Partial Functional Diversification of Drosophila melanogaster Septin Genes Sep2 and Sep5

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ABSTRACT The septin family of hetero-oligomeric complex-forming proteins can be divided into subgroups, and subgroup members are interchangeable at specific positions in the septin complex. Drosophila melanogaster has five septin genes, including the two SEPT6 subgroup members Sep2 and Sep5. We previously found that Sep2 has a unique function in oogenesis, which is not performed by Sep5. Here, we find that Sep2 is uniquely required for follicle cell encapsulation of female germline cysts, and that Sep2 and Sep5 are redundant for follicle cell proliferation. The five D. melanogaster septins localize similarly in oogenesis, including as rings flanking the germ line ring canals. Pnut fails to localize in Sep5; Sep2 double mutant follicle cells, indicating that septin complexes fail to form in the absence of both Sep2 and Sep5. We also find that mutations in septins enhance the mutant phenotype of bazooka, a key component in the establishment of cell polarity, suggesting a link between septin function and cell polarity. Overall, this work suggests that Sep5 has undergone partial loss of ancestral protein function, and demonstrates redundant and unique functions of septins.

Septins are a family of cytoskeletal GTP-binding proteins that form hetero-oligomeric rod-like complexes, which can further assemble into higher-order structures such as filaments or rings (John et al. 2007; Sirajuiddin et al. 2007; Bertin et al. 2008; DeMay et al. 2011). Septins play roles in cell division (Hartwell et al. 1970; Longtine et al. 1996), regulation of cell shape and membrane rigidity (Tooley et al. 2009; Mostowy et al. 2011; Gilden et al. 2012), restriction of lateral diffusion at membranes (Schmidt and Nichols 2004; Caudron and Barral 2009; Hu et al. 2010; Kwitny et al. 2010; Spilotis and Gladfelter 2012; Clay et al. 2014; Ewers et al. 2014), protein scaffolding (Hanrahan and Snyder 2003; Kozubowski et al. 2005; Kinoshita 2006; Hagiwara et al. 2011; Hall and Russell 2012; Feng et al. 2015), and maintenance of cell polarity (Barral et al. 2000; Takizawa et al. 2000; Spilotis et al. 2008; Berepiki and Read 2013). Animal septins are divided into four subgroups: SEPT2, SEPT6, SEPT7, and SEPT3 (Kinoshita and Noda 2001). Septin hetero-oligomeric complexes have a subgroup-specific linear order (Kinoshita 2003; Sirajuiddin et al. 2007, 2009; Bertin et al. 2008; Nakahira et al. 2010); for example, mammalian septin hexamers have a 7-6-2-6-7 subgroup organization. Septin subgroup members can be interchangeable (Kinoshita 2003), as observed for Saccharomyces cerevisiae septins Cdc11 and Shs1 (Bertin et al. 2008; Finnigan et al. 2015a,b) and mammalian septins SEPT6, SEPT8, and SEPT11 (Sellin et al. 2011a). However, subgroup members have distinct characteristics, such as protein interactions (Nakahira et al. 2010) and expression patterns (Cao et al. 2007; Tsang et al. 2008; Peterson and Petty 2010). The combination of interchangeability and distinct characteristics can lead to functionally distinct populations of septin complexes acting within cells and across tissues (Hernández-Rodríguez et al. 2014). Duplication and functional divergence of septin genes was likely important for generating functional diversity in the septin gene family.

Whereas mammals have 13 septin genes (Cao et al. 2007), Drosophila melanogaster has five (Adam et al. 2000); Sep1 and Sep4 (SEPT2 subgroup), Sep2 and Sep5 (SEPT6), and pnut (SEPT7). D. melanogaster septin complexes with Sep1, Sep2, and Pnut have been isolated (Field et al. 1996; Oegema et al. 1998). Protein–protein interaction data indicate that Sep5 also interacts with Sep1 and Pnut (Guruharsha et al. 2011), suggesting interchangeability of Sep2 and Sep5. Whereas Sep2 and Sep5 single mutants survive to adulthood, Sep5; Sep2 double mutants lack imaginal discs and die as prepupae (O’Neill and Clark 2013), similar to pnut mutants (Neufeld and Rubin 1994). Sep2 mutants also

KEYWORDS

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have oogenesis defects that are not rescued by overexpression of Sep5, showing that Sep2 and Sep5 have diverged in function (O’Neill and Clark 2013).

Here, we further explore Sep2 and Sep5 in gametogenesis, finding that Sep2 has a unique function for follicle cell encapsulation of female germline cysts, and is redundant with Sep5 for follicle cell proliferation and localization of Pnut. Further, Sep2 and Sep5 have similar subcellular localization in oogenesis. Heterozygosity for mutations in Sep2, Sep5, and pnut enhance the embryonic lethal phenotype of bazooka, a key regulator of epithelial cell polarity. Although several components of cell polarity are not perturbed in septin mutants, the interaction with bazooka suggests a connection between cell polarity and septin function in D. melanogaster. This work highlights the complexity of septin function in multicellular organisms, where septin subgroup members can have redundant functions as well as unique functions required in certain contexts.

