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Separating from the pack: Molecular mechanisms of *Drosophila* spermatid individualization

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Successful completion of gametogenesis is critical for perpetuation of the species. In addition to the inherent interest, studies of gamete development, in particular spermatogenesis, have yielded insight into diverse biological processes, including actin and microtubule organization, mitochondrial dynamics, plasma membrane remodeling, lipid signaling, apoptosis, and many others.

Mammalian sperm are formed from germline stem cells that reside near the basal surface of the seminiferous tubules.[1] Spermatogonia produced from these stem cells undergo amplifying mitotic divisions with incomplete cytokinesis to eventually produce interconnected chains of spermatocytes that synchronously transition into meiosis.[2,99] Cytokinesis of the meiotic divisions also is incomplete, such that cytoplasmic channels remain between sister spermatids after each division.[3,4] This allows for the sharing of cytoplasm between sister spematids, which synchronizes their development and protects them from the genetic effects of haploidy.[3,5,6] Following meiosis, the haploid spermatids undergo spermiogenesis, the terminal differentiation process wherein acrosomes are formed from Golgi, chromatin compacts, the nuclei are reshaped, and the flagella elongate.[7,8] After terminal differentiation, the cytoplasmic contents are removed and the cytoplasmic bridges connecting sister spermatozoa are dissolved.[9,10] This last process is dependent on the actin cytoskeleton and is essential for proper sperm function.[11-14] The spermatozoa are released from the testis into the epididymis, where their plasma membranes undergo molecular changes.[15,16] Epididymal activation is required for motility and fertilization.[17]

Spermatogenesis is strikingly similar in the fruit fly, and many molecular players are conserved between mammals and *Drosophila*.[18,19] A single *Drosophila* gonialblast, formed by division of a germline stem cell, undergoes four mitotic divisions and two meiotic divisions to produce 64 interconnected sister spermatids in a germline cyst.[20,97] As in mammals, incomplete cytokinesis leads to cytoplasmic sharing between sister spermatids, via intercellular bridges called ring canals.[3] Following nuclear compaction and formation of the flagella, the interspermatid bridges are dissolved concurrently with cytoplasm removal in an actin-dependent process called spermatid individualization.[21,22] Much has been discovered about this process in the 21st century.

Individualization is carried out by the individualization complex (IC), which first forms at the rostral end of the cyst, around the spermatid nuclei (**Figure 1**). The IC is composed of 64 actin cones, one for each germ nucleus of the cyst.[21,22] Actin filaments form a meshwork at the leading edge of the cones and are organized into parallel bundles at the rear of the cones.[23] The meshwork is formed by the Arp2/3 actin nucleating complex.[24,25] The actin motor Myosin VI works with unknown binding partners to localize Arp2/3 and to stabilize the meshwork at the front of the cones.[23,24,26] Other factors at the cone fronts include Actin Capping Protein and Cortactin, and the membrane binding protein Amphiphysin.[24] At the rear of the cones, the actin bundling proteins Quail/Villin, Chickadee/Profilin, and Singed/Fascin localize.[25] As individualization proceeds, the actin cones of the IC move synchronously away from the nuclei toward the caudal end of the cyst, traversing the spermatid flagella at an average speed of 3 μm/minute and finishing the 1.8 mm journey in 10 hours.[27] As it travels, the IC removes the cyst cytoplasmic contents and individualizes each spermatozoon in its own plasma membrane (**Figure 1**).[21] The cones accumulate actin during this process, especially at their front edges, and proper accumulation of actin filaments in the leading edge meshwork is required for cytoplasmic extrusion.[23,25] Extruded cytoplasmic contents are collected in a cystic bulge that forms around the IC.[21] When the IC and cystic bulge reach the end of the flagella, the actin cones and cytoplasmic contents find themselves in a waste bag, the contents of which are degraded.[21] It is not yet known what generates the force for IC movement. Although Myosins V and VI are important for this process, motor activity does not seem to power migration of the

