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# Oskar allows *nanos* mRNA translation in *Drosophila* embryos by preventing its deadenylation by Smaug/CCR4

Sophie Zaessinger, Isabelle Busseau and Martine Simonelig\*

Anteroposterior patterning of the *Drosophila* embryo depends on a gradient of Nanos protein arising from the posterior pole. This gradient results from both *nanos* mRNA translational repression in the bulk of the embryo and translational activation of *nanos* mRNA localized at the posterior pole. Two mechanisms of *nanos* translational repression have been described, at the initiation step and after this step. Here we identify a novel level of *nanos* translational control. We show that the Smaug protein bound to the *nanos* 3' UTR recruits the deadenylation complex CCR4-NOT, leading to rapid deadenylation and subsequent decay of *nanos* mRNA. Inhibition of deadenylation causes stabilization of *nanos* mRNA, ectopic synthesis of Nanos protein and head defects. Therefore, deadenylation is essential for both translational repression and decay of *nanos* mRNA. We further propose a mechanism for translational activation at the posterior pole. Translation of *nanos* mRNA at the posterior pole depends on *oskar* function. We show that Oskar prevents the rapid deadenylation of *nanos* mRNA by precluding its binding to Smaug, thus leading to its stabilization and translation. This study provides insights into molecular mechanisms of regulated deadenylation by specific proteins and demonstrates its importance in development.

**KEY WORDS:** CCR4-NOT complex, Deadenylation, *Drosophila*, P bodies, Translational control

## INTRODUCTION

Post-transcriptional mechanisms of gene regulation play a prominent role during early development. Because the oocyte and developing embryo go through a phase in which no transcription takes place, gene expression relies on a pool of maternal mRNAs accumulated during oogenesis and is regulated at the level of translation or mRNA stability. It has been shown in several biological systems that poly(A) tail shortening contributes to translational silencing, whereas translational activation requires poly(A) tail extension (Richter, 2000; Tadros and Lipshitz, 2005). Poly(A) tail shortening, or deadenylation, is also the first step in mRNA decay. Subsequent steps occur only after the poly(A) tail has been shortened beyond a critical limit (Meyer et al., 2004; Parker and Song, 2004). Rapid deadenylation of unstable RNAs is caused by destabilizing elements, for example AU-rich elements (AREs) found in the 3' UTRs of several mRNAs. A number of proteins have been identified that bind to destabilizing RNA sequences and accelerate deadenylation as well as subsequent steps of decay (Meyer et al., 2004).

In yeast, deadenylation is mostly catalyzed by the multi-subunit CCR4-NOT complex (Tucker et al., 2001), and this complex is also involved in deadenylation in *Drosophila* (Temme et al., 2004) and in mammalian cells (Chang et al., 2004; Yamashita et al., 2005). A second conserved deadenylase, the heterodimeric PAN2-PAN3 complex, appears to act before the CCR4-NOT complex (Yamashita et al., 2005). A third enzyme, the poly(A)-specific ribonuclease (PARN) (Korner and Wahle, 1997) is present in most eukaryotes but has not been found in yeast and *Drosophila*.

Translational regulation of maternal mRNAs in *Drosophila* is essential to the formation of the anteroposterior body axis of the embryo. During embryogenesis, a gradient of the Nanos (Nos) protein arises from the posterior pole (Gavis and Lehmann, 1994)

and organizes abdominal segmentation (Wang and Lehmann, 1991). This gradient results from translational regulation of maternal *nos* mRNA. The majority of *nos* transcripts is uniformly distributed throughout the bulk cytoplasm and is translationally repressed (Dahanukar and Wharton, 1996; Gavis et al., 1996; Smibert et al., 1996) and subsequently degraded during the first 2-3 hours of embryonic development (Bashirullah et al., 1999). A small proportion of *nos* transcripts is localized in the pole plasm, the cytoplasm at the posterior pole that contains the germline determinants (Bergsten and Gavis, 1999). This RNA escapes repression and degradation, and its translation product forms a concentration gradient from the posterior pole (Gavis and Lehmann, 1994). Both translation activation at the posterior pole and repression elsewhere in the embryo are essential for abdominal development, and head and thorax segmentation, respectively (Dahanukar and Wharton, 1996; Smibert et al., 1996; Wang and Lehmann, 1991; Wharton and Struhl, 1991).

Translation of *nos* mRNA is repressed in the embryo by Smaug (Smg), which binds two Smaug response elements (SREs) in the proximal part of the *nos* 3' UTR (Dahanukar et al., 1999; Dahanukar and Wharton, 1996; Smibert et al., 1999; Smibert et al., 1996). The SREs are also essential for the decay of *nos* mRNA (Bashirullah et al., 1999; Dahanukar and Wharton, 1996; Smibert et al., 1996). Repression of *nos* translation appears to be a multistep process, involving at least one level of regulation at the initiation step (Nelson et al., 2004) and another after *nos* mRNA has been engaged on polysomes (Clark et al., 2000; Markesich et al., 2000). Repression at the initiation step is thought to involve an interaction between Smg and the protein Cup. The latter associates with the cap-binding initiation factor eIF4E, displacing the initiation factor eIF4G (Nelson et al., 2004). Translation of *nos* mRNA at the posterior pole depends on Oskar (Osk) protein, although its mechanism of action has remained unknown (Ephrussi and Lehmann, 1992; Smith et al., 1992; Wang and Lehmann, 1991).

Bulk *nos* mRNA has a short poly(A) tail, and it was thought that *nos* translational control was independent of poly(A) tail length regulation (Gavis et al., 1996; Salles et al., 1994). More recently,

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Smg and its yeast homologue Vts1 were shown to be involved in the degradation of mRNAs (Aviv et al., 2003; Semotok et al., 2005). Smg induces degradation and deadenylation of *Hsp83* mRNA during early embryogenesis. This appears to result from recruitment by Smg of the CCR4-NOT deadenylation complex on *Hsp83* mRNA, although the Smg-binding sites in this mRNA have not been identified. However, *Hsp83* mRNA deadenylation was reported not to repress its translation (Semotok et al., 2005). Here, we show that *nos* mRNA is subject to regulation by active deadenylation by the CCR4-NOT deadenylation complex. This deadenylation depends on Smg and on the SREs in the 3' UTR of *nos* mRNA. We confirm the model of the CCR4-NOT complex recruitment by Smg, in that case, onto *nos* mRNA, using genetic interactions between mutants affecting *smg* and the CCR4 deadenylase, and showing the presence in a same protein complex of endogenous Smg and CAF1, a protein of the CCR4-NOT complex. We also show that active deadenylation of *nos* mRNA contributes to its translational repression in the bulk embryo and is essential for the anteroposterior patterning of the embryo. Moreover, we find that Osk activates translation of *nos* by preventing the specific binding of Smg protein to *nos* mRNA, thereby precluding active deadenylation and destabilization of *nos* mRNA.

## MATERIALS AND METHODS

### *Drosophila* stocks and genetics

The *w<sup>1118</sup>* stock was used as a control. *twin* mutants were *twin<sup>KG877</sup>* (Temme et al., 2004), and *twin<sup>I2209</sup>* and *twin<sup>8115</sup>* (Benoit et al., 2005), which were generated by DGSP (*Drosophila* Gene Research Project, Tokyo Metropolitan University). *P*-elements in *twin<sup>KG877</sup>* and *twin<sup>I2209</sup>* are inserted in a region that overlaps *twin* and the *cav* gene; we checked that these two *twin* alleles complement the null allele of *cav* (Cenci et al., 2003) for lethality and ovarian phenotypes. Two deficiencies overlapping *twin* were used, *Df(3R)crb-F89-4* and *Df(3R)Exel6198* (Bloomington Stock Center). *smg* mutants were *smg<sup>1</sup>* and a deficiency overlapping *smg*, *Df(Scj<sup>rk6</sup>)* (Dahanukar et al., 1999). *nos<sup>BN</sup>* mutant does not produce *nos* mRNA (Wang et al., 1994). *nos(ΔTCE)* stocks are transgenic lines containing a *nos* transgene in which the first 184 nucleotides (nt) of the 3' UTR have been removed (Dahanukar and Wharton, 1996). This 184 nt region includes both SREs. Two independent transgenic *nos(ΔTCE)* stocks were used with the same results. In embryos from *nos(ΔTCE)/+*; *nos<sup>BN</sup>* mothers, all *nos* mRNA is produced by the *nos(ΔTCE)* transgene. *smg* mutants, *nos<sup>BN</sup>* and *nos(ΔTCE)* stocks were gifts from R. Wharton. *osk<sup>34</sup>* is a null allele. Osk overexpression in embryos was performed using *UASp-osk-K10* (Riechmann et al., 2002) (gift from A. Ephrussi) and the germline driver, *nos-Gal4:VP16* (*nos-Gal4* stock) (Rorth, 1998).

