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The role of acroblast formation during *Drosophila* spermatogenesis

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Running title: acroblast-acrosome formation

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Key words: acrosome, acroblast, Golgi, *Drosophila*, spermatogenesis

28 **Summary Statement**

29 This study demonstrates that a functional acroblast is essential for normal nuclear elongation
30 and acrosome formation during *Drosophila* spermatogenesis.

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Abstract

Protein recycling is important for maintaining homeostasis of the Golgi and its cisternae. The Vps54 (Scat) protein, a subunit of the GARP tethering complex, is a central factor in retrograde transport to the *trans*-Golgi. We found the *scat^l* mutant to be male sterile in *Drosophila* with individualization problems during spermatogenesis. Another typically observed phenotype was the abnormal nuclear structure in elongated mutant cysts. Examining the structure and function of the Golgi a failure in acrosome formation and endosome-Golgi vesicular transport were found in the *scat^l* mutant. This acrosome formation defect was due to a fault in the *trans*-Golgi side of the acroblast ribbon. Testing a mutation in a second retrograde transport protein, Fws, a subunit of the COG tethering complex, the acroblast structure was again disrupted. *fws^P*, caused a similar albeit milder acrosome and sperm individualization phenotype as the *scat^l* mutant. In the case of *fws^P* the *cis* side of the acroblast ribbon was dispersed, in-line with the intra-Golgi retrograde function of COG. Our results highlight the importance of an intact acroblast for acrosome formation, nuclear elongation and therefore sperm maturation. Moreover, these results suggest the importance of retrograde tethering complexes in the formation of a functional Golgi ribbon.

51 **Introduction**

52

53 In most mammalian cell types the Golgi apparatus appears as a ribbon formed from
54 interconnected stacks of cisternae. However, this arrangement is not universal. For example,
55 in gastric parietal cells the Golgi appears in the form of mini-stacks dispersed throughout the
56 cytoplasm (Gunn et al., 2011). This scattered arrangement is common in most cell types of
57 the fruit fly *D. melanogaster* (Kondylis and Rabouille, 2009). Several possible roles have
58 been proposed for the assembly of Golgi stacks into a ribbon. These include an increase in
59 efficiency and uniformity of glycosylation (Puthenveedu et al., 2006), a necessity of the
60 ribbon for the secretion of large cargoes (Lavieu et al., 2014), and importantly, a role in
61 polarized secretion (Horton et al., 2005). Generation of the Golgi ribbon requires microtubule
62 mediated transport of stacks or vesicles forming them into the vicinity of the microtubule
63 organizing center (Wehland et al., 1983), followed by tethering and fusion of these into a
64 ribbon (Marra et al., 2007). Specialized cell types in non-vertebrates can also present an
65 assemblage of Golgi stacks in a perinuclear location. For example, the Golgi apparatus of
66 developing *Drosophila* spermatids is in such a peri-nuclear location just prior to and during
67 the nuclear elongation phase of spermatogenesis (Kondylis and Rabouille, 2009). This
68 specialized Golgi assemblage, known as the acroblast, is likely needed to organize the
69 secretory pathway in this highly polarized cell type of the fruit fly.

70 While it is clear that anterograde transport to the Golgi is essential for generation of a
71 polarized assembly of Golgi stacks, the role of retrograde transport in this process is less well
72 understood. Retrograde transport within and to the Golgi is coordinated by two multisubunit
73 tethering complexes, the Golgi associated retrograde protein (GARP) (Bonifacino and Hierro,
74 2011) and the conserved oligomeric Golgi (COG) (Miller and Ungar, 2012) complexes.
75 GARP is a four subunit complex of the CATCHR (Complexes Associated with Tethering
76 Containing Helical Rods) family (Hughson and Reinisch, 2010), composed of the Vps51,
77 Vps52, Vps53 and Vps54 proteins (Conibear and Stevens, 2000). Its primary role in
78 membrane trafficking is to direct retrograde carriers to the TGN (Conibear and Stevens,
79 2000), such as vesicles that recycle the mannose-6-phosphate receptor (M6PR) (Perez-
80 Victoria et al., 2008), or those carrying the SNARE protein Snc1 (Quenneville et al., 2006)
81 from endosomes to the TGN. Lack of Vps54 in mice causes the *wobbler* phenotype, which
82 manifests in progressive neurodegeneration and male sterility (Schmitt-John et al., 2005). In
83 *Drosophila* the GARP complex has been shown to require the Arl5 GTPase for correct