MATERIALS AND METHODS

Fly strains and culture

w^{1118}, y^{5} w^{5}; P[Sep2-GFP.GA]/CyO, y^{5} w^{5}; P[UASp-Sep1.GFP]3/CyO, w^{5}; P[UASp-Sep4.GFP]3/TM3 Sb^{5}, w^{5}; P[UASp-Sep5.GFP]3, w^{1118}; P[GA4:VP16- nos.UTR]CG6325MDV1, y^{5} w^{5}; P[Ad5C-GAL4]25FO1/CyO, y^{5} w^{5}; P[tubP-GAL4]1/LITM3 Sb^{5} Ser^{5}, y^{5} baz/FM7a, pnut^{XP}/T(2;3)SM6aTM6B T^{b}, P[hsFLP]22, w^{1118}, P[neoFRT]82B P[Ubi-GFP]6S5Tm6 3R/TM6B T^{b}, P[neoFRT]82B ca^{1} sr^{1} e^{1} ca^{1}, and y^{5} w^{5} P[PTT-GC] baz^{cco941}. Samples were obtained from Bloomington Drosophila Stock Center at Indiana University. Sep2^{5}, Sep5^{5}, P[UASp-Sep2]18A, and P[UASp-Sep5]33B were generated as described in O’Neill and Clark (2013). Flies were reared on standard cornmeal-molasses-agar medium or Equation 4-24 plain instant media (Carolina Biological Supply Company, Burlington, NC) at 25°C and 60% relative humidity. Crosses to generate mitotic clones for eye, female germline, and egg length analyses were between w^{5} hsFLP; Sep5^{5}; FRT P[Ubi-GFP, w^{5}] virgin females and w^{5}; Sep5^{5}; FRT ca^{1} sr^{1} e^{1} ca^{1}, w^{5}; FRT ca^{1} Sep2^{1}/TM6B, or w^{5}; Sep5^{5}; FRT ca^{1} Sep2^{1}/TM6B males. Mitotic clones were induced by heat shocking larvae at 38°C. Clones in eyes were generated by a 1 hr heat shock at the second instar. Areas of mitotic clone twin spots were measured from stereomicroscope digital images using Fiji (Schindelin et al. 2013). Clones for analyses of egg length and ovary phenotypes were generated by 1 hr heat shocks 3 and 4 d in a row, respectively, starting at the second instar.

Immunofluorescence

One-day-old females were aged for 2 d with yeast paste and males. Ovaries were dissected on ice in PBS (phosphate buffered saline) and fixed for 20 min in 4% w/v paraformaldehyde in PBS. Fixed samples were washed in PBST (PBS with 0.1% Triton-X-100) and then permeabilized for 2 hr in PBS with 1% Triton-X-100 and 2% normal goat serum (NGS; Jackson ImmunoResearch Laboratories Inc.). Samples were washed in PBST + 2% NGS and then incubated with primary antibody at 4°C overnight. Samples were washed six times in PBST + 2% NGS and then incubated for 2 hr with secondary antibody. Samples were washed with PBST and then incubated with 10 μM Draq5 (Cell Signaling Technology) and 10 μg/mL rhodamine phalloidin (Sigma-Aldrich) for 20 min. Samples were finally washed in PBS and stored in 90% glycerol:PBS at 4°C. The following primary antibody concentrations were obtained from the Developmental Hybridoma Studies Bank at The University of Iowa: anti-Orb 6H4 (diluted 1:20), anti-Pnut 4C9H4 (1:20), anti-α-spectrin 3A9 (1:100), anti-Hs-RC (1:100), anti-Disc large 4F3 (1:20), and anti-Armadillo (1:20). Anti-Annulin (1:50) was a gift from Julie Brill. The secondary antibodies Alexa Fluor 594-conjugated AffiniPure Goat Anti-Mouse IgG and Alexa Fluor 488-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) were purchased from Jackson ImmunoResearch Laboratories Inc. (diluted 1:500). Fluorescence images were acquired using a Leica SP2 Confocal microscope. Fiji (Schindelin et al. 2013) was used for image processing.

Male fertility

Males of various genotypes and wild-type (w^{1118}) virgin females were collected daily and separately aged for 3–4 d with yeast paste. Individual males and five virgin females were placed in single vials. After 5 d, males that produced larvae were scored as fertile.

Septin enhancement of baz

We tested for enhancement of the baz^{4} embryonic lethal defective cuticle phenotype similarly to Shao et al. (2010). Virgin females of genotypes baz^{4}/w^{5}; Sep2^{1}/Sep2^{1}, baz^{4}/w^{5}; Sep5^{5}/Sep5^{5}, baz^{4}/w^{5}; pnut^{XP}/pnut^{+}, and baz^{4}/w^{5} were crossed to w^{1118} males. Eggs were collected for 24 hr on grape agar plates spread with yeast paste, and then were aged for 48 hr. Of the baz^{4}/Y arrested embryos, half were heterozygous for a mutant septin allele and half were homozygous wild type, except for the control where all were homozygous wild type. Unhatched eggs were dechorionated in 50% bleach for 2–3 min, mounted on slides with 1:1 Hoyer’s Mountant:lactic acid, and baked at 60°C overnight. Embryonic cuticles were scored blind into six categories using a Leica Digital Light Microscope with 40× objective.