### Immunoprecipitations

Embryos 0-3 hours old were homogenized on ice in four volumes of DXB-150 (Nakamura et al., 2001) containing 1 mmol/l AEBSF, 1 μg/μl pepstatin, 1 μg/μl leupeptin, 10 μg/μl aprotinin. The homogenate was cleared by two centrifugations at 10,000 g for 5 minutes. Seven hundred microlitres of the cleared supernatant were mixed with 50 μl of wet protein-A sepharose beads and 5 μl of anti-Smg antibody (Dahanukar et al., 1999) or 5 μl of rabbit serum, in the presence of either RNasin (100 units, Promega) or RNase A (100 μg, Sigma), and incubated for 3 to 4 hours at 4°C on a rotator. The beads were washed six times with DXB-150. For western analyses, the beads were resuspended in one volume of SDS sample buffer. Antibodies were affinity-purified anti-CAF1 or anti-CCR4 (Temme et al., 2004), and anti-Smg. For RNA extraction, the beads were treated with phenol-chloroform, and RNA was resuspended in 11 μl water after isopropanol precipitation in the presence of glycogen.

### RNA

PAT assays were performed as described previously (Juge et al., 2002; Salles and Strickland, 1999) with the specific primers 5'-TTTTGTTTAC-CATTGATCAATTTTTC for *nos* mRNA and 5'-GGATTGCTACAC-CTCGGCCCGT for *sop* mRNA. RT-PCR were performed as reported

previously (Benoit et al., 2002), with the same RNA preparations used for PAT assays. Primers for RT-PCR were 5'-CTTGTTCATCGTCGTGGC-CG and 5'-GTTGAAATGAATACTTGCATACATG for *nos* mRNA, 5'-CCAAGCACTTCATCCGCCACCAGTC and 5'-TCCGACCAGTTA-CAAGAACTCTCA for *rp49* mRNA, and 5'-ATCTCGAACTCTTTG-ATGGGAAGC and 5'-CACCCCAATAAAGTTGATAGACCT for *sop* mRNA. RT-PCR was carried out on serial dilutions of the cDNA templates. PCR from dilution 1/5 are shown. RNA preparations were from 20 embryos each. RT-PCR following Smg immunoprecipitation was performed as follows: 2 μl RNA recovered from the beads was reverse transcribed (SuperScript II Reverse Transcriptase, Invitrogen) in 25 μl using oligo-dT<sub>12-18</sub> primer. Several dilutions of these cDNAs were used in PCR with two pairs of primers to amplify *nos* mRNA and either of *rp49* or *sop* mRNAs, in the same reaction. Two independent sets of immunoprecipitations were performed, followed in each case by several independent RT-PCR. PCR products were analysed on 2% agarose gels. Quantifications were done using ImageJ. Real-time PCR (QPCR) were performed with the Lightcycler System (Roche Molecular Biochemical) using primers 5'-CGGAG-CTTCCAATTCCAGTAAC and 5'-AGTTATCTCGACTGAGTGGCT for *nos* mRNA.

### Antibodies, western blots and immunostaining

Western blots and immunostaining were performed as reported (Benoit et al., 2005; Benoit et al., 1999). Antibody dilutions were 1/1000 for western blots and as follows for immunostaining: rabbit anti-Nos, 1/1000 (A. Nakamura, unpublished), anti-CCR4, 1/300 and anti-CAF1, 1/500 (Temme et al., 2004), guinea pig anti-Smg 1/1000 (C. Smibert, unpublished), anti-Pacman, 1/500 (Newbury and Woollard, 2004), anti-human Dcp1, 1/500 (van Dijk et al., 2002), anti-HtsRC, 1/1 (Robinson et al., 1994) (from Developmental Studies Hybridoma Bank).

### RNA in situ hybridization and cuticle preparations

Whole-mount in situ hybridization and cuticle preparations were performed by standard methods. The probe for in situ hybridization was an RNA antisense made from the pN5 *nos* cDNA clone (Wang and Lehmann, 1991).

## RESULTS

### *ccr4/twin* function is essential in the female germline

We previously characterized the *ccr4* gene (Temme et al., 2004), which was found to be identical to the gene called *twin* (Morris et al., 2005), genetically identified before (Spradling, 1993). We thus renamed *ccr4*, *twin*, according to FlyBase. Using the *twin<sup>KG877</sup>* allele (previously *ccr4<sup>KG877</sup>*), we showed that the CCR4 deadenylase is required for deadenylation of bulk mRNA in the soma, although *twin* function is not required for viability (Temme et al., 2004). However, a certain level of sterility and maternal effect embryonic lethality in *twin* mutant females (Fig. 1E,F) suggested that *twin* function might be required in the female germline for oogenesis and early embryonic development. To investigate this possibility, we characterized two new *P*-element alleles, *twin<sup>I2209</sup>* and *twin<sup>8115</sup>*, generated by DGSP (*Drosophila* Gene Search Project, Tokyo Metropolitan University; Fig. 1A). Both mutants were homozygous viable and showed a substantial decrease in CCR4 protein levels in ovaries, analysed by immunostaining (Fig. 1B-D). Maternal effect embryonic lethality and ovarian defects were examined for all three mutants either homozygous or in combination with a deficiency overlapping the *twin* locus (Fig. 1E,F). Based on these two phenotypes, the three alleles form an allelic series in which *twin<sup>KG877</sup>* is the weakest allele and *twin<sup>8115</sup>* the strongest. The most striking aspect of *twin* ovarian phenotypes concerns defects in cell division. *Drosophila* egg chambers result from four rounds of synchronous divisions of a cystoblast, which generate cysts of 16 germ cells interconnected by ring canals. From two cells, the pro-oocytes that are connected with four neighbours, one becomes the oocyte, whereas the remaining 15 cells become

polyploid nurse cells (Spradling, 1993). A frequent defect in *twin* egg chambers was that they contained more than 16 germ cells, including chambers with 32 germ cells containing an oocyte with five ring canals, which indicated that the cyst had undergone a fifth round of division (Fig. 1G,H,I,K). The remaining mutant egg chambers showed a germ cell number lower than 16 or degeneration (Fig. 1F,I). This is consistent with *twin* ovarian phenotypes described earlier (Morris et al., 2005) and indicates a role of *twin* in the control of cell division. An increase in the amount of either Cyclin A or Cyclin B protein causes the cyst to undergo a fifth division (Lilly et al., 2000). *Cyclin A* and *Cyclin B* mRNA poly(A) tails were shown to be longer in *twin* mutant ovaries, leading to elevated amounts of Cyclin A and B (Morris et al., 2005). Consistent with *twin* cell cycle defects in ovaries, DAPI staining of embryos from *twin* mutant females showed that syncytial blastoderm nuclei divided asynchronously (Fig. 1L,M).

These results show that, although *twin* function is not essential for viability, regulated deadenylation of specific target mRNAs by CCR4 is required for oogenesis and early embryonic development.