84 localization, loss of which results in defective recycling of Lerp, the fly homolog of M6PR
85 (Rosa-Ferreira et al., 2015). While COG is also involved in endosome to Golgi transport
86 (Whyte and Munro, 2001), its main function is the intra-Golgi retrograde trafficking of
87 resident Golgi proteins (Oka et al., 2004). The eight COG subunits can be grouped into two
88 lobes, with subunits Cog1-4 forming lobe A, and Cog5-8 lobe B (Ungar et al., 2002). While
89 loss of lobe A function causes defects in the recycling of early Golgi residents, lobe B is
90 mainly involved in late Golgi homeostasis (Oka et al., 2004; Willett et al., 2013; Wu et al.,
91 2004). Consequently, lobe A is essential for development of an organism, its loss is lethal in
92 yeast (Whyte and Munro, 2001) and during early development in *Drosophila* (Schnorrer et
93 al., 2010). In contrast, lobe B loss causes much milder phenotypes, for example loss of Cog5
94 in a human patient was shown to lead to relatively mild psychomotor retardation (Paesold-
95 Burda et al., 2009), while two loss of function alleles of its fly homolog, *fws*, cause male
96 sterility due to incomplete cytokinesis during spermatogenesis (Farkas et al., 2003).
97 Interestingly, COG interacts with the golgin TMF (Miller et al., 2013), which is a critical
98 factor for vesicular transport during late stages of mouse spermatogenesis (Lerer-Goldshtein
99 et al., 2010).

100 During *Drosophila* spermatogenesis, following meiotic division the 64 spermatids
101 undergo a dramatic differentiation program that leads to formation of the highly elongated
102 flagellated mature sperm (Fig. 1A). This process starts with rearrangement and fusion of
103 mitochondria to form the Nebenkern from two mitochondrial derivatives (Fig. 1A) (Tokuyasu
104 et al., 1972). At the same time the basal body is embedded into the nuclear envelope to
105 polarize the nucleus (Vogt et al., 2006). The Golgi apparatus, which is normally a collection
106 of scattered stacks throughout the cytosol, is then recruited to the nucleus at the opposing side
107 to the basal body (Fig. 1A,B) (Fuller, 1993). This polarization event is thought to be essential
108 for subsequent nuclear elongation. The change in nuclear shape is coincident with a major
109 reorganization of chromatin, which manifests in the replacement of histones with protamines.
110 This histone to protamine switch is critical for the proper elongation of the nuclei (Raja and
111 Renkawitz-Pohl, 2005). During the later stages of nuclear elongation the specialized Golgi
112 structure, the acroblast, is converted into the acrosome and the actin-based investment cones
113 are formed (Fig. 1A). These investment cones are also involved in the individualization of the
114 mature sperm when an enormous amount of new membrane is used for elongation, which
115 concludes spermatogenesis (Fabian and Brill, 2012).

116 The acroblast described above, contains all the markers of a typical Golgi apparatus,
117 such as the glycosylation enzyme mannosidase II (Farkas et al., 2003), the golgins

118 Golgin245, GM130 (Hirst and Carmichael, 2011) and Lava lamp (Farkas et al., 2003), the
119 COPI vesicle coat (Kitazawa et al., 2012) and the COG complex (Farkas et al., 2003). In
120 addition, the lysosomal protein Lamp1, and the acrosomal protein Sneaky also localize to the
121 acroblast (Wilson et al., 2006) (Fig. S1G,H). Yet, the acroblast is unusual in *Drosophila*, as it
122 forms a ribbon as opposed to the scattered stacks typical for Golgi architecture in other fruit
123 fly cells (Kondylis and Rabouille, 2009). The molecular determinants of acroblast formation
124 and its breakdown upon acrosome formation are not very well understood, but the Golgi
125 architecture leading to acrosome formation has been recently documented (Yasuno et al.,
126 2013). After meiosis the Golgi is organized around the nucleus and participates in the
127 formation of the acroblast (Fig. 1A,B). Once nuclei elongate the acroblast disassembles and
128 some of the Golgi components, such as Sneaky, together with lysosomal components
129 generate the acrosome, which maintains an apical positioning next to the nucleus (Fig. 1A,D).
130 At the same time the remaining Golgi components migrate to the posterior side of the nucleus
131 and appear as scattered stacks akin to somatic *Drosophila* cells (Fig. 1A,C). The known
132 molecular players that have so far been associated with the formation of the acroblast, and its
133 later breakdown have all been found to affect meiotic division as well (Belloni et al., 2012;
134 Farkas et al., 2003). It is therefore often difficult to tease out direct effects on Golgi
135 architecture from secondary effects due to delays in spermatogenesis and associated defects
136 in polarization. Such factors include microtubules (Yasuno et al., 2013), the
137 phosphatidylinositol transfer protein Giotto (Giansanti et al., 2006), the small GTPase Rab11
138 (Giansanti et al., 2007), the TRAPP II complex (Robinett et al., 2009), as well as the Cog5
139 and Cog7 subunits of COG (Belloni et al., 2012; Farkas et al., 2003).

