Smaug assembles an ATP-dependent stable complex repressing *nanos* mRNA translation at multiple levels

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The *nanos* (*nos*) mRNA encodes the posterior determinant of the *Drosophila* embryo. Translation of the RNA is repressed throughout most of the embryo by the protein Smaug binding to Smaug recognition elements (SREs) in the 3' UTR. Translation is locally activated at the posterior pole by Oskar. This paper reports that the SREs govern the time- and ATP-dependent assembly of an exceedingly stable repressed ribonucleoprotein particle (RNP) in embryo extract. Repression can be virtually complete. Smaug and its co-repressor Cup as well as Trailer hitch and the DEAD box protein Me31B are part of the repressed RNP. The initiation factor eIF4G is specifically displaced, and 48S pre-initiation complex formation is inhibited. However, later steps in translation initiation are also sensitive to SRE-dependent inhibition. These data confirm several previously untested predictions of a current model for Cup-dependent repression but also suggest that the Cup model by itself is insufficient to explain translational repression of the *nos* RNA. In the embryo extract, recombinant Oskar relieves translational repression and deadenylation by preventing Smaug's binding to the SREs.

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Introduction

Maturing oocytes, eggs, and early embryos are transcriptionally silent, and gene expression depends on mRNA provided maternally during oocyte development. Many maternal mRNAs are stored in a translationally inert form until they are activated at specific stages of development (Colegrove-Otero *et al*, 2005; Tadros and Lipshitz, 2005; Vardy and Orr-Waver, 2007). The term 'masking' describes the translational silencing and storage of such RNAs in the form of ribonucleoprotein particles (RNPs) (Spirin, 1966). Masked

mRNPs have been isolated from oocytes and eggs (Ilan and Ilan, 1978; Jenkins *et al*, 1978; Grainger and Winkler, 1987; Standart *et al*, 1990). Resistance of the repressed state to the time-consuming purification procedures and the dilutions involved implies a high stability of the masked RNP. This stability may reflect the need for very tight control of translation: As the developmental stages at which masking is required can last for very long periods of time, and masked mRNAs encode important regulators like cyclins or c-mos (Sheets *et al*, 1995; Gebauer and Richter, 1997; Groisman *et al*, 2002), even a low level of translation would probably be deleterious.

Translational regulation of maternal mRNAs can be coupled with their localization. The RNA is translationally repressed while it is transported to a particular localization, and then translation is activated locally (Johnstone and Lasko, 2001; Gavis *et al*, 2007). One RNA regulated in this manner is the *nanos* (*nos*) mRNA, which has a key function in the determination of the anteroposterior body axis of the *Drosophila* embryo. The *nos* mRNA is localized to the germ plasm at the posterior pole of the embryo and translated only at this position. A gradient of Nos protein developing by locally restricted translation and diffusion is essential for the formation of the anteroposterior body axis (Wang and Lehmann, 1991; Gavis and Lehmann, 1992, 1994). However, ~95% of the *nos* RNA remains distributed throughout the embryo (Bergsten and Gavis, 1999). This RNA is translationally silenced and decays over the first 2½ h of development (Gavis and Lehmann, 1994; Dahanukar and Wharton, 1996; Bashirullah *et al*, 1999). Whereas repression of non-localized *nos* mRNA in the oocyte depends on Glorund (Kalifa *et al*, 2006), the protein Smaug is responsible for repression of non-localized *nos* mRNA during embryonic development (Smibert *et al*, 1996, 1999; Dahanukar *et al*, 1999). Two Smaug recognition elements (SREs) in the *nos* 3' UTR are necessary and sufficient for repression (Dahanukar and Wharton, 1996; Gavis *et al*, 1996; Smibert *et al*, 1996). The SREs also direct the degradation of the *nos* RNA (Dahanukar and Wharton, 1996; Smibert *et al*, 1996; Bashirullah *et al*, 1999), and Smaug is known to recruit the CCR4–NOT complex, which catalyses deadenylation of *nos* and other mRNAs (Aviv *et al*, 2003; Temme *et al*, 2004; Morris *et al*, 2005; Semotok *et al*, 2005; Zaessinger *et al*, 2006; Kadyrova *et al*, 2007; Tadros *et al*, 2007). At the posterior pole, localized Oskar protein mediates the local accumulation of *nos* RNA and prevents both its deadenylation and translational repression (Ephrussi *et al*, 1991; Ephrussi and Lehmann, 1992; Smith *et al*, 1992; Dahanukar and Wharton, 1996; Zaessinger *et al*, 2006), presumably via a direct interaction with Smaug (Dahanukar *et al*, 1999).

Deadenylation is not only the first step in mRNA degradation but also strongly reduces the translational efficiency of *nos* mRNA. However, additional mechanisms of repression must exist that operate independently of deadenylation

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(Jeske *et al*, 2006). Two such mechanisms have been proposed. First, the protein Cup associates both with Smaug and the translation initiation factor eIF4E; by displacing eIF4G from 4E, Cup may prevent the initiation of *nos* translation (Nelson *et al*, 2004). While defects in *nos* regulation in *cup* mutant embryos support a role of Cup, key predictions of the model, like an inhibition of 48S pre-initiation complex formation, have not been tested. Second, sucrose gradient centrifugation showed a large fraction of *nos* mRNA to be associated with rapidly sedimenting particles identified as polysomes by a puromycin run-off experiment. Thus, translational repression was suggested to act during elongation (Clark *et al*, 2000). This agrees with the observation that the *bicaudal* mutation, which affects a subunit of the complex interacting with nascent polypeptides at the exit tunnel of the ribosome, allows ectopic translation of *nos* mRNA (Markesich *et al*, 2000). However, arguing against repression during elongation, a different study found only ~5% of *nos* RNA associated with polysomes, in agreement with the localized fraction (Qin *et al*, 2007).

We have used extracts prepared from *Drosophila* embryos to examine the mechanism of translational repression of *nos* mRNA. Repression is based on the slow and ATP-dependent formation of a stable repressor complex that can shut off translation almost completely. Displacement of eIF4G from the repressed RNP and inhibition of 48S pre-initiation complex formation support the proposed mechanism of Cup action. However, other features of SRE-dependent translation inhibition are not explained by this model. Oskar protein prevents both deadenylation and translation inhibition of *nos* mRNA in extracts by preventing the association with the SREs.

