Introduction

Germline cells of many species ranging from insects to mammals develop as syncytia (Matova and Cooley, 2001). This is also the case in *Drosophila melanogaster* oogenesis, in which germline cells stay connected by intercellular bridges, so-called ring canals. In the germarium of the *Drosophila* ovary, a germline stem cell divides asymmetrically to produce a daughter stem cell and a daughter cystoblast. The cystoblast undergoes four rounds of mitosis with incomplete cytokinesis, generating a 16-cell cyst with 15 nurse cells and one oocyte (Ong and Tan, 2010). The polyploid nurse cells contribute mRNAs, proteins, and organelles to the oocyte. Actin filaments are recruited to the ring canals together with several actin-binding proteins and proteins phosphorylated at tyrosine residues (phosphotyrosine [PY]). Hu-li tai shao–ring canal (Hts-RC) and Filamin also localize to the ring canals in a strictly controlled manner. Later, ring canals are further stabilized by the actin-bundling protein Kelch (Kel; Xue and Cooley, 1993; Kelso et al., 2002). Kel interacts with the ubiquitin E3 ligase Cullin-3, and both are essential for the growth of ring canals (Hudson and Cooley, 2010).

During the growth and expansion of the ring canals, proteins involved in ring canal assembly and maturation are subject to rapid turnover. One way to increase the turnover of proteins is by their degradation by the ubiquitin–proteasome system. Ubiquitinated proteins can be directly recognized by subunits of the 26S proteasome or by so-called ubiquitin receptors, which shuttle the ubiquitinated proteins to the proteasome. Ubiquitin receptors bind to ubiquitin via their ubiquitin-associated (UBA) domain and to the proteasome via their ubiquitin-like (UBQ) domain (Schauber et al., 1998; Bertolaet et al., 2001; Grabbe and Dikic, 2009).

In this paper, we present a molecular and phenotypic analysis of the *Drosophila* ubiquitin receptor CG4420, which we have named Rings lost (Rngo) according to its mutant phenotype in oogenesis. Like Ddi1/Vsm1, its homologue in yeast (Liu and Xiao, 1997; Lustgarten and Gerst, 1999), Rngo possesses fusion of germline cells. Rngo appears to be a ubiquitin receptor that possesses a ubiquitin-like domain, a ubiquitin-associated domain, and a retroviral-like aspartate protease (RVP) domain. Rngo binds to ubiquitin and to the 26S proteasome and colocalizes with both in germline cells, and its RVP domain is required for dimerization of Rngo and for its function in vivo. Our results thus show, for the first time, a function for a ubiquitin receptor in *Drosophila* development.
an N-terminal UBQ and a C-terminal UBA domain, which flank a central retroviral-like aspartate protease (RVP) domain (Krylov and Koonin, 2001). Ddi1/Vsm1 regulates the proteasomal degradation of a variety of proteins, including cell cycle regulators, homothallism endonuclease, and a member of the Skp–Cullin–F-box complex (Kaplun et al., 2005; Díaz-Martínez et al., 2006; Ivantsiv et al., 2006; Gabriely et al., 2008). In addition, Ddi1/Vsm1 controls SNARE-mediated membrane fusion events (Lustgarten and Gerst, 1999; Marash and Gerst, 2003). Ddi1/Vsm1 and its homologues are the only members of the ubiquitin receptor family that possess an RVP domain (Elsasser and Finley, 2005; Gabriely et al., 2008). The catalytic center of aspartate proteases is formed by two aspartate residues and an activated water molecule, which is needed for hydrolysis (Sirkis et al., 2006). Retroviral aspartate proteases only possess one catalytic aspartate, so these proteins have to form homodimers to build a catalytic center.

