„Deadenylation and Translation Regulation of *nanos* mRNA in *Drosophila* Embryo Extract“

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CONTENTS

1 ABSTRACT

During oogenesis and early embryogenesis, maternal mRNAs and proteins determine the developmental program. Over this period of time, transcription does not contribute to the regulation of gene expression, and translation control becomes eminently important. The maternal *nanos* mRNA specifies the development of posterior structures in the *Drosophila* embryo. Translation of *nanos* mRNA is restricted to the posterior pole of the embryo, and the vast majority of *nanos* mRNA within the embryonic cytoplasm is silenced. *nanos* translation repression is mediated by the protein Smaug, which can bind specific sequences, Smaug recognition elements (SREs), in the *nanos* 3′ UTR. At the posterior pole, translation of *nanos* mRNA is derepressed by the action of Oskar protein. *nanos* mRNA regulation had so far been investigated mainly by genetic approaches, and the aim of this thesis was to use *in vitro* analyses in order to obtain a more detailed mechanistic understanding of the underlying processes.

In this doctoral study, a cell-free system derived from *Drosophila* embryos was established that recapitulates the translational repression of *nanos* mRNA. Moreover, a rapid deadenylation of *nanos* mRNA that depends on the SRE-sequences was observed in these extracts. Biochemical characterization revealed that deadenylation of *nanos* mRNA is an exonucleolytic process and is not influenced by a cap structure on the RNA. The SRE-dependent deadenylation activity can be sedimented entirely by centrifugation of the extract. The active pellet fraction contains Smaug, all known components of the CCR4-NOT deadenylase complex, which is likely responsible for catalysis of the SRE-dependent deadenylation reaction, as well as P body components, such as the putative RNA helicase Me31B and the 5′ exonuclease Pacman. Unexpectedly, deadenylation activity depends on the presence of an ATP-regenerating system. Furthermore, SRE-dependent deadenylation contributes to repression of *nanos* mRNA but is not the only regulatory mechanism.

In addition, an almost complete SRE-mediated translation inhibition was detected in *Drosophila* embryo extracts. Biochemical analysis revealed that the assembly of the repressor complex on the SRE RNA is slow and that the complex displays a very high kinetic stability. Moreover, strong SRE-dependent translation repression required ATP, and preliminary results suggested that ATP might serve to ensure sequence-specific binding of the regulatory complex.

According to the current model of SRE-mediated repression, Smaug interferes with the interaction of the translation initiation factors eIF4E and eIF4G. This model is based on the finding that Smaug can associate with the eIF4E-binding protein Cup, which can displace 4G from 4E. Several important predictions resulting from this model were tested in this study. It was shown that the repressed SRE-containing RNA is not associated with the small ribosomal subunit, indicating a block of translation before 48S complex formation. In addition, Smaug and Cup bind to RNA in an SRE-dependent manner. Furthermore, eIF4E is part of the complex that assembles on the SRE, whereas eIF4G is excluded. Thus, the *in vitro* system provided strong biochemical evidence in favor of the hypothesis of a repression mechanism that involves Cup and interferes with the eIF4E – eIF4G interaction. However, both efficient SRE-mediated repression and the high stability of the repressor complex were independent of a cap structure on the reporter RNA, suggesting the existence of an additional inhibitory mechanism different from the interference of the eIF4E – eIF4G interaction. This is further supported by the observation that translation of CrPV IRES-containing reporter RNAs, which is independent of translation initiation factors, is still repressed in an SRE-dependent manner.

Besides Smaug and Cup, several other proteins associated with RNA in an SRE-dependent manner, including the catalytically active subunit CAF1 and other components of the *Drosophila* CCR4-NOT complex, consistent with the SRE-mediated deadenylation activity. Furthermore, two additional proteins that have been implicated in translational repression of other target mRNAs were enriched, namely Me31B and Trailer Hitch.

Finally, it was shown that recombinant Oskar protein bound in an RNase-insensitive manner to Smaug and prevented it from binding to RNA. Importantly, recombinant Oskar overcame both SRE-dependent deadenylation and translational repression in the *Drosophila* embryo extract.

2 INTRODUCTION

Most cells of an organism contain the complete set of genetic information. Whereas some genes are indispensable to every cell, others are necessary only in specified cell types. Gene expression underlies an exact spatial and temporal control. Some genes are activated or inactivated by environmental factors or endogenous signals, whereas other genes are constitutively expressed. Control of gene expression is achieved at multiple levels. Every single step on the way from a gene to its gene product can be subjected to regulation, such as mRNA synthesis and processing, transport of the mRNA into the cytoplasm and localization within the cell, as well as mRNA translation and degradation. Initiation of mRNA synthesis is the most important control point for a variety of genes. However, the impact of post-transcriptional regulation of gene expression becomes especially prominent in situations where the genome is not accessible to transcription. Such situations are often found during development, and one example is the early *Drosophila* embryo. *Drosophila* is one of the most important model organisms in molecular genetics, and mRNA localization and translation regulation have been shown to largely contribute to control of the first steps of early *Drosophila* embryogenesis.

Oogenesis and early embryogenesis of *Drosophila*

The *Drosophila* female reproductive tract consists of two ovaries, and each of them is composed of a cluster of 16 to 20 ovarioles. The ovarioles are the functional units of egg production. Each ovariole contains a chain of six to seven sequentially more mature egg chambers, called follicles. A follicle contains a cyst of 16 cells interconnected by cytoplasmic bridges, also known as ring canals (Fig. 1). One of these 16 cells will develop into the future oocyte, whereas the residual 15 cells become nurse cells. One layer of somatic follicle cells surrounds the individual 16-cell cluster. The nurse cells are polyploid and undergo massive transcription. They produce mRNAs, proteins, and organelles that are deposited via the ring canals into the maturing oocyte and are essential for growth and development of the oocyte. At the end of oogenesis, the nurse cells rapidly transport their complete cytoplasm into the oocyte, a process called nurse cell dumping, and they degenerate. The follicle cells are necessary for the synthesis of the eggshell, composed of vitelline membrane

and chorion, and degenerate as well at the end of oogenesis. The mature egg reaches one of the pairy oviducts, where it becomes fertilized with semen from a previous mating and is laid by the vulva of the female fly.

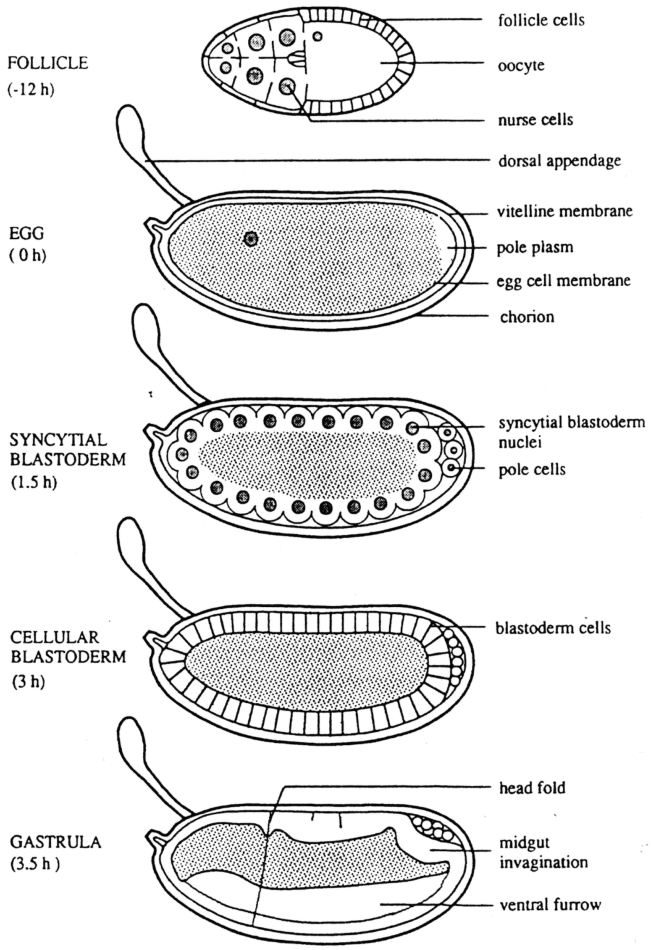

FIGURE 1. **Oogenesis and early embryogenesis of *Drosophila*.**
See text for explanation. Ring canals are depicted as notches of the cell membrane in the follicle. Figure taken from St Johnston *et al.* 1992.

Fertilization of the mature oocyte triggers 13 rapid and synchronous divisions of the nuclei. These early mitotic cycles are not followed by cytokinesis and, thus like oogenesis, early embryogenesis occurs within a syncytium. Initially, the nuclear divisions occur within the inner yolk region. Prior to the 10^{th} division cycle three to four nuclei migrate to the pole plasm at the posterior of the embryo. These nuclei cellularize precociously, and the newly formed cells divide two to three times to produce 20 to 30 pole cells, which are the germ-line precursor cells that will give rise to eggs or sperm. The remaining nuclei migrate to the surface at cycle 10 to 11 and undergo several more synchronous divisions, which result in formation of the

syncytial blastoderm (Fig. 1). During the 14th cycle the cell membrane grows inward from the egg surface to enclose each nucleus, thereby converting the syncytial blastoderm into a cellular blastoderm consisting of about 6000 separate cells (Fig. 1). Cellularization is completed about 2½ hours after fertilization and followed by the first gastrulation movements within the embryo.

