The Shot CH1 domain recognises a distinct form of F-actin during *Drosophila* oocyte determination

D. Nashchekin[1*], I. Squires[1†], A. Prokop[2] and D. St Johnston[1*]

Affiliations:

[1]The Gurdon Institute and the Department of Genetics, University of Cambridge; Tennis Court Road, Cambridge, United Kingdom.

[2]The University of Manchester, Manchester Academic Health Science Centre, Faculty of Biology, Medicine and Health, School of Biology, Manchester, United Kingdom.

*Corresponding author. Email: d.nashchekin@gurdon.cam.ac.uk d.stjohnston@gurdon.cam.ac.uk,

[†]Present address: Biosciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom.

Abstract

As in mammals, only one cell in a *Drosophila* multicellular female germline cyst is specified as an oocyte. The symmetry-breaking cue for oocyte selection is provided by the fusome, a tubular structure connecting all cells in the cyst. The *Drosophila* spectraplakin Shot localises to the fusome and translates its asymmetry into a polarised microtubule network that is essential for oocyte specification, but how Shot recognises the fusome is unclear. Here we demonstrate that Shot's actin-binding domain (ABD) is necessary and sufficient to localise Shot to the fusome and mediates Shot function in oocyte specification together with the microtubule-binding domains. The calponin homology domain 1 of Shot's ABD recognises fusomal F-actin and distinguishes it from other forms of F-actin in the cyst. By contrast, the ABDs of Utrophin, Fimbrin, Filamin, as well as Lifeact and F-tractin do not recognise fusomal F-actin. We therefore propose that Shot propagates fusome asymmetry by recognising a specific conformational state of F-actin on the fusome.

Introduction

Both male and female gametes differentiate inside cysts of interconnected germ cells. Whereas all male cells in the cyst become sperm, only one or few of the female germ cells are specified to become oocytes in most animals (Lu et al., 2017, Pepling and Spradling, 1998, Lei and Spradling, 2013). Since cells in the female germline cyst share cytoplasm through intercellular bridges, there must be specific mechanisms to select the future oocyte. This is only understood in *Drosophila*, where a polarised microtubule (MT) network that extends throughout the cyst directs the dynein-dependent transport of oocyte fate determinants into one cell (Theurkauf et al., 1993; Li et al., 1994). A similar mechanism could also be involved in oocyte specification in mouse cyst (Lei and Spradling, 2016; Niu and Spradling, 2022).

In *Drosophila*, cyst formation and oocyte specification occur in the germarium at the very tip of the fly ovary (Fig. 1A). Oocyte determination starts when a germline stem cell divides asymmetrically to produce a cyst progenitor, a cystoblast, that goes through 4 rounds of incomplete mitotic division to produce a cyst of 16 cells connected by intercellular bridges called ring canals (de Cuevas et al., 1997). The cystoblast contains a spherical structure inherited from the stem cell called the spectrosome, which contains endoplasmic reticulum, spectrins and actin-binding proteins (Lighthouse et al., 2008). At each subsequent division, new spectrosomal material forms in the ring canal connecting the two daughter cells and this fuses with the pre-existing spectrosome to form the fusome, which becomes a branched structure extending into all 16 cells of the cyst (Lin et al., 1994; De Cuevas and Spradling, 1998). Because one cell inherits the original spectrosome/fusome from the cystoblast, this cell contains more fusomal material than the others and this

ultimately specifies it as the pro-oocyte (Lin and Spradling, 1995; De Cuevas and Spradling, 1998).

The first step in the translation of fusome asymmetry into oocyte specification is the recruitment of the *Drosophila* spectraplakin, Shot (Roper and Brown, 2004; Nashchekin et al., 2021). Shot in turn recruits the MT minus end-binding protein Patronin (CAMSAP in mammals) to the fusome, where Patronin stabilises microtubule minus ends (Goodwin and Vale, 2010; Jiang et al., 2014; Nashchekin et al., 2021). The slight excess of Patronin in the future oocyte is then amplified by the dynein-dependent transport of Patronin and microtubule minus ends along the stabilised microtubules into this cell, leading to the formation of non-centrosomal microtubule-organising centres (ncMTOCs) in the future oocyte. Finally, these ncMTOCs nucleate a polarised MT network that directs the transport of oocyte determinants into this cell (Grieder et al., 2000; Bolívar et al., 2001; Nashchekin et al., 2021) (Fig. 1B).

Shot is a spectraplakin protein that belongs to a conserved family of actin-microtubule crosslinkers that include human Dystonin and MACF1/ACF7, which play important roles in cytoskeletal organisation during neurogenesis and in epithelia (Lee and Kolodziej, 2002; Voelzmann et al., 2017; Dogterom and Koenderink, 2018). A distinct feature of spectraplakins is the presence of an actin-binding domain (ABD) at the N-terminus and a MT-binding module at the C-terminus, separated by a long rod domain consisting of plakin and spectrin repeats (Fig. 1C). The ABD of spektraplakins consists of two tandem calponin homology domains, CH1 and CH2 and the MT-binding module is composed of the MT lattice-binding GAS2 domain and

an unstructured C-terminal domain containing two SxIP motifs that interact with the MT plus end-binding protein EB1 (Sun et al., 2001; Honnappa et al., 2009; Applewhite et al., 2010; Alves-Silva et al., 2012). Although Shot transmits fusome asymmetry to Patronin localisation and the formation of the polarised MT network that specifies the oocyte, how Shot recognises the fusome is not known. Previous work suggested that the Shot ABD is not involved (Roper and Brown, 2004). Here we show, however, that the Shot ABD is necessary and sufficient for localisation to the fusome, but this involves the recognition of a form of F-actin that is different in some way from other F-actin networks in the cyst. Both the actin and MT-binding domains are required for oocyte specification, consistent with Shot's role in organising the polarised microtubule network.

Results and Discussion

The Shot actin-binding domain localises to the fusome

To determine which domain(s) of Shot direct its localisation to the fusome, we expressed a mini-version of Shot that lacks the central rod domain, Shot-NC (Figure 1C). It has been shown previously that the NC version of ACF7 (a mammalian Shot homologue) is able partially substitute ACF7 function in cells (Wu et al., 2008). Like endogenous Shot and the full-length Shot transgene (Fig. 1D and 1E, respectively), Shot-NC localised to the fusome (Fig. 1F). This demonstrates that the fusome-binding activity of Shot resides in either its N- or C-terminal domains and that the central rod domain is dispensable for fusome localisation. Expression of either the N- or C-terminal domains alone showed that Shot-N binds efficiently to the fusome (Fig. 1G), whereas Shot-C forms foci in the cytoplasm and accumulates in one cell of the cyst (Fig. 1H), a pattern previously described for EB1 (Nashchekin et al., 2021). Live imaging of Shot-C-YFP in the germarium revealed that it forms EB1-like comets

(Video1) suggesting that the C-terminal domain of Shot associates with MT plus ends, and that the MT lattice-binding GAS2 domain is in an inhibited conformation, as shown for the mammalian Shot homologue, Dystonin, in Cos-7 cells (Kapur et al., 2012). Nevertheless, we decided to test whether the GAS2 domain has the potential to bind the fusome by expressing a portion of the Shot C-terminal domain containing the EF hand and the GAS2 domain that has strong MT binding activity (Maybeck and Roper, 2009). EF-GAS2-GFP localised to the fusome in the presence of endogenous Shot (Fig. 1I) but failed to do so in *shot* mutant cysts (Fig. S1). Fusome-associated MTs are largely lost in the absence of Shot, indicating that EF-GAS2-GFP localises to the fusome by binding to Shot-dependent MT (Roper and Brown, 2004). The GAS2 domain therefore cannot be responsible for the initial recruitment of Shot to the fusome.

