

Sperm plasma membrane breakdown during *Drosophila* fertilization requires Sneaky, an acrosomal membrane protein

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Fertilization typically involves membrane fusion between sperm and eggs. In *Drosophila*, however, sperm enter eggs with membranes intact. Consequently, sperm plasma membrane breakdown (PMBD) and subsequent events of sperm activation occur in the egg cytoplasm. We previously proposed that mutations in the *sneaky* (*snky*) gene result in male sterility due to failure in PMBD. Here we support this proposal by demonstrating persistence of a plasma membrane protein around the head of *snky* sperm after entry into the egg. We further show that *snky* is expressed in testes and encodes a predicted integral membrane protein with multiple transmembrane domains, a DC-STAMP-like domain, and a variant RING finger. Using a transgene that expresses an active Snky-Green fluorescent protein fusion (Snky-GFP), we show that the protein is localized to the acrosome, a membrane-bound vesicle located at the apical tip of sperm. Snky-GFP also allowed us to follow the fate of the protein and the acrosome during fertilization. In many animals, the acrosome is a secretory vesicle with exocytosis essential for sperm penetration through the egg coats. Surprisingly, we find that the *Drosophila* acrosome is a paternally inherited structure. We provide evidence that the acrosome induces changes in sperm plasma membrane, exclusive of exocytosis and through the action of the acrosomal membrane protein Snky. Existence of testis-expressed Snky-like genes in many animals, including humans, suggests conserved protein function. We relate the characteristics of *Drosophila* Snky, acrosome function and sperm PMBD to membrane fusion events that occur in other systems.

KEY WORDS: Fertilization, Male fertility, Acrosome, Paternal effect mutations, *Drosophila*

INTRODUCTION

Studies of fertilization have revealed the universal importance of membrane events to prepare and coordinate the gametes and ensure their successful union. These events have been most extensively studied in sperm of selected marine invertebrate and mammalian species and include dynamic changes in membrane proteins and lipids during sperm capacitation, acrosome exocytosis, binding, adhesion and membrane fusion with the egg (reviewed by Flesch and Gadella, 2000; Evans, 2002). The majority of molecules with confirmed roles in these processes are cell adhesion molecules, ion channels or signal transduction components with molecular mechanisms of action that have been studied in multiple cell types (reviewed by Talbot et al., 2003; Neill and Vacquier, 2004; Rubinstein et al., 2006). There is considerable interest in discovering proteins that act specifically during fertilization. The characterization of these molecules will contribute to a better understanding of the specialized properties of gametes and to the practical goal of identifying new targets for contraceptives.

To identify novel sperm molecules required for fertilization, we have pursued a genetic approach using *Drosophila melanogaster*. Previous studies of male sterile mutants indicated that those affecting fertilization might be relatively rare. Therefore, we isolated and categorized a large number of male-sterile mutations to find a subset that disrupt sperm-egg interactions or induce paternal effect defects (Fitch et al., 1998; Wakimoto et al., 2004). We previously described mutations in a gene called *sneaky* (*snky*), which met our

genetic criteria of specifically affecting fertilization (Fitch and Wakimoto, 1998). Mutations of *snky* showed detectable effects only on male fertility. Sperm produced by mutant males were competent to enter the egg but arrested before sperm nuclear decondensation and aster formation. We proposed that this sperm activation defect was due to a failure in breakdown of the sperm plasma membrane, a step that normally occurs immediately after sperm entry into the egg in *Drosophila*.

In this study, we provide phenotypic evidence of a role for Snky in affecting the integrity of the sperm plasma membrane during fertilization. We molecularly identify the *snky* gene and characterize its transcript and predicted protein to investigate how the protein might function. The results indicate that Snky is an acrosomal membrane protein that is contributed to the early embryo. In addition to suggesting a possible molecular role for Snky, these studies have implications for the function and the fate of the acrosome during *Drosophila* fertilization.

MATERIALS AND METHODS

Drosophila strains

Mutations are described in Fitch and Wakimoto (Fitch and Wakimoto, 1998) or FlyBase (<http://flybase.bio.indiana.edu>), except as noted below. The *snky*^{Z0566} and *snky*^{Z4482} alleles were recovered as ethylmethane sulfonate (EMS)-induced male sterile mutations from the Zuker collection (Koundakjian et al., 2004; Wakimoto et al., 2004). The strain *P{PZ} Syx13⁰¹⁴⁷⁰ ry⁵⁰⁶/TM3* (Bloomington *Drosophila* Stock Center) was used to generate deletions near the P-element by transposase-induced male recombination (Preston et al., 1996) and one *snky*⁻ chromosome, denoted *Df(3L) Syx13^{01470R5}*, was recovered. This chromosome is homozygous viable but poorly so due to deletion of genes other than *snky*⁺ (Fitch and Wakimoto, 1998). A transgenic line that expressed the secreted Green fluorescent protein (GFP^{secret}) of Pfeiffer et al. (Pfeiffer et al., 2000; Pfeiffer et al., 2002) under the control of the β 2t testis-specific promoter from the *tv3* vector (Wong et al., 2005) was generously provided by J. Brill.

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Molecular characterization of the *snky* gene, mutations and transcript

Molecular studies were carried out essentially as described by Sambrook and Russell (Sambrook and Russell, 2001). Genomic fragments flanking P-element insertions in the *P[PZ] Syx13⁰¹⁴⁷⁰* and *Df(3L) Syx13^{01470R5}* chromosomes were isolated and sequenced to identify the P-element insertion site and locate the deficiency breakpoints (Preston et al., 1996). These fragments were used as probes to select hybridizing genomic clones from a phage library (Tamkun et al., 1992). Overlapping clones that spanned the deleted region were used to probe genomic Southern blots containing DNA from *snky¹* and its parental *mwh red e* line to identify restriction site polymorphisms. The *snky^{Z0566}* and *snky^{Z4482}* mutations were identified using PCR to amplify and sequence selected regions from mutant and parental *bw*; *st* chromosomes.

For northern blots (Fig. 2), the lanes contained ~0.25 µg polyA+ RNA from testes (350 pairs of testes) or ~7.4 µg polyA+ RNA isolated from 40 males after removal of the testes. The probes were a ³²P-labeled 3.3 kb *AvaI* genomic fragment isolated from the clone BAC05N10 (UK-HGMP Resource Center) and a clone containing the ribosomal protein gene *rp49*.

We sequenced *snky* cDNA clones, which we generated from polyA+ RNA isolated from testes of *bw*; *st* males. Standard protocols were used for reverse transcription (Gibco Superscript II RNase H-Transcriptase Kit), 5' RACE (Ambion Choice RLM-RACE Kit), and cDNA cloning into the TOPO-TA vector (Invitrogen). Primers (Gibco) for cDNA amplification were: S1(CCTTCCTACTGGGACTCGTG), S2(GTTGTTGAAGCCGA-AAAGA), S3(CGAACTCCACGTCATTGAGA), S4(GTTGAGATCCT-CGGACAAT), S5(TTGTAACCTGCTTGCCATCAAAT), S6(GGC-AAAGCCACTGCACGTA).

