



Long-term interaction between *Drosophila* sperm and sex peptide is mediated by other seminal proteins that bind only transiently to sperm

Akanksha Singh^a, Norene A. Buehner^a, He Lin^{a,b}, Kaitlyn J. Baranowski^{c,1}, Geoffrey D. Findlay^{a,c}, Mariana F. Wolfner^{a,*}

^a Dept. of Molecular Biology & Genetics, Cornell University, Ithaca, NY, 14853, USA

^b East China Normal University, Shanghai, China

^c Dept. of Biology, College of the Holy Cross, Worcester, MA, 01610, USA

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ABSTRACT

Seminal fluid proteins elicit several post-mating physiological changes in mated *Drosophila melanogaster* females. Some of these changes persist for over a week after mating because the seminal protein that causes these changes, the Sex Peptide (SP), binds to sperm that are stored in the female reproductive tract. SP's sperm binding is mediated by a network of at least eight seminal proteins. We show here that some of these network proteins (CG1656, CG1652, CG9997 and Antares) bind to sperm within 2 h of mating, like SP. However, while SP remains bound to sperm at 4 days post-mating, none of the other network proteins are detectable at this time. We also observed that the same network proteins are detectable at 2 h post-mating in seminal receptacle tissue from which sperm have been removed, but are no longer detectable there by 4 days post-mating, suggesting short-term retention of these proteins in this female sperm storage organ. Our results suggest that these network proteins act transiently to facilitate the conditions for SP's binding to sperm, perhaps by modifying SP or the sperm surface, but are not part of a long-acting complex that stably attaches SP to sperm.

1. Introduction

Seminal fluid proteins (Sfps) are produced by male reproductive glands and are transferred to females during mating. Within mated females, insect Sfps affect female reproductive physiology and behaviors by interacting with the female reproductive tract and the central nervous system. Sfps have been extensively characterized in *Drosophila* (reviewed in Avila et al., 2011; Hopkins et al., 2017) as well as in other insects (e.g. tephritids: Davies and Chapman, 2006; mosquitoes: Boes et al., 2014; Dottorini et al., 2007; Rogers et al., 2009; Sirot et al., 2008; Sirot et al., 2011; honey bees: Baer et al., 2009; Collins et al., 2006; den Boer et al., 2010; Grassl et al., 2017; Nino et al., 2013; Peng et al., 2016; crickets: Andres et al., 2006; Andres et al., 2008; Braswell et al., 2006). In addition to their roles as regulators of post-mating responses, Sfps are of interest because of their potential contributions to the intra- and inter-sexual conflicts that can impact the evolution of reproductive traits (e.g., Bono et al., 2015; Castillo and Moyle, 2014; Wigby and Chapman, 2005).

The response of *D. melanogaster* females to Sfps can be divided into two phases: short-term (≤ 24 hr post-mating) and long-term (lasting

10–14 d; Kalb et al., 1993; Manning, 1962, 1967). The short-term response is dependent on receipt of Sfps (Kalb et al., 1993) such as the prohormone ovulin (Heifetz et al., 2000, 2005; Herndon and Wolfner, 1995; Rubinstein and Wolfner, 2013) and the sperm storage protein Acp36DE (Avila and Wolfner, 2009, 2015; Bloch Qazi and Wolfner, 2003), which are only detectable in females for a few hours after mating, in addition to the 36-amino acid seminal protein, sex peptide (SP) (Aigaki et al., 1991; Chapman et al., 2003; Chen et al., 1988; Liu and Kubli, 2003). Long term post-mating responses, including effects on egg production, mating receptivity, feeding, excretion, and sperm release from storage, are induced by SP and depend on SP's continued presence in the mated female (Apger-McGlaughon and Wolfner, 2013; Avila et al., 2010; Avila et al., 2015; Chapman et al., 2003; Liu and Kubli, 2003; Ravi Ram and Wolfner, 2007, 2009; Sitnik et al., 2016). The continued presence of SP in mated females is caused by its binding to sperm (Peng et al., 2005). Specifically, SP's N-terminal portion binds to sperm, while its C-terminal portion is gradually released by proteolytic cleavage and induces the post-mating effects. The action of a G-protein coupled receptor (the Sex Peptide Receptor, SPR; Yapici et al., 2008) is essential for these responses.

* Corresponding author.

E-mail address: mfw5@cornell.edu (M.F. Wolfner).

¹ Present address: Sanofi Genzyme, 153 2nd Ave, Waltham, MA 02451.

It is important to understand the mechanisms that bind SP to sperm for reasons that go beyond elucidating how SP prolongs post-mating responses in *Drosophila*. Binding of Sfps to sperm is a general phenomenon, beyond *Drosophila*: for example, bovine seminal plasma (BSP) proteins associate with sperm and facilitate sperm storage (Gwathmey et al., 2006). Understanding how sperm are modified to allow Sfp binding could yield insights into post-mating modification of sperm, including into phenomena such as capacitation, as well as having potential applications in allowing development of methods to attach important molecules to sperm to manipulate insect reproduction.

Genetic screens and an analysis of molecular coevolution identified several Sfps required for SP to associate with sperm: two predicted C-type lectins (CG1652 and CG1656), a serine protease (seminase (Sems)), three serine protease homologs (CG9997, aquarius (Aqrs) and intrepid), and two cysteine rich secretory proteins (CG17575 and Antares (Antr)) (Findlay et al., 2014; LaFlamme et al., 2012; Ravi Ram and Wolfner, 2009). These proteins act in a network (Fig. 1) in which each member is required for SP to accumulate in the female sperm storage organs and bind to sperm (Findlay et al., 2014; LaFlamme et al., 2012; Ravi Ram and Wolfner, 2009). For example, males that produce no (or little) CG9997, Antr, or Aqrs do not transfer the two lectins CG1652 and CG1656 efficiently. In turn, CG1656, CG1652, Antr and Aqrs are required in mated females to control the rate at which CG9997 is processed. CG17575 and Sems are required for CG1656, CG1652 and CG9997 to localize to the female's sperm-storage organs. In the absence of any one of these network proteins, SP is transferred to the female but is not retained over the long-term. In some matings, SP affects female physiology and behavior for ~24 h, but the female then reverts to a virgin-like state because SP is not retained (Findlay et al., 2014; Peng et al., 2005; Pilpel et al., 2008; Ravi Ram and Wolfner, 2007, 2009).

