Signal transduction during fertilization in the unicellular green alga, *Chlamydomonas*

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Sexual reproduction in the green alga, *Chlamydomonas*, is regulated by environmental conditions and by cell–cell interactions. After gametogenesis, flagellar adhesion between gametes triggers gamete activation, leading to cell fusion and zygote formation. Recent studies have identified new molecular events that underlie signal transduction during *Chlamydomonas* fertilization, including expression of a sex-determining protein, phosphorylation of a homeodomain protein, activity of a kinesin II and regulated translocation of an aurora/Ip11-like protein kinase from the cell body to the flagella.

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Abbreviations

IFT	intraflagellar transport
mt–	mating-type minus
mt+	mating-type plus

Introduction

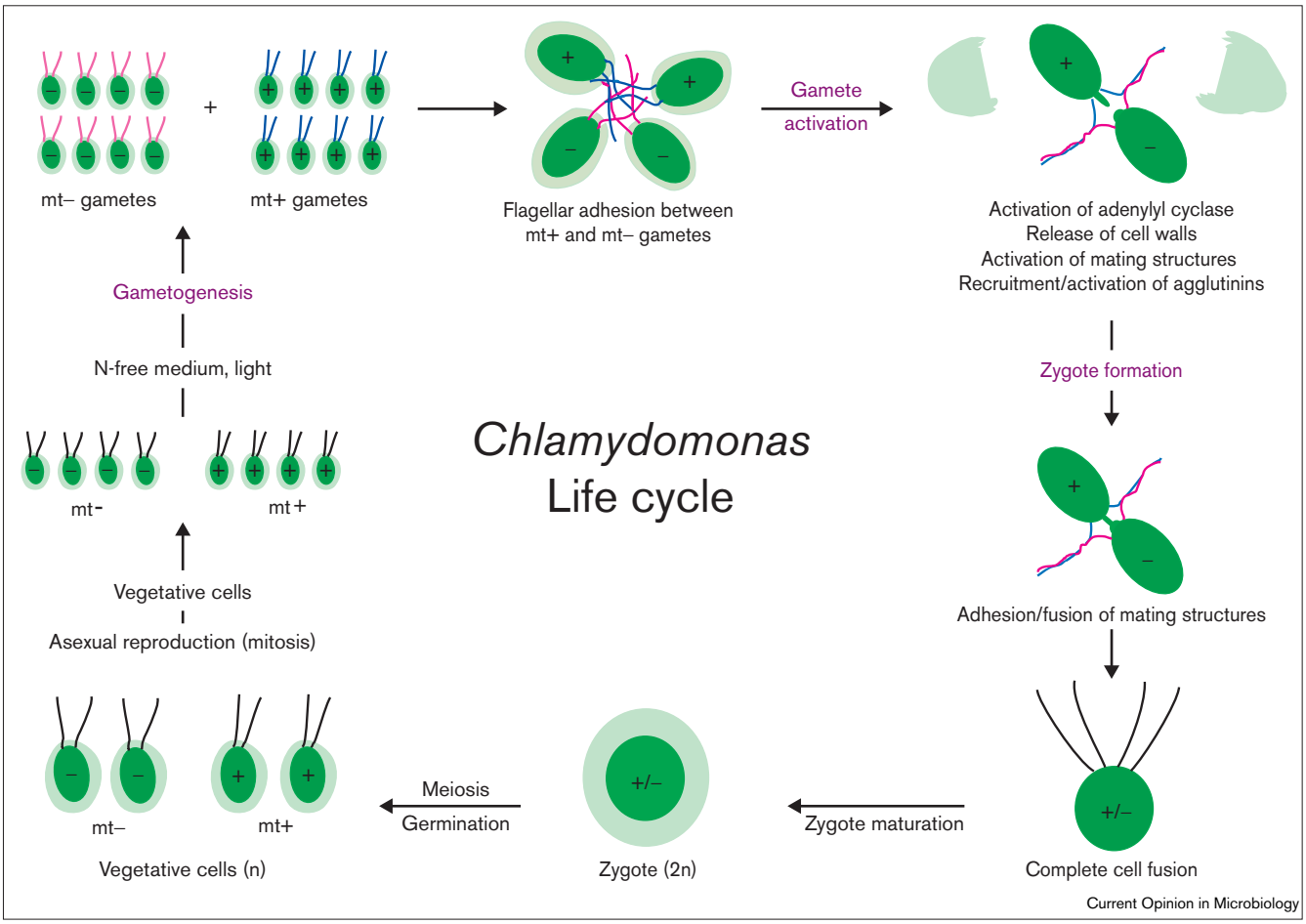
Not as complicated as in the corn smut *Ustilago* (which has over a 1000 different mating types) [1,2] and certainly not as sedate as in many non-motile fungi [3,4], sex in the unicellular green alga *Chlamydomonas* is a tumultuous affair. As soon as they are mixed together, the highly motile, biflagellated gametes of opposite mating types collide with each other and cling strongly to their partners by their flagella (even as they almost simultaneously contact additional flagellar adhesion partners). The interacting gametes shed their encumbering cell walls and form swirling masses of as many as 100–200 cells, each cell seemingly driven to make an intimate connection with the now naked cell body of its final fusion partner of the opposite sex. If roughly equal numbers of mating-type plus (mt+) and mating-type minus (mt–) gametes were mixed together, most of them would fuse to form quadriflagellated zygotes within five minutes of mixing. The newly formed diploid zygotes soon become more tranquil as they disengage from remaining non-fused gametes, resorb their flagella and assemble a thick zygotic cell wall. As the zygotes mature, they enter a dormant state that allows them to survive the nutrient-deficient conditions that initially induced gametogenesis (see below). Resuspension in nutrient-rich conditions along with the proper light regimen later lead to germination of the zygotes and formation of a new set of haploid mt+ and mt– vegetative cells ([5]; Figure 1).