The first 13 mitotic cycles of early embryogenesis are extremely rapid, occurring about every 10 minutes, and the zygotic genome is hardly accessible to transcription. Hence, control of the early steps of embryogenesis depends on maternally provided mRNAs and proteins. With the onset of cellularization, the control of development is transferred from the maternal to the zygotic genes. This process is referred to as maternal-to-zygotic transition in gene expression. The first zygotically programmed mitosis is the 14th, which is no longer synchronous. (Oogenesis, embryogenesis, and maternal-to zygotic transition are reviewed in St Johnston *et al.* 1992; Johnstone *et al.* 2001; Bastock *et al.* 2008; Cooperstock *et al.* 2001; Curtis *et al.* 1995; Micklem 1995; Tadros *et al.* 2007; Semotok *et al.* 2007.)

nanos determines the posterior development of the *Drosophila* embryo

The future embryonic body axes are specified before fertilization by a complex exchange of signals between the unfertilized oocyte and the surrounding follicle cells (Riechmann *et al.* 2001; Huynh *et al.* 2004; Roth *et al.* 2009). The embryonic anterior-posterior axis is formed through localization of *bicoid* mRNA to the anterior as well as *oskar* and *nanos* mRNA to the posterior of the oocyte. The dorso-ventral axis is established through dorsal localization and translation of *gurken* mRNA.

nanos belongs to the posterior set of maternal-effect genes, which comprises all genes that, when nonfunctional, lead to an abnormal phenotype of posterior patterning (Nüsslein-Volhard *et al.* 1987). During oogenesis, *nanos* mRNA is synthesized in the nurse cells and transported into the maturing oocyte (Wang *et al.* 1994). At the end of oogenesis, 4% of *nanos* mRNA are located at the pole plasm at the posterior tip of the egg (Fig. 2; Bergsten *et al.* 1999). Upon fertilization of the mature oocyte, translation of the localized fraction becomes activated, whereas *nanos* mRNA distributed throughout the cytoplasm remains inactive (Gavis *et al.* 1994). Due to the restricted protein synthesis and diffusion within the embryonic syncytium, a Nanos protein gradient forms emanating from the posterior pole (Fig. 2; Wang *et al.* 1991).

The *nanos* gene determines the development of the posterior body part. *Drosophila* females without a functional *nanos* gene produce embryos lacking an abdomen, and ectopic expression of *nanos* at the anterior part leads to development of a mirror-imaged abdomen at the expense of head and thorax (Fig. 2; Lehmann *et al.* 1991; Gavis *et al.* 1992). Furthermore, injection of synthetic *nanos* RNA into embryos lacking the function of *nanos* or any other posterior group gene rescues the segmentation defect (Wang *et al.* 1991).

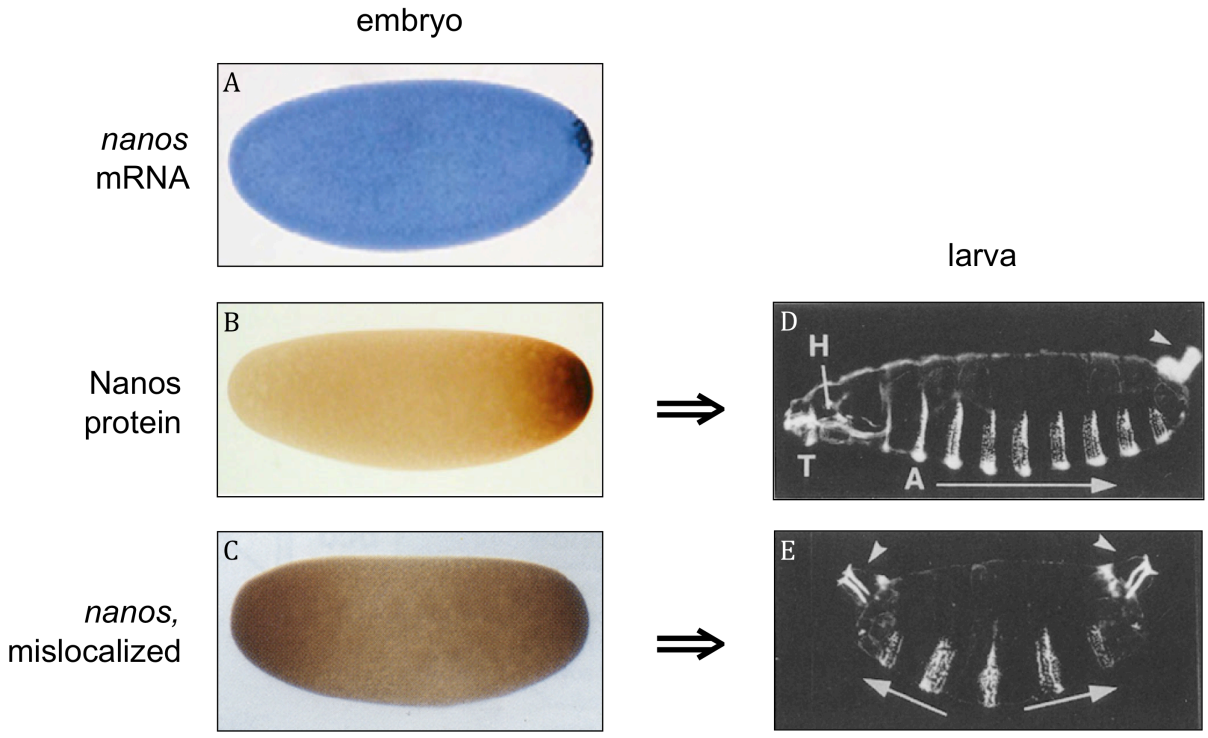

FIGURE 2. ***nanos* determines the posterior development of *Drosophila*.**
Localization of *nanos* mRNA to the pole plasm (**A**) results in a Nanos protein gradient (**B**) and is important for proper development into the larva (**D**). Mislocalization of *nanos* mRNA in the embryo is obtained by using females that carry an additional *nanos* transgene, where the 3' UTR is replaced by the *bicoid* 3' UTR. Whereas Nanos protein produced from the endogenous wt *nanos* gene forms a gradient at the posterior, Nanos protein expressed by the transgenic *nanos* RNA forms a gradient at the anterior (**C**). Hence, mislocalization of *nanos* mRNA results in equal distribution of Nanos protein throughout the embryo and lead to formation of a larva that has two abdominal parts but no head or thorax (**E**). Anterior is left and dorsal up. H, T, A, and arrowhead indicate head, thoracic segments, abdominal segments, and terminal structures, respectively. Images **A** and **B** were taken from Zaessinger *et al.* 2006 and **C** to **E** from Gavis *et al.* 1992.

Nanos is an RNA-binding protein, which is a component of the translational repressor complex acting on the maternal *hunchback* mRNA (Hülskamp *et al.* 1989; Irish *et al.* 1989; Struhl 1989; Tautz 1988; Wharton *et al.* 1991). Maternal *hunchback* mRNA and every known component of its repressor complex except Nanos are

distributed uniformly throughout the embryo (Macdonald 1992; Cho *et al.* 2006; Johnstone *et al.* 2001). Since Nanos protein is essential for translation inhibition of *hunchback* mRNA, Hunchback protein synthesis is restricted to the anterior part, and hence the Nanos protein gradient leads to an opposing gradient of Hunchback protein (Tautz 1988; Wang *et al.* 1991). Hunchback is a transcription inhibitor acting on several zygotic segmentation genes (Tautz *et al.* 1987; Hülskamp *et al.* 1990; Struhl *et al.* 1992). Its absence at the posterior is necessary and sufficient for proper expression of these genes at this position, which in turn permits correct progression of posterior patterning of the developing embryo (Hülskamp *et al.* 1990; Struhl *et al.* 1992).

Nanos is also involved in translational repression of *bicoid* mRNA in the posterior of the embryo, which is thought to serve as a fail-safe mechanism, in addition to other repressing mechanisms, to ensure that *bicoid* is only translated at the anterior (Wharton *et al.* 1989; Wharton *et al.* 1991; Gavis *et al.* 1992; Wreden *et al.* 1997; Gamberi *et al.* 2002). Furthermore, Nanos is part of the translational repression complex acting on *cyclin B* mRNA in the pole cells of the embryo, leading to mitotic arrest of the pole cells (Asaoka-Taguchi *et al.* 1999; Kadyrova *et al.* 2007).

In addition to its function in early embryonic development, *nanos* plays a role in germ-line development, dendrite morphogenesis, and regulation of sodium current (Ye *et al.* 2004; Kobayashi *et al.* 2005; Sato *et al.* 2007; Brechbiel *et al.* 2008; Muraro *et al.* 2008).

