A Mutation in *dVps28* Reveals a Link between a Subunit of the Endosomal Sorting Complex Required for Transport-I Complex and the Actin Cytoskeleton in *Drosophila*

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Submitted November 18, 2004; Revised February 7, 2005; Accepted February 12, 2005
Monitoring Editor: Suzanne Pfeffer

Proteins that constitute the endosomal sorting complex required for transport (ESCRT) are necessary for the sorting of proteins into multivesicular bodies (MVBs) and the budding of several enveloped viruses, including HIV-1. The first of these complexes, ESCRT-I, consists of three proteins: Vps28p, Vps37p, and Vps23p or Tsg101 in mammals. Here, we characterize a mutation in the *Drosophila* homolog of *vps28*. The *dVps28* gene is essential: homozygous mutants die at the transition from the first to second instar. Removal of maternally contributed *dVps28* causes early embryonic lethality. In such embryos lacking *dVps28*, several processes that require the actin cytoskeleton are perturbed, including axial migration of nuclei, formation of transient furrows during cortical divisions in syncytial embryos, and the subsequent cellularization. Defects in actin cytoskeleton organization also become apparent during sperm individualization in *dVps28* mutant testis. Because *dVps28* mutant cells contained MVBs, these defects are unlikely to be a secondary consequence of disrupted MVB formation and suggest an interaction between the actin cytoskeleton and endosomal membranes in *Drosophila* embryos earlier than previously appreciated.

INTRODUCTION

Multivesicular bodies (MVBs) are large vacuolar organelles with internal vesicles. These organelles serve as intermediates in several intracellular trafficking pathways: they harbor internalized ligands and receptors on their way to lysosomes (Futter *et al.*, 1996; Odorizzi *et al.*, 1998; Piper and Luzio, 2001), glycoproteins destined for melanosomes (Berson *et al.*, 2001), and mannose-6-phosphate receptors during their round-trip between the Golgi complex and late endosomes (Hirst *et al.*, 1998). The mechanisms that regulate MVB biogenesis and their biological role in different cell types are still poorly understood.

Some molecular components that participate in MVB biogenesis have recently been identified (Gruenberg and Stenmark, 2004). For example, lysobisphosphatidic acid is a lipid enriched in internal vesicles of MVBs and may play a role in their formation (Matsuo *et al.*, 2004). Furthermore, several genes necessary for the biogenesis of MVBs or the sequestration of cargo into their internal vesicles have been identified in yeast (Katzmann *et al.*, 2001) as a subset of genes

necessary for vacuolar protein sorting (*vps* genes). This subset, many members of which belong to the E class of *vps* genes, functions in the prevacuolar endosomal compartment (Rieder *et al.*, 1996). Proteins encoded by these genes associate into three distinct endosomal sorting complexes required for transport of cargo to the vacuole, called ESCRT-I, -II, and -III (Katzmann *et al.*, 2001; Babst *et al.*, 2002a,b; Bishop *et al.*, 2002; Katzmann *et al.*, 2003; Bowers *et al.*, 2004). Three proteins, Vps23p/Tsg101, Vps28p, and Vps37p, comprise the ESCRT-I complex in yeast and mammalian cells (Babst *et al.*, 2000; Bishop and Woodman, 2001; Bache *et al.*, 2004; Stuchell *et al.*, 2004).

In mammalian cells, the ESCRT-I complex functions in the down-regulation of cell surface receptors. Partial depletion of Tsg101 or interference by anti-hVPS28 antibody injections causes an inhibition of epidermal growth factor (EGF) degradation, enhanced recycling of EGF receptors, and prolonged activation of the downstream kinases extracellular signal-regulated kinase (ERK)1 and ERK2 (Babst *et al.*, 2000; Bishop *et al.*, 2002). Tsg101 is recruited to endosomal membranes via its interaction with pVps27/Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate). Hrs in turn binds to mono-ubiquitinated receptors and participates in their sorting into MVBs (Bache *et al.*, 2003; Katzmann *et al.*, 2003; Lu *et al.*, 2003).

The mechanism that drives invagination of internal vesicles of MVBs has gained additional interest because its elements are hijacked by enveloped viruses such as HIV-1 (Marsh and Thali, 2003). HIV-Gag mimics the function of Hrs in MVB formation by binding to Tsg101 and thereby recruiting the ESCRT complexes to the site of virus budding; this recruitment is required for HIV budding (Garrus *et al.*, 2001; Goila-Gaur *et al.*, 2003; Martin-Serrano *et al.*, 2003;

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-11-1013>) on February 23, 2005.

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Pornillos *et al.*, 2003; von Schwedler *et al.*, 2003; Stuchell *et al.*, 2004).

Surprisingly, the most drastic phenotype exhibited by Tsg101 mutant cells from knockout mice is an early cell cycle arrest (Ruland *et al.*, 2001; Wagner *et al.*, 2003). This early phenotype has not yet been reconciled with ESCRT-I function in MVB formation or virus budding, suggesting that this complex may mediate additional functions that remain unexplored.

As in mammalian cells, MVBs have been implicated in the regulation of several signaling pathways in *Drosophila* (Krämer, 2002; Seto *et al.*, 2002). For example, loss-of-function mutations in *Drosophila hrs* result in reduced EGF receptor degradation and increased mitogen-activated protein (MAP) kinase signaling (Lloyd *et al.*, 2002). To begin to explore the function of ESCRT proteins during development, we describe here a mutation in the *Drosophila* homolog of *vps28*. Our data indicate a requirement for dVps28 during spermatogenesis, the development of early embryos, and the compound eye. These defects were observed despite the continued presence of MVBs in mutant cells and revealed a surprising connection between the functions of Vps28 and the actin cytoskeleton.

MATERIALS AND METHODS

Expression Constructs and Transfections

Clones encompassing cDNAs for dVps28 (GH04443) or dTsg101 (GH09529) were identified in the collection of *Drosophila* expressed sequence tag sequences (Stapleton *et al.*, 2002). For expression in S2 cells, PCR-generated cDNAs encoding full-length dVps28 or dTsg101 were tagged with an N-terminal Myc or hemagglutinin (HA) epitope and expressed under control of the metallothionein promoter (Bunch *et al.*, 1988). For the generation of transgenic flies, cDNA GH04443 was cloned into the pUAST transformation vector (Brand and Perrimon, 1993).