From our results so far, we speculated that Shot recruitment to the fusome requires the Shot N-terminal region containing the two Calponin homology domains that constitute the ABD. We therefore expressed a construct containing the just ABD of Shot fused to GFP in the cyst and confirmed its localisation to the fusome (Fig. 1J). In contrast, full-length Shot lacking the ABD ($Shot^{\Delta ABD}$) did not localise to the fusome (Fig. 1K). Thus, the ABD of Shot is both necessary and sufficient for fusome localisation, presumably by interacting with fusome-associated F-actin.

Shot recognises a distinctive form of F-actin on the fusome

Several actin-binding proteins have been identified as components of the fusome, including b-spectrin, Hts/Adducin, Tropomodulin and Shot (De Cuevas et al., 1996; Lin et al., 1994; Lighthouse et al., 2008; Roper and Brown, 2004). However, actin

has never been detected in the fusome and Phalloidin does not label the fusome, staining only the ring canals and the cell cortex of germline cysts (Warn et al., 1985 and Fig. 1D-K). In contrast, Actin-GFP in the cyst showed staining in the fusome raising the possibility that absence of Phalloidin staining might be misleading (Fig. S2B). We therefore tested the presence of endogenous actin at the fusome by performing antibody stainings for actin using various fixation methods. Of all fixation methods used, only a combination of heat fixation followed by post- fixation with formaldehyde produced convincing actin staining at the fusome (Fig. 2A). As a staining control we used *hts* mutant cysts (Fig. S2A) where the fusome is absent (Lin et al., 1994), and confirmed that fusome-like actin staining was indeed missing under these conditions. We conclude that the fusome does contain F-actin, but not in a form that can be detected by Phalloidin. Since the Phalloidin-actin interaction is sensitive to the structure of F-actin filaments (McGough et al., 1997), this suggests that fusomal F-actin exists in a distinct structural conformation that can be bound by the Shot ABD but not by Phalloidin.

Both b-Spectrin and Shot are members of a large family of actin-binding proteins with ABDs formed by tandem CH domains (Korenbaum and Rivero, 2002; Yin et al., 2020) (Fig. S2C). To test whether other CH domain proteins can recognise fusomal F-actin, we analysed the distribution of over-expressed Utrophin ABD and endogenously-tagged Filamin and Fimbrin. None of these CH domain proteins recognised fusomal F-actin and they mainly concentrated at ring canals (Fig. 2B-D). F-tractin and LifeAct are commonly used F-actin markers (Melak et al., 2017) recognising a variety of F-actin structures (Riedl et al., 2008; Schell et al., 2001; Belin et al., 2014; Spracklen et al., 2014). When expressed in the germline cyst,

each showed a unique distribution, but neither localised to the fusome. F-tractin mainly localised to the cell cortex (Fig. 2E) whereas LifeAct recognised the cell cortex and ring canals (Fig. 2F). Moreover, substituting Shot's ABD with the LifeAct sequence (Shot$^{\Delta ABD}$-LifeAct) did not restore fusome recognition to full-length Shot (Fig. 2G). Thus, fusomal F-actin has a distinctive conformation that is only recognised by a subset of actin-binding proteins, including Shot's CH1-CH2 domain. The Shot ABD must therefore have structural features that allow it to bind preferentially to fusomal F-actin and to distinguish it from other F-actin structures in the cyst. This does not preclude Shot ABD binding to other forms of F-actin, since Shot re-localises to the cell cortex and ring canals in *hts* and *a-spectrin* mutant cysts where the fusome is not formed (Lin and Spradling, 1995; De Cuevas et al., 1996) (Fig. S3).

The spatial arrangement of tandem CH1 and CH2 domains can regulate the actin binding activity of CH-domain containing proteins, as the CH2 domain can sterically hinder some of the actin binding surfaces on the CH1 domain (Bañuelos et al., 1998; Galkin et al., 2010; Iwamoto et al., 2018). It has been shown that the transition between the "open" and "closed" conformations of the CH1-CH2 domain in ACF7 (the mammalian Shot homologue) is regulated by phosphorylation of a conserved Tyr located at the very end of the CH2 domain (Yue et al., 2016, Fig. 1C). Phosphorylation of the Tyr by Src/FAK kinases leads to an "open" conformation of the ACF7 ABD and enhances F-actin binding.

To test whether Shot binding to the fusome is regulated by Tyr phosphorylation, we expressed the Shot ABD containing phosphomimetic and non-phosphorylatable

versions of Tyr364 (ABD Y364D and ABD Y364F, respectively). Whereas ABD Y364F still bound to fusomal F-actin (Fig. 3B), ABD Y364D only did so in cysts in region 2a of the germarium and relocalised to ring canals in region 2b (Fig. 3A). This suggests that the open versus closed conformation of the CH domains alters the specificity of Shot's ABD for different forms of F-actin, rather than working as a simple on/off switch. It also suggests that the conformation of F-actin on the fusome differs between region 2a and 2b, which correlates with the transformation of the fusome from a thick tubular structure in 2a to a cable-like structure in 2b (Lin et al., 1994).

Since isolated ABDs could behave differently from the full-length protein, we introduced the same Tyr364 mutations into Shot-NC and full-length Shot. Whereas the Shot-NC Tyr364 mutants behave like the corresponding ABD mutants, the localisation of the full-length Shot was not affected by Tyr364 mutations (Fig. 3C-F). This suggests that either the open/closed transition does not affect the actin binding of full-length Shot or that sequences located between Shot's N- and C-terminal domains stabilise the ABD in the "closed" conformation, preventing the CH1-CH2 transition to an "open" state. The rod domain in Shot is unlikely function as a second fusome-binding domain itself, because full-length Shot^{DABD} does not localise to the fusome.

To test the importance of the CH1 and CH2 domains in Shot binding to the fusome, we analysed the localisation of full-length Shot lacking CH1 (Shot$^{\Delta CH1}$) or CH2 (Shot$^{\Delta CH2}$). Shot^{DCH2} still localised to the fusome (Fig. 3H), whereas the deletion of CH1 abolished fusome binding, leading to cytoplasmic localisation (Fig. 3G). These

results indicate that the CH1 domain mediates Shot binding to the fusome and that its intrinsic properties allow it to recognise a specific F-actin conformation that is invisible to several other CH-domain proteins and actin-binding molecules. This result also implies that recognition of fusomal F-actin by Shot does not require CH2 and is not regulated by the open/closed transition of the ABD.

Structural studies on the interaction of CH domains with F-actin have revealed that CH1 simultaneously interacts with two adjacent actin monomers and is sensitive to the torque/helicity of F-actin filaments (Hanein et al., 1998; Iwamoto et al., 2018; Kumari et al., 2020; Harris et al., 2020). Changes in the helical twist of F-actin filaments can be caused by mechanical tension, by interactions with actin-binding proteins (Harris et al., 2018; Jégou and Romet-Lemonne, 2020; Harris et al., 2020), or by the bending of F-actin filaments promoted by differences in the nucleotide states of actin monomers (Reynolds et al., 2022; Oosterheert et al., 2022). It is therefore possible that CH1 domains of different actin-binding proteins are predisposed to bind F-actin filaments with specific helical twists, which could explain their distinct but partly overlapping localisation patterns (Washington and Knecht, 2008; Harris et al., 2020; Jégou and Romet-Lemonne, 2021).