Construction of transgenic lines and assays for male fertility

The rat CD2 coding region was isolated as 1.1 kb *Clal/XhoI* fragment from a plasmid kindly provided by N. H. Brown (Dunin-Borkowski and Brown, 1995) and cloned downstream of the testis-specific β2-tubulin promoter in P-element transformation vector of Hoyle et al. (Hoyle et al., 1995). The construct also contained the *white^{+mc}* gene and was introduced into *y w¹¹¹⁸* flies using standard germline transformation techniques. A line carrying a transposon on chromosome 2 was used to construct the *y w¹¹¹⁸; P[w^{+mc} B2t::CD2] 2a; snky¹ e/TM3, y+ Ser e* strain.

The genomic region containing the CG11281 gene was isolated as a 7.3 kb *SalI* fragment from BACN05H10 and cloned into pCasPeR4, which carries the *w^{+mc}* gene (Thummel and Pirrotta, 1992). Three independent insertions, denoted *P[w^{+mc} snky^{+17.3}] A, B and C* were obtained. Two were on chromosome 3 and were introduced into a *snky⁻* background by recombination onto *Df(3L) Syx13^{01470R5}*.

A transgene expressing Snky-GFP fusion protein, denoted *P[w^{+mc} snky-GFP]* was created by inserting the Enhanced green fluorescent protein (EGFP) coding region from pEGFP-N3 (Clontec) into the pCasPeR4-7.3 S construct described above. The fusion protein retained the *snky⁺* gene promoter and flanking regions. It extended the *snky⁺* open reading frame (ORF) just before the stop codon to include: a 30 bp linker from pEGFP-N3, the EGFP coding sequences (amino acid 1 to 237) and, as a consequence of the cloning scheme, an in-frame duplication of the last seven codons of the *snky* ORF. The transgene was used to generate the *y w; P[w^{+mc} snky-GFP]; snky¹ e/TM3, y+ Ser e* lines.

Fertility assays were performed for males carrying *P[w^{+mc} snky^{+17.3}] or P[w^{+mc} snky-GFP]* in a *snky⁻* background to test for activity of the transgenes. Fertility was monitored in crosses of single males mated to three wild-type (*Canton-S*) females. Percentage of fertile crosses and progeny yield per male were compared to those of control crosses, which used *snky⁻* brothers that lacked the transgene or *snky¹/snky⁺* heterozygotes. Because *snky⁻* males produce one to five progeny on occasion (Fitch and Wakimoto, 1998), fertile crosses were defined as those yielding more than five offspring.

Assays for Snky-GFP and CD2 expression

To monitor Snky-GFP in testes, *y w; snky-GFP; snky¹ e* males were dissected in 2% paraformaldehyde in PBS (130 mmol/l NaCl, 7 mmol/l Na₂HPO₄, 3 mmol/l NaH₂PO₄). Testis squashes were prepared on poly-L-

lysine coated slides, frozen in liquid nitrogen, submerged in 95% cold ethanol, rinsed in PBS, then stained with 0.1 µg/ml DAPI (4',6-diamidino-2-phenylindole) to label nuclei. To label the mitochondrial derivative, larval testes were incubated with 400 mmol/l Mitotracker Dye (MT-CMXRos, Molecular Probes), then processed as described for adult testes. To assay Snky-GFP in sperm in the reproductive tracts of females and in eggs, wild-type females were mated to *y w; snky-GFP; snky¹ e* males. Sperm storage organs were dissected then prepared as described above. Eggs were collected within 15 minutes of deposition, dechorionated in 50% bleach, then fixed in 4% paraformaldehyde in PBS overlaid with heptane. After vitelline envelopes were removed by hand, eggs were processed without squashing as described above. All preparations were mounted in Vectashield (Vector Laboratories). Images were acquired at 600× magnification using a Bio-Rad Radiance 2000 confocal microscope equipped with a 488 nm Kr/Ar laser. Optical sections were typically 0.45 µm. Z-series stacks were assembled using NIH Image J (<http://rsb.info.nih.gov/ij/>) and images were edited using Adobe Photoshop.

Testes were isolated from adult males carrying the rCD2 transgene and fixed in 4% paraformaldehyde. For immunostaining, we used a 1:100 dilution of the primary OX-34 mouse anti-rat CD2 monoclonal antibody (Harlan Sera-Labs) and Alexa-488 goat anti-mouse antibody (Molecular Probes) as secondary antibody. Nuclei were counterstained with 1 µg/ml propidium iodide. The tissue was mounted in Vectashield for confocal microscopy. To track CD2 in embryos, wild-type females were mated to *snky¹* males carrying the rCD2 transgene. Eggs were collected within 20 minutes of deposition, aged for 30 minutes, then dechorionated in 50% bleach and transferred to poly-L-lysine coated slides. Eggs were squashed under a coverslip, and the slide was frozen in liquid nitrogen then submerged in 95% ethanol for 10 minutes. Tissue was fixed and incubated with the OX-34 antibody as described for testes. Secondary and tertiary fluorescent labeling of OX-34 was performed with Alexa 488 Signal-Amplification Kit (Molecular Probes) using the manufacturer's protocol. Nuclei were counterstained with DAPI. Images were obtained using a Nikon fluorescent microscope equipped with a CCD camera and edited with Adobe Photoshop.

RESULTS

Sperm produced by *snky* males retain a plasma membrane marker after entry into the egg

Males mutant for the *snky* gene are sterile despite normal spermatogenesis, successful transfer of normal quantities of sperm to females and efficient sperm entry into eggs (Fitch and Wakimoto, 1998). We previously reported that the *snky* defect is apparent shortly after the sperm enters the egg. The sperm nucleus remains tightly condensed and located at the egg cortex and no sperm aster is produced. We also reported that the sperm nucleus stains readily with DAPI, a membrane permeable with chromatin dye but variably so, or not at all, with membrane impermeable dyes. We therefore proposed that the defect is due to persistence of a plasma membrane around the head of *snky* mutant sperm, preventing access to activating factors in the egg cytoplasm. To obtain more direct evidence for this proposal, we expressed an integral membrane protein, the rat CD2 protein, during spermatogenesis to provide a sperm plasma membrane marker. Previous studies showed that rCD2, a single pass plasma membrane protein, can be expressed in *Drosophila* cells and detected with a specific monoclonal antibody (Dunin-Borkowski and Brown, 1995). Our transgenic line expressed the rCD2 gene under the control of a testis-specific promoter in *snky¹* homozygotes. Immunostaining showed that rCD2 was a membrane marker in primary spermatocytes and all subsequent stages of spermatogenesis. Localization was observed along the entire length of elongating spermatids and mature sperm, including the head (Fig. 1A,B). The rCD2 epitope was retained along the sperm after entry into the egg and for at least 30 minutes after egg deposition (Fig. 1C,D,E; n=9 eggs), providing evidence that *snky* mutant sperm fail in plasma membrane breakdown (PMBD).