140 Here we have analyzed two different male sterile P-element insertion mutations; one of
141 the GARP subunit Vps54 (*scat*), the other of the Cog5 (*fws*) subunit of COG. These mutants
142 have no defects in the meiotic phase of sperm development, but nuclear elongation and
143 acrosome formation are both affected. Mutant spermatids of *scat*^l and *fws*^P do not
144 individualize and therefore do not mature. We show that the main defect of these mutants is
145 in the organization of the acroblast and the ensuing completion of the spermatogenic
146 differentiation program. These results highlight an essential function of the GARP and COG
147 mediated retrograde transport processes in the establishment of a polarized Golgi ribbon,
148 which is important in nuclear elongation, individualization and acrosome formation during
149 *Drosophila* spermatogenesis.

150

151 **Results**

152

153 **The *scat*¹ mutant has a male fertility defect**

154

155 In order to probe the function of vesicle tethering complexes that act at the Golgi during
156 spermatogenesis, we investigated the Vps54 subunit of the GARP complex, encoded by the
157 *scat* gene. The *scat*¹ allele was identified as a male sterile mutant with scattered nuclei in a P
158 element screen (Castrillon et al., 1993). The P element is incorporated in the third exon of the
159 gene (Fig. 2A). Genetic characterization showed that homozygous *scat*¹ males were 100%
160 sterile and their seminal vesicle was devoid of mature sperm (Fig. S1 A,B). In contrast, all
161 females were fertile. We tested the *scat*¹ allele in complementation analysis and found male
162 sterility in a hemizygous combination with an overlapping *Df(2L)ED680* deficiency. The
163 male sterility of *scat*¹ was completely reversed by precise excision of the P element. To verify
164 the involvement of the *scat* gene a C-terminally RFP tagged *scat* transgene expressed from a
165 *P{UASp}* vector was used to rescue the male sterile phenotype. Expression of the *P{UASp-*
166 *Scat-RFP}* fusion protein using the germ line specific *Bam-Gal4* driver completely rescued
167 male sterility (Fig. S1 G-L). This proves that the P element insertion within *scat* is indeed
168 responsible for the male sterility and the Scat-RFP fusion protein correctly incorporates into
169 the GARP complex. A polyclonal antibody raised against Scat recognizes the protein at the
170 predicted molecular weight of 105 kDa, as well as the Scat-RFP fusion protein in extracts
171 from wild type or Scat-RFP transgenic testes (Fig. 2B first two lanes). In contrast, in
172 homozygous *scat*¹ mutant testis extracts the protein was absent from the immunoblot,
173 confirming that *scat*¹ is a null mutant (Fig. 2B right lane).

174

175 **Scat is Golgi localized throughout spermatogenesis**

176

177 Mouse Vps54 was shown to localize to both endosomes and Golgi, and to incorporate
178 into the fully developed acrosome (Berruti et al., 2010). This is in contrast with other Golgi
179 trafficking proteins, such as Golgin95 or Golgin97, which localize to the developing
180 acrosome only during the early steps of acrosomogenesis but do not label the testicular
181 spermatozoa (Moreno et al., 2000). We therefore tested the subcellular localization of Scat-
182 RFP during different stages of *Drosophila* spermatogenesis. Both in the early premeiotic and
183 in the late postmeiotic stages Scat localized to the Golgi (Fig. 2C-E), as is typical for the

184 GARP complex (Conibear et al., 2003). This was confirmed by co-staining with anti-
185 dGM130, a protein known to be restricted to the *cis*-Golgi cisternae, just as its mammalian
186 orthologue (Fig. 2C-E) (Sinka et al., 2008). We found that the Scat-RFP signal localized
187 close but slightly displaced from dGM130 in all stages of spermatogenesis, suggesting that
188 Scat is localized to the *trans* side of the Golgi (Fig. 2C-E). Early in the development process,
189 in primary spermatocytes, the RFP stained Golgi is randomly distributed throughout the
190 cytoplasm, similarly to the distribution found for GM130 and other medial/*trans* Golgi
191 markers (Yasuno et al., 2013) (Fig. 2C). Interestingly, after meiosis, during acroblast
192 formation the RFP staining marking the *trans* side of the Golgi was always positioned in the
193 proximity of the nuclei as opposed to the more distally positioned *cis*-Golgi side (Fig. 2D).
194 During nuclear elongation the Golgi localized Sneaky and the lysosomal Lamp then localized
195 to the acrosome (Fig. 3I, FigS2A), but Scat, like the *cis*-Golgi specific dGM130, and the
196 *trans*-Golgi specific dGolgin245 did not. Rather, Scat localizes with the rest of the Golgi
197 markers in the scattered Golgi-stacks that move to the basal side of the nucleus and are later
198 removed in the cystic bulge with the majority of the cytosol (Fig. 2E).