The actin- and MT-binding domains of Shot are required for the oocyte specification

In the absence of Shot, the oocyte is not specified leading to the formation of a 16 nurse cell follicle, but which domains of Shot are required for oocyte specification is not known (Roper and Brown, 2004). The cytoplasmic polyadenylation element

binding factor oo18 RNA-binding protein (Orb) is an early marker for oocyte specification which concentrates in the oocyte in regions 2b and 3 in wild-type germaria but it is uniformly distributed in *shot* null mutant cysts (Lantz et al., 1994; Roper and Brown, 2004) (Fig. 4A and B). To determine whether the presence of Shot actin- or MT-binding domains are sufficient for oocyte specification, we expressed Shot-N, Shot-C, Shot-NC and Shot-EF-GAS2 in *shot* null mutant cysts (Fig. 4C-F). Even though, Shot-N was able to bind to the fusome in the absence of endogenous Shot, it did not rescue Orb localisation and oocyte determination (Fig. 4C). Neither Shot-C or Shot-EF-GAS2 were localised to the fusome or rescued oocyte specification in *shot* mutant cysts (Fig. 4D-E, Fig. S1). However, expression of Shot-NC was sufficient to restore oocyte specification in the absence of full length Shot (Fig. 4F). Thus, both the ABD and MT-binding modules are necessary for Shot function during oocyte determination suggesting that Shot acts as an actin-microtubule cross-linker in this context.

It has been previously reported that Shot's ABD is not required for oocyte specification, since the oocyte is specified normally in cysts mutant for *shot*^{kakp1}, a P-element insertion in the intron upstream of CH1-domain-coding exon that is predicted to prevent the expression of CH1 domain containing isoforms of Shot (Roper and Brown 2004). However, as we show above, Shot's ABD is essential for the Shot localisation to the fusome and oocyte determination. To resolve this contradiction, we tested the requirement for Shot's CH1 and CH2 domains in the oocyte specification, by expressing Shot$^{\Delta ABD}$, Shot$^{\Delta ABD}$-LifeAct, Shot$^{\Delta CH1}$ and Shot$^{\Delta CH2}$ in *shot* mutant cysts (Fig. 4G-J). Shot truncations lacking the CH1 domain did not rescue oocyte specification, nor did substituting the Shot ABD with the actin-

binding activity of LiveAct (Fig. 4G-I). Only *shot* mutant cysts that expressed Shot$^{\Delta CH2}$ maintained correct Orb distribution and specified the oocyte (Fig. 4J). Thus, Shot binding to the fusome through the CH1 domain of its ABD is essential for the oocyte specification. Since the *shot*^{kakp1} mutant does not affect oocyte determination, we assume that this P-element insertion does not disrupt the expression of CH1-containing Shot isoforms in the germ line, although it does do so in somatic tissues (Roper and Brown 2004).

Previously, it has been proposed that Shot binds the fusome with unidentified domain and uses its GAS2 domain to bind and stabilise MT (Roper and Brown, 2004). Based on our results we propose an alternative model for Shot function in oocyte determination where it works as a classical actin-MT cytolinker by recognising fusomal F-actin with its ABD and using its C-terminal domain to attach MT to the fusome. Which part of the MT module is involved in this process is an open question. On the one hand expression of Shot-EF-GAS2 can recognise fusomal MT. On the other hand, expression of the whole Shot C-terminal domain showed that the GAS2 domain is not exposed in the full-length protein and Shot interacts only with MT plus ends through its EB1-binding motifs. Thus, Shot may guide the growth of MT plus ends along the fusome in a similar manner to that described for ACF7 in migrating cells (Kodama et al., 2003; Wu et al., 2008). The role of Shot in stabilising MT on the fusome could also be indirect, since it has been recently shown that Shot is required for fusome localisation of the MT minus-end stabilising protein Patronin/CAMSAP (Nashchekin et al., 2021). Moreover, fusome-associated MT are unstable in *patronin* mutant cysts even though Shot is still present. How Shot's C-terminus recruits microtubules to the fusome will therefore require further study.

Our evidence suggests that the asymmetry of the fusome is propagated to MT organisation in the *Drosophila* female germline cyst by formation of a distinct type of F-actin on the fusome, which then recruits the spectraplakin Shot. How fusomal F-actin is formed and the structural basis for its recognition by actin-binding proteins remain to be determined. Considering that Shot is a conserved actin binding protein, it is possible that a similar mechanism is used in other contexts where F-actin filaments in a specific mechanical state are recognised by only a subset of actin-binding proteins, leading to the establishment of asymmetry within the cell.

Material and methods

Drosophila stocks. The following previously described *Drosophila melanogaster* mutant alleles and transgenic lines were used: FRTG13 *shot*^{3} (Roper and Brown, 2004), *hts*^{1} and *hts*^{01103} (Yue and Spradling, 1992), *a-spectrin*$^{e2-26}$ (Hülsmeier et al., 2007), UAS-GFP-Actin42A (Roper et al., 2005), UAS-GFP-Shot EF-GAS2 (Maybeck and Roper, 2009); UAS-Shot$^{\Delta ABD}$-GFP, UAS-Shot$^{\Delta CH1}$-GFP, UAS-Shot$^{\Delta CH2}$-GFP and UAS-Shot$^{\Delta ABD}$-LifeAct-GFP (Qu et al., 2022), Shot-YFP (Nashchekin et al., 2016), UAS-F-tractin-tdTomato (Spracklen et al., 2014),Filamin-GFP trap line (gift from K. Röper, MRC-LMB, UK), Fimbrin-YFP (Cambridge Protein Trap Insertion line 100066, (Lowe et al., 2014), sqh>Utrophin ABD-GFP (Rauzi et al., 2010). UAS-Shot-GFP, UAS-ShotY364F-GFP, UAS-ShotY364D-GFP, UAS-Shot-NC-YFP, UAS-Shot-NCY364D-YFP, UAS-Shot-NCY364F-YFP, UAS-Shot-N-YFP, UAS-Shot-C-YFP, UAS-GFP-Shot ABD, UAS-GFP-Shot ABDY364F, UAS-GFP-Shot ABDY364D, UAS-LifeAct-RFP were generated for this study.

***Drosophila* genetics.** Germline clones of *shot*^{3} *and a-spectrin*$^{e2-26}$ were induced by incubating larvae at 37° for two hours per day over a period of three days. Clones were generated with FRT G13 nlsRFP and FRT 2A nlsRFP, (Bloomington Stock Center) using the heat shock Flp/FRT system (Chou and Perrimon, 1992). Germline expression of UAS transgenes was induced by nanos>Gal4.

