The actin-binding protein profilin is required for germline stem cell maintenance and germ cell enclosure by somatic cyst cells

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ABSTRACT

Specialized microenvironments, or niches, provide signaling cues that regulate stem cell behavior. In the Drosophila testis, the JAK-STAT signaling pathway regulates germline stem cell (GSC) attachment to the apical hub and somatic cyst stem cell (CySC) identity. Here, we demonstrate that chickadee, the Drosophila gene that encodes profilin, is required cell autonomously to maintain GSCs, possibly facilitating localization or maintenance of E-cadherin to the GSC-hub cell interface. Germline specific overexpression of Adenomatous Polyposis Coli 2 (APC2) rescued GSC loss in chick hypomorphs, suggesting an additive role of APC2 and F-actin in maintaining the adherens junctions that anchor GSCs to the niche. In addition, loss of chick function in the soma resulted in failure of somatic cyst cells to maintain germ cell enclosure and overproliferation of transiting-amplifying spermatogonia.

KEY WORDS: Profilin, Stem cells, APC2

INTRODUCTION

Adult stem cells are responsible for tissue homeostasis, generating short-lived but highly differentiated cell types and replenishing differentiated cells lost to injury. The molecular mechanisms that control stem cell function in vivo are crucial for regenerative medicine, and defects in these mechanisms may underlie degenerative diseases, tumor formation and aging. Short-range signals from the local microenvironment, the stem cell niche, maintain populations of adult stem cells over time through a balance between self-renewal and differentiation. The mechanisms by which stem cells recognize, attach to and orient towards their niche are essential for maintenance of regenerative capacity throughout the life of an individual.

The Drosophila testis stem cell niche supports germline stem cells (GSCs) and somatic stem cells (CySCs), both of which are attached to a group of non-dividing somatic cells: the hub. Hub cells express a secreted ligand, Unpaired, which activates the Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway in both GSCs and CySCs (Kiger et al., 2001; Tulina and Dinardo, 2008). Although a key pathway in both GSCs and CySCs (Kiger et al., 2001; Tulina and Dinardo, 2008), the STAT targets are a secreted ligand, Unpaired, which activates the Janus kinase–signal transducer and activator of transcription (JAK-STAT) pathway. The STAT loci are bound by activated STAT in Drosophila testes and required cell autonomously in germ cells to maintain GSCs at the hub, probably through effects on cell adhesion. In addition, chick function is required in somatic cyst cells for neighboring germ cells to differentiate.

RESULTS

chic is required cell autonomously for maintenance of germline stem cells in their niche

Loss of function of the single Drosophila profilin homolog, chic, caused failure to maintain male germline stem cells (GSCs) over developmental time, consistent with the effects of chic mutations on adult testes reported by Gönczy and DiNardo (1996). In third instar larvae, GSC number was markedly decreased in chic mutants compared with wild type (Fig. 1). Although null mutant combinations of chic alleles were embryonic lethal (Vethyey and Cooley, 1994; Baum and Perrimon, 2001), chic animals transheterozygous for either the hypomorphic chic11 and the strong loss-of-function chic1320 or for chic120 and the null chic121 survived to adulthood, so testes from these animals could be scored at larval stages. In wild-type late larval testes, a rosette of 12.0±2.6 GSCs (n=26 testes) surrounded the hub (Fig. 1A). However, only 3.6±2.8 GSCs (n=36 testes) were detected next to the hub in age-matched larval testes from chic11/chic1320 hypomorphs (Fig. 1B), and only 0.7±1.0 GSCs (n=12 testes) were observed in larval testes from chic121/chic1320 strong loss-of-function mutants (Fig. 1C). In most testes from third instar chic121/chic1320 larvae, the earliest germ cells observed were spermatocytes, suggesting that GSCs had been present at earlier stages in development but that GSCs were lost from the testsis tip during larval development (Fig. 1C). Consistent with progressive loss of GSCs over time, the number of GSCs touching the hub in chic11/chic1320 hypomorphs dropped from 3.6±2.8 GSCs per testis (n=56 testes) in late third instar larvae to 0.7±1.0 GSCs per testis (n=104 testes) in newly eclosed adults. As noted in other cases of germ cell loss, somatic cells expressing the cyst cell nuclear marker Tj moved in to fill the area adjacent to the hub when GSCs were lost in chic mutants (Fig. 1A-C).

