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A translation-independent role of *oskar* RNA in early *Drosophila* oogenesis

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The *Drosophila* maternal effect gene *oskar* encodes the posterior determinant responsible for the formation of the posterior pole plasm in the egg, and thus of the abdomen and germline of the future fly. Previously identified *oskar* mutants give rise to offspring that lack both abdominal segments and a germline, thus defining the 'posterior group phenotype'. Common to these classical *oskar* alleles is that they all produce significant amounts of *oskar* mRNA. By contrast, two new *oskar* mutants in which *oskar* RNA levels are strongly reduced or undetectable are sterile, because of an early arrest of oogenesis. This egg-less phenotype is complemented by *oskar* nonsense mutant alleles, as well as by *oskar* transgenes, the protein-coding capacities of which have been annulled. Moreover, we show that expression of the *oskar* 3′ untranslated region (3′UTR) is sufficient to rescue the egg-less defect of the RNA null mutant. Our analysis thus reveals an unexpected role for *oskar* RNA during early oogenesis, independent of Oskar protein. These findings indicate that *oskar* RNA acts as a scaffold or regulatory RNA essential for development of the oocyte.

KEY WORDS: *oskar*, Non-coding RNA, Polarity, Oogenesis, *Drosophila*

INTRODUCTION

In many organisms, determination of body axis relies on molecular asymmetries established during oogenesis or early embryogenesis. In *Drosophila melanogaster*, maternally provided mRNAs and proteins are transported into the growing oocyte and stored until they are required at later stages of development.

During *Drosophila* oogenesis, a germline stem cell located at the anterior tip of the germarium divides to generate a new stem cell and a sibling cystoblast (reviewed by Gilboa and Lehmann, 2004; Huynh and St Johnston, 2004). The cystoblast undergoes four rounds of divisions with incomplete cytokinesis, giving rise to a 16-cell cyst consisting of 15 nurse cells and an oocyte interconnected by cytoplasmic bridges called ring canals. The nurse cells provide the oocyte with most of the mRNAs and proteins required for its development, and for the development of the future embryo until the onset of zygotic transcription (reviewed by Lawrence, 1992; Spradling, 1993). One of these mRNAs encodes the posterior determinant Oskar (Ephrussi et al., 1991; Kim-Ha et al., 1991). During early oogenesis, *oskar* RNA is exported from the nurse cells into the oocyte cytoplasm, where the RNA accumulates as a translationally silent transcript. During mid-oogenesis (stage 8), *oskar* mRNA is transported towards the posterior pole, leading to its asymmetric localization (Ephrussi et al., 1991; Kim-Ha et al., 1991). *oskar* mRNA is exclusively translated at the posterior pole, where it initiates assembly of the pole plasm (Kim-Ha et al., 1991; Markussen, 1995; Rongo et al., 1995).

oskar mRNA produces two Oskar isoforms, Long Oskar and Short Oskar, generated by the use of two alternative start codons, called M1 and M2 (Markussen et al., 1995; Rongo et al., 1995). Together, the two Oskar proteins induce posterior pole plasm assembly and localization by recruiting the additional factors necessary for abdomen and germline formation in the future embryo (Ephrussi and Lehmann, 1992). Embryos from classical *oskar* mutant mothers fail to form posterior structures and lack germ cells (Lehmann and Nüsslein-Volhard, 1986), because they fail to recruit Vasa protein and *nanos* (*nos*) mRNA to the posterior pole (Ephrussi et al., 1991; Hay et al., 1990; Lasko and Ashburner, 1990). Vasa is a highly conserved component of the germline, and is required for abdomen and germline formation in *Drosophila* (Schüpbach and Wieschaus, 1986). Nanos, the abdominal determinant, acts by repressing translation of maternal *hunchback* mRNA in the posterior region of the embryo, allowing posterior activation of gap genes and, thus, formation of posterior structures (Gavis and Lehmann, 1992; Wang and Lehmann, 1991).

Classical *oskar* mutants were isolated in screens for maternal effect genes required for anteroposterior patterning of the embryo (Lehmann and Nüsslein-Volhard, 1986). These classical alleles all express a significant amount of *oskar* mRNA, but lack functional Oskar proteins and thus produce embryos lacking germ cells and abdomen (Ephrussi et al., 1991; Kim-Ha et al., 1991). Here, we describe two new *oskar* mutant alleles showing a strong reduction or complete absence of *oskar* mRNA, respectively. Intriguingly, these new *oskar* alleles cause a different, stronger *oskar* phenotype: the early arrest of oogenesis leading to a complete failure in egg production. Using a rescue approach with a number of transgenes unable to produce Oskar protein, we show that the *oskar* mRNA transcript, but not the protein, is required for early *oskar* function. In particular, its 3′UTR is sufficient to overcome the early oogenesis arrest, thus revealing an unexpected function for *oskar* mRNA.

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MATERIALS AND METHODS

Isolation and molecular characterization of the new *oskar* alleles

All *Drosophila* strains used in this study that have been previously reported are described in FlyBase. osk^{A87} was isolated on a *ru st e ca* chromosome in an EMS mutagenesis screen using a sensitized genetic background (D. St J., unpublished). osk^{187} was identified in a P element mutagenesis screen (Erdélyi et al., 1995). Although the mutations were isolated in EMS and P element mutagenesis, molecular analysis reveals that the osk^{A87} and osk^{187} mutations were induced by a ZAM (Leblanc et al., 1997) retrotransposon and an I element (Crozatier et al., 1988), respectively. Inverse PCR on a circularized *Ssp*I digest of genomic DNA purified from osk^{187} homozygous flies was performed by amplification with primers U8 (5′-TTGCGCCTGC TCTTTCGCCTTC-3′) and L5 (5′-TTTGTTCCCATTCGCCCACCAT-3′). The amplification product was cloned using a pCRScript kit (Stratagene) and an I element was identified in the sequence, which was obtained by sequencing using vector primers. $osk^{A87}/Df(3R)p^{XT103}$ genomic DNA was digested with *Sna*BI. Inverse PCR using L5 and oskSeq1 primers (5′-CGAAAAGCACCGTAAGTCTC-3′) was performed on the circularized template. The PCR product was cloned into Topo T/A plasmid (Invitrogen). Sequence analysis of the fragment revealed the insertion of a ZAM retrotransposon (Accession Number, AJ000387) in reverse orientation with respect to *oskar* transcription. Using ZAM element and the *oskar*-specific primers ZAM5′rev (5′-GTATGCGTTGTTCTGTCTGAG-3′) and circ osk 3′(5′-TAACTGCAGTTGGTCTTTTCATCCGTT-3′), the 5′ end of the ZAM element and the flanking *oskar* sequence were amplified. The insertion site of the ZAM element was precisely determined by cloning into Topo T/A vector and sequencing the PCR product.

