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RESEARCH ARTICLE

Src64 controls a novel actin network required for proper ring canal formation in the *Drosophila* male germline

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ABSTRACT

In many organisms, germ cells develop as cysts in which cells are interconnected via ring canals (RCs) as a result of incomplete cytokinesis. However, the molecular mechanisms of incomplete cytokinesis remain poorly understood. Here, we address the role of tyrosine phosphorylation of RCs in the *Drosophila* male germline. We uncover a hierarchy of tyrosine phosphorylation within germline cysts that positively correlates with RC age. The kinase Src64 is responsible for mediating RC tyrosine phosphorylation, and loss of Src64 causes a reduction in RC diameter within germline cysts. Mechanistically, we show that Src64 controls an actin network around the RCs that depends on Abl and the Rac/SCAR/Arp2/3 pathway. The actin network around RCs is required for correct RC diameter in cysts of developing germ cells. We also identify that Src64 is required for proper germ cell differentiation in the *Drosophila* male germline independent of its role in RC regulation. In summary, we report that Src64 controls actin dynamics to mediate proper RC formation during incomplete cytokinesis during germline cyst development *in vivo*.

KEY WORDS: Ring canal, Tyrosine phosphorylation, Src64B, *Drosophila* male germline, Incomplete cytokinesis, Actin

INTRODUCTION

Germ cell divisions in the *Drosophila* female and male germlines occur with incomplete cytokinesis, resulting in the formation of cysts in which germ cells are interconnected via stable intercellular bridges called ring canals (RCs) (Hime et al., 1996; Robinson and Cooley, 1996; Greenbaum et al., 2011) (Fig. 1A). RCs are required for fertility in both insects and mammals, but current knowledge of the regulation of these structures is limited (Robinson et al., 1994; Haglund et al., 2011). The scaffolding molecules Anillin and Cindr, the central spindle component Pavarotti, as well as actin and myosin have been shown to localize to RCs (Field and Alberts, 1995; Hime et al., 1996; Adams et al., 1998; Haglund et al., 2010; Eikenes et al., 2013), but little is known about the molecular mechanisms regulating incomplete cytokinesis *in vivo* (Ong et al., 2010; Greenbaum et al., 2011; Haglund et al., 2011; Mathieu et al., 2013; Yamamoto et al., 2013).

It has long been known that RCs in both the *Drosophila* female and male germlines are tyrosine phosphorylated (Robinson et al., 1994; Hime et al., 1996; Dodson et al., 1998; Guarnieri et al., 1998). During oogenesis, Src64 (Src64B – FlyBase) mediates tyrosine phosphorylation of the kinase Btk29 (Btk29A – FlyBase) and the actin-bundler Kelch to facilitate RC expansion during egg chamber growth following completion of the mitotic germ cell divisions (Robinson et al., 1994; Guarnieri et al., 1998; Roulier et al., 1998; Kelso et al., 2002; Lu et al., 2004). Hts-RC, Filamin, SCAR and the Arp2/3 complex also contribute to actin polymerization at the inner RC rim and are required for RC growth (Yue and Spradling, 1992; Robinson et al., 1994, 1997; Sokol and Cooley, 1999; Hudson and Cooley, 2002; Zallen et al., 2002; Petrella et al., 2007). Loss-of-function mutants of Src64 display egg chambers with reduced RC diameters and reduced fertility (Dodson et al., 1998; Guarnieri et al., 1998). However, the roles of RC tyrosine phosphorylation in the *Drosophila* male germline remain unexplored.

Here, we address the roles of RC tyrosine phosphorylation in the *Drosophila* male germline. We find a striking hierarchy of tyrosine phosphorylation on RCs in developing mitotic germ cell cysts in both the male and female germlines that positively correlates with RC age. We further show that Src64 is the major tyrosine kinase required for RC tyrosine phosphorylation in the male germline. Importantly, Src64 promotes the formation of an actin network around RCs that depends on Abl tyrosine kinase and the Arp2/3 pathway to ensure correct RC diameter. Together, our data provide insight into the regulation of incomplete cytokinesis during germline cyst development *in vivo*.

RESULTS

Drosophila germline RCs display a hierarchy of tyrosine phosphorylation within germline cysts

Because little is known about the nature and function of tyrosine phosphorylation of RCs during spermatogenesis we set out to address its role in the regulation of incomplete cytokinesis during the mitotic divisions of *Drosophila* male germline cysts. Phosphotyrosine (pTyr) epitopes could readily be detected on RCs in cysts of 4, 8 and 16 cells in wild-type testes as previously reported (Fig. 1B,C, arrows) (Hime et al., 1996). We labeled RCs M1 through M4, according to the mitotic division from which they originated (Fig. 1D) (Ong and Tan, 2010).

Strikingly, the relative abundance of pTyr on RCs within cysts was not uniform. From quantifications of relative pTyr intensity we found that the central RC, M1, showed the most intense staining with the pTyr antibody (Fig. 1C,E, Fig. S1). The RC diameters also displayed a hierarchy, where M1 in a 16-cell cyst (16-CC) had an average diameter of 1.92±0.3 µm (±s.d.) compared with 1.45±0.2 µm for M4 (Fig. 1F, green bars; $P<0.05$, Student's *t*-test). Thus, we detect a hierarchy of tyrosine phosphorylation of RCs in wild-type germ cell cysts, whereby the oldest RC with the largest diameter has the strongest pTyr intensity.

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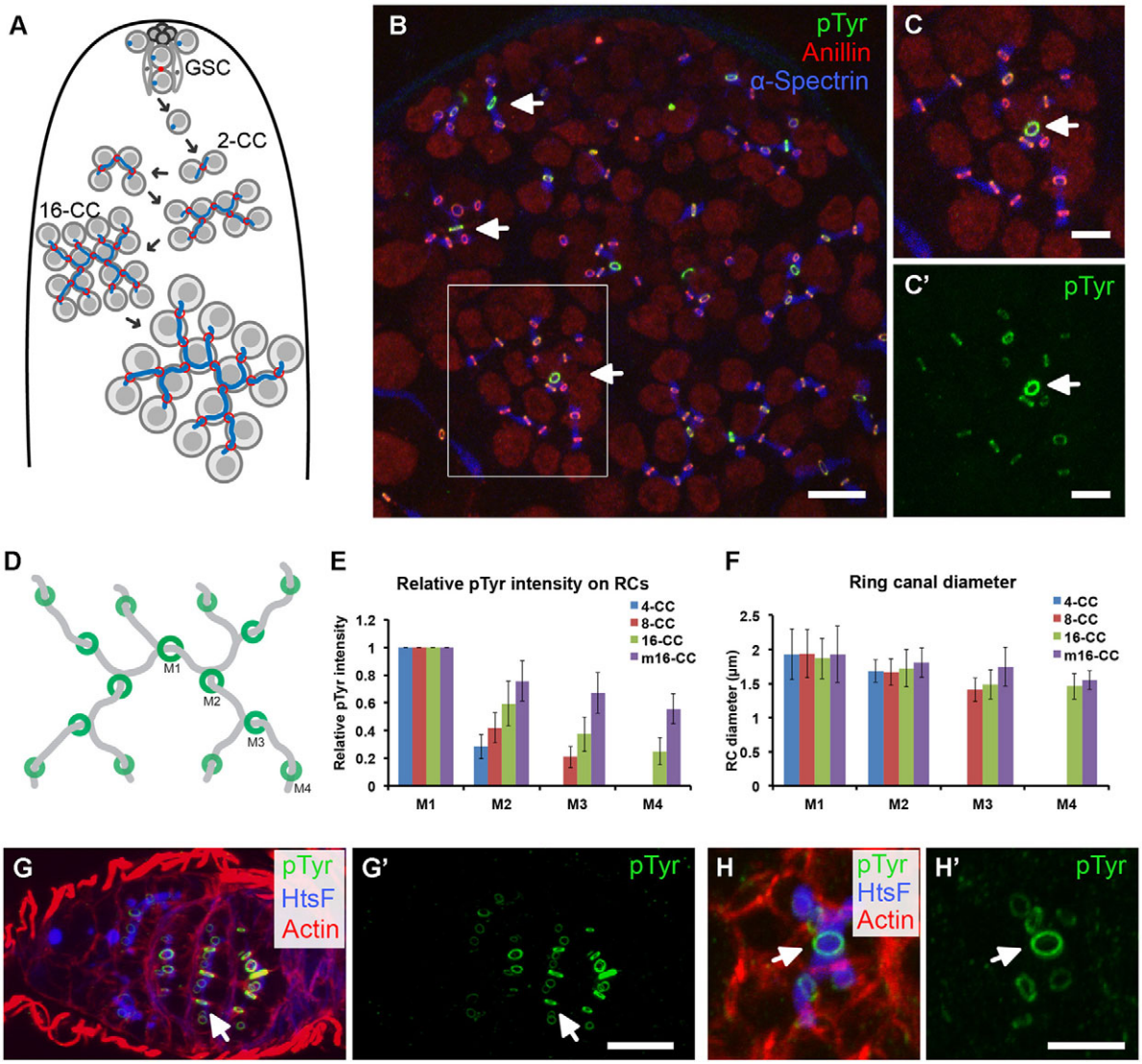