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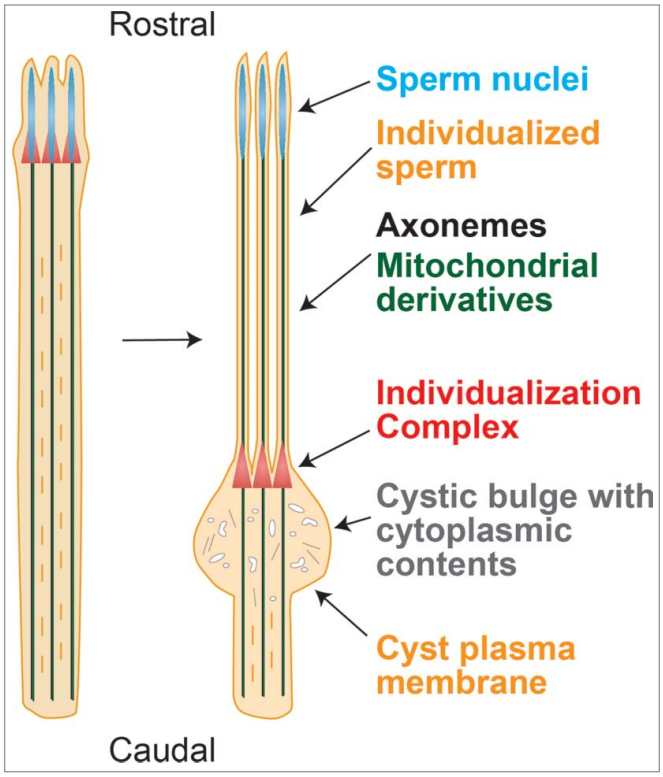

Figure 1. Spermatid individualization in Drosophila. The actin cones of the individualization complex (IC, red) form around the spermatid nuclei, which are located rostrally in the germline cyst. As the IC moves caudally down the spermatid flagella, it removes the cytoplasmic contents and invests each sperm in its own plasma membrane.

IC.[23,27-29] The observation that actin polymerization is essential for IC progression suggests that the IC moves by incorporating new actin filaments at the cones,[21,27] and experimental evidence indicates that the rear bundles specifically are involved.[25]

Classical genetic studies have identified at least 70 genes that mutate to give individualization phenotypes (**Table 1**). Grouping these factors according to function underlines several molecular pathways that contribute to the process. As expected, a number of actin binding proteins and regulators are required for individualization, many of which localize to the IC. Myosin V and the focal adhesion protein Lasp localize to the actin cones during their formation and are required for assembly of the cones around the nuclei.[29,30] Chickadee/Profilin is required for IC movement and localizes to the rear of the cones. In its absence, the cones are short, lack rear bundles, and do not progress away from the nuclei.[25] In contrast, ICs lacking leading edge proteins, such as Myosin VI or Arp3, have thin actin cones that are able to progress caudally, but these cones accumulate less actin, particularly in the front meshwork, do not remain in sync, and fail to successfully individualize the spermatids.[23,25] Intriguingly, the actin regulator Rotund, a GTPase activating protein (GAP) for the signaling protein Rac, also plays a role in this process, suggesting that a signal cascade initiates IC formation or movement.[31] The identity of such signal has yet to be discovered. Casein kinase might also be involved in transduction of the signal.[32]