Standing at the center of this complex process are two sets of events that define sex in all organisms: first, specific adhesion between gametes of opposite mating type (sex), which generates signaling pathways that activate the interacting cells for continued adhesion and for cell–cell fusion (collectively termed gamete activation for *Chlamydomonas*); and second, the cell–cell fusion event itself, which occurs at plasma membrane sites and through plasma membrane molecules that are distinct from those responsible for the initial recognition/adhesion between the two types of gametes [6]. Gamete fusion also generates signals that quickly render the gametes refractive to additional adhesive interactions and initiates zygote development. Here we present an overview of our current understanding of the cellular and molecular events that accompany and underlie fertilization in *Chlamydomonas*, focusing on the signal transduction pathways involved in its regulation. Table 1 presents a list of several molecules known or proposed to be involved in each of the three steps that comprise sexual reproduction in *Chlamydomonas* — gametogenesis, gamete activation and cell fusion/zygote formation.

Induction and regulation of gametogenesis

In the laboratory, gametogenesis is induced by separately resuspending vegetatively growing mt+ and mt– cells into nitrogen-deficient medium. For cells grown in liquid culture, gametes differentiate from vegetative cells within 10–14 hours, usually after 1–3 terminal cell divisions. The resulting transcriptionally active gametes bear gamete-specific and mating-type-specific adhesion molecules (high molecular weight glycoproteins) on their flagellar surfaces, termed the mt+ and mt– agglutinins. Mating type is controlled by the mating-type locus. Cells that receive the mt+ locus will differentiate into mt+ gametes during gametogenesis; those that receive the mt– locus will become mt– gametes. Interestingly, and with few exceptions, cells of both mating types contain all of the genes required to make a gamete of either mating type, even though each type of gamete expresses only a subset of these genes. Some are in each mating-type locus, and others are distributed at additional sites throughout the genome (e.g., a gene encoding an mt+ gamete-specific homeodomain protein discussed below). Two exceptions are the *MID* (for minus-dominance) gene and the *FUS1* gene. Ferris and Goodenough [7] have shown that the *MID* gene, which is present only at the mt– mating-type locus, is the master regulator for sex in *Chlamydomonas*. Cells that contain *MID* differentiate into mt– gametes; mt– cells with a lesion in the gene are pseudo-mt+ gametes. The *FUS1* gene, whose protein product is