We show that Rngo forms homodimers and binds to ubiquitin and to the proteasome. rngo is an essential gene, and the loss of rngo function in female germline cells affects the growth of ring canals and leads to cell fusion. Both the lethality and the oogenesis defects of rngo mutant animals can be fully rescued by transgenes encoding full-length (FL) Rngo-GFP and versions of Rngo lacking either the UBQ or the UBA domain. In contrast, the catalytic function of the RVP domain is essential for the function of Rngo. To our knowledge, this study is the first to demonstrate a specific function for a ubiquitin receptor in Drosophila development.

**Results and discussion**

**CG4420 is the Drosophila homologue of yeast Ddi1/Vsm1 and vertebrate Ddi1/Ddi2**

The Drosophila genome contains a single homologue of Ddi1/Vsm1 encoded by the CG4420 transcription unit on the X chromosome (Fig. 1 a). Because of its mutant phenotype (see following paragraphs), we named this gene *rngo lost. rngo* encodes an acidic protein (isoelectric point = 4.78) of 458 amino acids and a calculated molecular mass of 50,500 D. Rngo possesses an N-terminal UBQ, a central RVP, and a C-terminal UBA domain (Fig. 1 b). An alignment of the RVP domains of several eukaryotic Ddi1/Vsm1 homologues with the sequence of Rngo suggested that aspartate 257 most likely is the catalytic center.

**Rngo is a ubiquitously expressed nuclear and cytoplasmic protein that colocalizes with ubiquitin and the proteasome in the female germline**

To study the expression pattern and subcellular localization of Rngo, we raised an antibody against an FL GST-tagged Rngo protein. Rngo was ubiquitously expressed during embryogenesis and in larval and adult tissues (Fig. S2 f and not depicted). Because germline clones of the *rngo*<sup>GE3956</sup> null allele (see following paragraphs) showed severe defects during oogenesis, we focused our analysis on the subcellular localization of Rngo in ovaries. Rngo was ubiquitously expressed both in the somatic follicle cells and in the germline at all stages of development (Fig. 2, a–d; and Fig. S1). Rngo was uniformly localized in the cytoplasm, whereas in the nucleus, it was enriched in subnuclear structures. This was particularly obvious in the polyploid nurse cell nuclei and in the oocyte nucleus (Fig. 2, a and b). Because Rngo is a predicted proteasomal shuttle factor, we tested whether Rngo colocalized with ubiquitin and with the proteasome by performing double stainings against Rngo and ubiquitin (Fig. 2 c) and the proteasome (Fig. 2 d). These stainings showed that Rngo colocalized with ubiquitin and with the proteasome in female germline cells.

The specificity of the anti-Rngo antibody was tested in stainings of ovaries containing germline clones of the *rngo*<sup>GE3956</sup> allele (see following paragraphs; Fig. 2 e). The specificity of the anti-Rngo antibody was also tested in Western blots using protein extracts of *rngo*<sup>GE3956</sup> mutant L3 larvae in comparison with L3 larvae possessing the wild-type *rngo* allele (Fig. 2 f). In both stainings and Western blots, the signal obtained with the anti-Rngo antibody was completely abolished in tissues or protein extracts of *rngo*<sup>GE3956</sup> mutant animals, demonstrating that this antibody is highly specific for Rngo.

**Rngo binds to ubiquitin and to Rpn10, a regulatory subunit of the 26S proteasome**

Next, we asked whether Rngo physically associates with ubiquitin and with the proteasome. To test the binding to ubiquitin, we generated a series of FL and partially deleted Rngo fusion proteins N-terminally tagged with the maltose-binding protein (MBP) for expression in *Escherichia coli* (Fig. 3 a). The Rngo fusion proteins were incubated with agarose beads covalently attached to ubiquitin. Only variants of Rngo that possessed the UBA domain bound to ubiquitin (Fig. 3 b) as expected.