Fig. 1. A hierarchy of tyrosine phosphorylation of RCs in the *Drosophila* male and female germlines. (A) Schematic of the testes tip, where the apical hub organizes the stem cell niche. Germline stem cells (GSCs), a 2-cell cyst (2-CC) and a 16-CC are indicated (encapsulating cyst cells are omitted for clarity). (B) A wild-type testis tip stained with antibodies as indicated. Arrows indicate cysts of 4, 8 and 16 cells. (C,C′) A 16-CC (boxed in B), with the M1 RC indicated. (D) Graphical model of the fusome (gray) and RCs (green, M1 to M4) in a 16-CC. (E) The average relative pTyr intensities on RCs in mitotic cysts and primary spermatocyte cysts (m16-CC). The increase in relative pTyr on M2 is significantly different between 4-CCs and 16-CCs. $P<0.05$, Student's *t*-test. (F) The average diameters of indicated RCs. See Fig. S1 for details. (E,F) Error bars represent s.d. $n>14$ cysts quantified for each stage. (G,G′) Germarium stained as indicated. Note the different levels of pTyr intensity on RCs (arrow). (H,H′) An 8-CC from a germarium stained as in G. Arrow points to M1; note the gradient of pTyr levels within the cyst. See also Figs S1 and S2. Scale bars: 10 μm in B,G′; 5 μm in C,C′,H′.

Using transgene flies with premature or delayed entry into differentiation and thus with cysts of 8 or 32 germ cells (Insco et al., 2009), we observed either 7 or 31 RCs coded with appropriate pTyr levels (Fig. S2). This suggested that establishing the pTyr hierarchy is accomplished based on internal cues within the cyst.

To address whether a similar hierarchy is also present during early stages of oogenesis, we analyzed germaria stained with antibodies against pTyr and the fusome component HtsF (Fig. 1G,H). The pTyr hierarchy identified in mitotic male cysts (Fig. 1C′) could also be detected in female germline cysts (Fig. 1G,H).

We thus detect a hierarchy of tyrosine phosphorylation on RCs in germ cell cysts in both the male and female germlines in *Drosophila*. We next set out to investigate which kinase(s) might be responsible for generating RC tyrosine phosphorylation in the male germline.

Src64 controls RC tyrosine phosphorylation and diameter in the *Drosophila* male germline

During *Drosophila* oogenesis, Src64 and Btk29 control RC growth, and the strongest RC phenotypes are observed in the genetic null allele $Src64^{KO}$ (Cooley, 1998; Dodson et al., 1998; Guarnieri et al., 1998; O'Reilly et al., 2006). We first tested the importance of Src64 for RC phosphorylation in the male germline. Indeed, we observed a complete loss of pTyr staining on RCs at the testis tip of $Src64^{KO}$ males (Fig. 2A,B, Fig. S3A-F), as well as of Src64 protein (Fig. 2C,D).

In addition to the strong pTyr staining on RCs, close inspection of the pTyr signal at the testis tip revealed pTyr epitopes also around RCs (Fig. 2E,E′). pTyr epitopes extensively overlapped with Src64 and actin filaments both around RCs in 2-CCs and on RCs in older mitotic cysts (Fig. 2E, arrows), as well as at the hub-GSC interface

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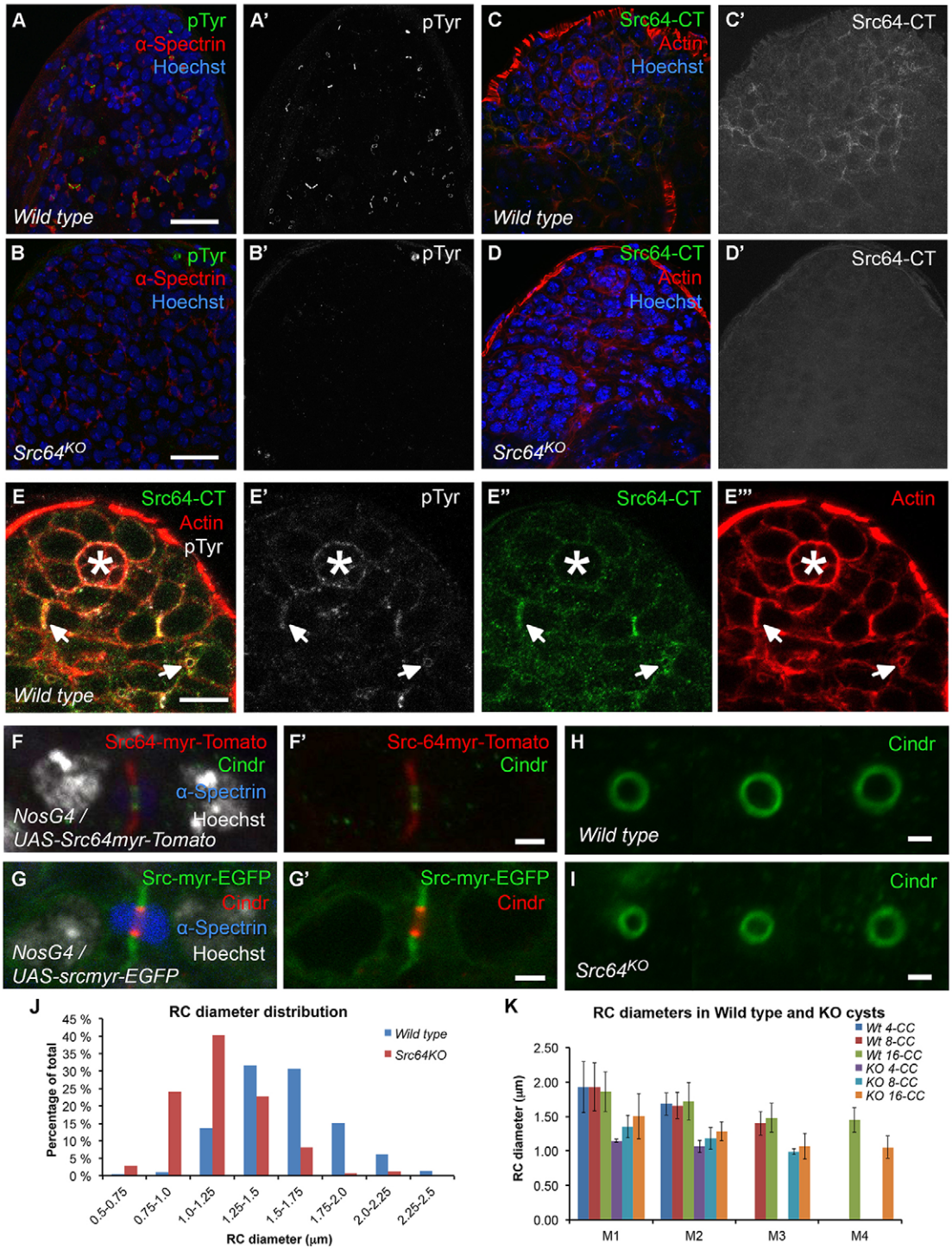

Fig. 2. Src64 is required for tyrosine phosphorylation and correct RC diameter in mitotic male germline cysts. (A-B′) Wild-type or *Src64*^{KO} testes stained as indicated. (C-D′) Wild-type or *Src64*^{KO} testes stained with an antibody against Src64 (green or gray) and with phalloidin to visualize actin filaments (red). (E-E‴) Wild-type testes stained with antibodies as indicated and with phalloidin. Asterisk marks the hub; the arrows point to a 2-CC (upper) and an RC in an 8-CC (lower). (F-G′) Confocal images of 2-CCs expressing fluorescently tagged myristoylation peptides from Src64 (red) or human SRC (green) stained with the antibodies indicated. Hoechst (white) labels nuclei. (H,I) Representative RCs visualized by Cindr staining of wild-type or *Src64*^{KO} testes. (J) Distribution of RC diameters measured in 16-CCs from wild-type or *Src64*^{KO} testes. n=212 wild-type RCs and n=171 *Src64*^{KO} RCs. P<0.05, Student's t-test. (K) RC diameters in 4-, 8- and 16-CCs measured in wild-type (*Wt*) and *Src64*^{KO} (*KO*) males. Error bars indicate s.d. The number of 4-, 8- and 16-CCs quantified were n=12, n=18 and n=16 cysts from wild-type testes and n=3, n=9 and n=9 cysts from *Src64*^{KO} testes, respectively. See also Fig. S3. Scale bars: 20 μm in A,B; 10 μm in E; 1 μm in F′,G′,H,I.