Fly strains are available upon request. Supplemental Material, Figure S1 shows Sep5^{5}; Sep2^{5} germline cysts have wild-type distribution of several proteins, Figure S2 shows Sep2-GFP and Sep5-GFP fusion proteins are functional, Figure S3 shows Sep1-GFP and Sep4-GFP localization in oogenesis, and Figure S4 shows Sep5^{5}; Sep2^{5} follicle cells have wild-type distribution of several cell polarity proteins.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS

Sep2 is required for follicle cell encapsulation of germline cysts

In Drosophila oogenesis (reviewed in Spradling 1993), a germline stem cell in the gerarium produces a daughter cystoblast that undergoes four rounds of incomplete division to produce a cyst of 16 cells with cytoplasm connected by stable ring canals. One of the 16 cells becomes the oocyte and the other 15 become nurse cells. Precursor follicle cells envelope the germline cyst to form an egg chamber (Horne-Badovinac and Bilder 2005). Follicle cells proliferate in the gerarium and early egg chambers. The egg chamber then exits the gerarium and travels posteriorly along the ovariole as it develops into a mature egg.

We previously found that Sep2^{5} mutants, which contain a large deletion of the Sep2 coding region and are thus expected to be null mutants, have egg chambers with abnormal numbers of nurse cells (O’Neill and Clark 2013). To determine if the Sep2^{5} egg chamber defects are due to abnormal cystoblast divisions or fusion of multiple cysts into a single egg chamber, we counted oocytes and nurse cells per egg chamber by staining ovaries for Orb, which accumulates in the oocyte (Lantz et al. 1994), and DNA, which highlights polytene nurse cell nuclei (Dej and Spradling 1999; Figure 1). Whereas wild-type egg chambers always contain a single cyst (i.e., one oocyte and 15 nurse cells), 17% (18/107) of Sep2^{5} egg chambers contain multiple cysts...
(e.g., two oocytes and 30 nurse cells) and 2% (2/107) have numbers of nurse cells indicating failure of cystoblast division (i.e., not a multiple of 15). The Sep2\(^{22}\) egg chamber phenotype was rescued by driving expression of a Sep2 cDNA transgene, but not a Sep5 cDNA transgene, using Act5C-GAL4, consistent with previous results (O’Neill and Clark 2013).

We also examined male fertility and found that Sep2\(^{22}\) males are sterile; however, Sep2\(^{22}\) male sterility is rescued by driving expression of either a Sep2 or Sep5 transgene using tubP-GAL4 (Figure 1E).

**Sep2 and Sep5 are redundant for follicle cell proliferation**

Sep5\(^{22}\) mutants, which have a deletion of the entire Sep5 coding region, are viable and fertile; however, Sep5\(^{22}\)/Sep2\(^{22}\) double mutants lack imaginal discs and arrest as prepupae (O’Neill and Clark 2013). We used mitotic clones to investigate Sep5\(^{22}\); Sep2\(^{22}\) adult structures. In eyes, Sep5\(^{22}\); Sep2\(^{22}\) mitotic clones (Figure 2A) fail to proliferate compared to single mutant and wild-type control clones (Figure 2B). Mitotic clone twin spot area ratios (dark orange:white) among the three Figure 2B genotypes, \(w^{+}hsFLP; Sep5^{22}/Sep5^{+}\); FRT \(P(\text{Ubi-GFP} \ w^{+})/\text{FRT} \ cu^{1} \ Sep2^{22}\) (mean ratio 1.34, \(N = 40\)); \(w^{+}hsFLP; Sep5^{22}\); FRT \(P(\text{Ubi-GFP} \ w^{+})/\text{FRT} \ cu^{1} \ e^{r} \ e^{c} \ cu^{1}\) (mean ratio 1.33, \(N = 31\)), and \(w^{+}hsFLP; Sep5^{22}/Sep5^{+}\); FRT \(P(\text{Ubi-GFP} \ w^{+})/\text{FRT} \ cu^{1} \ e^{r} \ e^{c} \ cu^{1}\) (mean ratio 1.43, \(N = 42\)), were not significantly different (one-way ANOVA, p-value = 0.41). The curvature of the eye and transparency of white ommatidia likely made the edges of white clones appear pigmented, thereby biasing the ratios of twin spot areas toward dark orange. In contrast, only 12 of 24 dark orange clones in \(w^{+}hsFLP; Sep5^{22}; FRT \ P(\text{Ubi-GFP} \ w^{+})/\text{FRT} \ cu^{1} \ Sep2^{22}\) had a white mutant twin spot, and the mean dark orange:white ratio was 18. Therefore, Sep5\(^{22}\); Sep2\(^{22}\) mutant clones have a growth disadvantage (Figure 2A).