Results

An SRE-dependent repressor complex assembles slowly

An *in vitro* system derived from early *Drosophila* embryos reproduces two key aspects of the regulation of *nos* mRNA, rapid deadenylation and translational repression. Both reactions depend on SREs in the 3' UTR (Jeske *et al*, 2006). For the following studies, firefly luciferase mRNAs were used that contained an 'internal' poly(A) tail followed by 40 additional nucleotides of mixed sequence. Such an internal tail stimulates translation as efficiently as a 'terminal' poly(A) tail, but cannot be removed by SRE-dependent deadenylation (Jeske *et al*, 2006) (Supplementary Table I). Translation of the luciferase RNA fused to the SRE-containing segment of the *nos* 3' UTR was compared with the translation of a similar RNA carrying an inactivating point mutation (Aviv *et al*, 2003, 2006; Johnson and Donaldson, 2006; Oberstrass *et al*, 2006) in each of the two SRE loop regions (luciferase *nos* versus luciferase *nos* SRE⁻ RNA). Translation of the same set of RNAs in rabbit reticulocyte lysate, which does not respond to SREs (Smibert *et al*, 1999; Clark *et al*, 2000; Jeske *et al*, 2006), was routinely used as a control.

With most batches of extract, SRE-dependent repression after a 1-h translation reaction was approximately three-fold (Table I). However, in time course experiments, translation of the control RNA proceeded linearly, whereas translation of the SRE-containing RNA decreased with time and came almost to a halt after 40 min (Figure 1A). This suggested a slow step in the repression mechanism. A simple two-step incubation procedure confirmed this and improved SRE-dependent

Table I Impact of 5' and 3' end modifications on SRE-dependent repression^a

Structure of reporter RNA	– Pre-incubation	+ Pre-incubation
Capped, polyadenylated	2.8 ± 0.4 (<i>n</i> = 4)	16.0 ± 6.8 (<i>n</i> = 10) ^b
Uncapped, polyadenylated	1.7 ± 0.3 (<i>n</i> = 4)	15.0 ± 6.8 (<i>n</i> = 5)
Capped, no poly(A) tail	n.d. ^c	5.4 ± 0.6 (<i>n</i> = 2)
Uncapped, no poly(A) tail	n.d.	4.2 ± 2.2 (<i>n</i> = 2)

^aRepression factors (*nos* SRE⁻/*nos*) based on translation for 1 h in embryo extract. '*n*' indicates the number of batches of extract tested.

^bTranslation in the presence of 27% protein buffer instead of potassium acetate (see Materials and methods) resulted in a repression factor of 44.5 ± 16.5 (*n* = 4).

^cNot done here. In a previous paper, the number was ~2 (Jeske *et al*, 2006).

repression. The reporter RNAs were pre-incubated for 30 min together with extract and buffer lacking an ATP-regenerating system. Omission of the ATP-regenerating system was sufficient to prevent translation. Then translation was started by the addition of ATP, creatine phosphate, and creatine kinase. With the pre-incubation, translation of *nos* mRNA was repressed from the very beginning of the translation reaction, and repression factors of 10-fold and higher were routinely obtained (Figure 1B; Table I). With a slight change in buffer conditions, almost 50-fold repression was achieved; in other words, *nos* RNA was essentially not translated at all (Table I). Thus, the translation repression observed *in vitro* was at least as efficient as that obtained when luciferase reporter RNAs were injected into *Drosophila* embryos (~13-fold repression (Nelson *et al*, 2004)).

The reporter RNA had to be present during the pre-incubation; pre-incubation of the extract in the absence of the mRNA did not improve the efficiency of repression. Repression also increased with the time of pre-incubation (Figure 1C). These observations suggest that a repressive protein complex assembles on the *nos* RNA during the pre-incubation. Alternative explanations, such as selective degradation, deadenylation or decapping of the *nos* RNA during pre-incubation, were excluded (Supplementary data; Supplementary Figure S1; see also below).

The *nos* RNA 3' UTR segment used here consists of 155 nucleotides and contains two SREs plus a binding site for the repressor Glorund, which controls *nos* translation in oocytes (Kalifa *et al*, 2006). When this RNA segment was replaced with three SRE motifs, translation of the reporter RNA was repressed as efficiently (~15-fold) as the one containing the larger *nos* segment (data not shown). Thus, sequences outside the SREs are not essential for repression.

Assembly of the repressor complex is ATP-dependent

SRE-mediated deadenylation depends on an ATP-regenerating system (Jeske *et al*, 2006). Therefore, ATP dependence of SRE-mediated translation repression was tested. The average ATP concentration in five batches of embryo extract, measured with a luminescence assay, was 680 μM and ATP did not drop below 200 μM under pre-incubation conditions (data not shown). To investigate a possible role for the remaining ATP in repressor complex formation, ATP was depleted from the pre-incubation mix by incubation with hexokinase and glucose for 5 min before reporter mRNA was added and pre-incubation was carried out. A control reaction received no hexokinase and glucose. At the end of the pre-incubation

Figure 1 Efficient repression depends on the slow assembly of a repressor complex. Capped (left panels) or uncapped (right panels) luciferase RNAs containing an internal poly(A) tract were incubated in embryo extract under translation conditions. At the time points indicated, the translation yield was determined by a luciferase assay. **(A)** Luciferase RNAs containing the *nos* 3' UTR fragment (circles) or the fragment with point mutations in the SRE sequences (*nos* SRE⁻, filled circles) were mixed with extract, and translation was started immediately. **(B)** The same experiment as in **(A)** was carried out, but luciferase RNAs were pre-incubated with extract for 30 min before translation was started by the addition of the ATP-regenerating system (see Materials and methods) at $t = 0$. **(C)** Luciferase RNAs containing the *nos* or the *nos* SRE⁻ 3' UTR fragment were pre-incubated with extract for the times indicated before translation was started. Luciferase activity obtained from the SRE⁻ RNA after 1 h of translation was set to 100%. The circle indicates the relative translation level obtained from a reaction where luciferase RNAs were added after the pre-incubation step.

period, ATP was restored by the addition of ATP, creatine kinase, and creatine phosphate in excess over glucose. In the control reaction without ATP depletion, the anticipated degree of translation repression was obtained. With the ATP depletion/restoration procedure, translation of the control RNA proceeded at a similar rate as without ATP depletion, albeit after a longer lag phase. In contrast, translation of the *nos* RNA was strongly increased by ATP depletion, and SRE-dependent repression was as low as was typically observed in reactions without any pre-incubation (Figure 2). We conclude that the relatively low level of ATP present in the extract is essential for the formation of the repressor complex.