Analysis of germline clones indicated that chic is required cell autonomously for GSC maintenance. GSCs were made homozygous mutant for chic and simultaneously marked by loss of green fluorescent protein (GFP) by FLP-mediated recombination induced by heat shock. For two different null alleles of chic, GFP-negative chic mutant GSC clones were detected next to the hub at 3 days post-clone induction (dpci) in 80% (chic22) and 96% (chicP320) of testes. However, the percentage of testes maintaining GFP-negative chic mutant GSC clones next to the hub decreased over time. By 11 dpci, none of the testes contained chic mutant GSC clones (Fig. 2A).
By contrast, control GSC clones induced in a genetic background wild type for chic were maintained over the 11-day period of observation (Fig. 2A). The chic mutant germ cells initiated differentiation and progressed to spermatocytes. However, no chic-null spermatid clones were observed, suggesting that mutant spermatocytes failed to complete terminal differentiation (supplementary material Fig. S1). Likewise, when function of chic was knocked down specifically in germ cells throughout development by RNAi under control of nanos-GAL4 at 18°C, testes from newly eclosed adults completely lacked GSCs, similar to the chic strong loss-of-function phenotype (Fig. 2B-G**). Wild-type testes displayed a gradient of differentiating germ cells beginning with GSCs at the apical tip and progressing through spermatogonia and spermatocytes (Fig. 2B,E-E’) to mature spermatid bundles at the distal end of the testis. By contrast, in testes in which chic function had been knocked down in early germ cells by RNAi, elongated spermatid bundles extended into the apical tip (Fig. 2C) in newly eclosed males, and spermatogonia and even spermatocytes were rare (Fig. 2F-F‘) or not detected, similar to the phenotype observed in adulthood from chic1221/chic221 strong loss-of-function mutants (Fig. 2D,G-G’).

**Overexpression of APC2 in early germ cells rescued loss of GSCs in chic hypomorphs**

The requirement of profilin for GSC maintenance raised the possibility that microfilament assembly and/or function might help maintain GSCs in their niche at the testis apical tip. Consistent with this, analysis of GFP-tagged F-actin expressed in GSCs under control of nanos-GAL4 revealed actin-GFP localized to the cell cortex under the GSC-hub interface (Fig. 3A’, arrowhead). Analysis of F-actin by phalloidin staining of wild-type testes also showed F-actin localized to the interface between GSCs and hub cells, juxtaposed with β-catenin/Armadillo (Fig. 3B’). In age-matched larval testes from chic11/chic1320 hypomorphs, F-actin localized to regions where GSCs touched hub cells but F-actin localization was decreased or lost where GSCs were absent (Fig. 3C,C’,C”). In testes completely lacking GSCs from strong loss-of-function chic1221/chic1320 larvae, F-actin localization around the perimeter of the hub was completely absent (Fig. 3D,D’,D”). Forced expression of actin-GFP subunits in germ cells of chic11/chic1320 or chic221/chic1320 animals was not sufficient to rescue GSC loss (data not shown), consistent with the known role of profilin in F-actin assembly from G-actin subunits.

Notably, in control larval testes, the border of the hub in contact with GSCs appeared concave, with V-shaped protrusions between GSCs and hub cells (Fig. 1A-A’, arrowheads indicate GSCs abutting the hub. Scale bar: 20 μm.)

**Fig. 1. Loss of germine stem cells in chic mutants.** (A-C”) Larval testis tips from (A-A”) wild-type, (B-B”) chic1320/homologous carriers, (C-C”) chic221/chic1320 strong loss-of-function animals with anti-Arm/β-catenin (blue) to mark hub cells (asterisk), anti-Vasa (green) to mark germ cells and anti-Tj (red) to mark early cyst cell nuclei. Arrows indicate Vasa-positive cells touching the hub that were scored as GSCs. Arrowhead indicates differentiating spermatocytes. Scale bar: 20 μm.

**Fig. 2. chic is cell autonomously required for maintenance of germline stem cells.** (A) Percentage of testes with at least one GFP-negative, homozygous control FRT40A (blue), chic221 (red) or chicP5205 (green) GSC over time after clone induction. Numbers next to data points reflect the number of testes scored for each genotype at each time point. Error bars indicate s.e.m. See also supplementary material Fig. S1. (B-G”) Adult testis tips from (B,E,E”) wild type, (C,F-F”) from nanos-GAL4/UAS-chicRNAi resulting in knockdown of chic in the germ line and from (D,G-G”) chic221/chic1320 strong loss-of-function mutant. (B-D) Phase-contrast images. White arrowheads indicate elongated spermatid bundles. Scale bar: 10 μm. (E-G”) Anti-Vasa to mark germ cells. Anti-Arm to mark hub cells. Yellow arrowheads indicate GSCs abutting the hub. Scale bar: 20 μm.
Adenomatous Polyposis Coli, suggesting that functional chic in GSCs may promote enrichment of APC2 at the hub-GSC interface. GSCs are attached to hub cells by E-cadherin-based adherens junctions, which provide an anchoring platform for concentration of APC2 (Fig. 3E) (Yamashita et al., 2003). Immunofluorescence analysis of testes from chic/+ control third instar larvae revealed APC2 protein localized mainly to the hub-GSC interface in GSCs but with localization observed around the GSC cell cortex in some cells (Fig. 4A’). In 66.7% of GSCs in chic/+ control larval testes, APC2 localized to the hub-GSC interface (Fig. 4E). By contrast, in larval testes from chic11/chic1320 hypomorphs, only 9.5% of GSCs scored displayed APC2 localized to the hub-GSC interface (Fig. 4B’). Similarly, immunofluorescence analysis of germ line clones from adult males 4 dpci demonstrated that 87.5% of homozygous chic221/chic1320 and 84.2% of homozygous chic11/chic1320 mutant GSC clones showed APC2 distributed around the cell cortex rather than localized to the hub-GSC interface, compared with 46.2% and 38.7%, respectively, of adjacent heterozygous control GSC clones from the same testes (Fig. 4C’,D’,F).