Post-transcriptional regulation at the level of translation and mRNA stability

Development of the posterior part of the embryo depends strictly on the proper expression of *nanos* mRNA. Almost no RNA synthesis and thus no transcriptional control take place within the early *Drosophila* embryo. Instead, maternally provided *nanos* mRNA is regulated on the post-transcriptional level to ensure translation at the pole plasm and translation inhibition in the remaining embryo. The next section gives a short overview of the mechanism of translation initiation (Fig. 3) and principles of translational repression in eukaryotes.

The active state of mRNA

During their synthesis in the nucleus, almost all eukaryotic mRNAs are equipped

with a 7-methyl guanosine (m^7G) cap structure on their 5′ ends and a poly (A) tail of about 50 to 300 nucleotides on the 3′ ends (Proudfoot *et al.* 2002). The presence of these modifications largely contributes to efficient translation initiation of an mRNA.

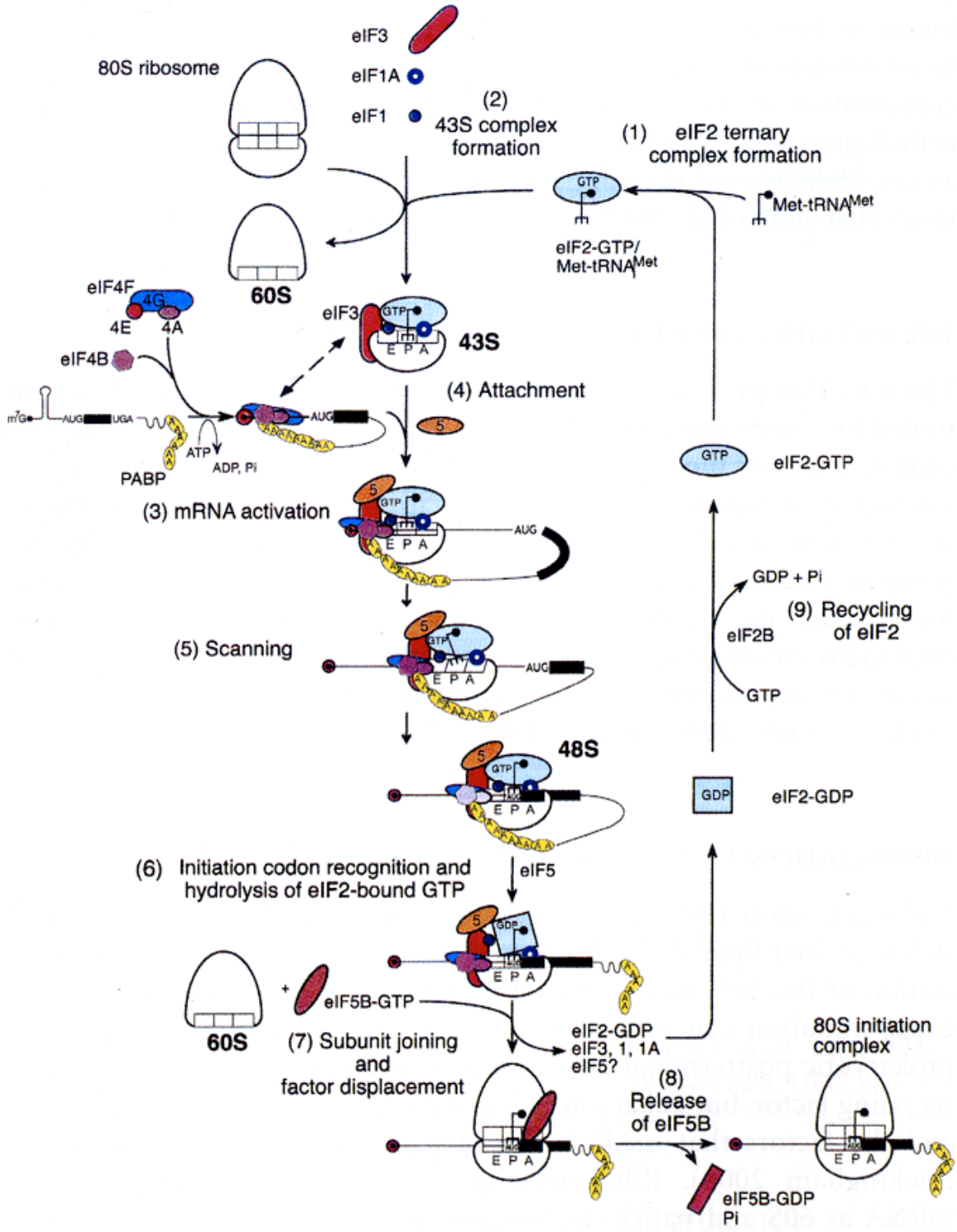

FIGURE 3. **Scheme of the translation initiation process on a capped and polyadenylated mRNA.**
Shown are the individual steps of translation initiation until assembly of the 80S ribosome on the mRNA. Eukaryotic initiation factors (eIFs) and individual steps are described in the text. Figure taken from Pestova *et al.* 2007.

After synthesis, mRNAs are dynamically associated with proteins that mediate different processes in the cell like nuclear export. Therefore, mRNAs are present in the cells as messenger ribonucleoproteins (mRNPs).

Most eukaryotic mRNAs initiate the process of protein synthesis by assembling the eukaryotic translation initiation factor (eIF) 4F on the 5′ cap structure (step 3 in Fig. 3). eIF4F is composed of the three subunits eIF4E, eIF4A, and eIF4G. eIF4E is the component that specifically recognizes the 5′ m^7G cap structure on the mRNA. eIF4G is an unspecific RNA-binding protein, which serves as a large scaffolding protein. eIF4G is involved in recruitment of the small ribosomal subunit (40S) to the mRNA. Upon binding of the 40S subunit to the mRNA, it scans along the 5′ untranslated region (UTR) until it reaches the first AUG codon. For the recruitment and scanning process, the 40S subunit is associated with a subset of initiation factors. The 43S preinitiation complex is the functional entity which is competent for being recruited to the mRNA and is composed of the 40S subunit, the 'ternary complex' and the eIFs 3, 1, and 1A (step 2 in Fig. 3). The ternary complex consists of GTP-bound eIF2 and the initiator tRNA coupled to the first amino acid, methionine, (Met-$_i$tRNA^{Met}) and is responsible for delivering the Met-$_i$tRNA^{Met} to the 40S subunit (step 1 in Fig. 3). Beyond other functions, eIF3 mediates attachment of the 43S complex to the mRNA by contacting eIF4G bound to the mRNA (step 4 in Fig. 3). The assembly of mRNA, 40S subunit and initiation factors is called the 48S complex. eIFs 1 and 1A together with those of the eIF4 group are involved in scanning and start codon recognition (step 5 in Fig. 3). eIF4A is an ATP-dependent RNA helicase that facilitates the scanning process by unwinding secondary structures within the mRNA's 5′ untranslated region (5′ UTR) that otherwise would block sliding of the small ribosomal subunit.

Upon recognition of the initiation codon of the mRNA, the 43S complex comes to a halt, and the large ribosomal subunit (60S) joins the small ribosomal subunit with the help of eIFs 5 and 5B to form the 80S ribosome on the mRNA (step 7 in Fig. 3). eIF5 induces hydrolysis of the eIF2-bound GTP, which leads to reduction of the affinity of eIF2 for Met-$_i$tRNA^{Met}. eIF2-GDP and other initiation factors are displaced from the interface of the 40S subunit by a combined action of the ribosome-dependent GTPase eIF5B and the 60S subunit during the actual joining event. After assembly of the 80S ribosome on the mRNA, protein synthesis begins and proceeds until the stop codon is reached. Protein synthesis terminates by binding of the release factors to the ribosome leading to release of the completed polypeptide. (Translation initiation and initiation factors are reviewed in Gingras *et al.* 1999; Gallie 2002; Prévôt

et al. 2003; von der Haar *et al.* 2004; Proud 2005; Pestova *et al.* 2007; Sonenberg *et al.* 2009.)

The poly(A) tail of an mRNA contributes to efficient translation of an mRNA. It is covered by the cytoplasmic poly(A)-binding protein (PABPC), which was shown to interact with eIF4G (Mangus *et al.* 2003; Kühn *et al.* 2004). Hence, simultaneous binding of eIF4E and PABPC to eIF4G increases the affinities of these molecules to each other and to RNA due to cooperative effects and is thought to allow circularization of the mRNA (Wells *et al.* 1998; Tarun *et al.* 1996; Tarun *et al.* 1997; Kühn *et al.* 2004; Amrani *et al.* 2008). This 'closed loop' structure enhances the affinity of eIF4E to the cap structure by lowering its dissociation rate and stimulates the binding activity of PABPC to the poly(A) tail (Le *et al.* 1997; Luo *et al.* 2001; Haghighat *et al.* 1997; Borman *et al.* 2000; Ptushkina *et al.* 1998). Furthermore, the 'closed loop' model is thought to allow a recycling of ribosomes from the 3' end to the 5' end (Mangus *et al.* 2003).

The cap structure and the poly(A) tail are not essential for translation of an mRNA, but their synergistic effect on translation initiation efficiency is thought to ensure preferential translation of intact, i.e. capped and polyadenylated, mRNAs in the cell (Gallie 1991; Bi *et al.* 2000).