To understand how the network proteins mediate the association of SP with sperm, it is important to know how long they persist in females and whether they co-localize with sperm. One can imagine at least three possibilities. First, network proteins could act transiently and/or catalytically to modify sperm and/or Sfps to allow for SP-sperm interactions within the male's ejaculate or in the female's bursa (uterus) immediately after mating. Second, network proteins could enter the female sperm-storage organs and bind to sperm transiently, to facilitate long-term binding of SP. Third, network proteins could bind to sperm for extended periods of time, similar to SP, potentially serving as a physical link between sperm and SP. Knowing which network proteins show which characteristics is critical for understanding how these proteins mediate SP's retention in mated females, but previous investigations of the fates

of the network proteins in mated females have been limited. To date, only two proteins, SP and CG1656, have been tested for direct binding to sperm: initially, both do and require the other network proteins to do so, but only SP persists on sperm for longer than one day (Peng et al., 2005; Ravi Ram et al., 2005, 2009). In addition to SP and CG1656, other network proteins have been shown to localize to the female's seminal receptacle (SR): CG1652, CG9997, and a small amount of Sems, but not CG17575 (LaFlamme et al., 2012; Ravi Ram et al., 2005). However, these additional proteins have not yet themselves been tested for sperm binding, and antibodies with which to test additional network proteins (such as Antr) were not available.

Here, we sought to more comprehensively determine the localization within the female tract and the potential interactions of the SP network proteins in order to determine by which of the three actions outlined above each network member contributes to SP-sperm association. Using Western blotting and immunofluorescence, we found that CG1652, Antr, and CG9997 – like CG1656 – are bound to sperm within the female SR by 2 h after the start of mating (ASM). However, by 4 days ASM, SP is the only network protein that can be detected as bound to sperm. Interestingly, we also found that the four network proteins that enter the SR with sperm are also detected in protein isolated from SRs from which sperm had been removed by dissection, suggesting the possibility that the proteins are retained by molecular association, or in crypts, in SR tissue. Finally, using new null alleles of network proteins that we generated using CRISPR/Cas9-based editing, we placed the action of Antr into the context of the other network proteins. Taken together, these results suggest that the SP network proteins act transiently in the hours after mating to set up SP-sperm interactions, through some combination of direct sperm binding, catalytic action on SP or sperm in the female reproductive tract, or interaction with the female sperm storage organ. While these proteins do not persist on sperm or in the female for nearly as long as SP does, their actions are nonetheless essential to set up the long-term SP response.

2. Methods

2.1. Flies and mating experiments

Sex peptide null mutant males ($SP^0/\Delta 130$) (Liu and Kubli, 2003) were generated by crossing an SP knockout line ($SP^0/TM3, Sb ry$) to a deficiency line ($\Delta 130/TM3, Sb ry$). For CG1656, CG1652, and CG9997 we generated knockout lines using CRISPR/Cas9-based genome editing. For each gene, we generated two guide RNAs (by using the tool: <http://>

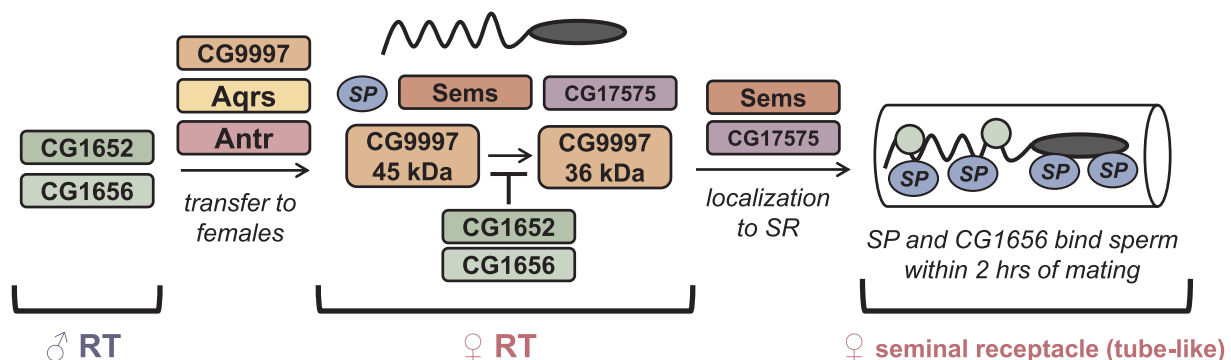


Fig. 1. A network of seminal proteins is required for sex peptide (SP) to bind stably to sperm within the female seminal receptacle. Colored shapes indicate proteins produced in the male accessory glands. CG1652 and CG1656 require fellow network proteins CG9997, Aqrs and Antr to be transferred to females. Once deposited in females, Sems and CG17575 are required for SP and CG1656 to localize to the seminal receptacle (SR), the major site of sperm storage in female *D. melanogaster*. In the SR, SP and CG1656 bind sperm within 2 h of the start of mating. Also, within the female reproductive tract (RT), the presence of CG1652 and CG1656 slows the rate at which CG9997 is processed from a 45 kDa form to a 36 kDa formed. One additional network protein, Intrepid, is not shown, since its position in the pathway is presently unknown. Loss of any one of these network proteins prevents SP accumulation on sperm in the SR. Colors indicate predicted protein functional classes: red/orange/yellow: serine proteases and protease homologs; pink/purple: cysteine-rich secretory proteins; green: C-type lectins. This model represents knowledge of the SP network prior to this study and is derived from Ravi Ram and Wolfner (2009), LaFlamme et al. (2012) and Findlay et al. (2014). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

flycrispr.molbio.wisc.edu/tools) to generate cuts at either end of the coding region (CG1652: 5'-CTTCGATCCCAGGAGCTCGCCCCG-3' and 5'-CTTCGGCGTGGCTCCGTCGGCGGC-3'; CG1656: 5'-CTTCGCCGG AATGGACCGTCATCA-3' and 5'-CTTCGGCGGACCAATGGACATT-3'; CG9997: 5'-CTTCGTATAGATCCACGCCGTGT-3' and 5'-

CTTCGCGATGGAGATGAGTGTTCG-3'). Following the methods of Gratz et al. (2013), these gRNAs were cloned into pU6-BbsI, which was then injected into *y[1] P{vas-Cas9.S}ZH-2A w[1118]* embryos by Rainbow Transgenics. Surviving injectants were crossed to *y w; Sco/Cy* and backcrossed to generate homozygous knockout flies. PCR was used to confirm that the knockout flies carried the expected deletion. Before using these flies for Western blotting and sperm-staining, we confirmed that they lacked the targeted proteins (Fig. S1, also Figs. 2–6) and that their phenotypes were like those of the near-complete knockdowns we had generated previously for these genes (Findlay et al., 2014; Ravi Ram and Wolfner, 2009): failing to maintain long-term responses in SP-mediated behavioral traits (remating receptivity and egg-laying; Fig. S1), and failing to retain SP in mated females (see Figs. 2 and 4). For our studies of *antr* (CG30488) we generated knockdown (KD) males by crossing transgenic flies carrying a UAS-driven RNAi-generating construct (VDRC ID 100513 (KK)) to a ubiquitous driver (*Tubulin-Gal4/TM3, Sb*); controls were the *TM3* siblings of the knockdown flies. For *sems* knockout males we used a knockout (null) line from the Bloomington Stock Center (stock ID 23408). We used Canton S (CS) males as our controls. Levels of the tested seminal proteins in CS males were either similar to or slightly higher than those in the background-matched controls for our knockdown/knockout males, but any differences were not to a degree that impacted interpretation of the presence/absence of the tested protein (Fig. S2).