Molecular Biology. To generate pUASP Shot-GFP, three fragments of the *shot* RE cDNA were amplified from pUAST Shot-GFP (Lee and Kolodziej, 2002) and cloned together with EGFP into the pUASPattb vector. pUASPattb-Shot-NC-YFP was generated by PCR amplifying fragments from pUAST Shot-GFP corresponding to the first 520 aa (Shot-N) and last 462 aa (Shot-C) of Shot PE, cloning them together into pUASP-YFP-Cterm and then re-cloning into pUASPattb. pUASPattb-Shot-N-YFP and pUASPattb-Shot-C-YFP were generated by amplifying Shot-N or Shot-C fragments from pUASPattb-Shot-NC-YFP and cloning them together with YFP into pUASPattb. Shot ABD cDNA (corresponding to 146-368 aa of Shot PE) was amplified from pUASPattb-Shot-NC-YFP and cloned together with EGFP into pUASP-attb to generate pUASP-attb GFP-Shot ABD. The Q5 Site-Directed Mutagenesis Kit (New England BioLabs) was used to generate pUASPattb-GFP-

Shot ABDY364F, pUASPattb-GFP-Shot ABDY364D, pUASPattb-Shot-NCY364D-YFP and pUASPattb-Shot-NCY364F-YFP. Shot-N with the Y364F or Y364D mutations was amplified from pUASPattb-Shot-NCY364F or pUASPattb-Shot-NCY364D, respectively, and cloned together with two fragments covering the rest of Shot RE cDNA and EGFP into pUASP-attb to generate pUASPattb-ShotY364F-GFP and pUASPattb-ShotY364D-GFP. pUASP-LifeAct-tagRFP was generated according to Riedl et al (2008). NEBuilder HiFi DNA Assembly (New England BioLabs) was used for most of the cloning. The primers used are listed in TableS1.

Immunohistochemistry. Ovaries were fixed for 20 min at room temperature in 4% paraformaldehyde and 0.2% Tween in PBS. Ovaries were then blocked with 1% BSA in PBS with 0.2% Tween for 1 hr at room temperature. Ovaries were incubated with the primary antibody for 16 hr with 0.1% BSA in PBS with 0.2% Tween at 4C° and for 4 hr with the secondary antibody at room temperature. For detection of fusomal F-actin, we used the heat fixation protocol described in Chen et al (2018) with some modifications. Ovaries were dissected in PBS, fixed for 10 sec in hot (95C°) 1xTSS (0.03% Triton X-100, 4 g/L NaCl), post-fixed in 8% paraformaldehyde and 0.1% Triton X-100 in PBS for 10 min at room temperature and then treated as above. We used the following primary antibodies: guinea pig anti-Shot at 1:1000 (Nashchekin et al., 2016), mouse anti-Orb at 1:10 (DSHB Hybridoma Products 4H8 and 6H4. Deposited to the DSHB by Schedl, P), mouse anti-a-Spectrin at 1:200 (DSHB Hybridoma Product 3A9. Deposited to the DSHB by Branton, D. / Dubreuil, R.), mouse anti-Actin at 1:200 (clone AC-40, Merck), rabbit anti-b-spectrin at 1:200 (Byers et al., 1989). Secondary antibodies conjugated with Alexa fluor dyes (Thermo Fisher Scientific) were used at 1:1000.

Imaging. Fixed preparations were imaged using a Leica SP8 (63x/1.4 HC PL Apo CS Oil) confocal microscope. Germaria were imaged by collecting 10–15 z sections spaced 0.5 µm apart. For live imaging, ovaries were dissected and imaged in Voltalef oil 10S (VWR International) on a Leica SP5 confocal microscope (63x/1.4 HCX PL Apo CS Oil) or on an Olympus IX81 inverted microscope with a Yokogawa CSU22 spinning disk confocal imaging system (100x/ 1.3 NA Oil UPlanSApo). Images were collected with Leica LAS AF software or MetaMorph and processed using ImageJ. The images are projections of several z sections.

Reproducibility of experiments and statistical analyses. Images are representative examples from at least three independent repeats for each experiment. The number of mutant cysts (region 2b to 3) analysed for the rescue experiments were as follows: Figure 4C (21), Figure 4D (28), Figure 4E (18), Figure 4F (26), Figure 4G (14), Figure 4H (19), Figure 4I (16), Figure 4J (21). No statistical methods were used to predetermine sample size, the experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

Supplemental material
Figures S1-S3, Video 1 and TableS1.

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Figure legends

Figure 1. Shot is recruited to the fusome by its actin-binding domain. (A) Schematic diagram of the *Drosophila* germarium showing germline cyst formation and oocyte specification. **(B)** Diagram of a germline cyst showing how Shot and

Patronin translate the fusome asymmetry into the polarised MT network that directs dynein transport of oocyte fate determinates into one cell that becomes the oocyte. Asterisk indicates the presumptive oocyte. Adapted from Nashchekin et al., 2021. **(C)** The domain structures of full-length Shot and Shot truncations. CH, calponin homology domain. ABD, actin-binding domain. **(D-K)** Endogenous Shot **(D)** and ectopically expressed full-length Shot-GFP **(E),** Shot-NC-YFP **(F),** Shot-N-YFP **(G),** Shot-EF-GAS2-GFP **(J),** Shot ABD-GFP **(J)** localise to the fusome, whereas Shot-C-YFP **(H)** and Shot$^{\Delta ABD}$ **(K)** do not. An enlargement of the fusome is shown on the right. a-Spectrin marks fusome. Phalloidin marks ring canals and the cell cortex. Scale bars, 10µm.

Figure 2. Standard F-actin labelling reagents fail to recognise fusomal F-actin.
(A) Detection of F-actin on the fusome**.** A confocal image of a germarium stained with anti-actin after heat fixation and post-fixation with fomladehyde (green) and anti-Shot antibody (red). **(B-D)** Localisation of CH domain-containing proteins (green) in cysts stained for a-spectrin (red) to label the fusome and cell cortices and Phalloidin (blue), which labels the ring canals. Utrophin ABD-GFP **(B)**, Filamin-GFP **(C)** and Fimbrin-YFP **(D)** localise to ring canals and the cell cortex but not recognise the fusome. **(E)** A confocal image of a living germarium expressing F-tractin-tdTomato (red) and Shot-YFP (green). Shot localises to the fusome, whereas F-tractin labels the ring canals. **(F-G**) LifeAct does not recognise the fusome. Confocal images of germaria expressing LifeAct-RFP (green) **(F)** and Shot$^{\Delta ABD}$-LifeAct-GFP stained for a-spectrin (red) and Phalloidin (blue) **(G).** The righthand panels show enlargements of the fusome region. Scale bars, 10µm.

Figure 3. The Shot CH1 domain recognises the fusome.
(A-D) The effects of phosphomimetic and non-phosphorylatable mutations in Tyr364, Y364D and Y364F. Y364D disrupts the localisation of Shot ABD and Shot-NC to the fusome in region 2b. Shot ABDY364D-GFP **(A)** and Shot-NCY364D-YFP **(C)** localise to ring canals in region 2b of the germarium. The localisation of Shot ABDY364F-GFP **(B)** and Shot-NCY364F **(D)** is not affected. **(E-F)** Mutations of Y364 in full length Shot do not affect fusome recognition. Germaria expressing Shot Y364D-GFP **(E)** and Shot Y364F-GFP **(F)**. **(H-I)** Deletion of the Shot CH1 domain abolishes

binding to the fusome, but CH2 is not required. Germaria expressing Shot$^{\Delta CH1}$-GFP **(H)** and Shot$^{\Delta CH2}$-GFP **(I)**. The righthand panels show enlargements of the fusome region. a-Spectrin (red in the lefthand panels) marks the fusome. Phalloidin (blue) marks the ring canals and cell cortex. Regions of the germarium are indicated on the top. Scale bars, 10µm.