Regulation of translation initiation

Regulation of translation of an mRNA is a critical step in post-transcriptional control and modulates protein synthesis after transcription has been occurred. Translation initiation is considered the rate-limiting step of protein synthesis (Mathews *et al.* 2007). Therefore, most known control mechanisms affect the translation initiation step.

Translation can be modulated globally as a response of cells to stress or growth stimuli. In most cases, the phosphorylation state of individual translation initiation factors or regulators that interact with them is changed. Common targets are eIF2, eIF4E, or 4E-binding proteins (4E-BPs) (Proud 2005; Goodfellow *et al.* 2008; Sonenberg *et al.* 2009). Global control affects translation of most cellular mRNAs in a similar way, but mRNAs that have specific *cis* elements in their 5' UTRs can individually respond to such general changes with hypersensitivity or resistance (Dever 2002).

mRNA-specific regulation of translation plays a role in processes like metabolism, cell differentiation, synaptic transmission, and embryonic patterning

(Hentze *et al.* 2007; Thompson *et al.* 2007; Gavis *et al.* 2007). Mostly, *trans*-acting factors bind to specific *cis* elements often located in the 5′ or 3′ UTR of such mRNA, which very commonly leads to inhibition of translation. Furthermore, mRNA repression plays a role in mRNA localization events. There exist several examples where mRNAs are transported in a translationally silent form until they reach their final destination and become translated (Lipshitz *et al.* 2000; Johnstone *et al.* 2001; Gavis *et al.* 2007; Besse *et al.* 2008; Kugler *et al.* 2009).

5′ UTRs of mRNAs can contain structured or unstructured sequences that when bound by a specific ligand (a metabolite or protein) reduce translation efficiency by interfering with recruitment of the 43S preinitiation complex to or scanning of the 43S complex along the 5′ UTR (Hentze *et al.* 2004; Hentze *et al.* 2007).

In most known cases however, translation is regulated by sequences that lie in the 3′ UTR of an mRNA. Such sequences can be structured or non-structured and are bound by specific RNA-binding proteins or micro RNAs (miRNAs), which mediate the repression process (Hentze *et al.* 2007; Thompson *et al.* 2007). Since the regulatory sites are situated downstream of the site of translation event, 3′ UTR-mediated repression mechanisms differ fundamentally from those mediated by 5′ UTR sequences. Repression can be achieved by affecting different individual steps of the translation initiation process (and there are even a few examples where later steps of the translation process are targeted).

Most of the repression mechanisms described so far depend on the idea of circularization of the mRNA, which brings the 3′ UTR and the site of translation initiation in close proximity. Most repressors are thought to mediate the conversion of the translation-competent into a translationally repressed closed loop. The 3′ UTR-bound repressor can associate, for example, with a cap-binding protein different from the translation initiation factor 4E. The alternative cap-binding protein competes with eIF4E for binding to the cap structure, but does not bind eIF4G and therefore can block translation initiation of the RNA at the very first step. In *Drosophila*, the 4E-homologous protein (4E-HP), for example, can interact with both the cap structure and the repressor Bicoid or Brain Tumor to inhibit translation of *caudal* or *hunchback* mRNA, respectively (Hernández *et al.* 2005; Cho *et al.* 2006; Cho *et al.* 2005).

Another mechanism of a repressed closed loop involves repressors that compete with eIF4G for binding to eIF4E. Such eIF4E-binding proteins target specific RNAs by binding to a second protein that has the specific RNA-binding domain. The *Xenopus* protein Maskin is a 4E-binding protein, which can interact with the

cytoplasmic polyadenylation element (CPE)-binding protein to repress mRNAs that bear the CPE in their 3′ UTR (Stebbins-Boaz *et al.* 1999). The *Drosophila* protein Cup is thought to act similarly. It was shown to interfere with the eIF4E – eIF4G interaction on *oskar* and *nanos* mRNA by binding the corresponding 3′ UTR-binding proteins Bruno and Smaug, respectively (see below; Nelson *et al.* 2004; Nakamura *et al.* 2004; Wilhelm *et al.* 2003; Zappavigna *et al.* 2004; Chekulaeva *et al.* 2006).

Two examples have been described where the 3′ UTR-bound repressor is thought to interfere with the last step of translation initiation, which is the joining of the 60S ribosomal subunit to the RNA-bound 43S complex (Ostareck *et al.* 2001; Hüttelmaier *et al.* 2005). However, the molecular mechanism of this kind of repression is not known so far.

Function of deadenylation in translation control

In the first sense, the degree of translation depends on the amount of mRNA in the cytoplasm, which is a result of the rates of mRNA synthesis, export, and decay. Regulation of an mRNA's steady-state level in the cytoplasm consequently regulates its translational output.

The half-life of an mRNA in somatic cells is mainly determined by the length of its poly(A) tail. Deadenylation is considered the first step in the decay of most eukaryotic mRNAs. Often, this process is rate limiting, because subsequent degradation steps, which proceed from either end of the mRNA, occur without detectable intermediates and are faster than deadenylation. Usually, deadenylation is followed by either of two different degradation pathways, which are conserved in eukaryotes. In yeast, deadenylation is followed in most cases by removal of the cap structure of the mRNA, which is catalyzed by the Dcp1/Dcp2 complex. Decapping relieves a 5′ monophosphate on the RNA, which then can be a substrate for the 5′ exonuclease Xrn1 (Pacman in *Drosophila*). In mammals, deadenylation is followed mostly by exonucleolytic degradation from the 3′ end of the mRNA, which is catalyzed by the exosome. 3′ exonucleolytic activity leaves the cap structure of the mRNA over, which is degraded by a scavenger-decapping enzyme DcpS. (mRNA turnover is reviewed in Parker *et al.* 2004; Meyer *et al.* 2004.)

Whereas stable mRNAs are deadenylated slowly unstable mRNAs are targeted for rapid deadenylation by *trans*-acting factors, which usually bind to their 3′ UTR (de Moor *et al.* 2005; Meyer *et al.* 2004). AU-rich elements (AREs), for example, are found in many mammalian mRNAs and, upon binding to ARE-binding proteins,

mediate both accelerated poly(A) shortening and subsequent RNA degradation (Barreau *et al.* 2006). PUF proteins belong to a highly conserved protein family of eukaryotes that were shown to bind to 3′ UTRs and to induce deadenylation of target mRNAs (Wickens *et al.* 2002; Goldstrohm *et al.* 2006; Thompson *et al.* 2007; Goldstrohm *et al.* 2007). miRNAs can also promote deadenylation of mRNAs by targeting their 3′ UTRs (Jing *et al.* 2005; Giraldez *et al.* 2006; Wu *et al.* 2006; Wakiyama *et al.* 2007; Iwasaki *et al.* 2009; Zdanowicz *et al.* 2009).

In principle, translation can also be inhibited by removal of at least one of the terminal mRNA modifications (i.e. cap and poly(A) tail) so that translation of the mRNA is no longer efficient.

There exist several pieces of evidence from yeast that decapping and translation initiation of an mRNA are competing processes (Coller *et al.* 2004). When translation initiation of an mRNA is decreased, its decapping rate increases and *vice versa* (Muhlrad *et al.* 1995; LaGrandeur *et al.* 1999; Schwartz *et al.* 1999). Furthermore, factors that have been described as decapping activators were shown to act as general repressors of translation (Coller *et al.* 2005).

In oocytes or early embryos, deadenylation of an mRNA is not followed by degradation, and the length of a poly(A) tail correlates with the translation status of an mRNA (de Moor *et al.* 2005): While translated mRNAs bear a long poly(A) tail, repressed mRNAs have a short or even lack a poly(A) tail. Since oocytes and early embryos are transcriptionally largely silent, it is important that mRNAs are kept in a translationally repressed form rather than degraded so that they can be re-activated through cytoplasmic polyadenylation events without *de novo* synthesis. 3′ UTR-mediated deadenylation and cytoplasmic polyadenylation events have also been observed in the early *Drosophila* embryo (Sallés *et al.*, 1994; Wreden *et al.*, 1997; Schisa *et al.* 1998; Coll *et al*. 2010).

Several different deadenylation enzymes have been described from a variety of organisms (Meyer *et al.* 2004; Goldstrohm *et al.* 2008). In *Drosophila*, the main deadenylase is the CCR4-NOT complex, which is active in default as well as in transcript-specific deadenylation (Temme *et al.* 2004; Morris *et al.* 2005; Behm-Ansmant *et al.* 2006; Kadyrova *et al.* 2007; Chicoine *et al.* 2007). The complex is composed of several subunits, namely CCR4, CAF1 (=POP2), CAF40, NOT1 to NOT3, and loosely associated NOT4, but the function of most of them is largely unknown (Temme *et al.* 2010). Knock-down of NOT1, NOT2, and CAF1 had a strong effect on both bulk poly(A) tail length and deadenylation of *Hsp70* mRNA in

Drosophila Schneider 2 cells, and the latter component is suggested to be the catalytically active subunit (Temme *et al.* 2010). Purified CAF1 homologs from human, mouse, and trypanosomes show adenosine-specific exonuclease activity *in vitro* (Bianchin *et al.* 2005; Viswanathan *et al.* 2004; Schwede *et al.* 2008).