### ***nos* mRNA degradation in the embryo depends on deadenylation by CCR4**

Because translational regulation of *nos* mRNA is essential for early embryogenesis, and progressive degradation of *nos* mRNA during the first hours of embryogenesis has been documented, we focused on this mRNA and asked whether its degradation in the bulk embryo resulted from deadenylation by CCR4. RNAs prepared from embryos spanning 1 hour intervals during the first 4 hours of embryogenesis were analysed by RT-PCR and PAT assays (Salles and Strickland, 1999), a technique that allows the measurement of poly(A) tail length of specific mRNAs. In wild-type embryos, *nos* mRNA is degraded, except in the polar plasm, after 2 hours of embryogenesis (Bashirullah et al., 1999) (Fig. 2A,C). Consistent with a role of deadenylation in mRNA degradation, *nos* mRNA degradation correlated with shorter poly(A) tails: an important pool of poly(A) tails of 40 nt in length was present in 0-1 hour wild-type embryos and decreased in 1-2 hour embryos. In embryos from *twin*<sup>KG877</sup>/*Df(3R)crb-F89-4* or *twin*<sup>12209</sup> homozygous females, *nos* mRNA was stabilized. It was still detected by RT-PCR in 3-4 hour embryos. This stabilization correlated with a lack of poly(A) tail shortening, as the pool of 40 nt poly(A) tails also remained up to 4 hours (Fig. 2A). RNA in situ experiments confirmed a stabilization of *nos* mRNA after 2 hours of development and showed that this stabilization occurred throughout the embryo, where *nos* mRNA was degraded in the wild type (Fig. 2C).

These data show that the progressive degradation of *nos* mRNA during the first hours of embryonic development depends on its deadenylation by the CCR4 deadenylase.

### **Smg recruits the CCR4-NOT complex onto *nos* mRNA to activate its deadenylation**

Smg is a repressor of *nos* mRNA translation and achieves this function through its binding to the SREs in *nos* 3' UTR. We asked whether Smg was involved in *nos* mRNA deadenylation and degradation. In embryos from *smg*<sup>1</sup>/*Df(Scf<sup>R6</sup>)* (the null allelic combination) females, *nos* mRNA was stabilized after 2 hours of development (Fig. 2B,C), and this stabilization correlated with elongated poly(A) tails of *nos* mRNA (Fig. 2B). This suggested that *smg* is involved in *nos* mRNA deadenylation and degradation. We next determined if Smg acted on *nos* mRNA deadenylation through its binding to the SREs. Two SREs with redundant function are present in the 5'-most region of *nos* 3' UTR (Dahanukar and

Wharton, 1996). Each SRE forms a stem-loop with a CUGGC loop sequence. We used a *nos* transgene, *nos*( $\Delta$ TCE), in which the first 184 nt of the 3' UTR including both SREs are deleted. *nos*( $\Delta$ TCE) RNA is stabilized throughout the embryo at stages when *nos* wild-type mRNA is present only in the pole plasm (Dahanukar and Wharton, 1996). Stabilization of *nos*( $\Delta$ TCE) mRNA up to 4 hours of development correlated with elongated poly(A) tails (Fig. 2B). Therefore, Smg is involved in *nos* mRNA deadenylation and degradation in the bulk embryo through its binding to SREs in the 3' UTR of *nos* mRNA.

To address whether Smg plays a role in *nos* mRNA deadenylation in conjunction with the CCR4 deadenylase, we looked for genetic interactions between *smg* and *twin* mutants. We found an interaction between the null allele of *smg*, *smg*<sup>1</sup> and the strongest *twin* allele, *twin*<sup>8115</sup>. Females double heterozygous for these two mutations produced embryos of which 32% did not hatch, whereas maternal effect embryonic lethality of females heterozygous for either mutation alone was not different from that of wild-type females (8%) (Fig. 3A). *nos* mRNAs were analysed by PAT assays in embryos from *smg*<sup>1</sup> +/+ *twin*<sup>8115</sup> double heterozygous females and found to be stabilized up to 4 hours of development, with elongated poly(A) tails in 2-4 hour embryos (Fig. 3B). This elongation was stronger in embryos produced by double homozygous *smg*<sup>1</sup> *twin*<sup>12209</sup> females. These results suggested that Smg and CCR4 acted together in *nos* mRNA deadenylation. We tested physical interactions between Smg and the CCR4-NOT complex by co-immunoprecipitation in embryo extracts. Upon immunoprecipitation of Smg, CCR4 co-immunoprecipitation was not detected. However, CAF1, another protein of the deadenylation complex (Temme et al., 2004), co-immunoprecipitated with Smg, independently of the presence of RNA (Fig. 3C). This is consistent with the reported co-immunoprecipitation of Smg with CCR4-HA and CAF1-HA overexpressed in embryos (Semotok et al., 2005).

Together, these results strongly suggest that Smg recruits the CCR4-NOT deadenylation complex onto *nos* mRNA by physical interactions, resulting in activated deadenylation and degradation of *nos* mRNA in the bulk embryo. Deadenylation by Smg/CCR4 is essential to early embryonic development, as a substantial number of embryos from *smg*<sup>+/-</sup> *twin*<sup>+/-</sup> double heterozygous females do not develop.

### **Deadenylation by CCR4 is required for translational repression of *nos* mRNA**

We determined whether active deadenylation of *nos* mRNA by Smg/CCR4 contributed to translational repression. Nos protein distribution was analysed in embryos from *twin* or *smg* mutant females during the first hours of development. The amounts of ectopic Nos protein resulting from a lack of translational repression in embryos from *smg* mutant mothers have been reported to be low during the first hour of development, although *nos* activity in the anterior is detectable (Dahanukar et al., 1999). We found that ectopic Nos protein in bulk embryos from *smg* mutant females was visible at 2-3 hours (Fig. 4A, right panels). The lack of *nos* mRNA deadenylation and decay in embryos from *twin* mutant females led to ectopic accumulation of Nos protein throughout the embryos, most visible at 2-3 hours (Fig. 4A). *nos* activity at the anterior of the embryo was assayed by head skeleton analysis. The presence of Nos protein at the anterior results in repression of *bicoid* and *hunchback* mRNA translation and head skeleton defects (Dahanukar and Wharton, 1996; Smibert et al., 1996; Wharton and Struhl, 1991). Cuticles of embryos from *twin* mutant females showed pleiotropic phenotypes (lack of, or pale, cuticle), but 15% ( $n=73$ ) of embryos