Fly Genetics

The *hook*¹¹ null allele has been described previously (Krämer and Phistry, 1999). Other fly stocks were obtained from the Bloomington Stock Center (Bloomington, IN). The line with the P-element insertion l(2)k16503 carried an additional unidentified lethal mutation that was removed by recombination. The mutation l(2)k16503 was reverted to viability by introducing P-element transposase (Robertson *et al.*, 1988). Small clones homozygous for *dVps28*^{l(2)k16503} were generated from heterozygous flies by using the FLP recognition target (FRT)/FLP technique (Xu and Rubin, 1993). To assess the effect of *dVps28*^{l(2)k16503} on eye color, clones were generated in a *white*⁺ background in *FRT42D GMR-Hid l(2)CL-R1/FRT42D dVps28*^{l(2)k16503} flies, thus eliminating cells in the eye that were heterozygous or homozygous wild type with respect to *dVps28* (Stowers and Schwarz, 1999). To induce germline clones, *FRT42A dVps28*^{l(2)k16503}/CyO females were crossed to *Hs-FLP*; *FRT42A ovo*^D males (Chou and Perrimon, 1996), and the progeny were exposed to a 1-h 37°C heat shock in the larval stages.

Histology

Antibodies against dVps28 were generated in rabbits. The C-terminal 134 amino acids of dVps28 (amino acids 83–236) were fused to glutathione S-transferase (GST). This fusion protein was expressed and purified using standard procedures before injection into rabbits (Ausubel *et al.*, 1994). Eye discs were stained as described previously (Krämer *et al.*, 1991) by using antibodies against Boss (anti-Boss NN1 at 1:3000; Krämer *et al.*, 1991), dVps28 (1:5000), Delta (mAb202 at 1:50; Parks *et al.*, 1995), Sevenless (1:2000; Banerjee *et al.*, 1987), EGF receptor (1:500; Lesokhin *et al.*, 1999), dpERK (1:500; Sigma-Aldrich, St. Louis, MO), and MYC epitope (9E10; 1:500; Covance Research Products, Berkeley, CA), and Alexa-labeled secondary antibodies (Molecular Probes, Eugene, OR). Signals were detected using a Bio-Rad MRC1024 confocal microscope with a 63×, 1.3 numerical aperture (NA) lens.

Embryos or testes were stained with TO-PRO-3 iodide (0.3 μM; Molecular Probes), phalloidin-Alexa488 (5 U/ml; Molecular Probes), or antibodies against AX-D5 (1:50; Karr, 1991), nuclear fallout (Nuf) (1:500; Riggs *et al.*, 2003), Rab11 (1:2000; Dollar *et al.*, 2002), or Dah (1:25; Zhang *et al.*, 2000) as described previously (Theurkauf, 1994; Sullivan *et al.*, 2000). To visualize F-actin with phalloidin-Alexa488, formaldehyde-fixed embryos were hand devitelinized. Staining in embryos was detected with a Leica DRM microscope equipped with a Zeiss AxioCam charge-coupled device camera with a 20× 0.5 NA lens or a Leica TCS SP2 confocal microscope with a 20× 0.7 NA

or a 40× 1.2 NA 1.2. All digital images were adjusted for gain and contrast in Photoshop. To test for sperm motility, testes were dissected into Schneider's medium and directly inspected by phase contrast microscopy (Fabrizio *et al.*, 1998).

For electron microscopy, adult eyes were fixed, embedded in plastic, sectioned as described previously (Van Vactor *et al.*, 1991), and imaged with a JOEL 1200EX electron microscope. MVBs were recognized by their characteristic morphology, and their diameters were measured directly on the electron microscope.

Biochemistry

S2 cells were grown and transfected as described previously (Krämer and Phistry, 1996). Extracts were prepared and used for coimmunoprecipitation assays as described previously (Sevrioukov *et al.*, 1999). dVps28 and associated proteins were precipitated using anti-dVps28 antibodies at 1:200, separated by SDS-PAGE, and detected on Western blots by using anti-HA or anti-Myc antibodies (1:1000; Covance Research Products) or anti-dVps28 (1:2000) and enhanced chemiluminescence (Pierce Chemical, Rockford, IL).

For detection of dVps28 proteins in mutant embryos and first instars, a green fluorescent protein (GFP)-marked balancer (Casso *et al.*, 2000) was used to distinguish homozygous and heterozygous animals. Extracts of 100 embryos or 50 larvae were prepared in 100 μl of SDS loading buffer, separated by SDS-PAGE, and Western blots were probed for dVps28 (1:5000) or tubulin (anti-tubulin DM1A 1:2000; Sigma-Aldrich). Eye pigments were measured as described previously (Ooi *et al.*, 1997).

RESULTS

CG12770 encodes the *Drosophila* Ortholog of *Vps28p*

In the *Drosophila* genome, a single gene, CG12770, exhibits significant homology to the yeast and mammalian Vps28 proteins (Figure 1A). The cDNA GH04443 is derived from this locus and encodes a predicted protein of 210 amino acids that is 62 and 35% identical to its human (hVPS28) and yeast (ScVPS28) counterparts, respectively. There are no similarities to other protein sequence motifs in the database. An antibody raised against dVps28 recognizes a protein of the expected size that is widely expressed during *Drosophila* development and also in cultured *Drosophila* S2 cells (Figure 1B). In S2 cells (our unpublished data) as well as in cells of the eye disk and in isolated spermatocysts (see below), dVps28 protein was uniformly distributed throughout the cytosol with no obvious enrichment in any organelle.

To test whether the homology of Vps28 proteins extends to their biochemical activity, we investigated its binding to Vps23p/Tsg101 (Babst *et al.*, 2000; Bishop and Woodman, 2001). The *Drosophila* homolog dTsg101 is encoded by cDNA GH09529 (Stapleton *et al.*, 2002). Because antibodies are not yet available against endogenous dTsg101 protein, we coexpressed epitope-tagged versions of dTsg101 and dVps28 to test their interaction in S2 cells. Immunoprecipitation of dVps28 from S2 cells resulted in the coprecipitation of expressed HA-dTsg101 (Figure 1C, lane 5), which was increased after coexpression of Myc-dVps28 (lane 4). The interaction was specific because it was not observed with preimmune serum (lane 3), and the unrelated Hook protein was not pulled down (lane 2). These results indicated that, like its yeast and mammalian orthologs, dVps28 bound specifically to dTsg101.