(Fig. 2E, hub marked with asterisk). Consistently, fluorescently tagged myristoylation peptides from either *Drosophila* Src64 or human SRC expressed using *Nanos-GAL4* were strongly recruited to the membrane areas surrounding the forming RCs in mitotic cysts (Fig. 2F,G). These results suggested that Src64 can be recruited to the membrane around the RC and/or to the RC itself and colocalize

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with pTyr and actin filaments at these sites in germline cysts during *Drosophila* spermatogenesis.

Given the hierarchies of RC pTyr levels and diameters observed in wild-type germline cysts (Fig. 1E,F) we next measured RC diameters in 16-CCs from wild-type and $Src64^{KO}$ testes. This analysis revealed a clear decrease in the diameter of $Src64^{KO}$ RCs, with representative examples shown in Fig. 2H,I. The average RC diameter in wild-type cysts (1.54±0.3 µm) was significantly reduced in $Src64^{KO}$ cysts (1.15±0.3 µm) (Fig. 2J; $P<0.05$, Student's *t*-test). Further analysis showed that all RC types (M1-M4), and not just a subset of RCs, had decreased diameters (Fig. 2K). Whereas wild-type M1 RCs displayed similar diameters in 4-, 8- and 16-CCs, $Src64^{KO}$ M1 diameters appeared to increase in size from the 4-CC to 16-CC stage (Fig. 2K). RCs in $Src64^{KO}$ 4- to 16-CCs did not collapse or close, indicating that the formation of an RC at a minimal diameter can be accomplished in the absence of tyrosine phosphorylation.

Upon quantification of RC pTyr levels and RC diameters in *Btk29* mutant compared with control cysts, we found similar pTyr intensities on RCs (Fig. S3G-I) and RC diameters to be slightly increased (Fig. S3J; $P<0.05$, Student's *t*-test). We conclude that Src64 is responsible for mediating tyrosine phosphorylation on and around RCs and ensures the formation of RCs of correct diameter in *Drosophila* male germline cysts.

Src64 overexpression results in reduced diameter and elongation of RCs

Examining the effects of Src64 overexpression we found a strong reduction of RC diameter accompanied by increased pTyr levels (Fig. 3A,B, Fig. S8A,C). The diameter of M1 in 16-CCs was significantly reduced from 1.8±0.1 µm in control cysts to 0.9±0.2 µm in cysts overexpressing Src64 using *Nanos-GAL4*, as were the diameters of all the RCs within such 16-CCs (Fig. 3C; $P<0.05$, Student's *t*-test). In addition, many cysts with excess Src64 levels displayed tubular elongation of the M1 RCs (Fig. 3D, arrow). Expression of Src64 using *Bam-GAL4* did not alter RC diameter (Fig. S8H), indicating that Src64 overexpression affects RCs early during germline cyst formation. These data illustrate that a critical balance of tyrosine phosphorylation is required to ensure proper RC stabilization during the mitotic divisions in *Drosophila* male germline cysts.

Localization of pTyr epitopes at the RC and relative to actin

While analyzing pTyr staining in germline cysts we noticed that it did not completely overlap with the RC marker Cindr, but instead was detected as two distinct bands at the RC edges (Fig. 3A, arrows). To validate these observations, we performed structured illumination microscopy (SIM), allowing high-resolution 3D reconstruction of fluorescent signals. This analysis indeed showed two distinct signals from the pTyr antibody positioned as two outer rings, located at the RC edges (Fig. 3E, arrows and Movie 1). Cindr localized between the pTyr signals, and its uneven appearance could potentially indicate a substructure within the RC (Fig. 3E).

We next investigated the spatiotemporal dynamics of tyrosine phosphorylation in developing 2-CCs. As shown in the representative example in Fig. 3F, 2-CCs displayed increasing pTyr on the RCs as they moved away from the hub (asterisk). In the early cyst closest to the hub, pTyr was found in equal intensities on the RC and at the membrane around the RC, together with actin filaments (Fig. 3G). In the more mature 2-CCs, the majority of the pTyr signal was found on the RC (Fig. 3H). Together, these observations suggest that tyrosine phosphorylation is first detected along the plasma membrane around the RC and on the RC, and as the 2-CCs develop further it accumulates as two distinct bands of pTyr on the RC.

Abl tyrosine kinase is important for correct RC diameter

To gain insight into mechanisms by which Src64 controls RC diameter we set out to identify Src64 substrates in the *Drosophila* male germline. Western blot analysis using an anti-pTyr antibody showed a marked decrease of tyrosine-phosphorylated proteins, including a major band at ∼180 kDa, in $Src64^{KO}$ compared with wild-type testes lysates (Fig. 4A).

We next performed immunoprecipitation from wild-type testes lysates using an anti-pTyr antibody, followed by mass spectrometry analysis. The proteins identified (Table S1) include Abl tyrosine kinase (171 kDa), a known Src substrate in mammalian cells (Plattner et al., 1999; Sirvent et al., 2007). The *Drosophila* Abl protein has 75% homology to the sequence flanking the phosphorylation site in the activation loop of human SRC, and a pSrc family antibody against this site has been shown to cross-react with phosphorylated *Drosophila* Abl (Tamada et al., 2012). Consistently, a ∼180 kDa band detected by another antibody against this site (pSrc-Y418) in wild-type *Drosophila* lysates was weaker in Abl^4/+ lysates, suggesting that it detects phosphorylated Abl (Fig. 4B, black arrow). In pTyr immunoprecipitates, the pSrc-Y418 antibody detected a ∼180 kDa band that was reduced in $Src64^{KO}$ compared with wild-type testes lysates, indicating that Abl is phosphorylated in an Src64-dependent manner in the male germline (Fig. 4B′). Analyzing the distribution of Abl-GFP expressed from the *Abl* promoter in germline cysts revealed Abl localization around RCs at the testis tip (Fig. 4C), very similar to what we observed for Src64, pTyr and F-actin (Fig. 2E). Abl-GFP also colocalized with pTyr adjacent to RCs in early 2-CCs (Fig. 4D). We next asked whether this localization of Abl-GFP was dependent on Src64. Indeed, the accumulation of Abl-GFP at the interfaces of the cells in wild-type 2-CCs was reduced in $Src64^{KO}$ testes (Fig. 4E,F, Table S2).

To address the functional importance of Abl during RC formation we performed clonal analyses and measured the diameters of RCs in *Abl* mutant 16-CCs. Comparing control clones with Abl^4 mutant germ cell clones showed a significant reduction in the average RC diameter from 1.30±0.2 µm to 1.15±0.2 µm (Fig. 4G-I; $P<0.05$, Student's *t*-test). pTyr levels on RCs in control and Abl^4 clones were, by contrast, similar (Fig. 4J-M), suggesting that Abl or something downstream of Abl does not represent the major pTyr substrate on RCs and that Src64 targets additional substrates in the male germline. Of note is that Src64 might itself be tyrosine phosphorylated when active at the RC, as reported in egg chambers (O'Reilly et al., 2006), and that Abl could still contribute to pTyr epitopes around the RCs. Together, our analyses indicate that Abl is one substrate of Src64 in the *Drosophila* male germline and that Abl is required for the formation of RCs of correct diameter.

Src64 and Abl promote the assembly of actin filaments around RCs

We next addressed how Src64 and Abl might regulate diameters of *Drosophila* male germline RCs. Because both these kinases regulate actin dynamics *in vivo* (Grevengoed et al., 2001; Fox and Peifer, 2007; Baruzzi et al., 2010; Tsarouhas et al., 2014) we asked whether Src64 and Abl were involved in actin regulation during RC formation in 2-CCs in *Drosophila* testes.

Visualizing actin filaments in 2-CCs by expression of the actin-binding fusion protein Lifeact-GFP using *Nanos-GAL4* revealed