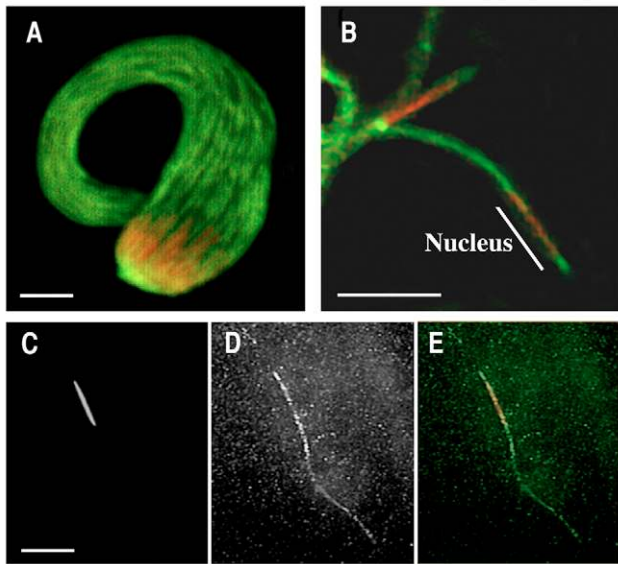


Fig. 1. The rCD2 protein marks the sperm plasma membrane. The panels show sperm produced by males homozygous for *snky*¹ and a transgene expressing rat CD2 gene. The rCD2 protein (green) was detected by immunolocalization and nuclei (red) by PI or DAPI staining. In a cyst of elongating spermatids (A) and mature sperm (B), rCD2 is located on the plasma membrane. After entry into the egg, sperm retain a condensed nucleus (C) and rCD2 (D). The merged image (E) shows overlapping rCD2 and nuclear staining consistent with retention of the plasma membrane surrounding the head. Scale bar: 10 μm.

The *snky* gene corresponds to predicted gene CG11281 and encodes a testis transcript

We previously mapped *snky* to the 69F-70A cytogenetic interval (Fitch and Wakimoto, 1998). To refine localization, we generated deficiencies in the region using P-element-mediated male recombination starting with the *P{PZ} Syx13⁰¹⁴⁷⁰* insertion. The strategy yielded a small deficiency, denoted *Df(3L) Syx13^{01470R5}*, that was homozygous viable and conferred the *snky*⁻ phenotype to surviving males. Molecular mapping of the deficiency breakpoints defined a 185 kb genomic interval within which we identified a restriction site polymorphism between *snky*¹ and its parent chromosome. The region overlapped with CG11281, a gene predicted by the *Drosophila* Genome Project but for which no expressed sequence tag or cDNAs had been reported.

Two additional observations showed that CG11281 corresponded to the *snky* gene. Northern analysis revealed a CG11281 transcript with an expression profile consistent with the specific effect of *snky* mutations on male fertility. As shown in Fig. 2A, a genomic fragment containing CG11281 hybridized to a rare 2.7 kb polyadenylated RNA that was found in testes, but not in carcasses (bodies lacking testes) of wild-type adult males. A corresponding transcript that was reduced in size and abundance was detected in testes of *snky*¹ males (Fig. 2B). A hybridizing transcript was absent in females (data not shown). Definitive evidence to assign CG11281 as the *snky* gene was achieved by transgenic rescue of the *snky*⁻ male sterility by a 7.3 kb genomic *Sall* fragment that included CG11281 but no other predicted genes. Three independent insertions of the transgene were obtained and each one conferred high levels of male fertility in a single dose (Table 1), indicating that the *Sall* fragment contains most, if not all, of the necessary sequences for the *snky*⁺ gene.

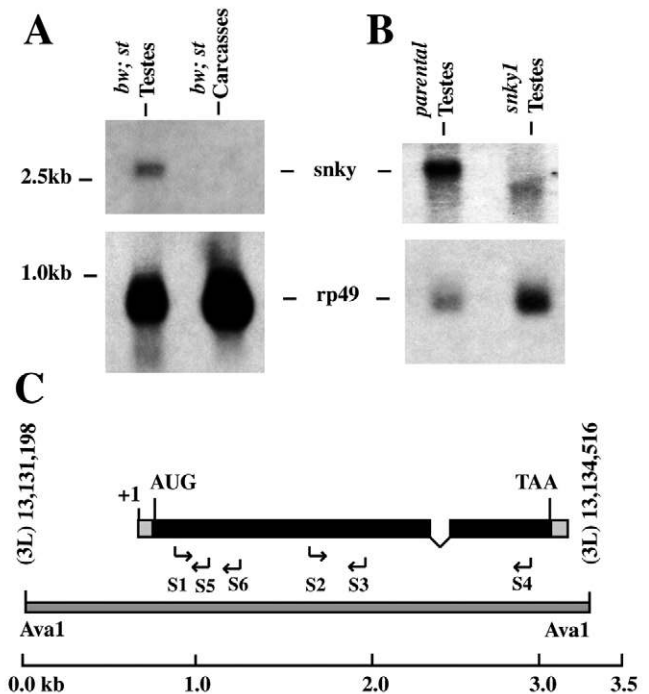


Fig. 2. Expression and structure of the *snky* transcript.

(A,B) Northern blots probed with a genomic fragment that contained CG11281 detected a 2.7 kb transcript that was present in testis, absent in male carcasses (A) and reduced in size and abundance in *snky*¹ testes compared to its parental strain (B). Bottom panel shows levels of rp49 mRNA in each lane as a loading control. (C) Transcript structure was determined by RT-PCR. Primers used for cDNA amplification are indicated by arrows. The transcript is contained within the *AvaI* genomic fragment, which was used as a probe for northern analysis. Fragment endpoints are labeled according to *Drosophila* Genome Project Release 4.0 coordinates. Transcription initiation site (+1) determined by 5' RACE is nucleotide 3L 13,131,973. Positions of the start and stop codons of the 2145 bp ORF (black box), the 72 bp intron and the predicted polyadenylation site are also indicated.