All flies were reared on yeast-glucose medium and a 12:12 light/dark cycle. All flies except for *antr* knockdowns/controls were raised and maintained at room temperature ($22 \pm 1^\circ\text{C}$); *antr* knockdown/controls were raised and maintained at $25 \pm 1^\circ\text{C}$ to improve knockdown efficiency. Mating experiments were carried out by singly-mating 3–5 day-old virgin CS females to 3–5 day-old virgin males of the appropriate genotype. For each genotype at each time point, 50–60 mated Canton S females were flash-frozen in liquid nitrogen at 2 h, 24 h or 4 d ASM and stored at -80°C before sample preparation for protein extraction or immunofluorescence.

2.2. Sample preparation and Western blot analysis

The presence of SP and other network proteins on sperm or in sperm-free SR tissue was analyzed at 2 h, 24 h and 4 d ASM. SRs were dissected from 50 to 60 frozen mated females from each time point. The mass of stored sperm was dissected from them and rinsed twice in $1 \times$ PBS, and then extracted for protein. The remaining SR tissue was torn open and placed into $10 \mu\text{L}$ of $1 \times$ PBS, then centrifuged for 5 min in an Eppendorf centrifuge, and the supernatant was removed. The pelleted SR tissue was resuspended in $10 \mu\text{L}$ of $1 \times$ PBS and proteins were extracted as in Findlay et al. (2008). Proteins from the mass of stored sperm, or from the SR tissue without sperm, were then resolved on gradient 5–15% polyacrylamide SDS gels and prepared for Western blot screening, all by minor modifications of the procedures in Ravi Ram et al. (2005). For primary antibodies we used affinity-purified rabbit antibodies against CG1656 (at 1:1000), CG1652 (1:250), CG9997 (1:500), CG17575 (1:250), SP (1:2000) (Ravi Ram and Wolfner, 2009), Sems (1:250) (LaFlamme et al., 2012), and mouse monoclonal anti-actin (Millipore Corp., catalog number #MAB1501MI at 1:3000). HRP-conjugated secondary anti-rabbit and anti-mouse antibodies were from Jackson ImmunoResearch. While we replicated the finding of LaFlamme et al. (2012) that low levels of Sems are detectable in intact SR at 2 h post-mating, the levels were too low for us to be able to determine whether Sems presence was due to its binding sperm or association with the SR, or both.

Rabbit polyclonal affinity-purified antibodies against Antr were

generated by methods like those described in Ravi Ram et al. (2005). Briefly, at Cocalico, rabbits were injected with a recombinant protein in which GST had been N-terminally fused to amino acids 54–233 of Antr. Antisera from these rabbits were affinity-purified against a recombinant protein with 6x-His N-terminally fused to the same region of Antr. We used the antibodies at 1:500 dilution for Western blotting.

Unless otherwise noted, positive controls for Western blots were the lower reproductive tracts ($n = 2$) of Canton S females mated to Canton S males at 1 h ASM, and 1 pair of male accessory glands (MAG) from Canton S males. Negative controls were 2 lower reproductive tracts from virgin Canton S females, and reproductive tracts from Canton S females that had mated to the appropriate knockout males (or knockdown males for *antr*).

2.3. Immunofluorescence

Immunofluorescence to detect sperm-bound Sfps was carried out according to the protocol of Ravi Ram and Wolfner (2009), with minor modifications. Sperm were dissected from SRs of Canton S females mated to control, knockout (SP, CG1652, CG1656, CG9997, *sems*), or knockdown (*antr*) males dissected at 2 h and 4 d ASM. Sperm were attached to poly-L-lysine-coated slides and were washed twice with $1 \times$ PBS. The slides were kept on ice until dissections were completed (15 min or less). Samples were fixed with 4% paraformaldehyde in $1 \times$ PBS for 15 min at room temperature, washed 3–4 times in $1 \times$ PBS, blocked with 0.2 M glycine in $1 \times$ PBS for 30 min, and then washed again 3 times with $1 \times$ PBS. Samples were then blocked with 1% non-fat milk in $1 \times$ PBS for 30 min, and then incubated with anti-SP (1:200), anti-CG1656 (1:100), anti-CG1652 (1:50), anti-CG9997 (1:100), anti-Sems (1:50) or anti-Antr (1:50) in 0.1% milk in PBS overnight at 4°C . On the next day, samples were washed again 3 times with $1 \times$ PBS and incubated for 2 h in mouse anti-rabbit IgG coupled to Alexa Fluor 488 (Invitrogen) at a concentration of 1:300 in $1 \times$ PBS for 2–3 h at room temperature in the dark, washed 3 times in $1 \times$ PBS, and then stained with DAPI (1:5000) for 15 min at room temperature in the dark. Finally, samples were washed 3 times in $1 \times$ PBS and mounted using antifade (0.2% n-propyl galate in 75% glycerol; Sigma). Fluorescence was visualized using a Zeiss 710 confocal microscope at the Cornell imaging facility. For the 2 h ASM time point, sample sizes ranged from 10 to 20 sperm masses per male genotype; for the 4 d ASM time point, sample sizes ranged from 8 to 10 sperm masses per male genotype.

