Journal Pre-proofs

Central role of *squid* gene during oocyte development in the Hemiptera *Rhodnius prolixus*

Agustina Pascual, Catalina Taibo, Rolando Rivera-Pomar

PII: S0022-1910(24)00107-0
DOI: https://doi.org/10.1016/j.jinsphys.2024.104719
Reference: IP 104719

To appear in: *Journal of Insect Physiology*

Received Date: 6 August 2024
Revised Date: 30 October 2024
Accepted Date: 31 October 2024

Please cite this article as: Pascual, A., Taibo, C., Rivera-Pomar, R., Central role of *squid* gene during oocyte development in the Hemiptera *Rhodnius prolixus*, *Journal of Insect Physiology* (2024), doi: https://doi.org/10.1016/j.jinsphys.2024.104719

Central role of *squid* gene during oocyte development in the Hemiptera *Rhodnius prolixus*

Agustina Pascual[1,2]; CatalinaTaibo[3]; Rolando Rivera-Pomar[1,2,4,5*]

[1]Centro de BioInvestigaciones (CeBio-CICBA), Universidad Nacional del Noroeste de la Provincia de Buenos Aires (UNNOBA). Pergamino, Argentina.

[2]Centro de Investigaciones y Transferencias del Noroeste de la Provincia de Buenos Aires (CITNOBA-CONICET). Pergamino, Argentina.

[3]Laboratorio de Microscopia Integral (LIM), (CICVyA, INTA). Hurlingham, Argentina

[4]Centro Regional de Estudios Genómicos, Facultad de Ciencias Exactas, Universidad Nacional de la Plata. La Plata, Argentina.

[5]Max Planck Institute for Multidisciplinary Sciences, Dept. Molecular Developmental Biology. Göttingen, Germany.

***Correspondence:** rrivera3@mpinat.mpg.de

Abstract: Oocyte polarity establishment is a conserved and crucial phenomenon for embryonic development. It relies on the precise spatial localization of maternal factors deposited during oocyte development, which is essential for establishing and maintaining cell polarity and subsequently specifying embryonic axes. The heterogeneous nuclear ribonucleoprotein (hnRNP) encoded by the *squid* (*sqd*) gene has been implicated in mRNA localization and embryonic axis establishment in *Drosophila melanogaster*. Comparative genomics allowed for the identification of a homologue in *Rhodnius prolixus*. In this study, we investigated the function of *Rp-sqd* during oogenesis and early embryonic development. We observed persistent expression of *Rp-sqd* during oocyte development, with localization in the cytoplasm of ovary germarium and growing oocytes in previtellogenic and vitellogenic stages. A Parental RNA interference (RNAi) experiment targeting *Rp-sqd* resulted in female sterility. The ovaries showed disrupted oocyte development, disarray of follicular epithelium, and affected nurse cells integrity. Immunostaining and microscopic techniques revealed microtubule disarray and a reduction in the presence of organelles in the trophic cords that connect the germarium with the oocytes. The *Rp-sqd* depletion impacted the transcript expression of maternal mRNAs involved in apoptosis, axis formation, oogenesis, and cytoskeleton maintenance, indicating a pleiotropic function of *Rp-sqd* during oogenesis. This study provides new insights into the genetic basis of *R. prolixus* oogenesis, highlighting the crucial role of *Rp-sqd* in oocyte development, fertility, and germarium integrity. These findings contribute to our understanding of insect developmental processes, provide a foundation

for future investigations into reproduction, and reveal the regulatory mechanisms governing the process.

Keywords: oogenesis, embryogenesis, insect, RNAi, gene expression.

1 Introduction

The establishment of oocyte polarity relies on the precise spatial localization of maternal factors deposited during oocyte development, a phenomenon conserved across diverse taxonomic groups (Fitch et al., 1998; Goto and Kinoshita, 1999; Nusslein-Volhard, 1991; Simpson, 1983; Tadros and Lipshitz, 2009). In insects, the molecular mechanisms underlying oogenesis derive from studies in *Drosophila melanogaster,* which represents one of the most derived lineages of insects in terms of oogenesis and embryogenesis (Davis and Patel, 2002; Liu and Kaufman, 2005; Lynch et al., 2011; Lynch and Roth, 2011). Thus, an understanding of the genetic and cellular processes of oogenesis in other species leads to the unveiling of their molecular evolution.

Although the genetic network responsible for establishing the body axes, both anterior-posterior (A-P) and dorsal-ventral (D-V) polarity, of the future embryo has not been fully identified outside Diptera (Driever and Nusslein-Volhard, 1988; Neuman-Silberberg and Schupbach, 1993; Schroder, 2003), advances in high-throughput sequencing and comparative genomics have enabled the inference of the identity of maternal genes in other insects (Bucher et al., 2005; Lynch et al., 2006; Lynch and Desplan, 2010; Ozuak et al., 2014; Pultz et al., 2005). These represent a substantial portion of the protein-coding genome, ranging from 54 to 65% (Lecuyer et al., 2007; Pascual and Rivera-Pomar, 2022; Preuss et al., 2012; Tadros et al., 2003).

The expression of maternal genes is regulated at virtually every stage of development through different mechanisms, including transcriptional control, splicing, mRNA localization and decay, translation, protein maturation, modification, and degradation (Riechmann and Ephrussi, 2001; Teixeira and Lehmann, 2019). Among these, localization is crucial for the asymmetric distribution of maternal mRNAs, which is essential for the establishment and maintenance of cell polarity (Cooperstock and Lipshitz, 2001). This process leads to the spatial regulation of gene expression, which specifies the embryonic axes (Fitch et al., 1998; Goto and Kinoshita, 1999; Lasko, 2012; Nusslein-Volhard, 1991; Roth and Lynch, 2009; Simpson, 1983). Several mechanisms have been described for maternal mRNA localization, which involve the transport along cytoskeletal tracks (Lipshitz and Smibert, 2000; St Johnston, 1995).

Previous studies in *D. melanogaster* have shown that the heterogeneous nuclear ribonucleoprotein (hnRNP) encoded by the *squid* (*sqd*) gene mediates the localization, maturation and translation of nascent RNAs (Caceres and Nilson, 2009; Clouse et al., 2008; Piccolo et al., 2014). In *D. melanogaster*, which possesses polytrophic ovary, *sqd* plays a pivotal role throughout oogenesis, contributing to the establishment of both A-P and D-V polarity (Delanoue et al., 2007; Kelley, 1993; Steinhauer and Kalderon, 2005; Weil et al., 2012). During the early and mid-stages of oogenesis, *sqd* is essential for the cytoskeletal reorganization, which produces a polarized microtubule network. This network is critical for the proper transport of determinant mRNAs (Steinhauer and Kalderon, 2005). *sqd* is required not only for active mRNA transport but also for their anchoring at their destination and for maintaining the integrity of sponge bodies (Delanoue et al., 2007). At earlier stages, *sqd* is involved in the dispersal of nurse cell chromosomes following several cycles of endoreplication, transitioning these chromosomes from a polytene, blob-like conformation to a dispersed state (Finger et al., 2023; Goodrich et al., 2004).

