Knock down analysis reveals critical phases for specific oskar noncoding RNA functions during Drosophila oogenesis

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Abstract

The oskar transcript, acting as a noncoding RNA, contributes to a diverse set of pathways in the Drosophila ovary, including karyosome formation, positioning of the microtubule organizing center (MTOC), integrity of certain ribonucleoprotein particles, control of nurse cell divisions, restriction of several proteins to the germline, and progression through oogenesis. How oskar mRNA acts to perform these functions remains unclear. Here, we use a knock down approach to identify the critical phases when oskar is required for three of these functions. The existing transgenic shRNA for removal of oskar mRNA in the germline targets a sequence overlapping a regulatory site bound by Bruno1 protein to confer translational repression, and was ineffective during oogenesis. Novel transgenic shRNAs targeting other sites were effective at strongly reducing oskar mRNA levels and reproducing phenotypes associated with the absence of the mRNA. Using GAL4 drivers active at different developmental stages of oogenesis, we found that early loss of oskar mRNA reproduced defects in karyosome formation and positioning of the MTOC, but not arrest of oogenesis. Loss of oskar mRNA at later stages was required to prevent progression through oogenesis. The noncoding function of oskar mRNA is thus required for more than a single event.

Keywords: noncoding RNA; oskar; karyosome; MTOC; oogenesis; shRNA

Introduction

Drosophila oskar (osk) mRNA is required, independent of Osk protein, during oogenesis (Jenny et al. 2006). Mutants in which osk mRNA levels are substantially reduced or eliminated exhibit myriad defects. Most conspicuous of these is female sterility, a consequence of arrested oogenesis. Even before the arrest, more subtle defects appear: egg chambers often have too many nurse cells, the condensation of oocyte chromosomes to form the karyosome is incomplete, positioning of the microtubule organizing center (MTOC) within the oocyte is abnormal, certain ribonucleoprotein particles are disorganized, and proteins normally restricted to the germline cells of the ovary can be detected at low levels in surrounding somatic follicle cells (Jenny et al. 2006; Kanke et al. 2015; Kenny et al. 2021). Discovery of additional defects seems likely, awaiting only the application of appropriate assays. The diversity of osk RNA null mutant phenotypes, with no discernible shared dependence on a particular cellular pathway downstream of osk itself, might suggest the existence of multiple different noncoding functions. However, each phenotype listed above is sensitive to mutation of the same defined functional sequences in the osk mRNA 3’ UTR: a tightly clustered combination of Bruno1 (Bru1) binding sites, the osk noncoding element (ONCE), and A-rich sequences (ARS) (Vazquez-Pianzola et al. 2011; Kanke et al. 2015; Kenny et al. 2021). Thus, these elements either mediate multiple events or interactions, or they perform a single function with multiple pathways affected by disruption of that event. An extreme version of the latter option would be an initial “gateway” event with an immediate outcome, such as karyosome formation or proper MTOC organization, being a prerequisite for later outcomes, such as progression through oogenesis.

One approach to explore the relationship of different osk RNA null defects is to disrupt osk ncRNA activity at different stages of oogenesis, and ask if specific defects can be induced separate from others. Classical application of this strategy has often relied on temperature-sensitive mutants (e.g., Jarvik and Botstein 1973). A more recent take on this concept relies on inducible gene activation or silencing (e.g., Gossen and Bujard 1992; Gossen et al. 1995; Kumar et al. 2009; Fenno et al. 2011). In Drosophila, a useful method is gene knockdowns (KDs), relying on transgenic shRNAs under UAS/GAL4 control (https://fgr.hms.harvard.edu/fly-in-vivo-rnai) and a variety of GAL4 drivers (Brand and Perrimon 1993) with different temporal and/or spatial patterns of expression. Here, we have taken the KD approach, commencing with the development of effective osk-shRNA transgenes to address a disabling limitation of the existing versions. We provide evidence of different critical periods for different osk ncRNA roles, and show that initial defects from early loss of osk mRNA do not have lasting effects if osk mRNA is restored.
Materials and methods
Flies and transgenes
To construct osk-shRNA transgenes, pairs of oligonucleotides were annealed and cloned into the NheI and EcoRI sites of the VALUM2 vector. Oligonucleotide pairs, all shown with 5’ to the left: osk-shRNA#1, CTACGCTAGATTCCTGCATACGAGTTATATATATATATACGACATATCAGGCG, and osk-shRNA#2, CTACAGCACATTATGCAGACCAGAATGTGGCG. All shRNAs were expressed from plasmids containing a cycleretaining the wild type intron sequences and encoding MATα4-GAL-VP16