Southern analysis of the osk^{A87} mutant DNA revealed a band whose size precisely reflected the insertion of a ZAM retrotransposon (data not shown). Northern blot analyses were performed using poly(A)$^+$ selected RNA and radiolabeled antisense *oskar* and *rp49* RNA probes (Roche RNA labeling kit), according to standard protocols.

For RT-PCR analysis, total RNA from the abdomen of three to six females was extracted using the Absolutely RNA RT-PCR Miniprep kit (Stratagene) including a DNAse digest. Oligo-dT primed cDNA was synthesized with Superscript II (Invitrogen) according to standard procedures. To show the absence of *osk* transcript in osk^{A87} (Fig. 1C), RT-PCR was performed on cDNAs from $osk^{A87}/Df(3R)p^{XT103}$ and $nos^{A10}/Df(3R)p^{XT103}$ females with primers A87RT (5′-TTGCTGAGCCACGCCCAGAA-3′) and Bi_osk_control (5′-ACATTGGGAATGGTCAGCAG GAAATC-3′) for 40 cycles (annealing temperature 55°C). Primers bcd_up (5′-AACGAGCAAG-AAGACGACGCTACAGTCTTG-3′) and bcd_rt (5′-GCGAATAGCG-TATTGCAGGGAAAGTATAGA-3′) were used as positive control.

Quantitative real-time RT-PCR (Fig. 4) was performed on cDNAs from pCogGal4:VP16/w^{1118}; UAS *osk-K10*/+; osk^{A87},NanosGal4:VP16/$Df(3R)p^{XT103}$, pCogGal4:VP16/UAS *osk*WT;; osk^{A87},NanosGal4:VP16$Df(3R)p^{XT103}$, and pCogGal4:VP16/UAS *osk3′UTR*;; osk^{A87}, NanosGal4:VP16/$Df(3R)p^{XT103}$ females using SYBR Green1 chemistry (Molecular Probes) on an ABI PRISM 7900HT real-time PCR apparatus. *oskK10* mRNA was amplified using primers oskK10_for (5′-CTCC-TGTCTAATCAACGAAAGG-3′) and K10_rev (5′-TTGACCATGGGT-TTAGGTATAATG-3′), and primers A87RT and circ osk 3′ were used to amplify total *osk* mRNA. For normalization, *bcd* mRNA was amplified using primers bcd_up (5′-AACGAGCAAGAAGACGACGCTACAGTCTTG-3′) and bcd_rt (5′-GCGAATAGCGTATTGCAGGGAAAGTATAGA-3′). Amplification efficiencies were comparable as determined using serial 10-fold dilutions of an initial *osk* PCR product as substrate.

In situ hybridization and immunohistochemistry

Whole-mount antibody staining and in situ hybridization using fluorescent RNA probes were performed as previously described (Hachet and Ephrussi, 2004; Tomancak et al., 1998). For whole-mount antibody staining, antigens were detected using the following primary antibodies: mouse anti-BicD (a mix of monoclonals 1B11 and 4C2, 1:10 dilution; gift of Beat Suter), rabbit anti-Staufen (pre-adsorbed, 1:2000 final dilution), rat anti-Bruno (1:5000 dilution) (Filardo and Ephrussi, 2003), mouse anti-Orb (a mix of 48H and 6H4 monoclonals, 1:20 dilution; Developmental Studies Hybridoma Bank) and rabbit anti-Par-1 (1:40 dilution) (Tomancak et al., 2000). For fluorescent detection, Rhodamine- or FITC-coupled goat anti-mouse, rabbit or rat secondary antibodies were used (1:500; Jackson Immuno Research Laboratories).

Construction of transgenes

P(*mM1 mM2stop*) was made in a two-step PCR reaction. The product of a first round of PCR, generated using primers linkerM2mut (5′-AGC-GAGAACAACGGTACCATCATCGAG-3′; ATG→GGT) and osk54Hind (5′-AAGAAAGCTTTCAAACATAAAGCTACTCACTCCTACTCACTG-ATGCTCGATATCGTGATT-3′) was used as primer for a second round of PCR together with M1BssHII (ATG→CGC). The product was then reamplified with the outside primers M1BssHII (5′-TAGGATCCAA-GAATATTGGATCACTTTCCTCCAAGCGCGCGCCGCAGTCACA-3′) and osk54Hind, then cloned into pBluescript (pBSNTL) and sequenced. The fragment was then used to replace the wild-type *Bam*HI (*oskar* promoter)–*Hin*dIII (first intron) fragment of pGem11go6.45, a pGEM11 vector with a 6.45kb *Xho*I-*Apa*I genomic DNA fragment encompassing the *oskar* locus. The mutated *oskar* gene was then transferred into pCasper4 (Pirrotta, 1988) as an *Xho*I-*Not*I fragment (pCaspNTL).