3. Results

3.1. Some network proteins bind to sperm and seminal receptacles by 2 h post-mating

Of the network proteins, SP, CG1656, CG1652, CG9997 and Sems were reported to enter the seminal receptacle by 1 h post-mating (LaFlamme et al., 2012; Ravi Ram and Wolfner, 2009). At this time, SP and CG1656 have bound to sperm, and SP is also detectable in SRs from which sperm have been removed (Peng et al., 2005; Ravi Ram and Wolfner, 2009). We wondered whether CG1652, CG9997, Antr, or Sems also bind to sperm and/or associate with SR tissue, and whether Antr enters the sperm storage organs and requires CG17575 or Sems. To address these questions, we examined the presence of each of these proteins by Western blot on protein extracted from sperm masses dissected from the SR, or the remaining, empty SRs, at 2 h ASM. We refer to these fractions as “SS” (for “stored sperm”) and “SR” (for “seminal receptacle”), respectively. Fig. 2 shows that in matings of wildtype females to wildtype (CS) males, SP, CG1656, CG1652, Antr, and CG9997 (in both its intact and processed forms, 45 kDa and 36 kDa, respectively; see Fig. 1) are found in the SS fraction, suggesting that these proteins are bound to sperm. All of these proteins were also detected in SRs from which sperm had been removed at this time (Fig. 2). CG1656, CG1652, CG9997 and Antr were also detected in the SS and SR fractions obtained from females mated to SP null males (Fig. 2), indicating that their binding to sperm and their localization to the SR do not require SP. In contrast, the association of CG1656 and CG1652 with the SS and SR

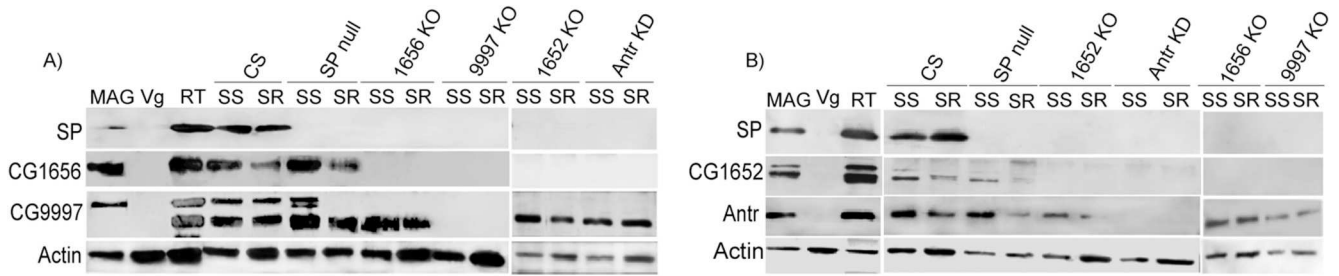


Fig. 2. Presence of (A) SP, CG1656, and CG9997, and (B) CG1652 and Antr in proteins extracted from stored sperm (SS) or to the indicated knockdown/knockout males. CG9997 is detected as a 45 kDa protein band in male accessory glands (MAG) and is processed to 36 kDa in mated females (Ravi Ram and Wolfner, 2009). The four lanes on the right of each panel are from a different blot, run and probed in parallel and with similar controls. MAG = accessory glands from a CS male, RT = reproductive tract of CS female, 1h after mating with a CS male, Vg = reproductive tract of an unmated CS female. A probing for actin is included as a loading control. Three or more independent repeats were done per treatment; results shown are representative examples.

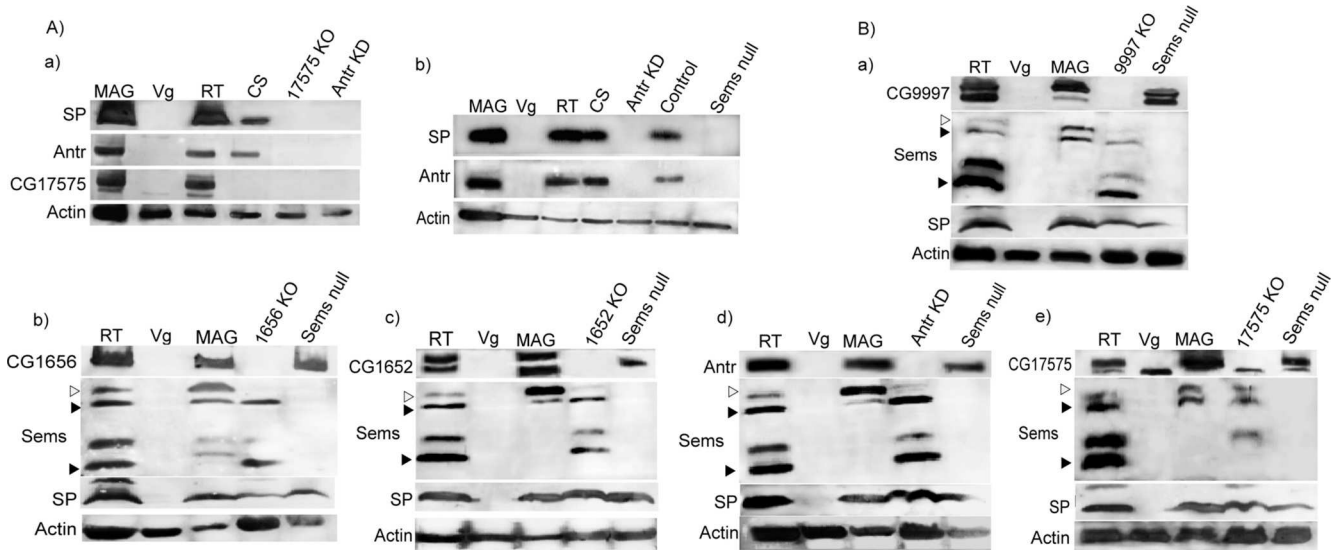


Fig. 3. A) The Western blots on the top left (a) and middle (b) show that Antr fails to localize to sperm-containing seminal receptacles of mated CS females 2 h after mating to males deficient in CG17575 or Sems, respectively. Matings of females to CS or to Antr knockdown males are included as positive and negative controls, respectively; actin is included as a loading control. B) Seminal transfer to females does not require other network proteins, and transfer of other network proteins does not require Sems. Western blots show protein from whole reproductive tracts of females 1 h after mating to males knocked out or knocked down for the indicated proteins. Open arrowheads indicate full-length Sems in or from males; filled arrowheads indicate the 27 kDa and 12 kDa products that occur after processing in females; a 15 kDa cross-reactive band is visible in several lanes. Normal amounts of Sems are detected in CS females mated to CG1656, CG1652 or CG9997 knockout males, or to *antr* knockdown males at 1 h after mating. Sems is also detected in CS females mated to CG17575 knockout males, but its 12-kDa processed product is reduced in abundance relative to control. These blots also show that transfer of the network proteins to females proceeds in the absence of Sems. Probing for the knocked down/out network protein and for SP are included as controls, and a probing for actin is included as a loading control. MAG = accessory glands from a CS male, Vg = reproductive tract of an unmated CS female, RT = lower reproductive tract of CS female 1hr after mating with a CS male. Three or more independent repeats were done per treatment; results shown are representative examples.