A Mutation in the *dVps28* Gene

The dVps28 coding unit CG12770 is contained within a single exon in the fly genome (Adams *et al.*, 2000). The P-element-induced allele l(2)k16503 carries a transposon in the 5' untranslated region (UTR) of *dVps28* (Spradling *et al.*, 1999) and is homozygous lethal. Because excision of the P-element reverts lethality, we suspected that the lethality was due to the disruption of *dVps28* function. We confirmed this by expressing a dVps28 cDNA under control of an arm-Gal4 driver (Sanson *et al.*, 1996), which rescued the lethality of the l(2)k16503 mutation, either homozygous or

Figure 1. Vps28 is conserved in *Drosophila*. (A) Comparison between the *Drosophila* dVps28 protein (NP_652053) and its homologs from *Anopheles gambiae* (aVps28; XP_315357), *Homo sapiens* (hVps28; NP_057292), and *Saccharomyces cerevisiae* (Vps28p; NP_015260). (B) An antibody raised against a GST-dVps28 fusion protein recognizes a single protein of the expected size in S2 cells (lane 1). On expression of a Myc-tagged dVps28 a second band is seen of the size expected for the tagged protein (lane 2). Vps28 protein (arrow) is detected during all stages of *Drosophila* development (E, embryos; L1, L2, L3, first, second, and third instars; P, pupae; M, males; and F, females). The identity of the cross-reacting band at ~35 kDa (asterisk) is not known. In a parallel blot, tubulin served as a loading control. (C) Coimmunoprecipitation demonstrated binding of dVps28 to dTsg101. S2 cells were transfected with Myc-dVps28, HA-dTsg101, HA-Hook, or combinations as indicated. Expressed proteins were detected in the input samples consisting of S2 cells lysates with immunoblots (IB) by using anti-dVps28 (top) or anti-HA (middle). Top, samples that were transfected with Myc-dVps28 (lanes 2, 3, 4, and 6) were diluted fivefold before loading to generate comparable signals. After immunoprecipitation (IP) with anti-dVps28 antibodies (bottom) only HA-dTsg101 (lanes 4 and 5) but

Figure 2. A *dVps28* mutation in *Drosophila*. (A) The dVps28 protein is encoded in a single exon in the *Drosophila* genome. The lethal P-element l(2)K16503 (Spradling *et al.*, 1999) is inserted in the 5' UTR of *dVps28* generating the *dVps28*^{l(2)K16503} allele (abbreviated *dVps28* in this figure). (B) At 20 h AEL, the level of dVps28 protein is reduced in embryos homozygous for *dVps28*^{l(2)K16503}, compared with heterozygous (*dVps28*/+) and wild-type (Ore-R) controls. At 50 h AEL, dVps28 protein is no longer detected in homozygous larvae. Loss of dVps28 protein was indistinguishable between homozygous (*dVps28*/dVps28) and hemizygous *dVps28*^{l(2)K16503} mutants in trans to the deficiency Df(2L)CA53 (*Df*^{CA53}/dVps28). Stripped blots were probed with anti-tubulin antibodies to control for loading.

hemizygous over the deficiency Df(2R)CA53, which removes the *dVps28* gene (Tearle and Nüsslein-Volhard, 1987). We will therefore refer to the l(2)k16503 mutation as the *dVps28*^{l(2)k16503} allele.

In *dVps28*^{l(2)k16503} mutants, expression of the dVps28 protein is severely reduced. In *dVps28*^{l(2)k16503} embryos 20 h after egg laying (AEL), the level of dVps28 protein is reduced compared with wild-type embryos (Figure 2B), and dVps28 protein becomes undetectable by 50 h AEL. The rate of disappearance of the protein is indistinguishable between *dVps28*^{l(2)k16503} homozygous and hemizygous larvae, indicating that *dVps28*^{l(2)k16503} is a strong mutation eliminating most or all of dVps28 function. Unless otherwise indicated, the *dVps28*^{l(2)k16503} allele was used in all subsequent experiments that refer to a *dVps28* mutation.

dVps28 Is Required for Proper Eye Development

Because the cellular phenotypes of mutations affecting membrane trafficking can be readily characterized in the compound eye (Lloyd *et al.*, 1998; Stewart, 2002), we used vari-

not HA-Hook (lane 2) was detected by Western blotting by using anti-HA. Endogenous dVps28 was sufficient to pull-down a small amount of dTsg101 (lane 5), but no coimmunoprecipitation was detected when preimmune serum was used (lane 3).

Figure 3. *dVps28* is required for eye development. Micrographs of wild-type (A), *dVps28*^{l(2)K16503} (B), *or*^{49h2} (C), or *dVps28*^{l(2)K16503} *or*^{49h2} (D) adult eyes demonstrate changes in eye color. Eyes consisting almost entirely of *dVps28*^{l(2)K16503} or *dVps28*^{l(2)K16503} *or*^{49h2} mutant cells were generated in a *white*⁺ background by using the FLP/FRT system as modified by Stowers *et al.* (1999). This method eliminates heterozygous and homozygous *dVps28*^{+/+} cells. Micrographs of plastic sections through wild-type (E) and *dVps28*^{l(2)K16503} mutant (F) eyes reveal a variety of defects in *dVps28*^{l(2)K16503} ommatidia, including the loss of photoreceptor cells (arrowheads), the appearance of superfluous or misplaced pigment cells (red asterisk), large vacuolar structures (arrow), and the misalignment of ommatidia relative to each other (e.g., red double arrow). Bar, in E, 175 μ m (A–D) and 15 μ m (E and F).

ations of the FLP/FRT technique that eliminate most heterozygote or wild-type cells (Stowers and Schwarz, 1999) to generate compound eyes almost entirely composed of *dVps28* mutant cells (Figure 3). In such *dVps28* mutant eyes, we noticed a disorganization of ommatidia that is externally visible as roughness of the compound eye (Figure 3, B and F) and a darker eye color (Figure 3B) compared with wild-type (Figure 3A). This subtle alteration in eye color is characteristic of rough eyes because roughness changes the light-guide effects of ommatidia (Franceschini, 1972; Pichaud and Desplan, 2001). Consistent with the idea that the observed eye color change is a consequence of the rough eye phenotype, we found that levels of both types of pigments, ommochromes and drospterins, were not significantly different in *dVps28* and wild-type eyes (our unpublished data).