Because Ddi1/Vsm1 binds to Rpn10, a regulatory subunit of the 26S proteasome (Saecki et al., 2002), we tested whether Rngo can also bind to Rpn10. To that aim, we incubated GST–FL Rngo together with MBP–Rpn10 and performed a pull-down with glutathione agarose beads. We detected MBP–Rpn10 in the precipitate with GST–FL Rngo but not in the control (Fig. 3 c), demonstrating that Rngo forms a complex with Rpn10 in vitro. The binding of Rngo to ubiquitin and to Rpn10 was also confirmed in vivo by coimmunoprecipitation (IP; co-IP; Fig. 3 d).

**Rngo homodimerization is mediated by the RVP domain and does not require the catalytic residue D257**

It was previously found that proteins containing an RVP domain can form homodimers to establish a catalytic center around two conserved aspartate residues (Miller et al., 1989). This is also true for Ddi1/Vsm1 (Sirkis et al., 2006; Gabriely et al., 2008). Thus, we used a series of Rngo constructs (Fig. 3 a) to determine the structural requirements for homodimerization by co-IP. We coexpressed FL Rngo tagged at the C terminus with GFP and either FL Rngo or one of the mutated versions, Rngo<sup>ΔUBA</sup>, Rngo<sup>ΔUBQ</sup>, Rngo<sup>ΔRVP</sup>, or Rngo<sup>Δ257A</sup>, tagged at the C terminus with the HA epitope in S2 cells and performed co-IP with an anti-GFP antibody. We found that only Rngo<sup>ΔRVP</sup>, which lacks the RVP domain, is unable to bind FL Rngo-GFP.
Rngo is required for proper growth of ring canals in germline cells

To investigate the requirement for rngo during female germline development, we generated germline clones of the mutant rngoGE3956 allele using the Flipase–Flipase recombination target technique (Harrison and Perrimon, 1993). Although control clones showed the wild-type staining pattern of Rngo, clones mutant for rngoGE3956 completely lacked Rngo staining in the germline (Fig. 2 e and Fig. S1). We noted that egg chambers with rngoGE3956 mutant germline cells did not develop beyond stages 8 and 9 of oogenesis because of degeneration and apoptosis of germline cells (Fig. S1 i). Closer analysis of the development of mutant egg chambers revealed severe abnormalities with respect to the localization of ring canal–associated proteins.
The subcellular localization of Rngo during oogenesis. (a) Rngo (red) is expressed in somatic cells and germline cells of egg chambers. It localizes to the cytoplasm and to the nuclei. The strongest staining can be seen in the nucleus of the oocyte (arrows), marked by the expression of Orb (green). (b–d) Costaining of Rngo with lamin (b, green), ubiquitin (c, green), and with the proteasome (d, green). (e) Germline cells homozygously mutant for rngo^{GE3956} (asterisk) completely lack Rngo staining. Clones are marked by loss of GFP expression (green). (f) L3 larval protein extracts were analyzed by Western blotting. In larvae possessing the wild-type rngo gene (right lanes), a band of 60 kD corresponding to FL Rngo is detectable, which is absent in the extract of rngo^{GE3956} mutant animals (left lane). α-Tubulin was used as a loading control.

In a–e, DNA was stained with DAPI (turquoise, only shown in the merged images). Egg chambers are at stages 6 and 7. Anterior is to the left. fc, follicle cell; nc, nurse cell; oc, oocyte. Bars: (a–d) 10 µm; (e) 20 µm.

and the shape of the germline cells. In wild-type egg chambers, Hts-RC, Kel, Cheerio (Cher; Drosophila Filamin), and PY epitopes exclusively localized to the ring canals in the germline (Fig. 4, a, d, and f; Video 1; and Video 3). In contrast, Hts-RC, Cher, and PY epitopes spread over the whole plasma membrane of the germline cells in rngo^{GE3956} mutant germline clones (Fig. 4, b, c, e, and g; Video 2; and Video 3). Kel still localized to remnants of ring canals and to the plasma membrane of germline cells but was also found in the cytoplasm (Fig. 4 e). The defects in ring canal morphogenesis ranged from an almost complete
larger than their wild-type counterparts and continued to grow until stage 8 (Fig. 4 i). To our knowledge, rngo is the first mutation that differentially affects the growth of ring canals between nurse cells and between nurse cells and the oocyte. Ring canal defects and the mislocalization of ring canal–associated proteins were already detectable at early stages of oogenesis (Fig. S1), demonstrating that Rngo function is required throughout oogenesis.