Regulation of *nanos* mRNA during early *Drosophila* development

Localization of *nanos* mRNA

Localization of *nanos* mRNA to the posterior pole is essential for its expression there and determines development of the abdominal segments of the later larvae (Gavis *et al.* 1992; Gavis *et al.* 1994). In contrast to several other asymmetrically distributed mRNAs, localization of *nanos* mRNA to the pole plasm is not mediated by an active transport along microtubules. Rather, its localization is accomplished by passive trapping of the diffusing mRNA by an anchor in the pole plasm ('diffusion trapping') (Glotzer *et al.* 1997; Forrest *et al.* 2003). This passive trapping is most likely the reason why localization of *nanos* mRNA to the pole plasm is very inefficient with 4% being localized (Bergsten *et al.* 1999). *nanos* mRNA localization requires ~550 nucleotides of the 3' UTR (Fig. 4), but the molecular mechanism of the process is largely unknown (Gavis *et al.* 1996, Curtis, *et al.* 1996). The F-actin cytoskeleton and the 3' UTR-binding protein Rumpelstilskin are thought to be involved (Lantz *et al.* 1999; Forrest *et al.* 2003; Jain *et al.* 2008).

Translational repression of *nanos* mRNA in the cytoplasm

Translational repression of *nanos* mRNA in the late oocyte and the early embryo is mediated by the first ~150 nucleotides of the 3' UTR (Fig. 4; Gavis *et al.* 1994; Gavis *et al.* 1996; Dahanukar *et al.* 1996; Smibert *et al.* 1996). During late oogenesis, translational repression of unlocalized *nanos* mRNA requires an AU-rich stem-loop structure located in this 3' UTR fragment and seems to involve binding of Glorund to this sequence (Crucs *et al.* 2000; Forrest *et al.* 2004; Kalifa *et al.* 2006). After fertilization of the egg the oocyte-specific repression of *nanos* mRNA in the bulk cytoplasm is replaced by an embryo-specific one.

Translational repression of *nanos* mRNA in the embryo is mediated by two stem-loop structures also located in the 150 nucleotide fragment of the 3' UTR. These structures can be bound by the protein Smaug and are referred to as 'Smaug

recognition elements' (SREs) (Smibert *et al.* 1996; Smibert *et al.* 1999; Dahanukar *et al.* 1999). *smaug* mRNA is synthesized in the ovaries of *Drosophila* females, and its expression is started after egg deposition (Dahanukar *et al.* 1999; Smibert *et al.* 1999). Smaug protein is homogenously distributed both in the cytoplasm and the pole plasm of the embryo (Dahanukar *et al.* 1999; Smibert *et al.* 1999). Smaug expression is restricted to the early embryo; it is not detected in the ovaries and it disappears 4 hours after egg deposition (Dahanukar *et al.* 1999; Smibert *et al.* 1999).

One SRE consists of a stem with a CUGGC loop sequence (Fig. 4; Dahanukar *et al.* 1996; Smibert *et al.* 1996; Aviv *et al.* 2003). Several different point mutations within the SREs abolish Smaug binding in UV cross-linking experiments (Smibert *et al.* 1996). Mutations in either the SREs or the *smaug* gene result in a phenotype resembling that of a Nanos overexpression suggesting a derepression of *nanos* mRNA translation (Smibert *et al.* 1996; Dahanukar *et al.* 1999). Embryos from transgenic females with point mutations in both SREs show a properly localized *nanos* mRNA but no translational repression of it, and hence these embryos lack the normal head structure (Smibert *et al.* 1996).

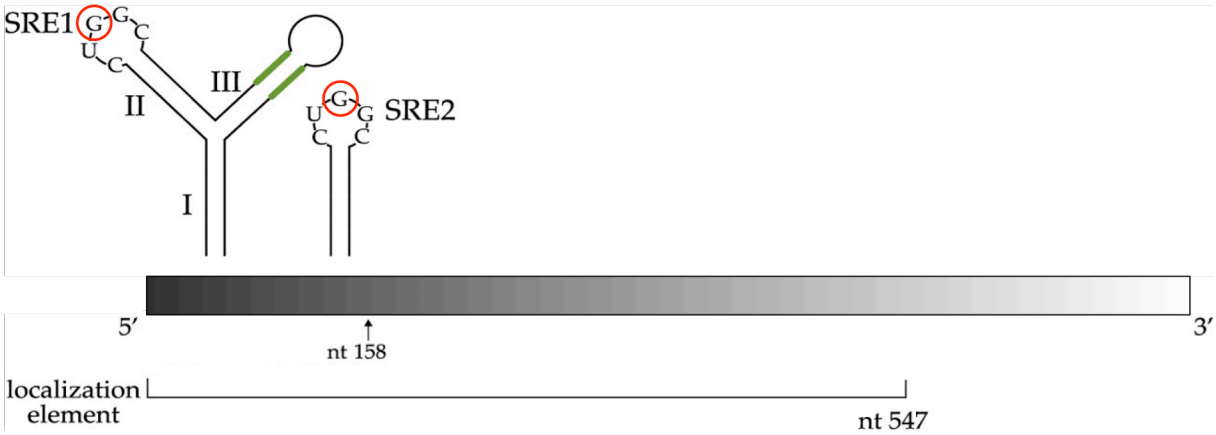

FIGURE 4. **Scheme of the *nanos* 3' UTR.**
The *nanos* 3' UTR consists of 872 nucleotides. Whereas the first 547 nucleotides of the 3' UTR are necessary for proper localization of the mRNA to the *Drosophila* pole plasm, only the first 158 nucleotides are sufficient to mediate repression of unlocalized *nanos* mRNA. The two SRE stem-loop structures, which mediate translational repression in the embryo, are indicated and the critical nucleotide within each of the two loop sequences is circled in red. The AU-rich stem is thought to mediate repression of *nanos* mRNA in the late oocyte (III, in green). Figure adopted and modified from Johnstone *et al.* 2001.

Smaug binds to the SRE via the 'sterile α motif' (SAM) domain (Green *et al.* 2003; Aviv *et al.* 2003). NMR structures of the SRE complexed with the SAM domain of the yeast homolog Vts1 reveal that G3 of the loop sequence (Fig. 4, red circled) is

the only base that is recognized specifically and that the stem-loop structure aids to expose this nucleotide properly to the SAM domain surface (Oberstrass *et al.* 2006; Johnson *et al.* 2006). The dissociation constant of the SRE – Smaug SAM domain complex determined by a fluorescence polarization assay was 40 nM (Aviv *et al.* 2003).

Smaug is thought to repress translation of *nanos* mRNA via its interaction with the eIF4E-binding protein Cup (see above; Wilhelm *et al.* 2003; Nelson *et al.* 2004; Nakamura *et al.* 2004; Zappavigna *et al.* 2004). Cup is expressed during oogenesis and is distributed uniformly throughout the embryo until cellularization (Keyes *et al.* 1997; Wilhelm *et al.* 2003). Cup is thought to interact with the RNA-binding domain of Smaug in an RNase-insensitive manner (Nelson *et al.* 2004). Embryos derived from *cup* mutant females showed a reduced level of SRE-dependent translational repression (Nelson *et al.* 2004) but a direct evidence of Cup in *nanos* mRNA regulation has not been shown yet. In addition to its binding to Smaug, Cup was shown to interact with the *oskar* mRNA repressor Bruno and the *hunchback* mRNA repressor Nanos *in vivo*, *in vitro*, and in yeast two-hybrid assays (see above; Verrotti *et al.* 2000; Nakamura *et al.* 2004). Cup is the *Drosophila* homolog of the human 4E transporter protein (4E-T), and both proteins were reported to shuttle between nucleus and cytoplasm (Dostie *et al.* 2000; Zappavigna *et al.* 2004).

While the 4E-Cup-Smaug interactions may block translation at the very early initiation step, there is also evidence for repression of *nanos* mRNA at a later step in translation: Repression of *nanos* mRNA was suggested to occur on the ribosome, due to the fact that *nanos* is ectopically expressed in embryos derived from females with a defect in the *bicaudal* gene, which codes for the β subunit of the nascent polypeptide-associated complex (NAC) (Markesich *et al.* 2000). The second evidence came from sucrose gradient centrifugations of embryonic extracts (Clark *et al.* 2000). Although only 4% of *nanos* mRNA is localized to the pole plasm and translated (Bergsten *et al.* 1999), more than 50% of endogenous *nanos* mRNA was co-sedimenting with translating polysomes, as shown by ribosome run-off assays in the presence of puromycin (Clark *et al.* 2000). The obvious contradiction in the mechanism of *nanos* mRNA regulation at an early and at a late step in translation might be explained by the fact that the *nanos* 3' UTR mediates repression by two different repressors in the early embryo (Nelson *et al.* 2004; Hentze *et al.* 2007). Glorund represses translation in the late oocyte and presumably in the early embryo until the onset of Smaug expression (Kalifa *et al.* 2006). Since the mechanism of repression by Glorund is not known, presence of repressed *nanos* mRNA in translating polysomes in the early

embryo is not excluded. However, another study that determined the translation profile of individual mRNAs by fractionation of embryo extract through sucrose gradients followed by microarray analysis found only 5% of *nanos* mRNA associated with polysomes, which is consistent with the fraction of *nanos* mRNA localized to the posterior pole of the embryo (Qin *et al.* 2007).