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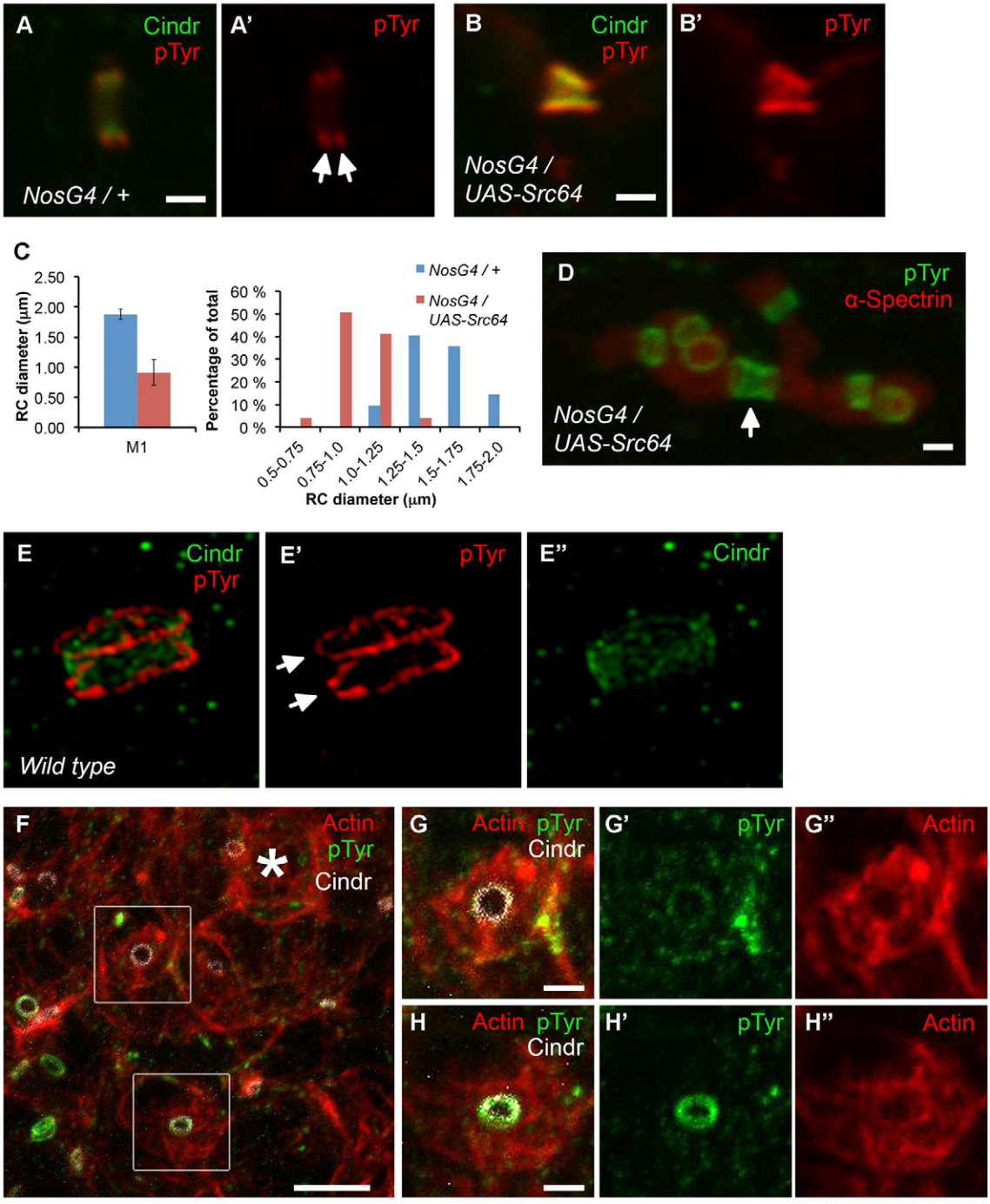

Fig. 3. Src64 overexpression alters RC morphology and pTyr localizes at the RC edges in germline cysts. (A-B′) M1 RCs of 16-CCs from control testes or testes overexpressing Src64 using *Nanos-GAL4* (*NosG4*) stained with the antibodies indicated. Arrows point to pTyr signals. (C) Quantification of M1 diameters from 16-CCs in control or testes expressing Src64 using *Nanos-GAL4* (n=6 RCs for each genotype) and distribution of RC diameters in control (n=84 RCs) and Src64-overexpressing (n=75 RCs) 16-CCs. P<0.05, Student's t-test. Error bars represent s.d. (D) Example of an 8-CC overexpressing Src64, stained as indicated. Note the broadened M1 RC (arrow). (E-E″) SIM image of an M1 RC from a 4-CC stained as in A, showing two distinct bands of pTyr lining the edges of the RC (arrows). (F) Wild-type testis tip stained as indicated. Two 2-CCs at different maturation stages and distance from the hub (asterisk) are indicated (boxed). (G-G″) Upper 2-CC from F. Note the presence of pTyr signal adjacent to the RC together with actin, and weak pTyr on the RC. (H-H″) Lower 2-CC from F. Note the strong pTyr signal on the RC. See also Movie 1. Scale bars: 1 μm in A,B,D; 5 μm in F; 2 μm in G,H.

strong defects in $Src64^{KO}$ 2-CCs compared with control cysts (Fig. 5A,B). The peri-RC localization of Lifeact-GFP seen in control cysts was abolished in $Src64^{KO}$ 2-CCs (Fig. 5A,B). $Src64^{KO}$ cysts instead appeared without a clear cell-cell contact surface, and the RC was often mispositioned in the cyst (Fig. 5A,B, arrows and Table S2). A tight membrane contact surface in wild-type cysts was also observed by electron microscopy (Fig. S4A). The RC diameters in 2-CCs in the $Src64^{KO}$ mutant were, moreover, significantly reduced compared with those in control cysts, from 1.5±0.2 μm to 1.1±0.2 μm (Fig. 5C; P<0.05, Student's t-test).

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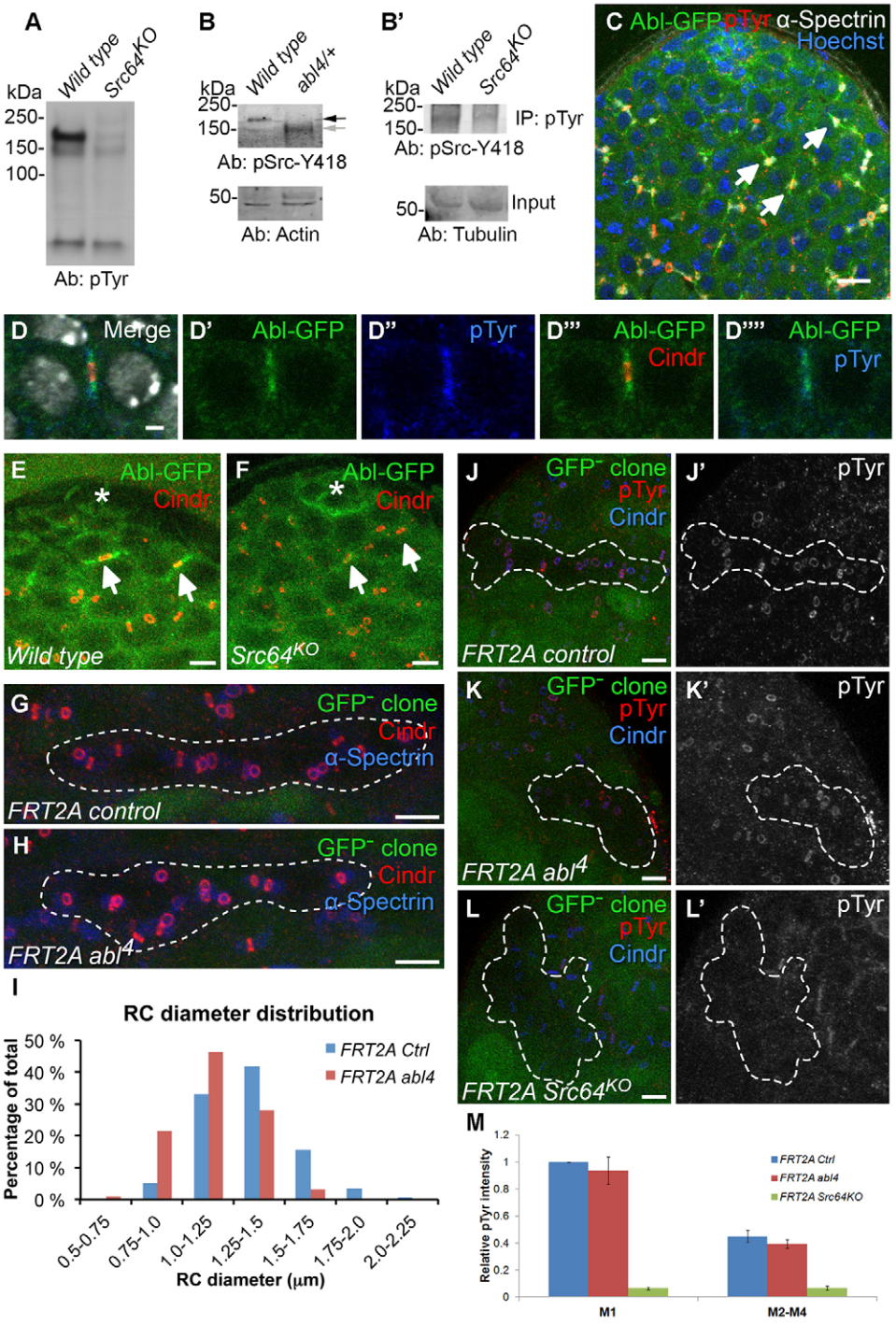