Figure 3. Rngo binds to ubiquitin and to the proteasome and forms homodimers. (a) A schematic diagram of Rngo fusion proteins used in this study. Numbers corresponding to amino acid residues indicate domain boundaries and the extent of the introduced deletions. (b) The UBA domain of Rngo is required for binding to ubiquitin. Ubiquitin-conjugated agarose beads were used to pull down the MBP-Rngo fusion proteins indicated above each lane. MBP was used as a negative control. (c) Direct binding of Rngo to the proteasomal subunit Rpn10. FL MBP-Rpn10 was pulled down by GST–FL Rngo but not by GST alone. (d) Rngo binds to ubiquitin (Ub) and Rpn10 in vivo. Extracts of wild-type ovaries (left) and ovaries expressing Rngo-GFP (right) were used for co-IP experiments with the indicated antibodies. The anti-MBP antibody was used as a negative control. (e) Rngo forms homodimers via its RVP domain. S2 cells were cotransfected with FL Rngo-GFP and either FL or mutated versions of Rngo tagged with the HA epitope. Cell extracts were used for IP with an anti-GFP antibody. IP with an anti-GST antibody was used as a negative control. Untransfected cells and cells transfected only with the respective HA-tagged version of Rngo were used as additional negative controls. White lines indicate that intervening lanes have been spliced out.

loss of ring canals (Fig. 4 c) to the formation of small, rudimentary ring canals connecting two nurse cells (Fig. 4, e and g) to the formation of unusually large ring canals connecting a nurse cell with the oocyte (Fig. 4 b). The quantification of these defects revealed that the growth of ring canals connecting two nurse cells was arrested at stage 5 in rngoGE3956 germline clones (Fig. 4 h), whereas ring canals connecting a nurse cell with the oocyte were always larger than their wild-type counterparts and continued to grow until stage 8 (Fig. 4 i). To our knowledge, rngo is the first mutation that differentially affects the growth of ring canals between nurse cells and between nurse cells and the oocyte. Ring canal defects and the mislocalization of ring canal–associated proteins were already detectable at early stages of oogenesis (Fig. S1), demonstrating that Rngo function is required throughout oogenesis.
Figure 4. \textit{Rngo} is required for ring canal growth and plasma membrane integrity in the female germline. (a–c) A control germline clone (glc, a) and \textit{rngoGE3956} germline clones (b and c) stained for F-actin (red) and Hts-RC (blue). Note the abnormally large ring canal (b, arrows) and the fusion of at least two nurse cells with the oocyte (c). Insets to the right of a and c show higher magnifications of the oocyte nucleus (red arrows) and an adjacent nurse cell. (d–g) Filamin (red) and Kelch (blue) localization in control (d and f) and \textit{rngoGE3956} germline clones (e and g). (h) Graph showing the number of ring canals connecting two nurse cells in control (blue) and \textit{rngoGE3956} (red) germline clones. (i) Graph showing the number of ring canals connecting a nurse cell with the oocyte in control (blue) and \textit{rngoGE3956} (red) germline clones. (j) Graph showing the number of nurse cell nuclei in the oocyte in control (blue), \textit{rngoGE3956} (red), two (green), and three (purple) nurse cell nuclei.
We wondered whether the mislocalization of ring canal–associated proteins in rngoGE3956 germline clones was accompanied by a change in their expression levels. To address this question, we performed Western blotting of ovaries dissected from wild-type females and from females with egg chambers carrying rngoGE3956 germline clones. Concomitant with a reduction in Rngo protein level, the levels of Hts–RC, Kel, Cher, and PY epitopes were significantly reduced, in contrast to the tubulin control (Fig. 5 a). This finding was confirmed by confocal analysis of control and rngoGE3956 germline clone egg chambers that were positioned next to each other in the same ovariole. Although the levels of Hts–RC, Kel, Cher, and PY epitopes were similar in adjacent wild-type and control germline clone egg chambers (Fig. 5, b, d, and f), they were strongly reduced in rngoGE3956 germline clone egg chambers (Fig. 5, c, e, and g).