Figure 4. The Shot CH1 and MT-binding domains are required for the oocyte determination. (A-B) The distribution of the oocyte specification marker Orb in wild type (WT) (**A**) and *shot*^{3} (**B**) germline clone mutant cysts marked by the loss of nlsRFP (blue). **(C-E)** Shot-N-YFP **(C)**, Shot-C-YFP **(D)** and Shot-EF-GAS2-GFP **(E)** do not rescue oocyte determination in *shot*^{3} germline clones. **(F)** Expression of Shot NC-YFP in *shot*^{3} mutant cysts restores oocyte specification. **(G-J)** Expression of Shot$^{\Delta CH2}$-GFP **(J)** but not Shot$^{\Delta ABD}$-GFP **(G)**, Shot$^{\Delta ABD}$-LifeAct-GFP **(H)** or Shot$^{\Delta CH1}$-GFP **(I)** rescues oocyte specification in *shot*^{3} germline clones. Arrows point to the future oocyte; cysts are marked by dashed lines; mutant cysts are labelled by the absence of nuclear localisation signal RFP (nlsRFP, blue). Scale bars, 10µm.

Figure S1. Shot-EF-GAS2 does not localises to the fusome in *shot* mutant cysts. A germarium expressing Shot-EF-GAS2-GFP (green) in wild type and *shot*^{3} mutant cysts. Cysts are marked by dashed lines; mutant cysts are labelled by the absence of nuclear localisation signal RFP (nlsRFP; blue). An enlarged view of the fusome is shown on the right. a-Spectrin (red in left panel) marks the fusome. Scale bars, 10µm.

Figure S2. (A) Fusomal actin is not detected in *hts* mutant cysts. A germarium from a *hts* mutant female stained with anti-Shot (red) and anti-Actin (green) antibodies after heat fixation followed by post-fixation in 8% formaldehyde. **(B)** Actin-42A localises to the fusome. A germarium expressing Actin-42A-GFP (green) stained with anti-a-Spectrin antibody (red) to mark the fusome. The righthand panel shows an enlarged view of the fusome. **(C)** Diagrams showing the domain structure of actin-binding proteins with calponin homology (CH) domains. ABD, actin-binding domain.

Figure S3. Shot localisation in *a-spectrin* and *hts* mutant cysts. Shot localises to the cell cortex and ring canals in *a-spectrin* germline clone cysts **(A)** and *hts* **(B)** mutant cysts, which lack the fusome. **(A)** A germarium with *a-spectrin* germline clones stained with anti-Shot (red), Phalloidin (blue) and anti-b-Spectrin (white) to label the fusome. Mutant cysts are labelled by the absence of nuclear localisation signal RFP (nlsRFP; blue). *a-spectrin* mutant cysts are marked by dashed lines. Arrows point to the fusome in a wild type cyst. **(B)** A germarium from a *hts* mutant female stained with anti-Shot (red), Phalloidin (blue) and anti-a-Spectrin (red) to label the fusome. Scale bars, 10µm.

Video legends

Video 1. A time-lapse video of Shot-C-YFP in a wild-type germarium. Related to Figure 1G. Images were collected every 1 second on a spinning disc confocal microscope. The video is shown at 15 frames/sec.

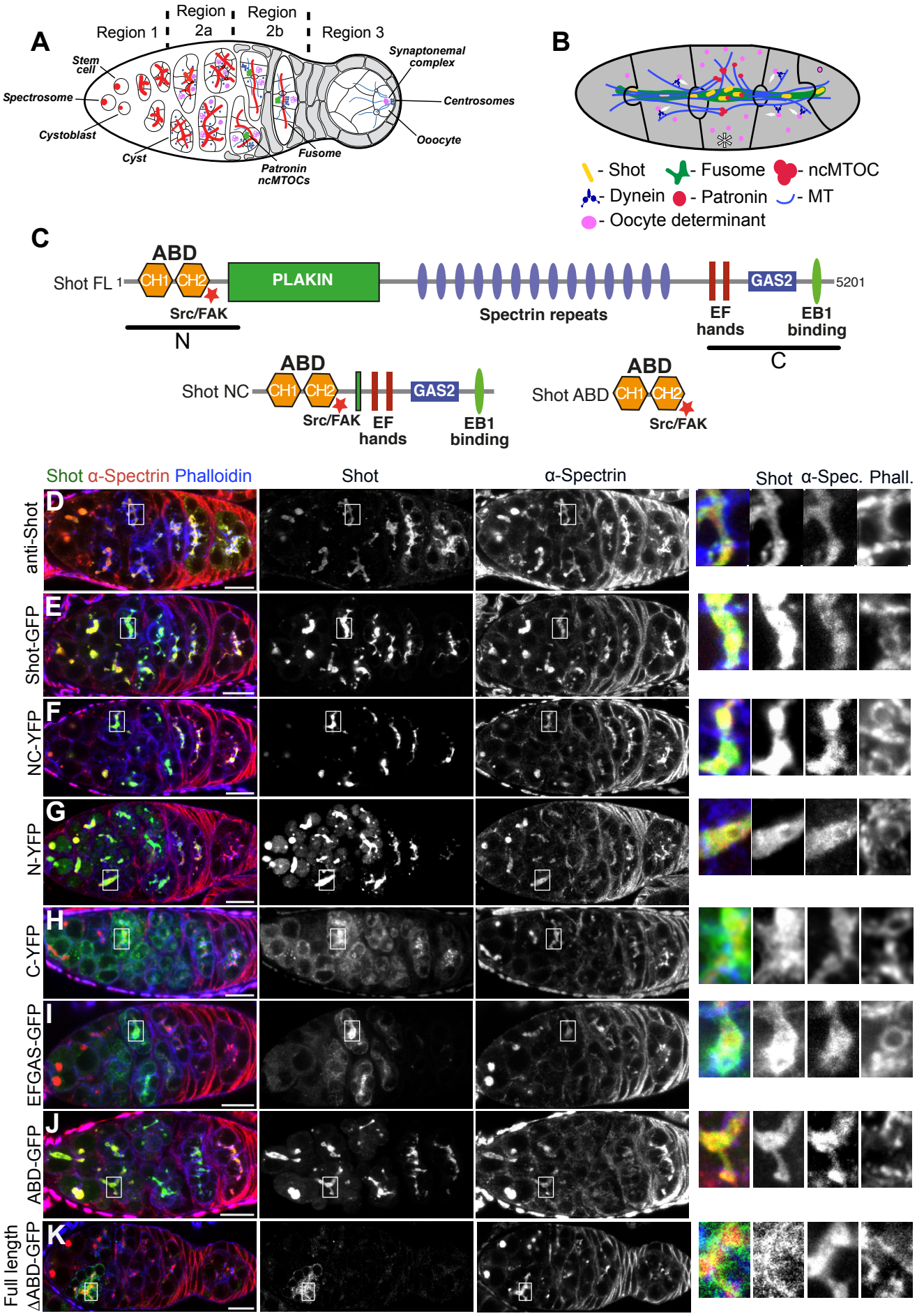


Figure 1. Shot is recruited to the fusome by its actin-binding domain.

