Role of Survivin in cytokinesis revealed by a separation-of-function allele

Edith Szafer-Glusman[a], Margaret T. Fuller[a], and Maria Grazia Giansanti[b]
[a]Departments of Developmental Biology and Genetics, Stanford University School of Medicine, Stanford, CA 94305;
[b]Istituto di Biologia e Patologia Molecolari del Consiglio Nazionale delle Ricerche, Dipartimento di Biologia e Biotecnologie C. Darwin, Università di Roma "Sapienza," 00185 Rome, Italy

ABSTRACT **The chromosomal passenger complex (CPC), containing Aurora B kinase, Inner Centromere Protein, Survivin, and Borealin, regulates chromosome condensation and interaction between kinetochores and microtubules at metaphase, then relocalizes to midzone microtubules at anaphase and regulates central spindle organization and cytokinesis. However, the precise role(s) played by the CPC in anaphase have been obscured by its prior functions in metaphase. Here we identify a missense allele of *Drosophila* Survivin that allows CPC localization and function during metaphase but not cytokinesis. Analysis of mutant cells showed that Survivin is essential to target the CPC and the mitotic kinesin-like protein 1 orthologue Pavarotti (Pav) to the central spindle and equatorial cell cortex during anaphase in both larval neuroblasts and spermatocytes. Survivin also enabled localization of Polo kinase and Rho at the equatorial cortex in spermatocytes, critical for contractile ring assembly. In neuroblasts, in contrast, Survivin function was not required for localization of Rho, Polo, or Myosin II to a broad equatorial cortical band but was required for Myosin II to transition to a compact, fully constricted ring. Analysis of this "separation-of-function" allele demonstrates the direct role of Survivin and the CPC in cytokinesis and highlights striking differences in regulation of cytokinesis in different cell systems.**

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INTRODUCTION

The chromosomal passenger complex (CPC), composed of the Ser-Thr kinase Aurora B and three partner proteins, plays several key roles in mitosis and meiosis, including regulation of attachment of kinetochores to microtubules, the spindle checkpoint that delays anaphase onset until all chromosomes are under tension on the spindle, regulation of sister chromatid cohesion, and cytokinesis (Ruchaud *et al.*, 2007a). To accomplish these different tasks, the Aurora B kinase must be exquisitely localized in space and regulated in time. During mitosis, Aurora B associates with the microtubule-binding protein Inner Centromere Protein (INCENP; Cooke *et al.*, 1987; Mackay *et al.*, 1993), Borealin/DASRA/CSC-1 (Romano *et al.*, 2003; Gassmann *et al.*, 2004; Sampath *et al.*, 2004; Hanson *et al.*, 2005; Klein *et al.*, 2006), and the small, multifunctional BIR-motif protein Survivin to form the CPC (Vagnarelli and Earnshaw, 2004).

Dissecting the role of individual CPC components has been hampered by the extraordinary interdependence of the four subunits (Ruchaud *et al.*, 2007a, 2007b); depletion of any single CPC protein by RNA interference knockdown in human cells affected the structural unit, localization, and function of the CPC (Adams *et al.*, 2001; Carvalho *et al.*, 2003; Honda *et al.*, 2003; Lens *et al.*, 2003; Gassmann *et al.*, 2004). The structural basis of this interdependence is evident in the crystal structure of the Survivin-Borealin-INCENP core of the CPC complex, in which Borealin and INCENP associate with the C-terminal helical domain of Survivin to form a tight three-helix bundle (Jeyaprakash *et al.*, 2007).

Strict localization of Aurora B by the CPC ensures that this kinase, which has multiple substrates, phosphorylates the correct targets at the proper points in cell cycle progression. Concentrated on chromosomes from G2, then at inner centromeres from prometaphase until the metaphase-to-anaphase transition, the CPC is required to regulate chromosome condensation, spindle formation and dynamics, kinetochore maturation, kinetochore–microtubule interaction, correct chromosome alignment, and control of the spindle

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Address correspondence to: Margaret T. Fuller (mtfuller@stanford.edu).

Abbreviations used: CPC, chromosomal passenger complex; CS, central spindle; MKLP1, mitotic kinesin-like protein 1.

checkpoint (reviewed in Ruchaud *et al.*, 2007a; Carmena, 2008). At anaphase onset the CPC then translocates to the central spindle (CS) midzone and equatorial cortex (Adams *et al.*, 2001; Vagnarelli and Earnshaw, 2004; Vader *et al.*, 2006) and is involved in CS formation (Kaitna *et al.* 2000; Severson *et al.* 2000; Giet and Glover, 2001; Carmena, 2008). At the CS Aurora B phosphorylates the centralspindlin components, Pav/mitotic kinesin-like protein 1 (MKLP1)/Zen-4 (Guse *et al.*, 2005; Neef *et al.*, 2006; Douglas *et al.*, 2010) and the RacGAP50/MgcRacGAP/Cyc-4 (Minoshima *et al.*, 2003). However, the mechanisms that target the CPC to the spindle midzone and equatorial cortex after onset of anaphase and the mechanisms by which the CPC regulates central spindle formation and cytokinesis are not understood. In addition, the requirements for CPC function for critical events in metaphase and at the metaphase–anaphase transition have complicated analysis of how the CPC is localized and functions at later stages for cytokinesis.

Here we characterize the role of *Drosophila* Survivin (dSurvivin), previously termed *deterin* and analyzed in its antiapoptotic activity (Jones *et al.*, 2000), as a regulator of cell division, identifying a missense mutation (*scapolo*) in the dSurvivin BIR domain that allows recruitment and function of the CPC in metaphase but disrupts CPC localization and function in anaphase and telophase. Our findings reveal that Survivin plays a role in targeting the CPC and centralspindlin to the central spindle and the equatorial cell cortex during anaphase. In spermatocytes, Survivin function is also required to localize Polo and localize the small GTPase RhoA to set up the contractile ring machinery at the onset of cytokinesis (Giansanti *et al.*, 2004; this study). In larval neuroblasts undergoing mitotic division, however, the *scapolo* mutant did not block initial accumulation of Rho to a band at the equatorial cortex, although it did cause failure of cytokinesis. The different requirements for Survivin function for equatorial accumulation of Rho in spermatocytes versus neuroblasts may reflect a fundamental difference in the series of steps that lead to formation of the contractile ring in these two cell types.

RESULTS

The *Drosophila* homologue of mammalian *survivin* (dSurvivin) is localized in a pattern typical of chromosomal passenger proteins during cell division

Immunofluorescence analysis of fixed preparations of both larval neuroblasts undergoing mitosis (Figure 1A) and spermatocytes undergoing meiosis (Figure 1B) stained with an anti-dSurvivin antibody showed that Survivin accumulated at kinetochores during metaphase, concentrated at the interdigitating central spindle microtubules upon anaphase entry, and was enriched in the central spindle midzone by late anaphase and telophase (Figure 1, A and B). Likewise, live imaging of primary spermatocytes expressing green fluorescent protein (GFP)–tagged Survivin encoded on a genomic transgene revealed dynamic localization of GFP-Survivin to kinetochores at prometaphase, metaphase, and early anaphase and to the equatorial cortex and the central spindle midzone during late anaphase and telophase (Figure 1C and Supplemental Movies S1 and S2). Imaging of spermatocytes expressing GFP-tagged Survivin also revealed GFP-Survivin weakly associated with separating chromosomes during anaphase and telophase during the male meiotic divisions (Figure 1C and Supplemental Figure S1), as previously shown for INCENP (Resnick *et al.*, 2006).