We determined the *snky* transcript structure using RT-PCR and 5' RACE. This analysis revised the CG11281 predicted structure and revealed a gene with a single intron and an ORF of 715 amino acids. Sequence analysis of two EMS-induced alleles and their parent *bw; st* line identified a single lesion in the ORF in both cases. In *snky*^{Z0566}, a change from *T* to *A* results in a missense mutation that replaces a Cys with a Ser at residue 258. In *snky*^{Z4482}, a transition mutation of *C* to *T* results in a stop codon at residue 379.

Snky is a predicted transmembrane protein with a RING finger domain

The Snky protein is predicted to be a protein with multiple transmembrane domains. The following predictive algorithms were used to examine potential topologies: TMHMM 2.0 (Krogh et al., 2001), HMMTOP 2.0 (Tusnady and Simon, 2001), PHOBIUS (Kall et al., 2004), SOSUI (Hirokawa et al., 1998) and ConPred II (Arai et al., 2004). These programs gave conflicting results probably due to the relatively high content of positively charged residues in Snky. However, all programs predict a minimum of four transmembrane domains and one, ConPred II, predicts eight transmembrane domains. The topology shown in Fig. 3A is based on the SOSUI prediction, which identifies a signal sequence within

Table 1. Rescue of the *snky* phenotype by transgenes

Strain	Genotype	n*	% Fertile crosses†	Average progeny yield
Control (<i>snky</i> +)	<i>y w^l Y; +/+; snky¹ / +</i>	20	100	113.2
Control (<i>snky</i> -)	<i>y w^l Y; +/+; snky¹</i>	20	0	0.0
Transgenic	<i>y w^l Y; P{w⁺mC, snky⁺t 7.3}A / +; snky¹</i>	20	100	99.8
Control (<i>snky</i> +)	<i>y w^l Y; Df snky / +</i>	20	95	98.3
Control (<i>snky</i> -)	<i>y w^l Y; Df snky / snky¹</i>	16	0	0.06
Transgenic	<i>y w^l Y; Df snky, P{w⁺mC, snky⁺t 7.3}B / snky¹</i>	20	85	110.0
	<i>y w^l Y; Df snky, P{w⁺mC, snky⁺t 7.3}C / snky¹</i>	20	75	68.4
Control (<i>snky</i> +)	<i>y w^l Y; snky¹ / +</i>	10	100	124.2
Control (<i>snky</i> -)	<i>y w^l Y; snky¹</i>	36	0	0.06
Transgenic	<i>y w^l Y; P{w⁺mC, snky-GFP} / +; snky¹</i>	19	89.5	85.8
	<i>y w^l Y; P{w⁺mC, snky-GFP}; snky¹</i>	22	86.4	88.4

Df snky=*Df(3L)Syx13^{01470R5}*.

*Number of single male crosses.

†Crosses yielding >5 progeny (see Materials and methods).

the first 18 residues and predicts the 6 hydrophobic membrane-spanning domain most commonly predicted by the suite of programs we used.

Snky has two regions with noteworthy Cysteine residue patterns (Fig. 3A,B). One region is located in the largest predicted extramembrane loop and contains six Cys that are separated by eight to 12 amino acids. The functional significance of this region, which we refer to as the patterned 6 Cys motif, is indicated by the *snky*²⁰⁵⁶⁶ mutation, which changes the second of these Cys to a Ser. The second region is located near the carboxyl end and consists of four pairs of C-X2-Cs (where X is any amino acid) that are linked by four to ten residues. This second pattern corresponds to a variant of the Pfam C3HC4 RING finger (*zf*-PF00097) (Finn et al., 2006). In *Snky*, a Cys is substituted for the His resulting in a C4-C4 variant. Additionally, a coiled-coil domain is predicted in *Snky* from residue 597 to 624.

***Snky* is a member of a protein family**

BLAST searches (*blastp*) (Altschul et al., 1997) of non-redundant sequences in NCBI identified a series of *Snky*-related proteins present in invertebrates and vertebrates. Of these, a set of proteins, which we refer to as the *Snky* family, not only have the highest level of amino acid identity across their entire length, but also share additional features. All are predicted to have multiple transmembrane domains. Each also has the patterned 6 Cys motif in a predicted extramembrane loop that follows the pattern: C-(10X)-C-(10X)-C-(12-13X)-C-(8X)-C-(9-12X)-C. These proteins also contain a RING finger with the consensus pattern: C-(2X)-C-(10-11X)-C-(2-4X)-C-(4X)-C-(2X)-C-(7X)-C-(2X)-C. The *Snky* proteins include: a mosquito protein (*Aedes aegypti*, EAT46485, conceptual translation) with 30% amino acid identity; a zebrafish protein (XP683798, conceptual translation) with 22.2% identity; a human protein (BAB71440) with 22% identity; and a mouse protein (XP994048) with 21.8% identity. The human and mouse protein sequences are supported by full-length testis cDNAs. Although expressed sequence tags have also been isolated from other tissues, comparison of available sequence indicates the possibility of testis-specific isoforms in humans. CLUSTAL W alignment (Thompson et al., 1994) revealed three regions, which are the most highly conserved among *Snky* family members. These regions are denoted a, b and c in Figs 3 and 4. Regions b and c overlap with the ends of the region that defines the DC-STAMP sequence family (Pfam PF07782). In *Snky*, the DC-STAMP-like region extends 192 amino acids, from amino acid 406-597.

Snky is more distantly related to other proteins that had been previously recognized as containing DC-STAMP-like regions. They include the human and mouse DC-STAMP-Domain Containing-2 Proteins, the *Caenorhabditis elegans* sperm protein SPE-42 (Kroft et al., 2005) and a *Drosophila* protein encoded by the CG6845 gene. These proteins contain a RING finger and a Cysteine-containing region spaced similar to the patterned 6 Cys motif in *Snky*. However, compared with proteins described above, they show a reduced level of identity with *Snky* at the amino acid level (10-17% identity), particularly in regions a, b and c. Included in this group is a predicted sea urchin protein (XP795449) with 15.1% identity to *Snky*. Even more distantly related are the human and mouse DC-STAMP, which contain the DC-STAMP domain but lack a RING finger and a patterned 6 Cys motif.

Localization of a *Snky*-Green fluorescent protein fusion during spermatogenesis

We generated transgenic flies that contained a *snky*-Green fluorescent protein fusion gene expressed from the *snky* promoter. The fusion protein contained the entire *Snky* protein and Enhanced GFP at the carboxyl terminus. Four independent transgenic lines were recovered and in each line, the transgene significantly rescued the *snky*⁻ male sterile phenotype in a single dose. The transgene used in all subsequent studies rescued fertility to a level comparable to that conferred by the *snky*⁺ transgene (Table 1). Hence, the *Snky*-GFP fusion protein was highly active and could be used as a tool to deduce the localization of the normal *Snky* protein.