The *orange* gene (*or*) encodes one of the subunits of the adaptor protein (AP)-3 complex in *Drosophila* necessary for transport to pigment granules (Lloyd *et al.*, 1998; Mullins *et*

al., 2000). Because in yeast the AP-3 complex is specifically required for a pathway to the vacuole distinct from the *vps28*-dependent route (Cowles *et al.*, 1997; Katzmann *et al.*, 2001), we tested the genetic interactions between *or*^{49h2} and *dVps28*. The phenotypes of these mutants are distinct, because *or*^{49h2} eyes exhibit loss of pigmentation but no roughness (Figure 3C). In double mutants, these genes exhibited no interactions; the *or*^{49h2} eye color defect was not modified in the *or*^{49h2}, *dVps28* double-mutant eyes (Figure 3D). Similarly, roughness of *dVps28* mutant eyes was not significantly enhanced by loss of AP-3 function. These data suggest that the AP-3- and *Vps28*-dependent pathways transport different sets of cargo in *Drosophila*.

The external roughness of the *dVps28* compound eye reflected a variety of cellular defects as revealed by thin sections of plastic-embedded mutant compound eyes. Some ommatidia were missing photoreceptor cells (Figure 3F, arrowheads) and others contained extra cells (an extra pigment cell is indicated by the asterisk in Figure 3F). Still others contained a full complement of cells, yet they were not oriented correctly (compare the ommatidia indicated by the double arrow in Figure 3F). All of these phenotypes are consistent with misregulation of EGF receptor signaling (Wolff, 2003). It is important to note, however, that the majority of ommatidia had no visible defects, indicating that loss of *dVps28* function had only minor effects on cell-cell interactions mediated through EGF receptor or other cell surface molecules during cell specification in the developing eye disk.

Mammalian cells with reduced function of the hVps28p-binding protein Tsg101 exhibit a prolonged activation of the MAP kinase pathway after EGF-induced activation (Babst *et al.*, 2000). To directly test such a role of *dVps28*, we generated patches of *dVps28* mutant cells in eye imaginal discs and assayed the levels of phosphorylated ERK (Figure 4). In eye discs, phosphorylated ERK detected by anti-dpERK antibodies exhibits a dynamic pattern that serves as a sensitive readout of receptor tyrosine kinase signaling (Wasserman *et al.*, 2000; Spencer and Cagan, 2003). The comparison between wild-type and *dVps28* mutant cells revealed no significant differences in the level of dpERK staining (Figure 4D). Furthermore, no significant difference was observed in ERK phosphorylation after immunoprecipitation from wild-type or *dVps28* mutant larvae (our unpublished data). These results demonstrated that any changes in MAP kinase signaling suggested by developmental defects in the fly eye were too subtle to be detected by these methods.

To directly assess trafficking from the cell surface to lysosomes, we analyzed the distribution of two ligands, Delta and Boss, that have been used previously to visualize changes in endocytic trafficking in the fly eye (Krämer, 2002). Much of the Delta ligand detected in eye discs is present in endosomes (Parks *et al.*, 1995), and levels are sensitive to mutations that alter lysosomal delivery (Krämer and Phistery, 1999; Lai, 2002; Le Borgne and Schweisguth, 2003). We did not observe a difference between Delta staining in wild-type and *dVps28* mutant cells (Figure 4E).

The Boss ligand is expressed in the central R8 cells in developing ommatidia, binds to the Sevenless receptor, and is internalized into neighboring R7 cells (Krämer *et al.*, 1991). Thus, the level of Boss detected in R7 cells is sensitive to mutations such as *hook* and *dor* that alter trafficking from the cell surface to lysosomes (Figure 4, F–H; Krämer and Phistery, 1996; Sevrioukov *et al.*, 1999; Chang *et al.*, 2002). When patches of *dVps28* mutant cells were compared with *dVps28*⁺ cells, no difference could be detected in the level of Boss staining in R7 cells of otherwise wild-type (Figure 4I) or

Figure 4. Cells mutant for *dVps28* are not deficient in lysosomal delivery. Clones of *dVps28* mutant cells in eye discs (A–E, J) were detected by the absence of *dVps28* staining (A, green in C) or the Myc epitope tag (B–J, red in C). No difference is visible in ERK phosphorylation (D) or Delta staining (E) between heterozygous (+/–) and *dVps28* homozygous mutant cells (–/–). The Boss ligand is expressed on the R8 cell surface and, upon binding to Sevenless, internalized into R7 cells (F, inset). The level of detectable Boss protein in R7 cells is a sensitive measure of changes in lysosomal delivery as revealed by the comparison between wild-type ommatidia (F), *hook¹¹* mutant ommatidia (G), which exhibit enhanced lysosomal delivery (Sunio *et al.*, 1999) and *dor⁸* homozygous mutant cells (labeled *dor⁸/dor⁸* in H) in which lysosomal delivery is blocked and as a consequence Boss accumulates in endosomes (Sevrioukov *et al.*, 1999). White arrowheads in F and H point to Boss ligand internalized into wild-type R7 cells, and orange arrows (H) indicate the exaggerated staining for Boss in *dor* mutant R7 cells. (I) No change is detected in the level of Boss ligand on the surface of R8 cells or internalized into R7 cells (arrows) between the heterozygous (–/+) and *dVps28* mutant cells (–/–) in an otherwise wild-type eye disc (I). Because in *hk¹¹* mutant R7 cells Boss staining is reduced due to enhanced lysosomal delivery, we attempted to use it as a sensitized system to detect minor reductions in lysosomal delivery in *dVps28* mutant cells. However, no changes were noted in *dVps28* homozygous mutant cells (–/–) in a *hk¹¹* background (J). Bar, in J, is 75 μ m (A–C), 10 μ m (D, E, I, and J), and 6 μ m (F–H).

Figure 5. MVBs are present in *dVps28* mutant cells. Electron micrographs of MVBs in wild-type (A) and *dVps28^{l(2)K16503}* mutant photoreceptor cells (B). Bar, 500 nm (A and B). (C) The diameter of the limiting membranes of MVBs was measured in thin sections. From three different *dVps28^{l(2)K16503}* or wild-type eyes, 20–80 MVBs were measured per eye, and the diameters were found to be significantly different ($p = 0.01$). (D) The diameter of internal vesicles were determined in seven MVBs from wild-type ($n = 271$ vesicles) and mutant cells ($n = 281$ vesicles) each. Bars indicate the means, and error bars denote SEM.

hk¹¹ mutant eye discs (Figure 4J). Similarly, staining of eye discs with antibodies against the Sevenless receptor (Banerjee *et al.*, 1987) or the EGF receptor (Lesokhin *et al.*, 1999) did not reveal differences between wild-type and *dVps28* mutant cells (our unpublished data). Together, these experiments demonstrated that despite the developmental defects observed in *dVps28* mutant ommatidia, any effect on the lysosomal delivery of these ligands or receptors were too subtle to be detected by these methods.