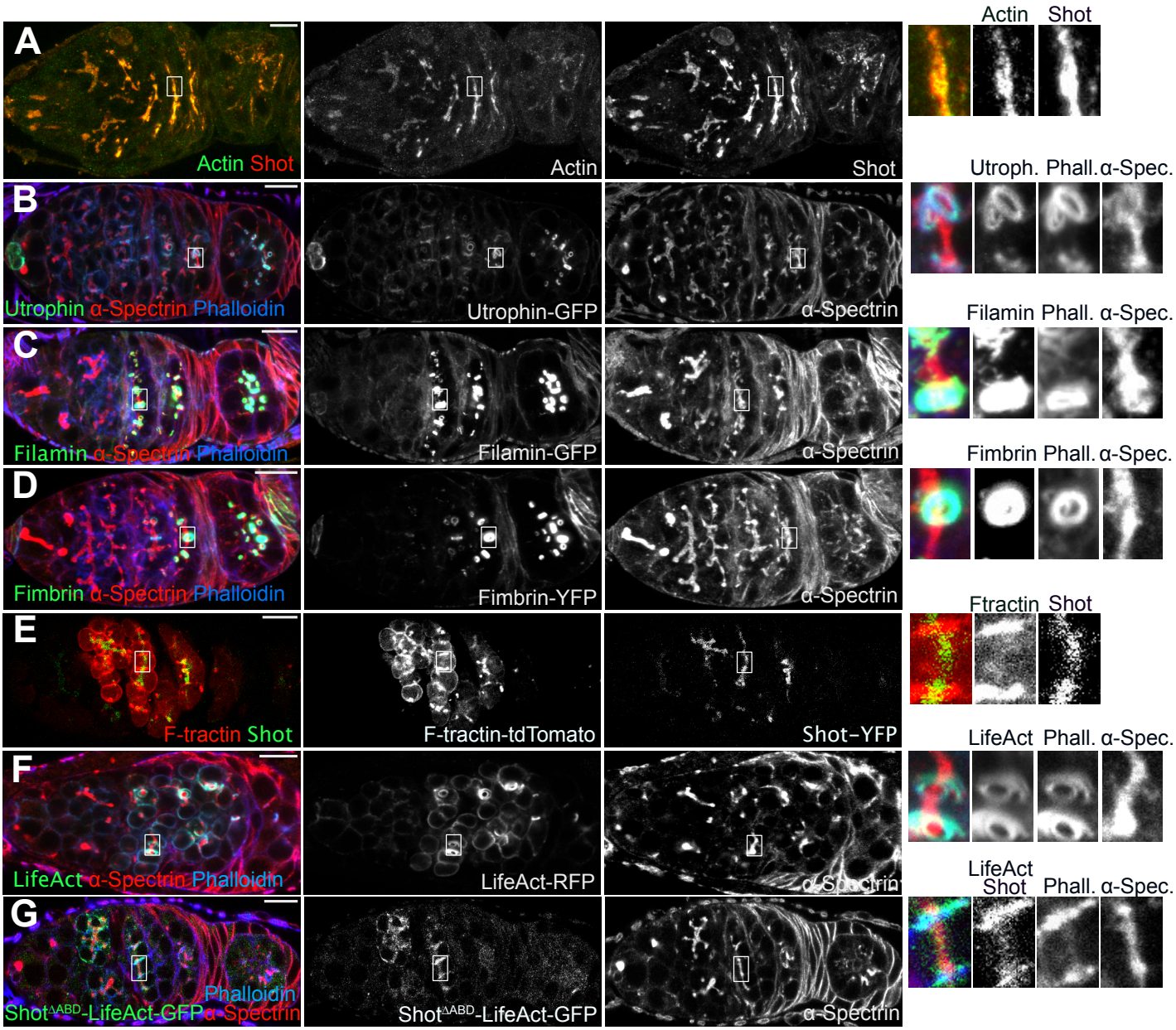


Figure 2. Standard F-actin labelling reagents fail to recognise fusomal F-actin

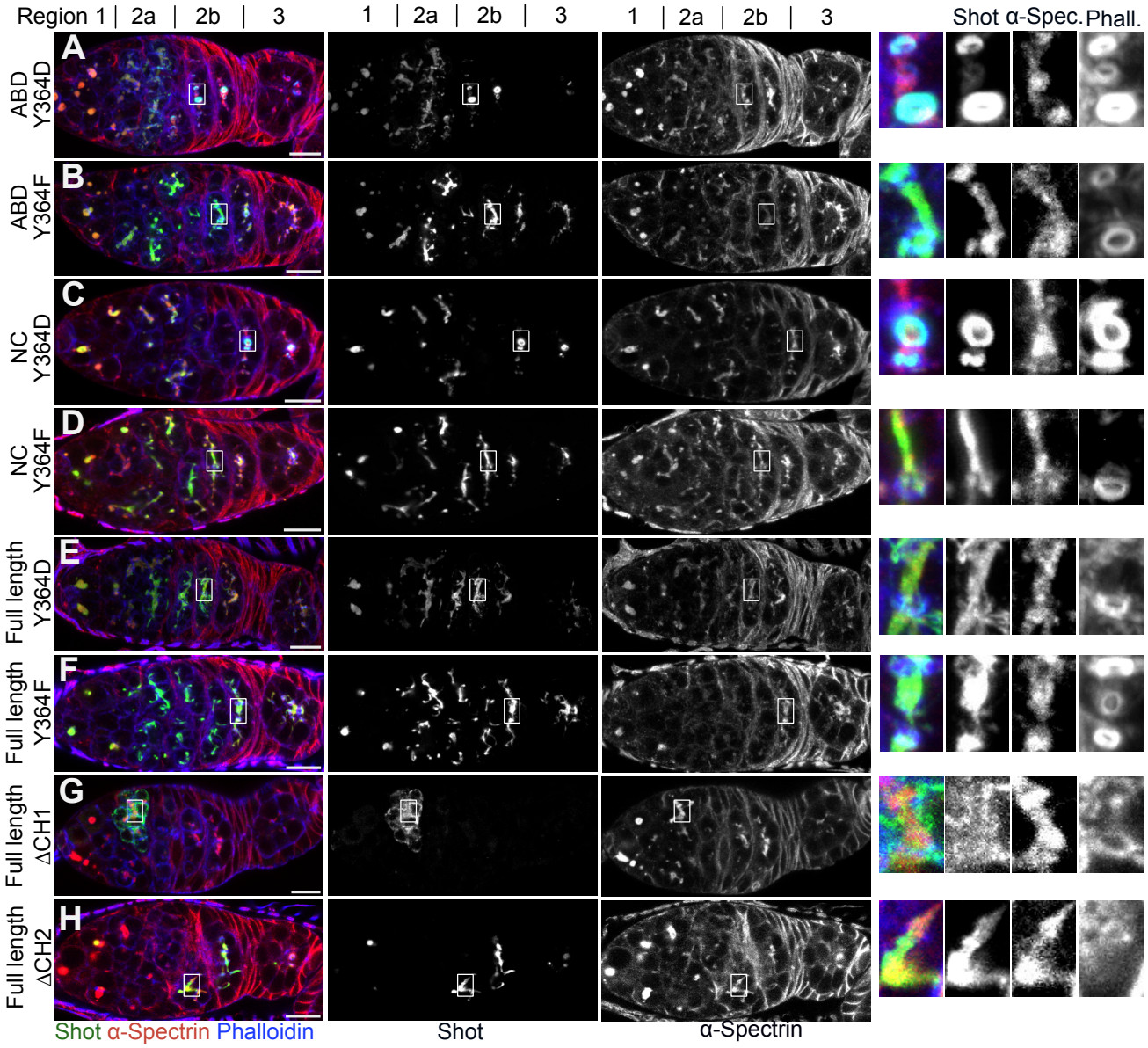


Figure 3. The Shot CH1 domain recognises the fusome