To generate P(*mM1SLmM2*), the hairpin HP7, which blocks scanning by small ribosomal subunits (Kozak, 1989a), was cloned as a blunt-ended *Bam*HI-*Hin*dIII fragment into the blunt-ended *Sph*I site of pBSNTL (pBSNTL HP7; orientation: reconstituted *Bam*HI site proximal to the *oskar* transcription start site). The *Bam*HI-*Xcm*I fragment of pGem11go6.45 was then replaced by the corresponding fragment of pBSNTL HP7 and the *Bst*XI site of the resulting plasmid was destroyed by cutting, filling and religation. This resulted in an additional frame shift (CCACTGG instead of CCACCTGG; sequence not canonical owing to fill-in artefact). The mutated *oskar* transgene was then transferred as an *Xho*I-*Not*I fragment into pCasper4, resulting in P(mM1SLmM2).

pUASp *oskWT* and pUASp *oskΔi(1,2,3)* were constructed by cloning genomic and cDNA versions of *oskar* as *Bam*HI/*Nsi*I fragments of pGem g.osk and pGem g/c.osk, respectively, into pUASp Casper (Rorth, 1998) digested with *Bam*HI/*Pst*I. pGem g.osk was constructed by subcloning a 6.45 kb *Xho*I-*Apa*I fragment of *oskar* genomic DNA into pGem11Zf (Promega). pGem g/c.osk was constructed by replacing a 2425 bp *Bss*HII-*Sac*II fragment of pGem g.osk containing all of the *oskar* introns with the equivalent 2024 bp fragment of the *oskar* cDNA of Blue-osk (Ephrussi et al., 1991).

Complementation and rescue analysis

Trans-heterozygous *oskar* mutant females (A87/54, A87/84, A87/346, 187/54, 187/84 and 187/346) were produced, as well as P*(transgene)*/SM6B; $osk^{187}/Df(3R)p^{XT103}$ and P(*transgene*)/SM6B; $osk^{A87}/Df(3R)p^{XT103}$ females [in which P(*transgene*) represents the various rescue constructs under *osk* promoter]. Rescue analysis using the UAS yeast inducible promoter was performed in the osk$^{A87}/Df(3R)p^{XT103}$ background using pCog-Gal4:VP16 and Nanos-Gal4:VP16 drivers simultaneously.

Flies of the following genotypes were analyzed:

pCog-Gal4:VP16/UAS *oskWT*;; osk^{A87},Nanos-Gal4:VP16/$Df(3R)p^{XT103}$;

pCog-Gal4:VP16/UAS *oskΔi(1,2,3)*;; osk^{A87},Nanos-Gal4:VP16/$Df(3R)p^{XT103}$;

pCog-Gal4:VP16/UAS *osk3′UTR*;; osk^{A87},Nanos-Gal4:VP16/$Df(3R)p^{XT103}$;

pCog-Gal4:VP16/w^{1118}; UAS *osk-K10*/+; osk^{A87},Nanos-Gal4:VP16/$Df(3R)p^{XT103}$.

Test females were collected as virgins and mated with Oregon-R (Fig. 3B), or w^{1118} males (Fig. 3D). The egg-laying capacity of at least 30 individual test females from each experiment was monitored over four days, in egg-laying blocks on apple-juice agar plates, at 25°C. Values (including the wild-type controls) were normalized to the average of eggs laid per day per Oregon-R or w1118 female. The standard deviation was calculated from normalized values.

RESULTS

oskar RNA null mutants fail to complete oogenesis

Two new *oskar* (*osk*) mutants, osk^{A87} and osk^{187}, were isolated in independent mutagenesis experiments. When transheterozygous with the *oskar* nonsense alleles osk^{54}, osk^{84} and osk^{346} (Kim-Ha et

al., 1991; Lehmann and Nüsslein-Volhard, 1986), both osk^{A87} and osk^{187} produce embryos that display the classical 'posterior group phenotype' (lack of an abdomen and germline; data not shown; see also Fig. 3A,B) and thus fail to complement previously known *oskar* alleles. Molecular analysis revealed the presence of I (Crozatier et al., 1988) and ZAM (Leblanc et al., 1997) transposable elements in the upstream regulatory sequences or 1st exon of *oskar* in osk^{187} and osk^{A87}, respectively (Fig. 1A). The position of these mutagenic elements suggested that they might affect the production or stability of *oskar* transcripts. Consistent with this hypothesis, northern analysis, in situ hybridization and RT-PCR of mutant osk^{187} egg chambers confirmed that only residual amounts of *oskar* RNA are present, which are even further reduced in hemizygous animals (Fig. 1B,D; data not shown). In the case of osk^{A87}/Df(3R)p^{XT103} egg chambers, we detected no *oskar* mRNA either by RT-PCR or by in situ hybridization. osk^{A87} therefore is a true RNA null allele of *oskar* (Fig. 1C,D).