Fig. 4. Abl is required for correct RC diameter in *Drosophila* male germline cysts. (A) Western blot of lysate from wild-type and $Src64^{KO}$ testes, blotted with a pTyr antibody. The band at 40 kDa indicates approximately equal loading. (B,B′) Western blots with the pSrc-Y418 antibody of (B) wild-type and Abl^4/+ larval lysates and (B′) pTyr immunoprecipitates (IP) from wild-type or $Src64^{KO}$ testes lysates. Anti-pSrc-Y418 detected a 180 kDa band in wild-type but not Abl^4/+ (B, black arrow), whereas a 150 kDa band (B, gray arrow) was detected in both lysates. Actin was used as loading control. In B′ 10% of input lysates were blotted with anti-tubulin. (C) Testes tip with Abl-GFP expressed from the *Abl* promoter, immunostained as indicated. Hoechst labels the nuclei (blue). Arrows indicate 2-CCs at the testis tip. (D-D′′′′) Confocal images of a 2-CC showing Abl-GFP, Cindr (red), pTyr (blue) and Hoechst (white). (E,F) Confocal images showing localization (arrows) of Abl-GFP adjacent to RCs in 2-CCs in wild-type but more weakly in $Src64^{KO}$ males. Cindr labels the RCs (red). (G,H) Representative confocal images of GFP-negative clones (outlined) 3 days after clonal induction in *FRT2A* control or *FRT2A* Abl^4 testes stained as indicated. A GFP-positive cell from a neighboring cyst is lying on top of the outlined *FRT2A* Abl^4 clone in H. (I) The distribution of RC diameters in *FRT2A* control and *FRT2A* Abl^4 16-CCs. n=172 and n=125 RCs, respectively, P<0.05, Student's t-test. (J-L′) Representative confocal images of GFP-negative control, Abl^4 and $Src64^{KO}$ 16-CC clones (outlined) 3 days after clonal induction. Testes were stained as indicated. (M) The relative average RC pTyr intensities in control, Abl^4 and $Src64^{KO}$ 16-CC clones based on three independent experiments. Error bars represent s.e.m. n=27, n=26 and n=15 16-CCs were quantified for control, Abl^4 and $Src64^{KO}$, respectively. Differences between control and Abl^4 clones are not statistically significant. Scale bars: 10 μm in C; 2 μm in D; 5 μm in E-H,J-L.

Importantly, we found Src64 and pTyr accumulating at the actin disk surrounding the RC in 2-CCs (Fig. 5D, Fig. 2E). Analyses of actin filaments using phalloidin confirmed the above finding that 2-CCs in the $Src64^{KO}$ mutant failed to properly assemble the actin disk around the RC (Fig. 5E,F, Table S2). We conclude that Src64 regulates the assembly of an actin network around RCs in male germline cysts.

We next examined how the septin Peanut (Field et al., 1996; Adam et al., 2000; Eikenes et al., 2013) and the Arp2/3 activator SCAR (WAVE1 in mammals) (Zallen et al., 2002), both of which are involved in actin dynamics, localize in relation to RCs in 2-CCs. Interestingly, both Peanut and SCAR accumulated around RCs in wild-type cysts, but were irregular in ~90% of $Src64^{KO}$ 2-CCs (Fig. 5G-J, Fig. S4B,C,F,G, Table S2). Similar results were observed in 4- to 16-CCs (data not shown). These results suggest that Src64 is important for the proper organization of proteins involved in the assembly of actin filaments around RCs in 2-CCs in the *Drosophila* male germline.

Recent studies have shown that actin polymerization contributes to positioning new adherens junctions after cell division (Herszterg et al., 2013; Morais-de-Sá and Sunkel, 2013). In 2-CCs, we observed the adherens junction protein E-cadherin (Shotgun – FlyBase) around the actin-surrounded RCs in wild-type testes but not in $Src64^{KO}$ mutant cysts (Fig. 5K,L, Fig. S4D,H, Table S2),

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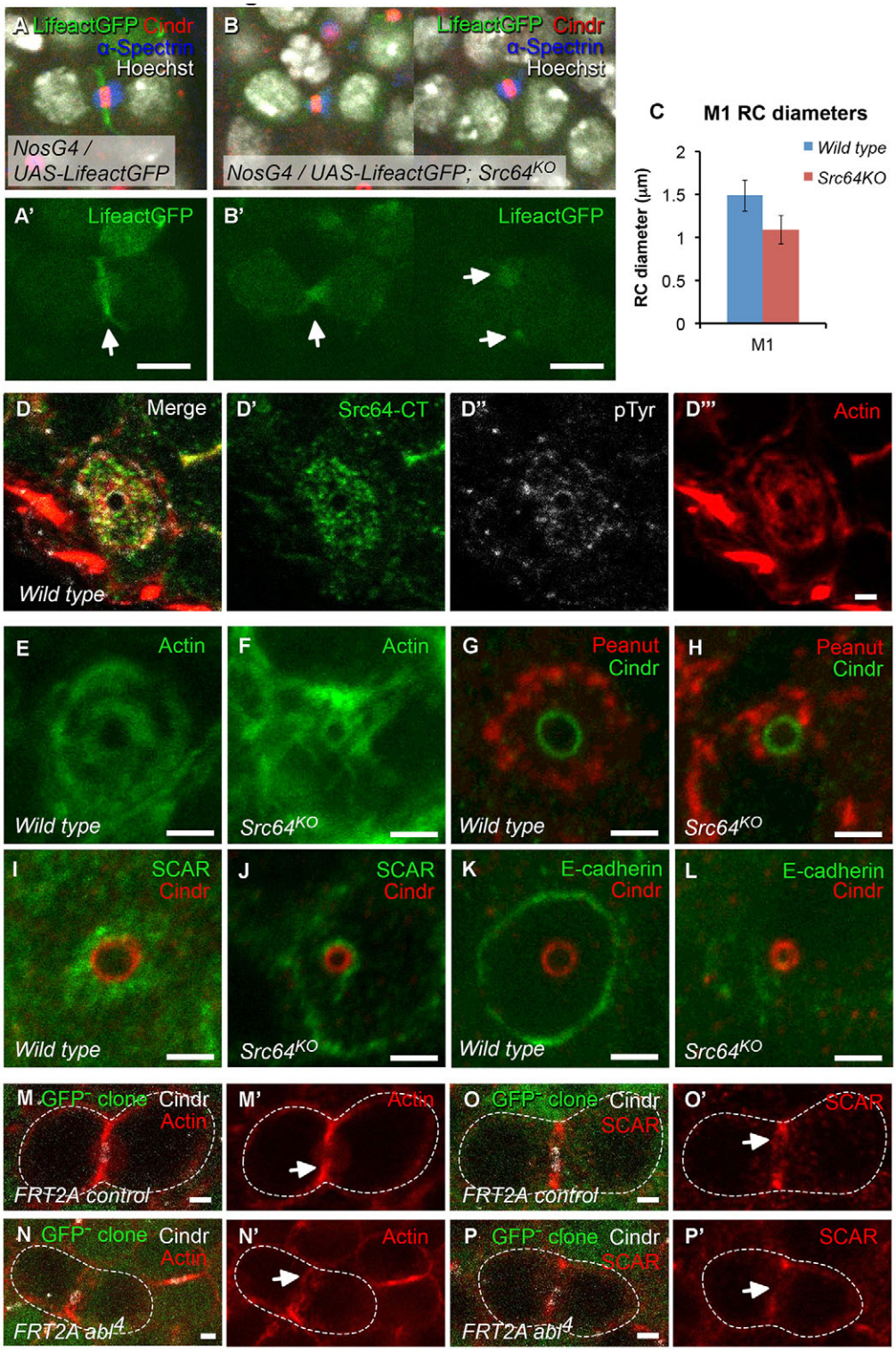

Fig. 5. Src64 is required for the generation of an actin network around RCs. (A-B′) Expression of Lifeact-GFP using *Nanos-GAL4* in wild-type and $Src64^{KO}$ 2-CCs, immunostained as indicated. Hoechst labels nuclei (white). Arrows indicate accumulation of actin. (C) The average diameters of M1 RCs from wild-type (n=23 RCs) and $Src64^{KO}$ (n=20 RCs) 2-CCs. Error bars indicate s.d. P<0.05, Student's t-test. (D-D‴) A wild-type 2-CC stained with the indicated antibodies and with phalloidin to visualize actin filaments (red). Src64 (green) and pTyr (white) both localize to the RC and to the adjacent membrane, together with the actin network. (E-L) RCs in 2-CCs from wild-type and $Src64^{KO}$ testes stained with phalloidin to visualize actin or the antibodies indicated. (M-N′) *FRT2A* control or *FRT2A* Abl^4 2-CC clones (outlined) 3 days after clonal induction, stained as indicated. F-actin was normal in *FRT2A* control clones (n=7 2-CCs), but irregular or absent in ~60% of *FRT2A* Abl^4 clones (n=17 2-CCs, 10 of which displayed irregular actin filaments). Note the neighboring GFP-positive cyst with normal actin localization (N). (O,P) *FRT2A* control or *FRT2A* Abl^4 2-CC clones (outlined) 3 days after clonal induction stained as indicated. SCAR localization was normal in *FRT2A* control clones (n=7 2-CCs), but irregular or absent in ~60% *FRT2A* Abl^4 clones (n=24 2-CCs, 14 of which displayed irregular SCAR staining). Arrows indicate actin (M′,N′) or SCAR (O′,P′). See also Fig. S4 and Table S2. Scale bars: 1 μm in A′,B′; 2 μm in D‴,E-L,M,N,O,P.

suggesting that adherens junctions do not form properly following loss of Src64 function in germline cysts.