Figure 1



Sexual reproduction during the life cycle of *Chlamydomonas* comprises three central phases – gametogenesis, gamete activation and cell fusion/zygote formation. Gametogenesis is induced by loss of a nitrogen source and brings about the light-dependent differentiation of vegetative cells into gametes. When gametes of opposite mating types are mixed together, flagellar adhesion triggers gamete activation – a collection of cellular and molecular events that readies the interacting gametes for cell–cell fusion. Fusion itself generates signals that turn off gamete-specific functions and activate the zygote developmental pathway. The cycle begins again when the appropriate environmental conditions stimulate the dormant zygote to undergo germination to produce new, haploid mt+ and mt– vegetative cells.

required to make a functional (fusogenic) mt+ fusion organelle, is present only at the mt+ mating-type locus [8]. An mt+ mutant gamete (*imp1*), which has a lesion in the *FUS1* gene, undergoes normal flagellar adhesion and gamete activation, but is unable to consummate cell fusion. An mt– cell with a lesion in *MID* differentiates into an mt+ gamete endowed with all of the molecules required of an mt+ gamete, except the FUS1 protein, which can be provided by transfection of the *FUS1* gene. To complete the story, an mt+ gamete transfected with the *MID* gene becomes a fully functional mt– gamete [7].

The mechanisms involved in induction of gametogenesis remain unknown. It is likely that nitrogen deprivation leads to expression of at least two types of regulatory molecules: those that control expression of gamete-specific genes common to both types of gametes, and those that control sex-specific genes. No candidates have emerged for the former, but the *MID* gene product and a recently discovered homeodomain protein [9], GSP1, are prime candidates for regulators of sex-specific genes. The MID protein does not contain any DNA-binding motifs, although it does have a leucine zipper element which might allow it to associate with transcription factors.

The GSP1 homeodomain protein, the first homeodomain protein to be identified in an alga, is expressed only in mt+ gametes and appears during gametogenesis around the same time that mt+ gamete-specific properties (e.g. mt+ agglutinins) appear [9]. The presence in GSP1 of a well-conserved homeodomain (which ia a DNA binding motif present in many transcription factors) along with its expression pattern are consistent with a possible role in mt+ gamete-specific gene expression. Expression of wild-type and altered forms of GSP1 in mt+ and mt– gametes should help to clarify its role in fertilization.

Table 1

Molecules in *Chlamydomonas* fertilization.

	Molecule	Role in fertilization
Gametogenesis	MID (novel protein) [7]; *ISO1* [49]	Sex determination
	GSP1 (homeodomain protein) [9,29••]	mt+ gamete-specific gene expression?
	Blue-light-responsive gene products [10,13]	Flagellar agglutinin activity and location
Gamete activation	mt+, mt– agglutinins (glycoproteins) [50]	Flagellar adhesion; adenylyl cyclase activation
	GSP1	Regulation of agglutinin gene expression?
	FLA10 (kinesin II motor protein) [34,51]	Coupling adhesion to adenylyl cyclase activation
	SKSC (protein kinase) [28,52]; *IMP3* [26]; calcium [53]	Regulation of adenylyl cyclase?
	Cyclic AMP [14,15]	Regulation of protein kinase A or ion channels?
	CALK (aurora/Ip11-like protein kinase) [32••]	Flagellar tip activation; motility; signaling?
	Actin [54]	Fertilization tubule formation
	GLE (metalloprotease) [55]; serine protease [56]	Cell wall degradation
Zygote formation	FUS1 (glycoprotein) [8]	Adhesion/fusion of mating structures
	GSP1	Expression of zygotic genes
	EZY1 (novel acidic protein) [57]	Chloroplast inheritance
	ZYS1 (novel protein) [39]	Chloroplast inheritance?
	ZYS3 (novel protein with WW domains) [40]	Zygote maturation
	Transglutaminase [58]	Zygote wall assembly
	GAS28 (hydroxyproline-rich glycoprotein) [11•]	Zygote wall assembly?