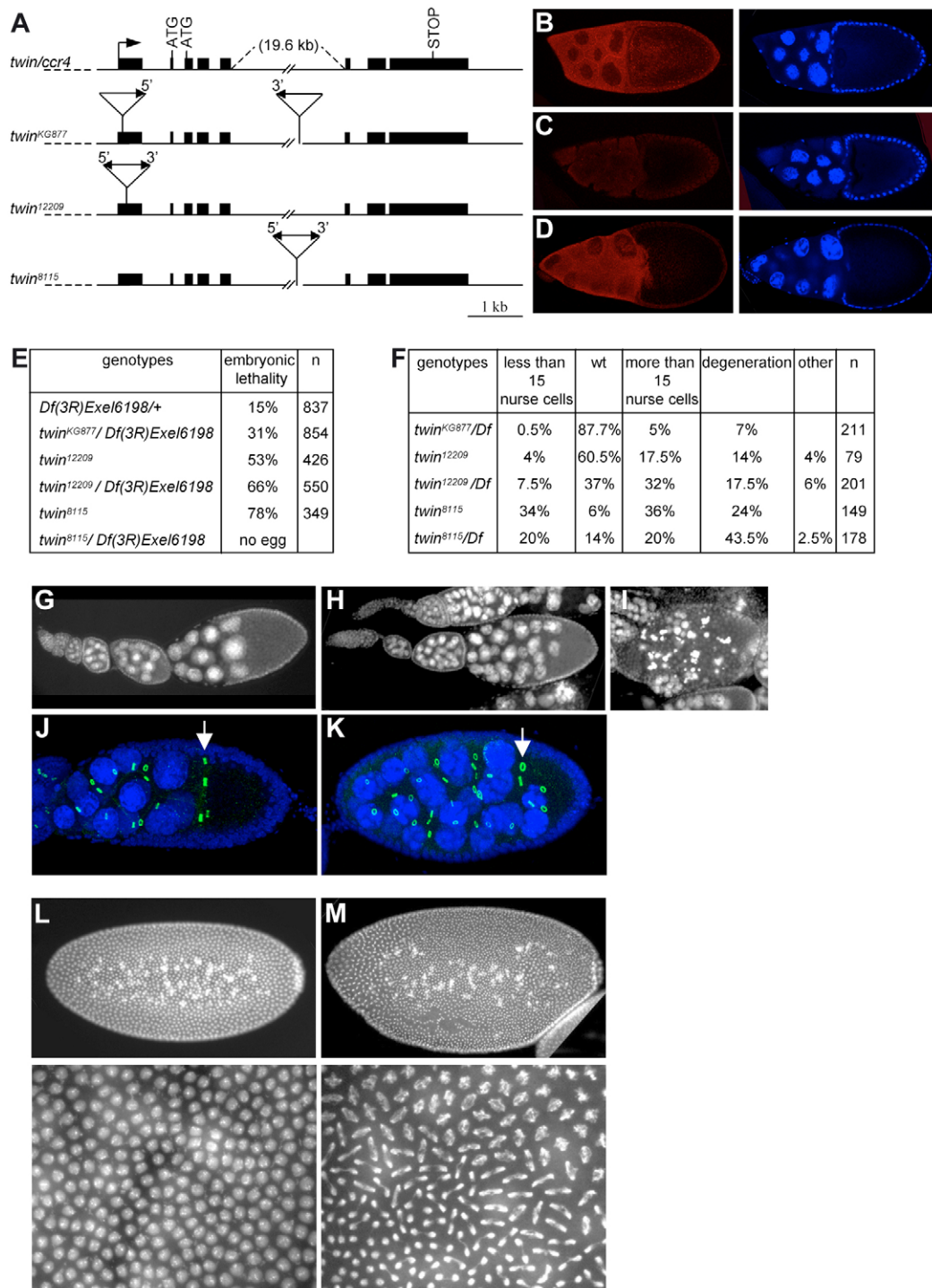


Fig. 1. See next page for legend.

that developed a cuticle had strong head defects, including a complete loss of head structures (Fig. 4B-D). These defects resemble some of those resulting from ectopic Nos protein synthesis following ubiquitous *osk* expression in the embryo (see below, Fig. 5).

We conclude that deadenylation of *nos* mRNA by CCR4 is absolutely required for complete translational repression of the pool of *nos* mRNA that is not localized at the posterior pole and for anteroposterior patterning of the embryo.

### Translation of *nos* mRNA results from the prevention of its binding to Smg by Oskar

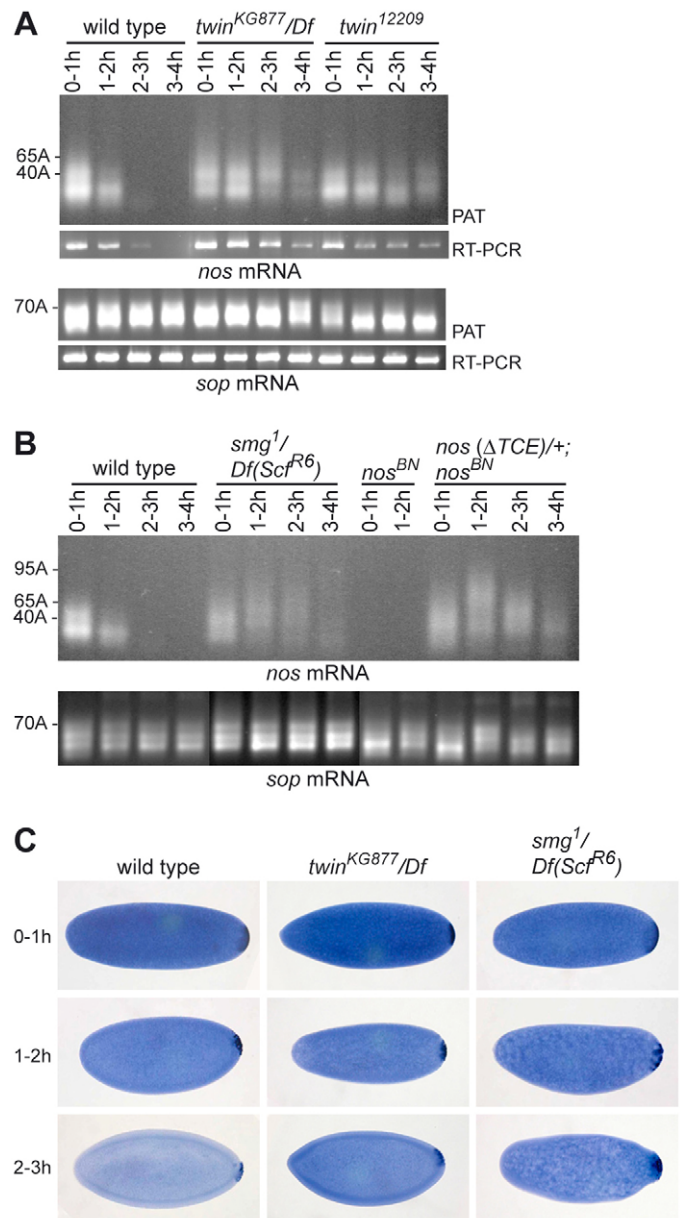
Translation of *nos* mRNA in the pole plasm is required for abdomen development and depends on *osk* function. As we found that deadenylation contributes to translational repression of *nos* in the bulk embryo, we asked whether Osk could activate *nos* mRNA translation in the pole plasm by preventing its deadenylation. PAT assays of whole embryos allow the measurement of poly(A) tail length of bulk *nos* mRNA that is unlocalized and translationally

**Fig. 1. Characterization of *twin* mutants and function in the germline.**

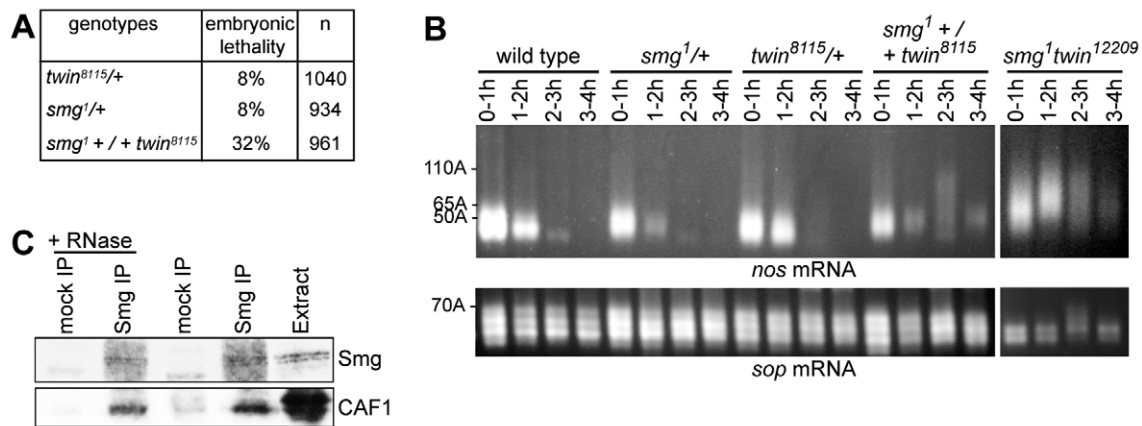
**(A)** Schematic representation of *twin* locus and mutants. Black boxes indicate exons. Intron 5 is 19.6 kb long according to genomic and EST sequences in FlyBase. The arrow indicates the transcription start site. *P* elements (not drawn to scale) inserted in the *twin* locus in the three alleles are shown. In *twin*<sup>12209</sup> and *twin*<sup>8115</sup>, insertions are *P-UAS-GFP* and *P-UAS*, respectively. Insertion sites were verified by DNA sequencing. Coordinates of the insertion sites, according to the AE003746 sequence in NCBI, are 189597 and 173089 for *twin*<sup>KG877</sup>, 189529 for *twin*<sup>12209</sup> and 174697 for *twin*<sup>8115</sup>.