In the early embryo, more than 95% of the *nanos* mRNA is degraded during the first 2 hours of development, and during gastrulation, the presence of both *nanos* mRNA and Nanos protein is restricted to the pole cells (Wang *et al.* 1991; Bashirullah *et al.* 1999). Decay of *nanos* mRNA requires SREs and Smaug function *in vivo* (Dahanukar *et al.* 1996; Smibert *et al.* 1996; Zaessinger *et al.* 2006). Furthermore, the CCR4-NOT complex, which was shown to interact with Smaug *in vivo* and in co-immunoprecipitation assays, is necessary for degradation of *nanos* mRNA in the embryo (Semotok *et al.* 2005; Zaessinger *et al.* 2006; Temme *et al.* 2010).

In addition to its specific function in regulating proper expression of *nanos* mRNA, Smaug has been reported to play a general role in several other processes in the *Drosophila* embryo as well, such as syncytial cell cycle control, maternal transcript destruction, zygotic gene activation, blastoderm cellularization, and gastrulation (Benoit *et al.*, 2009; Tadros *et al.*, 2007).

Obstruction of *nanos* mRNA repression at the posterior pole

Although Smaug is ubiquitously expressed in both the cytoplasm and the pole plasm (Smibert *et al.* 1999), *nanos* mRNA translation is only repressed in the cytoplasm but not in the pole plasm. Localization of *nanos* mRNA to the pole plasm is a prerequisite for activation of its translation there (Gavis *et al.* 1992; Gavis *et al.* 1994). Genetic experiments reveal that activation of *nanos* mRNA translation requires the gene functions of *oskar*, *tudor*, and *vasa* (Ephrussi *et al.* 1992; Smith *et al.* 1992). However, Oskar protein seems to be the limiting factor for activation for the following reasons. Firstly, Oskar presence is restricted to the pole plasm, whereas the other two proteins are enriched in the pole plasm but also present in the cytoplasm (Hay *et al.* 1990; Lasko *et al.* 1990; St Johnston *et al.* 1991; Bardsley *et al.* 1993; Kim-Ha *et al.* 1995; Markussen *et al.* 1995; Rongo *et al.* 1995). Secondly, *oskar* overexpression leads to ectopic translation of *nanos* mRNA in the cytoplasm (Ephrussi *et al.* 1992; Smith *et al.* 1992).

Oskar and Smaug seem to compete in the pole plasm. It was shown that Oskar-dependent translation activation of *nanos* mRNA in the pole plasm is blocked

by Smaug overexpression (Dahanukar *et al.* 1999). Furthermore, fragments of Oskar can interact with Smaug fragments *in vitro* and in yeast two-hybrid assays (Dahanukar *et al.* 1999). Smaug immunoprecipitation with extracts from wild type embryos or embryos overexpressing *oskar* revealed that *nanos* mRNA co-immunoprecipitation was reduced in extracts from embryos that overexpressed *oskar*, which indicated that Oskar acts by preventing Smaug binding to *nanos* mRNA (Zaessinger *et al.* 2006).

The aim of the thesis

The aim of this doctoral thesis was to investigate 3′ UTR-mediated translation regulation of *nanos* mRNA *in vitro* using cytoplasmic extracts of early *Drosophila* embryos. *In vitro* systems allow easy manipulation of RNA substrates or reporters and can be simply supplemented with reagents, such as recombinant proteins. Recapitulating translation regulation *in vitro* potentially allows for purification or isolation of the specific regulatory protein complex, which can help to identify new candidates that are involved in this process and to study their function.

nanos mRNA is a very good candidate to analyze translation regulation *in vitro*, because it is one of the mRNAs that have been most extensively studied in the early *Drosophila* embryo by means of genetic and microscopic approaches. Furthermore, due to the fact that more than 90% of *nanos* mRNA are regulated in the embryonic cytoplasm, cytoplasmic extract should have a great potential to regulate exogenously added reporter RNAs. Studying regulation of an mRNA that is essential for development of an organism further provides the basis to complement *in vitro* studies with genetic methods.

One of the main subjects of interest of the thesis was whether repression of *nanos* mRNA might be regulated by 3′ UTR-mediated deadenylation and/or destabilization. Bulk *nanos* mRNA has short poly(A) tails, and an SRE-dependent degradation in the embryo was reported (Sallés *et al.* 1994; Gavis *et al.* 1996; Dahanukar *et al.* 1996; Smibert *et al.* 1996). Furthermore, the observation that the yeast Smaug homolog Vts1 triggers degradation of SRE reporter RNAs in a CCR4-dependent manner (Aviv *et al.* 2003) led to the assumption of an SRE-mediated deadenylation of *nanos* mRNA in *Drosophila*. In this context the question arose whether translational repression of *nanos* mRNA is predominantly a consequence of a possible deadenylation or degradation. SRE-dependent translational repression was shown *in vitro* and immunodepletion of Smaug abolished repression (Smibert *et*

al. 1999). However, no mechanistic analysis followed, neither generally nor specifically concerning the short poly(A) tail of *nanos* mRNA found *in vivo*.

Independently of a possible regulation of *nanos* mRNA by an SRE-dependent deadenylation or accelerated degradation, there existed evidence of a more direct interference with the translation initiation process mediated by the co-repressor protein Cup. Although Cup was found to interact with Smaug genetically and in co-immunoprecipitation assays, a function of Cup in *nanos* mRNA regulation has not been shown directly (Nelson *et al.* 2004). Furthermore, indications that Cup might not be essential for repression of *nanos* mRNA came from another example, where translational repression in the *Drosophila* oocyte involves Cup: Translation inhibition of *oskar* mRNA was not completely abolished *in vitro* using extracts from ovaries that expressed a truncated Cup variant that was not able to associate with eIF4E (Chekulaeva *et al.* 2006; Nakamura *et al.* 2004). Studying translation regulation *in vitro* might help to understand *nanos* mRNA repression in more detail.

Oskar was suggested to be the limiting factor for activation of *nanos* mRNA translation in the pole plasm (Dahanukar *et al.* 1999). Due to the fact that the pole plasm is only a minor fraction of the *Drosophila* embryo, an *in vitro* system derived from these embryos should largely represent the cytoplasm. Supplementing the cell-free system with recombinant proteins that are candidates for induction of activation of *nanos* mRNA translation, such as Oskar, might permit studying the activation process *in vitro*.

3 RESULT SUMMARY & DISCUSSION

In the context of this doctoral study an *in vitro* system was established that displays several key activities of post-transcriptional regulation. The cell-free system is derived from a mixed population of 0-2 hours old *Drosophila* embryos and recapitulates *nanos* mRNA regulation mediated by a short fragment of the 3′ UTR (Jeske *et al.* 2008). New insights could be obtained that concern deadenylation, translational repression, and translation activation of *nanos* mRNA.

The *Drosophila* embryo extract recapitulates translational repression of *nanos* mRNA

To study translation regulation *in vitro,* capped and polyadenylated luciferase reporter RNAs containing different 3′ UTRs were incubated with *Drosophila* embryo extract under *in vitro* translation conditions, and the translation yield was determined by luciferase activity assays (RNA substrates are depicted in Fig. 1 in Jeske *et al.*, 2006). Incubation of a luciferase RNA with a non-regulated 3′ UTR sequence served as control. Translation of the luciferase with the *nanos* 3′ UTR fragment (nucleotides 7 to 161) was strongly reduced compared to the level of the control RNA (Fig. 7 & 8 in Jeske *et al.*, 2006). This difference in translation yield was not due to a difference in the stability of the RNA body and thus reflects regulation of translation. The *nanos* 3′ UTR fragment used contains three stem-loop structures: two Smaug recognition elements (SREs) and one secondary structure with an adenosine- and uracil-rich stem (AU-rich stem-loop). A point mutation in each of the two SREs largely restored translation. When the AU-rich stem-loop was mutated in addition, complete abolishment of repression was achieved. Thus, translation inhibition mediated by the *nanos* 3′ UTR primarily depends on the intact SREs, and the AU-rich stem-loop makes only a minor contribution to translation inhibition in the embryo extract. The AU-rich stem-loop is bound by the repressor Glorund and is thought to mediate translational repression of *nanos* mRNA in the late oocyte, where Smaug is not present (see INTRODUCTION, p. 14; Crucs *et al.* 2000; Kalifa *et al.* 2006). Smaug synthesis commences after egg deposition, and probably Glorund acts as repressor in the first minutes of embryogenesis until enough Smaug has been produced (Kalifa *et al.* 2006). Since the *Drosophila* extract used for the *in vitro* analysis

was prepared from a mixture of 0 to 2 hours old embryos, the observation of translational repression mediated by the AU-rich stem-loop is plausible.