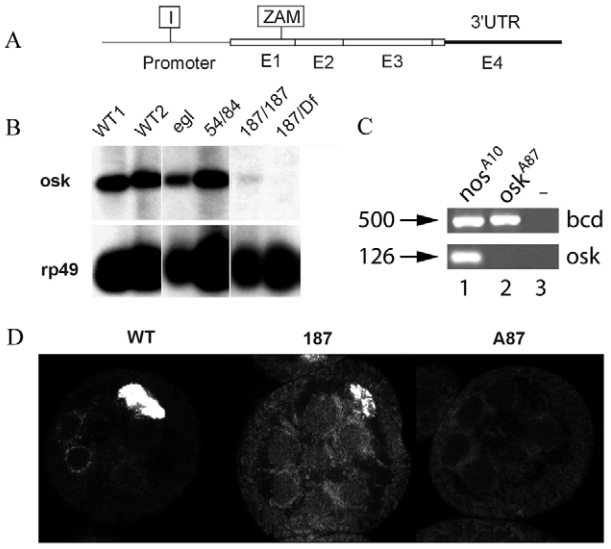

Fig. 1. Characterization of the osk^{A87} and osk^{187} alleles. (**A**) Schematic representation of the *oskar* locus and the insertion points of the transposable elements in the new *oskar* alleles. E1 through E4 represent the *oskar* exons. The first exon contains a 15 nucleotide untranslated region. In osk^{A87}, a ZAM retrotransposable element is inserted 51 bp upstream of the first intron, in reverse orientation relative to *oskar*. In osk^{187}, an I element is inserted 534 bp upstream of the *oskar* transcription initiation site, in the same orientation as *oskar*. (**B**,**C**) Northern blot and RT-PCR analysis of *oskar* mRNA in the new *oskar* mutants. (B) Northern blot: WT1 sample is RNA from wild-type (Oregon R) egg chambers of stages 1 to 14, and WT2 from wild-type egg chambers of stages 1 to 7-8. The egl^{RC12} sample represents ovaries whose development arrested during oogenesis at stages similar to the new *oskar* mutants. osk^{54} and osk^{84} are nonsense alleles of *oskar* (Kim-Ha et al., 1991). Only trace amounts of *oskar* mRNA are detected in homozygous osk^{187} egg chambers and no *oskar* mRNA is detected in osk^{187}/*Df(3R)*p^{XT103} egg chambers. (C) RT-PCR: in contrast to *bcd* (upper panel), no *oskar* mRNA is detected by RT-PCR in osk^{A87}/*Df(3R)*p^{XT103} egg chambers (lower panel, lane 2) while a 126 bp band is detected in a control amplification from nos^{A10}/*Df(3R)*p^{XT103} (lower panel, lane 1), an allele of *nanos* originating from the same genetic background as osk^{A87}. Lanes 3: negative controls without addition of template. (**D**) *oskar* RNA in situ hybridization in osk^{187} and osk^{A87}. Fluorescently-labeled antisense *oskar* RNA probe was used to visualize the presence of endogenous *oskar* RNA in stage 3-4 wild-type, osk^{187}/*Df(3R)*p^{XT103} and osk^{A87}/*Df(3R)*p^{XT103} mutant egg chambers. Compared with wild type, considerably less or no *oskar* RNA is detected in osk^{187}/*Df(3R)*p^{XT103} and in osk^{A87}/*Df(3R)*p^{XT103} egg chambers, respectively.

Surprisingly, and in contrast to females carrying the previously known nonsense alleles of *oskar*, osk^{54}, osk^{84} and osk^{346}, which do not express Oskar protein (as judged by western blots) and are thus considered strong loss of function alleles (Markussen et al., 1995; Rongo and Lehmann, 1996), females carrying only an osk^{A87} or osk^{187} allele [osk^{A87}/*Df(3R)*p^{XT103}, osk^{187}/*Df(3R)*p^{XT103}] fail to lay eggs and are sterile, owing to an early arrest of oogenesis. In both mutants, oocytes are determined and begin to develop, as indicated by the accumulation of BicD (Suter and Steward, 1991), Orb (Lantz et al., 1994), Bruno (Webster et al., 1997) and Par1 (Vaccari and Ephrussi, 2002) in the oocyte (Fig. 2D,F,H,J). Only Staufen, a RNA-binding protein (St Johnston, 1992), the localization of which within the oocyte is interdependent with that of *oskar* mRNA (St Johnston et al., 1991), is not detected in osk^{A87}/Df(3R)p^{XT103} oocytes (Fig. 2B), indicating that Staufen accumulation in the oocyte is mediated by *oskar* mRNA. Defects in osk^{A87}/Df(3R)p^{XT103} egg chambers first become evident at stage 2, when fragmentation of the normally compact karyosome is observed (Fig. 2L). Mutant egg chambers continue to develop until stage 7, when they begin to degenerate.

To further prove that the oogenesis defects are due to mutations in *oskar*, we performed genetic rescue experiments using three *oskar* transgenes, encoding either both of the Oskar isoforms (Markussen et al., 1995; Rongo et al., 1995), or each isoform individually (Fig. 3A). The first transgene, P*(osk+)* (Markussen et al., 1995), consists of a genomic DNA fragment that encompasses the *oskar* locus and encodes both Oskar isoforms. P*(M1L)* contains a mutation in M1, the first translation initiation site in *oskar* mRNA, and therefore produces only Short Oskar, the isoform responsible for pole plasm formation (Markussen et al., 1995). Conversely, P*(M139L)* produces only Long Oskar, owing to a mutation in M2, the second translation initiation site in *oskar* mRNA (Markussen et al., 1995). Long Oskar is essential for the cortical anchoring of *oskar* RNA and thus for correct localization of the pole plasm, but fails to rescue the abdominal and germ cell defects of the *oskar* protein null mutants (Markussen et al., 1995; Rongo et al., 1995; Vanzo and Ephrussi, 2002). All three transgenes, P*(osk+)*, P*(M1L)* and P*(M139L)*, fully complement the egg-less phenotype of osk^{A87}/Df(3R)p^{XT103} and osk^{187}/Df(3R)p^{XT103}, indicating that these are indeed *oskar* alleles (Fig. 3B).

oskar transcript but not Oskar protein is required for completion of oogenesis

The surprising observation that the osk^{54}, osk^{84} and osk^{346} nonsense mutant alleles (Kim-Ha et al., 1991) rescue the early oogenesis defect of the new *oskar* mutants (giving rise to the 'posterior group phenotype'; see also Fig. 3A,B), suggested that the early function of *oskar* might be mediated by *oskar* RNA, rather than by Oskar protein. To rule out the possibility that a truncated, unstable and thus undetectable Oskar peptide that is responsible for rescue of the oogenesis defects of osk^{A87} and osk^{187} is produced by the nonsense alleles, we constructed two translationally incapacitated, protein null *oskar* alleles, and tested their ability to rescue the new alleles. The first construct, P*(mM1 mM2stop)*, consists of an *oskar* gene identical to the osk^{54} nonsense allele, but whose capacity to produce a short peptide initiating from M1 or M2 was abolished by mutation of M1 and M2 to CGC and GGT, respectively (Fig. 3A). The second construct, P*(mM1SLmM2)*, also containing a mutated M1 and M2, was additionally designed to prevent the initiation of translation by blocking scanning small ribosomal subunits, as well

as to abolish putative translation of Oskar peptides that might initiate from in-frame methionine codons elsewhere in the *oskar* transcript. To this end, a sequence predicted to adopt a stable hairpin structure and that has been shown to block translation by stalling scanning ribosomes (Kozak, 1989b) was inserted between mutated M1 and M2, and a frame-shift was introduced downstream of M2 (Fig. 3A). Both P*(mM1 mM2stop)* and P*(mM1SLmM2)* fully complement the egg-less phenotype of osk^{A87}/Df(3R)p^{XT103} and osk^{187}/Df(3R)p^{XT103}, to the same extent as the original P*(osk+)* transgene (Fig. 3B). However, the embryos produced lack an abdomen, confirming the absence of Oskar protein. These results demonstrate that no feature of Oskar protein is required for early oogenesis, indicating that this function of *oskar* is mediated by another aspect of the gene.