Pre-incubation does not accelerate SRE-dependent deadenylation

In contrast to the slow assembly of the complex repressing translation, SRE-dependent deadenylation in *Drosophila*

embryo extract proceeds without a pronounced lag phase. However, deadenylation is also ATP dependent: It takes place in the presence of an ATP-regenerating system and is prevented by ATP depletion (Jeske *et al*, 2006). Deadenylation substrates were subjected to the same pre-incubation protocol as used for the translation experiments. Under these conditions of low ATP concentration, there was almost no deadenylated product at the end of the pre-incubation (Figure 3); thus, the low ATP concentration was insufficient to drive deadenylation. When deadenylation was then started by the addition of the ATP-regenerating system, it proceeded at the same rate as without any pre-incubation. Thus, the slow step in the formation of the translation repression complex is irrelevant for SRE-dependent deadenylation, suggesting that the protein complexes responsible for translation repression and deadenylation are not identical.

Figure 2 Translation repression is ATP dependent. (A) Scheme of the assay: embryo extract was incubated with hexokinase and glucose for 5 min for ATP depletion. Capped luciferase RNAs containing an internal poly(A) tract were then added and pre-incubated for 30 min. Subsequently, translation was started by addition of the ATP-regenerating system (ARS). The control reaction was performed similarly, but the extract was initially incubated for 5 min without addition of hexokinase and glucose. (B) Luciferase RNAs containing the *nos* (circles) or the *nos* SRE⁻ (filled circles) 3' UTR fragment were incubated as described in (A). For a better comparison, the ordinates of the two graphs are drawn in the same scale. The time scale refers to the start of translation.

SRE-dependent repression of translation is independent of the cap but partially dependent on the poly(A) tail

When uncapped RNAs were used for the translation assays, luciferase yield dropped by a factor of about 10 (Figure 1; Supplementary Table I), as anticipated from a cap-dependent initiation mechanism. However, SRE-dependent repression was functional with uncapped RNA (Figure 1; Table I). As reported before, translation of RNA lacking a poly(A) tail was 5- to 10-fold less efficient in the embryo lysate (Jeske *et al*, 2006) (Supplementary Table I). Translation of such RNAs was also repressed by the SREs, but repression was less efficient (Jeske *et al*, 2006). Even with a pre-incubation step, repression was approximately five-fold less efficient as compared with RNAs with the 'internal' poly(A) tail (Table I). Thus, while the repression mechanism is fundamentally poly(A) independent, the poly(A) tail appears to assist the formation of the repressor complex; the 5' cap is dispensable.

The repressor complex is stable and cap-independent

A short SRE-containing RNA mixed with the luciferase reporter was able to block repression of translation: 1 μ M competitor RNA was required to reduce repression to two-fold, and ~ 10 μ M completely eliminated repression. In contrast, addition of excess competitor after pre-incubation had only a very weak effect on SRE-mediated repression (Figure 4A), indicating that the repressor complex formed during pre-incubation is stable. Under these conditions, a slow increase in the rate of luciferase production from the *nos* RNA was detected (Figure 4C). Assuming equal stability of *nos* and mutant RNA during incubation, the data could be fitted to a first-order decay of the repressor complex

Figure 3 SRE-dependent deadenylation is not accelerated by a pre-incubation step. Polyadenylated, uncapped, and ³²P-labelled RNA substrates containing the *nos* or the *nos* SRE⁻ 3' UTR fragment were subjected to a 25-min pre-incubation with *Drosophila* embryo extract, then deadenylation was started by addition of the ATP-regenerating system (0 min; left half of the panel). After the time points indicated, RNA was isolated and separated on a denaturing polyacrylamide gel. The control reaction (right half of the panel) was performed in the same way but the pre-incubation step was omitted. S, unreacted substrate RNA. The percentage of fully deadenylated product (Jeske *et al*, 2006) is shown below each lane.

with half-lives of ~ 200 and ~ 230 min in two independent experiments (data not shown). That the repression is reversible, albeit slowly, provides additional evidence that the mechanism is not based on an irreversible SRE-dependent modification of the mRNA (e.g. degradation).

The chain of interactions postulated by the Cup model, SRE-Smaug-Cup-eIF4E-cap (Wilhelm and Smibert, 2005; Vardy and Orr-Waver, 2007) predicts that the interaction between the cap and eIF4E should contribute to the formation of the repressed RNP. However, the complex was kinetically stable even when it was formed on an RNA lacking a cap (Figure 4D). Irrelevance of the cap for formation of the repressor complex was confirmed by addition of increasing amounts of competitor RNA at the beginning of the pre-incubation. Very similar amounts were required to compete with the repression of capped and uncapped RNA (Figure 4B). In contrast, reporter RNA lacking a poly(A) tail was approximately five-fold more sensitive to the competitor, in agreement with less efficient repression of poly(A)⁻ RNAs (Table I). We conclude that the 5' cap does not have an important function in the formation of a stable repressed RNP, but the poly(A) tail contributes.

The repressor complex blocks formation of the 48S pre-initiation complex

The step at which translation of the *nos* RNA was blocked was investigated by sucrose gradient centrifugation of initiation complexes. RNAs with a short open reading frame of 27 amino acids and an 'internal' poly(A) tail were used that were otherwise similar to the luciferase reporter RNAs. In rabbit reticulocyte lysate, both *nos* RNA and *nos* SRE⁻ RNA formed 80S complexes in the presence of cycloheximide and 48S pre-initiation complexes in the presence of the non-hydrolyzable GTP analog GMPPNP with indistinguishable efficiencies (Figure 5A). When the same RNAs were incubated for 40 min in *Drosophila* embryo extract in the presence of GMPPNP and an ATP-regenerating system, 48S complexes were obtained, but their formation was less efficient with the *nos* RNA (Figure 5B). When the RNAs were pre-incubated for 10 min before GMPPNP and the ATP-regenerating system were added, almost no 48S complexes were formed on the

Figure 4 The assembled repressor complex is stable. (A) Capped luciferase RNAs containing an internal poly(A) tract and the *nos* or the *nos* SRE⁻ 3' UTR fragment were pre-incubated for 25 min and subsequently translated for 60 min. Increasing amounts of a short-competitor SRE RNA were added to the reaction either before (filled circles) or after (circles) the pre-incubation step. The percentage of translation of the SRE-containing RNA relative to the mutant RNA is plotted against the competitor concentration. (B) Luciferase RNAs containing an internal poly(A) tract with the indicated 5' and 3' end modifications were mixed with increasing amounts of the SRE competitor. Subsequently, the RNA mixture was pre-incubated for 25 min with embryo extract followed by a 60-min translation reaction. The percentage of translation of the SRE-containing RNA relative to the mutant RNA is plotted against the competitor concentration. (C) Capped luciferase RNAs containing an internal poly(A) tract and the *nos* (circles) or the *nos* SRE⁻ (filled circles) 3' UTR fragment were pre-incubated for 25 min with embryo extract to allow repressor complex formation on *nos* RNA. After pre-incubation, 12 μM of wild-type (left) or mutant (right) SRE competitor was added to the mixture together with the ATP-regenerating system ($t = 0$ min), and a time course of translation was measured. (D) The same experiment as described in (C) was performed using uncapped luciferase RNAs.