**(B-D)** Immunostaining of wild-type and *twin* mutant ovaries with anti-CCR4 antibody. (B) Wild-type, (C) *twin*<sup>12209</sup> and (D) *twin*<sup>8115</sup> stage 10 egg chambers, stained with anti-CCR4 (left) and DAPI to visualize DNA (right). **(E)** *twin* mutant females show maternal effect embryonic lethality. Females of the indicated genotypes were crossed with wild-type males and hatched and unhatched embryos were scored. A proportion of these embryos have a thin chorion. When possible, both *twin* homozygous females and *twin* alleles in combination with *Df(3R)Exel6198* were analysed. *twin*<sup>KG877</sup> homozygous are lethal at larval stage, due to an independent mutation on the chromosome (Temme et al., 2004). *Df(3R)Exel6198* is a deficiency overlapping the *twin* locus, which is independent and shorter than *Df(3R)crb-F89-4*. The phenotypes of *twin*<sup>12209</sup>/*Df(3R)Exel6198* and *twin*<sup>8115</sup>/*Df(3R)Exel6198* are stronger than that of homozygous *twin*<sup>12209</sup> and *twin*<sup>8115</sup> homozygous females, respectively, indicating that none of these alleles is null. **(F-K)** Ovarian phenotypes of *twin* mutant females were analysed by DAPI staining. (F) Stage 3 to 10 egg chambers were scored according to their numbers of cells. *Df* is *Df(3R)crb-F89-4*. Similar results were obtained when *Df(3R)Exel6198* was used (wt, wild type; other: phenotypes including no oocytes, two oocytes or one mislocalized oocyte). (G) Wild-type egg chambers. (H,I) *twin*<sup>12209</sup>/*Df(3R)crb-F89-4*. An example of 32 germline cell phenotype and of an apoptotic egg chamber are shown in H and I, respectively. (J,K) Staining with DAPI and anti-HtsRC antibody that recognizes ring canals. The oocyte is linked to four nurse cells by four ring canals in wild-type egg chambers (J, arrow). An example of *twin*<sup>12209</sup>/*Df(3R)crb-F89-4* egg chamber, where the oocyte is linked by five ring canals (K, arrow). **(L,M)** Mitosis defects in embryos from *twin* mutant females visualized by DAPI staining. Nuclear cleavages are synchronous in wild-type syncytial blastoderm embryos (L), whereas nuclei in all the phases of the cell cycle are found in a same embryo from *twin*<sup>KG877</sup>/*Df(3R)crb-F89-4* female (M). Bottom panels show high magnifications of images in L,M. Anterior is oriented toward the left in all panels.

repressed. By contrast, because the pool of translated *nos* mRNA localized at the posterior pole is very small (4% of total *nos* mRNA) (Bergsten and Gavis, 1999), it is likely to escape this analysis. Consistent with this, we did not observe increased *nos* mRNA deadenylation, in PAT assays of whole embryos from *osk* mutant females, compared to wild-type (Fig. 6A). Note that impaired deadenylation of unlocalized *nos* mRNA observed in embryos from *twin* mutant females was also independent of *osk* function: *nos* poly(A) tails were similar in embryos from *twin* and *osk twin* mutant females (Fig. 6A). We, therefore, expressed *osk* in the whole embryo using *UASp-osk-K10* (Riechmann et al., 2002) and the *nos-Gal4* germline driver (Rorth, 1998). *Osk* protein was overexpressed ubiquitously in *UASp-osk-K10/+; nos-Gal4/+* oocytes and early embryos, resulting in bicaudal embryos or the lack of head skeleton, due to ectopic *Nos* synthesis (Smith et al., 1992) (Fig. 5). In these embryos, *nos* mRNA was stabilized up to 4 hours of development, with long poly(A) tails (Fig. 6B). This demonstrates that *Osk* prevents deadenylation of *nos* mRNA. *Osk*



**Fig. 2. CCR4 and Smg are required for *nos* mRNA deadenylation and degradation.** **(A)** PAT assays and RT-PCR of *nos* mRNA showing its deadenylation and destabilization in the wild type and its stabilization in *twin* mutants during early embryogenesis. Females of the indicated genotypes were crossed with identical males. Note that, consistent with both maternal and zygotic contributions to mRNA destabilization in early embryos (Bashirullah et al., 1999), we found that embryonic lethality increased when *twin* females were crossed with males of the same genotype instead of wild-type males [e.g. 94% embryonic lethality ( $n=468$ ) from a cross between *twin*<sup>12209</sup> homozygous females and males]. *Df* is *Df(3R)crb-F89-4*. RNAs were from embryos spanning 1 hour intervals. The *sop* mRNA (bottom panels) was used as a control in A and B. **(B)** PAT assays of *nos* mRNA showing its stabilization with long poly(A) tails in *smg* mutants or from a *nos* transgene lacking the TCE. *nos*<sup>BN</sup> is a null mutant that does not produce *nos* RNA. Females of the indicated genotypes were crossed with wild-type males. RNA was prepared from the time intervals indicated. **(C)** In situ hybridizations revealing *nos* mRNA in embryos. *twin*<sup>KG877</sup>/*Df(3R)crb-F89-4* females were crossed with identical males and *smg*<sup>1</sup>/*Df(Scf<sup>R6</sup>)* females were crossed with wild-type males. Anterior is oriented toward the left.



**Fig. 3. Genetic and physical interactions between Smg and the CCR4-NOT complex of deadenylation.** (A) Genetic interaction between *smg* and *twin*. Females of the indicated genotypes were crossed with wild-type males, and hatched and unhatched embryos were scored. (B) PAT assays of *nos* and control *sop* mRNAs showing a wild-type pattern of *nos* mRNA poly(A) tails in embryos from *smg*<sup>1</sup>/*+* or *twin*<sup>8115</sup>/*+* females and a stabilization of *nos* mRNA with longer poly(A) tails in embryos from *smg*<sup>1</sup>/*+* *twin*<sup>8115</sup>/*+* double heterozygous females; poly(A) tails were still longer in embryos from double homozygous *smg*<sup>1</sup> *twin*<sup>12209</sup> mutant females (right panel). Females were crossed with identical males. (C) Co-immunoprecipitation of CAF1 protein with Smg in 0-3 hour embryo extracts. Proteins were immunoprecipitated with anti-Smg (Smg IP) or rabbit serum (mock IP) either in the presence or the absence of RNase A. Bound proteins were detected by western blots with anti-Smg or anti-CAF1. Extract before IP was also loaded.

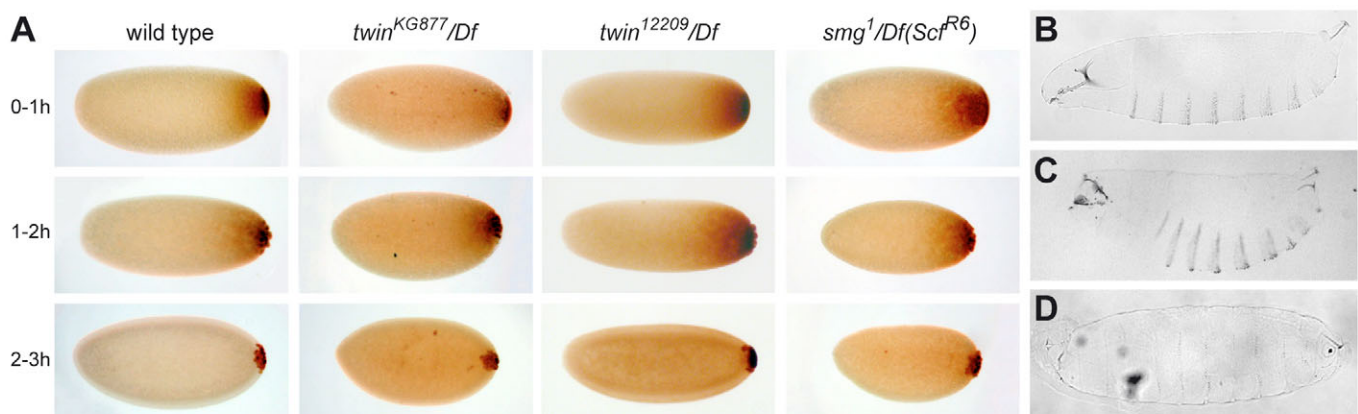
protein interacts with Smg in yeast two-hybrid assays and in GST pull-down experiments (Dahanukar et al., 1999). Therefore, Osk might affect Smg function in the pole plasm by disrupting either the physical interaction between Smg and the CCR4-NOT deadenylation complex or the interaction between Smg and *nos* mRNA. In both cases, this would prevent the active recruitment of the CCR4-NOT complex onto *nos* mRNA. We performed Smg immunoprecipitations in wild-type embryos and in embryos overexpressing Osk. Co-immunoprecipitation of CAF1 remained unaffected in embryos that overexpressed Osk (Fig. 6C), suggesting that Osk does not affect the association between Smg and the CCR4-NOT complex. *nos* mRNA levels were then quantified in the complexes immunoprecipitated with Smg. In wild-type embryos, *nos* mRNA was found to be enriched in Smg complexes, compared with control *sop* or *rp49* mRNAs, as

previously reported (Semotok et al., 2005). Strikingly, this enrichment decreased to background level in embryos overexpressing Osk (Fig. 6C,D).