Strikingly, forced expression of APC2 in germ cells rescued male GSC loss in chic hypomorphs. When APC2 was expressed in the germ line of chic11/chic1320 hypomorphs under control of nanos-GAL4, testes from newly eclosed adults had the normal spatial gradient of germ line differentiation from early germ cells to mature sperm restored (Fig. 5B), similar to chic/+ controls (Fig. 5C) and in contrast to the loss of early germ cells observed in chic11/chic1320 mutant siblings grown under the same conditions (Fig. 5A). Immunofluorescence revealed Vasa-positive GSCs (4.9±1.6 GSCs per testis; n=114 testes) attached to the hub in the rescued testes (Fig. 5F), compared with 0.7±1.9 GSCs per testis (n=104 testes) in chic11/chic1320 siblings (Fig. 5E). There was no significant difference in the number of Vasa-positive GSCs in chic/+ sibling controls without added APC2 (7.9±1.6 GSCs per testis; n=114 testes) (Fig. 5G) compared with chic/+ sibling controls with forced expression of APC2 (7.9±1.8 GSCs per testis; n=111 testes) (Fig. 5H), indicating that whereas forced expression of APC2 increased GSC number in chic11/chic1320 mutants, ectopic APC2 did not increase GSC number in control testes.

Expression of APC2 in the germ line also restored normal distribution of somatic cyst cells. Rather than many Tj-positive cyst cells clustered next to the hub in the absence of germ cells observed in testes from chic11/chic1320 mutants (Fig. 5L’), testis tips from chic11/chic1320, nanos-GAL4/UAS-APC2-GFP males had Tj-positive cells dispersed throughout the apical region (Fig. 5L’’) in a pattern similar to chic/+ controls (Fig. 5K,K’) and chic/+ controls with added APC2 (Fig. 5L,L’). Forced expression of APC2 in germ cells of strong loss-of-function chic221/chic1320 animals failed to rescue GSC loss (data not shown).

Function of profilin/chic was required cell intrinsically in germ cells for proper localization and/or stabilization of the adherens junction component E-cadherin to the hub-GSC interface. Expression of an E-cadherin-GFP transgene specifically in early germ cells using nanos-GAL4 allowed E-cadherin in the plasma membrane of GSCs to be distinguished from E-cadherin in the membrane of hub cells at the hub-GSC interface (Fig. 6). In early germ cells in late third instar larval testes from animals wild-type for chic, the E-cadherin-GFP localized to the GSC-hub interface (Fig. 6A-Aa) as observed previously for adult testes (Srinivasan et al., 2012). Forced expression of E-cadherin-GFP in early germ cells of chic11/chic1320 (Fig. 6B-Ba) or chic11/chic1320 animals (data not shown) failed to rescue GSC loss. Forced expression of E-cadherin-GFP in germ cells of chic11/chic1320 (Fig. 6B-Ba) or chic11/chic1320 animals with nanos-GAL4: UAS-E-cad-GFP stained with anti-Vasa lacked early germ cells (data not shown). Likewise, testes from late third instar chic11/chic1320; nanos-GAL4/UAS-Ecad-GFP larvae contained similar GSC numbers (4.75±2.0 GSCs; n=20 testes) to age-matched testes from chic11/chic1320 larvae without expression of E-cadherin-GFP (3.6±2.8 GSCs; n=36 testes, see Fig. 1), further demonstrating that forced expression of E-cadherin was not sufficient to rescue GSC loss in chic mutants.