von Gromoff and Beck [10] have shown that gametogenesis can be subdivided into light-independent and light-dependent phases, and they identified several cDNAs whose transcripts are regulated during gametogenesis. These transcripts are gamete-specific but not sex-specific, in that they appear in gametes of both mating types and are not expressed by vegetative cells of either mating type. Thus, they become candidates for molecules that are involved in common gamete functions such as adhesion- or fusion-induced signaling. Although transcripts for two of these gamete-specific molecules, GAS28 and GAS29, are highly upregulated during gametogenesis, Rodriguez *et al.* [11•] have proposed that they may not be used until after zygote formation. Pan, Haring and Beck [12,13] have also shown that light regulates the presence of active agglutinin molecules on the flagella through processes that are sensitive to inhibitors of protein kinases and protein phosphatases. Whether light regulates the location of the agglutinins or their activity (or both) remains an open issue.

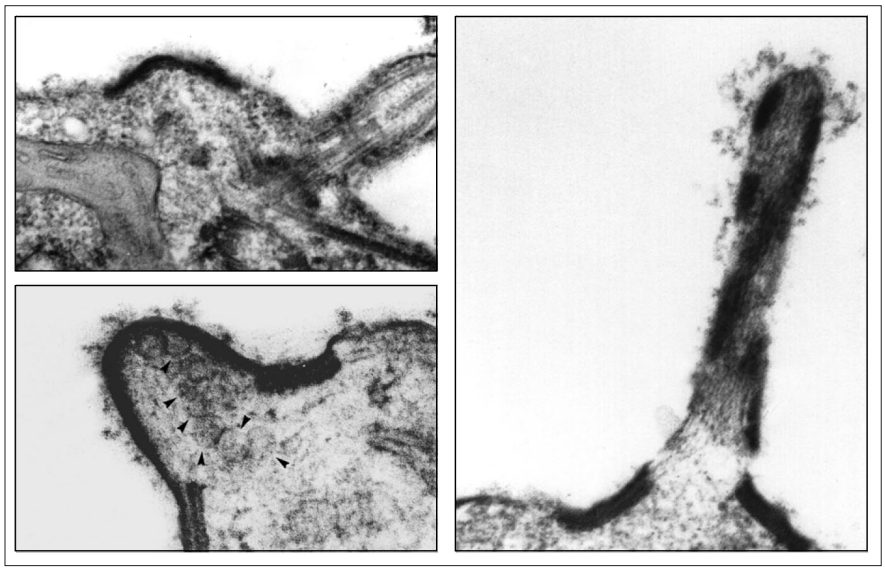
Gamete activation

Flagellar adhesion stimulates gamete activation — a set of remarkable cellular changes that enhances flagellar adhesiveness, exposes plasma membrane adhesion/fusion sites on newly forming cell-fusion organelles, and maintains the gametes in a state of constant sexual readiness.

In *Chlamydomonas*, gamete activation is triggered by interactions between the mt+ and mt– flagellar agglutinins. Binding between the agglutinins activates a gametic flagellar adenylyl cyclase in each of the interacting gametes, and the resulting nearly 10-fold increase of intracellular cAMP triggers dramatic alterations in the cells, most of which can be mimicked by incubation of gametes of a single mating type in the cell-permeable analog of cAMP, dibutyryl cAMP [14,15]. Flagellar motility is altered, and the adhesiveness of the flagellar surface is increased nearly eightfold by the translocation of inactive agglutinin molecules from the plasma membrane of the cell body onto the contiguous flagellar membrane where the agglutinins become active [16]. The translocated agglutinins also replace agglutinins that are lost from the flagella as a consequence of flagellar adhesion [17]. In a process termed ‘flagellar tip activation’, the tips of the flagella acquire amorphous material between the flagellar membrane and the underlying microtubules, and the flagellar tips extend as a consequence of an increase in length of the A microtubules in the nine outer doublet microtubules in the flagellar axoneme [18].