In oogenesis, double mutant follicle cell clones in \(w^{+}hsFLP; Sep5^{22}\); FRT \(P(\text{Ubi-GFP} \ w^{+})/\text{FRT} \ cu^{1} \ Sep2^{22}\) were less common and usually contained < 10 cells, compared to control clones in \(w^{+}hsFLP; Sep5^{22}\); FRT \(P(\text{Ubi-GFP} \ w^{+})/\text{FRT} \ cu^{1} \ e^{r} \ e^{c} \ cu^{1}\) and \(w^{+}hsFLP; Sep5^{22}/Sep5^{+}\); FRT \(P(\text{Ubi-GFP} \ w^{+})/\text{FRT} \ cu^{1} \ Sep2^{22}\), which were more common and always consisted of more than 10 cells (Table 1). The majority of Sep5\(^{22}\); Sep2\(^{22}\) follicle cell clones had some cells with pyknotic nuclei (Figure 2C) indicating cell...
Figure 2. Sep2 and Sep5 share a redundant function for follicle cell proliferation but are dispensable for cystoblast divisions. w+/hsFLP; Sep5\(^2\); FRT P[w\(^+\)]/w\(^+\); FRT Sep2\(^2\), w+/hsFLP; Sep5\(^2\); FRT P[Ubi-GFP]/FRT cu1 sr1 es ca1, w+/hsFLP; Sep5\(^2\)/Sep5\(^+\); FRT P[Ubi-GFP]/FRT cu1, w+/hsFLP; Sep5\(^2\); FRT P[Ubi-GFP]/FRT cu1 Sep2\(^2\), and w+/hsFLP; Sep5\(^2\); FRT P[Ubi-GFP]/FRT cu1 Sep2\(^2\) were used to generate wild-type control, wild-type in a Sep5\(^2\) background, Sep2 single mutant, and Sep5; Sep2 double mutant clones, respectively. (A) Sep5\(^2\); Sep2\(^2\) mitotic clones are small (A1, A2, arrowheads) or absent (A3) in eyes. Dark orange clones in a light orange background are the homozygous Sep2\(^+\) twin spots of Sep2\(^2\) clones that did not grow in a Sep5\(^2\) background. (B) Dark orange: white area ratios of Sep2\(^2\) clones in a Sep5\(^+\) background (B1), and Sep2\(^+\) clones in Sep5\(^2\)/Sep5\(^+\) (B2) and Sep5\(^+\) (B3) backgrounds are not significantly different. (C) Sep5\(^2\); Sep2\(^2\) follicle cells are often misshapen, have pyknotic nuclei (arrowhead), and often fail to maintain epithelial structure (e.g., stage six egg chamber shown in C1). (D) Some Sep5\(^2\); Sep2\(^2\) follicle cells have enlarged nuclei (e.g., stage eight egg chamber in D1, arrowhead in D2), indicating failure of cytokinesis. (E) Egg chambers with only Sep5\(^2\); Sep2\(^2\) follicle cells have severely reduced follicle cell numbers (E1 and E2 show focal planes through the middle and follicle cell layer of the same stage four egg chamber, respectively). (F) Sep5\(^2\); Sep2\(^2\) germline clones appear wild-type. (G) Genotypes of laid eggs were determined by presence of GFP (GFP negative eggs arose from either wild-type, Sep2\(^2\) or Sep5\(^2\); Sep2\(^2\) germline clones). Reduction in length of eggs from w+/hsFLP; Sep5\(^2\)/Sep5\(^+\); FRT P[Ubi-GFP]/FRT cu1 Sep2\(^2\) females is independent of the genotype of germline clone genotype, suggesting that follicle cell genotype determines egg length. Scale bar = 10 \(\mu\)m. GFP, green fluorescent protein; Sep, septin.
death, enlarged nuclei (Figure 2D) indicating failure of cytokinesis, and epithelial defects where cells had lost their position in the follicular epithelium (Figure 2C and Table 1). Some Sep2-GFP follicle cell clones in a Sep52/Sep5+ background also had enlarged nuclei (Table 1). Rare ovarioles were observed that had entirely Sep52; Sep2-GFP follicle cells; the few follicle cells surrounding cysts in these ovarioles were misshapen and appeared to have pyknotic nuclei and lack cell polarity, and these egg chambers were never observed beyond stage 4 (Figure 2E). In contrast, Sep52; Sep22 germ line clones were relatively common and did not have defects in cell number (Figure 2F). Sep52; Sep22 germ line clones have wild-type staining for Orb in the oocyte, α-spectrin at the fusionome, actin and Hts-RC at the ring canals, and anillin at the cytokinetic furrow (Figure S1).

We previously observed that Sep2-GFP eggs were shorter than wild-type eggs (O’Neill and Clark 2013), similar to pnut×P eggs (Adam et al. 2000) and a small egg or dumpless phenotype (Spradling 1993). To determine if abnormal egg morphology is due to loss of septins in the maternal germline, we induced mitotic recombination in germline mosaics, consistent with the failure of proliferation in Sep52; Sep2-GFP follicle cell clones (Figure 2, C and D).