The microtubule cytoskeleton, as well as the actin cytoskeleton, seems to be important for individualization. Loss of the microtubule binding protein Abnormal Spindle (Asp) results in many spermatogenesis defects, including failed individualization.[33] Mutations in components of the Dynein-Dynactin complex, including Cytoplasmic Dynein Intermediate Chain (CDIC) and two *Drosophila* Dynein Light Chains, DDLC1 and DLC90F, perturb synchronous movement of the actin cones, but they also perturb nuclear shaping and positioning.[34-36] Mutations in two other genes implicated in cytoskeletal dynamics, *yuri gagarin* and *merlin,* disrupt both nuclei and ICs as well.[37,38] Cytoplasmic microtubules adjacent to the nuclei are important for nuclear shaping,[39] which in turn may be required for the IC to assemble properly. Alternatively, microtubules in the vicinity of the nuclei might play independent roles in nuclear shaping and in aligning the actin cones during their formation. Recently, the individualization mutant *mulet* was mapped to a tubulin-specific chaperone E-like protein (TBCEL), again pointing to a role for microtubules.[40,41] Unlike the Dynein-Dynactin complex mutations, the *mulet* mutation disrupts IC translocation without affecting the nuclei.[22,40] The TBCEL protein can block microtubule assembly by disrupting tubulin heterodimers, and in the *mulet* mutant, cytoplasmic microtubules persist aberrantly in individualizing cysts, suggesting that these microtubules interfere with IC progression.[40] Altogether, these observations indicate that cytoplasmic microtubules are important for assembly of the IC around the nuclei but must be cleared in order for the IC to translocate. Experiments with microtubule depolymerizing or stabilizing drugs in cultured cysts suggest that cytoplasmic microtubules are not involved in IC movement per se, but it is not clear whether the progressing IC interacts with axonemal microtubules.[27] When axonemal microtubules are not properly post-translationally modified, individualization is affected.[42,43] Furthermore, the putative axonemal Dynein Intermediate Chain Dic61B is required for individualization.[44] However, other studies suggest that individualization can occur normally in the absence of certain axonemal components.[45] Finally, DDLC1 plays a role in actin accumulation on the cones, which is independent of the Dynein-Dynactin motor and could result from its association with Myosin V.[34,46]

The individualization process may require deposition of new membrane between the spermatids. Other processes that involve new plasma membrane deposition, such as cytokinesis and spermatid elongation, use vesicles to shuttle phospholipids from the Golgi.[47-49] However, visualization of membranes with fluorescent dye shows little vesicle trafficking at the cystic bulge during IC progression.[27] Despite this observation, a number of vesicle trafficking factors are required for individualization, including Auxilin, Clathrin Heavy Chain, Rab11, Shibire/Dynamin, Vps28, and the Vps54-like protein Scattered.[22,24,34,50-52] The cystic bulge contains numerous membraneous structures, and puncta within the cystic bulge stain positively for the endocytic adaptor α-adaptin.[21,24,27] Because most of the cytoplasmic contents are removed by the individualization process, perhaps

Table 1. Drosophila genes in spermatid individualization

Gene/protein	IC phenotype in mutant	EM[a] showing individualization defects?	Caspase staining?	Other spermatogenesis phenotypes	Reference	Molecular function	Cellular function
Ark/ Apaf-1	Scattered migrating ICs, reduced cystic bulges and waste bags	+	Active caspase staining reduced	None reported	[58,59]	Apoptosis effector	Apoptosis
Dcp-1	ND[b]	+	ND	None reported	[58]	Caspase	Apoptosis
Dredd	Scattered migrating ICs	+	No change[c]	None reported	[58]	Caspase	Apoptosis
Drice	ND	+	No change	None reported	[61]	Caspase	Apoptosis
Driceless	Normal ICs	+	Active caspase staining eliminated	None reported	[58]	Apoptosis effector	Apoptosis
Dronc	Scattered migrating ICs, reduced cystic bulges and waste bags	+	Active caspase staining reduced	None reported	[58,59]	Caspase	Apoptosis
Fadd	Scattered migrating ICs	+	No change	None reported	[58]	Apoptosis effector	Apoptosis
Hid	Scattered migrating ICs	+	No change	None reported	[58]	Apoptosis effector	Apoptosis
Tango7	Scattered migrating ICs	ND	Active caspase staining eliminated	None reported	[60]	Apoptosis effector	Apoptosis
Cytochrome-c-d	No migrating ICs	+	Active caspase staining eliminated	Axoneme microtubules are not properly polyglycylated, defective mitochondrial derivatives	[58,59, 62]	Apoptosis effector, electron transport chain	Apoptosis, mitochondria
Bruce	ICs do not form normally	ND	No change	Scattered misshapen nuclei	[62,63]	Inhibitor of apoptosis (IAP), ubiquitin conjugating enzyme (E2)	Apoptosis, ubiquitin-proteasome pathway
Arp3	Thin migrating actin cones, less actin density on ICs, reduced cystic bulges	+	ND	Defective minor mitochondrial derivative	[25]	Actin meshwork nucleator	Cytoskeleton
Abnormal spindle	ND	+	ND	Meiosis failure, abnormal spindles, defective mitochondrial derivatives	[33]	Cytoskeleton regulator	Cytoskeleton
Bug22	Scattered migrating ICs, reduced cystic bulges	+	No change	Defective mitochondrial derivatives, abnormal axoneme structure,	[42]	Ciliary protein	Cytoskeleton