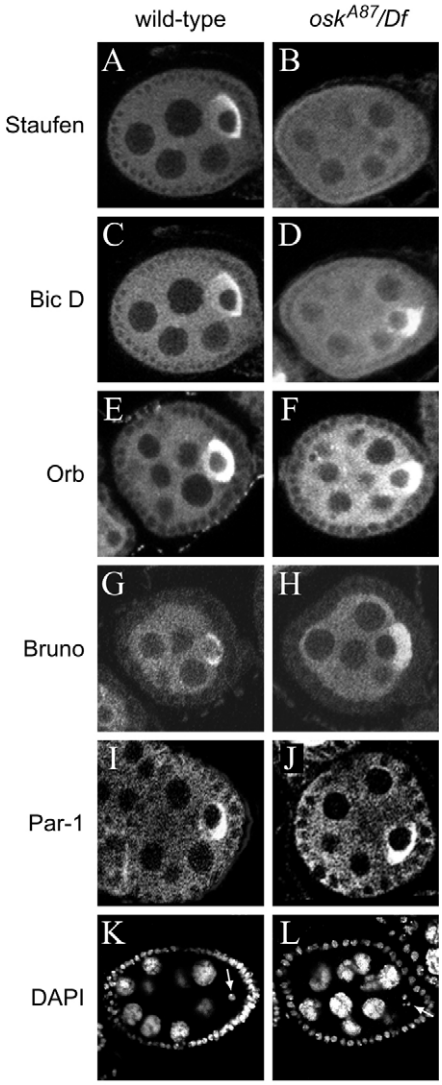

Fig. 2. An oocyte is specified in $osk^{A87}/Df(3R)p^{XT103}$. (**A**,**B**) Whole mount antibody staining of $osk^{A87}/Df(3R)p^{XT103}$ ovaries (B) shows that Staufen is not enriched in the osk^{A87}mRNA null oocytes compared with wild type (A). (**C-J**) In contrast, the oocyte markers BicD (Suter and Steward, 1991), Orb (Lantz et al., 1994), Bruno (Webster et al., 1997), and Par-1 (Shulman et al., 2000; Tomancak et al., 2000) accumulate in a single, posterior cell in both wild-type (C,E,G,I) and $osk^{A87}/Df(3R)p^{XT103}$ (D,F,H,J) egg chambers, indicating that early steps in oocyte specification occur normally in the mutant. (**K**,**L**) DAPI staining of chromatin in stage 4 wild-type (K) and $osk^{A87}/Df(3R)p^{XT103}$ (L) egg chambers. The compact structure of the karyosome (arrow) is detected within the wild-type oocyte nucleus. $osk^{A87}/Df(3R)p^{XT103}$ mutant oocytes show a fragmented karyosome structure (arrow).

The 3′UTR of *oskar* is sufficient to rescue the oogenesis arrest phenotype of osk^{A87}

All constructs that rescue the oogenesis arrest phenotype of the *oskar* RNA null alleles (see above) contain the endogenous *oskar* promoter, the coding region, the introns and the *oskar* 3′UTR. We therefore wanted to address which region of the *oskar* locus is required for its function in early oogenesis. To exclude the promoter region, we made use of a set of transgenes in which the *oskar* promoter was replaced by the yeast UAS promoter, thus placing the *oskar* gene under Gal4 transcriptional control (Brand and Perrimon, 1993). We first analyzed the UAS *oskWT* transgene which only differs from the P(*osk*+) transgene by the replacement of the *oskar* promoter region by the UAS promoter (Fig. 3C). When driven by a combination of pCogGal4:VP16 and NosGal4:VP16 drivers, UAS *oskWT* rescues the early oogenesis arrest of $osk^{A87}/Df(3R)p^{XT103}$ females, as well as the posterior group phenotype of the progeny (Fig. 3D; data not shown). This rules out an involvement of the promoter region in *oskar* rescue activity and demonstrates that these drivers are sufficient to drive expression of a rescuing transgene. To assess a possible role of *oskar* intronic sequences in early *oskar* function, we tested the rescue ability of a UAS *oskar* transgene called UAS *oskΔi(1,2,3)*, in which the three *oskar* introns were deleted (Fig. 3C). $osk^{A87}/Df(3R)p^{XT103}$ females expressing *oskΔi(1,2,3)* produce a normal number of eggs (Fig. 3D), indicating that the *oskar* introns are not essential for early *oskar* function.

We then tested whether the *oskar*-coding region or the 3′UTR are required to rescue the early oogenesis phenotype. Expression of a UAS *osk-K10* transgene in which the *oskar* 3′UTR was replaced by that of *K10* (Fig. 3C) (Riechmann et al., 2002) under the control of the same Gal4 drivers successfully used above, is unable to rescue the early oogenesis defect of the new *oskar* alleles, even when the flies are raised at 29°C in order to increase the expression level of the transgene (Fig. 3D and not shown). To confirm the functionality of this transgene, we tested whether osk^{A87}/osk^{+} heterozygous flies overexpressing UAS *osk-K10* produce delocalized Oskar activity during embryogenesis. Indeed, 83% of embryos laid by such females are bicaudal, showing that the UAS *osk-K10* transgene is functional. Furthermore, real-time PCR on cDNA of ovaries from $osk^{A87}/Df(3R)p^{XT103}$ females expressing the UAS *osk-K10* transgene under control of both Gal4 drivers confirmed that the UAS *osk-K10* transgene is expressed at early stages of oogenesis (before stage 7 when oogenesis arrests; Fig. 4C; see 4A and 4B for schematic and exact genotypes). The transcript is, however, present at lower levels than the rescuing oskWT transcript, probably because of the degeneration of the ovaries (note that in wild-type background, the transcript levels of UAS oskWT and UAS oskK10 are similar; data not shown). Nevertheless, antibody staining showed that the UAS *osk-K10* RNA is translated well before oogenesis arrests (Fig. 4D). The fact that UAS *osk-K10* fails to rescue early oogenesis confirms our observation that Oskar protein does not provide early *oskar* function. In addition, it indicates that the *oskar* 3′UTR might provide the early oogenesis function of *oskar*.