Activated gametes also secrete a serine protease that converts an extracellularly stored prometalloprotease to an active matrix-degrading enzyme [19]; the thick, multilayered, glycoprotein-containing extracellular matrix (the cell wall) that surrounds each cell is partially degraded and released from the cells. And perhaps most dramatically, within seconds to minutes after flagellar contact, mt+ gametes erect an actin-filled, 3-μm long, microvillous-like structure, the fertilization tubule, at their apical ends near the bases of the flagella; mt– gametes erect a similar, but shorter, actin-free, apically localized mating structure. Concomitant with formation of the fusion organelles is the appearance on their tips of active adhesion/fusion molecules, presumably delivered there by small membrane vesicles observed to fill the forming organelles (Figure 2). The fertilization tubule and cell–cell fusion itself are highly amenable to study in *Chlamydomonas*. Several fusion-defective mutants have been characterized, a strong candidate for an adhesion/fusion molecule has been cloned (FUS1) [8], and methods are in hand for isolating biochemical quantities of fertilization tubules that retain the ability to bind to mating structures on activated mt– gametes [20]. For cells that fail to find a fusion partner immediately, this same signaling

Figure 2



Electron micrographic analysis of the mt+ mating structure. The apical end of an unactivated mt+ gamete contains a crescent-shaped, membrane thickening – the mt+ mating structure (upper left panel). During early stages of gamete activation, vesicles appear within the structure as it begins to project from the cell surface (lower left). The fully formed, erect fertilization tubule (about 3 μm in length) is filled with actin filaments and decorated on its surface with fine, filamentous material called fringe (right panel). Adapted with permission from [59].

pathway maintains non-fused, activated gametes in a state of prolonged sexual readiness by upregulating synthesis of gamete proteins, including the agglutinins [21].

Activation of a gamete-specific, non-G-protein-dependent flagellar adenylyl cyclase

Surprisingly (but like a rat sperm adenylyl cyclase [22]), the gametic flagellar adenylyl cyclase whose activation is coupled to mt+ and mt– agglutinin interactions appears not to be regulated by G-proteins [15,23]. Instead, it is regulated by protein phosphorylation and de-phosphorylation. Zhang *et al*. [24,25] showed that the flagellar adenylyl cyclase in unactivated gametes was inhibited by a heat-labile, flagellar membrane-associated protein kinase activity. Although they reached somewhat different conclusions, Saito *et al*. [26] also presented data that were consistent with the presence of a heat labile, membrane-associated inhibitor of the gametic flagellar adenylyl cyclase. In addition, Zhang and Snell [25] found that activation of the adenylyl cyclase during flagellar adhesion was blocked by low (50 nM) concentrations of the protein kinase inhibitor staurosporine. Other experiments by our laboratory and by Saito *et al*. [26] showed that the gametic flagellar adenylyl cyclase exhibited regulatory properties that were significantly different from those of the vegetative flagellar enzyme. In related experiments to identify flagellar proteins whose phosphorylation was regulated by flagellar adhesion, Kurvari *et al*. [27] and Zhang *et al*. [28] identified a novel protein kinase, SksC, the *in vitro* phosphorylation of which was blocked by flagellar adhesion. The *in vitro* phosphorylation of SksC was insensitive to cAMP, which suggested that the protein kinase that phosphorylates SksC is upstream of adenylyl cyclase. The roles in gamete activation of SksC and of the soluble, adhesion-regulated protein kinase that phosphorylates SksC remain unclear.