fractions are interdependent: CG1652 and CG1656 are undetectable when fractions are obtained from mates of CG1656 and CG1652 knockout males, respectively (Fig. 2). Consistent with previous findings that CG1652, CG1656, and Antr regulate the processing of CG9997 (Fig. 1; Findlay et al., 2014; Ravi Ram and Wolfner, 2009), we observed only the 36-kDa form of CG9997 in the SS and SR fractions obtained from females mated to CG1652 null, CG1656 null or *antr* knockdown males (Fig. 2). Similarly, consistent with the requirement that males produce CG9997 and Antr in order to transfer CG1652 and CG1656, we do not detect CG1652 or CG1656 in SS or SR fractions from females mated to CG9997 null or *antr* knockdown males (Fig. 2A). Additionally, levels of Antr in the SS and SR fractions appear to be reduced in females that had mated to males null for CG1652 and CG1656 (Fig. 2). Finally, we find that CG17575 and Sems are needed for localization of Antr to the seminal receptacle (Fig. 3A), as they were reported to be for CG9997, CG1656, CG1652 (Ravi Ram and Wolfner, 2009). However, transfer of CG1656, CG1652, CG9997, CG17575, Antr and SP to females is not dependent upon Sems (Fig. 3B, top row in panels a–e). Likewise, the

transfer and processing of Sems was unaffected in males lacking CG1656, CG1652, CG9997 or Antr, and only its proteolytic processing was altered in females mated to CG17575 KO males (Fig. 3B, bottom rows in panels a–e).

Our Western blotting results indicated that five network proteins associate with sperm in females at 2 h ASM. To confirm these findings, we carried out immunofluorescence experiments on sperm dissected from the SR (Fig. 4). First, we observed that SP and CG1656 are detectable on sperm tails at this time point (Fig. 4A–C and 4E–G, respectively), consistent with or extending previous reports that both proteins are bound to sperm at 1 h ASM (Peng et al., 2005; Ravi Ram and Wolfner, 2009). We then observed that CG1652, CG9997, and Antr are also bound to sperm at this time (Fig. 4I–K, 4M–O, and 4Q–S, respectively). The specificity of antibody binding in all cases was confirmed by the lack of staining on sperm from the SRs of females mated to males lacking SP, CG1656, CG1652, or CG9997, or to males knocked down for Antr (Fig. 4, panels D,H,L,P,T). Interestingly, the sperm-binding patterns differed among the network proteins. As previously reported by Peng et al. (2005), we observed SP bound to sperm heads and

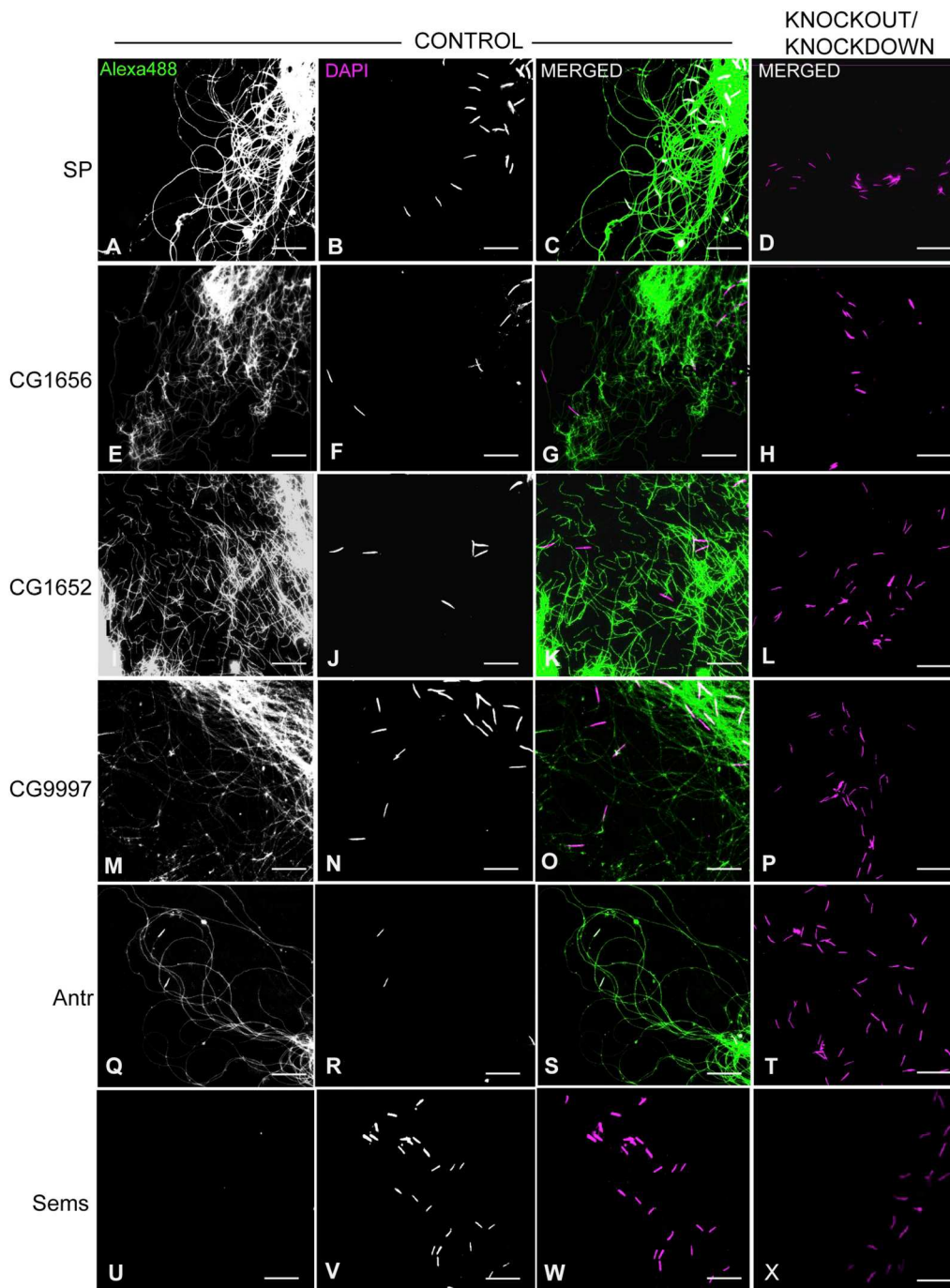


Fig. 4. Immunofluorescence showing the binding and distribution of SP, CG1656, CG1652, CG9997, Antr and Sems on sperm dissected from SRs of females 2 h after the start of mating to CS (“control” panels) or knockout/knockdown males (right panels). For each row, the two rightmost panels are merges, in which green shows the signal from the secondary anti-rabbit Alexa488 conjugated antibody, detecting the anti-Sfp primary antibody. DAPI staining, false-colored in magenta, shows sperm heads. For CG9997, its weak binding to sperm heads is particularly clearly seen on the sperm head that is close to the letter-labels on panels M–O. Representative images from the following sample sizes: $n = 20$ (SP), $n = 15$ (CG1656), $n = 13$ (CG1652) and $n = 10$ (Antr), $n = 12$ (Sems), $n = 10$ (CG9997). Bar = 20 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