Notably, E-cadherin-GFP failed to localize to the GSC-hub interface in chic mutant GSCs. Analysis of 78 control GSCs in nine late larval testes showed E-cadherin-GFP localized to the hub-GSC interface (Fig. 6A,AA). By contrast, 96.8% of chic11/chic1320; nanos-
GAL4/UAS-Ecad-GFP mutant GSCs analyzed (92/95) in 20 late larval testes displayed no detectable E-cadherin-GFP signal (Fig. 6B, Ba). Taken together, these observations suggest that F-actin underlying adherens junctions may help maintain GSC-hub attachment by localizing and anchoring junctional components such as E-cadherin and APC2, and that lowered activity of the actin regulator profilin in GSCs may weaken adherens junctions and allow somatic cells to outcompete germ cells for association with the hub. Increased levels of APC2 in germ cells may strengthen adherens junctions between GSCs and the hub, making up for partial impairment of F-actin assembly in chic hypomorphs.

The transcription factor STAT binds near the chic promoter region
The chic gene has three predicted alternate transcription start sites (TSS) (Fig. 7A), two of which were shown by 5′ RACE to be active in upd-overexpressing testes, which contain an abundance of GSCs and CySCs. A reporter construct containing a genomic DNA fragment extending from the ATG of chic to 2256 bp upstream of the ATG and including all three TSSs (Fig. 7A) was sufficient to drive expression of GFP in both germline and somatic lineages in the testis (Fig. 7C-C′′′), consistent with the ubiquitous role of profilin in regulating the actin cytoskeleton.

Chromatin immunoprecipitation (ChIP) analysis suggested that chic may be a direct target of the transcription factor STAT in GSCs and/or CySCs. A consensus STAT-binding motif was present in the chic promoter region, just downstream of the first TSS (Fig. 7A). ChIP from extracts of upd-overexpressing testes with antibodies against STAT (P-STAT) followed by quantitative PCR showed 13.7±3.8-fold enrichment of the genomic region spanning the consensus STAT motif relative to a control gene thioredoxin, whereas ChIP with normal rabbit serum showed no enrichment (0.8±0.2) relative to thioredoxin (Fig. 7B). However, mutation of the STAT motif in the reporter construct did not eliminate E-GFP expression in GSCs and CySCs in vivo (Fig. 7D-D′′′), suggesting either that STAT is not the only transcriptional regulator of chic expression in stem cells, or STAT may direct an increase in the levels of chic undetected by immunostaining of the GFP reporter.

Furthermore, forced expression of profilin in stat-depleted GSCs failed to rescue loss of attachment of GSCs to the apical hub, suggesting that chic is not the only important target of STAT for GSC attachment. To evaluate the role of STAT in chic-mediated GSC maintenance, germline-specific RNAi was performed to knock down function of stat in GSCs while profilin was ectopically expressed at the same time (supplementary material Fig. S2). Flies were grown to adulthood at 18°C then shifted to 30°C to induce expression of the RNAi hairpin. Testes were dissected at 14 days post-shift. As previously reported (Leatherman and Dinardo, 2010), stat-depleted GSCs detached from the hub and were displaced by somatic cells that surrounded the hub (supplementary material Fig. S2A-A′′). stat-depleted GSCs in which profilin was
between the clusters of small cells. Testes expressing the chic hairpin sometimes appeared wider than sibling controls, likely due to the massive numbers of small cells.

Fusome morphology, expression of cytoplasmic Bam and synchronous cell cycle progression all indicated that transit-amplifying germ cells failed to stop proliferation and differentiate into spermatocytes when profilin function was knocked down in somatic cyst cells. Although immunostaining of control testes with anti-Spectrin showed GSCs and gonialblasts with dot fusomes, and spermatogonial and spermatocyte cysts with branched fusomes (Fig. 8E,F′), the small cells that accumulated in c587-GAL4; UAS-chicRNAi animals mostly contained branched fusomes (Fig. 8F,F′), indicating their identity as transit-amplifying spermatagonia rather than GSCs or gonialblast-like cells.

Likewise, the spermatogonial protein, bag of marbles (Bam), normally expressed in 4-, 8- and 16-cell spermatogonial cysts but not detected in GSCs, gonialblasts or spermatocytes in controls (Fig. 8G,G′), was expressed in the small cells that accumulated throughout the testis in c587-GAL4; UAS-chicRNAi animals (Fig. 8H,H′). Consistent with failure of spermatagonia to cease mitotic division after loss of chic function in somatic cyst cells, analysis of cells in S phase after a short pulse of 5-ethyl-2′-deoxyuridine (EdU), showed EdU incorporation in germ cells far away from the apical tip in testes from c587-GAL4; UAS-chicRNAi animals, in contrast to EdU incorporation only near the testis apical tip in GSCs, 2-, 4- and 8-cell spermatogonial cysts in controls (Fig. 8I,I′). EdU-positive germ cells away from the tip in c587-GAL4; UAS-chicRNAi animals included singletons and doublets, suggesting some cells resembled GSCs or gonialblasts. However, the majority of EdU-positive germ cells divided in 4-, 8-, 16- and >16-cell clusters (Fig. 8J,J′), indicating spermatogonial cell identity and failure to exit the transit-amplifying program.