199

200 **Nuclear elongation is disrupted in *scat*¹ males**

201

202 All early steps of spermatogenesis, such as the maintenance of germ stem cells, the
203 formation of primary spermatocytes, and meiotic divisions were normal in *scat*¹ testes.
204 Nucleus to Nebenkern ratio was 1:1 in all round spermatids of the *scat*¹ mutant, suggesting
205 normal cytokinesis (Fig. S1 D,E). We therefore focussed on the post meiotic stages of
206 spermatogenesis to understand how the loss of Scat function perturbs spermatogenesis.
207 Investigating the elongating spermatid nuclei their majority were found in late canoe stage
208 and hardly any were observed as needle shaped in the *scat*¹ mutant cysts (Fig. 3A,B,D,E,G,
209 H, I, J). The lack of needle shaped, fully elongated nuclei correlated with the appearance of
210 scattered spermatid bundles in the *scat*¹ mutant post-meiotic cysts (Fig. 3A,B). Elongation
211 and chromatin condensation occur parallel to each other. As in mammals, chromatin
212 condensation is achieved by a histone to protamine switch during nuclear elongation in
213 *Drosophila* (Raja and Renkawitz-Pohl, 2005). This switch is normal in the *scat*¹ mutant (Fig.
214 3D, E), suggesting that the observed nuclear elongation defect is independent of the
215 chromatin condensation process. The scattering of nuclei could also be caused by defects in
216 basal body formation (Texada et al., 2008). However, visualization of the basal body with

217 GFP-PACT failed to reveal any abnormalities in elongated cysts of the *scat*¹ mutant (Fig.
218 3G,H).

219 Vps54 mutant mice that are male sterile are missing acrosomes (Paiardi et al., 2011).
220 These are normally formed during the later stages of nuclear elongation, so we wondered
221 whether acrosome formation was normal in *scat*¹ mutants. Two different acrosomal markers,
222 Snky-GFP and Lamp1-GFP (Fabian and Brill, 2012; Wilson et al., 2006) both showed
223 acrosomal localization at the tips of elongated nuclei in WT spermatids (Fig. 3I and FigS2
224 A). Yet the GFP signal was diffuse without any recognizable acrosome staining in the same
225 stage of *scat*¹ mutant spermatids (Fig. 3J and FigS2 B).

226 Individualization starts with the formation of 64 actin-rich investment cones adjacent to
227 the nuclei, which move together towards the distal end of the individualizing cyst (Fig 3A)
228 (Fabrizio et al., 1998). In the case of the *scat*¹ mutant we hardly observed any investment
229 cones and the process of individualization did not start. Occasionally we could detect a very
230 faint Phalloidin signal, which could be due to investment cone remnants or the investment
231 cones in the process of degradation, but these were always scattered (Fig. 3B). Following
232 failed individualization the elongated cysts lost their integrity, the cells scattered and died.

233 Thus the earliest defect in spermatogenesis in *scat*¹ mutants is their failure to fully
234 elongate the nuclei. While this is not accompanied with a defect in chromatin condensation, it
235 does lead to a defect in individualization.

236

237 **Acrosome defects are the consequence of the abnormal acroblast formation**

238

239 The failures in nuclear elongation, acrosome formation and individualization all point to
240 a defect following acroblast disassembly. This could be caused by an inherent defect of the
241 acroblast itself in the mutants. The GARP complex is known to contribute to the recycling of
242 M6PR between endosomes and the TGN (Perez-Victoria et al., 2008) in mammals, and
243 therefore we wondered if this trafficking pathway was for example defective at the acroblast.
244 A transgenic line was established with testis specific expression of the *Drosophila* M6PR,
245 *P{tv3-GFP-Lerp}*. This showed acroblast localization in WT spermatids (Fig. 4A), while it
246 had a more dispersed localization in the *scat*¹ mutant (Fig. 4B). The *trans*-Golgi marker
247 Golgin245 showed co-localization with GFP-Lerp in WT, but not in the *scat*¹ mutant
248 postmeiotic round spermatids (Fig. 4A,B). Thus similar to effects in mammalian cells, a
249 faulty GARP complex causes defective retrograde trafficking of M6PR from endosomes to

250 the TGN. Such a defect in the endosome-Golgi trafficking route marked by the M6PR could
251 affect acroblast integrity.