Separation of function of *Drosophila* Survivin in metaphase versus cytokinesis

A missense mutation in dSurvivin, identified in a large screen for ethyl methyl sulfonate–induced male sterile mutations that cause

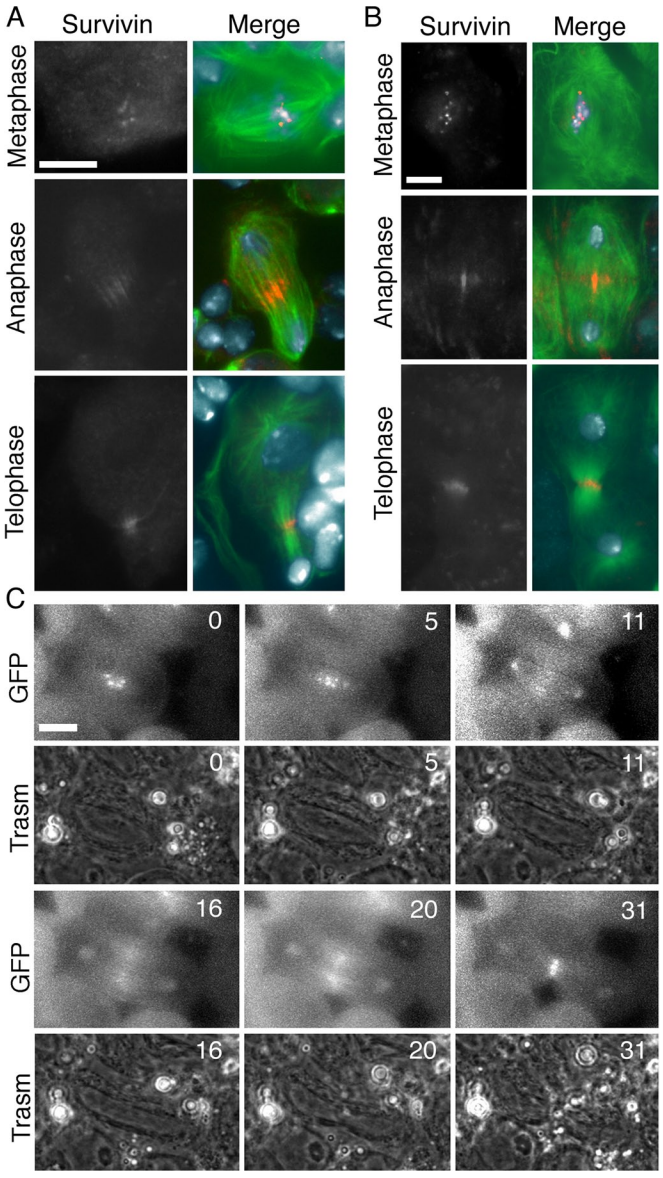

FIGURE 1: *Drosophila* Survivin protein localization in dividing cells in a pattern typical of chromosomal passenger proteins. Immunofluorescence images of wild-type (A) larval neuroblasts undergoing mitosis and (B) primary spermatocytes undergoing meiosis, stained for Survivin (red), tubulin (green), and DNA (blue). Left, Survivin channel only, in grayscale. (C) Selected snapshots from Supplemental Movies S1 and S2 showing the dynamic behavior of wild-type *Drosophila* Survivin-GFP protein in a dividing spermatocyte. Top, GFP; bottom, phase-contrast image. Same cell viewed throughout. Scale bar, 10 μm.

cytokinesis failure (Giansanti *et al.*, 2004), differentially affected Survivin localization at metaphase versus ana/telophase, allowing investigation of Survivin function at the onset of cytokinesis. Genetic mapping and molecular cloning (*Materials and Methods*) revealed the *scapolo* (*scpo*) mutant $scpo^{z2775}$ as a missense allele of the *Drosophila* orthologue of Survivin. Both a transgene carrying the wild-type *survivin* locus on a 2.5-kb genomic fragment and a transgene encoding GFP-survivin (used in Figure 1C and Supplemental Figure S1) fully rescued the *scpo* male sterile phenotype(s).

The temperature-sensitive, hypomorphic *scpo* mutation caused severe disruption of cytokinesis but only mild defects in chromosome

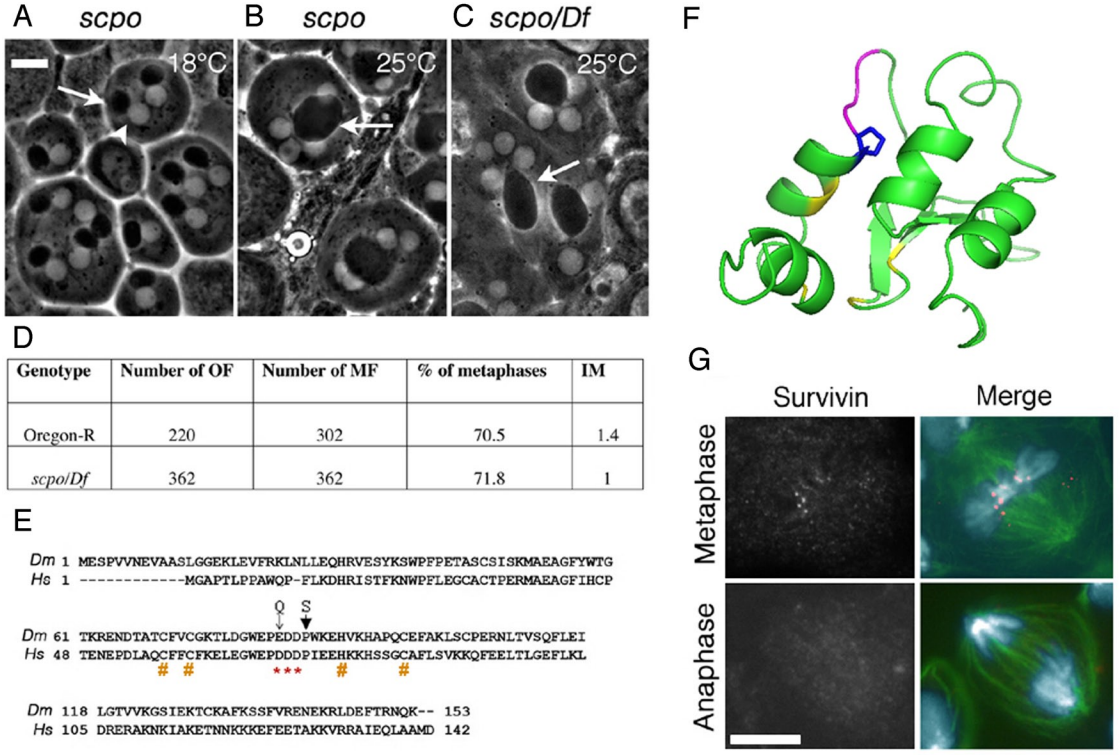

FIGURE 2: The *scpo* mutation separates action of survivin in cytokinesis from earlier functions. (A–C) Onion-stage spermatids from (A) *scpo*^{z2775} mutant males raised at 18°C showing normal spermatids, each containing a mitochondrial derivative (arrow) associated with a nucleus of similar size (arrowhead); (B) *scpo*^{z2775} males raised at 25°C; and (C) *scpo*^{z2775}/*Df(3L)Exel5780* males raised at 25°C. In B and C most spermatids contained a large mitochondrial derivative (arrow) associated with two or four nuclei of similar size. (D) Mitotic parameters in larval brain preparations from *Drosophila* Oregon-R and *scpo*^{z2775}/*Df(3R)Exel5780* mutants stained for tubulin and DNA. OF, optic field: the circular area defined by a phase-contrast Neofluar 100X Zeiss objective, using 10× oculars and the Optovar set at 1.25. MF, Number of mitotic cells scored. MI, mitotic index: average number of mitotic figures per optic field. (E) Alignment of *Drosophila* Deterin/Survivin with human Survivin. Arrowheads indicate amino acids mutated in the *scapolo* male sterile allele: open arrowhead, permissive substitution of glutamine for glutamic acid in position 83, also present in the background chromosome; solid arrowhead, substitution of Ser for wild-type proline at position 86 that underlies the *scpo* phenotype. Asterisks, acidic patch near the Pro substitution; #, residues involved in Zn coordination. (F) Ribbon representation of dSurvivin (amino acids 20–106 of the 152 amino acid–long protein) modeled with Swiss Model and visualized with PyMOL. Blue sticks, Pro-86; magenta ribbon, acidic patch; yellow ribbon, amino acids in the Zn coordination loop: Cys-70 and -73 and His-90 and -93 (Jeyaprakash *et al.*, 2007). (G) Immunofluorescence images of *scpo*^{z2775}/*Df(3R)Exel5780* larval neuroblasts stained for Survivin (red), tubulin (green), and DNA (blue). Scale bar, 10 μm.

segregation during male meiosis. Testes from *scpo*^{z2775} homozygous males grown at 25°C displayed postmeiotic early (onion stage) spermatids with multiple equal-sized nuclei associated with an enlarged mitochondrial derivative (Figure 2B), indicating failure of cytokinesis during both meiotic divisions. Testes from *scpo*^{z2775} homozygous males grown at 29°C also had multinucleate spermatids, with 5% of the nuclei varying in size, suggesting defects in both chromosome segregation and cytokinesis in a small number of meiotic divisions. In contrast, onion-stage spermatids from *scpo*^{z2775} homozygous males grown at 18°C appeared normal, indicating no obvious cytokinesis defects during meiosis (Figure 2A). Hemizygous individuals carrying the *scpo*^{z2775} allele in combination with *Df(3R)Exel5780*, a genomic deficiency that uncovers the *survivin* locus (*Drosophila* Genetic Resource Center stock number 150046), failed to hatch and survived only to larval stages. Spermatids from *scpo*^{z2775}/*Df(3R)Exel5780* larval males grown at 25°C were also multinucleate with an enlarged mitochondria derivative (Figure 2C). DNA staining in spermatocytes undergoing anaphase of the first meiotic division confirmed mild defects in chromosome segregation during meiosis: chromosomes segregated properly in 83% of *scpo*^{z2775}/*Df(3R)Exel5780* mutant cells scored (N = 103; Supplemental Figure S2).

