and raised the possibility that the two forms were differentially glycosylated. HAP2 contains one predicted site for O-glycosylation and nine for N-glycosylation. We also used the N-glycosylation inhibitor tunicamycin to examine the relationship between the two forms. We reasoned that we should be able to detect the non-glycosylated form (~120 kDa) if N-glycosylation of HAP2 were inhibited during constitutive turnover in gametes. As shown in Fig. 5B, incubation of gametes in tunicamycin led to a gradual disappearance of both isoforms (Fig. 5B) and a new, more rapidly migrating form of ~120 kDa appeared. Thus, the formation of both forms from the single 120 kDa precursor required protein N-glycosylation.

Moreover, although neither form was sensitive to O-glycosidase, incubation of lysates with the glycosidase PNGase F (Maley et al., 1989) led to alteration (detected as a change in migration on SDS-PAGE) of only the lower form (Fig. 5C). Taken together, the above results indicated that both forms of HAP2 were glycosylated and that they were glycosylated differently.

Finally, RT-PCR experiments (not shown) failed to detect multiple forms of the HAP2 transcript, further confirming that the two isoforms of HAP2 were generated from a single transcript. The full-length *HAP2* cDNA sequence (3420 bp; GeneBank accession number EF397563) was assembled from multiple overlapping sequences obtained by sequencing several PCR products of *HAP2* cDNA obtained from wild-type *minus* gametes (strain *6145c*). The predicted molecular mass of the HAP2 protein is 115 kDa. These results indicated that HAP2 is synthesized as a single polypeptide. Through as yet uncharacterized mechanisms, approximately half of the HAP2 becomes N-glycosylated in a PNGaseF-sensitive manner and remains internal. The remaining portion of the protein becomes N-glycosylated to a non-PNGase-sensitive form and becomes localized on the surface of the mating structure.

Having failed to find evidence that the upper form was converted to the lower form during fusion, we searched for HAP2 degradation products in the fertilization medium. In experiments not shown, we detected small amounts of the upper and lower forms in the medium of gametes, but we found similarly low amounts in the medium of non-fusing activated gametes. Presumably, small amounts are released during gamete activation. Conversely, close examination of immunoblots of the cells, such as the one shown in Fig. 3A, indicated the transient appearance during fertilization of HA-



**Fig. 5. HAP2 modifications.** (A) HAP2 is glycosylated. Left panel: HAP2-HA is present as two isoforms (arrowhead) in whole cell lysates of transformants. The left lane is a control of wild-type *minus* gametes. Middle and right panels: immunoprecipitated HAP2-HA protein detected with silver staining (middle panel) or a glycoprotein staining kit (right panel). (B) Both isoforms of HAP2-HA were lost in gametes incubated in tunicamycin. *hap2-HAP2-HA minus* gametes incubated in tunicamycin were analyzed by immunoblotting. A new, more rapidly migrating, presumably non-glycosylated form of HAP2-HA appeared in tunicamycin-treated samples (arrow). (C) The lower form of HAP2-HA is sensitive to PNGase F but not to O-glycosidase. The first lane on the left was treated with glycosidase reaction buffer only. (D) HAP2 is degraded after fusion. *hap2-HAP2-HA minus* gametes mixed with wild-type *plus* gametes (left set of lanes) or *fus1 plus* gametes (right set of lanes) were analyzed by immunoblotting. Both short exposure and long exposure of the same blot are shown, indicating the two isoforms of HAP2-HA (upper panel, which is an image of the upper region of the gel from a short exposure of the immunoblot membrane) and transiently appearing degradation products of HAP2-HA (middle panel, which is an image of a longer exposure of the lower region of the immunoblot membrane). The arrowheads indicate two HAP2-HA degradation products that appeared transiently in zygotes. The arrow indicates a degradation product that appeared in gametes and presumably was due to constitutive degradation of HAP2-HA. The lower panel is a loading control.

reactive material at an apparent molecular mass of ~60-65 kDa (the asterisk in Fig. 3A indicates the position of a faintly stained band visible on the original immunoblot).