To determine whether Abl acts on a similar pathway as Src64 to control RC diameter we further examined the F-actin, SCAR and E-cadherin distribution in control and Abl^4 mutant clones. In over 50% of the Abl^4 mutant clones analyzed, actin filaments or SCAR were irregular or absent (Fig. 5M-P), and E-cadherin staining was irregular in ~50% of Abl^4 2-CCs (Fig. S4I-K). We conclude that Src64 and Abl regulate proper localization of the actin regulator SCAR, E-cadherin and actin filament polymerization around RCs in *Drosophila* male germline cysts.

The Arp2/3 complex and Rac GTPases are required for correct RC diameter in male germline cysts

Because SCAR depends on both Src64 and Abl to localize around RCs in 2-CCs, we next investigated the effect of SCAR depletion on

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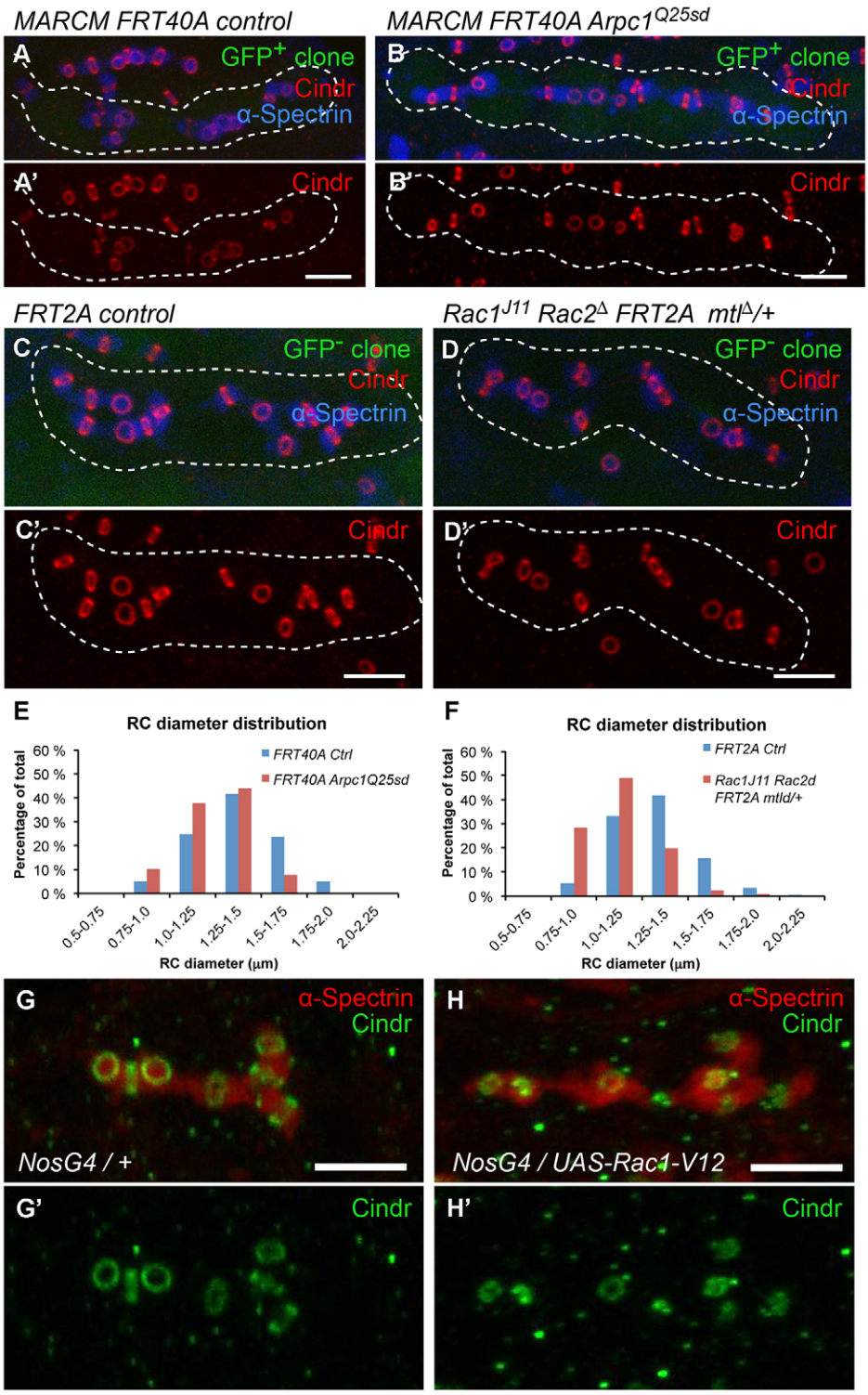

Fig. 6. Rac and the Arp2/3 complex are required for correct RC diameter. (A-B′) GFP-positive clones (outlined) in *MARCM FRT40A* control and *MARCM FRT40A Arpc1*^{Q25sd} testes stained as indicated. (C-D′) GFP-negative clones (outlined) in *FRT2A* control and *Rac1*^{J11} *Rac2*$^{\Delta}$ *FRT2A Mtl*$^{\Delta}$/+ testes stained as indicated. (E) The distribution of RC diameters measured in 16-CC clones as in A and B (*n*=101 and *n*=177 RCs, respectively). (F) The distribution of RC diameters measured in 16-CC clones as in C and D (*n*=172 and *n*=127 RCs, respectively). (E,F) *P*<0.05, Student's *t*-test. (G-H′) Confocal images of germline cysts from *Nanos-GAL4*/+ and *Nanos-GAL4*/*UAS-Rac-V12* testes stained as indicated. See also Fig. S5. Scale bars: 5 μm.

RC diameter. Loss of SCAR function reduced RC diameters in mitotic germ cell cysts (Fig. S5A,B,E; *P*<0.05, Student's *t*-test). Another Arp2/3 activator, Cortactin, did not affect RC diameter (Schenck et al., 2004; Somogyi and Rørth, 2004) (Fig. S5C,D,F).

The Arp2/3 complex is involved in actin polymerization and RC growth during oogenesis (Hudson and Cooley, 2002; Zallen et al., 2002; Stradal and Scita, 2006). The GTPase Rac acts upstream of the Arp2/3 complex together with Abl and Src kinases to regulate branched actin polymerization (Leng et al., 2005; Ardern et al., 2006; Stradal and Scita, 2006; Yamazaki et al., 2007; Sanz-Moreno et al., 2008). We next investigated whether loss of Arp2/3 or Rac GTPases would affect RC diameter by generating clones of *Arpc1* or the three *Drosophila* Rac genes *Rac1*, *Rac2* and *Mtl* and analyzing the RC diameters in 16-CCs (Fig. 6A-D). Compared with appropriate genetic control clones, loss-of-function mutation of the Arp2/3 complex or loss of the Rac GTPases significantly reduced the average RC diameter from 1.41 μm to 1.26 μm or from 1.30 μm to 1.15 μm (±0.2 μm in all cases), respectively (Fig. 6E,F; both

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$P<0.05$, Student's *t*-test). Further, expression of constitutively active Rac1-V12 using *Nanos-GAL4* resulted in small and abnormally shaped RCs in germline cysts (Fig. 6G,H), resembling the RC phenotype in egg chambers mutant for SCAR or Arp2/3 (Hudson and Cooley, 2002; Zallen et al., 2002; Zobel and Bogdan, 2013). Together, these results reveal that appropriate levels of SCAR, the Arp2/3 complex and Rac GTPases, all of which are regulators of branched actin filaments, are required to ensure the correct diameter of RCs during the mitotic germ cell divisions in the male germline. The actin network that we have identified around RCs thus seems to be important for proper RC diameter in *Drosophila* male germline cysts.

Loss of Src64 results in delayed entry into differentiation of male germline cysts

In addition to reduced RC diameters and associated actin defects in the $Src64^{KO}$ testes, we observed an expansion of the mitotic area as well as tumors or testes filled with mitotic cells (Fig. 7A,B). About 30% of $Src64^{KO}$ mutant testes contained mitotic tumors and over 60% were filled with mitotic cells (Fig. 7B,B′, Fig. S6A). Expression of Src64 in the germline using *Nanos-GAL4* rescued this phenotype so that ∼75% of the testes displayed normal morphology (Fig. 7C, Fig. S6A).

Quantification of spermatocyte cysts (Insco et al., 2009) confirmed the above observation, as the 60% of cysts with 32 or more cells in the $Src64^{KO}$ mutant testes (described as normal or tumor in Fig. 7B) were efficiently rescued upon germline expression of Src64 (Fig. S6B-E). Re-expression of Src64 in the germline restored pTyr on the RCs, but also led to severe malformations of the RCs, similar to overexpression of Src64 in the wild-type background (Fig. 3A,B, Fig. S6F-H, Fig. S8A,C). Nucleotide labeling or Anillin (Scraps – FlyBase) staining showed that mitotic cysts in both wild-type and $Src64^{KO}$ testes divided synchronously (Fig. 7D,E, Fig. S7A,B,D,E). The $Src64^{KO}$ mutant cysts, however, displayed delayed accumulation of Bam protein (Fig. 7F,G), suggesting a delay in entry into differentiation or faster proliferation of germline cysts in the $Src64^{KO}$ mutant.