Although the adenylyl cyclase that is activated during adhesion is in the flagella, many of the most striking responses to increased levels of cAMP, occur within the cell body, albeit probably at the apical ends of the cell in the region of the flagellar basal bodies. But is the cell-body adenylyl cyclase involved in gamete activation, or is the flagellar adenylyl cyclase solely responsible for the dramatic increase of intracellular cAMP observed during flagellar adhesion? Results from our laboratory [24] and from Saito *et al*. [26] indicated that the flagellar form, but not the cell-body form of the enzyme, could be activated 10–70-fold by prior treatment at elevated temperatures. These results, consideration of the specific and total activities of the cell body and flagellar enzymes, and the data from Saito *et al*. [26] that showed that the cell-body enzyme was not activated by cAMP (<0.2-fold stimulation by 100 nM cyclic nucleotide) suggest that the flagellar enzyme alone is responsible for generating the cAMP required for gamete activation.

Gamete activation stimulates phosphorylation of the GSP1 homeodomain protein

Although several morphological changes stimulated by gamete activation in *Chlamydomonas* have been characterized, only recently have we identified activation-related alterations in the properties of cloned cytoplasmic molecules. Wilson *et al*. [29••] showed that the GSP1 homeodomain protein, which is a soluble, cytoplasmic molecule, became phosphorylated within minutes after mt+ and mt– gametes were mixed together. Cell fusion was not required for phosphorylation, and incubation of mt+ gametes in dibutyryl cAMP was sufficient to induce this post-translational modification. Although we do not yet know the function of the GSP1 phosphorylation, it may be a key signal for upregulation of synthesis of molecules that undergo rapid turnover during gamete activation.

For example, in earlier investigation with non-fusing *imp1* mutant gametes, Snell and Moore [21] and Goodenough [30] showed that the agglutinin molecules are rapidly depleted from cells during flagellar adhesion. We also determined that protein synthesis was required to replace the lost agglutinins. Related to this gamete-activation-induced upregulation of synthesis of agglutinin molecules, Kurvari *et al.* [9] found that the transcript levels of GSP1 were dramatically upregulated during gamete activation in the absence of cell fusion. Moreover, Kamiya *et al.* have found that other gamete related transcripts are upregulated during gamete activation (R Kamiya *et al.*, personal communication). We should note here that GSP1 is a member of a class of transcription factors known to regulate mating-type-specific genes in other eukaryotic microorganisms [3], and that phosphorylation regulates the activity of many transcription factors [31]. On the basis of these ideas, we have speculated that phosphorylated GSP1 may be involved in transcriptional regulation of agglutinins during gamete activation.

Gamete activation induces translocation of CALK from the cell body to the flagella

A second protein to undergo gamete-activation-induced changes is CALK (*Chlamydomonas* aurora/Ip11-like protein kinase), a newly discovered aurora/Ip11-like protein kinase [32••]. Members of this protein kinase family in other organisms are associated with cell division, centrosome duplication, and microtubules [33]. In *Chlamydomonas*, the protein exists as a doublet of 78/80 kD in the cell bodies of vegetative cells and unactivated gametes of both mating types, and is barely detectable in the flagella. Within minutes after gametes are activated by flagellar adhesion or by incubation in dibutyryl cAMP, however, substantial amounts of CALK translocate to the flagella. Intriguingly, only the 78 kD form of CALK moves to the flagella.

The ability to translocate 78 kDa CALK appears to be unique to gametes, as CALK has not been detected in vegetative flagella under any circumstances. This may be a novel example of regulated translocation of a cell-body protein into an intact cilium/flagellum. The role of CALK, if any, in gamete activation is unknown, but, as an aurora protein kinase from a *Xenopus* gamete (oocyte) binds to microtubules and phosphorylates a kinesin, one possibility is that CALK regulates microtubule-related functions in the flagella, such as flagellar tip activation. It will be interesting to find out whether gamete-activated CALK translocation might also be related to gamete-activation-induced translocation of agglutinin molecules from the cell body to the flagella.