Function of chic is required for maintenance of the somatic cyst cell lineage

Testes from c587-GAL4; UAS-chicRNAi adults shifted to restrictive temperature showed loss of cyst cells over time. By day 4/5 post-shift, zinc-finger homeodomain-1 (Zfh-1)-positive somatic cyst stem cells (CySCs) in testes from c587-GAL4; UAS-chicRNAi adults were mostly absent (Fig. 9B′) compared with sibling controls (Fig. 9A′). Tj-positive cyst cells also decreased in number and sometimes exhibited abnormal morphology: nuclei appeared larger than normal and had de-condensed chromatin (Fig. 9B′). The number of Eya-positive cyst cells also declined (Fig. 9B′′′) compared with sibling controls (Fig. 9A′′′).

Somatic cells lacking chic function were unable to maintain germ cell enclosure, visualized by labeling cyst cell membranes using a membrane-bound UAS-Green Fluorescent Protein (UAS-mGFP). UAS-chicRNAi and UAS-mGFP were both expressed under control of somatic-specific c587-GAL4, enabling evaluation of cyst cell enclosure of germ cells. In control testes, GFP was detected on intact, continuous membranes of somatic cells surrounding germ cells (Fig. 9C′,C′′,E,E′). By contrast, in testes from c587-GAL4/UAS-mGFP; UAS-chicRNAi/+ animals held at restrictive temperature for 5 days, the membrane-targeted GFP revealed either truncated, diffuse strands or complete failure to surround the clusters of small germ cells (Fig. 9D,F) whether a Tj-positive somatic cell was present or not (Fig. 9D′,F′), suggesting cyst cell cytoplasmic extensions were fragmented, not established or had disintegrated. Similar results were obtained by staining c587-GAL4; UAS-chicRNAi testes for Arm, which labels the cell membranes of cyst cells (Fig. 9A′′′,B′′′; supplementary material Fig. S3A′′′,B′′′).
DISCUSSION
Profilin/Chickadee in the germ line
Chickadee, which encodes the only Drosophila profilin homolog, is required cell intrinsically for GSC maintenance in the testis. As profilin is a regulator of actin filament polymerization (Cooley et al., 1992; Theriot and Mitchison, 1993; Verheyen and Cooley, 1994) and filamentous actin (F-actin) plays a crucial role in the development and stabilization of cadherin-catenin-mediated cell-cell adhesion (Desai et al., 2013; Nola et al., 2011; Jamora and Fuchs, 2002), profilin likely maintains attachment of Drosophila male GSCs to the hub through its effect on F-actin, which concentrates at the hub-GSC interface where localized adherens junctions anchor GSCs to hub cells. We propose that profilin-dependent stabilization of F-actin at the GSC cortex next to the hub may help localize E-cadherin and APC2 to the junctional region. E-cadherin and APC2 in turn may recruit β-catenin/Armadillo, stabilizing the adherens

Fig. 6. chic is required in germ cells for E-cadherin localization to the hub-GSC interface. (A-Ba′′′) Larval testes expressing E-cad-GFP in early germ cells. (A-Aa′′′)+/+; nanos-GAL4/UAS-Ecad-GFP controls, (B-Ba′′′) chic11/chic1320; nanos-GAL4/UAS-Ecad-GFP animals. Anti-GFP (green) to highlight expression of the E-cad-GFP transgene, anti-Vasa (red) and anti-Arm (blue) to outline hub cells, and anti-Tj (blue) to mark somatic cyst cells. Yellow arrowheads indicate E-cadherin-GFP localized to hub-GSC interface. Scale bars: 20 μm in A-A′′′,B-B′′′; 10 μm in Aa-Aa′′′,Ba-Ba′′′.