To determine if Src64 is required in germ cells to enter into differentiation, we next performed a clonal analysis in germ cells and quantified spermatocyte cysts. Surprisingly, less than 10% of the $Src64^{KO}$ clones contained 32 cells – far fewer than the 60% of cysts with excess cell numbers in the full $Src64^{KO}$ mutant (Table S3). We then asked whether loss of any of the downstream components required for RC diameter could lead to delayed differentiation in a cell-autonomous manner. Clones of Abl^{4}, $Rac1^{J11}$, $SCAR^{\Delta 37}$ and $Arpc1^{Q25sd}$ were analyzed and compared with appropriate genetic controls. Loss of Abl gave rise to a similar percentage of 32-CCs as the $Src64^{KO}$ clones (less than 10%), whereas none of the other mutations caused delayed differentiation (Tables S3 and S4). This suggests that control of RC diameter is not directly linked to germ cell differentiation. We next used either *Nanos-GAL4* or *Bam-GAL4* to overexpress Src64, but this did not result in any alterations in cyst size (Fig. S8A-J). Together, our data show that although Src64 promotes germ cell entry into differentiation at the correct time, increased kinase levels specifically in the germline are not sufficient to modulate the timing of germ cell differentiation.

Loss of Src64 results in the formation of asynchronous tumors

Tumors detected in $Src64^{KO}$ mutant testes were often detached from the mitotic zone (Fig. 7B, arrow). In comparison to cysts from $bam^{\Delta 86}$ heterozygote testes, where cysts also undergo additional rounds of mitosis, $Src64^{KO}$ tumors often contained RCs that were severely reduced in diameter and often closed entirely (Fig. 7H,I, arrows). RCs in overgrown cysts in $bam^{\Delta 86}$/+ males had an average diameter of 1.6±0.3 µm, whereas that in the $Src64^{KO}$ tumor was significantly smaller at 1.2±0.3 µm (Fig. 7J; $P<0.05$, Student's *t*-test), which is comparable to the average RC diameter in $Src64^{KO}$ 16-CCs (1.15±0.3 µm) (Fig. 2J, Fig. S7F). We also detected Anillin localization at the cell periphery in dispersed cells in $Src64^{KO}$ mutant tumors, indicative of metaphase cells and asynchrony within the tumors, a feature not observed in $bam^{\Delta 86}$/+ cysts (Fig. 7H,I, Fig. S7C). Labeling $Src64^{KO}$ testes with EdU to identify S-phase cells confirmed the tumors as asynchronous (Fig. 7K, Fig. S7D,E). A minimal RC diameter might thus be required for proper germ cell cyst synchronization in the *Drosophila* male germline.

DISCUSSION

Here we show that Src64 controls tyrosine phosphorylation on and around RCs and proper RC diameter in *Drosophila* male germline cysts (Figs 2 and 3). Src64 regulates the localization of Abl and SCAR around the RCs, which are required to assemble a network of actin filaments around RCs during the mitotic germline cyst divisions (Figs 4 and 5). Dysregulation of this actin network results in smaller RCs in germline cysts (Figs 2, 5 and 6). Moreover, we show that loss of Src64 results in the formation of germ cell tumors (Fig. 7).

Src64 controls RC tyrosine phosphorylation in the *Drosophila* male germline

The role of tyrosine phosphorylation of RCs during incomplete cytokinesis in the *Drosophila* male germline has remained an unanswered question for nearly two decades. Tyrosine phosphorylation of egg chamber RCs depends on both Src64 and Btk29 (Dodson et al., 1998; Guarnieri et al., 1998; Roulier et al., 1998). We found that Src64, but not Btk29, is responsible for RC tyrosine phosphorylation in *Drosophila* male germline cysts (Fig. 2, Fig. S3G-I). Our study shows that Src64 controls the assembly of an actin network outside of the RCs during the mitotic germ cell divisions in the *Drosophila* male germline (Fig. 5). This is distinct from its role in promoting the accumulation of actin filaments on the inside of RCs to mediate RC and egg chamber growth following completion of the mitotic germ cell divisions in the female germline (Dodson et al., 1998; Guarnieri et al., 1998). Thus, Src64 controls a previously uncharacterized actin network around RCs during the mitotic divisions in the *Drosophila* male germline.

Src64 and Abl control RC diameter

We identified Abl as a tyrosine phosphorylated protein in the male germline and that Abl localization around the RC depends on Src64 (Fig. 4A-C). Both Src64 and Abl are required to ensure correct RC diameter during the mitotic germ cell divisions (Fig. 4D-H) and to control the localization of the Arp2/3 activator SCAR and the formation of the actin network around the RC in 2-CCs (Fig. 5). We propose that Src64 acts upstream of Abl to promote the formation of this actin network during incomplete cytokinesis in *Drosophila* male germline cysts. Src64 and Abl also affect E-cadherin, and it will be important to address whether the actin network acts upstream or downstream of E-cadherin.

Actin regulation around RCs in the *Drosophila* male germline

pTyr levels on RCs change during progressive 2-CC development, as manifested by a clear increase in pTyr levels on RCs and a decrease in actin-associated pTyr epitopes around RCs (Fig. 1E, Fig. 3F-H). Src64 substrates might thus first be recruited and

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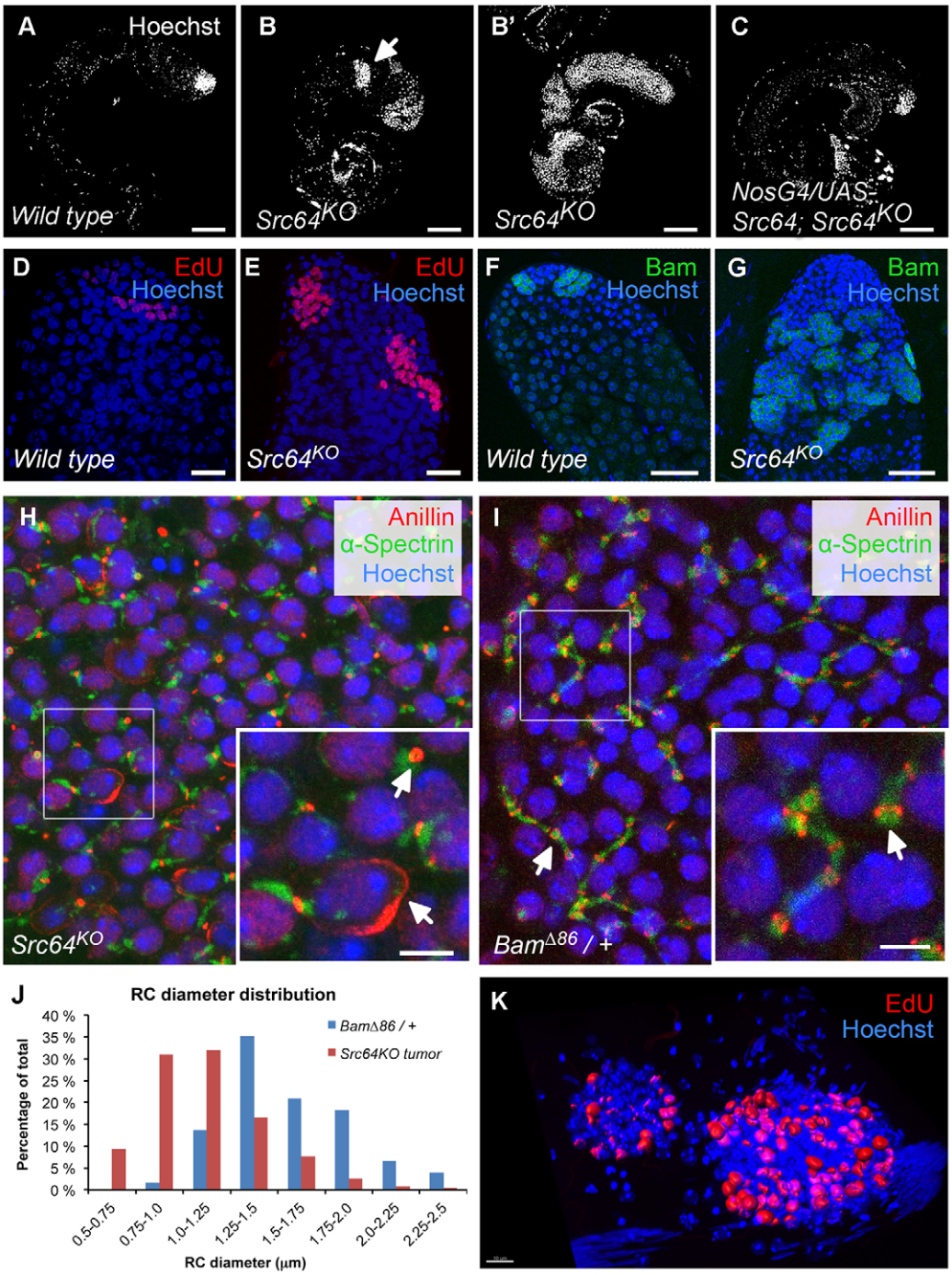