nos RNA, whereas only a slight reduction was visible with the control RNA (Figure 5C). It is important to note that, on the control RNA, stable 48S complexes were formed only during the second phase of the incubation, that is upon the addition of GMPPNP and an ATP-regenerating system; no stable complexes were detectable by gradient centrifugation at the end of the pre-incubation phase (data not shown). Thus, the SRE-dependent repressor complex assembled during the pre-incubation prevented pre-initiation complex formation during the second incubation.

Reduction of the 48S peak was accompanied by an increase of radioactivity in the RNP fractions of the gradient, possibly

reflecting the formation of the repressed *nos* RNP. This is supported by the observation that the *nos* RNP sedimented slightly faster than the RNP formed on the control RNA (Figure 5B and C). In contrast, the slight reduction of the 48S peak formed with the control RNA was accompanied by increased radioactivity in the very top fractions of the gradient presumably containing degraded material.

The repressor complex contains Smaug, Cup, and other repressor proteins and excludes eIF4G

For an analysis of proteins participating in repressor complex formation, short biotinylated RNAs similar to those used for

Figure 5 Repressed *nos* RNA does not form 48S complexes. (A) ^{32}P -cap-labelled RNAs containing an internal poly(A) tract and the indicated 3' UTR were incubated with rabbit reticulocyte lysate in the presence of cycloheximide (left panel) or GMP-PNP (right panel) under translation conditions. Resulting ribosomal complexes were resolved by centrifugation in 5–25% linear sucrose gradients (25% sucrose on left). After fractionation from the bottom to the top of the gradient, the radioactivity was monitored, expressed as percentage of total counts recovered and plotted against the fraction number. (B) Scheme of ribosomal complex assembly reaction using *Drosophila* embryo extract. ^{32}P -cap-labelled RNAs containing an internal poly(A) tract were incubated under translation conditions in the presence of GMP-PNP either with or without a preceding pre-incubation step for 10 min. ARS, ATP-regenerating system. (C) Substrate RNAs with the indicated 3' UTR were allowed to assemble ribosomal complexes as described in (B) and analysed as described in (A).

sucrose gradient analysis were immobilized on streptavidin beads and incubated in extract under pre-incubation conditions either with or without ATP depletion (Figure 6A). After pull down and washing, the protein complexes formed on the RNA were analysed by western blotting. As expected, Smaug was enriched on the *nos* RNA as compared with the mutant control. More Smaug was recovered in the presence of ATP, and recovery also improved with the time of incubation. This indicates an ATP- and time-dependent increase either in the amount of Smaug–RNA complex or in complex stability (Figure 6B). As a control, the cytoplasmic poly(A)-binding protein (PABPC) bound equally well to both RNAs in a time- and ATP-independent manner. Other proteins accumulated on the *nos* RNA in a similar manner as Smaug: recovery improved with the time of incubation, and the presence of ATP either increased the yield or improved the specificity for the *nos* RNA. These proteins included Not2 and Caf1, two

subunits of the CCR4–NOT complex responsible for *nos* deadenylation; the DEAD box protein Me31B, a known translational repressor (see Discussion); Trailer Hitch (Tral), one of several proteins associating with Me31B; and Cup, which interacts not only with Smaug (Nelson *et al*, 2004), but also with Me31B or the Tral–Me31B complex (Nakamura *et al*, 2004; Wilhelm *et al*, 2005; Barbee *et al*, 2006; Tritschler *et al*, 2008). We conclude that Tral, Me31B, and the CCR4–NOT complex are components, together with Smaug and Cup, of the SRE-dependent repressor complex.

The postulated chain of interactions, cap–eIF4E–Cup–Smaug–SRE, predicts that binding of all three proteins should be affected by both the cap and the SRE, and that eIF4G should be competed off eIF4E by Cup. The predictions were confirmed in a pull-down experiment (Figure 6C). Whereas the control protein PABPC bound equally well to all RNAs, Smaug was strongly enriched on the *nos* RNAs as compared

Figure 6 RNA pull-down assays reveal components of the repressed RNP: exclusion of eIF4G. **(A)** Scheme of the RNA pull-down assay. Biotinylated and ³²P-labelled RNA substrates containing an external poly(A) tail were immobilized on streptavidin beads (upper panel). Embryo extract was incubated for 5 min in the presence of hexokinase and glucose for ATP depletion or without these reagents as a control. Immobilized RNA was added and incubated with the extract for the times indicated (lower panel). The beads were washed and bound proteins eluted by boiling in SDS sample buffer. Proteins were separated on an SDS-polyacrylamide gel and analysed by western blotting. **(B)** Proteins were detected in an RNA pull-down assay as described in **(A)** using capped *nos* and *nos* SRE⁻ RNA substrates. In all, 36 µl of embryo extract were used for each time point of the pull down. For comparison, various amounts of extract were analysed in the same western blot (shown in the first six lanes). **(C)** eIF4G is excluded from the repressed RNP. An RNA pull-down assay was carried out as described in **(A)** except that no ATP depletion was carried out. Capped and uncapped substrate RNAs carrying the *nos* or the *nos* SRE⁻ sequences were compared as indicated. Immobilized RNAs were incubated with embryo extract for 30 min. Beads without immobilized RNA were used as a background control.

with the SRE mutant RNAs (compare lanes 2 and 4 with 3 and 5), and also bound slightly better to the capped as compared with the uncapped *nos* RNA (lanes 2 and 4). Cup was enriched on the capped *nos* RNA (lane 2). Binding to the uncapped *nos* RNA was below the detection limit in this experiment (lane 4), but a low level of binding was visible in others. No binding was visible with the SRE mutant RNAs (lanes 3 and 5). eIF4E was not detectable on the RNA lacking both a cap and an SRE (lane 5), the levels were intermediate on RNAs containing either a cap or an SRE (lanes 3 and 4), and highest on the RNA containing both (lane 2). In agreement with the model, this suggests that eIF4E can associate with the RNA either via the cap or via Smaug and Cup. Most importantly, eIF4G binding was seen only on the RNA that contained a cap but lacked an SRE and, thus, did not bind Smaug and Cup (lane 3). This strongly supports displacement of eIF4G by Smaug.