252 The distribution of *cis*- and *trans*-Golgi markers was therefore tested throughout
253 spermatogenesis. In the early stage the *cis*- and *trans*-Golgi markers GM130 and Golgin245
254 are localized close to each other in both WT and *scat¹* mutant spermatocytes (FigS2 D,F). In
255 WT post-meiotic spermatids these *cis*- and *trans*-Golgi markers appear closely apposed to
256 each other in perinuclear localization, consistent with the perinuclear ribbon like Golgi
257 formed by the acroblast (Fig. 4C). However, in the *scat¹* mutants of the same stage,
258 localization of the *trans*-Golgi marker is much more diffuse (Fig. 4D). The failure of GM130
259 and Golgin245 to co-localize in the *scat¹* mutants persists during nuclear elongation when the
260 Golgi travels to the basal side of the nucleus (FigS. 2E,G). However, the high degree of cell
261 death at this stage precludes far reaching conclusions to be drawn from this last stage. Overall
262 these results suggest that a functional GARP complex is necessary for normal acroblast
263 organization. Our data also imply that proper perinuclear organization of the acroblast is
264 required for completion of spermatogenesis, including individualization, acrosome formation
265 and the final stage of nuclear elongation.

266

267 **The intra-Golgi retrograde transport factor Fws is also necessary for acroblast integrity** 268 **and completion of the late stages of spermatogenesis**

269

270 Involvement of GARP-dependent trafficking in acrosome formation has been
271 demonstrated in mice (Paiardi et al., 2011). The fruit fly spermatogenesis model provides a
272 unique opportunity to study the involvement of Golgi ribbon biogenesis in acrosome integrity
273 and formation, since the acroblast is the only true ribbon-like Golgi structure in the
274 developing *Drosophila* sperm. Generation of a Golgi-ribbon is known to require the
275 microtubule mediated transport of Golgi elements to the perinuclear region (Wehland et al.,
276 1983). Our finding that GARP complex function is required for the correct formation of the
277 peri-nuclear Golgi known as acroblast raised the intriguing possibility that other known
278 retrograde transport factors could also be important contributors of Golgi-ribbon formation. A
279 second retrograde trafficking factor, the Fws subunit of the intra-Golgi transport specific
280 COG complex was therefore also investigated. Fws has previously been characterised during
281 spermatogenesis using two EMS alleles (*fws^{z-0161}* and *fws^{z-1201}*). Transheterozygotes of these
282 two alleles were shown to manifest in spermatocyte cytokinesis and spermatid elongation
283 defects (Farkas et al., 2003). Given the strong defect in these EMS mutants during

284 cytokinesis, it is unclear whether the spermatid elongation and associated acrosome formation
285 defects are secondary consequences of the meiotic defect. We therefore decided to investigate
286 the phenotype of a new P element insertion line $fws^{KG02853}$ (fws^P).

287 fws^P contains a P element insertion in the first exon of the fws gene. This disruption of
288 the fws gene results in 74% male sterility in homozygotes and 90% in hemizygotes over the
289 *DfBSC148* deficiency. These numbers show that this mutant is possibly a hypomorphic allele
290 of fws . The sterile homozygous fws^P mutants' seminal vesicles were devoid of mature sperm
291 (Fig1S C). Remobilisation of the P element in fws^P reverted the male sterile phenotype to
292 recover complete fertility. The male sterile phenotype of fws^P was also rescued with the wild
293 type GFP- fws genomic rescue construct (Farkas et al., 2003), suggesting that the P element
294 insertion in fws is indeed responsible for the male sterile phenotype of fws^P (FigS1 M-O).
295 Importantly, in contrast to the previously reported EMS alleles, meiotic cytokinesis in fws^P
296 homozygous testes was normal (Fig. S1D,F) and proper elongation of the post meiotic cysts
297 was observed (Fig. S1F). Phenotypic characterisation of developing spermatids showed
298 scattered nuclei and investment cones in the fws^P mutant cysts (Fig. 3C), but similarly to the
299 *scat^l* mutant the histone protamine transition was again found to be normal (Fig. 3F). Using
300 the acrosomal markers Snky-GFP and Lamp1-GFP showed that the majority of the elongated
301 spermatids do not form acrosomes in the fws^P mutant (Fig. 3K and FigS. 2C). Yet, in contrast
302 to the *scat^l* mutant, in some cases we could observe an acrosomal signal with both transgenes
303 (Fig. 3K and FigS. 2C arrowhead), some of the acrosomes decorating non-scattered nuclei.
304 This is in line with the fertility results suggesting that the fws^P mutant has an overall milder
305 defect than the *scat^l* mutant, likely due to fws^P being a hypomorphic rather than a null-allele.
306 Given the good correlation between the extent of the fertility and acrosome defects it seems
307 that the most sensitive effect of fws disruption is on acrosome formation and nuclear
308 elongation rather than cytokinesis (Farkas et al., 2003).