Analysis of mitotic chromosomes in *scpo*^{z2775}/*Df(3R)Exel5780* larval brain squashes (see *Materials and Methods*) indicated defects in cytokinesis also occurred during CNS neuroblast divisions: 23% of metaphase figures were polyploid in the mutant (n = 238), compared with 0.3% in wild type (n = 298). However, the *scpo* mutation did not appear to delay the metaphase-to-anaphase transition, as the frequency of prometaphases/metaphases relative to all the mitotic figures was not significantly increased in *scpo*^{z2775}/*Df(3R)Exel5780* compared with wild type in larval brain preparations stained for tubulin and DNA (Figure 2D). Rather, the mitotic index was slightly lower than in controls (Figure 2D). Analysis of anaphase figures from *scpo*^{z2775}/*Df(3R)Exel5780* larval neuroblasts failed to detect lagging chromosomes or aberrant chromosome separation to the poles (Figure 2G), consistent with defects mainly in cytokinesis rather than in chromosome segregation.

Substitution of serine for the wild-type Pro-86 affects localization of Survivin protein during anaphase

Sequencing of the *scpo*^{z2775} allele revealed two point mutations in the *dSurvivin* open reading frame compared with the original reference sequence for *Drosophila melanogaster deterin* (FlyBase), causing substitution of glutamine for a conserved glutamic acid at position 83 and serine for a conserved proline at position 86 (Figure 2E). However, the glutamic acid–to-glutamine substitution is not likely to be the cause of the mutant phenotype, as the glutamic acid 83–to-glutamine substitution was also present in the original fly line prior to mutagenesis (Zuker line; Koundakjian *et al.*, 2004), and flies homozygous for the Zuker screen–background chromosome did not show evidence of cytokinesis defects. In addition, transgenic flies carrying the *survivin* genomic region with the glutamic 83–to-glutamine mutation fully rescued the *scpo* phenotype. The replacement of glutamic acid 83 by glutamine disrupts only one of several amino acids in a conserved patch of acidic amino acids (residues E83, D84, and D85 in *Drosophila* Survivin) in a solvent-exposed loop of the BIR domain (Figure 2E, asterisks; Figure 2F, magenta ribbon; Jeyaprakash *et al.*, 2007) important for Survivin function at centromeres (Cao *et al.*, 2006; Lens *et al.*, 2006; Yue *et al.*, 2008; Kelly *et al.*, 2010; Wang *et al.*, 2010) and so is not likely to strongly affect function of Survivin in binding to Aurora B (Cao *et al.*, 2006). In contrast, a *survivin* genomic transgene with the Pro-86–to-serine substitution failed to rescue the scapolo phenotype, suggesting that the defects in cytokinesis in *scpo* mutants are due to this amino acid change.

The conserved Pro-86 (Figure 2E, solid arrowhead; Figure 2F, blue residue) is positioned right before a short alpha helix that helps coordinate a structural Zn atom (Jeyaprakash *et al.*, 2007; Figure 2E, yellow hatches, and Figure 2F, yellow residues, indicate residues involved in Zn coordination), raising the possibility that the alpha helix might help stabilize the Zn coordination loop. It is possible that, with increasing temperature, a conformational change imparted due to the *scpo* mutation (perhaps a slight displacement of the helix) may affect coordination of the Zn atom and reduce structure stability. Alternatively, the mutant may affect Survivin function because it creates a consensus site for phosphorylation not present in the wild-type protein. Of interest, the mutant sequence generated by substitution of serine for the wild-type proline at position 86 of dSurvivin (Figure 2E, DD-*S*-WKE) conforms exactly to the consensus motif for phosphorylation by Polo kinase, D/E-X-S/T-Φ-X-D/E (with Φ indicating hydrophobic residue (Nakajima *et al.*, 2003), raising the possibility that phosphorylation by Polo of the mutant Ser-86 might cause abnormal conformation or spatial hindrance that affects dSurvivin activity.

Immunostaining of larval neuroblasts from *scpo*^{z2775}*/Df(3R)Exel5780* animals with antibodies against Survivin showed that the mutant protein localized correctly to kinetochores in dividing mutant cells during metaphase (Figure 2G), similar to wild type (Figure 1A). However, Survivin failed to localize to the equatorial midzone in *scpo*^{z2775}*/Df(3R)Exel5780* larval neuroblasts (Figure 2G), suggesting that substitution of serine for the wild-type Pro-86 might impede localization of Survivin during anaphase without compromising its localization at earlier steps of division.

Substitution of serine for Pro-86 in Survivin caused defects in localization of Aurora B and INCENP to the equatorial midzone during anaphase

The chromosomal passenger proteins Aurora B and INCENP behaved similarly to the defective Survivin protein in *scpo* mutant spermatocytes and neuroblasts. Immunofluorescence analysis of spermatocytes from *scpo*^{z2775}*/Df(3L)Exel5780* males showed that INCENP and Aurora B localized correctly to kinetochores at metaphase (N = 37 for INCENP; N = 32 for Aurora B), as in wild type (N = 22 for INCENP; N = 24 for Aurora; Figure 3, A and B). However, both CPC proteins failed to accumulate to the central spindle midzone during ana/telophase in *scpo* spermatocytes (Figure 3, A and B; for INCENP, N = 46 mutant cells, N = 28 wild-type cells; for Aurora B, N = 55 mutant cells, N = 33 wild-type cells). Similarly, immunostaining of larval neuroblasts indicated that Aurora B accumulated at kinetochores in metaphase cells in both wild type (N = 100) and *scpo*^{z2775}*/Df(3L)Exel5780* (N = 147) (Figure 3C) but failed to relocalize to the spindle midzone at anaphase–telophase in *scpo*^{z2775}*/Df(3R)Exel5780* (N = 39). In wild-type larval neuroblasts, in contrast, Aurora B consistently relocalized to the spindle midzone at anaphase–telophase (N = 24) (Figure 3C).

Consistent with the indication that the *scpo*^{z2775} allele of *survivin* does not compromise the function of the CPC during metaphase but specifically affects CPC function during later stages, histone H3S10 phosphorylation on metaphase chromosomes, which depends on Aurora B activity, appeared similar in *scpo* mutant metaphase spermatocytes and in wild type (Figure 3D),

A role for Survivin in central spindle organization in dividing cells

Dynamic live-cell analysis of microtubules in wild-type versus *scpo*^{z2775}*/Df(3R)Exel5780* spermatocytes expressing enhanced GFP (EGFP)–tagged β-tubulin revealed that the *scpo* mutant impairs assembly of the central spindle during anaphase and telophase. In wild type (N = 10), centrosome-nucleated microtubules extended from the poles to contact the cell cortex at the equatorial region in anaphase. On contact, the pole-to-equator microtubules bundled and became stabilized at the cortex (Figure 4A, 0–5 min, and Supplemental Movie S3). This initial bundling was rapidly followed by cell elongation and organization of cytoplasmic microtubules into interdigitated arrays at the cell midzone (Figure 4A, 5–11 min, and Supplemental Movie S3). With ingression of the cleavage furrow the arrays of microtubules compacted into the characteristic telophase central spindle (Figure 4A, 11–25 min, and Supplemental Movie S3).

In *scpo*^{z2775}*/Df(3R)Exel5780* mutant spermatocytes (N = 6), in contrast, although microtubules extended from the poles and contacted the cortex at the equator during anaphase, (Figure 4B, 0–11 min), upon initial contact the microtubules either failed to bundle and stabilize or did so only transiently (Figure 4B, cortical dots at 11 min, and Supplemental Movie S4, cortical dots at 22–24 min). In addition, internal pole-to-equator microtubules failed to organize robust interdigitating arrays at the cell midzone (Figure 4B, 11–25 min, and Supplemental Movie S4). Finally, there was no evidence of furrowing at the equator in *scpo*^{z2775}*/Df(3R)Exel5780* mutant spermatocytes, and the cells failed to elongate (Figure 4B, 15–25 min, and Supplemental Movie S4).