SRE-dependent repression also affects steps subsequent to eIF4G binding and 48S complex formation

The results obtained so far confirm the inhibition of 48S pre-initiation complex formation predicted by the Cup model of SRE-dependent repression. In order to test whether subsequent steps are also inhibited in an SRE-dependent manner, the internal ribosome entry site from the cricket paralysis virus intergenic region (CrPV IRES) was fused to the reporter RNAs. CrPV IRES-dependent initiation operates by 80S ribosome formation directly on the IRES, independently of initiation factors (Wilson *et al*, 2000; Pestova and Hellen, 2003); eIF4G and its interaction with eIF4E are not required for CrPV IRES-dependent translation (Deniz *et al*, 2009; Garrey *et al*, 2010). Thus, if a displacement of eIF4G were the only mechanism of SRE-dependent repression, CrPV IRES-dependent translation should be resistant. At elevated

Figure 7 CrPV IRES-dependent translation is repressed in an SRE-dependent manner. Uncapped luciferase reporter RNAs containing the CrPV-IRES, the indicated 3' UTR and internal poly(A) tails were pre-incubated for 30 min with embryo extract under the conditions described in Materials and methods. Subsequently, translation was started by the addition of an ATP-regenerating system, and luciferase activity was measured at the time points indicated.

salt concentrations, CrPV IRES-dependent translation in embryo extract was 180-fold more efficient than cap-dependent translation and insensitive to cap competition, as expected (Supplementary Figure S2). CrPV IRES-dependent translation was repressed approximately three-fold by the SREs (Figure 7). At the salt concentrations used, cap-dependent translation was inhibited to the same low extent. Repression was only observed with a pre-incubation (data not shown). Thus, the SRE-dependent repressor complex inhibits not only eIF4G binding and 48S complex formation but also at least one later step in translation.

One possibility to explain the inhibition of CrPV IRES-dependent initiation would be that the SREs affect nascent peptide chains (see Introduction). To test this hypothesis, capped mRNAs encoding flag-tagged luciferase were translated in embryo extract in the presence of ³⁵S-methionine. In addition to the serine protease inhibitor used routinely during extract preparation, the proteasome inhibitor MG132 was included. Products derived from the reporter RNAs were separated from the background of endogenous translation products by selection on anti-flag beads and then analysed by gel electrophoresis. Both flag-luciferase *nos* and flag-luciferase *nos* SRE⁻ RNAs yielded mostly full-length products; no significant increase in partial translation products was detectable with the repressed RNA (Figure 8). Quantitative assays showed that the incorporation of ³⁵S was inhibited to the same extent (7.1 ± 0.7 -fold) as luciferase activity (6.8 ± 0.3 -fold). This confirms that the proportion of partial translation products, which contribute to ³⁵S incorporation but not to luciferase activity, was not different between *nos* and *nos* SRE⁻ RNAs (data not shown). Thus, an effect of the SREs on nascent peptide chains was not detectable, in agreement with earlier data (Clark *et al*, 2000).

Oskar overcomes deadenylation and translation repression

In the embryo, translational repression of the *nos* RNA is overcome at the posterior pole by a mechanism involving Oskar. In the embryo extract, the addition of Short Oskar (Markussen *et al*, 1995) fused to GST (Supplementary Figure S3) was sufficient to prevent SRE-dependent translation inhibition (Figure 9A). Translation of the control RNA was not affected, and the addition of GST had no effect on either RNA. GST-Oskar was unable to relieve repression when it was

Figure 8 SRE-dependent repression has no effect on nascent peptide chains. Capped luciferase reporter RNAs with an N-terminal flag tag and an internal poly(A) tract were translated as in Figure 1B. ³⁵S-Methionine was added at 2 μ Ci/reaction. Translation reactions were stopped with 5 μ l 50 mM EDTA and 1 mg/ml RNase A, incubated for 20 min at 30°C and mixed with 85 μ l IP buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5% Triton X-100, 1 mg/ml yeast total RNA). The mixture was transferred to 20 μ l anti-FLAG agarose (Sigma) and incubated for 40 min at 8°C with shaking. The agarose was washed five times with 200 μ l IP buffer. Proteins were sequentially eluted with FLAG peptide (1 mg/ml in IP buffer) and SDS-loading buffer and separated on a 10% SDS-PAGE. The picture shows the autoradiogram of the gel. 'Mock' is a translation reaction without added reporter RNA.

added after the pre-incubation during which the repressor complex was formed (data not shown). In agreement with *in vivo* observations (Zaessinger *et al*, 2006), GST-Oskar also prevented SRE-dependent deadenylation (Figure 9B). Higher Oskar concentrations were necessary for the inhibition of deadenylation compared with the relief of translation repression. Note that the luciferase reporter RNAs used for the translation assays carried an internal poly(A) tail resistant to deadenylation; thus, Oskar affects translation independently of the inhibition of deadenylation.

In a GST pull-down assay, GST-Oskar added to embryo extract bound endogenous Smaug, in agreement with a previously reported interaction of an Oskar fragment with Smaug prepared by *in vitro* translation (Dahanukar *et al*, 1999). The Oskar-Smaug association was resistant to RNase (Figure 10A). Pull down of the SRE-containing RNA showed that GST-Oskar inhibited the association of Smaug with the RNA, confirming a previous observation (Zaessinger *et al*, 2006) (Figure 10B). CAF1 was also not associated with the immobilized RNA when Smaug binding was prevented by GST-Oskar (Figure 10B), in agreement with the inhibition of deadenylation by GST-Oskar and with a recruitment of the CCR4-NOT complex by Smaug. As a control, binding of PABPC was not affected. Whether the effect of Oskar on deadenylation and translational repression reflects a simple interaction with Smaug or depends on additional proteins remains to be seen.