To optimize detection of transient, fusion-related degradation products, we mixed *HAP2-HA minus* gametes with wild-type *plus* gametes and, in a control experiment, separately mixed an aliquot of *HAP2-HA minus* with *fus1 plus* gametes. Immunoblotting showed, as expected, that the upper form of HAP2-HA disappeared only in the samples in which the cells formed zygotes (left lanes of top panel of Fig. 5D). When the blot was exposed for a longer time, however, we observed two degradation products of ~60 and 65 kDa that transiently appeared in the zygote samples (Fig. 5D, arrowheads, left lanes of middle panel) and that did not appear in the samples of activated gametes (right lanes).

In the parallel experiment in which *HAP2-HA minus* gametes were mixed with the fusion-defective *fus1 plus* gametes and the 60 kDa and 65 kDa HAP2 forms failed to appear, the amounts of both the original upper and lower forms increased substantially, as expected during gamete activation (Fig. 5D, top and middle panels, right lanes). Several lower-molecular-mass bands, including a dominant band of ~70 kDa (arrow), also appeared in these samples of activated gametes, which presumably were products of the constitutive degradation of HAP2-HA that occurs in gametes. Thus, our results indicated that fusion uniquely activated cleavage of the upper form of HAP2 to intermediate forms of ~65 kDa and 60 kDa, which were further and rapidly cleaved.

## DISCUSSION

Our work demonstrates the presence in *Chlamydomonas* of a mechanism that ensures that zygotes form from fusion of just one *plus* and one *minus* gamete. The degradation of FUS1 and HAP2 and the loss of fusion capacity triggered by fusion are consistent with the genetic evidence showing that mutants lacking either protein are incapable of fusion. Neither gamete activation alone nor mating structure adhesion alone activated the degradation of FUS1 or HAP2 and the loss of fusion capacity, but membrane fusion was required. To our knowledge, this is the first evidence in any organism that proteins essential for the membrane fusion reaction are rapidly lost after gamete fusion.

## Restricting gamete fusion in *Chlamydomonas*

At least three previously well-established features of gamete interactions during fertilization in *Chlamydomonas* could contribute to restricting fusion to just two gametes. First, fusion is temporally and spatially restricted and occurs only at the activated, apically localized mating structures. Second, progression from membrane adhesion to complete membrane fusion and coalescence of the two gametes is rapid. Using electron microscopy, we and others have been unable to capture membrane adhesion between mating structures of live wild-type gametes of opposite mating types under standard culture conditions (Goodenough et al., 1982; Forest, 1983; Forest, 1987; Liu et al., 2008). Third, soon after a zygote is formed, its flagella become non-adhesive and therefore the frequency of cell-body contacts between zygotes and non-fused gametes is dramatically reduced.

Although the three features listed above presumably contribute to restricting multi-gamete fusions, several considerations argue for the need of additional mechanisms. For example, flagellar adhesion is not a requirement for gamete fusion and activated, agglutinin-defective mutant gametes are capable of fusion (Pasquale and Goodenough, 1987; Misamore et al., 2003). Furthermore, even though the fusion sites are highly localized, gametes are capable of

forming simultaneous, multi-cell interactions at the mating structures. Presumably, the distribution of FUS1 along the entire length of the activated *plus* mating structure (Misamore et al., 2003) and the overall length of the structure accounts for its ability to provide access to and bind to the mating structures of multiple *minus* gametes. Thus, our findings that tri-cell mating structure interactions are possible, and even common (~30% tri-cell clusters, Fig. 1G), in experiments designed to detect them, yet formation of triploid zygotes is rare (0.6% triploid zygotes, Fig. 1D), suggested that cells must possess a mechanism for blocking supernumerary incipient membrane fusion reactions once a zygote forms.

### HAP2 and FUS1 regulation in gametes

The activation-induced increase in HAP2 (Fig. 3C) was similar to the activation-induced increase in agglutinin synthesis (Snell and Moore, 1980), and similar to the activation-induced increases in FUS1 transcripts we have seen in unpublished experiments (Liu and Snell). Presumably, the strong selection pressure for a gamete to maintain the capacity to form a zygote in the face of the many failed attempts that occur within the large clumps of adhering gametes that form prior to fusion underlies the activation-induced increase in expression of the flagella adhesion proteins and the fusion-essential proteins. The finding of a single new form of HAP2-HA in tunicamycin-treated cells and the RT-PCR analysis showing only a single transcript argue strongly for a common biosynthetic origin of the two isoforms. Notwithstanding that both forms are glycosylated, as shown by their glycoprotein staining properties (Fig. 5A) and tunicamycin sensitivity (Fig. 5B), they differ in details of glycosylation because only the lower form was sensitive to PNGase. Because we found no evidence for interconversion of the two forms, in future experiments it will be useful to determine whether the lower form has a distinct cellular function or is a misfolded or improperly processed form.