In addition to defects in ring canal morphogenesis, we found that the oocyte nucleus frequently was misshapen in rngoGE3956 mutant germline clones. Instead of being round and compact as in wild type (Fig. 4 a, inset), the nucleus in rngoGE3956 mutant oocytes was enlarged and elongated (Fig. 4 c, inset), pointing to abnormal packing of the chromatin. This phenotype was fully penetrant after stage 7 of oogenesis.

Compared with wild-type controls, rngoGE3956 mutant egg chambers showed an abnormal organization of cortical F-actin. Although wild-type egg chambers always contained 15 nurse cells and one oocyte that were separated from each other by plasma membranes with an associated cortical actin filament network, egg chambers with rngoGE3956 germline clones frequently had <16 germline cells based on the counting of cell outlines (Fig. 4 c), whereas the number of nuclei was unaffected. Consequently, germline cells with more than one nucleus were frequently observed in rngoGE3956 germline clones. This was particularly obvious for the oocyte, which in addition to the oocyte nucleus, always contained one or more nurse cell nuclei at later stages of oogenesis (Fig. 4, c and j). This phenotype most likely results from the fusion of germline cells, which may be caused by decreased stability of the cortical actin cytoskeleton. Loss of rngo function in the follicle epithelium did not cause any obvious abnormalities in actin organization (Fig. 4 e), pointing to a germline-specific function of rngo in oogenesis.

The proteolytic activity of the RVP domain is essential for the function of Rngo

To test which domains of Rngo are required for its function in vivo, we performed rescue experiments using the upstream activating sequence (UAS)–GAL4 system (Brand and Perrimon, 1993). To that aim, we ubiquitously expressed FL Rngo-GFP and different GFP-tagged mutant versions of Rngo in animals mutant for rngoGE3956. Although RngoΔUBQ-GFP and RngoΔUBA-GFP (Fig. 3 a) rescued both the lethality and the oogenesis defects of rngoGE3956 mutant animals to the same extent as FL Rngo-GFP (Fig. S2, a–d), neither RngoΔRVP-GFP (Fig. 3 a) nor RngoD257A-GFP, a point mutation that is predicted to destroy the catalytic activity of the RVP domain, showed any rescue. These results demonstrate that binding to ubiquitin and to the proteasome and its ability to form homodimers (Bertolaet et al., 2001; Sirkis et al., 2006; Gabriely et al., 2008). In contrast to Ddl1/Vsm1, which is dispensable for the growth and survival of yeast (Lustgarten and Gerst, 1999; Clarke et al., 2001), rngo is an essential gene and is required for proper development of female germline cells. The defects observed in rngo mutants were less severe than those of hts mutations but were stronger than mutations in kel and cher (Yue and Spradling, 1992; Robinson et al., 1994; Li et al., 1999; Sokol and Cooley, 1999; Hudson and Cooley, 2010). Egg chambers with rngo germline clones showed mislocalization of Hts–RC, Cher, Kel, and PY epitopes and, unlike the aforementioned mutations, never developed beyond stage 9 of oogenesis and showed fusion of germline cells. These phenotypic differences between rngo and other genes involved in ring canal morphogenesis point to additional functions of Rngo, possibly in membrane trafficking.