309 Finally, to test if the primary defect in the fws^P mutant, as in the *scat^l* mutant, is in
310 acroblast organization, the distribution of *cis*- and *trans*-Golgi markers was investigated in
311 fws^P from early stages up to cyst elongation (Fig. 4E and FigS. 2H,I). Similar to the *scat^l*
312 mutant we found a defect in the perinuclear acroblast (Fig. 4E). However, in this instance it
313 was the GM130 marker that showed a more dispersed staining, while Golgin245 remained
314 compact (Fig. 4E). This is in line with the COG complex, involved in intra-Golgi transport
315 (Miller and Ungar, 2012), affecting more the formation of the *cis* side of the Golgi ribbon,
316 while the GARP complex, involved in retrograde transport to the late Golgi (Conibear and
317 Stevens, 2000), affecting the *trans* side.

318

319 **Discussion**

320

321 Our work sheds light on the interplay between vesicle trafficking, Golgi structure,
322 acrosome formation and sperm development. The primary defect during sperm development
323 in the analyzed mutants is disruption of the acroblast structure, which in turn causes defects
324 in acrosome formation, nuclear elongation and individualization. As opposed to previously
325 characterized Golgi trafficking mutants that have been shown to exhibit acroblast
326 abnormalities (Farkas et al., 2003; Giansanti et al., 2006; Robinett et al., 2009), the *scat^l* and
327 *fws^P* mutants exhibit no meiotic cytokinesis defects at all. The Giotto, and TRAPP mutants,
328 as well as the EMS mutagenesis alleles of *fws* did show acroblast defects, but this could have
329 been caused by a generic delay and consequent disruption of spermatogenesis due to
330 defective cytokinesis. Our study is therefore the first clear demonstration that both Scat and
331 Fws and therefore the GARP and COG complexes are essential for establishing correct
332 acroblast morphology. Consequently, the lack of acrosomes in the here described mutants is,
333 again, a more direct demonstration of the need for acroblast homeostasis in order to generate
334 the acrosome and complete nuclear elongation, since other spermatogenesis stages up to the
335 canoe stage of nuclear elongation are normal. The acrosome has been shown to contain
336 elements of the TGN as well as the late endosome/lysosome (Herms et al., 2010). We find
337 that the correct organization of the rest of the Golgi, including both the *cis* and *trans* sides is
338 essential for acrosome formation, despite these Golgi components not being incorporated into
339 the acrosome (Fig. 5). The mutants we describe will therefore be valuable tools in the future
340 to study acrosome biogenesis during *Drosophila* spermatogenesis.

341 Several steps during post-meiotic spermatogenesis occur in parallel or close succession.
342 These include chromatin condensation, basal body formation, acroblast formation, nuclear
343 elongation and acrosome formation. The mutants characterized in this study allow us to place
344 these in a hierarchy of dependence. It is clear that acroblast formation is not required for
345 chromatin condensation, basal body formation and the initial phase of nuclear elongation. At
346 the same time, formation of the acrosome and the elongation to needle shape nuclei cannot
347 proceed even where chromatin has condensed and the basal body formed unless the acroblast
348 is fully functional (Fig. 5). The most important function of the acroblast's intact ribbon
349 during nuclear elongation and acrosome formation is its influence on the polarization of the
350 cyst, which ultimately leads to normal individualization. However, molecular details of the

351 links between acroblast formation and function and the process of individualization remain to
352 be identified.

353 The acroblast is a very special form of the Golgi apparatus in *Drosophila*, since it forms
354 a perinuclear ribbon as opposed to the scattered stacks found in other cells of the fruit fly
355 (Kondylis and Rabouille, 2009). The two mutants analyzed in this study show normal Golgi
356 distribution in cells where the scattered stack morphology is predominant (FigS 2F, H). This
357 implies that the retrograde transport routes defined by GARP and COG are not essential for
358 the formation and maintenance of Golgi stacks in spermatocytes. While Golgi defects are
359 common in mammalian COG mutants (Reynders et al., 2009; Ungar et al., 2002), a loss of
360 Golgi stacks is not observed. Similarly, the ribbon of the acroblast is seriously malformed in
361 both the *scat*¹ and the *fws*^P mutants. This implies that ribbon formation may indeed need the
362 retrograde transport pathways established for GARP (Bonifacino and Hierro, 2011) and COG
363 (Miller and Ungar, 2012) in addition to the well-known contributions of microtubule
364 mediated anterograde transport (Wehland et al., 1983). The fact that the observed disruption
365 in the acroblast is most prominent on either the *trans* side (for GARP) (Conibear and Stevens,
366 2000) or the *medial/cis* side (for COG) (Miller et al., 2013) is in line with the respective
367 known destinations of the transported vesicles (Bonifacino and Hierro, 2011; Willett et al.,
368 2013). Several candidates for the associated machinery that could act together with COG
369 have already been flagged up by other studies, such as the golgins TMF (Schmitt-John et al.,
370 2005) and GMAP210 (Kierszenbaum et al., 2011) that are both essential for acrosome
371 formation in mouse testes. Yet, future studies are required to address what it is that has to be
372 delivered to the particular Golgi areas by COG and GARP mediated retrograde transport in
373 order to generate specific parts of the ribbon: Is it the whole vesicle that is needed, is it a very
374 specific transport factor or factors that have to be recycled, or is it the general protein
375 homeostasis within cisternae, maintained through recycling, that is essential for ribbon
376 maintenance in the acroblast?