We, thus, directly tested the capacity of the *oskar* 3′UTR to rescue the $osk^{A87}/Df(3R)p^{XT103}$ early oogenesis arrest. Remarkably, expression of the *oskar* 3′UTR alone from the UAS *osk3′UTR*

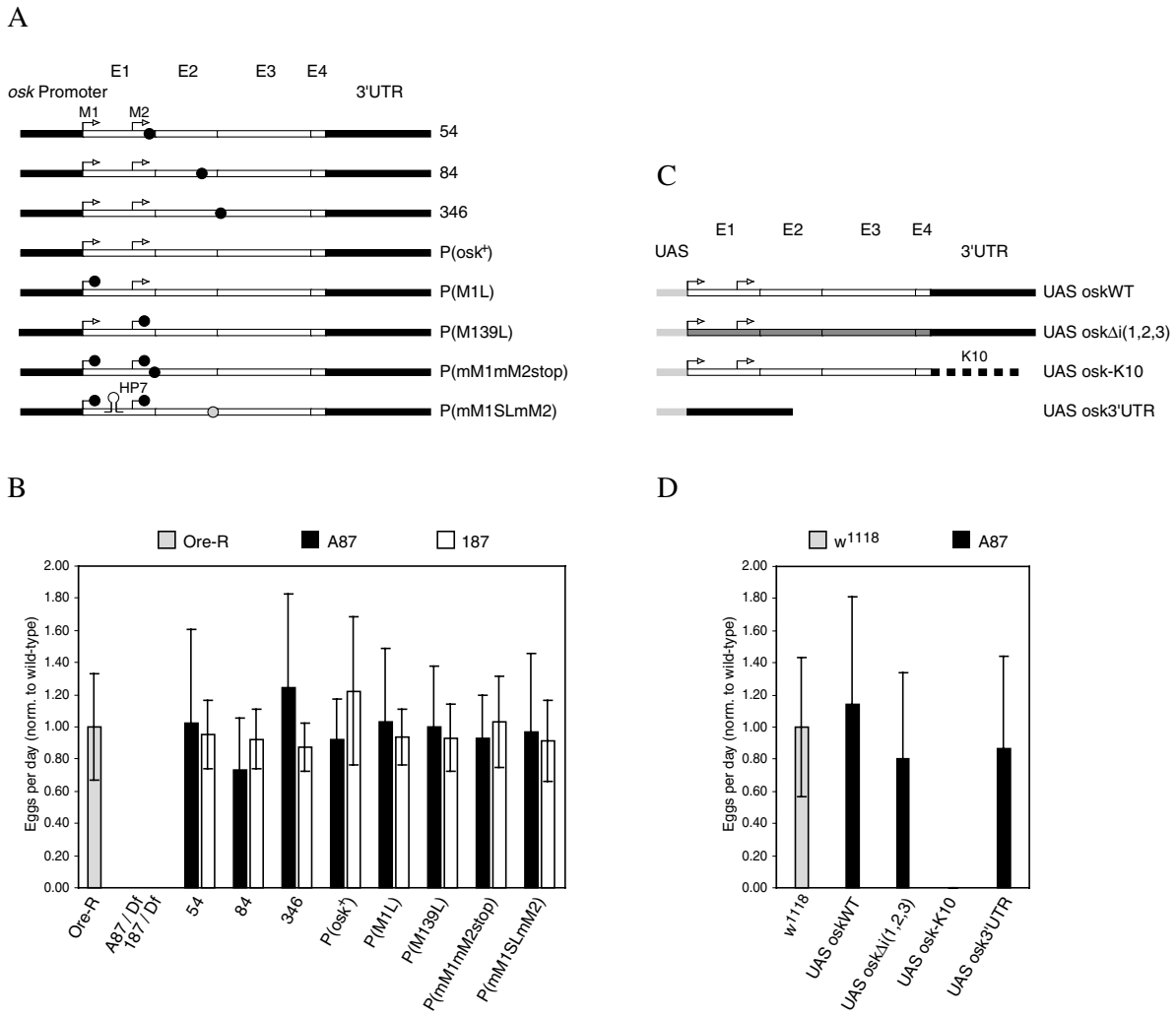

Fig. 3. The *oskar* 3′UTR is sufficient to rescue the oogenesis arrest phenotype of *oskar* RNA null alleles. (**A**,**C**) Schematic of *oskar* alleles and transgenes. Solid black bars represent the *oskar* promoter and 5′UTR (left) and the 3′UTR (right). M1 and M2 are the two translation initiation sites of *oskar*. E1 through E4 indicate the *oskar* exons. Black dots in *osk*^{54}, *osk*^{84}, *osk*^{346} and P(*mM1 mM2stop*) transcripts show the positions of the stop codons in the nonsense alleles and the transgene. P(*osk*+) is a wild-type *oskar* transgene that encodes both the long and short Oskar isoforms and fully rescues the *oskar* strong loss of function alleles (Markussen et al., 1995). Black dots at M1 and M2 in P(*oskM1L*), P(*oskM139L*), P(*mM1 mM2stop*) and P(*mM1SLmM2*) indicate the mutated translation initiation sites. HP7 represents the hairpin loop sequence (Kozak, 1989a) inserted between M1 and M2, and the grey dot shows the frame-shift mutation inserted in P(*mM1SLmM2*). (C) Light grey bars represent the UAS promoter. UAS *oskWT* expresses the *oskar* gene (including introns) under the control of the yeast UAS promoter. UAS *oskΔi(1,2,3)* expresses an *oskar* RNA whose three introns have been deleted (dark grey bar), under control of the UAS promoter. Note that all UAS-driven transcripts contain an intron derived from the pUASp vector. Dashed line in UAS *osk-K10* indicates the K10 3′UTR. UAS *osk3′UTR* expresses only the 3′UTR of *oskar* without any *oskar* coding region under control of UAS. To ensure continued high-level expression throughout oogenesis, expression of all UAS transgenes was driven by pCOG:Gal4VP16 and nanos:Gal4VP16 simultaneously. Flies were grown at 25°C. In the case of the UAS osk-K10 transgene, rescue was also tested at 29°C, at which Gal4-induced expression is maximal. (**B**,**D**) Average number and standard deviation produced daily by females of the genotype indicated underneath (normalized to the average number of eggs laid by wild-type females). See text for details. (B) Grey bar: Oregon-R control. Black and white bars: alleles or transgenes in *osk*^{A87}/Df(3R)*p*^{XT103} and *osk*^{187}/Df(3R)*p*^{XT103} background, respectively. (D) Grey bar: *w*^{1118} control. Black bars: transgenes in *osk*^{A87}/Df(3R)*p*^{XT103} background.