How could these phenotypes relate to the biochemical functions assigned to Rngo? The predicted function of Rngo as a ubiquitin receptor implies that it binds to ubiquitinated proteins and either shuttles them to the proteasome or prevents their proteasomal degradation by interfering with the recognition of the ubiquitin modification by a proteasomal ubiquitin receptor. This may be important for the proper assembly of ring canals because their growth requires the dynamic remodeling of the actin cytoskeleton and a high turnover of actin-binding proteins. Evidence supporting this hypothesis comes from a recent study that demonstrated a function for Kel as a component of an E3 ubiquitin ligase complex together with Cullin-3 (Hudson and Cooley, 2010). This study proposed that Kel is required as an adapter protein to ubiquitinate various ring canal–associated proteins, which need to be degraded to disassemble the actin network in the boxed regions. Note the elongated shape of the oocyte nucleus in c. (d and e) A control germline clone (d) and rngoGE3956 germline clone (e) stained for Filamin (red) and Kel (blue). Note the rudimentary ring canals (arrowheads) and the diffuse cytosolic localization of Kel in e. (f and g) A control germline clone (f) and rngoGE3956 germline clone (g) stained for PY epitopes (blue). Rudimentary ring canals in g are marked by arrowheads. DNA was stained with DAPI (turquoise). Germline clones are marked by an absence of GFP (green) expression. Note that for imaging of Hts–RC, Kel, Filamin, and PY in rngoGE3956 germline clones, a much higher gain of the photomultiplier was used than in the respective wild-type controls. Egg chambers are at stages 7 and 8. Anterior is to the left. (h and i) Quantification of outer ring canal diameter of ring canals connecting nurse cells (h) and a nurse cell with the oocyte (i). (j) Quantification of the fusion between nurse cells and the oocyte. In h, n = 40 for stages 3–7, and n = 19 for stage 8. In i, n = 16 for stages 3–7, and n = 6 for stage 8. The statistical significance of the differences was calculated with the Student’s t test; ***, P < 0.01. In j, n = 15 for all stages. Error bars represent SDs from three independent experiments. Bars: 20 µm; [insets] 10 µm.
Materials and methods

Fly stocks and genetics

The following stocks were used in this study: P[EP]CG4420^{Ge3956}/FM7, renamed rngo^{Ge3956} in this study [GenExel], y^arm^w^*/FM7c, ovo[D2] P[FRT(w^*)]9-2/Y; P{hsFLP} (stock no. 1,843), p{ftz/lacZ}YH1 (stock no. 616), P{FRT(w^*)}9-2; P{hsFLP} (stock no. 1,843), daughterless-GAL4 (stock no. 5,460), and y^w^N^/FM7c, P[GAL4-twi.G]108.4, P(UAS-2xEGFP)AX (stock no. 6,873; Bloomington Drosophila stock center). Expression of UASP-Rngo-GFP, UASP-RngoUBA-GFP, UASP-RngoUBQ-GFP, and UASP-RngoDVP-GFP in transgenic flies was performed with the UAS-GAL4 system [Brand and Perrimon, 1993]. Germline clones and follicle cell clones for rngo^{Ge3956} were generated as previously described using a heat shock promoter-driven flipase on the second chromosome (Chou and Perrimon, 1996). To obtain hemizygous rngo^{Ge3956} mutant L3 larvae, living embryos lacking GFP fluorescence derived from the FM7[twi::GFP] balancer chromosome were separated from their GFP-positive siblings under a fluorescent stereo microscope and raised in separate vials. To rescue the lethality and phenotype of hemi- or homozygous rngo^{Ge3956} mutant flies, rngo^{Ge3956}/FM7 females were