377

378 **Materials and methods**

379

380 **Fly stocks, mutants and transgenes**

381 Flies were crossed and maintained on standard cornmeal agar medium at 25°C. Oregon-
382 R stock was used as wild type control. Fertility tests were performed by crossing single males
383 with four wild type females. The progeny was counted in every tube and an average
384 calculated from 30-50 males.

385 The following lines were obtained from the Bloomington Stock Center: *scat*^l,
386 *Df(2L)ED680*, *fws*^P (*fws*^{KG02853}), *Df(2L)ED1175*, *P(His2Av-EGFP.C2)*, *P(protamineB-eGFP)*.
387 Flies carrying the *Snky-GFP*, *GFP-PACT*, *Lamp1-GFP*, *bam-Gal4* and *GFP-fws* transgenes
388 have been described previously (Farkas et al., 2003; Martinez-Campos et al., 2004; Wilson et
389 al., 2006). *Snky-GFP*, *Lamp1-GFP* and *GFP-fws* transgenes were recombined onto the 2nd
390 chromosome with *scat*^l and *fws*^P. Remobilization of the P element in *scat*^l and *fws*^P was done
391 according to Engels et al., 1990 (Engels et al., 1990). Revertant lines were tested for fertility
392 and the precise excisions of the P elements were confirmed by PCR. The C-terminal
393 *P{UASp-Scat-RFP}* construct was generated using the Gateway® cloning system
394 (Invitrogen) according to the manufacturer's instructions, using *scat* cDNA. Transgenic lines
395 were established and the *bam-Gal4* testis specific driver was used to express the transgene in
396 wild type and *scat*^l mutant backgrounds. The *P{tv3-Arl1-mCherry}* and *P{tv3-GFP-Lerp}*
397 transgenic constructs were generated by amplifying the *arl1* and *lerp* cDNAs, and cloning the
398 PCR fragments into a modified testis-vector3 (Wong et al., 2005) containing an insertion of
399 mCherry or GFP to create a C- or N-terminal fusion protein. Transgenic flies were generated
400 on a *w*¹¹¹⁸ background.

401

402 **Staining and microscopy**

403 For immunostaining, intact or partially squashed testes from 2-4 days old wild type and
404 mutant flies were processed as described earlier (White-Cooper, 2004). DAPI (1µg/ml) was
405 used for DNA staining and Texas Red®-X Phalloidin (Invitrogen) was used in 1:250 dilution
406 for actin visualization. Primary antibodies used were: rabbit anti-dGM130 (Abcam) and goat
407 anti-dGolgin245 (1:100, gift of Sean Munro, Riedel et al., 2016).

408 Alexa Fluor 488 and Alexa Fluor 594 conjugated anti-rabbit secondary antibodies were
409 from Invitrogen. The samples were mounted in Fluoromount (Southern Biotech) and imaging
410 was done with an Olympus BX51 fluorescent microscope or an Olympus FV 1000 confocal
411 microscope. Nuclear length was measured by ImageJ, statistical significance of differences
412 determined using a one-way ANOVA on ranks with a Tukey post-hoc test.

413

414 **Antibody generation and western blotting**

415 Polyclonal antibody was raised in guinea pigs immunised using purified His-tagged
416 fusion protein containing the N-terminal 200 residues of Scat expressed from the pET28b
417 vector (Novagen). For immunoblotting analysis adult testes from 40 individual males of each

418 genotype were homogenised in 100 µl of Lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM
419 NaCl, 0.5 mM EDTA, 0.5% NP40, 1 mM PMSF, 1× Protease inhibitor Cocktail) at 4°C.
420 Samples were separated on 8% SDS polyacrylamide gels (Bio-Rad) and transferred to PVDF
421 membrane for immunoblotting. Blocking and antibody incubations were in Tris-buffered
422 saline (Sigma-Aldrich) with 0.05% Tween-20 (TBST) containing 4% nonfat dry milk.
423 Primary antibodies were anti-Scat diluted 1:2000 and anti-tubulin (Abcam). HRP-linked
424 secondary antibodies (Millipore) were used at 1:5000. After incubation with the antibodies,
425 blots were washed in TBST and imaged on X-ray film using an ECL detection kit (GE
426 Healthcare).