Intraflagellar transport may be central to gamete activation

One of the few mutants with a defect in gamete activation is the *fla10* mutant [34], which has a temperature-sensitive lesion in the heterotrimeric kinesin, FLA10 (or KHP1) [34]. Both vegetative and gametic *fla10* cells bear flagella of normal length at 23°C, but at 33°C cells begin to shorten their flagella after 60–90 minutes at the restrictive temperature. Piperno *et al.* [34] have reported that when *fla10* gametes are switched to the restrictive temperature they lose their ability to undergo fertilization before they begin to resorb their flagella. Wild-type cells transferred to 33°C show no such changes in fertilization ability. Although Piperno *et al.* did not indicate at which step in fertilization the *fla10* gametes were blocked, our unpublished experiments (J Pan, WJ Snell, unpublished data) suggest that flagellar adhesion is normal, and that the defect is in gamete activation.

One exciting aspect of this lesion in *fla10* cells is that the FLA10 protein is a central element in an increasingly exciting cellular process known as intraflagellar transport (IFT), which is proposed to deliver flagellar precursor molecules to the flagellar tip [34,35••,36,37]. This kinesin-driven IFT motility is visualized as the rapid (2–4 μm/s) movement of particles underneath the flagellar membrane. Diener, Rosenbaum and colleagues [35••,36] have suggested that IFT may also be critical for flagellar signaling pathways. It will be important to determine whether FLA10 and CALK interact, whether IFT is involved in CALK translocation, and whether, like FLA10, CALK is involved in gamete activation. Not only can such studies shed new light on fertilization in *Chlamydomonas*, but also they have the potential to reveal new features about the important process of intraflagellar transport in general.

Cell–cell fusion itself regulates gamete and zygote processes

Within minutes after gametes have fused to form zygotes, several events are initiated. Flagellar agglutinins are inactivated *in situ*, although they still can be detected using an *in vitro* bioassay [38]. Soon after, the flagellar and cell body agglutinins are lost from the cells; flagella are resorbed; and transcripts for several classes of zygote molecules are upregulated, including transcripts for zygote cell-wall proteins and several cytoplasmic molecules [39,40]. One of these latter molecules is EZY1, a protein intimately involved in regulation of maternal chloroplast inheritance [41]. EZY1, which is encoded at the mt+ locus, becomes localized to mt– chloroplasts soon after cell fusion. In elegant studies using optical tweezers and single-cell PCR Nishimura *et al.* [42••] demonstrated that mt– but not mt+ chloroplast DNA molecules are digested over a 10-minute period between 30 and 90 minutes after zygote formation.

Conclusions and future directions

From the perspective of the mechanisms involved in fertilization, *Chlamydomonas* stands between yeast and multicellular organisms. Similar to many fungi, a homeodomain protein seems to be a central feature in the process. As with the sperm and eggs of most multicellular animals, the adhesion/fusion molecules responsible for cell–cell fusion are exposed on specialized, actin-filled plasma membrane structures that extend several micrometers from the

cell surface [43]. Not surprisingly, protein phosphorylation and dephosphorylation regulate fertilization in each of these systems. Key molecules involved in the initial sex-cell recognition and adhesion in yeast, *Chlamydomonas* and mouse have been identified [6], but the adhesion/fusion molecules that consummate fusion of the plasma membranes of interacting gametes have largely eluded characterization [44–48]. Continued studies on *Chlamydomonas* FUS1 to localize it and explore its functional domains, combined with *in vitro* bioassays for fertilization tubule adhesion and fusion, should reveal exciting new features of this defining event in fertilization.

Finally, understanding the mechanisms and signaling pathways that couple agglutinin interactions to activation of adenylyl cyclase will be greatly accelerated by the recent cloning (PJ Ferris, UW Goodenough, personal communication) of the mt+ and mt– agglutinins and when the elusive adenylyl cyclase is cloned and characterized. Learning more about the protein kinase function of CALK and the mechanisms of its translocation, as well as elucidating the role of FLA10 and IFT in gamete activation, should also provide important new insights into this complex, multistep process at the center of fertilization in *Chlamydomonas*.

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