As a member of the highly conserved family, a bona fide maternal *sqd* homolog has been identified in other insects, including *Tribolium castaneum* and *Rhodnius prolixus* (Pascual and Rivera-Pomar, 2022; Preuss et al., 2012). However, there is no evidence regarding its role.

Unlike *D. melanogaster*, *R. prolixus* exhibits telotrophic ovaries, with the germarium located at the anterior end of each ovariole. The germarium houses oocytes arrested in prophase I of meiosis, which are produced during the fifth instar nymph stage, along with the nurse cells (Huebner and Anderson, 1972a, c). Following a blood meal, oogenesis is activated, and the oocytes migrate in an orderly manner towards the posterior region, where they become surrounded by follicle cells (Huebner and Anderson, 1972b, c). The oocytes remain connected to the germarium via specialized tubular bridges, termed trophic cords, which mediate the transport of RNAs and nutrients from the nurse cells to the developing oocytes through a network of polarized microtubules (Harrison and Huebner, 1997; Valdimarsson and Huebner, 1989). Despite these, the genetic and molecular mechanisms that drive oogenesis in *R. prolixus* remain largely unclear

Our results show that *Rp-sqd* is a maternal gene with a crucial role during oocyte development. We identify the expression dynamics of *Rp-sqd* mRNA throughout the different stages of the oogenesis and perform parental RNAi experiments. Our results provide evidence that *Rp-sqd* acts at the germinal level for oocyte development, ovarian morphology and DNA integrity.

2 Materials and methods

2.1 Insect rearing

A colony of *R. prolixus* was maintained in our laboratory in a 12:12 hours light/dark period at 28 °C and 80% relative humidity in controlled environment incubators. Under these conditions, embryogenesis is complete in 14 ± 1 days. Insects were regularly fed using an artificial feeder and sterile defibrinated horse blood (Alfredo C. Gutiérrez Laboratory). The insects were housed, cared, fed and handled in accordance with resolution 1047/2005 (National Council of Scientific and Technical Research, CONICET) regarding the national reference ethical framework for biomedical research with laboratory, farm, and nature collected animals, in accordance with the international standard procedures of the Office for Laboratory Animal Welfare, Department of Health and Human Services, NIH and the recommendations established by the 2010/63/EU Directive of the European Parliament, related to the protection of animals used for scientific purposes. Biosecurity rules fulfill CONICET resolution 1619/2008, which is in accordance with the WHO Biosecurity Handbook (ISBN 92 4 354 6503).

2.2 cDNA synthesis and PCR

Total RNA was extracted from adult female ovaries using TRIzol reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized using the RevertAid Reverse Transcriptase (ThermoFisher) protocol and utilized as a template for polymerase chain reaction (PCR). Specific primers were designed for *Rp-sqd* (RPRC007924) using Primer3Plus (Untergasser et al., 2007) and PCR Primer Stats (Stothard, 2000) to amplify two independent templates (Table S1). PCR cycling was performed as follows: 2 minutes (min) at 94 °C, followed by 35 cycles of 92 °C for 30 seconds (s), 56 °C for 30 s and 72 °C for 45 s, and a final extension step of 4 min at 72 °C (Taq Pegasus, Productos Bio-

Lógicos, Argentina). The amplicons were analyzed by electrophoresis in a 1% agarose gel and sequenced to confirm their identity (Macrogen Inc.). Primers containing T7 promoter sequence (CGACTCACTATAGGG) at the 5′-end were designed for *in vitro* transcription of double-stranded RNA (dsRNA) or RNA probes.

2.3 *In situ* hybridization

DNA templates used to synthesize *in situ* hybridization probes were obtained by RT-PCR using oligonucleotides carrying T7 promoter sequence at the 5'-end. These templates, with the T7 promoter in either the sense (control) or antisense direction, were *in vitro* transcribed using the DIG RNA labeling kit (Roche) to produce the respective probes. *In situ* hybridization was detected with an alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche) antibody, as described in Pascual et al. (2021). Female ovaries (n = 8) were dissected in phosphate-buffered saline (PBS 1×) at days one and five post-blood feeding. The fixation was performed on ice in 4% paraformaldehyde (PFA) in PBT (PBS 1× + 0.1% Tween-20) for 30 min, then washed three times in PBT and stored at 4 °C until use.

2.4 Parental RNA interference

PCR product was used as template for a second PCR round to add the T7 promoter sequence at both ends. dsRNA was synthesized using the T7 RNA polymerase (ThermoFisher), following the manufacturer's instructions. The template was removed by RNase-free DNase digestion (Qiagen, Hilden, Germany). dsRNA integrity was assessed by agarose gel electrophoresis, and quantified using the Fiji package (Schindelin et al., 2012). A negative control was included using the *ß-lactamase* gene (dsRNA$^{\beta lac}$) amplified from a pRSETb plasmid (Lavore et al., 2012). dsRNA^{sqd} was quantified and injected into virgin females, at different concentrations (ranging from 1 to 3 µg/female), as previously described (Pascual et al., 2021). Forty-eight hours later, once the females had recovered from injection injury, they were fed to ensure proper blood intake, inducing oogenesis. Subsequently, mating was initiated. Oviposition (i.e., the number of eggs laid per female) was recorded every 48 h during three ovipositional periods of 16 days. Female ovaries were dissected at 5 days post-feeding during the vitellogenic stage, following the protocol described in Pascual et al. (2021).

2.5 Ovary manipulation and image acquisition.

Control (dsRNA$^{\beta lac}$) and depleted (dsRNA$^{sqd2\mu g}$) adult females, which had recently molted to the adult stage, were fed to induce oogenesis, and their ovaries were dissected in PBS 1×. Ovaries were fixed using different methods depending on the subsequent analysis, as described in Pascual et al. (2021). For histological sections, ovaries (n = 3 females per treatment) were fixed on ice in 4% paraformaldehyde (PFA) in PBT (PBS 1× + 0.1% Tween-20) for 30 min, washed with Millonig's buffer, dehydrated in graded series of ethanol and xylene (100%), and embedded in paraffin (Wanderley-Teixeira et al., 2006). Five-micrometer-thick sections were cut in a rotary microtome (Leica) and stained using standard hematoxylin-eosin procedure, mounted and photographed using an A1 Zeiss microscope. For transmission electron microscopy (TEM), ovaries (n = 2 females per treatment) were fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide in Millonig's buffer at pH 7.4. This was followed by dehydration in a graded series of ethanol and acetone (100%), after which the ovaries were infiltrated and embedded in epoxy resin (Durcupan ACM, Fluka AG, Switzerland). Ultrathin sections were examined

using a Zeiss EM109T (LANAIS-MIE). For fluorescence microscopy, ovaries (n = 15 females per treatment) were fixed on ice in 4% PFA for 30 min, followed by three washes in PBT. Nuclei were stained with Hoechst (Sigma-Aldrich, USA, 1 µg/ml) to determine cell distribution, while the lipophilic styryl dye FM 4-64FX (Thermo Fisher Scientific) was used to label cell membranes and nascent endosomes extending into the cytoplasm. Fluorescence and differential interference contrast (DIC) images were acquired using a Zeiss Axio Imager A2 fluorescence microscope.