Discussion

We have examined the mechanism of SRE-dependent translational repression of the *nos* RNA. Three results are unusual:

Figure 9 Recombinant Oskar prevents both SRE-dependent translation repression and deadenylation. **(A)** Translation reaction mixtures lacking RNA and the ATP-regenerating system were incubated with increasing amounts of recombinant GST-Oskar (left panel) or GST (right panel) for 15 min. Capped luciferase RNAs containing an internal poly(A) tract and with the indicated 3' UTR fragments were then added, and the incubation continued for another 25 min before translation was started by the addition of the ATP-regenerating system and allowed to proceed for 60 min. Luciferase activity is plotted against concentration of the protein indicated. When no protein was added (0 μM), protein buffer was used instead. **(B)** Deadenylation reaction mixtures were incubated for 15 min with GST-Oskar, GST, or protein buffer in the absence of RNA and an ATP-regenerating system. Deadenylation was started by addition of polyadenylated, ³²P-labelled substrate RNAs with the 3' UTR fragments indicated and buffer containing the ATP-regenerating system. At the time points indicated, RNA was isolated and separated on a denaturing polyacrylamide gel. S, unreacted substrate RNA.

Formation of the repressor complex is slow and ATP dependent; once formed, the repressor complex is very stable; and repression is almost complete, ~50-fold under favourable conditions. In agreement with a previously proposed model (Nelson *et al*, 2004), the protein Cup is incorporated into the repressor complex, and eIF4G is displaced. Consequently, translation is inhibited at the step of 48S pre-initiation complex formation. However, a late step in translation initiation is also inhibited. This, together with the ATP dependence of repressor complex formation and the presence of Me31B and Tral in the repressor complex indicates that the Cup model is not sufficient to explain SRE-dependent translation repression.

The unusual stability of the repressor complex and its potency in translation repression match the properties expected for a 'masked' RNP tightly repressing the synthesis of important regulatory proteins over extended periods of time. One feature linking the SRE-dependent repressor complex and previous analyses of masked RNPs is the presence of Me31B, a conserved translational repressor. Its *Xenopus* orthologue, Xp54, is a component of masked RNPs and is able to inhibit translation (Ladomery *et al*, 1997; Minshall *et al*, 2001, 2009). In flies, Me31B has a function in the translational repression of *oskar* and *bicaudal D* (Nakamura *et al*, 2001) and of mRNAs in neuronal cells (Barbee *et al*, 2006). Me31B and mammalian p54/RCK/DDX6 are involved in miRNA-dependent repression (Chu and Rana, 2006; Eulalio *et al*, 2007b). In yeast, *dhh1Δ/pat1Δ* double mutants have a general defect in translational repression, and Dhh1p (the yeast orthologue of Me31B) prevented 48S pre-initiation

complex formation *in vitro*, independently of specific RNA sequences. CrPV IRES-driven translation was also inhibited (Coller and Parker, 2005). Interestingly, the time- and ATP-dependent formation of a repressed mRNP with a protein fraction from *Xenopus* oocytes has been described (Yurkova and Murray, 1997). The protein fraction was later reported to contain Xp54 (personal communication cited in Minshall *et al* (2001)). While this reaction was independent of specific RNA sequences, the phenomenon is strongly reminiscent of our observations and suggests that we are dealing with a conserved mechanism. The association of Me31B with Tral and Cup is also conserved. For example, RAP55 and eIF4E-T, the *Xenopus* orthologues of Tral and Cup, are found, together with Xp54, in protein complexes and mRNPs in oocytes, and both RAP55 and eIF4E-T can repress translation in tethering assays or upon overproduction (Ferraiuolo *et al*, 2005; Tanaka *et al*, 2006; Minshall *et al*, 2007). The function of Me31B, Tral, and Cup is reflected in their localization in P bodies (Parker and Sheth, 2007; Eulalio *et al*, 2007a) and other RNP granules (Standart and Minshall, 2008). A role for Me31B and Tral in SRE-dependent repression is likely but remains to be proven.

While the kinetic stability of the SRE-Smaug complex has not yet been examined, the long time required for the formation of the repressor complex, its ATP dependence and its kinetic stability suggest the possibility that the repressor complex may be formed by an energy-consuming, catalysed assembly process and is not governed by simple association-dissociation equilibria. Both efficient repression of translation and specific protein binding in pull-down assays were de-

Figure 10 Recombinant Oskar associates with Smaug and prevents its binding to the SRE. **(A)** Oskar binds to Smaug independently of RNA. Embryo extract was incubated with GST-Oskar or GST in the presence or absence of RNases (see Materials and methods). After capture of the proteins on glutathione beads and washing, bound proteins were eluted by boiling in SDS sample buffer. Samples were separated on an SDS-polyacrylamide gel and analysed by western blotting. Different amounts of the input extract were loaded for comparison. **(B)** Oskar prevents binding of Smaug to SREs. An RNA pull-down assay was carried out as described in Figure 6 **(A)** using capped RNAs with the 3' UTR fragments indicated. Embryo extract was incubated with GST-Oskar or GST for 15 min, immobilized RNAs were added and incubated with the mixture for another 30 min. Beads without RNA were used as background control. Bound proteins were analysed by western blotting with antibodies against Smaug, CAF1, and PABPC as indicated. Different amounts of the input extract were loaded for comparison.

pendent on the presence of ATP. Interestingly, ATP not only promoted binding of proteins to the SRE-containing RNA but also appeared to reduce binding of the same proteins to the control RNA. Thus, ATP might be consumed in RNP remodeling reactions (Jankowsky and Bowers, 2006) that disassemble complexes formed on the 'wrong' RNA. One might speculate that this kind of proofreading is necessary because of the stability and potent repressor activity of the complex once it has formed. A pre-incubation comparable to the one used here also functions in cell-free systems for miRNA-dependent repression, although an ATP requirement has so far only been found for deadenylation (Mathonnet *et al*, 2007; Thermann and Hentze, 2007; Iwasaki *et al*, 2009; Zdanowicz *et al*, 2009).

According to the 'closed loop' model of translation initiation, eIF4G, which is essential for the recruitment of the small ribosomal subunit, is bound cooperatively by simultaneous interactions with PABPC occupying the poly(A) tail and with eIF4E associated with the cap (Tarun and Sachs, 1996; Jackson *et al*, 2010). Alternative 5'-3' protein assemblies can be used for translational repression. Repressor proteins bound to sites in the 3' UTR engage the cap in a manner that excludes eIF4G (Stebbins-Boaz *et al*, 1999; Cao and Richter, 2002; Cho *et al*, 2005; Minshall *et al*, 2007;

Standart and Minshall, 2008). SRE-dependent repression of *nos* translation has been proposed to rely on a similar mechanism. According to this model, SRE-bound Smaug associates with Cup, which in turn binds eIF4E and evicts eIF4G (Nelson *et al*, 2004). The *oskar* RNA is believed to be regulated in a similar manner, except that Cup is recruited by the RNA-binding protein Bruno (Wilhelm *et al*, 2003; Nakamura *et al*, 2004). This model is based on the well-established interaction between eIF4E and Cup and on genetic evidence that Cup is required for *nos* and *oskar* repression (Wilhelm *et al*, 2003; Nakamura *et al*, 2004; Nelson *et al*, 2004; Zappavigna *et al*, 2004).