Fig. 7. The STAT transcription factor binds near the chic promoter region. (A) Screen shot of chic locus from UCSC Genome Browser. Black boxes indicate exons. Hatched lines indicate introns. Purple circles indicate transcription start sites active in upd-overexpressing testes. Red bar indicates consensus STAT site. Black square indicates quantitative PCR (qt-PCR) amplicon. chic reporter containing a genomic DNA fragment extending from the ATG of chic to 2256 bp upstream, EGFP-coding sequence and SV40 3′UTR/terminator sequence. (B) Average enrichment for P-STAT (red) or normal rabbit serum (green) binding in the region flanking the STAT site relative to thioredoxin for three biological replicates. Error bars indicate s.e.m. P values calculated using the two-tailed Student's t-test. (C-D′′′) Adult testes from chic-EGFP transgenic flies containing (C-C′′′) wild-type or (D-D′′′) mutated consensus STAT-binding motifs stained with anti-GFP to highlight reporter expression, with anti-Vasa to mark germ cells, with anti-Tj to mark early cyst cell nuclei and with anti-FasIII to mark hub cells (blue asterisk). Scale bar: 50 μm.
junctions that attach GSCs to the hub. Chickadee may thus facilitate maintenance of GSCs through a cascade of interactions leading to localization and/or retention of both E-cadherin and β-catenin at the hub-GSC interface.

E-cadherin plays a crucial role in maintaining hub-GSC attachment. GSC clones mutant for E-cadherin are not maintained (Voog et al., 2008; Inaba et al., 2010). In addition, germline overexpression of E-cadherin delayed GSC loss in stat-depleted GSCs (Leatherman and Dimando, 2010). Our results indicate that profilin function is required in GSCs for proper localization of E-cadherin to the hub-GSC interface. Several studies have shown that the actin cytoskeleton plays a crucial role in assembly and stability of adherens junctions (Carramusa et al., 2007; Chu et al., 2004; Imamura et al., 1999). A favored model in the field is that actin filaments indirectly anchor and reinforce E-cadherin-mediated cell junctions by forming an intracellular scaffold for E-cadherin molecules (Lambert et al., 2007; Mège et al., 2006). Indeed, binding to F-actin stabilized E-cadherin and promoted its clustering (Hong et al., 2013). Furthermore, the actin cytoskeleton participates in proper localization of E-cadherin molecules to cell-cell contacts (Collinet and Lecuit, 2013). In chic/profilin mutant GSCs, disruption of actin polymerization at the cell cortex leading to local F-actin disorganization may destabilize E-cadherin and reduce its ability to localize to the GSC-hub junction, form clusters and build adequate adherens junctions.

Destabilization of E-cadherin may contribute to the mislocalization of APC2 seen in chic mutant GSCs, as E-cadherin recruits APC2 to cortical sites in GSCs (Inaba et al., 2010). Raising possibilities of a more direct link, actin filaments have been shown to be required for association of APC2 with adherens junctions in the Drosophila embryo and ovary (Yu et al., 1999; Townsley and Bienz, 2000). Treatment of embryos with actin-depolymerizing drugs resulted in complete delocalization of APC2 from adhesive zones and diffuse APC2 staining throughout the cell (Townsley and Bienz, 2000). Moreover, in ovaries of chic120/chic120 females, APC2 was substantially delocalized from the plasma membranes of nurse cells and their ring canals, and increased levels of cytoplasmic APC2 staining were observed (Townsley and Bienz, 2000). Similarly, we found that APC2 was delocalized from the hub-GSC interface in larval testes of chic1/chic1 hypomorphs.

In several studies, delocalization of APC2 from junctional membranes correlated with detachment of β-catenin/Armadillo from adherens junctions. APC2 co-localizes with Armadillo and E-cadherin at adherens junctions of Drosophila epithelial cells (Yu et al., 1999), nurse cells in Drosophila ovaries (Townsley and Bienz, 2000) and at the hub-GSC interface in Drosophila testes (Yamashita et al., 2003). Disruption of APC2 function resulting in significant reduction of junctional APC2 was accompanied by delocalization of APC2 to cortical sites in GSCs (Inaba et al., 2010). Raising possibilities of a more direct link, actin filaments have been shown to be required for association of APC2 with adherens junctions in the Drosophila embryo and ovary (Yu et al., 1999; Townsley and Bienz, 2000). Treatment of embryos with actin-depolymerizing drugs resulted in complete delocalization of APC2 from adhesive zones and diffuse APC2 staining throughout the cell (Townsley and Bienz, 2000). Moreover, in ovaries of chic1/chic1 females, APC2 was substantially delocalized from the plasma membranes of nurse cells and their ring canals, and increased levels of cytoplasmic APC2 staining were observed (Townsley and Bienz, 2000). Similarly, we found that APC2 was delocalized from the hub-GSC interface in larval testes of chic/chic hypomorphs.
junctional Armadillo and increased levels of free cytoplasmic Armadillo in embryonic epithelial cells (Yu et al., 1999) and ovaries (Townsley and Bienz, 2000). In the Townsley and Bienz study, which used chic\textsuperscript{120}/chic\textsuperscript{221} strong loss-of-function mutants, the delocalizing effect on junctional Armadillo was variable, presumably due to incomplete penetrance of chic mutant effects. Although we did not observe significant disruption in Armadillo staining along the hub-GSC interface of testes from chic hypomorphs, this may be due to incomplete penetrance. In addition, the Armadillo protein detected could be localized to the cortex of hub cells rather than GSCs.