MVBs Are Present in *dVps28* Cells

Because Vps28 is a subunit of the ESCRT-I complex implicated in sorting of proteins into MVBs, we examined the ultrastructure of *dVps28* mutant cells. We analyzed mutant cells in the retina by electron microscopy and compared them with cells in wild-type eyes. The most notable difference was the presence of large multivesicular structures in *dVps28* cells (Figure 5). We measured the diameter of these MVBs along the longest axis in ultrathin sections from three wild-type and three *dVps28* mutant eyes. The average diameter of wild-type MVBs was 395 ± 8 nm (mean $\pm$ SEM), whereas their size was significantly increased to 722 ± 36 nm in *dVps28* cells (Figure 5C). This 1.8-fold increase in average diameter corresponds to a more than fivefold increase in the volume of MVBs. Vacuoles of increased size have been observed in *hrs* mutant cells presumably due to a decrease in the formation of internal vesicles (Lloyd *et al.*,

2002). Importantly, in MVBs of *dVps28* mutant cells, internal vesicles were abundant (Figure 5B). Some of the internal vesicles looked larger than those in wild-type MVBs, but this was not a statistically significant difference; in wild-type cells, the diameter of internal vesicles averaged 67 ± 13 nm (45 ± 23 vesicles per MVB, $n = 7$ MVBs) compared with 69 ± 14 nm (40 ± 14 vesicles per MVB, $n = 7$ MVBs) in *dVps28* cells. The abnormal size of MVBs that we described is consistent with a function of *dVps28* in MVBs, but our data indicate that, in *Drosophila*, *dVps28* may not be strictly required for the biogenesis of MVBs.

dVps28 Is Required in the Female Germline

The lethal phase of *dVps28*^{l(2)k16503} homozygous or hemizygous *dVps28*^{l(2)k16503} mutants was during the first instar. Mutant embryos hatched, but most died as first instars (81%, $n = 80$). Some larvae had initiated (9%) or completed (9%) the formation of the second instar mouth hooks but died before molting. This developmental requirement is consistent with the increase in expression of *dVps28* from first to second instar (Figure 1B).

We reasoned that a maternal contribution might be sufficient to mask an embryonic requirement for *dVps28*. Using the FLP/FRT *ovo*^D system (Chou and Perrimon, 1996), we generated germline mosaics to eliminate the maternal *dVps28* contribution. Embryos produced from homozygous *dVps28* germ cells in which the maternal contribution was knocked out (mKO *dVps28*) exhibited major defects early in development (Figure 6). About one-half of these eggs (41–68% in different collections) were not fertilized as indicated by the absence of mitotic nuclear divisions 3 h AEL. We confirmed that arrested eggs were not fertilized by staining with the AX-D5 antibody (Karr, 1991) that recognizes a sperm-tail antigen (Figure 6D, inset). Note that the entire sperm tail enters the egg in *Drosophila* fertilization (Karr, 1991). The AX-D5 epitope was absent in all embryos that failed to undergo mitotic nuclear divisions.

Sperm entry and fertilization can be prevented by mutations that cause malformed micropyles, the openings through which *Drosophila* sperm enters an egg (Suzanne *et al.*, 2001). In *dVps28* eggs, we found that micropyles were not obviously malformed. In the majority of mKO *dVps28* mutant eggs (96% mKO *dVps28* mutant and wild-type embryos), the pore in the micropyle through which the sperm enters was visible by DIC imaging (Figure 6, A and B, insets), indicating that this was not the cause of the failure of these eggs to be fertilized.

The development of mKO *dVps28* embryos that did undergo nuclear divisions was blocked at various stages after fertilization but before complete cellularization. A frequently observed phenotype was an irregular distribution of nuclei in the embryo (compare Figure 6A to C and D). The subset of mutant embryos that initiated cellularization exhibited two additional phenotypes. First, pole cells were frequently absent; <3% of mKO *dVps28* embryos had the usual complement of cells in the characteristic position of pole cells (Figure 6, E and F). Similar phenotypes have previously been observed as a consequence of incomplete dumping of nurse cell content into the developing oocyte (e.g., in *armadillo* mutant germline; Cox *et al.*, 1996). We therefore examined *dVps28* germline clones but did not detect any dumping phenotypes (McKearin and Krämer, unpublished data). Nevertheless, due to the variability of mKO *dVps28* embryonic phenotypes, it is possible that some reflect earlier defects that originated during oogenesis.

Cells that formed during cellularization of mKO *dVps28* embryos displayed a droplet-like shape (Figure 6F) that

Figure 6. Maternally provided *dVps28* is necessary for early embryogenesis. Embryos lacking maternal *dVps28* protein (mKO *dVps28*) were generated using the FRT, *ovo*^D system (Chou and Perrimon, 1996) aged for 2–4 h, stained for DNA content (A–D), or visualized by DIC (E and F). A and E show wild-type embryos. B, C, D, and F show mKO *dVps28* embryos. The most frequent phenotype was a lack of fertilization (B), characterized by an arrest of cell divisions and the absence of the sperm-tail antigen AX-D5 (Karr, 1991). Sperm enters embryos through the micropyle, which seemed fully formed (compare insets in A and B). In a subset of embryos in which sperm tails were detectable (D, inset), migration of nuclei to the periphery was defective; nuclei clustered together (C) or were unevenly distributed (D). Presumably as a consequence of the failure of nuclear migration, pole cells, which are the first cells to form in wild-type embryos (E, arrow), were missing in most mKO *dVps28* embryos that initiated cellularization (F). In those embryos, cells were misshapen (compare cells marked by yellow arrowheads in E and F). Bar, in F, 100 μ m (A–D), 40 μ m (E), and 60 μ m (F).

differed from the cuboidal shape of wild-type cells (Figure 6E). This phenotype suggests a lack of membrane tension during the late phase of cellularization (Foe *et al.*, 1993). It is interesting to note that three phenotypes observed in embryos lacking *dVps28*, disorganized nuclear migration, lack of pole cells, and misshapen cells correspond to cellular processes that require the function of the actin cytoskeleton (Hatanaka and Okada, 1991; Riggs *et al.*, 2003). This points to a possible connection between *dVps28* function and the regulation of actin dynamics.