Results and discussion
Development and validation of new osk KD reagents
One approach to defining the critical phase or phases of osk ncRNA activity is to remove osk RNA by knock down (KD), relying on GAL4 drivers with different temporal activities to express a transgenic shRNA that targets osk mRNA. However, the maternal triple driver (MTD-Gal4) in combination with the osk shRNA transgenic reagent for germ-line expression (TRiP GL01101) from the Transgenic RNAi Project (TRiP) is not effective at mimicking the most dramatic osk RNA null phenotype, arrest of oogenesis, as egg laying is not substantially reduced (Liu and Lasko 2015). Similarly, the matalpha4-GAL-VP16 driver (henceforth referred to as MAT; abbreviations used for other drivers are given in MATERIALS AND METHODS) in combination with TRiP.GL01101 only modestly reduced the level of ovarian osk mRNA (Figure 1B) and failed to substantially reduce egg laying (Figure 1C).

Detection of RNAs and proteins
RNA levels were determined by qPCR as described (Kenny et al. 2021). In situ hybridization to detect osk mRNA made use of tiled short DNA oligonucleotides (smFISH) 3’-end labeled with Quasar 670 fluorophore (LGC Biosearch Technologies) and used at 1.5 nM. Assays were performed as described (Abbaszadeh and Gavis 2016). Immunodetection of proteins in ovaries was as described (Ryu and MacDonald 2015), with a different fixation protocol for gamma-tubulin (Kenny et al. 2021). TO-PRO-3 Iodide (Molecular Probes) was used for all subsequent osk KD experiments. For all imaging experiments, the samples were obtained from at least five flies, typically many more. Quantification of in situ hybridization data was done in Fiji, with germlaria or egg chambers traced and maximum pixel intensity measured. Measurements of oocyte areas bounded by gamma-tubulin foci were performed as described (Kenny et al. 2021).
overlapping. It would not be surprising if a similar effect underlies a subset of other ineffective shRNAs, and compilations of shRNA effectiveness could potentially be mined for information contributing to the identification of recognition sites for other RNA binding proteins.

Effectiveness of GAL4 drivers in reducing osk ncRNA activity

Several GAL4 drivers active in the female germ line were tested with osk-shRNA#3 to monitor effects on osk ncRNA activity, focusing initially on egg laying (Figure 2A). For comparison, KDs of bru1 were also performed. Females mutant for strong alleles of bru1 produce no eggs and display an early arrest of oogenesis, the expected phenotype for an effective KD. Three of the drivers tested, NGV, MAT, and 34C10, substantially reduced egg laying in the osk KDs; each must be active at or before the stage when osk ncRNA activity is required for progression through oogenesis. When used for bru1 KDs each of these drivers was also effective at arresting oogenesis. One driver, NGT, caused arrest of oogenesis in bru1 KDs, but did not interfere with progression through oogenesis in osk KDs. Although the osk and bru1 KDs are similar in causing arrest of oogenesis, the nature of the arrest is quite different. Whereas the osk KD allows progression through the early stages, the bru1 KD arrest is associated with overproliferation of germ cells in pseudo egg chambers (Supplementary Figure S1), as observed in mutants lacking bru1 activity (Parisi et al. 2001).

Each of these drivers was used in combination with a UAS-GFP transgene to compare periods of activity during oogenesis. Identical imaging settings were used for all genotypes. To detect lower levels of activity, the original images (Figure 2B) were adjusted identically in the green (GFP) channel to increase sensitivity (Figure 2B'). Only those drivers with substantial effects on egg laying in the osk KD were strongly active for UAS-GFP expression during the middle stages of oogenesis.

Both MAT and 34C10 drivers produced GFP at substantial levels from stage 3 though at least stage 8 (the endpoint of this analysis). In principle, the continual appearance of GFP could arise from an initial phase of expression and persistence of the protein later in development, or from continual expression. Two
observations suggest that persistence of GFP protein did not make a substantial contribution to the observed patterns. First, the half life of GFP appeared to be short relative to the time required for progression through oogenesis: GFP produced by the NGT driver in the germarium did not persist (Figure 2B'). Second, the intensity of the GFP signal from the MAT and 34C10 drivers remained high and even increased from stages 3–8 (Figure 2, B and B'), despite a more than 10-fold increase in the volume of the egg chamber in that developmental period (King 1970). If there was only a short period of expression, the resulting GFP should have become progressively more dilute as volume increased.