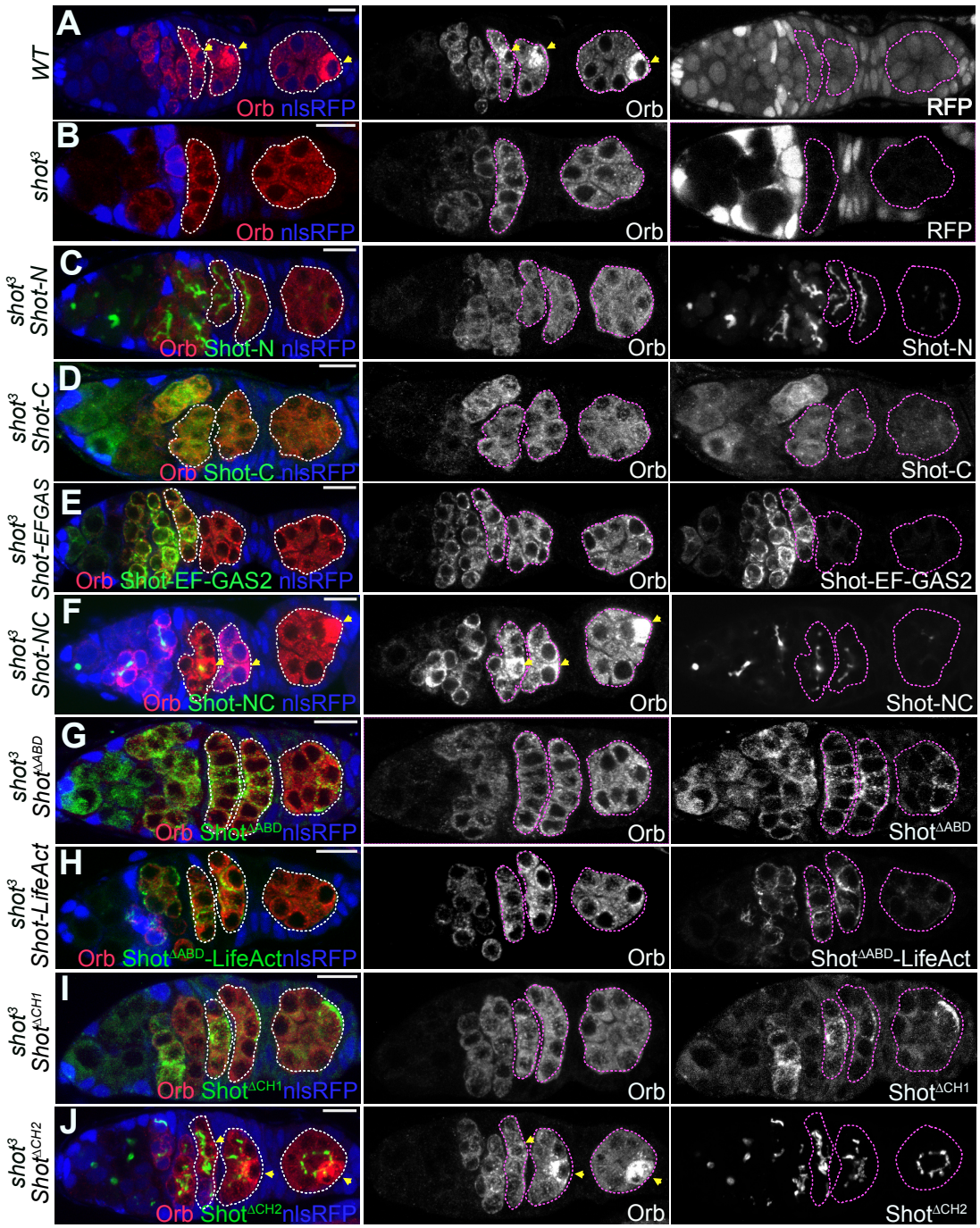


Figure 4. The Shot CH1 and MT-binding domains are required for the oocyte determination

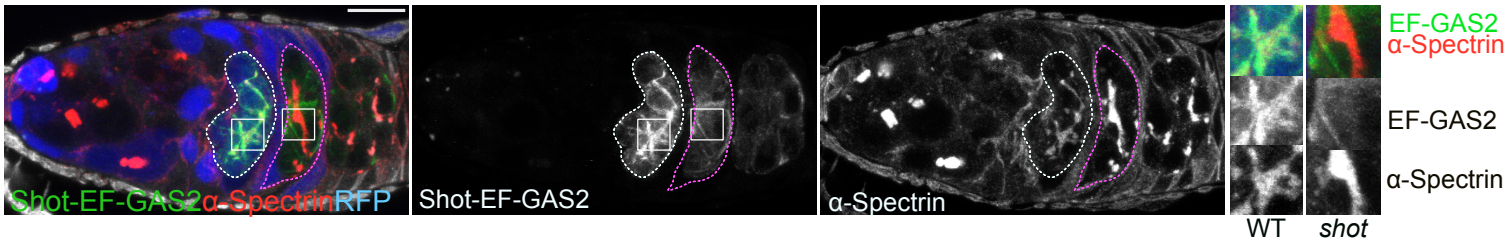
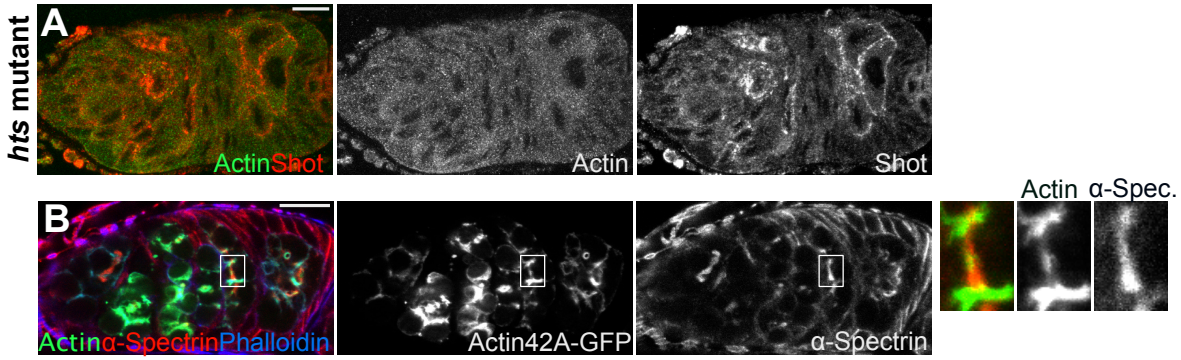
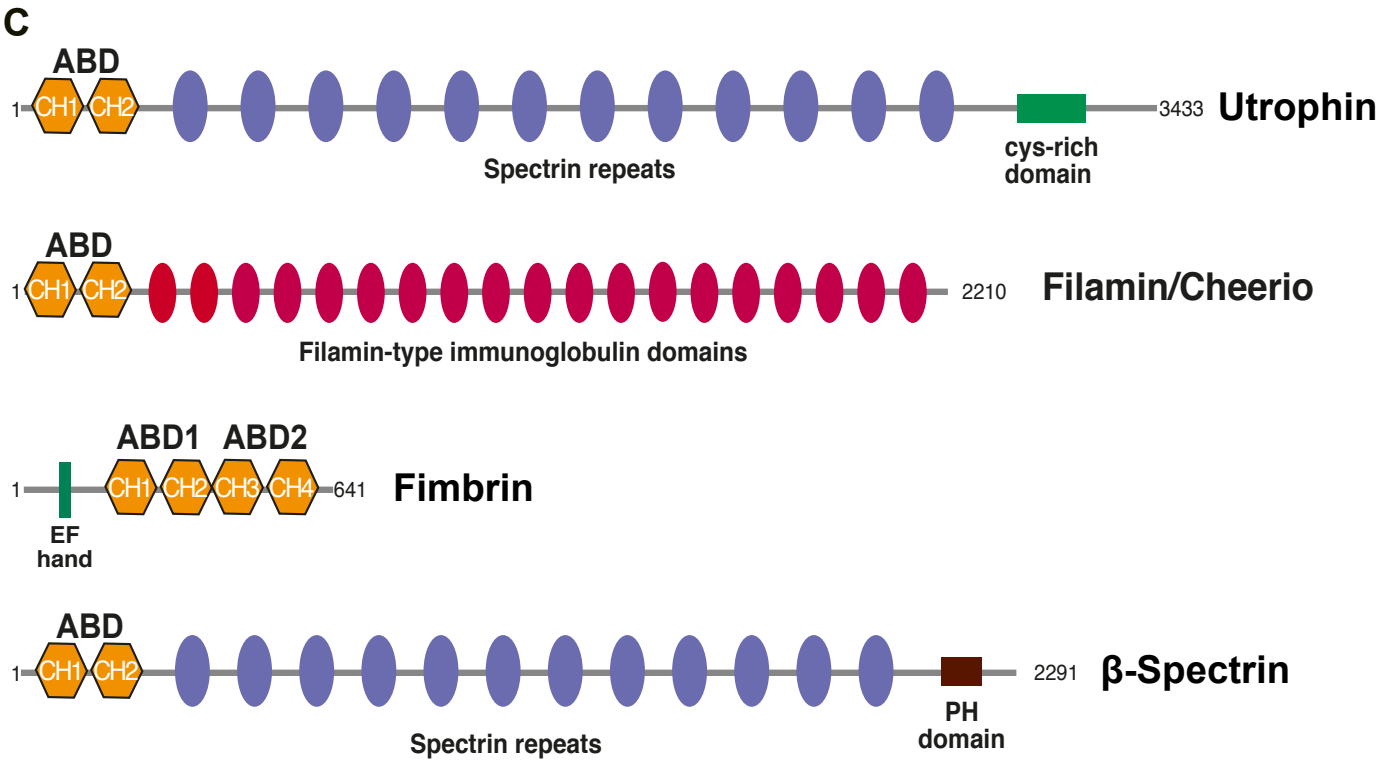