Our data confirm several key predictions of the Cup model: Cup is present in the repressed RNP. This is specific, as it is SRE dependent and, therefore, presumably Smaug dependent. Cup binding is also cap dependent, consistent with its association with the cap-binding protein eIF4E. eIF4G is specifically displaced, whereas eIF4E remains part of the repressed RNP; thus, the eIF4F complex is disrupted, consistent with the proposed role of Cup. Finally, 48S pre-initiation complex formation is prevented, as would be expected from the displacement of eIF4G.

However, a number of observations either contradict the Cup model or are not explained by it. First, the 5' cap is dispensable for repression. RNAs lacking a cap are efficiently repressed, and the stability of the repressor complex is independent of the cap. Second, inhibition of translation is not restricted to the displacement of eIF4G and prevention of 48S complex formation; a late step in translation is also affected, as shown by the inhibition of CrPV IRES-dependent translation. Third, the Cup model does not explain the ATP requirement in the formation of the repressor complex. Fourth, the repressed RNP contains proteins not accommodated by the model of Cup interfering with the eIF4E-eIF4G interaction, most of all Me31B. We are not aware of genetic evidence for a Me31B requirement in *nos* regulation, but such evidence does exist for *oskar* (Nakamura *et al*, 2001). Cup might have a second role in repression of *nos* mRNA by recruiting Me31B to the SRE via its interaction with Tral (Wilhelm *et al*, 2005; Tritschler *et al*, 2008). In summary, while our data suggest that key aspects of the Cup model are correct, the model is not sufficient to explain the repression of *nos* mRNA translation. Experiments on *oskar* regulation support the view that the Cup model only covers one aspect of translational repression of the *oskar* and *nos* mRNAs (Chekulaveva *et al*, 2006). It has recently been suggested that subunits of the CCR4-NOT complex may repress translation independently of the deadenylation process (Cooke *et al*, 2010); thus, the CCR4-NOT complex might also have a function in translational repression of *nos*.

The inhibition of multiple steps in translation initiation can be explained in two ways. It is possible that components of the repressor complex target multiple individual steps through specific protein-protein interactions, similar to the inhibition of the eIF4E-eIF4G interaction by Cup. Alternatively, the repressor complex might sequester the mRNA in a manner that makes it generally inaccessible. This hypothetical mechanism has been termed 'structural masking' and compared to the condensation of chromatin (Spirin, 1994). 'Structural masking' of the mRNA might most easily account for the effect of the repressor complex on CrPV IRES-dependent initiation, which provides few if any

targets for specific protein–protein interactions. *In vitro*, ribosome assembly on the IRES can proceed from separate subunits in the absence of any initiation factor (Wilson *et al*, 2000; Pestova and Hellen, 2003; Spahn *et al*, 2004). *In vivo*, preformed 80S ribosomes might associate with the IRES or separate subunits might assemble with the help of eIF5B (Pestova *et al*, 2004). An SRE-dependent inhibition acting on nascent chains, which might also explain repression of CrPV IRES-dependent translation, was not detectable *in vitro*.

The SREs direct both translational repression and deadenylation of the *nos* mRNA (Dahanukar and Wharton, 1996; Gavis *et al*, 1996; Smibert *et al*, 1996; Bashirullah *et al*, 1999). A role for Smaug in both processes has been shown by genetic evidence (Dahanukar *et al*, 1999; Nelson *et al*, 2004; Zaessinger *et al*, 2006). However, the protein complexes responsible for the two processes differ. Deadenylation proceeds without a pronounced lag phase, is not accelerated by a pre-incubation step, and requires a high concentration of ATP. In contrast, formation of the translation repressor complex is slow and can proceed at a lower ATP concentration. Furthermore, deadenylation proceeds efficiently with up to 50 nM RNA, whereas translational repression works well only at 1–10 nM. Finally, we have repeatedly had batches of extract that were active in SRE-dependent deadenylation but deficient in translational repression and *vice versa*. Thus, it appears that Smaug recruits different effector proteins that affect either deadenylation or translation; factors required for the latter may be less abundant in the extract.

Materials and methods

DNA templates for *in vitro* transcription

Plasmids coding for luciferase RNAs, deadenylation substrates, and SRE-competitor ('SRE only') RNAs have been described (Jeske *et al*, 2006). Construction of templates for RNAs used for sucrose gradients and pull downs is described in the Supplementary data (Supplementary Text; Supplementary Table II). These RNAs (1-AUG *nos*, 1-AUG *nos* SRE[−]) contained a short open reading frame coding for 27 amino acids. The construction of templates for the flag tag-luciferase RNAs and for those containing the IRES of the cricket paralysis virus intergenic region (a gift of Nicola Gray via Antje Ostareck) is also described in the Supplementary data. All DNA constructs contained a poly(A) sequence of 72 nucleotides downstream of the 3' UTR. Cutting the plasmids with *Bam*HI, *Hind*III, or *Kpn*I prior to *in vitro* transcription resulted in RNAs that carried no poly(A) tail, an external poly(A) tail, or an 'internal' poly(A) tail followed by 40 nucleotides of a mixed sequence, respectively.

In vitro transcription

RNA synthesis was carried out as described (Jeske *et al*, 2006). 3'-O-methyl-m⁷GpppG cap dinucleotide (New England Biolabs) was incorporated instead of m⁷GpppG. The SRE-competitor RNAs were neither capped nor polyadenylated. RNA substrates for sucrose gradient analyses were produced by *in vitro* transcription of *Kpn*I-digested 1-AUG templates in the absence of a cap dinucleotide and radioactive nucleotides. In all, 6 pmol of the purified RNA was used in an *in vitro* capping reaction according to the 'alternate cap 0 capping protocol' supplied with the ScriptCapTM m⁷G Capping System (Epicentre), except that the GTP concentration was 4 μ M, 1 μ M [α -³²P] GTP (3000 Ci/mmol, Hartmann Analytics, Braunschweig, Germany) was added, and the enzyme concentration was doubled. The resulting cap-labelled RNA was purified as described (Jeske *et al*, 2006). The capping efficiency was at least 55%. RNA substrates for RNA pull-down assays were synthesized by *in vitro* transcription of *Hind*III-digested 1-AUG templates in the presence of [α -³²P] UTP, 80 μ M Biotin-16-UTP (Roche) and 1 mM UTP.