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Table 1. Drosophila genes in spermatid individualization *(Continued)*

Gene/protein	IC phenotype in mutant	EM[a] showing individualization defects?	Caspase staining?	Other spermatogenesis phenotypes	Reference	Molecular function	Cellular function
				abnormal centrioles, axoneme microtubules are not properly polyglycylated			
CDIC/ Short wing	Scattered migrating ICs	ND	ND	Scattered nuclei	[34]	Microtubule motor	Cytoskeleton
Dynein intermediate chain 61B	Scattered migrating ICs	ND	ND	Defective mitochondrial derivatives, abnormal axoneme structure	[44]	Microtubule motor	Cytoskeleton
Dynein light chain 1/ Cut up	Scattered migrating ICs, less actin density on ICs	+	ND	Scattered nuclei, spermatid elongation abnormal	[34,35]	Microtubule motor	Cytoskeleton
Dynein light chain 90F/ Tctex	Scattered migrating ICs	ND	ND	Scattered misshapen nuclei, defective basal body positioning	[36]	Microtubule motor	Cytoskeleton
Lasp	ICs do not form normally, less actin density on ICs	+	ND	Hub is displaced from apical tip, premature sperm coiling, cyst degeneration	[30]	Actin binding protein	Cytoskeleton
Merlin	Scattered migrating ICs	+	ND	Mild cytokinesis defects, cyst polarization defects, scattered misshapen nuclei, defective mitochondrial derivatives	[38]	Cytoskeleton regulator	Cytoskeleton
Mulet	Scattered migrating ICs	ND	ND	Cytoplasmic microtubules persist	[22, 40, 41]	Tubulin-specific chaperone E-like	Cytoskeleton
Myosin V	ICs do not form normally, scattered migrating ICs	ND	ND	None reported	[29]	Actin motor	Cytoskeleton
Myosin VI/ Jaguar	Scattered migrating ICs, less actin density on ICs, reduced cystic bulges	+	No change	Mild nuclear scattering	[23, 28, 62]	Actin motor	Cytoskeleton
Profilin/ Chickadee	No migrating ICs, short actin cones	ND	ND	Cytokinesis defects	[25]	Actin bundling regulator	Cytoskeleton

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Table 1. Drosophila genes in spermatid individualization *(Continued)*