The *scpo* mutant affected localization of the centralspindlin component Pavarotti but not the microtubule-bundling protein Feo to the anaphase central spindle

Immunofluorescence staining of spermatocytes undergoing meiotic division with antibodies against the microtubule bundling protein Fascetto/PRC1 (Feo; Verni *et al.*, 2004) showed initial accumulation of Feo protein to microtubules at the midzone in *scpo*^{z2775}*/Df(3R)Exel5780* anaphases (N = 16 control cells; N = 17 mutant

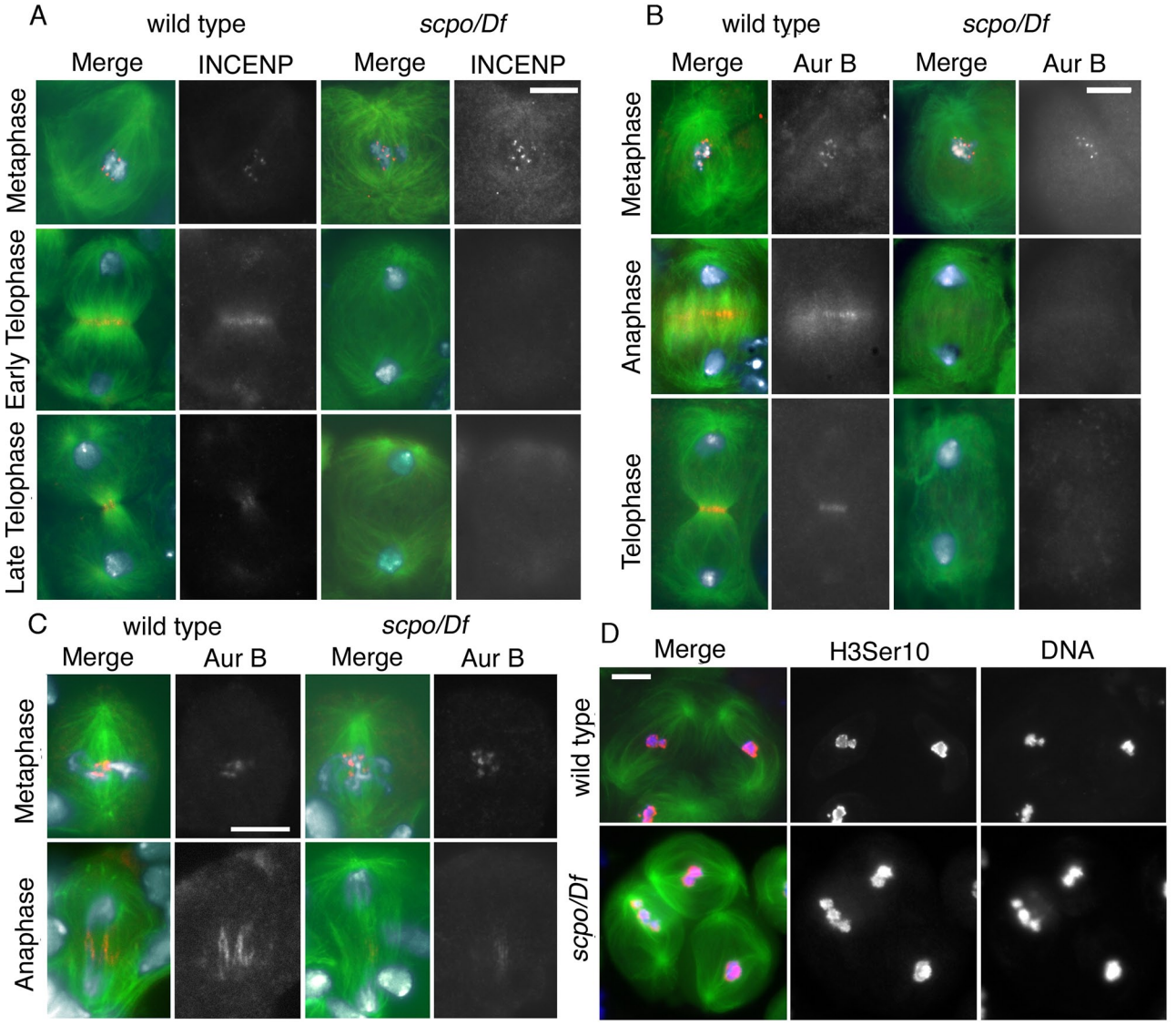

FIGURE 3: Aurora B complex components reflect the localization of survivin in *scpo* mutant cells. (A–C) Wild-type and *scpo*^{z2775}/*Df(3R)Exel5780* (A, B) spermatocytes or (C) neuroblasts stained for tubulin (green), DNA (light blue), and either (A) INCENP (red) or (B, C) Aurora B (red). Aurora B and INCENP were enriched at metaphase centromeres of both wild-type and *scpo*^{z2775}/*Df(3R)Exel5780* spermatocytes and neuroblasts but failed to accumulate at the spindle midzone of mutant ana/telophases. (D) Wild-type and *scpo*^{z2775}/*Df(3R)Exel5780* mutant testes stained for tubulin (green), phosphohistone H3 Ser-10 (red), and DNA (blue). Scale bar, 10 μm.

cells) (Figure 5A), indicating that pole-to-equator microtubules were present and arranged with their plus ends overlapping at the cell midzone in mid anaphases in mutant spermatocytes. However, Feo protein failed to concentrate to a tight equatorial midzone in telophases in *scpo*^{z2775}/*Df(3R)Exel5780* mutant spermatocytes (N = 24 control cells; N = 26 mutant cells) (Figure 5A), consistent with the failure of contractile ring formation and constriction in these cells.

In contrast, immunofluorescence staining with antibodies against a centralspindlin component, the kinesin-like PAV-KLP (Adams *et al.*, 1998), revealed that anaphase spindles in *scpo*^{z2775}/*Df(3R)Exel5780* spermatocytes were either devoid of Pav-KLP or displayed only very faint Pav-KLP concentration at the central spindle (N = 18 control cells; N = 18 mutant cells; Figure 5B). In telophase cells, Pav-KLP was completely absent from cortical microtubules and central spindle in all *scpo*^{z2775}/*Df(3R)Exel5780* spermatocytes examined (N = 22 control cells; N = 24 mutant cells).

In *scpo*^{z2775}/*Df(3R)Exel5780* neuroblasts, similar to spermatocytes, Feo protein was present marking microtubule plus ends at the cell equator during anaphase and early telophase (N = 20 control cells; N = 18 mutant cells; Figure 6A). In contrast to spermatocytes, however, some initial constriction of the cleavage furrow and some irregular bundling of microtubule plus ends marked by Feo occurred in telophase *scpo*^{z2775}/*Df(3R)Exel5780* neuroblasts (Figure 6A), although the Feo signal did not concentrate into the normal tight ring by late telophase (N = 18 control cells; N = 16 mutant cells; Figure 6A). In contrast, Pav-KLP displayed only faint or no accumulation to the spindle midzone of anaphases and telophases from *scpo*^{z2775}/*Df(3R)Exel5780* neuroblasts (N = 18 control cells; N = 16 mutant cells; Figure 6B).

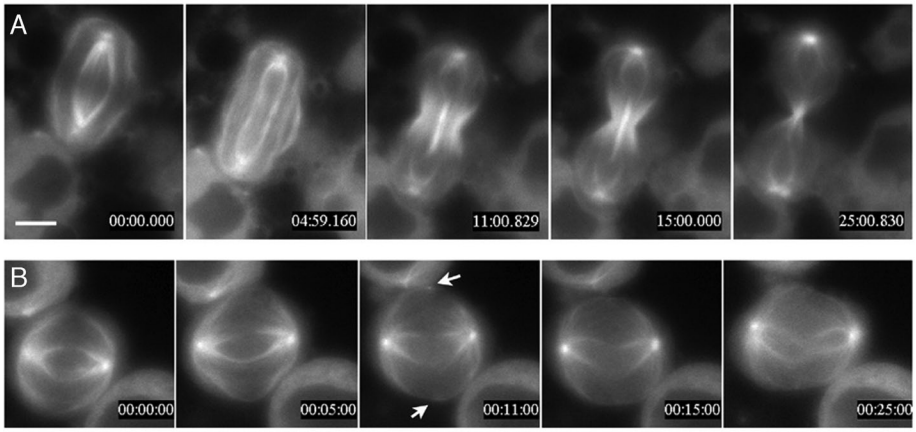

FIGURE 4: Pole-to-equator microtubules initially contact the cortex but fail to form a stable central spindle in *scpo* mutant primary spermatocytes undergoing meiosis. Time-lapse imaging of central spindle formation in wild-type and *scpo*^{z2775}*/Df(3R)Exel5780* mutant primary spermatocytes expressing EGFP–β-tubulin imaged every 60 s. (A) Wild type (selected frames from Supplemental Movie S3): a robust central spindle formed in anaphase (frame 4.59) and constricted during telophase (frames 8.59, 15.00, and 25.00). (B) *scpo*^{z2775}*/Df(3R)Exel5780* mutant (selected frames from Supplemental Movie S4): microtubules bundled only transiently (see dots in frame 11), and a robust central spindle failed to form. Scale bar, 10 μm.