To examine tissue expression of *Snky*-GFP, we examined larvae and adults that were homozygous for a *snky*-GFP transgene and the *snky*¹ mutation. We detected expression only in the testis. To determine when *Snky*-GFP is expressed, we distinguished the different stages of spermatogenesis by the characteristic morphology of nuclei and mitochondrial derivatives (A. D. Bates, PhD thesis, University of Leiden, 1971) (Fuller, 1993). Nuclei were monitored by DAPI staining and mitochondrial derivatives were visualized by MitoTracker Red-CMXRos staining. *Snky*-GFP was first detected in primary spermatocytes as a diffuse cytoplasmic signal (Fig. 5A), consistent with localization to the endoplasmic reticulum and as expected for an integral membrane protein. Following meiosis, at the onion stage, which is characterized by round spermatid nuclei and comparably sized mitochondrial derivatives, a prominent cluster of *Snky*-GFP spots was seen just adjacent each nucleus (Fig. 5B). The structures at this stage corresponded in size and location to the aggregated Golgi complexes that Bates (A. D. Bates, PhD thesis,

University of Leiden, 1971) referred to as the acroblast. With continued differentiation, as nuclear condensation began and mitochondrial derivatives and sperm tails elongated, Snky-GFP was present as multiple irregular spots throughout the cytoplasm. However, one spherical Snky-GFP signal, generally larger than the others, was located adjacent to each nucleus (Fig. 5C). This site of Snky-GFP was the only visible signal retained as spermatids became fully elongated (Fig. 5D,E). In individualized mature sperm, the Snky-GFP signal exhibited a thin oval shape located at the tip of the sperm, with its distal end slightly overlapping with apical end of the needle-shaped nucleus (Fig. 5F). This pattern of localization and the predicted structure of Snky suggest that it is an integral membrane protein targeted to the acrosome during spermatogenesis.

The fate of Snky-GFP and the acrosome during fertilization

Snky-GFP allowed us to examine the fate of Snky and the acrosome during normal fertilization. In *Drosophila*, females store sperm after mating. We examined seminal receptacles (sperm storage organs)

from wild-type females mated to *snky*¹ males that carried two copies of the Snky-GFP transgene. The Snky-GFP signal we observed in stored sperm appeared identical to that seen in mature sperm from the testis (data not shown).

Fertilization occurs within the reproductive tract of females. Sperm are released from the seminal receptacle and enter the egg as it passes down the uterus. The events from sperm entry into the egg to the completion of the first embryonic cell cycle occur rapidly (estimated <17 minutes) and are often completed before the egg is laid. To capture the earliest stages, eggs fertilized by sperm from Snky-GFP males were collected and fixed within 15 minutes of egg deposition, counterstained with DAPI and examined by confocal microscopy. Even with this rapid processing, the majority of embryos examined had progressed beyond cycle 2. Of the 17 fertilized eggs and embryos captured before metaphase of cycle 1, we observed a Snky-GFP signal in 16. The signal was located in close proximity to the sperm nucleus before visible evidence of nuclear decondensation (Fig. 6A, *n*=1), and more distantly as decondensation progressed (Fig. 6B, *n*=3). During pronuclear migration and pronuclear apposition, Snky-GFP was seen peripheral to what was judged to be the male pronucleus based on the state of chromatin condensation or position in the egg (Fig. 6C, *n*=11, with one without a GFP signal). During prometaphase of cycle 1, the Snky-GFP signal was observed near the condensing chromosomes (Fig. 6D, *n*=2). Although the position of the Snky-GFP signal relative to the sperm chromatin changed during these stages of fertilization, neither the shape nor the intensity of the signal appeared to change. Five embryos were found at stages between metaphase of cycle 1 and 2 and only one, which was at anaphase of cycle 1, exhibited a GFP signal (data not shown). We did not detect GFP signals in control eggs (fertilized by sperm of males lacking Snky-GFP), which were captured before prometaphase of cycle 1 (*n*=7, data not shown).

These studies revealed two surprising discoveries. The first of these was that sperm contributed Snky-GFP to the egg. The second remarkable finding was that the GFP signals observed in the egg and early cycle 1 embryos consistently appeared similar to those observed in mature sperm. We considered two possibilities to account for the retention of bright GFP signals in the egg cytoplasm. The paternally inherited structure could be an acrosomal vesicle, with the Snky-GFP signal marking the boundaries of an intact acrosome. Alternatively, the GFP signal could reflect retention of a large membrane remnant from a spent acrosome. To distinguish between the two possibilities, we obtained a transgenic line that expressed a soluble form of GFP in the acrosome. This line (gift of J. Brill) expresses the GFP^{secr} protein under the control of a testis-specific promoter. GFP^{secr} was designed and characterized by Pfeiffer et al. (Pfeiffer et al., 2000; Pfeiffer et al., 2002), and it contains the signal peptide from the Wingless protein fused to GFP. When expressed in embryonic or imaginal disc epithelial cells, GFP^{secr} is targeted to the secretory pathway, packaged in secretory vesicles and released into the extracellular space upon secretion (Pfeiffer et al., 2002). These properties predict that if GFP^{secr} is present in the acrosome, the GFP signal will dissipate upon acrosome exocytosis.

We examined eggs fertilized by sperm expressing GFP^{secr}. Of the 22 eggs captured between earliest stages of sperm nuclear decondensation (Fig. 6E) to prometaphase of cycle 1 (Fig. 6H), 21 showed a GFP^{secr} signal. In each case, the appearance was similar to that observed with Snky-GFP (compare higher magnification insets of Fig. 6A-H). These data are consistent with the retention of an intact acrosomal vesicle by the sperm during fertilization, the release