Together, these results strongly suggest that Osk prevents Smg binding to *nos* mRNA, thus inhibiting the recruitment of the deadenylation complex onto *nos* mRNA by Smg. This results in a lack of *nos* mRNA deadenylation and its stabilization and translation.

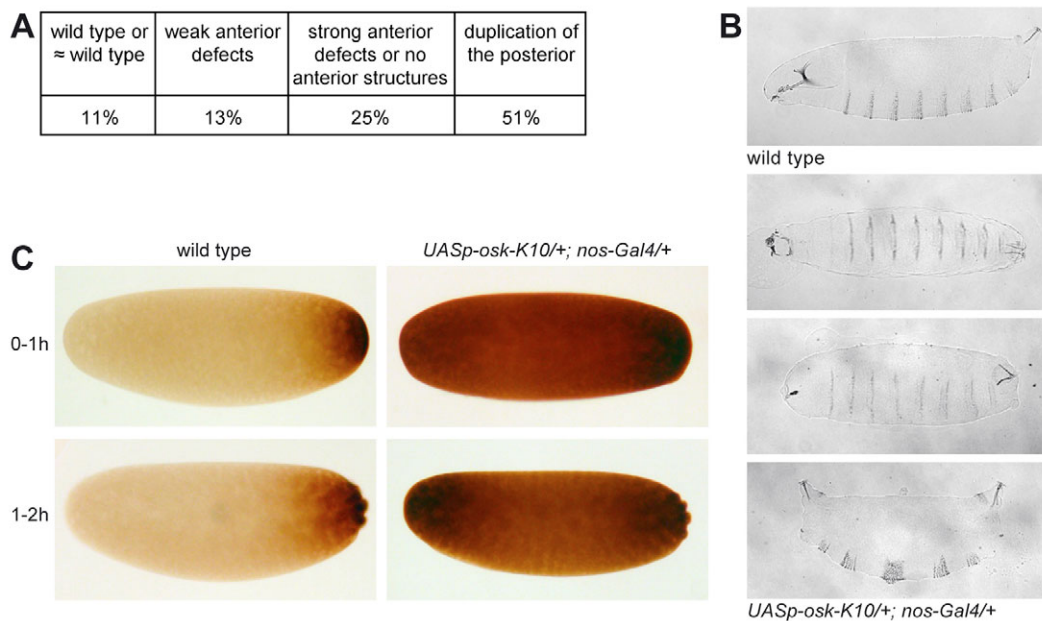
#### Colocalization of Smg and the CCR4-NOT complex in discrete cytoplasmic structures

CCR4 and CAF1 are concentrated in cytoplasmic foci in *Drosophila* ovaries (Temme et al., 2004) and CCR4 was reported to be present in P (processing) bodies in mammalian cells (Cougot et al., 2004). P bodies are cytoplasmic structures containing decapping and



**Fig. 4. Deadenylation of *nos* mRNA by CCR4 contributes to its translational repression.** (A) Immunostaining of embryos with anti-Nos antibody during the first hours of embryogenesis. The increase in amounts of Nos protein was visible in bulk embryos from *twin*<sup>KG877</sup>/*Df*(3*R*)*crb*-*F89-4* and *twin*<sup>12209</sup>/*Df*(3*R*)*crb*-*F89-4* females. *twin* mutant females were crossed with identical males and *smg*<sup>1</sup>/*Df*(*Scf*<sup>R6</sup>) females were crossed with wild-type males. (B-D) Cuticle preparations of embryos showing strong head defects. (B) Wild type. (C,D) Embryos from *twin*<sup>12209</sup>/*Df*(3*R*)*crb*-*F89-4* females crossed with wild-type males; (C) head replaced by a hole; (D) no head structures. Anterior is oriented toward the left.





**Fig. 5. Characterization of embryonic phenotypes caused by overexpression of *osk* with *UASp-osk-K10*.** (A,B) Cuticle of embryos from *UASp-osk-K10/+; nos-Gal4/+* females were prepared and classified from their defects ( $n=155$ ). Examples of the different phenotypes are shown in B. Two different examples of strong head defects (25%) are shown: head replaced by a hole (middle top panel), no head structures (middle bottom panel). The phenotype of mirror posterior duplication (51%) is also shown (bottom panel). (C) Immunostaining of embryos from wild-type or *UASp-osk-K10/+; nos-Gal4/+* females with anti-Nos antibody, showing that Nos protein accumulates in the whole embryo when *osk* is overexpressed ubiquitously. Anterior is oriented toward the left.

degradation enzymes as well as translational repressors and are thought to be the actual sites of translational repression and mRNA degradation (Bregues et al., 2005; Collier and Parker, 2005; Newbury et al., 2006). We analysed the intracellular distribution of Smg, CCR4 and CAF1 in 1-2 hour embryos, which show strong *nos* deadenylation. As in ovaries, CCR4 and CAF1 had a non-homogenous cytoplasmic distribution with foci of higher concentration (Fig. 7). Smg showed a similar distribution with, in addition, larger structures often localized at the periphery of nuclei. Colocalization of CCR4 or CAF1 with Smg was partial and occurred in medium size foci, seldom in larger Smg foci. To analyse the relationships between these structures and P bodies, the distribution of two bona fide components of yeast and mammalian P bodies was analysed in embryos (Fig. 7). Dcp1 is involved in decapping and Xrn1 (Pacman in *Drosophila*) is the 5'-3' exonuclease. Pacman distribution and colocalization with Smg were similar to that of CCR4 and CAF1. Unexpectedly, colocalization between Dcp1 and Smg, although still partial, was higher than with the other proteins and also occurred in large Smg foci.

These data are in agreement with Smg-dependent deadenylation in discrete cytoplasmic structures. Related structures, larger in size and containing Smg and Dcp1 but not the deadenylation complex, could be the sites of deadenylation-independent translational control.