Defective recruitment of Polo kinase to the spindle midzone in spermatocytes but not in larval neuroblasts from *scpo*^{z2775}*/Df(3R)Exel5780*

Localization of the mitotic kinase Polo to the central spindle in *scpo*^{z2775}*/Df(3R)Exel5780* larval neuroblasts was comparable with Feo (Figures 6A and 7B), consistent with the finding that recruitment of Polo to the central spindle in *Drosophila* S2 cells requires function of the microtubule plus end–bundling protein Feo but is independent of Pav/MKLP1 localization/activity (D'Avino *et al.*, 2007). In the *scpo* mutant neuroblasts, Polo protein was present at the cell equator at late anaphase as in wild type (N = 18 control cells, N = 18 mutant cells), although Polo failed to concentrate into the normal tight band during telophase (N = 16 control cells, N = 16 mutant cells; Figure 7B). However, although localization of Feo to anaphase I *scpo* spermatocytes appeared normal (Figure 5A), no Polo localization was detected in any of the *scpo*^{z2775}*/Df(3R)Exel5780* mutant spermatocytes in late anaphase (N = 29 control cells, N = 25 mutant cells; Figure 7A) and telophase scored (N = 25 control cells, N = 22 mutant cells; Figure 7A). As discussed later, this may reflect fundamental differences between spermatocytes and neuroblasts in the sequence of steps leading to contractile ring assembly and organization prior to ring constriction.

Survivin activity is required for localization and stabilization of Rho 1 and myosin to the equatorial cortex in spermatocytes but not in neuroblasts

Action of Survivin may play a key regulatory role enabling initial localization and perhaps maintaining the dynamic activation state of the F-actin assembly regulator Rho 1 at the equatorial cortex of dividing spermatocytes. Immunofluorescence staining of wild-type spermatocytes revealed that Rho 1 became concentrated in a narrow ring at the cell equator during late anaphase (N = 18; Figure 8A), and then the ring constricted as the cells proceeded through late telophase, forming a tight accumulation at the midbody (N = 28; Figure 8A). In *scpo*^{z2775}*/Df(3R)Exel5780* spermatocytes, in contrast, accumulation of Rho 1 was never detected at the cell midzone in either late anaphase (N = 16) or telophase (N = 24; Figure 8A), correlating with the failure of *scpo* mutant spermatocytes to initiate assembly of the F-actin ring (Giansanti *et al.*, 2004).

Loss of function of Survivin also resulted in failure to form the cortical ring of myosin II complex proteins in spermatocytes undergoing anaphase, visualized by the *Drosophila* myosin regulatory light chain Spaghetti squash tagged with GFP (Sqh-GFP). In wild-type spermatocytes undergoing anaphase, Sqh-GFP first appeared as a thin cortical band at the cell equator; then the band constricted to a tight ring as the cleavage furrow ingressed (Figure 8B). In contrast, no ring of Sqh-GFP was detected in anaphase or telophase *scpo*^{z2775}*/Df(3R)Exel5780* spermatocytes (Figure 8B).

scpo mutant neuroblasts undergoing mitosis differed from *scpo* mutant spermatocytes undergoing meiosis in the concentration of Rho1 and myosin at the equatorial cortex, perhaps reflecting differences in the mechanisms that first assemble these proteins at the cortex during anaphase in the two cell types. Immunostaining of *scpo*^{z2775}*/Df(3R)Exel5780* late anaphase–early telophase neuroblasts for either Rho1 (N = 18 control cells; N = 12 mutant cells; Figure 9A) or myosin II (N = 15 control cells; N = 10 mutant cells; Figure 9B) revealed normal accumulation of the contractile ring components to a broad band at the cortex, in spite of the absence of strong central spindle microtubule arrays (Figure 9B). However, the initial bands of Rho1 and myosin II failed to resolve into the typical tight rings in *scpo*^{z2775}*/Df(3R)Exel5780* late telophase neuroblasts, and, in spite of considerable constriction, completion of cytokinesis was impaired (Figure 9B).

DISCUSSION

A missense mutation leading to substitution of serine for the wild-type Pro-86 of *Drosophila* Survivin uncouples the function of Survivin in metaphase from function during anaphase and telophase, indicating a direct requirement for Survivin and the chromosomal passenger complex in orchestrating the profound reorganization of the cortical cytoskeleton at the cell equator at the onset of cytokinesis. This "separation-of-function" allele allowed analysis of Survivin and CPC function during cytokinesis, which is normally obscured by the better-known roles of the CPC at centromeres during metaphase, when it facilitates alignment of chromosomes to the spindle equator and mediates the spindle checkpoint (Carmena, 2008). Our finding that a point mutation in the BIR domain disrupts activity of Survivin during cytokinesis challenges the model that the C-terminal domain of Survivin is sufficient for cytokinesis function (Lens *et al.*, 2006) and indicates that residues in the BIR domain are important for localization and activity of Survivin at the central spindle.

Survivin associates with kinetochores and the central spindle with different dynamics, being highly mobile in prometaphase and metaphase and strongly immobile at the anaphase central spindle (Beardmore *et al.*, 2004; Delacour-Larose *et al.*, 2004). This change in dynamics may underlie the largely normal localization and function of *scpo* mutant Survivin at metaphase but the fully penetrant effect on assembly of the F-actin contractile ring and cytokinesis observed in *scpo* mutants (Giansanti *et al.*, 2004; this study).

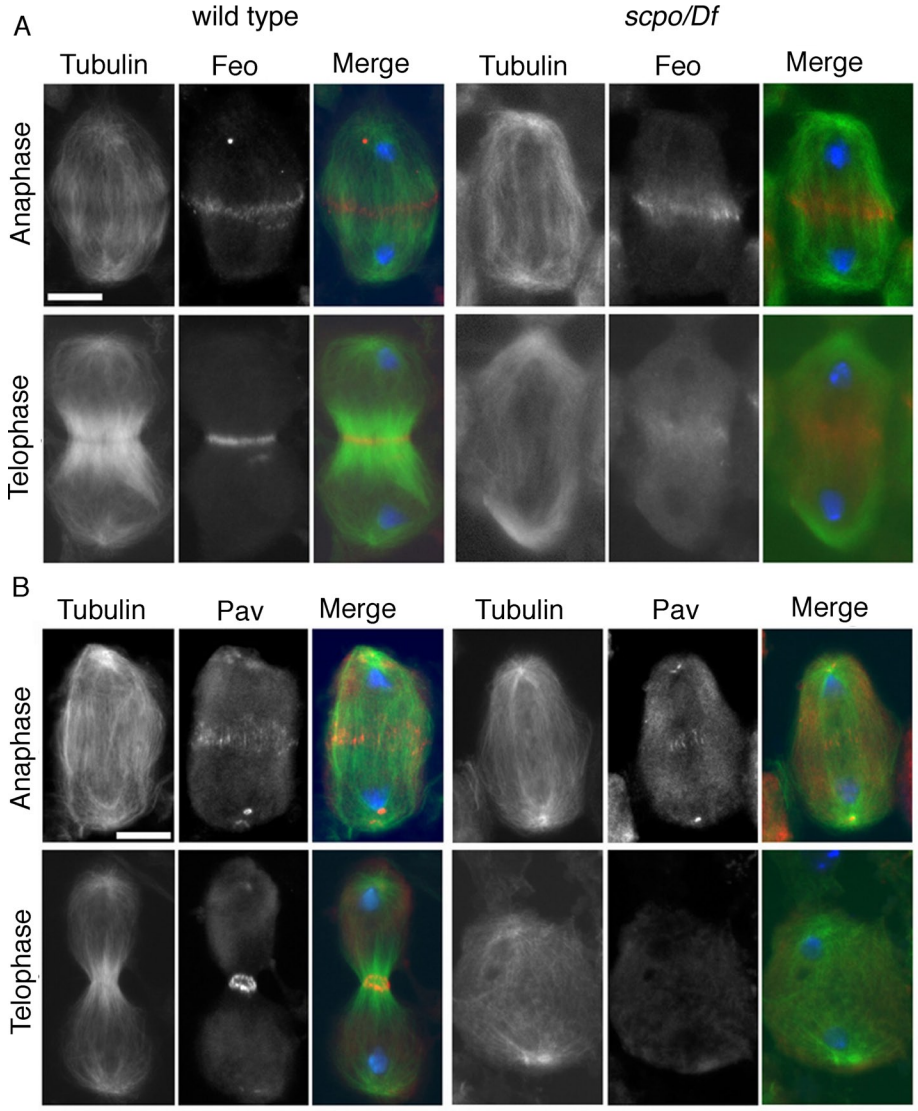

FIGURE 5: Loss of Survivin function during anaphase affects localization of the centralspindlin component Pav but not the microtubule-bundling protein Feo in *scpo* mutant spermatocytes. Wild -type and *scpo*^{z2775}/*Df(3R)Exel5780* mutant spermatocytes stained for tubulin (green), DNA (blue), and either (A) Feo (red) or (B) Pav (red). Scale bar, 10 μm.