Gene/protein	IC phenotype in mutant	EM[a] showing individualization defects?	Caspase staining?	Other spermatogenesis phenotypes	Reference	Molecular function	Cellular function
RotundRacGAP	No migrating ICs	+	ND	Spermatid elongation abnormal, reduced interspermatid membranes, randomly oriented axonemes, defective mitochondrial derivatives, disorganized cytoplasmic microtubules	[31]	GTPase activating protein	Cytoskeleton
Tubulin Tyrosine Ligase-like 3B	Scattered migrating ICs	+	ND	Swollen testis tips, axonemes degenerate	[43]	Glycylase	Cytoskeleton
Yuri gagarin	ICs do not form normally	+	ND	Scattered misshapen nuclei, coiling defects, defective mitochondrial derivatives, defective basal body positioning	[37]	Cytoskeleton regulator	Cytoskeleton
Fan	Scattered migrating ICs	ND	Ectopic caspases behind IC	None reported	[55]	ER resident, OSBP binding	Lipid metabolism
Noa	Scattered migrating ICs	ND	ND	Small testes, scattered nuclei	[90]	Fatty acid elongase	Lipid metabolism
NPC1	Scattered migrating ICs	+	Ectopic caspases behind IC	Scattered misshapen nuclei, mild cytokinesis defects	[56]	Sterol transport	Lipid metabolism
Osbp	Scattered migrating ICs	+	Ectopic caspases behind IC	None reported	[55]	Sterol transport	Lipid metabolism
Oys and Nes	Scattered migrating ICs	ND	No change	None reported	[57]	Lysophospholipid acyltransferase	Lipid metabolism
Pxt	Scattered migrating ICs	ND	ND	None reported	[98]	Cyclooxygenase	Lipid metabolism
Hsp60B	Scattered migrating ICs	ND	ND	None reported	[91]	Heat shock protein, chaperone	Mitochondria
Mitoferrin	ICs do not form normally	+	ND	Scattered nuclei, defective mitochondrial derivatives, spermatid elongation abnormal	[72]	Mitochondrial carrier protein	Mitochondria
Parkin	Scattered migrating ICs	+	ND	Defective mitochondrial derivatives, mild nuclear	[65, 67-69]	Ubiquitin ligase (E3)	Mitochondria

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Table 1. Drosophila genes in spermatid individualization *(Continued)*

Gene/protein	IC phenotype in mutant	EM[a] showing individualization defects?	Caspase staining?	Other spermatogenesis phenotypes	Reference	Molecular function	Cellular function
				scattering, disorganized cytoplasmic microtubules			
Pink1	ND	+	ND	Defective mitochondrial derivatives	66-68	Kinase	Mitochondria
Tafazzin	ND	+	ND	None reported	70	Cardiolipin transacylase	Mitochondria
Topo IIIα	Scattered migrating ICs	ND	ND	Scattered nuclei, germline stem cell loss	71	Topoisomerase	Mitochondria
Ago2	Scattered migrating ICs	ND	ND	Scattered nuclei	81	RISC complex	RNAi pathway
Blanks/ Lump	Scattered migrating ICs, arrested ICs	ND	ND	Scattered nuclei	82,83	dsRNA binding	RNAi pathway
Dcr2	Scattered migrating ICs	ND	ND	Scattered nuclei	81	dsRNA processing	RNAi pathway
HpRNA1	Scattered migrating ICs	ND	ND	Mild nuclear scattering	81	Non-coding hairpin RNA	RNAi pathway
Crossbronx	Scattered ICs	ND	ND	Scattered nuclei	22	Ubiquitin conjugating enzyme (E2)	Ubiquitin-proteasome pathway
Cullin-3	ND	ND	Active caspase staining eliminated	None reported	64	Ubiquitin ligase complex	Ubiquitin-proteasome pathway
Klhl10	ND	ND	Active caspase staining eliminated	None reported	64	Ubiquitin ligase complex	Ubiquitin-proteasome pathway
Nutcracker	ICs do not form normally	+	Active caspase staining eliminated	None reported	92	Ubiquitin ligase complex	Ubiquitin-proteasome pathway
Prosα6T	Scattered migrating ICs	ND	Active caspase staining reduced	Scattered misshapen nuclei	93	Proteasome core subunit	Ubiquitin-proteasome pathway
Purity of essence	Reduced cystic bulges, less actin density on ICs	ND	No change	Mild nuclear scattering	22, 62	Ubiquitin ligase (E3)	Ubiquitin-proteasome pathway
Roc1b	ND	ND	Active caspase staining reduced	None reported	64	Ubiquitin ligase complex	Ubiquitin-proteasome pathway
Scotti	Scattered migrating ICs, arrested ICs, reduced cystic bulges	ND	Elevated active caspase staining	None reported	63	Ubiquitin ligase inhibitor	Ubiquitin-proteasome pathway
Auxilin		+	ND		50	Clathrin regulator	Vesicle transport