Translation of a luciferase reporter RNA in *Drosophila* embryo extract was strongly stimulated by the presence of both a 5′ m^7G cap structure and a poly(A) tail (Jeske *et al.*, 2006; Supplementary Table I in Jeske *et al.* 2010). Thus, decapping and deadenylation events can contribute to translational repression by decreasing the translation efficiency of an mRNA. At the time the studies were started, there existed indications for a possible deadenylation mediated by the SREs. It had been shown that both Smaug and its yeast homolog Vts1p bind RNA via their SAM domain and share the same RNA sequence binding specificity (Aviv *et al.* 2003). Furthermore, Vts1p had been shown to induce degradation of an SRE-containing reporter construct *in vivo* through a mechanism that involved the catalytic activity of the yeast CCR4-NOT deadenylase complex (Aviv *et al.* 2003).

In order to investigate a potential SRE-mediated deadenylation in *Drosophila* embryo extract, truncated versions of the luciferase reporter constructs were designed that lack a large fragment of the luciferase-coding region. The deadenylation substrates contained a poly(A) tail of 72 nucleotides and a radioactively labeled RNA body. After their incubation with *Drosophila* embryo extract under deadenylation conditions, the reporter RNAs were isolated and separated by denaturing polyacrylamide gel electrophoresis. Use of the truncated RNA constructs allowed clear detection of a size difference originating from a removal of the poly(A) tail. Indeed, it turned out that the substrate with the *nanos* 3′ UTR fragment was rapidly deadenylated, whereas the control RNA was stable over a time period of 1 h (Fig. 2A in Jeske *et al.*, 2006). The SREs were required and sufficient for mediating deadenylation, whereas the AU-rich stem-loop was shown to have no influence (Fig. 2A, D & E in Jeske *et al.*, 2006). Since the deadenylation assay conditions were nearly identical to the *in vitro* translation conditions an SRE-dependent deadenylation could occur during the *in vitro* translation reaction. Due to the strong reduction of translation efficiency of an mRNA lacking a poly(A) tail in the *Drosophila* extract, SRE-dependent deadenylation might fully account for the observed SRE-mediated translation inhibition. However, it turned out that luciferase RNAs that are resistant to deadenylation were still repressed in an SRE-specific manner, indicating that deadenylation is not the only mechanism of translational repression of *nanos* mRNA (see below).

After SRE-dependent deadenylation had been observed in this study, an interaction of Smaug with the CCR4-NOT complex was reported (Semotok *et al.*

2005). Furthermore, a function of the CCR4-NOT complex-mediated deadenylation of *nanos* mRNA in translational repression *in vivo* was described (Zaessinger *et al.* 2006).

Analysis of the SRE-dependent deadenylation

Mechanistic studies of the deadenylation

From the mechanistic point of view, deadenylation of the RNA substrates used in the experiment is unexpected: The poly(A) tail of the constructs is encoded within the plasmid, and run-off transcription of the linearized DNA template is expected to result in an RNA with a poly(A) tail followed by three additional non-adenosine residues (GCU), which derive from the restriction site used for template linearization. CAF1 is the catalytically active subunit of the *Drosophila* CCR4-NOT complex (Temme *et al.* 2010). The human and mouse CAF1 enzymes were shown to act as 3′ – 5′ exonucleases possessing a pronounced preference for adenosine (Viswanathan *et al.* 2004; Bianchin *et al.* 2005). Thus, the non-adenosine 3′ end is expected to be a poor substrate for this deadenylase. Substrates that have a poly(A) tail followed by seven or 40 additional nucleotides of a mixed sequence remained resistant to deadenylation, confirming that SRE-dependent deadenylation is an exonucleolytic process (Fig. 3 in Jeske *et al.*, 2006; data not shown). Thus, three but not seven or more additional non-adenosine residues 3′ of the poly(A) tail can be degraded (presumably) by the deadenylation machinery, which could have several reasons.

This observation could be explained by the assumption that hydrolysis of non-adenosine residues is very slow, and three but not seven of such nucleotides can be removed from the 3′ end in the given time window to allow detection of a subsequent deadenylation. Alternatively, this observation might also be explained by a stimulating function of the cytoplasmic poly(A)-binding protein (PABPC) in the deadenylation process. Since the poly(A) tail of an mRNA is covered by PABPC, it seems likely that poly(A)-bound PABPC supports the poly(A) recognition process of the deadenylase, which could be important especially at the 3′ end. Although PABPC needs an oligo(A) stretch of 12 nucleotides for high affinity binding, it covers approximately 25 nucleotides (Kühn *et al.* 2004). Hence, PABPC bound to the terminus of the poly(A) sequence might cover three, but not seven or more additional nucleotides. In this scenario, PABPC stimulates the deadenylation

machinery to hydrolyze three additional non-adenosine residues, but not seven or more such nucleotides. Indeed, there exists evidence for a stimulating role of PABPC in deadenylation by the human CCR4-NOT complex (Fabian *et al.* 2009). On the contrary, PABPC was shown to inhibit the activity of the CCR4-NOT complex from different species (Tucker *et al.* 2002; Viswanathan *et al.* 2004). Thus, the function of PABPC in the deadenylation process is not easily explained and might involve antagonistic activities. Finally, the observation that three but not seven or more non-adenosine residues 3′ of the poly(A) tail can be degraded by the deadenylase might be explained by the constitution of the enzyme. The catalytic site of the deadenylase might be separated from the specificity site, which recognizes adenosine residues, and upon recognition stimulates the enzymatic activity. A poly(A) tail that is followed by three additional non-adenosine residues could be properly recognized, whereas a stretch of seven additional residues would lead to occupation of the specificity site by non-adenosine residues, leading to a decreased deadenylase activity.

Detailed analysis of the SRE-specific deadenylation using RNase H digestion assays suggested that removal of the poly(A) tail was followed by degradation of another ~10 nucleotides (Fig. 2B & C in Jeske *et al.* 2006). Further truncation after deadenylation has been observed for other maternal mRNAs *in vivo* as well, but it remains to be investigated if the deadenylase or an additional enzyme catalyzes this reaction (data not shown; Wreden *et al.* 1997; Wharton *et al.* 1991). Purified mouse and human CAF1 were shown to degrade parts of the RNA body after deadenylation (Viswanathan *et al.* 2004; Bianchin *et al.* 2005). However, the possibility exists that deadenylation is not followed by further shortening and such an interpretation arose by mistake due to incomplete digestion of the poly(A) tail by the RNase H treatment in the presence of oligo(dT), which served as a size control. This option might be excluded by subjecting the deadenylation product to sequencing.

Usually, deadenylation initiates mRNA degradation (Meyer *et al.* 2004). However, deadenylation of the *nanos* mRNA in *Drosophila* extracts is not followed by complete degradation of the RNA body, which is a phenomenon repeatedly observed during early developmental stages (Fig. 2A in Jeske *et al.* 2006; de Moor *et al.* 2005). The stable deadenylation product could be explained by the lack of degrading activities in the *Drosophila* embryo extract, such as decapping by Dcp2 and exonucleolytic digestion by Pacman or the exosome. In fact, deadenylated *nanos* reporter RNA displays a higher overall stability than the control RNA, suggesting that the assembly of the regulatory protein complex protects the RNA from further

degradation. The appearance of a stable deadenylation product facilitates data analysis and quantification of deadenylation and thus is advantageous for practical reasons.

SRE-dependent deadenylation is neither stimulated nor inhibited by the canonical 5′ cap structure on the RNA (Fig. 4 in Jeske *et al.*, 2006). Thus, the deadenylation process seems to be independent of translation, which is also supported by the fact that deadenylation is not prevented by the presence of the translation inhibitors cycloheximide or GMP-PNP (Freudenreich 2006; data not shown). The human CCR4-NOT complex was also reported to catalyze a translation-independent deadenylation (Fabian *et al.* 2009).

Deadenylation requires an ATP-regenerating system

An unexpected finding was that deadenylation in *Drosophila* embryo extract depends on the presence of an ATP-regenerating system (ARS) (Fig. 5 in Jeske *et al.* 2006). The ARS is composed of 20 mM creatine phosphate, 0.8 mM ATP, and creatine kinase, an enzyme that catalyzes the transfer of the phosphoryl group between creatine phosphate and any nucleoside diphosphate (data not shown; Voet *et al.* 1995). In the absence of an ARS almost no deadenylation was observed, and depletion of ATP from the extract completely abolished deadenylation (data not shown; Fig. 5 in Jeske *et al.* 2006). Depletion of ATP from the extract was achieved by addition of hexokinase and glucose. Hexokinase catalyzes the transfer of the terminal phosphoryl group between ATP and glucose (or other hexoses) (Voet *et al.* 1995).

Addition of ATP and/or creatine phosphate to a reaction that lacks creatine kinase was not sufficient to drive deadenylation. Increasing the ATP concentration from the routinely added 0.8 mM to 5 mM had no effect on deadenylation in the presence of creatine kinase, but did not allow for deadenylation in the absence of creatine kinase (data not shown).

Apparently, the regeneration process is essential for deadenylation to occur. Presumably, the addition of ATP alone leads to increasing ADP and AMP levels over time by ATP-consuming processes in the extract, whereas the regenerating system assures an almost constant high ATP to ADP (or AMP) ratio. As known for other ATP-dependent processes, increased concentrations of ADP or AMP could also inhibit the ATP-dependent step(s) of the deadenylation process in the extract. In this context, it is important to note that the product of deadenylation is most likely AMP (Bianchin *et al.* 2005), which might inhibit the deadenylase by product inhibition.