The Actin Cytoskeleton in Early Embryos Requires dVps28

To directly address the possibility that *dVps28* plays a role in actin dynamics, we visualized the F-actin distribution in mKO *dVps28* embryos. Actin accumulates in characteristic patterns in precellular blastoderm embryos (Figure 7 A and B, inset; Foe *et al.*, 1993). These patterns were aberrant in mKO *dVps28* embryos that developed to this stage. In many of these embryos, large regions were devoid of F-actin (Figure 7, B–E, arrowheads). These correlated with the appearance of prominent, enlarged actin bundles (Figure 7E, ar-

Figure 7. Defects in the actin cytoskeleton in mKO *dVps28* embryos. Confocal images of wild-type (A) and mKO *dVps28* embryos (B–F) stained for F-actin (green) and DNA content (red). In precellular blastoderm embryos actin undergoes stereotypic arrangements during mitotic cycles 10–13. Actin forms caps above the nuclei (A and F) and later, through mitosis (B, inset shows a wild-type embryo), accumulates at furrows between the nuclei. In mKO *dVps28* embryos, furrows that lack actin are abundant (B and C, arrowheads). Those gaps often are accompanied by irregular actin accumulations (D, star; E, arrowhead) and enlarged nuclei (E, stars; and F). Some uneven distribution of actin also is evident in the actin caps during interphase (F). Bar, 60 μ m (A and C), 40 μ m (B and D), 30 μ m (E), and 20 μ m (F).

rows). Even in embryos in which such gross abnormalities were not apparent, closer examination revealed an uneven distribution of actin (Figure 7F, arrowheads). We noticed that defects in the actin cytoskeleton often correlated with enlarged nuclei (Figure 7, E and F, asterisks) suggestive of failures in nuclear divisions. Such nuclei seem to be displaced toward the interior of the embryos (Figure 7F and 6, C and D). This is similar to other mutants in which nuclear

divisions fail with the resulting oversized nuclei either remaining at or retreating to the interior of the embryo (Sullivan *et al.*, 1993). Importantly, such a failure of nuclear divisions is not a prerequisite for the actin gaps that also were found in the presence of normal-looking interphase (Figure 7C) or metaphase nuclei (Figure 7B). These data therefore suggest that loss of *dVps28* function alters the actin cytoskeleton, and the defects in nuclear division, distribution, and axial migration are secondary defects.

Mutations in three genes have previously been demonstrated to have defects in the localization of actin to invaginating furrows, similar to our observations with mKO *dVps28* embryos. Defective actin hexagon (Dah) is a membrane-associated protein that localizes to invaginating furrows in syncytial blastoderm embryos and during cellularization. In embryos lacking maternal Dah protein, actin fails to properly localize to the furrow structures (Zhang *et al.*, 1996). Nuf and Rab11 are two additional proteins involved in this process (Rothwell *et al.*, 1998; Dollar *et al.*, 2002; Pelissier *et al.*, 2003). They are necessary for the delivery of vesicles from recycling endosomes to the invaginating furrows. In their absence, Dah is not recruited to furrows and as a consequence similar actin defects were observed as those induced by the lack of Dah protein itself (Riggs *et al.*, 2003).

Prompted by the similarity of these phenotypes to our observations, we investigated the distribution of these three proteins in mKO *dVps28* embryos. We found that in mKO *dVps28* embryos, Nuf and Rab11 accumulated around centrosomes apical to prophase nuclei during late cortical divisions, indistinguishable from wild-type embryos (Figure 8, A–D). By contrast, Dah distribution in mKO *dVps28* embryos is altered. Rather than being recruited to the invaginating furrows, as it is in wild-type embryos (Figure 8, E and G, an example is indicated by the arrow in the cross-sectional view in G), Dah remains distributed in small puncta and is found in aberrant accumulations within the mKO *dVps28* embryos (Figure 8H, arrows). These data indicate that *dVps28* is required for the normal localization of Dah to furrows by a mechanism that is independent of Rab11 and Nuf's localization to recycling endosomes.

dVps28 Is Required in the Male Germline

A connection between *dVps28* function and the actin cytoskeleton also was observed during spermatogenesis. Lethality of *dVps28* was rescued by expression of *dVPS28* under control of arm-gal4 driver (Sanson *et al.*, 1996), which provides widespread expression. Transgenic rescue restored viability and female fertility. Rescued males, however, lacked *dVps28* expression in the male germ line (Figure 9, A–D). In wild-type spermatocysts, *dVps28* is strongly expressed and found throughout the cytoplasm (Figure 9A). This staining was absent in the testis of *dVps28* males that were rescued by expression of *dVps28* under arm-Gal4 control. When such males were crossed to wild-type virgin females, 100% of the eggs were unfertilized as judged by AX-D5 staining (Karr, 1991), indicating that these males did not produce functional sperm.

The earliest stage of spermatogenesis visibly affected by the *dVps28* mutation was sperm individualization. Expression of *dVps28* was relatively low in the early stages of spermatogenesis in wild-type cells. Although we cannot rule out subtle earlier defects, spermatogenesis proceeded through these stages in *dVps28* mutant testis to yield a syncytium containing a bundle of spermatid nuclei indistinguishable from wild-type cells (Figure 9). Such syncytial nuclei are resolved into separate sperm during sperm individualization (Fabrizio *et al.*, 1998). This process encom-

Figure 8. Defects in Dah localization in mKO *dVps28* embryos. Confocal images of wild-type (A, C, E, and G) and mKO *dVps28* embryos (B, D, F, and H) stained for DNA (blue in merged images) and Rab11 (red in merged images), and Nuf (green in merged images A–D) or Dah (green in merged images E–H). Embryos are shown during prophase of late cortical divisions when Rab11 and Nuf are enriched around centrosomes (Riggs *et al.*, 2003). Pairs of colocalized Nuf and Rab11 flanked many nuclei in the surface views in A and B. Pericentriolar Nuf and Rab11 were located apical to the nuclei as shown in the cross sections (C and D). Localizations of Nuf and Rab11 were not altered in mKO *dVps28* embryos (B and D) compared with wild-type (A and C). During late cortical divisions, Dah accumulated at the furrows invaginating between the nuclei of wild-type embryos (E and G; Zhang *et al.*, 2000; Riggs *et al.*, 2003). The arrow in G points to one example. In mKO *dVps28* embryos (F and H), Dah failed to accumulate at the furrows. Instead, Dah is found in internal accumulations (H. arrows). Bar, 25 μ m in all images.

passes a dramatic reorganization of the plasma membrane driven by an actin-dependent synchronous movement of a structure called the cystic bulge (Rogat and Miller, 2002; Noguchi and Miller, 2003). Its progress along the spermatid tails can be visualized by fluorescently-labeled phalloidin. This staining highlights the characteristic actin cones that first assemble around the bundle of 64 spermatid nuclei before moving toward the distal end (Figure 9, E and F). In wild-type testis the moving actin cones were tightly synchronized (Figure 9G), whereas they seemed disorganized in *dVps28* mutant testis (Figure 9H). As a consequence, individual motile sperm were absent in the majority of dissected *dVps28* testes, and the remaining rare males had only a few motile sperm. Our observation that *dVps28* males were completely sterile indicates a strict requirement of *dVps28* in spermatogenesis.