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Table 1. Drosophila genes in spermatid individualization *(Continued)*

Gene/protein	IC phenotype in mutant	EM[a] showing individualization defects?	Caspase staining?	Other spermatogenesis phenotypes	Reference	Molecular function	Cellular function
	IC formation delayed, scattered migrating ICs			Mild nuclear scattering, abnormal spermatid plasma membranes, mild cytokinesis defects			
Clathrin heavy chain	No migrating ICs	+	ND	Scattered nuclei, abnormal spermatid plasma membranes	[22,50]	Vesicle coat	Vesicle transport
Dynamin/Shibire	ICs do not form normally, less actin density on ICs	ND	ND	None reported	[24,34]	GTPase, endocytosis	Vesicle transport
Rab11	Less actin density on ICs, scattered migrating ICs	ND	ND	None reported	[52]	GTPase	Vesicle transport
Scattered	Scattered migrating ICs	ND	ND	Scattered misshapen nuclei	[22]	Vps54-like, Golgi Associated Retrograde Protein (GARP) complex	Vesicle transport
Vps28	Scattered migrating ICs	ND	ND	None reported	[51]	ESCRT-I complex	Vesicle transport
Asunder	Scattered migrating ICs	ND	ND	Cytokinesis defects, meiosis failure, abnormal spindles, defective basal body positioning, scattered misshapen nuclei	[94]	Integrator complex	RNA metabolism
eIF4E-3	ICs do not form normally	ND	ND	Cytokinesis defects, abnormal meiotic segregation, scattered misshapen nuclei	[86]	Translation initiation factor	RNA metabolism
GLD2	ICs do not form normally	ND	ND	Scattered nuclei, abnormal chromatin compaction, defective basal body positioning	[88]	Cytoplasmic poly(A) polymerase	RNA metabolism
Novel spermatogenesis regulator	Scattered migrating ICs, reduced cystic bulges	+	ND	Coiling defects, abnormal axoneme structure	[87]	RNA binding protein	RNA metabolism
Orb2	ICs do not form normally, scattered migrating ICs	ND	ND	Meiosis failure, scattered misshapen nuclei, defective nebenkerne, spermatid elongation	[85]	Cytoplasmic Polyadenylation Element Binding protein	RNA metabolism

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Table 1. Drosophila genes in spermatid individualization *(Continued)*

Gene/protein	IC phenotype in mutant	EM[a] showing individualization defects?	Caspase staining?	Other spermatogenesis phenotypes	Reference	Molecular function	Cellular function
				abnormal, swollen testis tips			
Polypyrimidine tract-binding protein/ hnRNP1/ Hephaestus	Scattered migrating ICs	ND	No change	Scattered nuclei, swollen testis tips	[62,89]	RNA binding protein	RNA metabolism
Dud	Scattered migrating ICs	ND	ND	Mild nuclear scattering, lacking nebenkerne	[22]	ND	
Gilgamesh	Scattered migrating ICs	+	ND	Scattered nuclei	[32]	Casein kinase γ1	Signaling
Gudu	Scattered migrating ICs	ND	ND	None reported	[95]	ND	
Long island expressway	Scattered migrating ICs	ND	ND	Scattered nuclei	[22]	ND	
Mozzarella	ICs do not form normally	ND	ND	Scattered misshapen nuclei	[22]	ND	
Nanking	ICs do not form normally	ND	ND	Mild nuclear scattering, defective nebenkerne	[22]	ND	
Rae1	ICs do not form normally	ND	ND	Nuclear herniation, abnormal chromatin compaction, abnormal spindles, meiosis failure, defective nebenkerne, scattered misshapen nuclei	[96]	WD40 protein	
Thousand points of light	Scattered migrating ICs	ND	ND	Scattered misshapen nuclei	[22]	ND	