Figure S1. Shot-EF-GAS2 does not localises to the fusome in *shot* mutant cysts.


hts mutant
A
Actin Shot
Actin
Shot
B
Actin α-Spectrin Phalloidin
Actin42A-GFP
α-Spectrin
Actin α-Spec.


C
ABD
CH1
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3433
Utrophin
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cys-rich domain
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CH1
CH2
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2210
Filamin/Cheerio
Filamin-type immunoglobulin domains
ABD1
ABD2
CH1
CH2
CH3
CH4
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PH domain

Figure S2

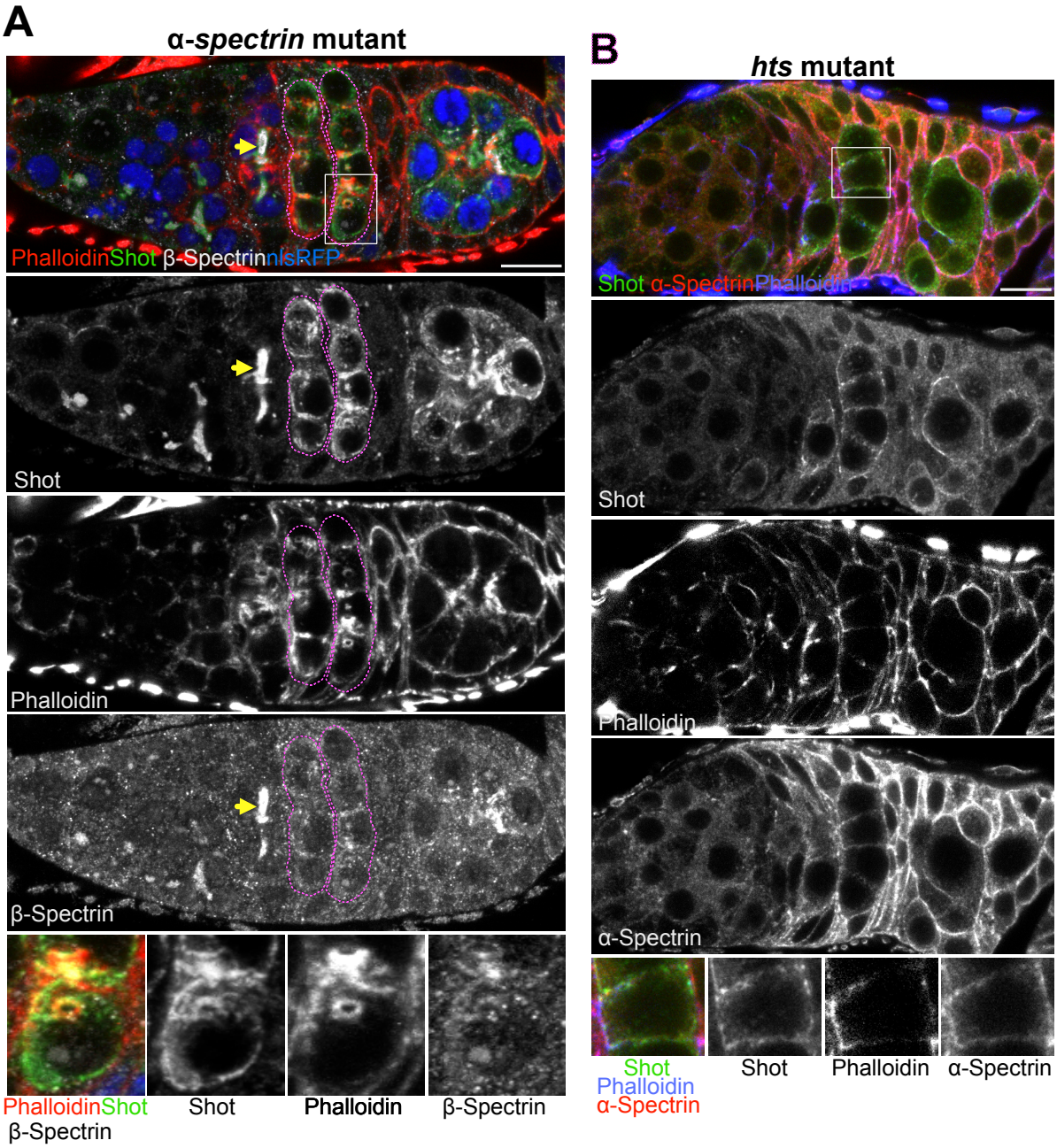

Figure S3. Shot localisation in *α-spectrin* and *hts* mutant cysts