Drosophila embryo extracts and *in vitro* assays

Extracts were prepared as described (Jeske and Wahle, 2008) except that centrifugation was for 30 min at 20 800 $\times$ g instead of 20 min at 14 500 $\times$ g. Extracts from 0.5- to 2.5-h-old embryos were mainly used.

Translation assays using *Drosophila* embryo extract have been described (Jeske *et al*, 2006). Luciferase RNAs were used at 1 nM, and magnesium acetate was 1 mM. GTP and spermidine were omitted, and incubation was at 22°C. When GST-Osk or GST was added, only 20% extract was used. ATP depletion was carried out and confirmed as described (Jeske *et al*, 2006) except that 5 mM glucose was used. The ATP-regenerating system consisted of creatine kinase, creatine phosphate, and ATP (Jeske *et al*, 2006). For translation of CrPV-IRES containing luciferase RNAs, magnesium acetate was increased to 1.5 mM and potassium acetate was used at 150 mM. MG132 (Calbiochem) was used at 1 μ M. Luciferase activities (RLU/s) reported in the figures correspond to 10 μ l of translation reaction.

Deadenylation assays were performed as described but spermidine was omitted (Jeske and Wahle, 2008). In all, 20% *Drosophila* embryo extract and 10 nM substrate RNAs were used.

Sucrose gradient analysis

In all, 13.5 nM of ³²P-cap-labelled 1-AUG RNA substrates were incubated with embryo extract under translation conditions in a volume of 10 μ l for the time indicated. GMPNP was used at 5 mM. After incubation, the reactions were diluted with 50 μ l gradient buffer (24 mM Hepes-KOH, 100 mM potassium acetate, 4 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.4) and loaded on top of an 11-ml 5–25% (w/v) sucrose gradient in gradient buffer. Gradients were centrifuged in an SW40Ti rotor (Beckman) at 38 000 r.p.m. and 4°C for 2 h followed by an $\sim$ 40 min deceleration without braking. Fractions of $\sim$ 500 μ l were collected from bottom to top of the gradient with continuous monitoring of the optical density at 280 nm, and the radioactivity was determined by Čerenkov counting.

Sucrose gradient analysis using rabbit reticulocyte lysate (untreated; Promega) was performed similarly with the following modifications. In the presence of 5 mM GMP-PNP, 10 nM RNA was incubated in a volume of 25 μ l, and the reaction was diluted with 75 μ l of gradient buffer. In the presence of 1 mM cycloheximide, 13.5 nM RNA and a volume of 10 μ l were used and diluted with 50 μ l buffer. RNA substrates were incubated for 30 min at 30°C using the same buffer mixture as for translation in *Drosophila* embryo extract.

Protein purification

The coding region of short Oskar (fly base accession number CG10901-PC) was cloned into the pGEX-6P-1 vector (Promega) as described in the Supplementary data. GST and GST-Oskar were expressed in *Escherichia coli* and purified on glutathione sepharose 4FF (GE Healthcare). Lysis and washing buffer consisted of 20 mM Tris-HCl, 500 mM potassium acetate, 0.1 mM EDTA, 0.05% (v/v) Tween 20, 1 mM DTT, pH 8.0. Elution buffer was 100 mM Tris, 200 mM potassium acetate, 50 mM glutathione, 1 mM DTT, pH 8.0. Eluted proteins were concentrated, glutathione was removed and the buffer changed to protein buffer (100 mM Tris-acetic acid, 200 mM potassium acetate, pH 8.0) by Centricon filtration. The concentrations of GST and GST-Oskar were determined from the UV spectra based on calculated extinction coefficients. Due to the presence of shorter fragments, the measured concentration overestimates the concentration of full-length Oskar.

RNA pull-down assay

Biotinylated and ³²P-labelled RNA substrates were incubated with Dynabeads[®] M-270 Streptavidin (Invitrogen) in 10 $\times$ SSC buffer supplemented with 0.5 μ g/ μ l methylated BSA for 1 h at 15°C with constant agitation. Repressor complex assembly was performed in a reaction containing 10 nM immobilized RNA, 40% *Drosophila* embryo extract, 250 μ g/ml yeast tRNA (Roche), 16 mM Hepes-KOH pH 7.4, 1 mM magnesium acetate, 27% (v/v) protein buffer, and 30 μ g/ml heparin at room temperature with mixing at 10 s intervals. (The concentration of heparin used did not affect SRE-dependent deadenylation. Concentrations up to 20 μ g/ml did not affect translation repression; higher concentrations could not be tested as translation was inhibited.) The tube was transferred onto ice, and the beads were washed twice with a buffer consisting of 16 mM Hepes-KOH pH 7.4, 150 mM potassium chloride, 1 mM

magnesium acetate, 27% (v/v) protein buffer, 30 µg/ml heparin, and 1 mM dithiothreitol. During the second washing step, the beads were transferred into a fresh tube. This reduced the background probably caused by proteins bound to the tube wall. When GST-Osk or GST was tested in the RNA pull-down assay, only 20% of extract were used and extra protein buffer was omitted.

GST pull-down assay

A measure of 90 µl reactions were assembled on ice and contained 20% *Drosophila* embryo extract, 250 µg/ml yeast tRNA, 1 mM magnesium acetate, 16 mM Hepes-KOH pH 7.4, 30 µg/ml heparin, and 26.2 µM GST-Oskar or GST protein. After incubation for 50 min at 22°C, reactions were diluted with 200 µl protein buffer and supplemented with 750 µM CaCl₂. Each reaction was split; one half was supplemented with 1500 U micrococcal nuclease (Fermentas) and 1 µg RNase A, the second half was left without RNases. GST-Oskar or GST were captured on 20 µl packed volume of MagneGST™ Glutathione Particles (Promega) equilibrated in protein buffer by incubation in the cold room for 5 h with head-over-tail-rotation. Beads were washed three times with 300 µl protein buffer. During the last washing step, the mixture was transferred to a fresh tube. Proteins were eluted by boiling in 30 µl of SDS sample buffer. In all, 40% of the eluted proteins were separated in a 4–12% gradient SDS–polyacrylamide gel and analysed by western blotting.

Western blotting

The procedure and antibodies to Caf1 and Not2 have been described (Jeske *et al*, 2006). Other antibodies were kind gifts of

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: MJ, BM, and AA did the experiments; MJ, BM, and EW designed the experiments and interpreted the results; and MJ and EW wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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