DISCUSSION

The ESCRT-1 complex is necessary for the sorting of mono-ubiquitinated cargo into the internal vesicles of MVBs and the budding of HIV particles (Katzmann *et al.*, 2002; Raiborg *et al.*, 2003). Vps28's role in these processes has been addressed by several approaches. In yeast, loss of *vps28* function causes malformed prevacuolar endosomal compartments (Rieder *et al.*, 1996) and defects in protein sorting to the interior vesicles within MVBs (Katzmann *et al.*, 2001). Consistent with these findings, injection of anti-hVps28 antibodies into mammalian cells resulted in reduced lysosomal delivery of EGF receptors and accumulation of ubiquitinated cargo (Bishop *et al.*, 2002). Vps28p binds directly to other ESCRT proteins (Babst *et al.*, 2000; Bishop and Woodman, 2001; Martin-Serrano *et al.*, 2003; Bowers *et al.*, 2004), and the Vps28 binding site in Tsg101 is necessary for ESCRT-1 function (Martin-Serrano *et al.*, 2003). Together, these experiments point to a critical requirement for Vps28p in ESCRT-1 function.

Here, we describe a mutation in *dVps28*, which encodes one of the ESCRT-I subunits in *Drosophila*. Consistent with the complex's conserved function from yeast to mammalian cells (Babst *et al.*, 2000; Bishop and Woodman, 2001; Bishop *et al.*, 2002; Bache *et al.*, 2003; Katzmann *et al.*, 2003), loss of *dVps28* function caused morphological changes in MVBs and developmental defects in the compound eye that indicate a subtle misregulation of cell signaling molecules. However, for the ligands and receptors tested, we did not detect significant changes in their cell surface levels or their delivery to lysosomes in *dVps28* cells, indicating that any changes were too subtle to be detected by the immunofluorescence methods used.

One possible explanation for this observation is that some Vps28 functions are partially fulfilled by another protein. It is not likely that this hypothetical protein is similar to Vps28p in sequence because we did not identify another Vps28-like molecule in the completed genome sequences of *Drosophila melanogaster*. Another possibility is that the *dVps28*^{l(2)k16503} allele does not completely inactivate *dVps28* function. Although we cannot rule out this possibility, it is unlikely for two reasons. First, the *dVps28*^{l(2)k16503} allele is a strong mutation that causes lethality early in development at the transition from first to second instar, much earlier than a null mutation in the *hrs* gene, which regulates the sorting of receptors into MVBs (Lloyd *et al.*, 2002). Furthermore, the lethal phase and the loss of *dVps28* protein were indistinguishable between larvae homozygous for *dVps28*^{l(2)k16503} and those that were hemizygous over a deficiency of the

Figure 9. dVps28 is required late in spermatogenesis. Arm-Gal4 driven expression of a dVps28 cDNA restored viability and expression of dVps28 in somatic tissues but not in testis. Compared with wild-type (A and C), staining for dVps28 protein (green) was dramatically reduced in isolated spermatocysts (B) and whole-mount testis (D) derived from *dVps28^{l(2)k16503}* mutants. The insets in A and B show dVps28 staining for the cells indicated in the rectangle. Staining for dVps28 protein seemed distributed through the cytoplasm and not

region. This indicates that the *dVps28^{l(2)k16503}* allele removes most if not all of dVps28 function.

Another possibility for the relatively mild phenotypes is the perdurance of dVps28 protein. This is suggested by the dramatic phenotypes after removal of the maternal contribution in *mKO dVps28* embryos: many remained unfertilized and the remaining embryos were arrested in their development before reaching the cellular blastoderm. It is interesting to compare this phenotype to that of embryos lacking any maternal Hrs contribution. Hrs binds to mono-ubiquitinated membrane proteins, whose sorting into MVBs is thought to be mediated by Hrs binding to ESCRT-1 (Bishop *et al.*, 2002; Bache *et al.*, 2003; Katzmann *et al.*, 2003; Lu *et al.*, 2003). Consistent with this notion, *mKO hrs* embryos exhibited a reduced down-regulation of cell surface receptors and a resulting enhanced activation of the MAP kinase pathway (Lloyd *et al.*, 2002). Importantly, these defects were observed after cellularization has completed and during gastrulation. Our finding that *mKO dVps28* embryos exhibited major defects before completion of cellularization indicates functions of dVps28 in addition to the down-regulation of membrane proteins mediated by the interaction of ESCRT-I with Hrs.

Surprisingly, the earliest defect we found in fertilized eggs was an uneven distribution of nuclei. In *Drosophila*, the first 13 nuclear divisions occur without accompanying cell divisions. The first five of these syncytial divisions occur close to the center of the embryo, with nuclei subsequently spreading out along the anterior-posterior axis. This process, referred to as axial expansion, depends on the function of the actin cytoskeleton (Hatanaka and Okada, 1991; von Dassow and Schubiger, 1994; Wheatley *et al.*, 1995). In *mKO dVps28* embryos, this process often was disorganized resulting in an uneven distribution of nuclei.

Other *dVps28* phenotypes emerged during cellularization. After axial expansion, the nuclei move to the embryo's cortex where the last four of the syncytial nuclear divisions occur. The resulting nuclei are surrounded by invaginating membranes in a process that requires the actin-myosin network (Foe *et al.*, 1993; Royou *et al.*, 2004). At this stage, two defects were evident in embryos lacking dVps28. Many of the cells that formed were droplet shaped instead of the usual cuboidal shape. The actin cytoskeleton is required for normal cellularization and interfering with its function, by cytochalasin (Royou *et al.*, 2004, and references therein), or altering the activity of the Rho or Cdc42 GTPases (Crawford *et al.*, 1998), interferes with normal cellularization. Furthermore pole cells, usually the first cells to form, were absent in most embryos. The lack of pole cells has previously been

enriched in any particular compartment we could identify. The staining in the nucleus most likely represented cross-reactivity because it was not changed in the mutant testis. Spermatocysts are generated from stem cells at the tip of the testis (marked with asterisk) and subsequently undergo incomplete mitotic and meiotic divisions which generate a syncytium containing 64 spermatid nuclei. In *dVps28* mutant testis, spermatogenesis proceeded to the formation of bundles of syncytial nuclei (D, arrow; also see F). These nuclei are separated by an actin-myosin-driven process called individualization (Rogat and Miller, 2002). (E-H) Phalloidin labeling (green) revealed actin in the early cystic bulge as it formed around the spermatid nuclei (E and F). In wild-type testis, actin cones drive the cystic bulge in synchrony along the spermatid axonemes (G) toward the distal end of the spermatids (Noguchi and Miller, 2003). By contrast, the appearance of the actin cones was disorganized in mutant spermatids (H). Bar, 30 μ m (A and B), 100 μ m (C and D), and 15 μ m (E-H).

observed in embryos in which axial expansion is perturbed upon interference with the actin cytoskeleton (Hatanaka and Okada, 1991).