Cytokinesis depends on the assembly of an equatorial actomyosin ring regulated by local activation of the small GTPase RhoA at the cortex, in turn catalyzed by the RhoGEF Ect2/Pebble (Yuce *et al.*, 2005; Zhao and Fang, 2005; Kamijo *et al.*, 2006; Nishimura and Yonemura, 2006). It has been proposed that association of RhoGEF/Pebble with centralspindlin promotes local RhoA activation at the cortex (Somers and Saint, 2003; Piekny *et al.*, 2005; Chalamalasetty *et al.*, 2006; Nishimura and Yonemura, 2006). In addition, the kinase polo (PLK1) has been implicated in RhoGEF localization and Rho activation, at least in part by phosphorylation of the centralspindlin component MgcRacGAP (Brennan *et al.*, 2007; Santamaria *et al.*, 2007; Burkard *et al.*, 2009; Wolfe *et al.*, 2009). Our observations that the *Drosophila* RhoA homologue, Rho1, failed to accumulate at the equatorial cortex in *scpo* mutant spermatocytes implicate Survivin and the CPC in the mechanism(s) that localize and activate RhoA at the equatorial cortex in these cells. This requirement may in part act through effects on Polo kinase. Failure to localize Polo to the central spindle in *scpo* mutant spermatocytes could prevent localization of RhoGEF by the centralspindlin complex and the consequent activation of Rho at the cortex. In this model, failure to localize Polo may contribute to the failure to form an equatorial ring of localized Rho1 and, in consequence, the inability to form a localized ring of myosin regulatory light chain and F-actin in *scpo* mutant male germ cells undergoing meiotic division. This mechanism may also explain the failure to maintain pole-to-equatorial microtubules observed in *scpo* mutant spermatocytes (Supplemental Movie S4 and Figure 4). It is likely that Rho-mediated activation of the Formin Dia helps stabilize microtubule arrays at the equatorial cortex of dividing cells, as active Rho and Formin (mDia) regulated stabilization of microtubule arrays at the cortex of migrating fibroblasts (Palazzo *et al.*, 2001; Bartolini *et al.*, 2008). Consistent with this model, we found that microtubules reached the plasma membrane at the equator of *scpo* dividing spermatocytes, but the bundles were transient and failed to form stable arrays at the cortex.

A striking finding of our work is the difference in requirement for Survivin function for localization of the Polo kinase and RhoA in anaphase neuroblasts versus spermatocytes. This difference raises two possibilities: either Survivin is not part of a universal signaling mechanism that directs cytokinesis, or different semiredundant mechanisms can drive cytokinesis, similar to redundancy between astral pulling and sliding of central spindle microtubules for anaphase B, and different cell types rely more strongly on one mechanism or the other. Indeed, consistent with the latter possibility, spermatocytes and neuroblasts display different cytoskeletal architectures during cytokinesis (Giansanti *et al.*, 2006). In neuroblasts, actomyosin initially accumulates in a broad cortical band, presumably because this is the region of the cell cortex that escapes repression of Rho associated with the plus ends of astral microtubule (Werner *et al.*, 2007; Chen *et al.*, 2008; Foe and von Dassow, 2008; von Dassow *et al.*, 2009). This initial wide band gradually narrows into a tight equatorial ring as the cell progresses into telophase (Giansanti *et al.*, 2006). Thus assembly of the contractile apparatus in neuroblasts proceeds, as proposed by Lewellyn *et al.* (2011) for *Caenorhabditis elegans* embryos, in "two genetically separable steps" in which localization of contractile machinery is initially independent of the central spindle. In support of this model, we found that Rho1 accumulated in a broad cortical band in *scpo* mutant neuroblasts, suggesting that the first stage can occur independent of Survivin and CPC localization to the central spindle.

Spermatocytes, in contrast, do not form an initial wide equatorial band of contractile ring components. Instead, from their first appearance in early anaphase, the actomyosin rings in spermatocytes are tightly focused at the cell equator (Giansanti *et al.*, 2006). We speculate that this restricted initial localization of contractile ring components and the apparent lack of a preceding wide equatorial

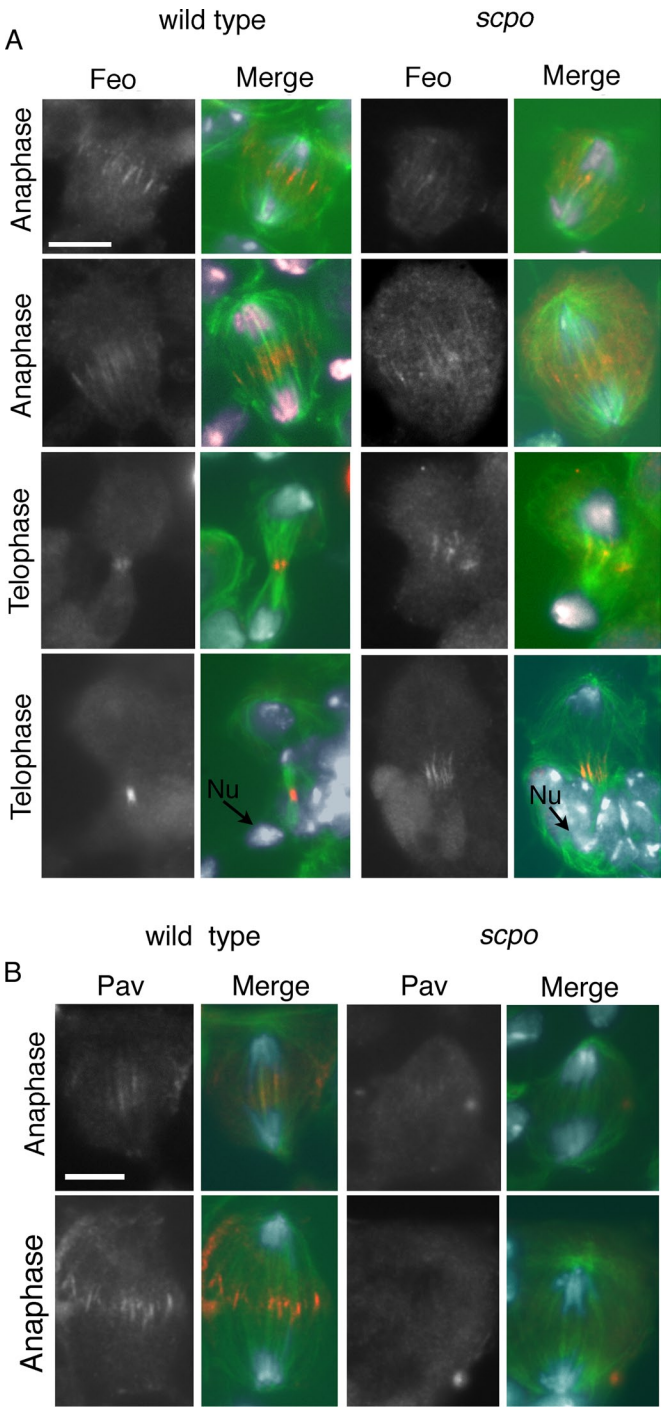

FIGURE 6: Loss of Survivin function disrupts localization of the centralspindlin component Pav but not accumulation of the microtubule-bundling protein Feo in *scpo*^{z2775}/*Df(3R)Exel5780* neuroblasts. Localization of Fascetto (Feo) and Pavarotti (Pav) in wild-type and *scpo*^{z2775}/*Df(3R)Exel5780* mutant neuroblasts stained for tubulin (green), DNA (blue), and either (A) Feo (red) or (B) Pav (red). Arrows in A point to telophase nuclei. Scale bar, 10 μm.