For quantification of nurse cell nuclei, the Fiji/ImageJ package was used. The total number of cells was counted based on Hoechst (Sigma-Aldrich, USA, 1 µg/ml) nuclear staining, using a plugin included in ImageJ (Schindelin et al., 2012). The measurements were adjusted to a region of interest in order to analyze the germarium in the same zone for each experiment. A total of 11 samples were acquired using a wide-field microscope. Briefly, the image was initially converted to 16-bit, after which the threshold was adjusted. The binary option was then selected, followed by the watershed option to separate clustered nuclei into individual ones. Finally, the cell count results were obtained after performing particle analysis.

2.6 Immunohistochemistry

Immunofluorescence was performed as described by Pascual et al. (2021) to localized tubulin, the constituent protein of microtubules (Huebner and Anderson, 1972a). Ovaries (n = 6 females per treatment) were dissected and fixed on ice in 4% formaldehyde (PFA) for 30 min. Primary antibody mouse α-tubulin (Sigma-Aldrich, 1:200) was incubated in buffer PBST (PBS 1x + 0.1% Triton X-100) overnight at 4°C. Next, the samples were washed three times in PBST, incubated 2 h with the fluorescent secondary antibody Cyanine3 -conjugated anti-mouse IgG (diluted 1:200 in PBST; Invitrogen, Life Technologies), washed and counterstained with Hoechst (Sigma-Aldrich, USA, 1 µg/ml). Image acquisition was conducted with a ZEISS Axio Imager A2 fluorescence microscope and optical sections, when necessary, obtained using a Zeiss LSM800 confocal microscope.

2.7 Real-time quantitative PCR

Total RNA samples were extracted from ovaries dissected from control (dsRNA$^{\beta lac}$) and depleted (dsRNA$^{sqd2\mu g}$) adult females, at one and seven days post-blood feeding. Ovaries (n = 3 per biological replicate) were dissected in PBS 1x and immediately stored in TRIzol reagent (Invitrogen). Total RNA was isolated using TRIzol reagent (Invitrogen) and treated with DNase (QIAGEN, Germany). First-strand cDNA synthesis was performed using the oligo $(dT)_{18}$ primer (TransGen) and RevertAid Reverse Transcriptase (Thermo Scientific), following the manufacturer's protocol. RT-qPCR was carried out in technical triplicates (three wells per cDNA sample), in a 10 µl final volume reaction using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and a Bio-Rad CFX-96 thermocycler. PCR cycling consisted of 30 s at 95 °C, followed by 35 cycles of 15 s at 95 °C, 30 s at 55 °C, and 45 s at 72 °C (annealing and extension). Melting curve analysis was performed at the end by using instrument default settings (65–95 °C, 0.5 °C temperature increment). A no-template control was included in all batches. All primer pairs (Table Supplementary 1) were designed with Primer3Plus (Untergasser et al., 2007) and PCR Primer Stats (Stothard, 2000), and tested for dimerization, efficiency, and amplification of a single product. The relative differences in transcript levels were calculated considering the amplification efficiency of each primer pair using the Pfaffl

method (Pfaffl, 2001). Expression values were normalized using *ribosomal protein L13A* (*Rp-Rpl13A*) gene, a reference chosen after screening of several housekeeping-gene candidates, as it provided consistent results on the ovaries analyzed.

To perform the analysis of the dynamic of *Rp-sqd* transcript expression total RNA was extracted from ovaries dissected from control adult females at one (T1), five (T5), and seven (T7) different days post-blood feeding, as well as from ovaries dissected from non-fed females (T0). Each sample was grouped in pools of three organs (n =3 per biological replicate), placed in TRIzol reagent (Invitrogen), and kept frozen until use. First-strand cDNA synthesis and RT-qPCR reactions were performed as described previously.

2.8 Statistical Analysis

Statistical analyses were performed using GraphPad Prism v6.0 software (GraphPad Software, CA, USA, www.graphpad.com). For RT-qPCR analysis, Shapiro-Wilk and D'Agostino & Pearson normality tests were applied to assess the normality of the data set. One-way ANOVA followed by Bonferroni multiple comparison *post-hoc* test was performed to analyze group differences. Data that did not meet the assumptions of normality were subjected to a Kruskal–Wallis test followed by Dunn's multiple comparison test for pairwise comparison. A two-way ANOVA was applied for the number of eggs laid, with 'treatment', 'time', and 'interaction' as fixed effects. Predicted values were compared with a significance level of 5%. For nuclei quantification, a t-test was performed to assess significance, with a significance level of 5%.

3 Results

3.1 mRNA expression of *Rp-sqd* gene during oocyte development

Quantitative analysis of *Rp-sqd* mRNA expression across different stages of oocyte development showed an increase in the abundance of the *Rp-sqd* transcripts on days one (T1) and seven (T7), although these differences were not statistically significant among other stages (Fig 1A). Based on this expression profile, T1 and T7 were selected for *in situ* hybridization. In both stages, *Rp-sqd* transcripts were clearly detected in the germarium and developing oocytes. Also, transcripts with lower abundance were observed in the somatic follicle cells (Fig 1B-D). These results show that *Rp-sqd* expression occurs at stages one, five and seven, suggesting the presence of the transcripts throughout all stages of oocyte development, beyond germline tissues. These findings prompted the analysis of the potential role for *Rp-sqd* in the oogenesis process.

3.2 *Rp-sqd* it is necessary for female fertility

In order to understand the function of *Rp-sqd* in *R. prolixus*, non-fed virgin females (n = 16) were injected with different concentrations of dsRNA^{sqd} (1 µg, 2 µg and 3 µg) and dsRNA$^{\beta lac}$, as a control. After feeding and mating, females were evaluated for egg deposition and embryo lethality. Compared to the control, females depleted with dsRNA$^{sqd1\mu g}$, dsRNA$^{sqd2\mu g}$, and dsRNA$^{sqd3\mu g}$ deposited significantly fewer eggs, close to zero throughout the experiments (Fig 2A). The few eggs laid corresponded to the first period analyzed for females following administration of dsRNA$^{sqd1\mu g}$. None of these eggs hatched, and after egg dissection, showed no distinguishable embryonic structures, indicating that early embryogenesis was affected (data not shown).

To confirm the silencing of *Rp-sqd*, transcript levels were quantified at different times after feeding. The results showed a significant reduction of *Rp-sqd* transcripts on day seven (nine days after dsRNA administration), with nearly 11-fold decrease in depleted females compared to the control group (Fig 2B). The reduction of transcript levels correlates with the decrease in ovipositions, indicating that *Rp-sqd* plays a critical role in female fertility.

3.3 *Rp-sqd* is required for oocyte development progression during oogenesis

To understand the sterility resulting from *Rp-sqd* depletion, we performed an analysis of ovarian morphology. In comparison to the control group, the ovaries of the depleted females showed oocytes that failed to progress to the choriogenic stage, instead undergoing reabsorption and/or degeneration (Fig 3A-B, Fig S1). Although the somatic follicular epithelium was present in the ovaries of depleted females (Fig 3B), its organization was disrupted. The nuclei of follicle cells exhibited a lack of their typical spherical shape, an irregular distribution, and fragmented DNA material (Fig 3C-D).