**Table 1 Mosaic egg chamber phenotypes**

| Genotype  | GFP –ve Germline | GFP –ve Follicle Cells | Small Clones | Pyknotic Nuclei | Epithelial Defects | Large Nuclei |
|-----------|-----------------|------------------------|--------------|----------------|------------------|-------------|
| Sep52 control | 0.27 (8/30) | 0.8 (24/30) | 0 | 0 | 0.04 (1/24) | 0 |
| Sep22 control | 0.26 (8/31) | 0.68 (21/31) | 0 | 0 | 0.05 (1/21) | 0.19 (4/21) |
| Sep52; Sep22 | 0.21 (11/52) | 0.38 (20/52) | 0.85 (17/20) | 0.75 (15/20) | 0.7 (14/20) | 0.65 (13/20) |

Stage seven to eight mosaic egg chambers from three genotypes were scored. Sep52 control was w+ hsFLP; Sep52; FRT P{Ubi-GFP}/FRT cu1 sr1 e1 ca1. Sep22 control was w+ hsFLP; Sep52; FRT P{Ubi-GFP}/FRT cu1 Sep22. Sep52; Sep22 was w+ hsFLP; Sep52; FRT P{Ubi-GFP}/FRT cu1 Sep22.

- Showing proportion of egg chambers with GFP negative follicle cells that had at least one GFP negative follicle cell with a given phenotype.
- GFP negative follicle cell clones with < 10 cells.
- GFP negative follicle cells that were either outside the follicle cell epithelium or inside the space occupied by the germline.

We tested whether Sep2-GFP, which contains upstream and downstream noncoding sequences of Sep2 to drive its expression (Silverman-Gavrilova et al. 2008), and Sep5-GFP, which has a UAS and therefore requires GALA4 for expression (Su et al. 2013), encode functional proteins by performing rescue experiments (Figure S2). Sep2-GFP rescues the Sep22 nurse cell phenotype. A few (3.5%, 4/113) Sep2-GFP Sep22/ Sep22 egg chambers had only 13–14 nurse cells, which could indicate that Sep2-GFP has a weak dominant negative effect on cystoblast divisions. Both Sep2-GFP and Act5C-GAL4 > Sep5-GFP rescue the Sep52; Sep22 prepupal lethal phenotype (note that double mutants expressing Act5C-GAL4 > Sep5-GFP arrest at the end of metamorphosis, but this is attributed to GAL4 sensitivity because Sep2-GFP transgenics with GAL4 also arrest). Two copies of Sep2-GFP increase lethality at the end of metamorphosis, further suggesting a weak dominant negative effect of the transgene. Together, these results indicate that the Sep2-GFP and Sep5-GFP transgenes encode functional proteins and should therefore be useful for investigating Sep2 and Sep5 localization.

We characterized the localization of Sep2-GFP and Sep5-GFP (Figure 3), as well as Sep1-GFP and Sep4-GFP (Figure S3), and Pnut immunostaining (Figure 4), in oogenesis, finding that they localize similarly. Note that, except for Sep2-GFP which contains its own promoter and regulatory region, the septin-GFP transgenes were expressed using nos-GAL4; although RNA-sequencing of whole ovaries shows that Sep5, Sep1, and Sep4 are expressed (modENCODE tissue RNA-seq presented on FlyBase.org. Attrill et al. 2016), their expression patterns in ovaries are unknown. In proliferating germline cells, septin-GFPs are localized cytoplasmically, with a higher concentration at the cell cortex and cytokinetic furrows (Figure 3, B and F). They remain at the outer rim of ring canals throughout oogenesis, appearing as double rings flanking the germline ring canals from around stages four to nine of egg chamber development (Figure 3, C and G). At around stage four of egg chamber development, Sep2-GFP and Sep5-GFP localize as double rings flanking the germline ring canals (Figure 3, C and G), indicating that they do not form a single continuous structure that spans the cell-cell boundary. Only a single ring is observed after egg chamber expansion around stage nine (not shown). We observed these double rings in unfixed Sep2-GFP egg chambers, showing that they are not an artifact of fixation (data not shown). In spermatogenesis, Sep2-GFP also localizes to germline ring canals, occasionally appearing as double rings, and also appears to localize to the fusome (Figure 3E). Septin-GFPs, particularly those driven by nosGALA4, also localize as cytoplasmic puncta and rings in both germline and follicle cells throughout oogenesis (example Figure 3F). Sep2-GFP localizes to the cortex of follicle cells in oogenesis (Figure 3C). After stage ten, Sep2-GFP is more strongly concentrated at the cortex of the oocyte relative to nurse cells, and is strongly expressed in border cells (not shown), as found for Sep1 (Fares et al. 1995).

Pnut localization in oogenesis, which was previously described by Adam (1999), is similar to that of the septin-GFPs (Figure 4, A and B) and colocalizes with Sep2-GFP (Figure 4C); however, Pnut differs from Sep2-GFP in that it is localized more strongly to the basal cortex compared to the lateral cortex in follicle cells (Figure 4B). In Sep22 mutants, Pnut is less concentrated in the germlarium, although it still weakly localizes to ring canals (Figure 4D) and in older egg chambers (Figure 4E). However, in Sep52; Sep22 clones, Pnut fails to localize to ring canals of germline cysts (Figure 4F) or in follicle cells (Figure 4G). Sep52 mutant cells appear to have wild-type Pnut (compare GFP-positive cells in Figure 4, G and B). Therefore, Sep2 and Sep5 are redundant for Pnut localization at female germ cell ring canals and the cortex of follicle cells.