transgene (Fig. 3C) (Filardo and Ephrussi, 2003) driven by the combination pCogGal4:VP16 and NosGal4:VP16 is sufficient to rescue the early oogenesis and egg-less phenotypes (Fig. 3D). As expected, the 3′UTR also rescues the karyosome defect (Fig. 5D), but does not rescue the late *oskar* phenotype of the resulting embryos, which display the ‘posterior group phenotype’ (data not shown). This demonstrates that the function of *oskar* during early oogenesis is mediated by *oskar* RNA, independent of Oskar protein, and demonstrates that the *oskar* 3′UTR is sufficient to perform this function.

We also analyzed the localization of Staufen in the ovaries of *osk*^{A87}*/Df(3R)p*^{XT103} females expressing either the UAS *osk3′UTR* or the UAS *osk-K10* transgene. In the case of UAS *osk-K10*, no Staufen protein was detected in the oocyte, indicating that Staufen fails to associate with *osk-K10* RNA (Fig. 5B). By contrast, expression of the *oskar* 3′UTR alone was sufficient to restore Staufen accumulation in the oocyte, showing that Staufen associates with *oskar* RNA though its 3′UTR (Fig. 5C). However, during late oogenesis, Staufen and *oskar* 3′UTR RNA fail to localize at the posterior of *osk*^{A87}*/Df(3R)p*^{XT103} oocytes [compare

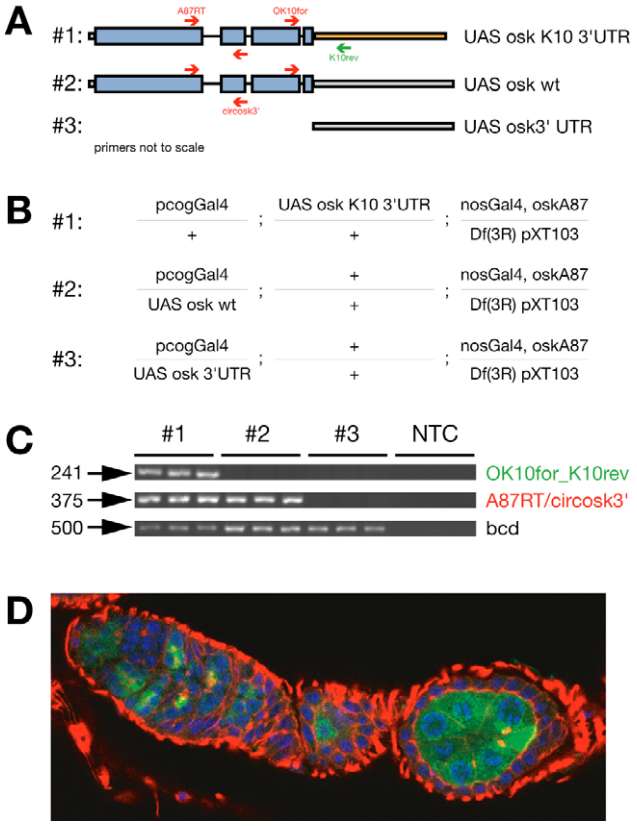

Fig. 4. UAS *osk-K10* produces Oskar protein before oogenesis arrest in *osk^{A87}/Df(3R)p^{XT103}*. (**A**) Scheme showing transgene structure and priming sites of PCR primers used for Real-time PCR. Large blue boxes represent *oskar* coding region. Grey and orange thin boxes represent *oskar* and *K10* UTRs, respectively. Thin lines correspond to introns. Oskar specific primers are in red, K10 specific primer is in green. (**B**) Genotypes of ovaries analyzed. Numbers correspond to numbers in panels A, C. (**C**) Two percent agarose gel showing end products of Real-time PCR reactions performed in triplicate using cDNA of early stage ovaries of genotype numbered on top. *bicoid* (*bcd*) was used for normalization. UAS oskK10 is about 15 fold less abundant than UAS osk WT. NTC: non-template control. (**D**) Antibody staining of non-rescued ovaries from females expressing UAS *oskK10* (genotype #1) show that Oskar protein (green) is expressed as early as in germaria. F-actin and DNA are in red and blue, respectively.

Fig. 5F,H with 5E,G (UAS *osk* WT)]. These observations confirm our previous results showing that the *oskar* 3′UTR is not sufficient for *oskar* RNA localization at the posterior of the oocyte (Hachet and Ephrussi, 2004). It therefore appears that the function supplied by the *oskar* 3′UTR for oocyte progression past early oogenesis is independent of the assembly of the *oskar* RNP localization complex that mediates its transport to the oocyte posterior in mid-oogenesis.