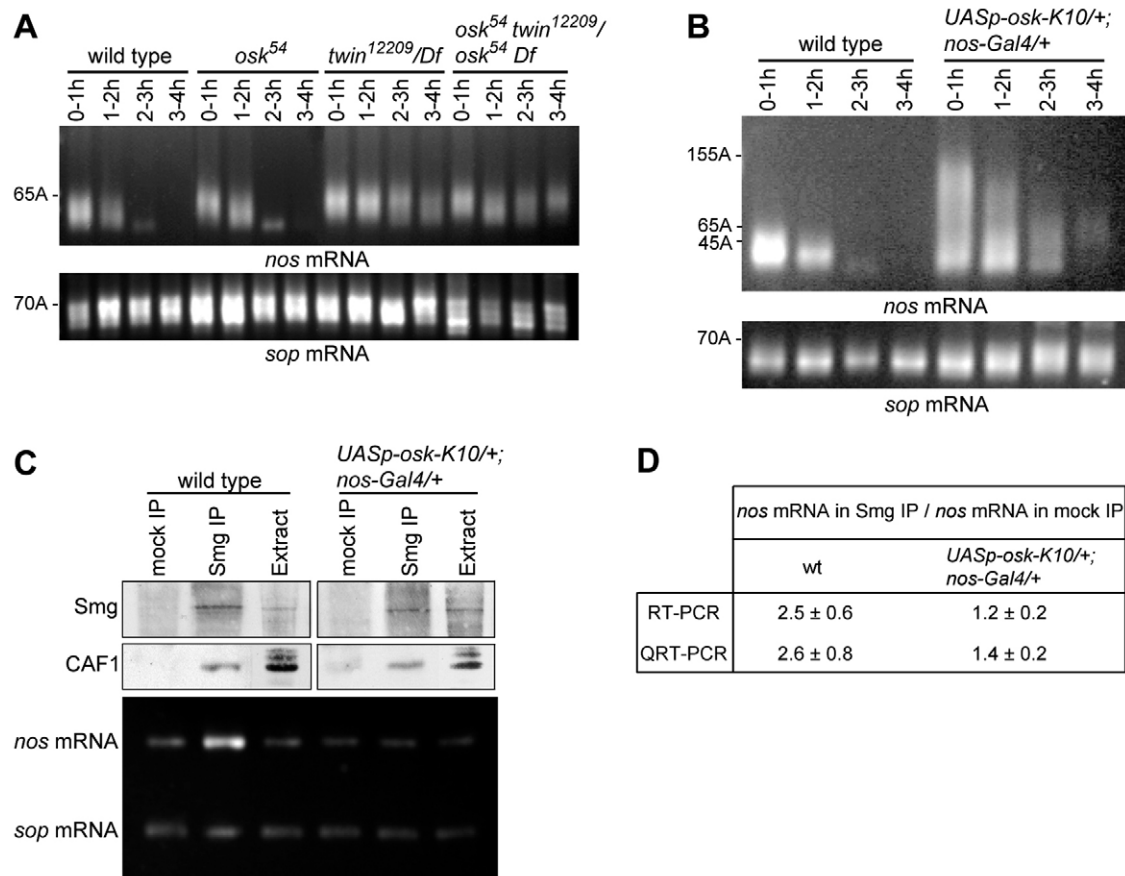
## DISCUSSION

### Importance of poly(A) tail length control in *nos* translational regulation

In this paper, we show that poly(A) tail length regulation is central to *nos* translational control. Poly(A) tail length regulation is a major mechanism of translational control, particularly during early development. *nos* translational control was reported previously to be

independent of poly(A) tail length. This conclusion came from the absence of *nos* poly(A) tail elongation between ovaries and early embryos (Salles et al., 1994), and the lack of *nos* poly(A) tail shortening between wild-type and *osk* mutant embryos in which *nos* mRNA is not translated at the posterior pole (Gavis et al., 1996). However, later studies suggested that this lack of poly(A) tail change was not unexpected, as *nos* mRNA translation starts in ovaries (Forrest et al., 2004), and the pool of translationally active *nos* mRNA in embryos is very small (4%) (Bergsten and Gavis, 1999) and remains undetected among the amount of translationally repressed *nos* in whole embryos. We now find that *nos* mRNA deadenylation by the CCR4-NOT complex, recruited to the 3' UTR by Smg, is required for *nos* translational repression in the bulk embryo. In addition, our data also suggest that *nos* translation at the posterior pole depends on the prevention of this deadenylation. *nos* mRNA is regulated at several levels, including localization, degradation, translational repression and translational activation. Localization at the posterior pole depends on two mechanisms: an actin-dependent anchoring at late stages of oogenesis, after nurse cells dumping (Forrest and Gavis, 2003) and localized stabilization. Localization and translational control are coupled in that the localized RNA escapes both translational repression and degradation. We propose a mechanism for this coupling. Translational repression and RNA degradation both involve Smg-dependent deadenylation. Deletion of the SREs in a *nos* transgene, as well as mutations in *smg* or in *twin*, which encodes the major catalytic subunit of the deadenylating CCR4-NOT complex, abrogate poly(A) tail shortening. Lack of deadenylation prevents the timely degradation of the RNA and also relieves translational repression. Deadenylation could repress *nos* mRNA translation by two mechanisms. Interaction of the cytoplasmic poly(A) binding protein (PABP) with mRNA poly(A) tails is important for the





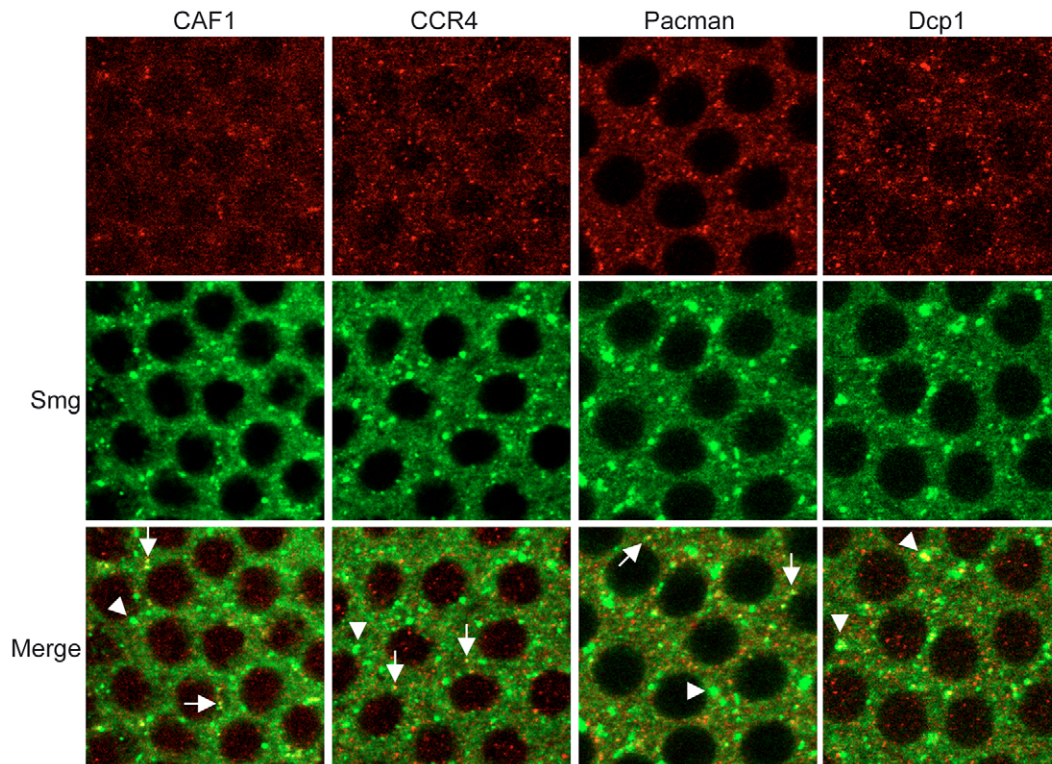
**Fig. 6. Osk prevents *nos* mRNA deadenylation by preventing the binding of Smg to *nos* mRNA.** (A) PAT assays of *nos* mRNA showing that poly(A) tails of bulk *nos* mRNA are not affected by the lack of Osk. *Df* is *Df(3R)Exel6198*. *osk* females were crossed with wild-type males and *twin* or *osk twin* females were crossed with identical males. *sop* mRNA was used as a control in A and B. (B) PAT assays showing that *nos* mRNA has longer poly(A) tails and is stabilized in embryos where Osk protein is overexpressed ubiquitously (*UASp-osk-K10/+; nos-Gal4/+*). Females were crossed with wild-type males. (C) Smg immunoprecipitations in 0-3 hour embryo extracts, either from wild-type embryos, or from embryos in which *osk* is overexpressed (*UASp-osk-K10/+; nos-Gal4/+*). Ribonucleoprotein complexes were precipitated with anti-Smg (Smg IP) or rabbit serum (mock IP) in the presence of RNasin. Bound proteins were revealed by western blots with anti-Smg and anti-CAF1 (top and middle panels). Ubiquitous expression of *osk* does not affect CAF1 co-immunoprecipitation with Smg. Bound RNAs were analysed by RT-PCR to visualize and quantify *nos* mRNA versus *sop* or *rp49* mRNAs used as controls. *nos* and the control mRNA (*sop* or *rp49*) were analysed in the same PCR reaction. An example of *nos* mRNA enrichment in Smg IP is shown (wild type); this enrichment is lost in Smg IP from embryos where *osk* is overexpressed (*UASp-osk-K10/+; nos-Gal4/+*) (bottom panel). Protein and RNA extracts before immunoprecipitation were also loaded (Extract). (D) Quantification of *nos* mRNA enrichment in Smg IP. PCR were performed on several dilutions of the RT reactions. The levels of *sop* or *rp49* control mRNAs were set at 1 and the levels of *nos* mRNA were calculated (*nos* mRNA/*sop* or *rp49* mRNA). The fold enrichment of *nos* mRNA in Smg IP compared to mock IP is indicated (ratio of *nos* mRNA level in Smg IP to that in mock IP). Quantifications were from four RT-PCR and three QRT-PCR in one set of immunoprecipitations. Similar results were obtained from an independent set of immunoprecipitations (mean of six RT-PCR: 2.9 in wild-type embryos, versus 1.3 in embryos overexpressing *osk*).

activation of translation initiation (Kahvejian et al., 2005; Wakiyama et al., 2000). Therefore, poly(A) shortening of *nos* mRNA would lead to PABP dissociation and inhibition of translation. In addition, deadenylation leads eventually to *nos* mRNA decay, which should also contribute to translational repression. Consistent with the Smg-dependent deadenylation of *nos* mRNA that we describe in embryos, a recent study documented SRE-dependent deadenylation of chimeric transcripts containing the 3' UTR of *nos* mRNA in cell-free extracts from *Drosophila* embryos. In this system, deadenylation of the chimeric RNAs also strongly contributes to translational repression, along with at least another deadenylation-independent mechanism (Jeske et al., 2006).