In contrast to control females, the follicle cells of depleted females showed a punctate FM 4-64FX dye pattern within their cytoplasm, indicating the presence of nascent endosomes. This pattern was not observed in the control group (Fig 4A-D).

These results suggest that *Rp-sqd* plays a crucial role in maintaining the integrity of the follicular epithelium and for ensuring proper oocyte development.

3.4 *Rp-sqd* is necessary for the structure of germarium and integrity of nurse cells

The morphology of the germarium, as observed under DIC optics, reveals subtle yet significant differences between control and depleted females (Fig 3A-B). A significant difference was observed in the number of Hoechst-positive nurse cell nuclei (Fig 5A). In particular, the germarium of depleted females exhibited a significantly reduced density of nurse cells, with nuclei that were smaller and irregularly shaped in comparison to those observed in control females (Fig 5B-C; Fig S2). Additionally, the histological examination of germarium sections highlighted remarkable differences in the trophic cords. In depleted females, the longitudinal arrangement of the trophic cords was lost, and the characteristic tubular structure was disrupted, resulting in a compacted tissue appearance (Fig 5D-E).

The observed effect of *Rp-sqd* depletion on trophic cord structure led us to investigate the localization of α-tubulin, a constituent protein of microtubules. Compared to the control group, the germarium of depleted females showed a sharp reduction of α-tubulin immunostaining in trophic cords (Fig 6A-D; Fig. S3). This reduction does not necessarily mean a decrease in protein levels. However, we propose that this reduction reflects a disarray of microtubules, attributing the observed decrease in intensity to the dilution of monomeric α-tubulin in the cytoplasm. To further investigate this, we analyzed cell ultrastructure by transmission electron microscopy. In control females, the cytoplasm adjacent to the trophic core of the germarium showed a large number of microtubules that were organized in directional groups. In depleted females, this arrangement of microtubules could not be identified (Fig 6E-F). Furthermore, the presence of ribosomes and mitochondria interspersed between the microtubules were also diminished in depleted females, compared to the control (Fig 6F; Fig S2). Our results suggest that *Rp-sqd* is required in the germarium for proper trophic cord structure.

3.5 *Rp-sqd* changes the dynamics of maternal genes expression during oogenesis

In order to investigate the molecular basis of *Rp-sqd* function, we studied the expression of maternal mRNAs previously identified in *R. prolixus* (Pascual and Rivera-Pomar, 2022) and known to be involved in different pathways of oogenesis in other insects, namely D-V polarity and mRNAs transport (Roth and Lynch, 2009; Vazquez-Pianzola et al., 2017). Also, we studied the expression of apoptosis related genes and the once associated with cytoskeleton constitution (Levine, 2020). We measured the transcript level at one and seven days after blood meal in both control and depleted females. We selected two apoptosis-related genes, *tumor suppressor p53* (*Rp-p53*) and *licorne* (*Rp-lic*), two involved in D-V axis formation *cactus* (*Rp-cact*) and *dorsal* (*Rp-dl*), five involved in oogenesis, *maternal expression at 31B* (*Rp-me31B*), *Bicaudal D* (*Rp-BicD*), *egalitarian* (*Rp-egl*), *Bicaudal C* (*Rp-BicC*), *cappuccino* (*Rp-cappu*) and one gene associated with cytoskeleton constitution and maintenance, *actin* (*Rp-actin*). All of them showed changes upon *Rp-sqd* depletion compared to the control (Fig 7A-J).

Relative gene expression of *Rp-p53* and *Rp-lic* genes, by day seven, had significantly decreased in depleted females compared to the control ones (Fig 7A-B). The genes involved in D-V axis formation were similarly reduced. The mRNA level of both *Rp-cact* and *Rp-dl*, exhibited a significant reduction in depleted females on day seven compared to the controls (Fig 7C-D). The genes involved in oogenesis showed different effects. The *Rp-me31B* gene displayed a four-fold decrease in expression on day one in depleted females, and this significant difference was maintained at day seven, compared to the controls (Fig 7E). *Rp-BicD* increased expression from day one to seven in control females, in contrast to depleted ones that showed a significantly repressed increase (Fig 7F). *Rp-egl*, *Rp-BicC* and *Rp-cappu* showed the same expression dynamics, control females maintained a low level at both days one and seven, while in depleted females a significant increase in expression was observed on day seven (Fig 7G-I). Additionally, *Rp-actin* showed a significant decrease of expression by day seven in depleted females compared with control ones (Fig 7J).

Taken together, these results show that gene expression is modulated by *Rp-sqd* depletion, suggesting a pleiotropic function during oogenesis, in agreement with the morphological changes observed in the ovary of depleted females.

4 Discussion

Early embryonic patterning involves both maternal and zygotic gene expression. The embryonic axes are established during oogenesis by a series of events that load maternal mRNAs and proteins into the oocyte during egg formation (Latham and Schultz, 2001; Tadros et al., 2003; Tadros and Lipshitz, 2009). These maternal factors are responsible for embryonic development until zygotic genome activation takes place at the mid blastula transition.

Klaus Sander's work on *Euscelis plebejus* was the first to demonstrate the requirement of maternal inputs in establishing embryonic axes (Sander, 1976). Building on this foundation, the genetics components responsible for anterior-posterior (A-P) and dorsal-ventral (D-V) polarity were characterized in *Drosophila melanogaster* (Lynch and Roth, 2011; Rivera-Pomar et al., 1995) and, later on, identified in other insect species, including *Tribolium castaneum* and *Nasonia vitripennis* (Schmidt-Ott and Yoon, 2022). In the Hemiptera *R. prolixus*, a large number of maternal transcripts have been identified, with

different expression profiles during oogenesis and the early stages of embryonic development (Pascual and Rivera-Pomar, 2022). However, the genetic basis of this process remains poorly understood.

One gene of interest is *Rp-sqd*. Previous studies have identified transient mRNA expression levels of *Rp-sqd* from oogenesis to germ band extension (Pascual and Rivera-Pomar, 2022), confirming a maternal nature and suggesting a role during oocyte development. Squid belongs to the highly conserved family of RNA-binding proteins that associate with nascent RNAs leading to localization, maturation and translation. It also plays different roles in cell signaling, telomere biogenesis, DNA repair, as well as in the regulation of gene expression at both the transcriptional and translational levels (Chaudhury et al., 2010; Dreyfuss et al., 2002; Dreyfuss et al., 1993; Han et al., 2010; He and Smith, 2009; Piccolo et al., 2014). The only information on the function of Sqd comes from *D. melanogaster*. *Dm-sqd* plays a central role in establishing both the A-P and D-V axes (Kelley, 1993; Steinhauer and Kalderon, 2005), as well as in the reorganization of microtubules during oocyte development (Delanoue et al., 2007). In relation to embryonic patterning, *Dm-sqd* has been demonstrated to be involved in the transport of maternal mRNAs to the developing oocyte, mRNA anchoring, and the integrity of cytoplasmic structures known as sponge-bodies (Delanoue et al., 2007). The role of *sqd* has been extensively studied for mRNAs present in the Diptera (Clouse et al., 2008; Lall et al., 1999; Lynch et al., 2011; Norvell et al., 2005), however, a conserved function for other insects has not been ruled out.