Fig. 7. Loss of Src64 results in delayed differentiation and asynchronous germline tumors. (A-C) Testes from wild-type, *Src64*^{KO} and *Nanos-GAL4/UAS-Src64;Src64*^{KO} males stained with Hoechst (white). Note the enlarged mitotic zone, tumor (arrow) and overproliferation in the *Src64*^{KO} testes (B,B′), and the rescue of mutant phenotype (C). (D-G) Testes from wild-type and *Src64*^{KO} males labeled with EdU (red, D) or stained as indicated. (H,I) Large cysts in *Src64*^{KO} mutant or *bam*$^{\Delta 86}$/+ testes stained with antibodies as indicated. Arrows point to closed RCs and cortical Anillin in *Src64*^{KO} mutant and to an RC with normal morphology in *bam*$^{\Delta 86}$/+ cysts. (J) Quantification of RC diameters in large cysts in *bam*$^{\Delta 86}$/+ and *Src64*^{KO} testes (n=182 and n=278 RCs, respectively). P<0.05, Student's t-test. (K) Imaris image of a tumor from the *Src64*^{KO} mutant showing unsynchronized EdU incorporation. See also Figs S6, S7, S8 and Tables S3 and S4. Scale bars: 100 µm in A-C; 20 µm in D-G; 5 µm in H,I (insets); 10 µm in K.

activated at the membrane to contribute to actin filament branching, and then translocate to the RC. Conversely, separate pools of pTyr substrates might also explain these spatial differences.

Src64 kinase itself, as well as Abl-GFP and SCAR, localized around RCs (Figs 4 and 5, Table S2). Because, in addition to Src64 and Abl, we found SCAR, Arp2/3 and Rac GTPases to be required for correct RC diameter, the formation of an actin network around RCs appears to be required for the generation of RCs of proper diameter. Interestingly, both Src64 and Abl can phosphorylate the SCAR/WAVE complex to activate Arp2/3-mediated actin polymerization in human cells (Leng et al., 2005; Ardern et al., 2006; Mendoza, 2013). Src64 and Abl may thus directly target actin regulators, including SCAR, to promote the formation of the actin network around the RCs in *Drosophila* male germline cysts.

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Loss of Src64 results in asynchronous tumors in the *Drosophila* male germline

In addition to reduced RC diameters, $Src64^{KO}$ mutant testes displayed delayed differentiation of germline cysts. Our data uncoupled the RC diameter and cyst differentiation phenotypes observed in the $Src64^{KO}$ mutant testes and indicated that the delayed germ cell differentiation is non-cell-autonomous (Tables S3 and S4), possibly owing to a function of Src64 in the somatic cells of the testis. That $Src64^{KO}$ and *Abl* mutant clones exhibited a low rate of 32-CC production (Table S3) indicates, moreover, that Src64 and Abl may cell-autonomously assist in the number of germ cell mitoses, but are not the driving factors.

Delayed germ cell differentiation or overproliferation can originate from both germ and somatic cells in the *Drosophila* testis (Gonczy et al., 1997; Schulz et al., 2002; Insco et al., 2009; Hudson et al., 2013; Qian et al., 2015). For example, dysregulation of the Bam protein gradient gives rise to mitotic germline tumors (Gonczy et al., 1997; Insco et al., 2009). In $bam^{\Delta 86}/+$ testes with delayed differentiation, RCs remained open (Fig. 7I), whereas in $Src64^{KO}$ tumors many RCs were closed (Fig. 7H). Upon loss of Src64 function, the closed RCs, altered signaling in germ or somatic cells and/or impaired fusome development may contribute to disrupting cyst synchrony. Male germline RCs are thought to promote intercellular germ cell communication and synchronization of, for example, mitotic germ cell divisions (Greenbaum et al., 2011; Haglund et al., 2011). However, the cell biological and developmental roles of RCs in male germline cysts remain elusive (Greenbaum et al., 2011; Haglund et al., 2011) and it will be important to address the physiological consequences of smaller RCs in germline cysts.

Src64 regulates an actin network around RCs in *Drosophila* male germline cysts

We propose a model in which Src64 controls an actin network around RCs during incomplete cytokinesis in *Drosophila* male germline cysts. This actin network appears to be important to ensure proper RC diameter in germline cysts. Src64 mediates tyrosine phosphorylation of substrate(s) at the plasma membrane around and on RCs. We propose that Src64 acts upstream of Abl and the Rac/SCAR/Arp2/3 pathway to promote actin assembly around RCs. We thus uncover a novel role for Src64 in coordinating an actin network around RCs, thereby ensuring correct RC diameter during incomplete cytokinesis in *Drosophila* male germline cysts.

MATERIALS AND METHODS

Drosophila stocks and husbandry

Fly crosses were performed at 25°C unless otherwise noted. w^{1118} was used as wild-type control. A list of stocks is provided in the supplementary Materials and Methods. Clonal induction of *FRT2A* control and *FRT2A* mutant animals was performed by subjecting pupae and young males to one 2-h heat shock at 37°C. Clonal induction of *FRT40A* control and *FRT40A* mutant animals was performed by subjecting 0- to 2-day-old males to one 2-h heat shock at 37°C for RC diameter measurements, or three 1-h heat shocks over 3 days during pupation for clonal analysis of spermatocyte cysts. Flies were returned to 25°C for 3 days before clonal analysis.

Immunostaining

Antibodies used are listed in the supplementary Materials and Methods. Experiments were performed using the testes squash protocol, with fixation in ice-cold methanol and acetone as described elsewhere (Cenci et al., 1994) for Fig. 1, Fig. 2A,B, Fig. 3D,E, Fig. 7H,I, Figs S1, S2 and Fig. S3A-F. Otherwise, testes and ovaries were dissected in PBS and fixed in 4% formaldehyde on ice for 30 min and stained as previously described (Haglund et al., 2010).

Western blotting and immunoprecipitation

Testes from wild-type and $Src64^{KO}$ males were lysed in a HEPES lysis buffer supplemented with protease and phosphatase inhibitors. Lysates were mixed with Laemmli buffer containing a final concentration of 0.1 M DTT and boiled before loading onto a 4-20% gradient SDS-PAGE gel, followed by protein transfer onto a PVDF membrane and probing with a rabbit anti-pTyr antibody. To assess the levels of tyrosine-phosphorylated Abl in testes, pTyr proteins were immunoprecipitated using a hot lysis protocol before SDS-PAGE. The western blot was performed with an anti-pSrc-Y418 antibody. For further information, see the supplementary Materials and Methods.

Microscopy

Confocal images were acquired using a Zeiss LSM 780 confocal microscope equipped with a 63× NA 1.4 oil DIC III (Plan-Apochromat) objective. Further information and details of structured illumination and electron microscopy are provided in the supplementary Materials and Methods.

Mass spectrometry

One thousand wild-type males were dissected and lysed in a HEPES buffer supplemented with fresh protease and phosphatase inhibitors. Proteins were immunoprecipitated using an agarose-conjugated anti-pTyr antibody and separated on an SDS-PAGE gel, stained with Coomassie G-250 and in-gel digested using 0.1 µg trypsin in 25 µl 50 mM ammonium bicarbonate pH 7.8. Proteins were identified by mass spectrometry analysis as described in the supplementary Materials and Methods.

pTyr intensity and RC diameter quantification

Quantification of relative pTyr intensity and RC diameter was performed using line scan in the Zen 2010 software (Carl Zeiss). A single line was drawn through the largest diameter of the RC at its peak intensity in the *z*-stack, and the intensity of the pTyr signal and the diameter were noted. In Figs 1, S1 and S2, pTyr intensity levels were related to the central RC within the cyst and comparisons were made with relative values. In Fig. 4M and Fig. S3I, pTyr intensity levels for M1 and M2-M4 were calculated for each cyst and average pTyr intensities for each genotype were related to the central RC within control cysts in each experiment.

Statistical analyses

Statistical analyses were performed using a two-tailed Student's *t*-test. Apart from statistical testing comparing M1 with M4 in Fig. 1E, where a paired analysis was used, all other statistical tests were performed using an unpaired Student's *t*-test. To confirm the appropriateness of the statistical testing, selected test results were checked by also using mixed effect models with experiments as random effect. Results were similar.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Å.H.E. and K.H. designed and conceived the study, performed experiments, analyzed the data and wrote the manuscript. L.M., A.L.-J., C.S.W. and A.B. performed experiments and analyzed the data. K.L. performed statistical analyses. H.S. analyzed the data. H.S. and K.H. supervised the study. All authors edited the manuscript.

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Supplementary information

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