[a]EM, electron micrograph.
[b]ND, no data.
[c]No change indicates that active caspases are still seen, but in many of these mutants, cystic bulges are not normal.

vesicle trafficking prior to IC progression segregates cellular components destined for degradation from those that will remain in the mature spermatozoa. Vesicles within the cystic bulge could provide specific lipids for incorporation into sperm membranes as well (see below). *shibire* mutants show additional defects in actin accumulation on the ICs, suggesting that Shibire/Dynamin plays a role in IC assembly as well as translocation.[24,34] Dynamin could anchor the plasma membrane to the IC, possibly in concert (or in parallel) with Amphiphysin, Cortactin, and Myosin VI.[24,26,53] Alternatively, Dynamin could play a role in actin deposition independent of the membrane, perhaps with DDLC1.[34,54] Some of the other vesicle trafficking mutants show nuclear defects, suggesting that they may also be required for IC assembly.[22,50] Thus, it is not clear whether vesicle trafficking plays a direct role in IC movement.

Several lipid metabolism factors are required for individualization. In the absence of the sterol trafficking proteins OSBP, Fan, and NPC1, the actin cones do not migrate synchronously.[55,56] Using filipin dye, sterols can be visualized in puncta within the cystic bulge, suggesting that trafficking of specific lipids occurs during this process.[55] In mammals, the molecular composition of the sperm plasma membrane changes during maturation, and proper composition is required for fertility.[15,16] Furthermore, failure to remove the cytoplasm can lead to peroxidation of membrane lipids and infertility.[13,14] Perhaps a similar process occurs during *Drosophila* individualization, wherein the molecular composition of the sperm membranes is determined during migration of the IC. In this case, membranes and vesicles within the cystic bulge may act as a depot for the lipids. Specific lipids might also tether the IC to the membrane. In addition to cholesterol, phospholipid metabolism pathways contribute to individualization. In the absence of the lysophospholipid acyltransferases Oys and Nes or the cyclooxygenase Pxt, the actin cones do not migrate properly, suggesting that prostaglandin-like lipids generated from membrane phospholipids are important for this process.[57,98] When phospholipid levels are genetically manipulated, no effect is seen, indicating that specific molecules, rather than bulk phospholipids, are critical.[98] Whether these lipids play structural or signaling roles remains to be determined.

The discarded cytoplasm undergoes an apoptosis-like program during the process of individualization. Numerous apoptotic proteins are required for individualization to proceed correctly, including the apoptosis effectors Tango7, Fadd, and Hid and the apoptosome component Ark/Apaf-1.[58-60] These proteins activate the pathway via initiator caspases Dronc and Dredd and effector caspases Drice and Dcp-1.[58,59,61] The spermatid apoptosis program seems to be limited by the inhibitor of apoptosis (IAP) Bruce, and Bruce in turn is localized by the ubiquitin-proteasome system.[62-64] Ubiquitylation of Bruce by the Klhl-10/Cullin-3 ubiquitin ligase complex at the rostral end of the cyst reduces Bruce levels, either by degradation or redistribution, which permits apoptosis initiation there.[63,64] At the caudal end of the cyst, the ubiquitin ligase inhibitor Scotti protects Bruce by preventing its ubiquitylation, thereby preventing apoptosis initiation.[63] Thus, by this mechanism, the apoptosis pathway is limited to the region of the cystic bulge, which begins at the rostral end. However, the spermatid nuclei also reside at the rostral end, and it is not known how they are protected from apoptotic degradation. Several mutants that disrupt movement of the IC have no effect on apoptosis initiation, suggesting that activation of this program is independent of other individualization events.[62] However, mutation of apoptosis components disrupts migration of the IC, indicating that faulty apoptosis can disturb the entire process.