In this study, we demonstrated that *Rp-sqd* depletion affects the proper oocyte development. As the oocytes progressed through the ovariole, they became folded and wrinkled. The follicular epithelium surrounding the oocyte lost its regular organization. However, the constitution and polarity of the follicle cells did not exhibit alteration in depleted females, which is consistent with the results reported for *D. melanogaster* (Steinhauer and Kalderon, 2005). The disorganization of the follicular epithelium in depleted females coincided with changes in *Rp-BicC* mRNA levels, which are crucial for the proper function of the follicular epithelium, affecting yolk uptake and chorion patterning in the triatomine *R. prolixus* (Pascual et al., 2021). The increased expression of *Rp-BicC* in depleted females suggests a potential interaction -direct or indirect- between these genes, not reported to date.

The oocytes did not form an egg, therefore there we could not assess any embryonic function. However, *Rp-dl* and *Rp-cact,* transcripts involved in D-V patterning (Berni et al., 2014) were found to be suppressed in *Rp-sqd* depleted females. This suggests that the regulation of embryonic patterning processes in *R. prolixus* may be conserved, as has been described for *D. melanogaster* (Kelley, 1993).

The integrity of the nurse cells is essential for the proper loading of maternal factors into the oocyte (Agapov et al., 2022). In *D. melanogaster Dm-Sqd* is required for the self-renewal and proliferation of nurse cells, as well as for mediating the dispersal of their chromosomes during oogenesis (Finger et al., 2023; Goodrich et al., 2004). In this species, nurse cell nuclei undergo multiple endoreplication cycles, followed by a process of dispersal (Dej and Spradling, 1999; Edgar and Orr-Weaver, 2001). In females devoid of *Dm-sqd* the nurse cell chromosomes fail to disperse and maintain a blob-like conformation (Goodrich et al., 2004). Although no significant conformational changes have been described so far in nurse cells during the progression of *R. prolixus* oogenesis, nuclear enlargement of cells from the anterior to the posterior pole of the germarium has

been observed (Huebner and Anderson, 1972a). Additionally, prominent basophilic nucleoli and nuclear extrusions into the cytoplasm have been reported by Huebner and Anderson (1972a). In our study, *Rp-sqd* depleted females showed a reduced number of nurse cells with altered nuclear shape and size. Furthermore, the germarium did not show evidence of the presence of nuclear extrusion in the cytoplasm. These results support the idea that Sqd plays a conserved role in the constitution and maintenance of nurse cells in triatomines. Alterations in nuclear architecture and chromatin organization can significantly impact gene expression, disrupting the transcriptional machinery and leading to changes in mRNAs levels (Misteli, 2007). Our findings support this notion, as *Rp-sqd* depletion resulted in changes of expression of several genes involved in oocyte development (Berni et al., 2014; Pascual and Rivera-Pomar, 2022; Pascual et al., 2021).

Also, in the germarium, the nurse cells share a common cytoplasm that transports specific mRNA to the oocytes. The transport is mediated by cytoplasmic extensions - trophic cords- that connect the nurse cells with the developing oocytes (Huebner and Anderson, 1972a). In *Rp-sqd* depleted females, we observed alterations in the trophic cords and a significant reduction in cytoplasmic components, including mitochondria, ribosomes, and microtubules.

In *D. melanogaste*r, microtubule-mediated transport is a mediator of long-distance transport due to its rapidity and efficiency, being Sqd a key player (Delanoue et al., 2007; Goodrich et al., 2004; Steinhauer and Kalderon, 2005). Our results provide evidence that *Rp-sqd* is required for the proper constitution of trophic cords and reorganization of microtubules during oocyte development, suggesting that it plays a conserved role in the organization of the maternal components during oogenesis. Moreover, the orthologues of *egl* and *BicD*, two genes that act together with Sqd during microtubule-mediated transport in Diptera (Delanoue et al., 2007), were also modulated in *Rp-sqd* depleted females of *R. prolixus*. These results support our previous data on gene expression during oogenesis and early embryogenesis, suggesting the conservation of the components of the localization machinery (Pascual and Rivera-Pomar, 2022). However, gene expression analysis revealed differences with *D. melanogaster*. *Rp-egl* mRNA level increases and *Rp-BicD* decreases in the ovaries of *Rp-sqd* depleted females. On the contrary, in *D. melanogaster*, the absence of *BicD* causes altered localization and diminished expression of *egl* mRNA in developing oocytes (Vazquez-Pianzola et al., 2017). Interestingly, this observation was independent of the expression analysis of *sqd*. We hypothesize that the absence of *Rp-sqd* affects the genes related to the localization machinery, a phenomenon not previously identified. The establishment of proper cytoskeletal organization and cytoplasmic streaming are crucial for the precise localization of polarity determinants. A key gene involved in preventing premature onset of fast cytoplasmic streaming in *D. melanogaster* is *cappu* (Theurkauf, 1994). Our results provide evidence that *Rp-cappu* mRNA level increases in the absence of *Rp-sqd*, supporting the idea of distinct functionality, as reported in *D. melanogaster* during microtubule reorganization in oocyte development (Steinhauer and Kalderon, 2005). Furthermore, reduced expression of *Rp-me31B* mRNA in depleted females provides evidence of alterations in the sponge-bodies composition, as indicated by this specific marker (Weil et al., 2012). Sponge-bodies are known to encompass several functions, including mRNA transport, storage, translation control, and processing (Anderson and Kedersha, 2006; Delanoue et al., 2007; St Johnston, 2005). Our results reflect the involvement of *Rp-sqd* in maintaining these sponge-bodies, in agreement with its reported role in the Diptera (Delanoue et al., 2007; Weil et al., 2012).

At first glance, the morphological changes also appear to be associated with the activation of programmed cell death (Levine, 2020; Zhang et al., 2015). However, the expression levels of *Rp-p53* and *Rp-lic* in the ovaries of depleted females reveal the opposite effect. While these genetic markers were not overexpressed, the presence of DNA damage and endomembrane formation in depleted females does not rule out the possibility that the activation of programmed cell death may be mediated by other genes involved in the process (Wyllie, 2010). Also, the expression levels observed in control females during the choriogenic stage indicate a clear activation of this program, consistent with previous reports concerning chorion establishment and nurse cell renewal, which is necessary for proper progression of oogenesis in the *R. prolixus* (Huebner and Anderson, 1972a, b). During oogenesis in *D. melanogaster*, p53 is also required to protect genome integrity (both germline and soma) and to mediate oocyte quality control (Chakravarti et al., 2022). In this study, the DNA lesions observed in the follicular epithelium of depleted females were similar to the results reported for *D. melanogaster* (*Dm-p53)*. Regarding *lic*, a secondary role in the establishment of D-V axes in *D. melanogaster* embryos has been reported (Suzanne et al., 1999), acting together with *sqd.* The parallel reduction of *Rp-sqd* and *Rp-lic* expression in the depleted females may indicate a conserved gene interaction in *R. prolixus.*

Taken together, our results contribute to the understanding of the molecular network underlying oogenesis in a hemipteran. *Rp-sqd* appears to participate in multiple processes throughout oogenesis. *Rp-sqd* does not affect cell polarity but rather the progression of the oocyte to the choriogenic stage and consequently, the overall physiology of egg formation. *Rp-sqd* alters the integrity of the nurse cell nuclei and, consequently, the transcription of maternal mRNAs, such as *Rp-dl* and *Rp-cact,* which determine the D-V axis, *Rp-BicC*, necessary for chorion formation, and others such as *Rp-p53, Rp-lic*, *Rp-egl*, *Rp-me31b* and *Rp-BicD*, involved in oocyte development. Although the nature of the regulation –whether direct or indirect- remains to be investigated, this is the first report demonstrating genetic interactions between these genes in hemimetabolous insects. The identification of gene networks involved in oogenesis is fundamental to understanding the evolution of insect reproduction.