tails (Fig. 4A–C). We observed that Antr and CG9997 are also bound to both the heads and the tails of sperm (Fig. 4M–S), although the binding of CG9997 to heads is relatively weak. However, CG1656 and CG1652 are only detected on the sperm tail, and their binding appears threadier than that seen for SP (Fig. 4E–L). We were unable to detect Sems bound to sperm (Fig. 4U–X).

Thus, we find that shortly after entry into the female, network proteins CG1652, CG1656, CG9997, and Antr accompany SP into the seminal receptacle and, like it, bind to sperm. However, the lectins in the network only bind to sperm tails, in contrast to SP and the protease homologs, which bind to both head and tail. Finally and unexpectedly, we see that all network proteins that enter the seminal receptacle in significant amounts also can be detected with SR tissue dissected free of sperm, suggesting that they might associate with SR cells and/or be trapped in crypts in the tissue's lining.

3.2. By 4 days after mating, the network proteins are no longer detectable on sperm (or in the female)

Our finding that CG1652, CG1656, CG9997, and Antr all enter the SR with sperm and, like SP, all are bound to sperm by 2 h ASM suggested two hypotheses for their role in SP's binding to sperm. One possibility is that some or all of these network proteins could directly connect SP to sperm, as adaptors or bridging molecules. An alternative possibility is that some or all of them could be present only temporarily, acting transiently to modify SP or the sperm surface so that SP could be stably bound to sperm.

These hypotheses can be distinguished by determining whether the network proteins are detectable in the female at a later time point after mating and remain sperm-bound, as SP does. Previous studies showed that the network protein CG1656 did not persist beyond 4 h in females (Ravi

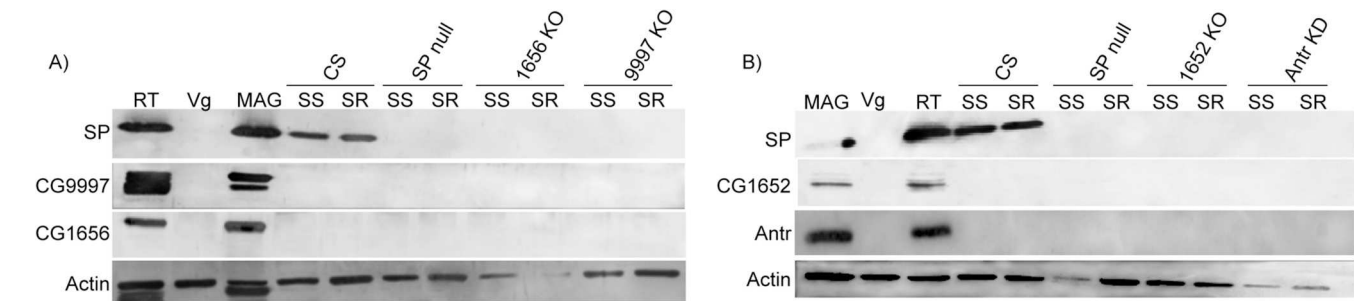


Fig. 5. Western blots for the presence of SP and other network proteins in proteins isolated from SS or sperm-free SRs of CS females 4 days after mating to CS or knockout/knockdown males (as indicated). In all blots, each pair of SS and SR lanes contain protein derived from 50 to 60 dissected females. For positive and negative controls, we included proteins from two lower reproductive tracts (without ovaries) of CS females mated to CS males 1 h ASM (RT) or virgin CS females (Vg), or the accessory glands (MAG) of one CS male. Three or more independent repeats were done per treatment; results shown are representative examples.

Ram et al., 2005), but the other network proteins were not tested. To test whether they persisted on sperm and in females, we used the procedures described above to examine the network proteins in females dissected at 4 d ASM. We first confirmed that SP remained detectable at this time point

(Peng et al., 2005) by Western blots in SS and SR fractions (Fig. 5) and on sperm by immunofluorescence (Fig. 6A–D). In contrast to these SP results, we did not detect CG1652, CG1656, CG9997 or Antr at 4 d ASM by Western blot (Fig. 5) or immunofluorescence (Fig. 6E–T). In fact, by 24 h ASM, the