**Sep22, Sep52, and pnutXP enhance baz4 defective embryonic cuticle phenotype**

Baz encodes a scaffolding protein important for cell polarity (Macara 2004; Margolis and Borg 2005; Wang and Margolis 2007). Baz4 hemizygotes are embryonic lethal and have cuticle defects (Bilder et al. 2003). A screen for enhancers of this baz4 phenotype identified Sep5...
(Shao et al. 2010). Here, we also find that the baz⁴ embryonic cuticle phenotype is significantly enhanced in Sep⁵, Sep⁴, and pnut⁴ heterozygotes compared to baz⁴ alone (Figure 5). The extent of enhancement by Sep⁴ or Sep⁵ is not significantly different, whereas the enhancement by pnut⁴ is significantly greater than Sep⁴ and Sep⁵.

To ask whether septins are required for the establishment or maintenance of cell polarity, we investigated the localization of several markers for cell polarity in Sep⁵; Sep⁴ follicle cells, finding that the cell polarity components Armadillo and Discs-large and the cytoskeletal component a-spectrin localize correctly, and that Sep⁴ mutant follicle cells have wild-type localization of Baz-GFP (Figure S4).

Figure 3 Sep2-GFP and Sep5-GFP localize similarly in oogenesis. Sep2-GFP (A–D) and nosGAL4 > UASp-Sep5-GFP (E–G) were used to characterize Sep2 and Sep5 localization. In the germarium (A, B, E, and F) and egg chambers (e.g., stage six egg chambers in C and G), Sep2-GFP and Sep5-GFP localize cytoplasmically with a concentration at the cell cortex, and are concentrated at the outer rim of ring canals (B, C, F, and G, arrowheads) and as cytoplasmic puncta (F, arrow). Beginning at around stage four of egg chamber development, septin-GFPs appear to form rings that flank either side of the ring canal (C and G, arrowheads). Sep2-GFP is localized to the cell cortex of follicle cells (C). In testes (D), Sep2-GFP has a cytoplasmic localization, is localized at ring canals (in some cases as double rings), and appears to localize to the fusome (for example, structure enclosed in large white box, and highlighted with a dotted outline in right panel). B and F show magnifications of white boxes in A and E, respectively. Scale bar = 10 μm. GFP, green fluorescent protein; Sep, septin.

DISCUSSION

Sep2 and Sep5 are redundant for Pnut localization

We found that Pnut fails to localize in Sep⁵; Sep⁴ cells (Figure 4, F and G). This suggests that both Sep2 and Sep5 interact with Pnut by occupying the SEPT6 positions of the D. melanogaster septin complex. Sep⁵ cells had lower levels of Pnut immunostaining at germline ring canals and follicle cells compared to wild-type or hsFLP; Sep⁵; FRT P{Ubi-GFP}/FRT Sep⁴ cells, consistent with the higher expression level of Sep2 compared to Sep5 in oogenesis (modENCODE Anatomy RNA-seq; Attrill et al. 2016). In mammals, SEPT2 and SEPT6 subgroup members initiate complex assembly, followed by binding of SEPT7 (Sellin et al. 2011a); our results are consistent with this model of assembly, where the D. melanogaster SEPT7 subgroup member Pnut can only bind after the initiating septins, which presumably include Sep2 or Sep5, have assembled. Requirement of one septin for the localization of another has been found previously, including tissue-specific requirements; for example, in embryos pnut is required for localization of Sep1 (Fares et al. 1995) but not Sep2 (Adam et al. 2000), whereas in dorsal pupal epithelial cells pnut is required for localization of Sep2 (Founounou et al. 2013).

Septins are dispensable in germline

All five D. melanogaster septins localize to ring canals (Figure 3, Figure 4, and Figure S3), and Sep⁵ egg chambers show numbers of nurse cells suggestive of occasional defects in cystoblast division (O’Neill and Clark 2013). Consistent with previous observations for pnut mutant germline clones (Adam et al. 2000), we also found that Sep⁵; Sep⁴ female germline clones are relatively common and do not show cell number defects. Sep⁵; Sep⁴ germline cysts can fully develop into eggs,
although we did not determine if these eggs are viable. Together, these observations suggest that septins have a subtle or nonessential role in the development of female germline cysts.