5 References

Agapov, A., Olina, A., Kulbachinskiy, A., 2022. RNA polymerase pausing, stalling and bypass during transcription of damaged DNA: from molecular basis to functional consequences. Nucleic Acids Res 50, 3018-3041.

Anderson, P., Kedersha, N., 2006. RNA granules. J Cell Biol 172, 803-808.

Berni, M., Fontenele, M.R., Tobias-Santos, V., Caceres-Rodrigues, A., Mury, F.B., Vionette-do-Amaral, R., Masuda, H., Sorgine, M., da Fonseca, R.N., Araujo, H., 2014. Toll signals regulate dorsal-ventral patterning and anterior-posterior placement of the embryo in the hemipteran Rhodnius prolixus. Evodevo 5, 38.

Bucher, G., Farzana, L., Brown, S.J., Klingler, M., 2005. Anterior localization of maternal mRNAs in a short germ insect lacking bicoid. Evol Dev 7, 142-149.

Caceres, L., Nilson, L.A., 2009. Translational repression of gurken mRNA in the Drosophila oocyte requires the hnRNP Squid in the nurse cells. Dev Biol 326, 327-334.

Chakravarti, A., Thirimanne, H.N., Brown, S., Calvi, B.R., 2022. Drosophila p53 isoforms have overlapping and distinct functions in germline genome integrity and oocyte quality control. Elife 11.

Chaudhury, A., Chander, P., Howe, P.H., 2010. Heterogeneous nuclear ribonucleoproteins (hnRNPs) in cellular processes: Focus on hnRNP E1's multifunctional regulatory roles. RNA 16, 1449-1462.

Clouse, K.N., Ferguson, S.B., Schupbach, T., 2008. Squid, Cup, and PABP55B function together to regulate gurken translation in Drosophila. Dev Biol 313, 713-724.

Cooperstock, R.L., Lipshitz, H.D., 2001. RNA localization and translational regulation during axis specification in the Drosophila oocyte. Int Rev Cytol 203, 541-566.

Davis, G.K., Patel, N.H., 2002. Short, long, and beyond: molecular and embryological approaches to insect segmentation. Annu Rev Entomol 47, 669-699.

Dej, K.J., Spradling, A.C., 1999. The endocycle controls nurse cell polytene chromosome structure during Drosophila oogenesis. Development 126, 293-303.

Delanoue, R., Herpers, B., Soetaert, J., Davis, I., Rabouille, C., 2007. Drosophila Squid/hnRNP helps Dynein switch from a gurken mRNA transport motor to an ultrastructural static anchor in sponge bodies. Dev Cell 13, 523-538.

Dreyfuss, G., Kim, V.N., Kataoka, N., 2002. Messenger-RNA-binding proteins and the messages they carry. Nat Rev Mol Cell Biol 3, 195-205.

Dreyfuss, G., Matunis, M.J., Pinol-Roma, S., Burd, C.G., 1993. hnRNP proteins and the biogenesis of mRNA. Annu Rev Biochem 62, 289-321.

Driever, W., Nusslein-Volhard, C., 1988. The bicoid protein determines position in the Drosophila embryo in a concentration-dependent manner. Cell 54, 95-104.

Edgar, B.A., Orr-Weaver, T.L., 2001. Endoreplication cell cycles: more for less. Cell 105, 297-306.

Finger, D.S., Williams, A.E., Holt, V.V., Ables, E.T., 2023. Novel roles for RNA binding proteins squid, hephaesteus, and Hrb27C in Drosophila oogenesis. Dev Dyn 252, 415-428.

Fitch, K.R., Yasuda, G.K., Owens, K.N., Wakimoto, B.T., 1998. Paternal effects in Drosophila: implications for mechanisms of early development. Curr Top Dev Biol 38, 1-34.

Goodrich, J.S., Clouse, K.N., Schupbach, T., 2004. Hrb27C, Sqd and Otu cooperatively regulate gurken RNA localization and mediate nurse cell chromosome dispersion in Drosophila oogenesis. Development 131, 1949-1958.

Goto, T., Kinoshita, T., 1999. Maternal transcripts of mitotic checkpoint gene, Xbub3, are accumulated in the animal blastomeres of Xenopus early embryo. DNA Cell Biol 18, 227-234.

Han, S.P., Tang, Y.H., Smith, R., 2010. Functional diversity of the hnRNPs: past, present and perspectives. Biochem J 430, 379-392.

He, Y., Smith, R., 2009. Nuclear functions of heterogeneous nuclear ribonucleoproteins A/B. Cell Mol Life Sci 66, 1239-1256.

Huebner, E., Anderson, E., 1972a. A cytological study of the ovary of Rhodnius prolixus. Cytoarchitecture and development of the trophic chamber. J Morphol 138, 1-40.

Huebner, E., Anderson, E., 1972b. A cytological study of the ovary of Rhodnius prolixus. I. The ontogeny of the follicular epithelium. J Morphol 136, 459-493.

Huebner, E., Anderson, E., 1972c. A cytological study of the ovary of Rhodnius prolixus. II. Oocyte differentiation. J Morphol 137, 385-415.

Kelley, R.L., 1993. Initial organization of the Drosophila dorsoventral axis depends on an RNA-binding protein encoded by the squid gene. Genes Dev 7, 948-960.

Lall, S., Francis-Lang, H., Flament, A., Norvell, A., Schupbach, T., Ish-Horowicz, D., 1999. Squid hnRNP protein promotes apical cytoplasmic transport and localization of Drosophila pair-rule transcripts. Cell 98, 171-180.

Lasko, P., 2012. mRNA localization and translational control in Drosophila oogenesis. Cold Spring Harb Perspect Biol 4.

Latham, K.E., Schultz, R.M., 2001. Embryonic genome activation. Front Biosci 6, D748-759.

Lavore, A., Pagola, L., Esponda-Behrens, N., Rivera-Pomar, R., 2012. The gap gene giant of Rhodnius prolixus is maternally expressed and required for proper head and abdomen formation. Dev Biol 361, 147-155.

Lecuyer, E., Yoshida, H., Parthasarathy, N., Alm, C., Babak, T., Cerovina, T., Hughes, T.R., Tomancak, P., Krause, H.M., 2007. Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell 131, 174-187.