Many other ubiquitin-proteasome pathway components have been identified that participate in individualization (see Table 1). Their targets are not currently known. There seems to be large-scale degradation of cellular components following cytoplasm extrusion.[21] Therefore, it is not clear if the individualization defects observed in these mutants are due to the persistence of specific targets or to a general failure of protein degradation.

Bruce removal alone may not be sufficient to initiate apoptosis. Similarly to mammalian apoptosis pathways, the mitochondria also play a role in apoptosis initiation in spermatids, via Cytochrome c-d.[58,59,62] Intriguingly, mutations that disrupt the mitochondria prevent proper individualization, including those in the genes *pink1*, *parkin*, *mitoferrin,* mitochondrially-targeted *topoisomerase IIIα*, and the cardiolipin transacylase gene *tafazzin.*[65-72] It has yet to be determined if this effect is mediated by Cytochrome c-d.

Spermiogenesis, in particular spermatid individualization, appears to be easily disrupted. Mutagenesis screens have discovered many genes that block spermatogenesis at this late step.[73-75,97] This may be because the process is complex, requiring many factors, as detailed above. Another hypothesis, not mutually exclusive, is that this step represents a checkpoint for the removal of improperly differentiated spermatids.[76] Support for this idea is found in flies experiencing meiotic drive, e.g. heterozygotes for a *Segregation Distorter* (*SD*) second chromosome that prevents formation of viable sperm carrying the other, normal second chromosome by interfering with proper chromatin condensation.[77-79] In heterozygous cysts, in which half of the 64 sister spermatids carry the *SD* chromosome and half carry the normal homolog, the spermatids carrying the normal homolog are blocked at the individualization step, while their sisters are properly individualized and released from the testis.[21,78] Individualization also is very sensitive to temperature, suggesting that cellular stress can halt the process.[98] Other cell stressors have not been tested, but *Wolbachia* infection has been seen to induce mild individualization defects in some cases.[80] Recently, it was found that genetic perturbation of the RNAi pathway causes individualization phenotypes.[81-83] RNAi pathway mutations also perturb cytoskeletal reorganization of the oocyte in a checkpoint-mediated process.[84] This seems to be a way for the oocyte to abort development when chromosomal integrity is disturbed by unregulated transposon activity. Perhaps a similar mechanism operates in spermatogenesis. Some, but not all, mutants that disrupt individualization show other spermatogenesis phenotypes; thus their effects on individualization may be indirect.

In conclusion, genetic studies have identified numerous genes required for individualization of the differentiated spermatozoa, the final step of spermatogenesis. Many of these genes have been

characterized molecularly, and they have highlighted important mechanisms at play during this process, including actin and microtubule dynamics, plasma membrane reorganization, and apoptotic elimination of the cytoplasmic contents. Many questions still persist, including: What are the signals that initiate individualization? How is the membrane reorganized, structurally and molecularly? How is membrane reorganization coordinated with IC movement? What propels IC movement? How are cytoplasmic components correctly partitioned into the cystic bulge? What protects the nucleus from the apoptosis pathway? Do all mutations that perturb individualization do so directly? How is gene expression coordinated at this developmental stage? It is likely that many factors are regulated post-transcriptionally, as the spermatid nuclei are highly condensed by this time, and indeed, RNA metabolism proteins play important roles in this process.[85-89] Furthermore, several genes necessary for individualization have not been characterized molecularly yet, and more genes acting in the process likely will be discovered. Future studies will elucidate a more coherent model that will undoubtedly reveal interesting molecular mechanisms and shed light on human fertility and infertility as well.

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