Septin localization at cytokinematic furrows and ring canals was detected previously. Cytokinematic furrow localization was observed for Sep1, Sep2, and Pnut in Drosophila S2 cells (Longtine et al. 1996; D'Avino et al. 2008), for Pnut and Sep2-GFP in pupal dorsal epithelium (Founounou et al. 2013), Pnut-mCherry in embryonic epithelium (Guillot and Lecuit 2013), and for Sep2-GFP in follicle cells (Morais-de-Sá and Sunkel 2013). Sep1, Sep2, and Pnut were all previously detected in germline ring canals during spermatogenesis (Hime et al. 1996). The presence of septins at germline ring canals during oogenesis was not entirely clear; concentrations of Sep2, Pnut, and Sep1 were sometimes observed at female germline ring canals (Fares et al. 1995; Adam 1999). The double ring localization of septin-GFPs is similar to the localization of phospho-tyrosine immunostaining found at male germline ring canals (Eikenes et al. 2015), and is reminiscent of the double septin ring at the bud neck of S. cerevisiae (Jiménez et al. 1998; Renz et al. 2016) and the double ring localization of Hts-RC in this study (Figure S1, F and G); thus, double ring localization may be an aspect of germline ring canal structure and a function that warrants further investigation. Although septins are not required for ring canal formation in the female germline, it is conceivable they might have a nonessential role at their outer rims, perhaps by maintaining membrane shape or rigidity (Tanaka-Takiguchi et al. 2009; Tooley et al. 2009; Mostowy et al. 2011; Gilden et al. 2012), regulating membrane remodelling (Sellin et al. 2011b), acting as a lateral diffusion barrier (Schmidt and Nichols 2004; Caudron and Barral 2009; Hu et al. 2010; Kwitny et al. 2010; Spiliotis and Gläßfelter 2012; Clay et al. 2014; Ewers et al. 2014), or protein scaffolding (Hanrahan and Snyder 2003; Kozubowski et al. 2005; Kinoshita 2006; Hagiwara et al. 2011; Hall and Russell 2012; Feng et al. 2015). Although septins can alter microtubule organization (Kulic et al. 2008; Spiliotis 2010), and act as scaffolding for posttranslational modifiers of microtubules and motor proteins (Kremer et al. 2005;

Figure 4 Sep2 and Sep5 are redundant for Pnut localization. In wild-type (w^1118), Pnut localizes cytoplasmically and at ring canals in the germarium (A, with magnification of white box in right panel) and later egg chambers (not shown). In follicle cells of later stage egg chambers (e.g., stage six egg chamber in B), Pnut is most concentrated basally. Pnut colocalizes with Sep2-GFP (C). Pnut is present cytoplasmically and at ring canals in Sep2^2 mutants (D, with magnification of white box in right panel with arrowheads indicating ring canal staining), and in egg chamber follicle cells (e.g., stage six egg chamber in E), although it is less concentrated compared to w^1118 (A and B). Compared to GFP positive cells, which appear to have wild-type Pnut, Sep5^2; Sep2^2 cells (GFP negative, highlighted with yellow lines) lack wild-type Pnut localization; in particular, Pnut still has some cytoplasmic signal, but fails to localize to ring canals (F) and the basal side of follicle cells (G) in Sep5^2; Sep2^2 double mutant cells. All images were collected and processed identically. Scale bar = 10 μm. GFP, green fluorescent protein; Sep, septin.
consistently with previous results (O'Neill and Johnston 2000), occurs normally in Sep3; Sep2 oocytes, suggesting that transport via microtubules (at least in early stage egg chambers) is not septin-dependent.

Sep2 and Sep5 are required in follicle cells
Consistent with previous results (O'Neill and Clark 2013), the Sep2 mutants have egg chambers with abnormal numbers of nurse cells (Figure 1). Here, we determined that the majority of these were fused egg chambers where multiple germline cysts were encapsulated by follicle cells to form a single egg chamber. Although the Sep2 flies are Sep5+, the expression level and pattern of Sep5 in oogenesis is not known. Overexpression of a Sep5 cDNA transgene did not rescue the Sep2 egg chamber phenotype, whereas the Sep2 cDNA did, thus showing that the function of Sep2 in oogenesis is unique and not redundant with Sep5. This contrasts with the observation that overexpressing Sep5 rescues Sep2 male sterility, which shows that Sep2 and Sep5 proteins are redundant for male fertility. Wild-type Sep5 expression is normally low in testes compared to Sep2 which is moderately high (Chintapalli et al. 2007; Attrill et al. 2016), so Sep2 male sterility is probably due to loss of septin complex function via reduced expression of SEPT6 septins generally rather than Sep2 specifically.

Sep5; Sep2 follicle cell clones are typically small, and egg chambers with only double mutant follicle cells have reduced follicle cell numbers (Figure 2, C and E and Table 1). Further, double mutant follicle cells often have pyknotic nuclei, indicating cell death, or enlarged nuclei, indicating cytokinesis failure (Figure 2, C and D). These results suggest that Sep2 and Sep5 are redundant for follicle cell proliferation and maintenance. Consistent with this result, pnut is required for cytokinesis in follicle cells (Morais-de-Sá and Sunkel 2013). Further, this is reminiscent of the loss of imaginal cell proliferation in Sep5; Sep2 double mutants and mutant clones (O’Neill and Clark 2013; Figure 2A) and pnut mutants (Neufeld and Rubin 1994), and the requirement for pnut in planar cell division of the pupal dorsal epithelium (Fournonou et al. 2013) and actomyosin ring formation and constriction in embryonic epithelium (Guillot and Lecuit 2013). The irregular shape of Sep5; Sep2 follicle cells may simply be due to a failure to proliferate and consequent stretching of cells as egg chambers grow, or it could represent a loss of apicobasal polarity in addition to proliferation defects. In mammalian epithelial cells, SEPT2 depletion leads to fibroblast-like cell shape and lack of polarity (Spiliotis et al. 2008). Although septins are implicated in the development and extension of cellular processes (Finger et al. 2003; Shinoda et al. 2010) and the coordination of cell movements (Chacko et al. 2005), Sep5; Sep2 follicle cells are able to envelope germline cysts, suggesting that septins are not required for the formation of the cellular processes and the migration required for the encapsulation of germline cysts. Thus, it appears that septins are required for specific types of cell division in D. melanogaster, as they are in mammals (Menon et al. 2014), including epithelial cell divisions (Fournonou et al. 2013). Further, we found that Sep2 mutant egg morphology is independent of the genotype of germline, suggesting that mutant follicle cells are responsible; thus, it is possible that septins play a role in follicle cell rotation during oogenesis, which is required for egg elongation (Haigo and Bieder 2011).