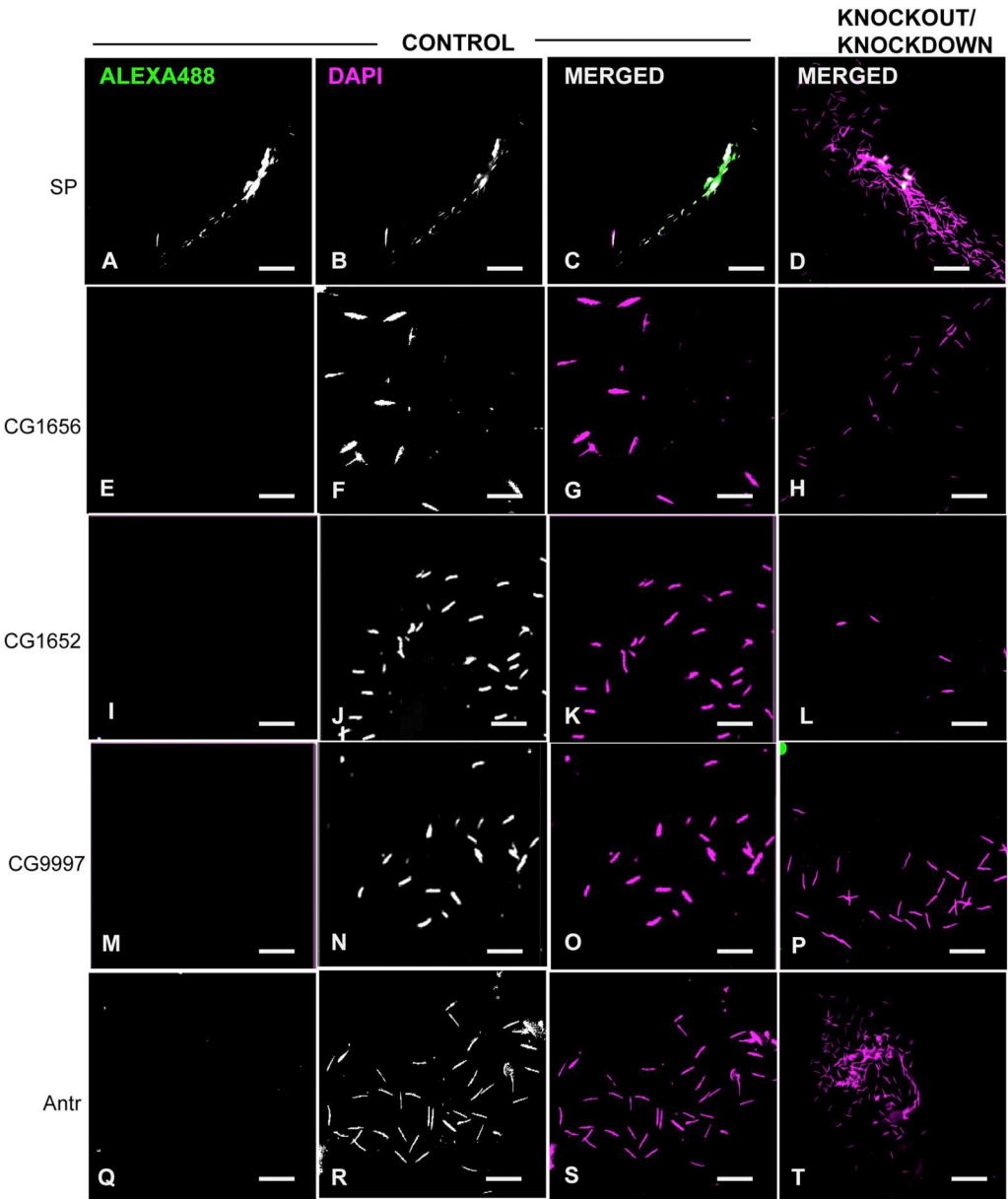


Fig. 6. Immunofluorescence showing the binding and distribution of SP, CG1656, CG1652, CG9997 and Antr on sperm dissected from SRs of females 4 d ASM to CS ("control" panels) or knockout/knockdown males (right panels). For each row, the two rightmost panels are merges, in which green shows the signal from the secondary anti-rabbit Alexa488 conjugated antibody, detecting the anti-Sfp primary antibody. DAPI staining, false-colored in magenta, shows sperm heads. Representative images from the following sample sizes: n = 9 (SP), n = 8 (CG1656 and CG1652), n = 9 (CG9997), n = 10 (Antr). Bar = 20 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

levels of most of these proteins were too low for detection by Western blot (Fig. S3), except for CG1652, for which only very small amounts were still detected. Thus, the presently known network proteins must act transiently, within the first ~ day ASM, to facilitate SP binding to sperm, but are no longer needed to maintain its binding thereafter.

3.3. Action of Antares within the SP network

Previous studies of the network proteins placed their actions into the pathway shown in Fig. 1 by examining each protein's transfer, stability and processing in females mated to males knocked down for other network proteins (Findlay et al., 2014; Ravi Ram and Wolfner, 2009). Experiments using *antr* knockdowns had placed it into the network at the same position as CG9997 and a less-well-characterized gene, *aqrs*. Specifically, *antr* is needed for the normal transfer of CG1656 and CG1652 (Findlay et al., 2014). Our data are consistent with this, in showing a lack of detectable CG1652 and CG1656 in SRs of females 2 h ASM to *antr* knockdown males (Fig. 2). However, the effects of other network proteins on the transfer, stability or localization of Antr itself had not previously been testable, because of the lack of an anti-Antr antibody. Here, to test how other pathway members affect the transfer and stability of Antr, we used a new polyclonal antibody against this protein to examine Antr's presence in knockouts or knockdowns of other pathway members (Fig. 2). We observed Antr in both the SS and SR fractions from dissected female reproductive tracts, even in females mated to males that did not produce SP, CG1656, CG1652 or CG9997. In addition, knockdown of two additional new network members, *aqrs* and *intrepid* (Findlay et al., 2014), did not reduce Antr transfer (Fig. S4). These data further support that Antr acts at the upstream-most step in the network, facilitating CG1652 and CG1656 transfer, and that Antr activity does not depend on any other known member of the network for its transfer, stability, or localization in mated females.

4. Discussion

SP exerts long-lasting behavioral effects on females, but only if it can bind stably to the sperm stored in their seminal receptacles (Peng et al., 2005) and then be proteolytically released to interact with the SP receptor (Yapici et al., 2008) on specific neurons, or other cells, in the

female reproductive tract (see Avila et al., 2011; Hopkins et al., 2017 for reviews; Apger-McGlaughon and Wolfner, 2013; Avila et al., 2015; Cognigni et al., 2011; Denis et al., 2017; Dove et al., 2017; Ferguson et al., 2015; Findlay et al., 2014; Garbe et al., 2016; Hasemayer et al., 2009; Haussmann et al., 2013; Reiff et al., 2015; Rezaval et al., 2012; Sitnik et al., 2016; Yang et al., 2009). SP's binding to sperm requires a network of at least eight other seminal proteins (Findlay et al., 2014; Ravi Ram and Wolfner, 2007, 2009). Members of this network could act transiently to facilitate SP binding to sperm, or they could associate with sperm and SP long-term, for example by facilitating stable SP-sperm interactions. Our results presented here show that all known network members act transiently, and thus potentially catalytically – though several also bind to sperm in the process (Fig. 7).