In our analysis, *twin* and *smg* mutants, although both impaired in *nos* mRNA poly(A) tail shortening, did not show the same defects. *twin* mutants fail to show *nos* poly(A) tail shortening during

embryogenesis, whereas in *smg* mutant embryos or when poly(A) tails are measured from *nos*( $\Delta$ TCE) transgene, a poly(A) tail elongation is visible. This suggests that *nos* mRNA is also regulated by cytoplasmic polyadenylation which balances the deadenylation reaction, and that Smg binding to the RNA reduces the polyadenylation reaction. Consistent with a dynamic regulation of poly(A) tail length of maternal mRNAs resulting from a tight balance between regulated deadenylation and polyadenylation, we found that in mutants for the GLD2 poly(A) polymerase that is involved in cytoplasmic polyadenylation, *nos* mRNAs are precociously degraded in 0-1 hour embryos (Perrine Benoit and M.S., unpublished).

We showed that ectopic expression of *osk* in the bulk cytoplasm of the embryo is sufficient to impair *nos* mRNA binding to Smg and its deadenylation and destabilization. Therefore, we propose that, in



**Fig. 7. Presence of Smg and of the CCR4-NOT complex in discrete cytoplasmic foci related to P bodies.** Immunostaining of wild-type syncytial blastoderm embryos (90-110 minute development) with anti-CAF1, anti-CCR4, anti-Pacman or anti-Dcp1, costained with anti-Smg and DAPI (not shown). Pacman and Smg are exclusively cytoplasmic, whereas CAF1, CCR4 and Dcp1 are present in low amounts in nuclei, in addition to their cytoplasmic distribution. All proteins show a diffuse cytoplasmic distribution and accumulation in cytoplasmic foci variable in size. Arrows indicate medium size foci where either CAF1, CCR4 or Pacman colocalize with Smg. Arrowheads indicate large size Smg foci that do not contain CAF1, CCR4 or Pacman, but do contain Dcp1.

wild-type embryos, Osk at the posterior pole inhibits Smg binding to the anchored *nos* mRNA, preventing deadenylation, decay and translational repression. This results in localized *nos* stabilization and translation. Osk might achieve this by a direct binding to Smg, as it was shown to interact with Smg *in vitro*, through a region overlapping the RNA-binding domain in Smg (Dahanukar et al., 1999). Alternatively, Osk could prevent Smg function independently of its binding to Smg, through its recruitment by another protein in *nos*-containing mRNPs. Consistent with a potential presence of Smg and Osk in the same protein complex, we were able to co-immunoprecipitate Osk with Smg in embryos overexpressing Osk (data not shown).

### Multiple levels of *nos* translational repression

Two mechanisms of *nos* translational repression have already been described. A first mode of translation inhibition appears to act during elongation, as suggested by polysome analysis (Clark et al., 2000) and by the involvement of the Bicoid protein, which corresponds to a subunit of the nascent polypeptide associated complex (Markesich et al., 2000). The second mode of repression involves Smg and is thought to affect initiation. It requires the association of Smg with the protein Cup, which also binds eIF4E. The association of Cup with eIF4E competes with the eIF4E/eIF4G interaction, which is essential for translation initiation (Nelson et al., 2004). We identify deadenylation by the CCR4-NOT complex as a novel level of *nos* translational repression, also involving Smg. Smg protein synthesis is probably induced by egg activation during egg-laying. Smg is absent in ovaries and accumulates during the

first hours of embryogenesis, with a peak at 1-3 hours (Dahanukar et al., 1999). Its amount is low during the first hour and possibly nonexistent during the first 30 minutes. This correlates with the presence at that time of high levels of *nos* mRNA in the bulk embryo that are not destabilized. *nos* translational repression is active, however, as this pool of mRNA is untranslated. Thus a Smg-independent mode of repression must be efficient during the first hour of development. This might correspond to repression at the elongation step and/or involve the Glorund protein, a *Drosophila* hnRNP F/H homologue newly identified as a *nos* translational repressor in the oocyte (Kalifa et al., 2006). Glorund has a role in repression of unlocalized *nos* mRNA in late oocytes and has been suggested to also act at the beginning of embryogenesis while Smg is accumulating to ensure the maintenance of translational repression at the oogenesis to embryogenesis transition (Kalifa et al., 2006). Analysis of *glorund* mutants revealed that the embryonic phenotypes are less severe than expected and led to the proposal that at least an additional level of *nos* translational repression is active in oocytes (Kalifa et al., 2006). We found that overexpression of Osk in the germline with *nos-Gal4* results in long poly(A) tails of *nos* mRNA, even in 0-1 hour embryos in which Smg protein is poorly expressed. This suggests that the short poly(A) tail of *nos* mRNA in 0-1 hour wild-type embryos could in part result from active deadenylation during oogenesis, which would depend on a regulatory protein different from Smg. Deadenylation could therefore be involved in *nos* regulation during oogenesis, and would also be prevented by Osk in the pole plasm, as in embryos.

Genetic evidences indicate that all three levels of translational repression are additive. Although the importance of the Smg/Cup/eIF4E mode of *nos* translational repression for the anteroposterior patterning of the embryo has not been addressed, the other two levels of repression are essential, as ectopic Nos protein leads to disruption of the embryo anteroposterior axis in *twin* (this study) or *bicaudal* mutants (Markesich et al., 2000). This demonstrates that none of the three levels of repression is sufficient by itself and suggests that all three regulations are required to achieve complete translational repression of *nos*. As Osk acts by preventing the binding of Smg to the *nos* 3' UTR, it is likely to inhibit both Smg-dependent mechanisms of translational repression.

### Subcellular localization of *nos* mRNA regulation

The presence of Smg in discrete cytoplasmic foci and its partial colocalization in these foci with components of the CCR4-NOT deadenylation complex, and with components of P bodies, suggest that Smg-dependent deadenylation and translational control of *nos* occur in P bodies. P body dynamics and function have not been addressed in a complete organism during development. Consistent with the apparent complexity of P body function, including mRNA decay and translational repression, we identified in embryos different subsets of Smg-containing structures that either do or do not contain the CCR4-NOT deadenylation complex and the Xrn1 5'-3' exonuclease. This suggests the existence of different types of P bodies that may have distinct functions.

### Functions and regulation of the CCR4-NOT deadenylation complex

We have shown previously that the CCR4-NOT complex is involved in default deadenylation of bulk mRNAs in somatic cells (Temme et al., 2004). We now find that the same deadenylation complex has a role in active, sequence-specific deadenylation of a particular mRNA. Activation of deadenylation by CCR4-NOT results from the recruitment of the deadenylation complex by a regulatory RNA-binding protein to its specific mRNA target (this study) (Semotok et al., 2005). Several RNA-binding proteins are expected to interact with the CCR4-NOT complex to regulate the deadenylation of different pools of mRNAs in different tissues. CCR4 controls poly(A) tail lengths of *Cyclin A* and *B* mRNAs during oogenesis (Morris et al., 2005); the regulatory protein has not been identified, but it cannot be Smg, which is not expressed in ovaries. A similar mode of active deadenylation involving the recruitment of the deadenylation complex by ARE-binding proteins has been proposed in mammalian cells (Lykke-Andersen and Wagner, 2005). More recently, a study in yeast has identified the PUF (Pumilio/FBF) family of RNA-binding proteins as activators of CCR4-NOT-mediated deadenylation through a direct interaction between PUF and POP2 (the CAF1 homologue) (Goldstrohm et al., 2006). Although default deadenylation by CCR4 is not essential for viability (Temme et al., 2004), active deadenylation by CCR4 of specific mRNAs is essential for certain developmental processes, in particular during early development.

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