Our finding that germline specific overexpression of APC2 in chic\textsuperscript{11}/chic\textsuperscript{120} hypomorphs partially rescued GSC loss is consistent with a previously proposed model that actin filaments shuttle APC2 to adherens junctions and APC2 in turn recruits cytoplasmic Armadillo to junctional membranes, reinforcing the adherens junctions (Townsley and Bienz, 2000). It is possible that in chic\textsuperscript{221}/chic\textsuperscript{120} hypomorphs, residual actin filaments associated with adherens junctions between the hub and GSCs are sufficient to shuttle the increased amounts of cytoplasmic APC2 to adherens junctions. This APC2 may in turn recruit free cytoplasmic Armadillo to the hub-GSC interface, locally stabilizing the adherens junctions and anchoring GSCs to their niche. Notably, however, germline specific overexpression of APC2 in testes of strong loss-of-function chic\textsuperscript{120}/chic\textsuperscript{221} mutants failed to rescue GSC loss. Thus either, adequate levels of actin filament polymerization may be required for the proposed translocation of junctional proteins to the plasma membrane, or APC2 function/localization may not be the only or even the major cell-autonomous target of profilin function important for maintaining GSCs. Indeed, loss of APC2 function did not lead to GSC loss (Yamashita et al., 2003). We suggest that the hub-GSC interface, best candidate for the most direct target of localized cortical F-actin underlying adherens junctions at the GSC-hub interface, is a plausible target, although it remains to be directly tested.

**Materials and Methods**

**Fly stocks and husbandry**

Flies were grown on standard cornmeal molasses agar medium with crosses performed at 25°C unless indicated otherwise. Strains are described in FlyBase (http://flybase.org) or otherwise specified. y\textsuperscript{w} flies or sibling controls were used as wild type. P{FRT42A}chic\textsuperscript{11} [Bloomboming Drosophila Stock Center (BDSC)] is a hemopoietic allele generated by P-element insertion (Castrillon et al., 1993). P{PZ}chic\textsuperscript{120}/chic\textsuperscript{221} (BDSC), a strong loss-of-function allele generated by P-element insertion (Cooley et al., 1992), did not behave as strongly as a null in heteroallelic combinations in our studies. The null allele chic\textsuperscript{221} (BDSC) is a deletion of S\textsuperscript{+} non-coding and some chic-coding sequences (Verheyen and Cooley, 1994). chic\textsuperscript{120}\textsuperscript{01320} is a null allele (Baum and Perrimon, 2001). The chic UAS-dsRNA strain (#102759) was obtained from the Vienna Drosophila RNAi Center (VDRc). To drive expression of UAS transgenes in early germ cells Sco/Cyo; nanos-Gal4VP16 (Van Doren et al., 1998) or chic\textsuperscript{120}/Cyo; nanos-Gal4VP16 virgin females were crossed to chic\textsuperscript{120}/Cyo; UAS-transgene or chic\textsuperscript{221}/Cyo; UAS-transgene males at 25°C. Transgenes included UAS-APC2-GFP (BDSC), UAS-Actin-GFP (BDSC), UAS-EGFP-chic from A. Prokop (Gonçalves-Pimentel et al., 2011); and UAS-DEFL\textsuperscript{6-1}, i.e. UAS-Ecadherin-GFP (Oda and Tsukita, 1999), from the Drosophila Genetic Resource Center, Kyoto.

**Immunostaining**

Immunostaining was performed as described previously (Srinivasan et al., 2012). Primary antibodies used were: rabbit anti-GFP (1:500-1:3000, Invitrogen); sheep anti-GFP (1:1000, AbD Serotec); rabbit anti-APC2 (1:5000, from M. Bienz, Cambridge, UK); rabbit anti-zinc-finger homeodomain-1 (Zfh-1) (1:5000, from R. Lehmann, New York, USA); guinea pig anti-Traffic Jam (Tj) (1:5000, from D. Godt, Toronto, Canada); goat anti-Vasa (1:100-1:200, DC-13 from Santa Cruz Biotech); mouse anti-Chickadee chi 1J [1:5, Developmental Studies Hybridoma Bank (DSHB)]; mouse anti-Armadillo (Arm) (1:10, DSHB); mouse anti-3A9 Alpha Spectrin (1:10, DSHB); mouse anti-Fas II 7G10 (1:10, DSHB); mouse anti-Eya 1H6 (1:10, DSHB); mouse anti-Barn (1:5, DSHB); and rat E-cadherin (1:40, DSHB). Secondary antibodies (Jackson Immunolabals) were used at 1:200-1:500. All images were taken using a Leica SP2 AOBS confocal system and processed with Adobe Photoshop.
**Clonal analysis**