Two of the network proteins either do not enter the SR (CG17575; Ravi Ram and Wolfner, 2009), or enter it at very low levels (Sems; LaFlamme et al., 2012). Consistent with these observations, we showed that neither binds sperm. However, both proteins are required for SP and the other network members to localize to the SR. Thus, these proteins must exert their influence very early after mating, within the ejaculate and/or the bursa. These proteins may modify network proteins or SP, or they may alter the sperm's surface to generate conditions that allow SP to bind. In contrast, we show here that the other network proteins – CG1652, CG1656, CG9997, and Antr – enter the SR with sperm and bind to sperm, as does SP. Unlike SP, however, the sperm-binding of these proteins is short-lived: most are undetectable on sperm by 1 d ASM, and none are detectable by 4 d ASM, a time when significant SP remains on the sperm (Peng et al., 2005; this study) and when SP's effects on the female persist. It is possible that the surface of sperm is “sticky”, binding these several Sfps and carrying them into the seminal receptacle, but that only the binding of SP is stable. Alternatively, the action of the network Sfps to facilitate SP's binding to sperm may be mediated by their own sperm-binding – but in a short-term, catalysis-like way. Interestingly, there are differences in the regions of binding of network proteins to sperm. SP, CG9997 and Antr bind to heads and tails of sperm, whereas CG1652 and CG1656 are detected only on tails. In this context, it is interesting that Peng et al. (2005) reported that SP release from sperm has different characteristics on head vs. tail: SP is released steadily from the sperm tail, but either more slowly or not at all from the sperm head. Together, these data suggest that there are compositional or structural differences on the

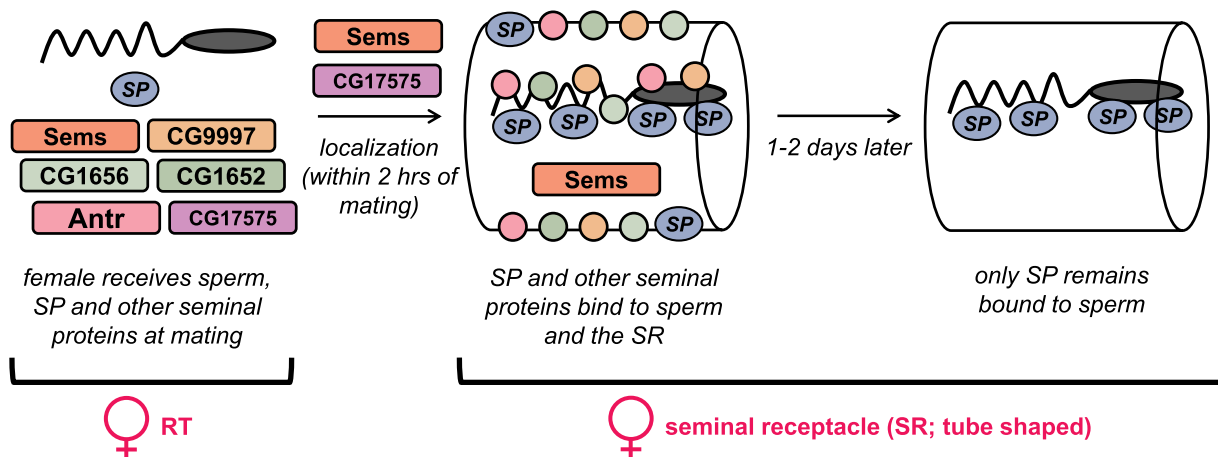


Fig. 7. An expanded model of the SP network to include the results from this study. The model indicates the transience of network Sfps, and their sperm or SR binding (as appropriate) are shown.

surfaces of the head vs. tail of *Drosophila melanogaster* sperm, causing differences in what can bind at, and the rate of release from, each region.

At present, we do not know of a network protein that persists in the female as long as SP does. Knowledge of the network's composition is not yet saturated; many of the > 200 Sfps have yet to be tested for roles in mediating SP's binding to sperm. Future functional analyses of additional seminal proteins are likely to add more players, and perhaps more steps, to the pathway that binds SP to sperm. It is possible, for example, that a Sfp(s) will be found that associates with sperm for as long as SP does and acts as an essential linker between SP and sperm. Alternatively, it is possible that SP binds directly to a sperm constituent, and that the role of the network Sfps is to assist in this binding, perhaps by exposing or processing the SP binding site on sperm or modifying SP to make it capable of sperm-binding.

We have also found that the network protein Antr, which was previously known only to be needed for the transfer of CG1652 and CG1656 to the female (Findlay et al., 2014), localizes to the SR, binds to sperm after mating and does not require any other known network protein to do so. Thus, its action in the network is at an early step, although its sperm binding might reflect a later, second, action. It is interesting that interference with transfer of some network proteins (by knockdown of *antr*, *CG9997*, or *aqrs*; Findlay et al., 2014; Ravi Ram and Wolfner, 2009) does not impair the transfer of all Sfps, including SP, suggesting a mechanism that imparts selectivity on the transfer or secretion of certain Sfps.

We report here that all of the network Sfps that enter the SR not only bind to the sperm within this organ, but that significant amounts of each protein remain associated with the SR even after sperm have been dissected out. Binding of SP to sperm-free SR had been reported by Ravi Ram and Wolfner (2009), but it was unexpected that all of the network Sfps that enter the SR would bind, particularly since the bound Sfps are in different predicted functional/biochemical classes. Perhaps the walls of the SR are “sticky,” or perhaps they have crypts in which proteins or protein complexes are trapped. The role of these proteins' binding to the SR is also unclear. They could be trapped non-specifically, as envisioned above, or they could be retained there to carry out actions that modify sperm or SP to facilitate or stabilize the latter's binding. Alternatively, these Sfps could potentially exert effects on the SR itself, perhaps to protect sperm or their bound SP from damage. However, since these Sfps are undetectable by 4 days post-mating, these latter actions would have to either be catalytic or replaced by actions of other (potentially female) proteins, since SP binding, and fertility, persist well beyond this time. The latter possibility is particularly intriguing to consider in light of the apparent co-option of duplicates of female reproductive protein genes to serve roles in seminal fluid (Sirot et al., 2014).

5. Conclusions

Binding of SP to sperm in mated *Drosophila* females is essential for persistence of this Sfp in the female reproductive tract and thus for the long-term maintenance of the post-mating effects that it induces. We previously showed that eight other seminal proteins are required to bind the SP to sperm. Here, we have shown that those seminal proteins act transiently to mediate SP's sperm-binding. Several of these “network proteins” bind to sperm initially, but are lost from the sperm and from the female within 1–2 days. By contrast, SP remains sperm-bound within the female for at least 7 days (Gligorov et al., 2013; Peng et al., 2005). These kinetics suggest that the known network proteins may act transiently to modify SP, or the sperm surface, to allow SP-sperm binding, rather than themselves forming adaptors that directly mediate the binding of SP to sperm. Future studies to examine the mechanisms of network protein actions in promoting SP binding to sperm will be of interest both from the perspective of reproductive biology (how are sperm and Sfps modulated within mated females, and how do non-sperm components stick to sperm cells?) and, given the rapid evolution

of many Sfps, from the perspective understanding the mechanics of intra- and intersexual conflicts in the evolution of reproductive traits.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ibmb.2018.09.004>.

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