427

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436

437 **Competing interest**

438 We have no competing interests.

439

440 **Author Contributions**

441 Conceived and designed the experiments: RS, DU. Performed the experiments: KF, ST,
442 Wrote the paper: RS, DU.

443

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610

611

612

613

614 **Figure legends**

615

616 **Figure 1. Post-meiotic *Drosophila* spermatogenesis.**

617 (A) Schematic representation of spermatogenesis highlighting round and individualizing
618 spermatids. (B) Fluorescent images of spermatids co-expressing Arl1-mCherry (acroblast)
619 (red), GFP-PACT (basal body) (green) and Histone-GFP (nucleus) (green). (C) Confocal
620 images of elongated cysts with antibody staining of the *cis*-Golgi marker GM130 (red)
621 localizing at the basal end of the cysts. (D) Fluorescent images of Snky-GFP (green) in
622 elongated 64 cell cysts marking the formed acrosome. Nuclei labelled with DAPI (blue).
623 Scale bars, 10 μ m.

624

625 **Figure 2. *Scat*^l is a null mutant and Scat protein shows Golgi and acroblast localization**
626 **in *Drosophila* testis.**

627 (A) Schematic representation of the *scat* gene with the inserted P element in the third exon.
628 (B) Immunoblots of testis lysates from WT, *scat*^l mutant, and WT expressing a Scat-RFP
629 transgene. α -Tubulin is used as a loading control. (C-E) Confocal images of Scat-RFP (red)
630 expressing testes immunostained for dGM130 (green). (C) primary spermatocytes, (D)
631 meiotic spermatids, (E) elongated spermatids. Nuclei labelled with DAPI (blue). Scale bars,
632 10 μ m.

633

634 **Figure 3. The main defects are the failure to complete nuclear elongation followed by**
635 **scattering of the nuclei and defective acrosome organization in *scat*^l and *fws*^P mutants.**

636 (A-C) Investment cones (arrow) visualized by confocal microscopy using Phalloidin staining
637 (red) and DAPI (blue) in WT, *scat*^l and *fws*^P spermatids containing elongated nuclei. (D-F)
638 Protamine-GFP (green) expressed in elongating spermatids visualized in WT (D), *scat*^l (E)
639 and *fws*^P (F) mutants using confocal microscopy. (G, H) Basal bodies visualized with GFP-
640 PACT (green) in WT (G) and *scat*^l mutant (H) cysts. (I-K) Fluorescence images of elongated
641 spermatids visualized with Snky-GFP in WT (I), *scat*^l (J) and *fws*^P (K) mutants. Arrowheads
642 label acrosomes in the WT and *fws*^P mutant. Arrows label the lack of acrosome in *scat*^l and
643 *fws*^P mutant. Nuclei labelled with DAPI (blue). Scale bars, 10 μ m. (L) Measurement of
644 nuclear length in elongated spermatids. n=100 in each genotype,***: p<0.001. Length is
645 indicated in μ m.

646

647 **Figure 4. Integrity of the Golgi ribbon is compromised at the acroblast stage in the *scat*¹**
648 **and *fws*^P mutants.**

649 (A, B) Confocal micrographs of round spermatids expressing GFP-Lerp (green)
650 immunostained for dGolgin245 (red) in WT (A) and in *scat*¹ (B) mutant. (C-E) Confocal
651 micrographs of WT (C), *scat*¹ (D) and *fws*^P (E) round spermatids stained with the Golgi
652 markers Golgin245 (green) and GM130 (red). Nuclei are marked with DAPI (blue). Scale
653 bars, 10µm. Arrowheads label acroblast.

654

655 **Figure 5. Schematics depicting the involvement of tethering complexes in acroblast**
656 **integrity.**

657 Under wild type conditions (top) the functions of the COG and GARP complexes in
658 retrograde traffic are needed for the proper morphology of the Golgi ribbon known as the
659 acroblast during spermatogenesis. This functional acroblast is then used to form the
660 acrosome. When COG or GARP are non-functional, such as in the *fws*^P and *scat*¹ mutants,
661 the Golgi ribbon spreads probably due to a lack of appropriate retrograde traffic – at the *cis*
662 side in COG mutants, or the *trans* side in GARP mutants. This results in defective acrosome
663 formation, nuclear elongation and ultimately failed spermatogenesis.