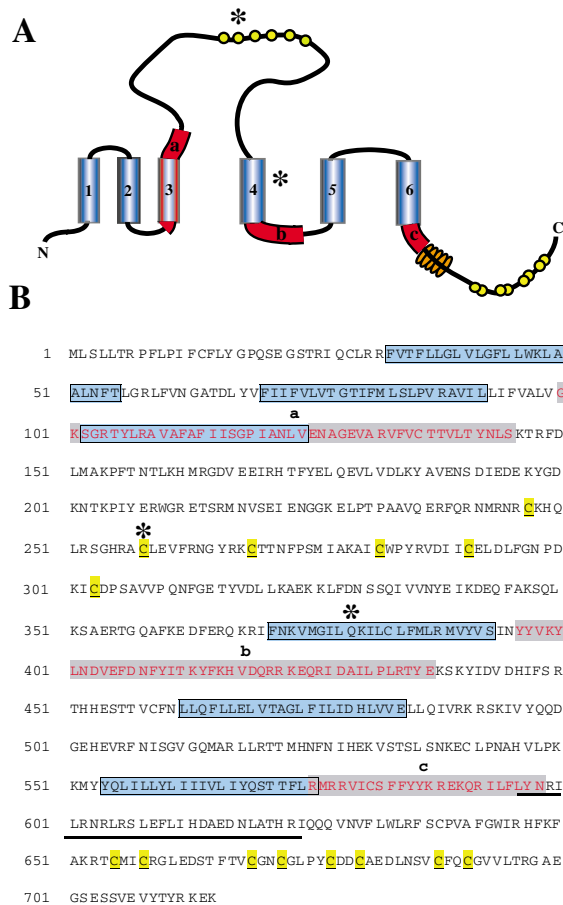


Fig. 3. Predicted topology and deduced amino acid sequence of Snky. (A) Predicted features of Snky are shown with six transmembrane (TM) domains (blue cylinders) as predicted by the SOSUI program (Hirokawa et al., 1998), three highly conserved regions a-c (red bars), a predicted coiled-coil domain (orange) and conserved Cys residues (yellow circles). A C4-C4 RING finger is located near the carboxyl terminus. Asterisks (*) indicate location of residues altered in *snky*² mutants. (B) Deduced amino acid sequence showing features diagrammed above. TM domains (boxed blue), highly conserved regions a-c (red), coiled-coil domain (underlined) and conserved Cys residues (C, boxed yellow).

Region a				
Snky		100	GKSGRTYLRVAVLAFIFLISGPIANLVENAGEVARVFCITVLTYNLS	145
Mosquito	EAT46485	94	GKAGRSMKKAIVLTCILTGPINNGSLNAREVVRVFCSTVLTYNLS	139
Zebrafish	XP683798	78	GSHGRTFLMVFVHGLTQGPINAGOHNVQDVASSMCCIDLOTHS	123
Human	BAB71440	112	GRGRLEVLGYALAAIVGPPANRRHNLNVTASLGCCTVELQINN	158
Mouse	XP994048	141	SKRGRLEVMGYALAAIVGPPANRRSINNEVTASLGCCTVELQINN	186
Region b				
Snky		400	YLNIVBENDFYITKVFKHVDORRKEORIDALPLRITYE	437
Mosquito	EAT46485	370	YLKKIDFENHVIDVFNHVDORRARYGKLLHLLPLRRIE	407
Zebrafish	XP683798	348	YCRDILEDNVYITTYFRQIDARRIHAERHLLPLRKAE	385
Human	BAB71440	398	YNHDIREFDNIYISTYFCQIDARRKRLGKRTLPLRKAE	436
Mouse	XP994048	425	YNWDIREFDNIYISTYFCQIDARRKRLGKQSLPLRKAE	462
Region c				
Snky		575	RLRRVLCSEFYMKREKORILFLYN	598
Mosquito	EAT46485	544	RRRRLCAWFPKREKORILFLYN	567
Zebrafish	XP683798	522	RLRRVIAAFYFPKREKRIILFLYN	545
Human	BAB71440	573	RLRRVIAAFYFPKREKRIILFLYN	596
Mouse	XP994048	599	RLRRVIAAFYFPKREKRIILFLYN	622

Identities Consensus (>60%) Similarities

Fig. 4. Multiple sequence alignment of Snky and related proteins. Three regions, denoted a, b and c, are the most highly conserved among Snky family members and are aligned here. Their locations in Snky are shown in Fig. 3. The sequence of Snky (*D. melanogaster*), the mouse (*M. musculus*) and human proteins are based on full-length cDNAs. The mosquito (*A. aegypti*) and zebrafish (*D. rerio*) sequences are based on conceptual translations of predicted genes.

of the vesicle into the egg cytoplasm after PMBD, and the persistence of the acrosome at least as late as prometaphase of the first embryonic cycle.

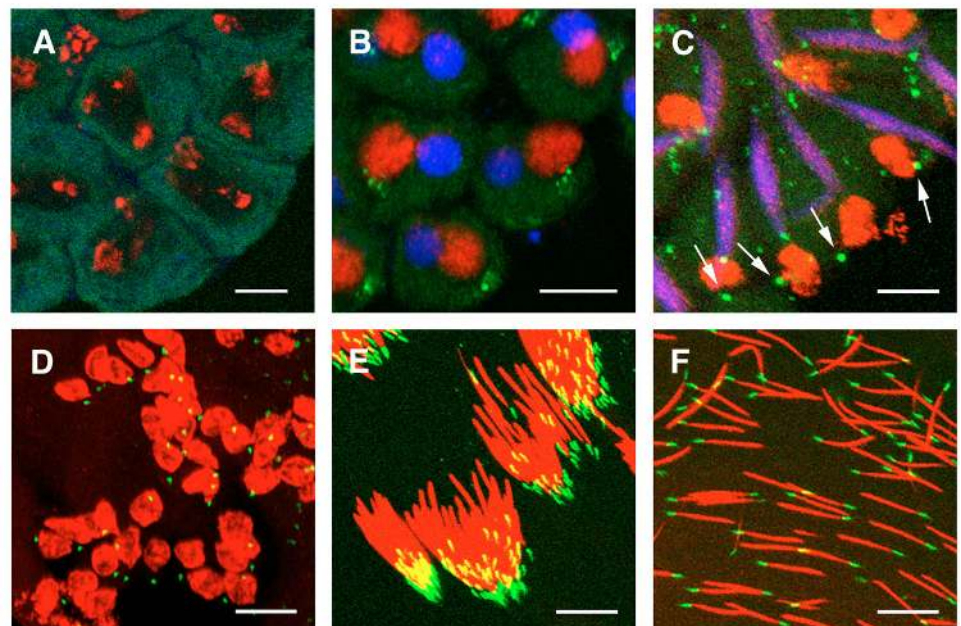
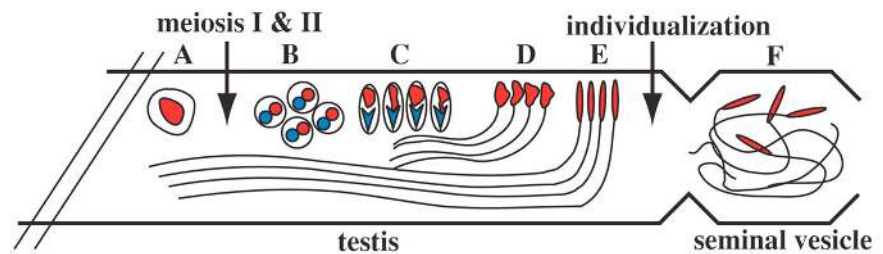
DISCUSSION

Fertilization in *Drosophila melanogaster* is unorthodox in that it does not involve a membrane fusion event between the sperm and egg. The mechanism used by the sperm to penetrate through the egg plasma membrane remains an interesting mystery. In her ultrastructural studies of fertilization in *D. melanogaster* and *D.*

montana, Perotti (Perotti, 1975) observed that the entire sperm enters the egg, including the excessively long tail surrounded by its plasma membrane. She noted that there was no evidence for 'extra' membranes that would support uptake into the egg by endocytosis. While her studies did not capture the sperm before nuclear decondensation, Perotti described one case in which a decondensing sperm nucleus was surrounded by what appeared to be remnants of the sperm plasma membrane. Her studies support the idea that the *Drosophila* sperm head must undergo PMBD in the egg cytoplasm. This step is required for the sperm nucleus to gain access to

Fig. 5. Localization of Snky-GFP during spermatogenesis.