Levine, A.J., 2020. p53: 800 million years of evolution and 40 years of discovery. Nat Rev Cancer 20, 471-480.

Lipshitz, H.D., Smibert, C.A., 2000. Mechanisms of RNA localization and translational regulation. Curr Opin Genet Dev 10, 476-488.

Liu, P.Z., Kaufman, T.C., 2005. Short and long germ segmentation: unanswered questions in the evolution of a developmental mode. Evol Dev 7, 629-646.

Lynch, J.A., Brent, A.E., Leaf, D.S., Pultz, M.A., Desplan, C., 2006. Localized maternal orthodenticle patterns anterior and posterior in the long germ wasp Nasonia. Nature 439, 728-732.

Lynch, J.A., Desplan, C., 2010. Novel modes of localization and function of nanos in the wasp Nasonia. Development 137, 3813-3821.

Lynch, J.A., Ozuak, O., Khila, A., Abouheif, E., Desplan, C., Roth, S., 2011. The phylogenetic origin of oskar coincided with the origin of maternally provisioned germ plasm and pole cells at the base of the Holometabola. PLoS Genet 7, e1002029.

Lynch, J.A., Roth, S., 2011. The evolution of dorsal-ventral patterning mechanisms in insects. Genes Dev 25, 107-118.

Misteli, T., 2007. Beyond the sequence: cellular organization of genome function. Cell 128, 787-800.

Neuman-Silberberg, F.S., Schupbach, T., 1993. The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. Cell 75, 165-174.

Norvell, A., Debec, A., Finch, D., Gibson, L., Thoma, B., 2005. Squid is required for efficient posterior localization of oskar mRNA during Drosophila oogenesis. Dev Genes Evol 215, 340-349.

Nusslein-Volhard, C., 1991. Determination of the embryonic axes of Drosophila. Dev Suppl 1, 1-10.

Ozuak, O., Buchta, T., Roth, S., Lynch, J.A., 2014. Dorsoventral polarity of the Nasonia embryo primarily relies on a BMP gradient formed without input from Toll. Curr Biol 24, 2393-2398.

Pascual, A., Rivera-Pomar, R., 2022. Dynamics of maternal gene expression in Rhodnius prolixus. Sci Rep 12, 6538.

Pascual, A., Vilardo, E.S., Taibo, C., Sabio, Y.G.J., Pomar, R.R., 2021. Bicaudal C is required for the function of the follicular epithelium during oogenesis in Rhodnius prolixus. Dev Genes Evol 231, 33-45.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29, e45.

Piccolo, L.L., Corona, D., Onorati, M.C., 2014. Emerging roles for hnRNPs in post-transcriptional regulation: what can we learn from flies? Chromosoma 123, 515-527.

Preuss, K.M., Lopez, J.A., Colbourne, J.K., Wade, M.J., 2012. Identification of maternally-loaded RNA transcripts in unfertilized eggs of Tribolium castaneum. BMC Genomics 13, 671.

Pultz, M.A., Westendorf, L., Gale, S.D., Hawkins, K., Lynch, J., Pitt, J.N., Reeves, N.L., Yao, J.C., Small, S., Desplan, C., Leaf, D.S., 2005. A major role for zygotic hunchback in patterning the Nasonia embryo. Development 132, 3705-3715.

Riechmann, V., Ephrussi, A., 2001. Axis formation during Drosophila oogenesis. Curr Opin Genet Dev 11, 374-383.

Rivera-Pomar, R., Lu, X., Perrimon, N., Taubert, H., Jackle, H., 1995. Activation of posterior gap gene expression in the Drosophila blastoderm. Nature 376, 253-256.

Roth, S., Lynch, J.A., 2009. Symmetry breaking during Drosophila oogenesis. Cold Spring Harb Perspect Biol 1, a001891.

Sander, K., 1976. Specification of the basic body pattern in insect embryogenesis. Adv Insect Physiol 12, 125–238.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat Methods 9, 676-682.

Schmidt-Ott, U., Yoon, Y., 2022. Evolution and loss of ss-catenin and TCF-dependent axis specification in insects. Curr Opin Insect Sci 50, 100877.

Schroder, R., 2003. The genes orthodenticle and hunchback substitute for bicoid in the beetle Tribolium. Nature 422, 621-625.

Simpson, P., 1983. Maternal-Zygotic Gene Interactions during Formation of the Dorsoventral Pattern in Drosophila Embryos. Genetics 105, 615-632.

St Johnston, D., 1995. The intracellular localization of messenger RNAs. Cell 81, 161-170.

St Johnston, D., 2005. Moving messages: the intracellular localization of mRNAs. Nat Rev Mol Cell Biol 6, 363-375.

Steinhauer, J., Kalderon, D., 2005. The RNA-binding protein Squid is required for the establishment of anteroposterior polarity in the Drosophila oocyte. Development 132, 5515-5525.

Stothard, P., 2000. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques 28, 1102, 1104.

Suzanne, M., Irie, K., Glise, B., Agnes, F., Mori, E., Matsumoto, K., Noselli, S., 1999. The Drosophila p38 MAPK pathway is required during oogenesis for egg asymmetric development. Genes Dev 13, 1464-1474.

Tadros, W., Houston, S.A., Bashirullah, A., Cooperstock, R.L., Semotok, J.L., Reed, B.H., Lipshitz, H.D., 2003. Regulation of maternal transcript destabilization during egg activation in Drosophila. Genetics 164, 989-1001.

Tadros, W., Lipshitz, H.D., 2009. The maternal-to-zygotic transition: a play in two acts. Development 136, 3033-3042.

Teixeira, F.K., Lehmann, R., 2019. Translational Control during Developmental Transitions. Cold Spring Harb Perspect Biol 11.

Theurkauf, W.E., 1994. Premature microtubule-dependent cytoplasmic streaming in cappuccino and spire mutant oocytes. Science 265, 2093-2096.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A., 2007. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res 35, W71-74.

Vazquez-Pianzola, P., Schaller, B., Colombo, M., Beuchle, D., Neuenschwander, S., Marcil, A., Bruggmann, R., Suter, B., 2017. The mRNA transportome of the BicD/Egl transport machinery. RNA Biol 14, 73-89.

Wanderley-Teixeira, V., Teixeira, A.A., Cunha, F.M., Costa, M.K., Veiga, A.F., Oliveira, J.V., 2006. Histological description of the midgut and the pyloric valve of Tropidacris collaris (Stoll, 1813) (Orthopetera: Romaleidae). Braz J Biol 66, 1045-1049.

Weil, T.T., Parton, R.M., Herpers, B., Soetaert, J., Veenendaal, T., Xanthakis, D., Dobbie, I.M., Halstead, J.M., Hayashi, R., Rabouille, C., Davis, I., 2012. Drosophila patterning is established by differential association of mRNAs with P bodies. Nat Cell Biol 14, 1305-1313.

Wyllie, A.H., 2010. "Where, O death, is thy sting?" A brief review of apoptosis biology. Mol Neurobiol 42, 4-9.

Zhang, B., Rotelli, M., Dixon, M., Calvi, B.R., 2015. The function of Drosophila p53 isoforms in apoptosis. Cell Death Differ 22, 2058-2067.