### The rapid degradation of FUS1 and HAP2 is specific to zygotes

The *in vivo* degradation of FUS1 and HAP2 that accompanies the fusion-triggered extinction of gamete fusion capacity is consistent with the fusion-defective phenotypes of mutant gametes lacking the proteins. *fus1* gametes are incapable of mating structure adhesion and fail even to initiate the membrane fusion reaction. *hap2* gametes fail to progress from membrane adhesion to membrane fusion. Thus, the *in vivo* loss of either of these proteins would concomitantly render the cells incapable of fusion. Moreover, even though *plus* and *minus* gametes undergo multiple complex changes after they adhere to each other by their flagella, none of these events triggered rapid loss of FUS1 or HAP2. Our results are similar to those in mammals, in which neither sperm binding nor pretreatment of eggs with agents that induce fertilization-related calcium oscillations bring about a block to polygamy (Wortzman-Show et al., 2007). Therefore, as would be expected for a block to polygamy, rapid degradation of proteins essential for fusion is tightly regulated and requires fusion.

Our studies comparing the constitutive degradation of both proteins that occurs in gametes with their degradation in zygotes demonstrated that loss of FUS1 and HAP2 in zygotes was not a passive consequence of fusion-induced cessation of their synthesis coupled with constitutive degradation, and that their loss after fusion was much more rapid than could be accounted for by constitutive degradation. Finally, our results demonstrating the appearance of zygote-specific HAP2 degradation products (Fig. 5D) confirmed that zygotes possess a mechanism for HAP2 degradation that is absent in *minus* gametes.

That degradation of the two proteins occurred so rapidly after fusion raises the possibility that pre-existing rather than newly synthesized activities bring about degradation. In this regard, we should note that the merging of two phenotypically distinct cells into a single cell brings previously separate, cell-type specific molecules together, thereby providing a unique mechanism for developmental regulation. One model for the degradation we observed is that degradation of HAP2 in the zygote is brought about by an activity synthesized only by *plus* gametes and degradation of FUS1 is brought about by an activity synthesized only by *minus* gametes. A related model is suggested by a novel regulatory mechanism for regulating transcription of zygote-specific genes in *Chlamydomonas* uncovered by work from our laboratory (Zhao et al., 2001) and the Goodenough laboratory (Lee et al., 2008). Each gamete brings a mating type-specific homeodomain protein to the zygote cytoplasm and only the heterodimer formed from the interaction between the two can activate the zygote developmental pathway. Thus, it will be interesting in future experiments to determine whether degradation of FUS1 and HAP2 is brought about by a protein complex that forms only in zygotes and is composed of subunits uniquely synthesized in each gamete.

Although the existence of a membrane block to polygamy has been established in many organisms, the dearth of information about proteins essential for the membrane fusion reaction has made it difficult to gain insights into the molecular mechanisms that underlie the block. This *Chlamydomonas* fertilization system, in which two fusion-essential proteins are known – one species-specific and one widely conserved – along with the newly documented loss of the proteins that occurs after fusion, should be useful in continued studies on the fundamental mechanisms of gamete fusion and blocks to polygamy. In particular, understanding the fate of HAP2 after fertilization is of potentially broad relevance because the protein functions during an ancient cellular process in our most important agricultural plants and in a devastating human pathogen.

### Acknowledgements

We thank Dr Yan Li for mass spectrometry analysis (Protein Chemistry Technology Center at UT Southwestern Medical Center); Dr Kate Luby-Phelps and Abhijit Bugde (UT Southwestern Medical Center Live Cell Imaging Core) for guidance on microscopy; and Dr Charlene Forest (Brooklyn, NY, USA) for providing the *gam-10* strain. We also thank Meredith Williams for assistance in the gamete activation experiments and Dr Fred Grinnell for helpful discussions and comments on the manuscript. We are grateful to Dr Qian Wang for insightful discussions. This work was supported by grants from the NIH (National Research Service Award GM-20329 to M.J.M. and GM-56778 to W.J.S.). Deposited in PMC for release after 12 months.

### Competing interests statement

The authors declare no competing financial interests.

### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.044743/-/DC1>

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