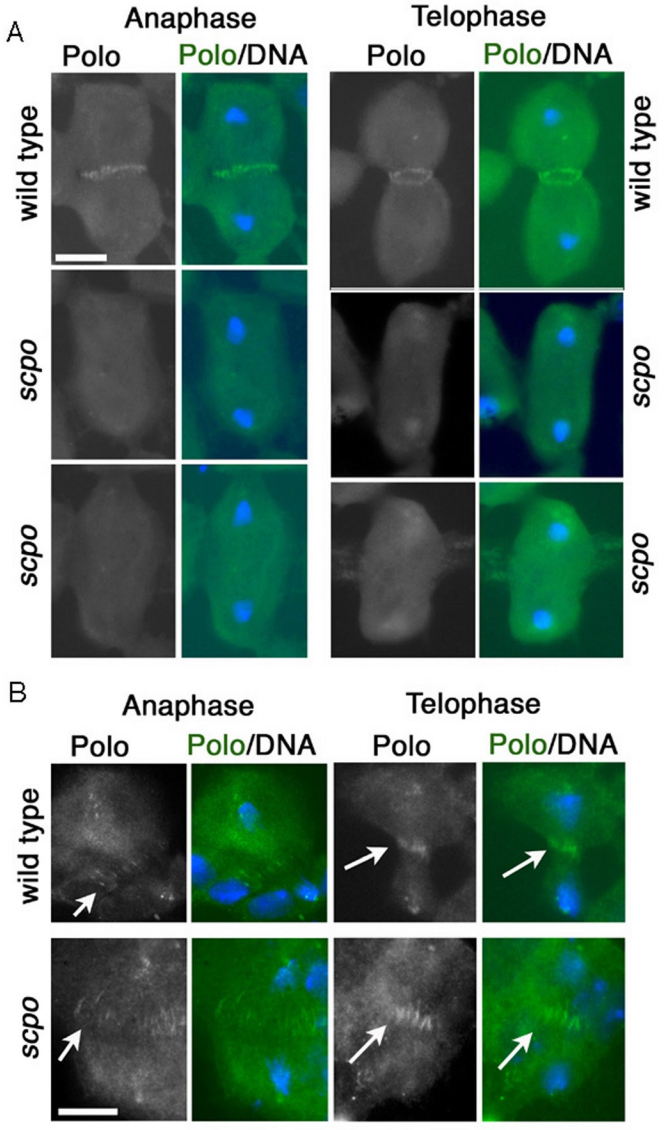

FIGURE 7: Recruitment of Polo to the midzone is impaired in spermatocytes but not in neuroblasts from *scpo*^{z2775}/*Df(3R)Exel5780* mutants. Wild-type and *scpo*^{z2775}/*Df(3R)Exel5780* (A) spermatocytes or (B) neuroblasts stained for DNA (blue) and Polo (green). Polo is present at the midzone of anaphase wild-type and mutant neuroblasts but fails to form a tight band in telophase of *scpo* mutant neuroblast (arrows in B). Scale bar, 10 μm.

band may be a consequence of a more stringent global block to Rho1 activation at the cortex in spermatocytes than in neuroblasts (Canman *et al.*, 2003; Foe and von Dassow, 2008; von Dassow *et al.*, 2009; Canman, 2009). We propose that this global block is eventually overridden by positive regulation of Rho1 by local concentration of RhoGEF, in turn facilitated by CPC-dependent events associated with and/or localized by central spindle microtubules (Burgess and Chang, 2005; Piekny *et al.*, 2005; this study). Rho1 activation would then occur within a narrow peak exactly at the site where pole-to-equator microtubules interact to maximize RhoGEF deposition/concentration at the cortex (Somers and Saint, 2003; Piekny *et al.*, 2005; Chalamalasetty *et al.*, 2006; Nishimura and Yonemura, 2006). Indeed, F-actin ring assembly occurs locally and cytokinesis initiates immediately after the pole-to-equator microtubules contact the cortex in *Drosophila* spermatocytes (Supplemental Movie S4 and Figure 4A). We propose that, according to this model, the defects in Survivin lead to lack of CPC activity and abnormal centralspindlin, resulting in absence of Rho1 and Polo kinase from the equator of *scpo* mutant spermatocytes.

In neuroblasts, where a more permissive cortex allows a broad belt of Rho1 activation at the cell equator, Survivin and CPC appear

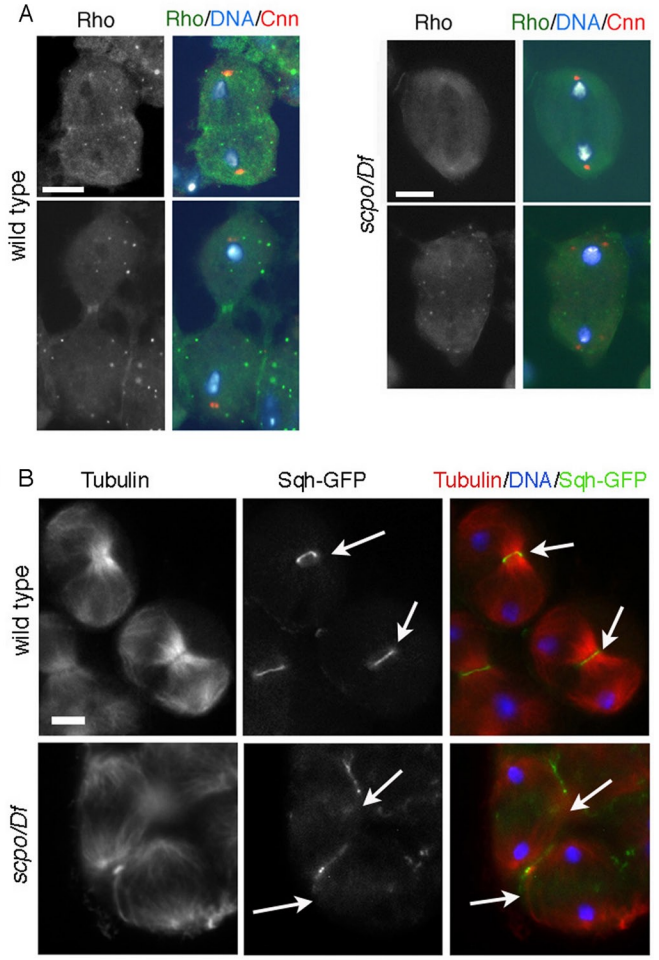

FIGURE 8: Rho1 and Sqh-GFP localization to the midzone is disrupted in spermatocytes from *scpo*^{z2775}/*Df(3R)Exel5780* mutants. Wild-type and *scpo*^{z2775}/*Df(3R)Exel5780* spermatocytes stained for DNA (blue), centrosomin (red), and Rho1 (green) (A) and for DNA (blue), microtubules (red), and Sqh-GFP (green) (B). Arrows in B point to the cells' equator. Scale bar, 10 μm.

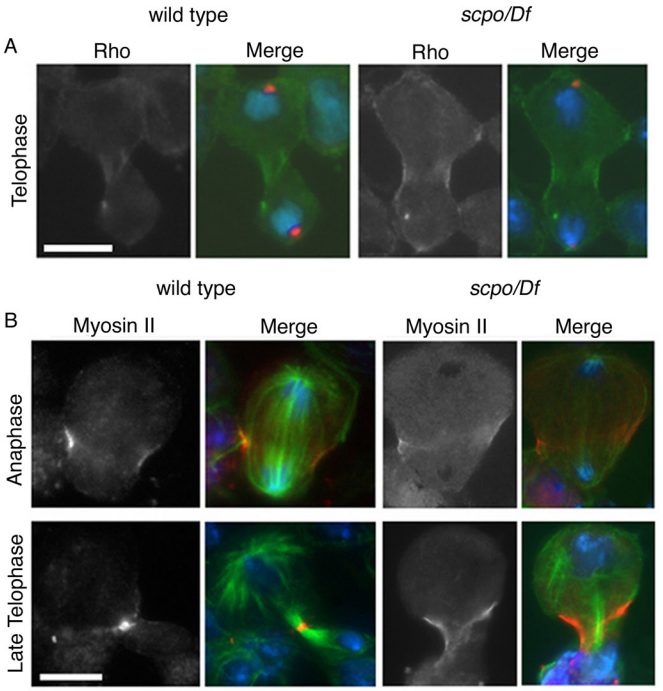

FIGURE 9: Loss of Survivin function does not impair the recruitment of Rho1 and Myosin II to the cortex of *scpo*^{z2775}/*Df(3R)5780* mutant neuroblasts but affects the formation of a compact, fully constricted ring. Localization of Rho1 (A) and myosin II (B) in wild-type and *scpo*^{z2775}/*Df(3R)Exel5780* mutant neuroblasts stained for Rho1 (green in A), centrosomin (red), tubulin (green in B), DNA (blue), and myosin II (red in B). Scale bar, 10 μm.

to promote gradual convergence of the initial broad band into a narrow ring centered at the maximum of RhoGEF activity at the cortex. In *scpo* mutants, which display irregular anaphase central spindles devoid of Pav, the broad Rho1 cortical band fails to narrow, the cells fail to form a focused, narrow ring of myosin, and cell division proceeds with inefficient and incomplete constriction.