The diagram depicts stages of spermatogenesis in which Snky-GFP expression was observed. Letters correspond to confocal images below (A-E). Primary spermatocytes (A) undergo two meiotic divisions to yield round spermatids (B). Differentiation follows (C-E), as spermatids undergo gradual condensation of nuclei (red), elongation of mitochondrial derivatives (blue) and sperm tails (black lines). Individualized, mature sperm are released from the base of the testis into the seminal vesicle (F). Confocal images show stages of spermatogenesis in males that were homozygous for *snky*¹ and carried two copies of a transgene expressing Snky-GFP (green). Nuclei were visualized by DAPI-staining (red). In A-C, mitochondrial structures were detected with the dye MT-CMXRos (blue). (A) In primary spermatocytes, Snky-GFP and the mitochondrial dye give diffuse cytoplasmic signals. (B) In post-meiotic spermatids at the onion stage, clusters of Snky-GFP-containing spheres are adjacent to nuclei and mitochondrial derivatives. (C) Snky-GFP is visible in clusters of spheres along the length of elongating spermatids, but a prominent spot (arrow) is associated with each nucleus. (D) A single slightly oval Snky-GFP signal is associated with each spermatid nucleus as nuclear condensation begins. (E) In cysts of late spermatids, Snky-GFP appears as a thin oval signal distal to the needle-shaped sperm nucleus. (F) Individualized sperm present in the seminal vesicle contain Snky-GFP signal located at the apical tip and consistent with localization to the acrosome. Scale bars: 10 μ m.



activating cytoplasmic factors that promote nuclear decondensation and replication, and for the centrosome to elaborate the sperm aster (Fitch et al., 1998). Our studies support this sequence of events and implicate Snky function in an event upstream of PMBD. Our original proposal that *snky* mutant sperm fail in sperm activation because they arrest before PMBD was based on their poor accessibility to membrane impermeant chromatin dyes (Fitch and

Wakimoto, 1998). Further support was obtained by Ohsako et al. (Ohsako et al., 2003), who used an antibody to a proposed cell surface proteoglycan to demonstrate retention on the sperm head before and after insemination. In our current study, we ectopically expressed the rat CD2 protein on the sperm plasma membrane and showed that immunoreactivity to this specific integral membrane protein was retained on the head of *snky*¹ sperm for at least 30 minutes after entry into the egg. These observations provide strong support for the proposal that *snky* sperm do not shed the plasma membrane surrounding the head. They also suggest that understanding the function of the Snky protein should provide clues about how PMBD is initiated or accomplished at the molecular level.

Molecular studies reported here show that Snky is a member of a family of related membrane proteins that are present in animals but are apparently absent in other lineages. Conservation among Snky family members points to three domains of potential functional significance. The most striking motif is the C4-C4 RING finger, which has been noted in at least four other proteins (Hanzawa et al., 2001; Kellenberger et al., 2005; Pugh et al., 2006; Arioli et al., 1998). In one of these, hNOT4, the C4-C4 RING has been shown to fold and bind zinc in a cross-brace fashion similar to the more common C3HC4 RING finger (Hanzawa et al., 2001), suggesting structural and functional similarity between these variants. Although RING finger proteins have been implicated in a broad variety of cellular functions, recent studies suggest that their unifying role is in mediating protein-protein interactions in large multiprotein assemblages (Borden, 2000). Thus, Snky may play a role in organizing and holding together macromolecular complexes at the membrane via its C4-C4 RING. The second Cys-containing region is the patterned 6 Cys motif, the functional significance of which is indicated by conservation and by the recovery of a male sterile *snky* allele that mutates the second Cys to a Ser. Cysteines in this region may form disulfide bonds to create a binding pocket or otherwise allow interactions with additional proteins. The third region is the DC-STAMP-like region, named after Dendritic cell specific transmembrane protein, a plasma membrane protein that was first described for its expression in human dendritic cells (Hartgers et al., 2000). Thus far, the function of only a few DC-STAMP-domain-containing proteins have been examined through the analysis of mutations or other knockdown strategies. The general classification of these proteins has been as members of a new class of putative receptors. Mouse DC-STAMP is upregulated in differentiating osteoclasts, and required for osteoclast fusion to form multinucleate cells (Kukita et al., 2004; Yagi et al., 2005). The *C. elegans* SPE-42 is required in sperm and is proposed to act at a step in fertilization just before or during sperm-egg membrane fusion (Kroft et al., 2005). Snky is the third DC-STAMP domain-containing protein to have its function studied and, like Spe-42, it has a role in sperm function. Although the precise molecular function of the DC-STAMP domain remains unknown, these three examples support a role in mediating membrane-membrane interactions.

The predicted structure of Snky, with its multiple transmembrane domains, and our Snky-GFP localization studies provide evidence that Snky is an acrosomal membrane protein. This localization presents the intriguing question of how an acrosomal membrane protein is able to influence the integrity of the sperm plasma membrane. The findings also have broader implications for acrosome function. The acrosome is a Golgi-derived membrane-bound organelle found at the apical end of sperm, and its function has been extensively studied in marine invertebrates and mammals. In these organisms, the acrosome is best known as a specialized secretory vesicle that undergoes exocytosis. Like many secretory