For clonal analyses using FLP/FRT mitotic recombination, y,w, hs-FLP22; FRT40A, Ubi-GFP/Cyo virgin females were crossed to w; FRT40A/Cyo or chic221, FRT40A/Cyo or chicP205, FRT40A/Cyo males at 25°C. Clones were induced by heat shocking pupae for 2 hours on days eight and nine post-lay in a 37°C circulating water bath. Male flies were collected 2 days post-clone induction (dpc). The following genotypes were scored: y,w, hs-FLP22; FRT40A, Ubi-GFP/FRT40A (control clones were wild type for chic function); y,w, hs-FLP22; FRT40A, Ubi-GFP/chic221, FRT40A (chic-null clones); and y,w, hs-FLP22; FRT40A, Ubi-GFP/chicP205, FRT40A (chic-null clones). GSCs divide approximately every 12-16 hours, and we therefore assessed GSCs with APC2 localized around the cell cortex. All clonal analyses were repeated three independent times with data shown as an average of the independence experiments.

**Inducible RNAi knockdowns**

For germline RNAi knockdown, w; UAS-chicRNAi (VDRC 102759) males at 18°C, allowing the RNAi hairpin to be expressed throughout progeny development. Tests from newly eclosed adults were analyzed in live squashes by phase contrast and in immunostained preparations by confocal microscopy. Knockdown of chic expression in early germ cells by RNAi appeared effective because it caused loss of GSCs, similar to the results seen in homozygous mutant clones. We could not assess Chic protein or RNA levels because early germ cells were lost in the knockdown tests. For somatic RNAi knockdown, c587-Gal4; Soco/Cyo; tubGAL80P/TM6B virgin females were crossed to w; UAS-chicRNAi males at 18°C. Progeny were shifted to 30°C upon eclosion. Tests from 4- to 5-day-old adults were analyzed. To test RNAi efficiency in somatic cells, immunostaining with anti-Profilin was performed in clonal assays for mutations in the hub-GSC interface as described previously, and data analyzed using Microsoft Excel. Enrichment, defined as (IP/Input)target site/(IP/Input)non-target site, where the non-target site is the predicted STAT-binding sites versus an unoccupied site in the genome, was calculated independently for P-STAT and IgG reactions.

**Quantitative PCR**

ChIP DNA was analyzed by q-PCR to assess relative enrichment at predicted STAT-binding sites versus an unoccupied site in the thio- redoxin gene. Primers that amplify 80-100 bp genomic DNA centered over the predicted target or non-target site were designed. Primer sequences listed 5′ to 3′ included: chic target (S), accaaccacgttagaag; chic target (A), caggtgccagctcatggtgct; thio-redoxin (S), caaatccagctgactctga; and thio-redoxin (A), ggtggctgctgctttca. Production of real-time PCR products was monitored using SYBR Green. Reactions containing either 10 ng input or ChIP DNA were run in triplicate using the default 40-cycle PCR protocol with dissociation curves on an Applied Biosystems 7300 thermocycler, and data analyzed using Microsoft Excel. Enrichment, defined as (IP/Input)target site/(IP/Input)non-target site, where the non-target site is thio-redoxin, was calculated independently for P-STAT and IgG reactions.

**Transgenic reporters**

**pattB-chic-EGFP** consisted of genomic region (chr2L: 5979503-5981759) extending from the ATG of chic to 2,256 bp upstream PCR-amplified from y,w genomic DNA, subcloned upstream of E-GFP coding sequence and SV-40 3′UTR/terminator. pattB-Chic-EGFP was derived from pattB-chic-EGFP by amplifying the chic sequence in two segments using primers containing engineered mutations that mutate the predicted STAT-binding site from the consensus TCCAGGGGA to GACAGTTC. The two resulting PCR products were ligated, subcloned into pBS-KS+, sequenced to ensure the necessary mutation was introduced, then moved into pattB-EGFP. Clones were sequenced following each subcloning step to ensure that sequences, orientation and number of inserts were correct. Transgenic fly lines were generated by BestGene (Chino Hills, CA) using the PhiC31 integrase system (http://flyc31.frontiers-in-genetics.org/). Independent transgenic lines containing insertions on chromosomes X, 2 and 3, respectively, were analyzed. Immunostaining was performed on testes carrying wild-type and mutated versions of the transgenic reporter constructs, and samples were imaged sequentially using the same settings on a Leica SP2 AOBS confocal microscope.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**


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