A key difference between neuroblasts and spermatocytes that may account, at least in part, for the differences in behavior of Rho1 and myosin complex proteins we observed in *scpo* mutant mitotic versus male meiotic cells is in the relationship between Polo kinase and the CPC. In spermatocytes, Polo and the CPC are interdependent and Polo colocalizes with the CPC along its full journey from metaphase through anaphase and telophase (Goto *et al.*, 2006; Vuoriluoto *et al.*, 2011; this study). In neuroblasts, in contrast, Polo localization during cytokinesis appears to be independent of the CPC and centralspindlin, at least at early stages of cell division, but Polo appears to colocalize with Feo (this work). A second difference between neuroblasts and spermatocytes may be the recently described, spindle-independent backup system that can localize myosin to a broad band at the cell cortex near the future cleavage plane under control of the neuroblast cell polarity system (Cabernard *et al.*, 2010). The broad localization of myosin to the cell cortex observed in ana/telophase neuroblasts in *scpo* mutants may be in part due to these redundant mechanisms.

MATERIALS AND METHODS

Fly strains, husbandry, and transgenes

Flies were raised on cornmeal molasses or dextrose medium at 25°C. The *scapolo* allele of *survivin* was from the C. Zuker collection of ethylmethane sulfonate–mutated lines that were screened for male sterility (Giansanti *et al.*, 2004). The Sqh-GFP stock was a gift from R. Karess (Royou *et al.*, 2002). The β-tubulin–EGFP strain was kindly provided by M. Savoian and D. Glover (Inoue *et al.* 2004). The P-elements and chromosomal deficiencies used in the mapping of *scpo* were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN) and the Szeged University *Drosophila* Stock Center (Szeged, Hungary). Transgenic flies were generated by P-element–mediated transformation of sequences cloned into pCaSpeR4. For the *survivin* rescue construct, a 2.5-kb fragment containing *survivin* was PCR amplified from the genomic BAC RP98-48C17 clone (BACPAC Resources, Oakland, CA) and cloned into pCaSpeR4. The survivin transgenes containing glutamine 83–to-glutamic substitution and Pro-86–to-serine substitutions were generated by PCR site-directed mutagenesis on the survivin rescue construct and cloned into pCaSpeR4. For the *survivin*-GFP fusion construct, *survivin* was fused in-frame to the 3′ end of EGFP using endogenous promoter and genomic sequences and cloned in pCaSpeR4.

Mapping *scapolo*

The *scapolo* locus was initially mapped by meiotic recombination to a region between the markers e and Pr on chromosome 3R

(Giansanti *et al.*, 2004). Deficiency complementation tests showed that Exelixis *Df(3R)Exel5780* and *Df(3R)Exel5781* (from the Harvard Stock Center, Cambridge, MA) failed to complement the *scpo* mutant phenotype. Male recombination was used to narrow the *scpo* genomic region to the distal of P-element insertion P{w[+mC] y[+mDint2] = EPgy2}EY02138 and to the proximal of P{EPgy2}EY07690, leaving a region of 100 kb. *scpo* was identified by comparing sequences of candidate genes in the region in *scpo* homozygous to sequences of the original Zuker-background chromosome.

Antibody generation

Antibodies were generated against a glutathione *S*-transferase (GST)–full-length dSurvivin fusion protein. *survivin* cDNA was PCR amplified from EST LP03704 (Berkeley *Drosophila* Genome Project, Berkeley, CA) and cloned in-frame into a pGEX-3X vector (Amersham Biosciences, Piscataway, NJ). The 50-kDa GST–Survivin fusion protein was expressed in *Escherichia coli* BL21 strain and purified on glutathione–Sepharose beads (Sigma-Aldrich, St. Louis, MO) according to the *GST Gene Fusion System Handbook* (Amersham Biosciences). Antibodies against the GST–Survivin fusion protein were produced in rabbits (GenScript, Piscataway, NJ).

Microscopy and immunofluorescence

Squashed adult or larval testes were imaged by phase contrast on a Zeiss Axioskop (Thornwood, NY) as described by Regan and Fuller (1988). Cytological preparations for immunostaining were made with testes or brains from third-instar larvae or pupae. For all the preparations except for those shown in Figure 2, A and B, we used testes or brains from *scpo*^{z2775}*/Df(3R)Exel5780* individuals raised at 25°C. To visualize tubulin and Aurora B, INCENP, or Survivin in spermatocytes, larval testes were fixed and stained according to Starr *et al.* (1998). To visualize α-tubulin along with either Scpo-GFP or α-tubulin with Ser-10 H3 histone, larval testes were fixed with 4% formaldehyde as described by Farkas *et al.* (2003). For all other immunostaining, preparations were fixed using 3.7% formaldehyde in phosphate-buffered saline and then squashed in 60% acetic acid according to Giansanti *et al.* (1999). To visualize mitotic chromosomes, larval brains preparations were dissected in NaCl, 0.7%, treated with hypotonic solution for 7 min, fixed in 45% acetic acid processed as per Giansanti *et al.* (1999), and stained with 4′,6-diamidino-2-phenylindole (DAPI). Monoclonal antibodies were used to stain α-tubulin (T6199, diluted 1:100; Sigma-Aldrich), Rho1 (diluted 1:100; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), and Polo mouse monoclonal anti-Polo M294 (diluted 1:20; a gift from D. Glover [Tavares *et al.*, 1996]). Polyclonal antibodies were used to stain Pavarotti (diluted 1:200; a gift from D. Glover (Adams *et al.*, 1998)], Fascetto (diluted 1:200; a gift from F. Verni [Verni *et al.*, 2004]), anti–Aurora B (diluted 1:200; a gift from P. P. D'Avino and D. Glover [Giet and Glover, 2001]), anti-Survivin (diluted 1:500; this study), anti-DmINCENP (diluted 1:400; a gift from J. Wakefield), anti–myosin II (diluted 1:400; a gift from R. Karess [Royou *et al.*, 2002]), and anti–Ser-10 H3 histone (diluted 1:100; Cell Signaling Technology, Danvers, MA). Secondary antibodies Alexa 488/Alexa 555–conjugated anti–rabbit immunoglobulin G (Molecular Probes, Eugene OR) and rhodamine/fluorescein isothiocyanate–conjugated anti-mouse (Jackson ImmunoResearch, West Grove, PA) were used at dilution of 1:250 and 1:20, respectively. Slides were mounted in Vectashield medium with DAPI (Vector Laboratories, Burlingame, CA). Images were captured with a Photometrics (Tucson, AZ) cooled charge-coupled device camera connected to a Zeiss Axiophot epifluorescence microscope. Grayscale images were collected separately and were pseudocolored and merged in Photoshop 6 (Adobe, San Jose, CA).

Live imaging

Time-lapse imaging of living spermatocytes was carried out according to the protocol described in Inoue *et al.* (2004). Testes isolated from adult flies or third-instar larvae were dissected under 10S Voltalef oil (Elf Atochem, Philadelphia, PA) onto a clean coverslip attached to the underside of an aluminum slide. Cells were examined with a Zeiss Axiovert 20 microscope equipped with a 63×, 1.25 numerical aperture (NA) or a 63×, 1.4 NA objective and a filter wheel combination (Chroma Technology, Bellows Falls, VT). Images were acquired with a CoolSnap HQ camera (Photometrics), using a 2 × 2 bin controlled through Metamorph software (Molecular Devices, Sunnyvale, CA). Images were collected at 1-min intervals; 11 fluorescence optical sections were captured at 1-μm z-steps. Movies were created using the Metamorph software and show the maximum-intensity projection of all of the sections.

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