Consequences of removing osk ncRNA activity during the early stages of oogenesis

The NGT driver was active only very early in oogenesis: expression of the UAS-GFP reporter was detected in the germarium and in stage 2 egg chambers, in a developmental profile roughly complementary to MAT activity (Figures 2B' and 3A). Despite the early activity of NGT, there was no effect on egg laying in the osk KD (Figure 2A). Thus, either NGT is not effective for the osk KD, or osk mRNA is not required at early stages for the progression through oogenesis. To address the effectiveness of the NGT KD of osk, we monitored osk mRNA levels by in situ hybridization. In the germarium, including stage 1 egg chambers not yet budded off, osk mRNA was largely eliminated (Figure 3, B and C). By stage 4 the osk mRNA levels had begun to recover: a substantial fraction of osk mRNA signal: (Figure 3, B and C). By stage 4 the osk mRNA levels had begun to recover: a substantial fraction.

Figure 2 Comparison of GAL4 drivers for activity during oogenesis. (A) Rates of egg laying by females in which the indicated GAL4 driver was present in combination with transgenes for the KD of osk or brul. (B) Expression patterns from the combination of UAS-GFP and the indicated GAL4 driver. Each panel shows a single ovariole from germarium (left) to stage 8 (right). Panel (B') is the same set of panels with gain increased in the RGB green channel. Scale bar is 100 μm.

Figure 3 osk mRNA can be removed by KD early in oogenesis. (A) MAT and NGT driver activity in early oogenesis. Shown are the most anterior portions of ovarioles, with the germarium to the left and individual egg chambers to the right. Scale bar is 10 μm. Only the RGB green channel is shown in the panels at right. (B) In situ detection of osk mRNA at early stages of oogenesis. For both stages the osk mRNA signal from the left column is shown by itself in the right column. For stage 4 the levels of osk mRNA in the KD with the NGT driver were variable and two examples indicative of this variation are shown. Scale bars are 10 μm. (C) Quantitation of osk mRNA levels at early stages of oogenesis from in situ hybridization images (representative examples in panel B). For the stage 4 analysis imaging conditions were chosen to best reveal differences in low levels of osk mRNA in the osk mutant and KDs; consequently, there was signal saturation (pixel intensity of 255) for the wild-type sample. P-values were derived from the Wilcoxon rank-sum test. For all pairwise comparisons P < 0.01, with two exceptions: in the germarium/Stage 1 samples P < 0.1 for w1118 vs MAT>osk-shRNA#3 and for oskΔ vs NGT>osk-shRNA#3.
driver further narrow the definition of the critical phase for the requirement of osk ncRNA activity in progression through oogenesis. Because a significant fraction of stage 4 egg chambers in the osk KD with NGT still have extremely low levels of osk mRNA, yet there is no reduction in egg laying, osk ncRNA activity must be required only later. We conclude that at some point between stage 5 and the time of arrest at stage 6/7 osk mRNA performs the function that allows further progression through oogenesis.

Arrest of oogenesis is only one manifestation of absence of osk mRNA. Other osk RNA null phenotypes, such as altered organization of the MTOC (Kenny et al. 2021) and defective formation of the karyosome (Jenny et al. 2006; Kanke et al. 2015), can appear prior to the time of arrest. These processes are expected to be sensitive to loss of osk mRNA early in oogenesis from KD. Indeed, the distribution of osk mRNA, when present in stage 4 egg chambers after osk KD with NGT (Figure 3B), suggests an MTOC defect. In wild-type oocytes the MTOC from stages 2 to 6 is positioned at the posterior of the oocyte (Theurkauf et al. 1992), and this distribution underlies the posterior enrichment of multiple mRNAs including osk itself (Clegg et al. 1997). By comparison to wild-type stage 4 oocytes, in the osk KD with NGT the posterior enrichment of osk mRNA appeared to be reduced.