All of these early phenotypes are consistent with a role of dVps28 in directly or indirectly organizing the actin cytoskeleton. A role for endosomes in actin remodeling during cellularization has previously been established in embryos mutant for *nuf* or *rab11* (Riggs *et al.*, 2003). The small GTPase Rab11 localizes to recycling endosomes (Sonnichsen *et al.*, 2000; Dollar *et al.*, 2002), and Nuf is a homolog of Arfo2 that directly binds Rab11 and acts in recycling endosomes (Hickson *et al.*, 2003). Mutants eliminating either of these proteins cause gaps in which actin fails to be recruited to the furrows during cortical nuclear divisions, similar to our observations in mKO dVps28 embryos (Figure 7). In all three of these mutants, the actin defects may be a consequence of the failure to recruit Dah protein to invaginating furrows (Figure 8; Rothwell *et al.*, 1999; Riggs *et al.*, 2003). Dah has significant similarity to dystrobrevin and dystrophin (Zhang *et al.*, 1996). Dystrophin plays a critical role in anchoring the actin cytoskeleton to membranes (Michalak and Opas, 1997), and consistent with a similar function for Dah, embryos lacking maternally contributed Dah fail to properly assemble the actin cytoskeleton at furrows (Zhang *et al.*, 1996).

A subset of the defects in *rab11* mutant embryos has been linked to a requirement for trafficking through the recycling endosome during cellularization (Pelissier *et al.*, 2003). Consistent with this notion, defects in *rab11* and *nuf* embryos only become apparent during cortical divisions (Rothwell *et al.*, 1998; Riggs *et al.*, 2003). In mKO dVps28 embryos, by contrast, defects were detected in the distribution of nuclei, long before cellularization initiates (Figure 6). This indicates a function of dVps28 in actin remodeling independent of recycling to the cell surface and independent of Rab11 and Nuf. This is consistent with our finding that in mKO dVps28 embryos, recycling endosomes are not affected, as judged by Rab11 and Nuf localization (Figure 8), suggesting that dVps28 is required for furrow localization of Dah downstream or in parallel to Rab11 and Nuf's function in recycling endosomes. Such a model is difficult to reconcile with the canonical function of Vps28 as an ESCRT-1 subunit involved in targeting proteins into the interior vesicles of late endosomes (Katzmann *et al.*, 2002; Raiborg *et al.*, 2003).

It is unlikely that Dah mediates all effects of dVps28 on the actin cytoskeleton. No defects are observed in embryos lacking Dah before cycle 10 (Zhang *et al.*, 1996), long after defects have become apparent in mKO dVps28 embryos (Figure 7). Furthermore, Dah is not required during spermatogenesis, another developmental context in which we observed effects of dVps28 on the organization of the actin cytoskeleton. Spermatogenesis in dVps28 mutant testis progresses until bundles of 64 syncytial spermatids are formed (Figure 8). These spermatids are separated by a process called individualization that requires complex membrane rearrangements (Fabrizio *et al.*, 1998). At the site of these rearrangements, syncytial membrane, cytoplasm, and vesicles accumulate in the cystic bulge (Noguchi and Miller, 2003). In wild-type testis, an actin-dependent process drives the cystic bulge away from the 64 spermatid nuclei toward the distal end. The loss of synchrony in the movement of the cystic bulge is the first detectable defect during spermatogenesis in dVps28 mutant testis (Figure 8). Because *dah* mutants have no phenotype in males (Zhang *et al.*, 1996), these results indicate an independent connection between dVps28 function and the actin cytoskeleton.

Actin acts at many stages in the endocytic pathway (Engqvist-Goldstein and Drubin, 2003). In yeast, the initial in-

ternalization step requires actin polymerization (Kubler and Riezman, 1993; Wendland *et al.*, 1998; Kaksonen *et al.*, 2003), and in mammalian cells, early endosomes move on the tip of actin tails (Merrifield *et al.*, 1999). Additionally, late endocytic organelles require the actin cytoskeleton for fusion in yeast (Eitzen *et al.*, 2002) and mammalian cells (Kjeken *et al.*, 2004). Furthermore, a screen of the yeast genome for mutations interfering with protein sorting to the vacuole identified several regulators of the actin cytoskeleton (Bonangelino *et al.*, 2002).

Importantly, several of these mutants identified on the basis of a vacuolar sorting phenotype also exhibited defects in the organization of the actin cytoskeleton. For example, aberrant actin patches and a reduction of actin cables were observed in yeast lacking Vps36p (Bonangelino *et al.*, 2002), one of the subunits of the ESCRT-II complex (Babst *et al.*, 2002b). It will be interesting to see whether a similar functional connection between the actin cytoskeleton and the ESCRT-I complex may underlie the enigmatic phenotypes of Tsg101 mutations in mice that cause cell cycle arrest and early embryonic lethality (Ruland *et al.*, 2001; Krempler *et al.*, 2002).

ACKNOWLEDGMENTS

We are grateful to Dr. Tim Megraw for advice with sperm motility assays, Dr. Dennis McKeearin for help in evaluating germline clones in ovaries, and Dr. Mohammed Ali Akbar for help with the coimmunoprecipitations. We thank Drs. Ellen Lumpkin, Dennis McKeearin, Tim Megraw, and Adam Haberman for valuable comments on the manuscript. We thank Drs. Nick Baker, Robert Cohen, Tao-shih Hsieh, Bill Sullivan, Larry Zipursky, Bloomington Stock Center, and the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) for fly stocks and reagents. This work was supported by grants to H. K. from The Welch Foundation (I-1300) and the National Institutes of Health (EY-10199 and NS-43406).

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