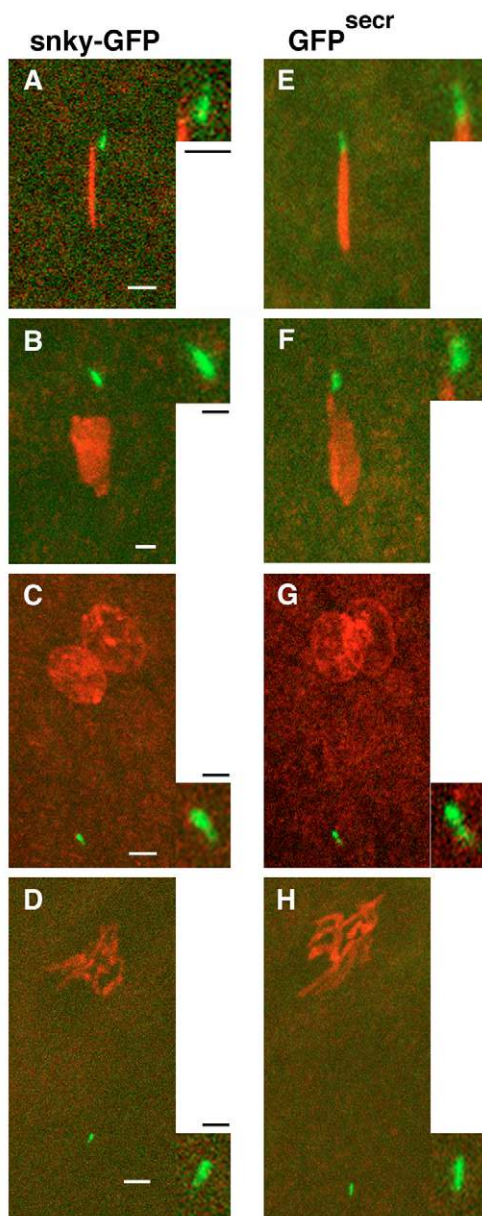


Fig. 6. Localization of Snky-GFP and GFP^{secr} during fertilization. (A-D) GFP (green) was monitored in eggs fertilized by sperm from *snky*¹ males that carried two copies of a *snky-GFP* transgene. Nuclei (red) were stained with DAPI. Insets to the right of each picture show higher magnification view of the GFP signal. (A) A single GFP signal is observed just apical to the condensed sperm nucleus and (B) more distantly as the sperm nucleus decondenses. (C) GFP is seen near the apposing male and female pronuclei and (D) by the chromosomes during prometaphase of the first embryonic cycle. (E-H) In eggs fertilized by sperm from males expressing GFP^{secr}, the GFP signals appear similar to those described for Snky-GFP. Scale bars: 3 μ m in A,B,E,F; 6 μ m in C,D,G,H; 2 μ m in the higher magnification insets.

events, a rise in intracellular Ca^{2+} is required for acrosome exocytosis. This increase in Ca^{2+} requires influx into the sperm as well as efflux from internal stores. The acrosome has been identified as an internal source of Ca^{2+} and is believed to release Ca^{2+} to contribute to its own exocytosis (Walensky and Snyder, 1995; Herrick et al., 2005). Ultrastructural studies show that exocytosis involves vesiculation of the outer acrosomal membrane and overlying plasma membrane. This results in the release of contents of the acrosome, which include hydrolytic enzymes and other components that facilitate binding and penetration through the egg coats. Exocytosis also exposes the inner acrosomal membrane, resulting in a new membrane patch and associated acrosomal molecules on the surface of the sperm. In marine invertebrates, this newly exposed region is the site of binding and membrane fusion with the egg (Colwin and Colwin, 1967; Lopo et al., 1982), providing a direct physical link between the requirement for acrosome exocytosis and membrane fusion. In mammals, acrosome exocytosis is also a prerequisite for sperm to bind to and fuse with the egg. However, the plasma membrane that lies over the equatorial segment of the acrosome, and not the acrosome membrane itself, is believed to be the point of membrane fusion with the egg (Yanagimachi, 1994). Experimental studies of hamster sperm by Takano et al. (Takano et al., 1998) suggest that fusion competency of this specialized region of mammalian sperm requires changes that occur immediately before or during acrosome exocytosis, as well as the contents of the acrosome.

Considering these known acrosome functions, it is interesting that acrosomes are not universal features of animal sperm. Acrosomes are not present in the amoeboid sperm of nematodes, and they are occasionally absent or reduced in species that otherwise possess the flagellated sperm typical of animals (Baccetti, 1979). For instance, the absence or reduction of an acrosome in mature sperm of teleosts (Coward et al., 1996) and certain insect species (Baccetti, 1972; Dallai et al., 2003) is well documented and is generally considered a derived condition (Baccetti, 1979). Hence the requirement for the acrosome during fertilization has been eliminated or bypassed in some lineages during the course of evolution. Ultrastructural studies show that an acrosome is typical of insect sperm (Baccetti, 1972). However, few studies have examined the role of the insect acrosome during fertilization. Studies of fertilization in the house fly *Musca domestica* suggested 'loosening or loss' of the sperm plasma membrane before entry into the micropyle of the egg, followed by exocytosis of acrosomal contents during passage of the sperm through the micropyle (Degrugillier and Leopold, 1976).

Our studies revealed a different fate for the *Drosophila* acrosome. The observation that Snky-GFP was a paternally contributed molecule to the early egg was a surprising finding. The persistence of a single prominent Snky-GFP structure, with an intensity and shape in eggs that appeared similar to those seen in mature sperm, suggests the possibility that the inherited structure was an intact acrosome. This possibility was further supported by studies tracking GFP^{scer} , a soluble protein that is secreted into the extracellular space when expressed in other *Drosophila* cells (Pfeiffer et al., 2002). Its robust and identical appearance to Snky-GFP argues against exocytosis, at least until after prometaphase of the first embryonic cell cycle.

These cytological observations of the fate of the acrosome during normal fertilization, combined with the defect in PMBD observed in *snky*⁻ mutant sperm, suggest that the acrosome may be acting primarily as a signaling vesicle to elicit changes in the overlying sperm plasma membrane. This activity requires the Snky acrosomal membrane protein, but occurs without acrosome

exocytosis. Snky may be serving as a receptor that permits communication between the acrosome and plasma membrane. Alternatively, Snky or its associated proteins may serve to initiate or maintain contact between the membranes, or modify membrane lipids or proteins in preparation for PMBD. In this sense, Snky, DC-STAMP and SPE-42 may share a common mechanism in promoting membrane interactions (Yagi et al., 2005; Kroft et al., 2005). However, in the case of Snky, the pathway would lead to breakdown of the overlying plasma membrane, rather than fusion between two membranes.

In *Drosophila*, Snky is required specifically for male fertility. It will be interesting to determine whether Snky family members are required for sperm function in zebrafish, which have sperm that lack acrosomes, and for acrosome function in marine invertebrate and mammalian sperm. If human Snky-like proteins are specifically required for sperm function, then they may be potential targets for male contraceptives. More immediately, our studies of Snky provide tools for further investigating how the membrane events that occur during *Drosophila* fertilization compare to conventional views of membrane dynamics during sperm activation and fertilization in animals. Research on the acrosome has focused primarily on its function as a specialized secretory or Ca^{2+} storage vesicle. Our studies suggest a primary role as signaling vesicle in *Drosophila*, with a newly identified acrosomal membrane protein communicating directly or indirectly with the plasma membrane to affect changes in membrane integrity. Comparisons among species should continue to shed light on the intriguing ways in which sperm structures and fertilization molecules, such as Snky, may be selected for conservation or diversification during the course of evolution.

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