Figure legends

Fig. 1. Gene expression of *Rp-sqd* in *R. prolixus* ovaries. **A** Transcript levels of *Rp-sqd* were measured in ovaries dissected at one (T1), five (T5), seven (T7) days post-blood feeding, and from non-fed females (T0). Each bar represents the mean and standard error of three biological replicates mean (SEM) (ANOVA, Bonferroni test, $\alpha = 0.05$). **B-D** *In situ* hybridization in control ovaries at T7 (n = 4) (**B**) using sense (control) RNA probe for *Rp-sqd* gene, and in control ovaries at T1 (n = 4) (**C**) and T7 (n = 4) (**D**) using antisense RNA probe for *Rp-sqd* gene. Key: (pV) previtellogenic oocyte, (V) vitellogenic oocyte, (Fc) follicle cells. Scale bar: 200 µm common to all panels.

Fig. 2. Oviposition and mRNA expression levels of *Rp-sqd* in *R. prolixus* females after dsRNA administration. A Scatter plot representing the number of total eggs per concentration. The treatment factor significantly affects the response variable *** $p < 0.0001$. **B** Transcript levels of *Rp-sqd* during the course of the experiment. Expression was measured 1- and 7-days post- blood feeding, following administration of $dsRNA^{\beta\text{-}lac}$ (blue bars) or $dsRNA^{sqd}$ (pink bars). All values were referred to as the *Rp- Rpl13A* expression at 1 day in females treated with $dsRNA^{\beta\text{-}lac}$. Each bar represents the mean and standard error of three biological replicates mean (SEM). ** $p < 0.0005$ (ANOVA, Bonferroni test, $\alpha = 0.05$).

Fig. 3. Effect of $dsRNA^{sqd}$ administration on the follicular epithelium of *R. prolixus*. **A-B** Ovarioles of control (**A**) and depleted (**B**) females observed by differential interference contrast (DIC) microscopy. Scale bar: 100 µm. **C-D** Hoechst staining of the follicular epithelium of vitellogenic oocytes from control (n = 11) (**C**) and depleted (n = 11) (**D**) females showing the nuclei distribution. Note the altered pattern of nuclei distribution in the somatic follicular epithelium of depleted females. Scale bar: 50 µm. Key: (pV) previtellogenic oocyte, (V) vitellogenic oocyte, (Fc) follicle cells, (Tch) trophic cords (*) fragmented DNA.

Fig. 4. *Rp-sqd* depletion affects follicle cell integrity. **A-B** Follicular epithelium from control (n = 4) (**A**) and depleted (n = 4) (**B**) females showing the nuclei distribution by Hoechst staining. **C-D** Localization of the FM4-64X probe in the cytoplasm of control (**C**) and depleted (**D**) follicle cells. Note the presence of punctate structures present in depleted females (*). Scale bar: 50 µm, applicable to all panels.

Fig. 5. Effect of $dsRNA^{sqd}$ administration on *R. prolixus* nurse cells and trophic cords. **A** Quantification of positive nuclei of nurse cells in the germarium of vitellogenic females after administration of $dsRNA^{\beta\text{-}lac}$ or $dsRNA^{sqd}$ (t-test, Welch's correction) ***

$p < 0.0001$. **B-C** Nuclei distribution of nurse cells in control (n = 11) (**B**) germarium staining by Hoechst. **C** Nuclei distribution of nurse cells in depleted (n = 11) (**C**) germarium staining by Hoechst. Scale bar: 50 µm. **D-E** Histological staining (hematoxylin-eosin) of germarium from control (n = 3) (**D**) and depleted (n = 3) (**E**) females. Note the zoom in of each histological section. Scale bar: 200 µm.

Fig. 6. *Rp-sqd* depletion alters microtubule distribution. **A-B** Immunostaining with anti-α-tubulin antibody to determine the distribution of microtubules (visualized in red) in a germarium of control (n = 6) (**A-C**) and depleted (n = 6) (**B-D**) females. Nuclei distribution staining by Hoechst. (**A-B**) Scale bar: 100 µm. (**C-D**) Scale bar: 50 µm **E-F** Transmission electron microscopy (TEM) of a germarium from control (n = 2) (**E**) and depleted (n = 2) (**F**) females. Arrow indicates the microtubules. Key: (tco) trophic core, (r) ribosomes. Scale bar: 0.2 µm.

Fig. 7. Transcript levels of mRNAs during the progression of the experiment. Expression was measured 1- and 7-days post- blood feeding, after administration of $dsRNA^{\beta\text{-}lac}$ (blue bars) or $dsRNA^{sqd}$ (pink bars). All values were referred to as the *Rp-Rpl13A* expression at 1 day in females treated with $dsRNA^{\beta\text{-}lac}$. Each bar represents the mean and standard error of three biological replicates mean (SEM). * $p < 0.05$ ** $p < 0.005$ *** $p < 0.0005$. (ANOVA, $\alpha = 0.05$). (**A**) *Rp-p53* (RPRC003641), (**B**) *Rp-lic* (RPRC014787), (**C**) *Rp-dl* (RPRC003790), (**D**) *Rp-cact* (RPRC017349), (**E**) *Rp-me31B* (RPRC009336), (**F**) *Rp-BicD* (RPRC000632 + RPRC000704 + RPRC004076), (**G**) *Rp-egl* (RPRC012064), (**H**) *Rp-BicC* (RPRC001612 + RPRC001613), (**I**) *Rp-cappu* (RPRC003459) and (**J**) *Rp-actin* (RPRC015176); Sequence available in VectorBase (https://vectorbase.org).

Supplementary Materials:

Supplementary Table 1. Primers used for dsRNA synthesis, RNA probes and RT-qPCR.

Supplementary Fig 1. Ovaries of control and depleted females. A-B. Complete ovariole of control ($dsRNA^{\beta\text{-}lac}$) (**A**) and depleted ($dsRNA^{sqd}$) (**B**) females showing the nuclear distribution by Hoechst staining. Previtellogenic oocyte (pV), vitellogenic oocyte (V), choriogenic oocyte (Ch). Scale bar: 200 µm.

Supplementary Fig. 2. Ultrastructural analysis of the nurse cells present in the germarium of control and depleted females. A. Germarium of control ($dsRNA^{\beta\text{-}lac}$) females. **B** Germarium of depleted ($dsRNA^{sqd}$) females. Nucleoli (Nu), mitochondria (M), nuclear emissions (NE), trophic core (TCO), necrotic nuclei *, cytoplasmic projections (arrow). Scale bar: 2 µm.

Supplementary Fig 3. **Microtubule distribution in the germarium of control and depleted females**. Immunostaining with anti-α-tubulin antibody to determine the distribution of microtubules in a germarium of control (A) and depleted (B) females. Sections were obtained using LSM800 confocal microscopy. Scale bar: 50 µm.

The authors declare no competing interests in the matter of this project.

Agustina Pascual: Conceptualization, investigation, funding acquisition, writing - Original Draft **Catalina Taibo**: Resources. **Rolando Rivera-Pomar:** Conceptualization, supervision, funding acquisition, writing- reviewing and editing.