MTOC organization was monitored using gamma-tubulin as a marker, initially focusing on stage 3 and 4 egg chambers. Posterior enrichment is lost in the absence of osk mRNA, with multiple foci of gamma-tubulin present throughout the oocyte (Kenny et al. 2021) (Figure 4A). A similar defect was found in the early osk KD with the NGT driver (Figure 4, A and B).

Figure 4 Disruption of MTOC from KD of osk in early oogenesis. (A) Representative stage 3-4 egg chambers from females with the genotypes indicated. Each egg chamber is oriented with posterior, the position of the oocyte, to the right. Left column: Complete egg chambers stained for gamma-tubulin (green) and DNA (red). Scale bar is 10 μm. Middle column: Images from the left column with only the gamma-tubulin signal (white). Right column: enlargements of the regions outlined in yellow in the middle column, containing the oocyte. (B) Measurements of the areas bounded by foci of gamma-tubulin in the oocyte (see Material and methods). At least 19 oocytes were analyzed for each genotype. The P-values were derived from the Wilcoxon rank-sum test: *** P < 0.01. (C) Representative stage 5-6 egg chambers from females with the genotypes indicated. Each egg chamber is oriented with posterior to the right. Left: complete egg chambers stained for gamma-tubulin (green) and DNA (red). Scale bar is 10 μm. The posterior portion with the oocyte is shown to the right with only the gamma-tubulin signal (white).
The loss of osk mRNA from the NGT KD did not persist as oogenesis progressed. Consequently, we could ask if MTOC organization recovered as osk mRNA reappeared from ongoing transcription. In wild type stage 5 and 6 egg chambers, the clusters of gamma-tubulin transitioned into one or two predominant foci (Figure 4C). In the absence of osk mRNA, the abnormal distribution of gamma-tubulin persisted at later stages of oogenesis (Figure 4C). By contrast, in the early osk KD with the NGT driver the normal pattern was restored, with no significant difference from wild type by stages 5/6.

We also monitored karyosome formation at the same early and mid stages of oogenesis. At stage 2 of oogenesis, chromosomes appear throughout the oocyte nucleus. With the completion of meiotic recombination the oocyte chromosomes condense to form the karyosome, a single compact cluster within the nucleus (King 1970) (Figure 5). Karyosome formation is defective in the absence of osk ncRNA activity, with the chromosomes partitioned into two or more foci (Jenny et al. 2006; Kanke et al. 2015) (Figure 5). KD of osk mRNA with the NGT driver produced the same defect (Figure 5A), statistically indistinguishable from the osk0 mutant (Figure 5C).

The karyosome defects from the early osk KD with the NGT driver were substantially corrected as oogenesis proceeded and osk mRNA levels increased, although a significant fraction of egg chambers still had abnormal karyosomes at stages 5/6 (Figure 5, B and D; the example shown in Figure 5B is one in which the karyosome remains abnormal). Because the initial failure to form the karyosome or to correctly position the MTOC did not prevent at least partial recovery when osk mRNA levels began to recover, the critical period for osk ncRNA activity in these two processes is ongoing and the role played by osk mRNA must occur repeatedly and not only once. The differing extents to which these defects were corrected could reflect different modes of action for osk ncRNA activity in each process, or different sensitivities to loss of osk mRNA. By either explanation, it seems unlikely that osk mRNA performs a single initial event, which then feeds into the different pathways.

**Figure 5** Disruption of karyosome formation from KD of osk in early oogenesis. (A) Representative stage 3–4 egg chambers from females with the genotypes indicated. Each egg chamber is oriented with posterior to the right. Left: complete egg chambers stained for DNA (red) and lamin (green) to outline the nuclei. Scale bar is 10 μm. Right: enlargements of the oocyte nuclei from the left images. (B) Representative stage 5–6 egg chambers from females with the genotypes indicated. Presented as in panel A, except that the scale bar is 20 μm. (C) Proportion of stage 3–4 oocytes of the indicated genotypes with normal karyosomes, defined as a single cluster of DNA in the oocyte nucleus. The number (n) of oocytes analyzed for each genotype is indicated. The P-values were derived from the Wilcoxon rank-sum test: ***P < 0.01. (D) Proportion of stage 5–6 oocytes of the indicated genotypes with normal karyosomes, defined as a single cluster of DNA in the oocyte nucleus. The P-values were derived from the Wilcoxon rank-sum test: ***P < 0.01.
Data availability

The data underlying this article are available in the article and in its online supplementary material.

Supplementary material is available at G3 online.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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