Figure 5. The levels of ring canal–associated proteins are reduced in rngo^{Ge3956} germline clones. (a) Western blot of extracts from wild-type ovaries (left) and ovaries with rngo^{Ge3956} germline clones (glc, right). α-Tubulin was used as a loading control. (b and c) A control germline clone lacking GFP shows the same level of Hts-RC (blue) as the adjacent wild-type egg chamber (b), whereas Hts-RC levels are strongly reduced in an rngo^{Ge3956} germline clone egg chamber (c). (d–g) The same is true for PY epitopes (d and e, blue), Filamin (f and g, red), and Kel (f and g, blue). Germline clones are marked by the absence of GFP (green). Images are projections of z stacks. Egg chambers are at stages 5–7. Anterior is to the left. Bars, 20 μm.
crossed to males that were homozygous for one of the UASP-Rngo-GFP constructs recombined with the daughTerless-GAL4 driver on the third chromosome and carried the FM7 balancer. Rescued hemizygous males carrying the rngo<sup>265Ee</sup> allele and the GFP construct were crossed to rngo<sup>265Ee</sup>/FM7 females also carrying the GFP construct to establish a viable and fertile stock homozygous for the mutant rngo<sup>265Ee</sup> allele.

**Antibodies and immunohistochemistry**

An antibody against Rngo was generated by immunizing a rabbit with a GST fusion protein containing the FL Rngo protein (E. coli). First bleed serum was affinity purified and used for immunohistochemistry. For immunohistochemical stainings of ovaries the following antibodies were used: rabbit anti-Rngo, affinity purified at 1:1,000, mouse anti-26S proteasome (Rpn10) at 1:400 (Santa Cruz Biotechnology, Inc.), mouse anti-PY at 1:1,000 (Sigma-Aldrich), rabbit anti-GFP at 1:1,000 (Invitrogen), mouse anti-antibiquitin at 1:6,000 (FK2, Enzo Life Sciences), rat anti–N-filamin at 1:3,000 (Sokol and Cooley, 1999), provided by L. Cooley, Yale University, New Haven, CT, mouse anti–Hts-RC at 1:20 (Hts-RC; Developmental Studies Hybridoma Bank), mouse anti-Kel at 1:1 (KelB; Developmental Studies Hybridoma Bank), and mouse anti-Orb at 1:20 (OrbE; Developmental Studies Hybridoma Bank). Secondary antibodies conjugated to Cy2, Cy3 (Jackson Immunoresearch Laboratories, Inc.), and Alexa Fluor 647 (Invitrogen) were used at 1:200. To visualize F-actin, ovaries were incubated with 2 µL rhodamine-conjugated phallloidin in PBS with 0.1% Triton X-100. DNA was stained with DAPI. Ovaries were fixed in 4% formaldehyde–PBS and stained according to standard procedures. Images were taken on a confocal microscope (Leica Meta; Carl Zeiss) using 25× Plan-Neofluor, NA 0.8, and 63× Plan-Apochromat, NA 1.40, objectives and processed using Photoshop (Adobe).

**Western blots and co-IP**

Lysates of I3 larvae, ovaries, and S2 cells were prepared in TNT buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 0.5% Triton X-100), added to 15 µl ubiquitin–agarose fusion proteins were diluted in 500 µl binding buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 0.5% Triton X-100), added to 1.5 µl ubiquitin–agarose (Enzo Life Sciences) and incubated for 2 h at 4°C. The beads were then washed with binding buffer and eluted by adding 15 µl of 2× SDS sample buffer and boiling for 10 min at 96°C. The supernatants were separated by SDS-PAGE and analyzed by Western blotting.

**Online supplemental material**

Fig. S1 is related to Fig. 2 and Fig. 4 and shows the requirement for Rngo already at the earliest stages of oogenesis and also the degeneration of rngo mutant egg chambers after stages 8 and 9 of oogenesis. Fig. S2 is related to Fig. 4 and shows stainings and Western blots of rngo mutant animals rescued by different Rngo-GFP transgenes. Video 1 is related to Fig. 4 and shows the 3D reconstruction of the wild-type staining pattern of Hts-RC in an egg chamber at stage 8. Video 2 is related to Fig. 4 and shows the 3D reconstruction of the staining pattern of Hts-RC in two adjacent egg chambers, one at stage 6 that is wild type for rngo and the other one at stage 8 that is mutant for rngo. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201009142/DC1.

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**References**