DISCUSSION

The new *oskar* alleles *osk^{A87}* and *osk^{187}* reveal that *oskar* plays a crucial role during early oogenesis. This early function of *oskar* is unusual, as it is mediated by *oskar* RNA, and not by Oskar protein. The low but detectable amount of *oskar* RNA observed in *osk^{187}* mutants indicates that a threshold exists, below which egg chambers fail to develop. Our findings raise the possibility that many protein-coding genes may fulfill additional non-coding functions and thus increase the spectrum of gene function.

What might the function of *oskar* RNA be during early oogenesis? Oskar protein serves as a scaffold for the assembly of cytoplasmic structures essential for germline development, the polar granules, at the posterior pole of the oocyte and embryo. During early oogenesis, *oskar* RNA might provide a similar scaffold function for assembly of cytoplasmic complexes essential for the progression of oocyte development. RNAs are associated with proteins in ribonucleoprotein complexes during most of their existence, from their emergence as nascent transcripts in the nucleus, during nucleo-cytoplamic transport, to their final cytoplasmic localization, translation or degradation (Shyu and Wilkinson, 2000).

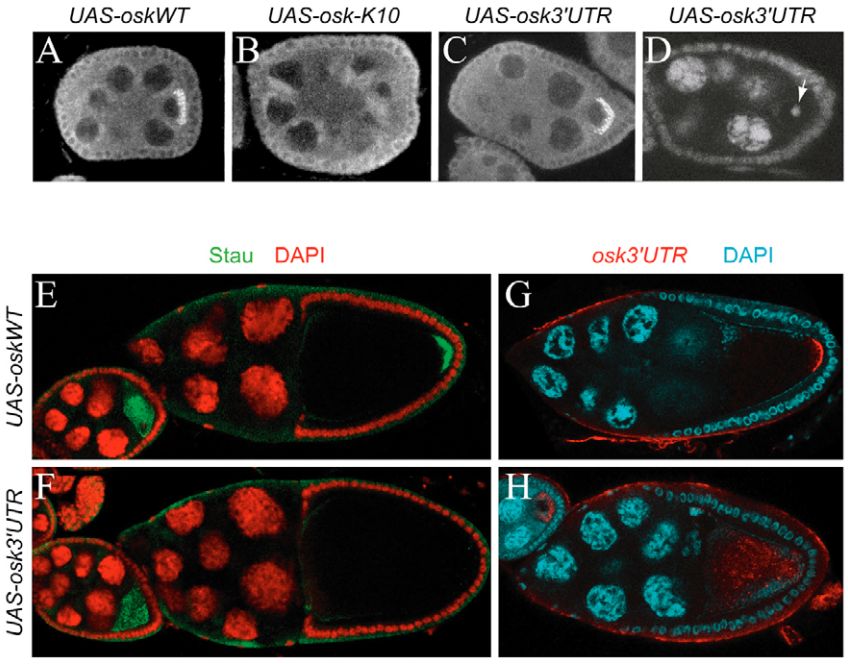

Fig. 5. The *oskar* 3′UTR is sufficient for Staufen transport to the oocyte, but not for *oskar* RNA localization at the posterior pole. (**A-C**) Staufen revealed by immunofluorescence in stage 4 *osk^{A87}/Df(3R)p^{XT103}* egg chambers expressing pCogGal4:VP16;NosGal4:VP16-driven UAS *oskWT* (A), UAS *oskK10* (B), and UAS *osk3′UTR* (C). (**D**) The karyosome defect is rescued in egg chambers expressing pCogGal4:VP16;NosGal4:VP16-driven UAS *osk3′UTR*. White arrow marks an intact karyosome revealed by DAPI stain. (E-H) Top and bottom panels show stage 10 *osk^{A87}/Df(3R)p^{XT103}* egg chambers expressing UAS-driven *oskWT* and *osk3′UTR*, respectively. (**E**,**F**) Staufen detected by immunofluorescence is shown in green; DAPI staining is in red. (**G**,**H**): *oskar* RNA detected by fluorescent in situ hybridization is shown in red; DAPI staining is in blue.

Staufen protein requires *oskar* RNA for its transport from the nurse cells into the oocyte (Fig. 2B). In addition, we have shown that it is the *oskar* 3′UTR that mediates accumulation of Staufen protein in the oocyte (Fig. 5). This reveals that the well-known mutual interdependence of Staufen and *oskar* mRNA in their localization during oogenesis is mediated by interaction of Staufen with the *oskar* 3′UTR. Of the candidate proteins we examined, only Staufen showed a clearly altered distribution in the *oskar* RNA null mutant, yet Staufen itself does not appear to play a role during early oogenesis (St Johnston et al., 1991). It is therefore reasonable to assume that *oskar* RNA acts as a structural partner for the transport into the oocyte of additional, so far unidentified proteins or RNAs essential for its development. In this regard it is interesting to note that both *VgT* RNA and the non-coding *Xlsirts* RNA have been shown to mediate anchoring of several RNAs at the vegetal pole of the *Xenopus* oocyte (Heasman et al., 2001; Kloc and Etkin, 1994; Kloc et al., 2005).

Alternative functions of the *oskar* 3′UTR are also plausible. In particular, the *oskar* 3′UTR might bind and sequester a negative regulator that, in its free form (i.e. in an *oskar* RNA null background), inhibits early oogenesis. One candidate that has been shown to bind to the *oskar* 3′UTR is the translational regulator Bruno (Kim-Ha et al., 1995). However, overexpression of Bruno – at least in the presence of wild-type levels of *oskar* mRNA – does not cause a phenotype similar to that of the *oskar* RNA null mutant (Filardo and Ephrussi, 2003). Thus, to fully understand the mechanism of oogenesis arrest resulting from absence of *oskar* mRNA, it will be important to identify other proteins and RNAs binding to the *oskar* 3′UTR that are required for egg chamber development.

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