Punctate septins
Punctate or ring-like cytoplasmic localization of septins (Figure 3, Figure 4, and Figure S3) has also been previously reported. In D. melanogaster, apically distributed puncta of Sep2-GFP, Sep5-GFP, and Pnut were observed in epithelia (Fournonou et al. 2013), and all septin-GFP fusions localized as puncta during cellularization of blastoderm.
embryos (Su et al. 2013). In human K562 cells, septin discs of ~0.8 μm diameter were observed during interphase; these discs were dependent on microtubules, and were disrupted during fixation (Sellin et al. 2011b). Larger and more prominent puncta were observed for nos-GAL4 driven septin-GFPs compared to Sep2-GFP and Pnut immunostaining, suggesting that overexpression can lead to septin-GFP aggregation. However, Pnut immunostaining does have a punctate appearance, indicating that septin puncta are not entirely artifactual. It is not clear whether these puncta have a specific cytoplasmic function, such as cytoplasmic cytoskeletal organization, or if they act as a septin reserve that can be rapidly deployed at the onset of cytokinesis or membrane deformation.

A link between cell polarity and septin function

Baz encodes a scaffolding protein that is important for cell polarity (Macara 2004; Margolis and Borg 2005; Wang and Margolis 2007); for example, in the D. melanogaster embryonic epithelium, Baz is apically localized, is required to establish apicobasal polarity, and forms complexes with Par-6αPKC and cadherin (Harris and Peifer 2004, 2005). While a previous screen found that the only septin to enhance the mutant baz embryonic cuticle defect was Sep5 (Shao et al. 2010), we found that at least three septins, Sep2, Sep5, and pnut, are enhancers. The lower level of baz enhancement by Sep2 and Sep5 is consistent with redundancy of Sep2 and Sep5, both SEPT6 septins, compared to pnut, which is the only SEPT7 septin in D. melanogaster.

The enhancement of the baz embryonic cuticle phenotype by septin mutants (Figure 5), the irregular shape and frequent loss of epithelial structure of Sep5; Sep2 follicle cells (Figure 2C and Table 1), and polarized localization of D. melanogaster septins found here (Figure 4B) and by others (Shao et al. 2010; Fournonou et al. 2013) suggest a link between septins and cell polarity in flies. In mammalian epithelial cells, septins associate with specific microtubule tracks to facilitate polarized transport of vesicles, and septin depletion leads to loss of apical and basolateral membrane protein localization (Spilioti et al. 2008). We reasoned that septins might be involved in the establishment or maintenance of cell polarity in D. melanogaster epithelial cells. However, whereas Sep5; Sep2 clones had disrupted localization of Pnut, several components of cell polarity pathways localized normally, suggesting that cell polarity does not require septins, and that septins function downstream of cell polarity or in an independent pathway. Potential connections between Baz and septins should be further explored. Baz interacts with the lipid phosphatase PTEN to regulate actin cytoskeleton organization and generate an apical enrichment of phosphatidylinositol (4,5) bisphosphate (von Stein et al. 2005). In humans, phosphatidylinositol (4,5) bisphosphate is bound by SEPT4 and required for septin filament formation (Zhang et al. 1999). So, it is possible that septin localization and function depend on proper localization of Baz.

Conclusions

The relationship between septin complex assembly and the diversity of the various septin subgroups raises the intriguing idea that the functional characteristics of individual septin complexes depend on their subunit composition, thus providing a mechanism to allow multiple distinct populations of septin complexes to operate independently within a single cell (Kinoshita 2006; Cao et al. 2009; Hernández-Rodríguez et al. 2014). Our investigation of Sep2 and Sep5 finds that they are redundant in imaginal tissues and follicle cells, yet Sep2 maintains a unique function in follicle cells. Thus, assuming that Sep2 ancestral function is conserved, we suggest that, after Sep5 arose via retropseudification, it underwent partial loss of this ancestral protein function. Whether the diversification of Sep2 and Sep5 is representative of septin evolution generally, and of septin functional diversity in other lineages, such as mammals with 13 septin genes, is unclear; it is worth noting that most human septins arose before the divergence of tetrapods from fish (Cao et al. 2007) and have thus had significantly more time for functional diversification than Sep2 and Sep5. This work highlights the importance of considering subgroup member redundancy and the potential diversity of functions across tissues when studying animal septins.

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