Importantly, the nucleoside triphosphate necessary for deadenylation activity must not necessarily be ATP. Creatine kinase-catalyzed transfer of the phosphoryl group is not specific for the nucleobase adenine (see above), and the nucleotides and nucleoside diphosphates present in the extract participate in exchange reactions catalyzed by the base- and sugar-unspecific nucleoside diphosphate kinase (Voet *et al.* 1995). Thus, addition of ATP or ARS to extract that contains nucleoside diphosphate kinase and any of the nucleotide diphosphates will lead to high levels of any nucleoside triphosphate. Taken together, an ARS is essential for the deadenylation process in *Drosophila* embryo extract, but the exact nature of the molecule(s) needed and why ATP alone cannot substitute for the regenerating system remains to be solved.

Dependence of the deadenylation on an ARS suggests that at least one step in this process is energy consuming. However, it is not clear why ATP (regeneration) is essential for deadenylation. Neither binding of Smaug to the SRE (Aviv *et al.* 2003; Green *et al.* 2003; Smibert *et al.* 1996; Dahanukar *et al.* 1999) nor catalysis of the deadenylation reaction using (semi) purified catalytically active components of the CCR4-NOT complex are ATP-dependent (Bianchin *et al.* 2005; Temme *et al.* 2004; Tucker *et al.* 2001; Viswanathan *et al.* 2004; Goldstrohm *et al.* 2008). The ARS dependence of the SRE-mediated deadenylation process thus implicates the involvement of a so far unknown factor or ATP-dependent step. miRNA-mediated deadenylation in *Drosophila* embryo extract was also shown to depend on an ARS, which makes the ATP dependence likely to be a general property of deadenylation in *Drosophila* embryo extract (Iwasaki *et al.* 2009; Zdanowicz *et al.* 2009). A possible role for ATP in the deadenylation process is analyzed and discussed in the Additional results section (p. 63 ff.).

Sedimentation of the deadenylation activity

Centrifugation of the extract was performed in order to test whether heavy or dense particles, such as ribosomes, can be sedimented, and hence separated from the SRE-specific deadenylation activity. This procedure was planned to serve as a pre-clearing method prior to purification of the sequence-dependent deadenylation complex by chromatographic approaches. However, after centrifugation of the extract for 1 hour at ~100.000 x g the SRE-specific deadenylation activity was found exclusively in the pellet fraction (Fig. 6A in Jeske *et al.* 2006). The active pellet fraction contained Smaug, all known components of the CCR4-NOT deadenylase complex,

which is likely responsible for catalysis of the SRE-dependent deadenylation reaction, as well as P (processing) body components like the putative RNA helicase Me31B and the 5' exonuclease Pacman (Fig. 6B in Jeske *et al.* 2006). Apparently, the deadenylation activity resides entirely in heavy and/or dense particles similar or identical to P bodies. P bodies are cytoplasmic structures, which contain proteins that are involved in mRNA regulatory processes, such as mRNA degradation and translation inhibition, together with their mRNA targets (Eulalio *et al*. 2007). Indeed, it has been described that components of the *Drosophila* CCR4-NOT complex localize, although not primarily, to P bodies (Lin *et al.* 2008; Temme *et al.* 2004; Zaessinger *et al.* 2006) and Smaug is present in medium-size cytoplasmic particles together with CCR4-NOT complex components (Zaessinger *et al.* 2006). However, sedimentation of the proteins mentioned above is not affected by a mild RNase treatment, whereas the integrity of P bodies from yeast cells was shown to be RNase-sensitive (Fig. 7, p. 69; Teixeira *et al.* 2005).

Further analysis revealed that even milder centrifugation conditions (i.e. 1 h at 20.800 x g) are sufficient to sediment the complete deadenylation activity (Fig. 8, p. 70). Interestingly, under these conditions only a small fraction of the CCR4-NOT complex subunit NOT2 was found in the pellet, whereas the majority remained in the supernatant (data not shown). Similarly, after centrifugation of extract through a 5 to 25% sucrose gradient, several components of the CCR4-NOT complex were primarily detected in the light RNP fractions and only smaller amounts were found in denser fractions co-migrating with the 80S ribosomal peak (Fig. 10E, p. 74). Again, no deadenylation activity was observed in the light fractions but only in denser fractions, such as the 80S ribosome-containing and adjacent fractions (Fig. 10C & D, p. 74). Interestingly, the P body marker protein Me31B was present in the heavier fractions that contained deadenylation activity. Thus, these experiments again suggest that deadenylation activity resides in large complexes, which could possibly be P bodies. Interestingly, the deadenylation activity found in the dense fractions was neither SRE-specific nor ATP-dependent. Thus, whereas centrifugation at 20.800 x g resulted in a pellet fraction containing SRE-specific activity, sucrose gradient centrifugation apparently separated deadenylase activity from the factors conferring SRE specificity and ATP dependence.

Taken together, in several experiments deadenylation activity resided exclusively in fast-sedimenting particles. Eventually, integration of deadenylase components into large entities is a prerequisite for deadenylation activity. However, several other explanations exist, such as selective enrichment of deadenylase inhibitors in lighter

fractions (see also Additional results section, p. 68 ff.). The nature of the heavy particles remains to be investigated.

Analysis of the SRE-mediated translational repression

Assays in *Drosophila* embryo extract recapitulate the translational repression of *nanos* mRNA using polyadenylated luciferase reporter RNAs containing the *nanos* 3′ UTR fragment (see above). Repression is primarily conferred by the SREs, while the AU-rich stem-loop has only a minor effect. Potentially, the rapid deadenylation induced by the SREs might be the primary cause for translational repression of the luciferase reporter RNA (see above). To investigate if the SREs are able to mediate a translation inhibition independent of deadenylation, capped luciferase reporter RNAs were used that contained a poly(A) tail followed by 40 additional nucleotides of a mixed sequence. This internal poly(A) stretch stimulates translation similar to a poly(A) tail at the 3′ end but is resistant to deadenylation (Jeske *et al.* 2006). In the following experiments, the effect of the AU-rich stem-loop was not investigated, and translation was analyzed using luciferase RNAs with the *nanos* 3′ UTR fragment (including the AU-rich sequence) containing either the wild type or mutant SRE sequence.

Furthermore, extracts were prepared from ~ ½ to 2 ½ h old embryos, which are supposed to show an increased Smaug contribution and a decreased Glorund contribution in the translational repression of *nanos* mRNA.

Influence of the poly(A) tail on repression

Interestingly, the SREs triggered a translation inhibition of the luciferase transcript with the internal poly(A) sequence, and the extent of repression was similar to that of the luciferase containing the terminal poly(A) tail (Jeske *et al.* 2006). The observation that repression of the reporter RNAs with the terminal poly(A) tail was not significantly stronger might be due to the possibility that the effect of deadenylation during *in vitro* translation may not be strong enough to be detected in the given time window. SRE-mediated translational repression was also observed with luciferase constructs lacking a poly(A) tail, suggesting that a poly(A) tail is not absolutely required for repression (Jeske *et al.* 2006; Supplementary Table I in Jeske *et al.* 2010). However, this repression was less pronounced, which indicates that a poly(A) tail enhances repression. Taken together, deadenylation contributes to translational

repression of *nanos* mRNA, but there must exist at least one additional SRE-dependent mechanism that is independent of deadenylation.

The contribution of the poly(A) tail could be explained by a function of PABPC in the repression mechanism. PABPC bound to the poly(A) tail can mediate circularization of an mRNA by an association with eIF4G at the 5′ end (Amrani *et al.* 2008; Tarun *et al.* 1996; Kühn *et al.* 2004). Close proximity of the two mRNA termini could facilitate formation of a repressor complex that originates from the SREs in the 3′ UTR but interferes with translation initiation events that occur at the 5′ end. Indeed, Smaug bound to the SREs in the 3′ UTR of the *nanos* mRNA is thought to affect the interaction between the cap-binding protein eIF4E and eIF4G at the 5′ end (see INTRODUCTION, p. 16). PABPC could also play a more direct role in the repression mechanism as was shown for both miRNA- and Sex-lethal-mediated translational repression (Duncan *et al.* 2009; Zekri *et al.* 2009; Fabian *et al.* 2009; Tritschler *et al.* 2010). To investigate whether PABPC contributes to SRE-mediated repression, the effect of PABPC depletion on translational repression should be assayed. Alternatively, PABPC can be displaced from the poly(A) tail by addition of PABP-interacting protein 2 (Paip2) to *in vitro* translation assays. Paip2 has been shown to interfere with the PABPC – poly(A) interaction (Khaleghpour *et al.* 2001; see also the Additional results section, p. 66 ff.).

Kinetic analysis of the repressor complex

Analysis of *in vitro* translation time-courses revealed that the SRE-containing RNA was not repressed from the very beginning (Smibert *et al.* 1999; Fig. 1A in Jeske *et al.* 2010). Rather, translation of the regulated RNA appeared to be repressed completely only after half an hour or more (Jeske *et al.* 2010; Smibert *et al.* 1999), suggesting a slow repressor complex assembly on the SRE RNA. This assumption was tested by a modification of the *in vitro* translation protocol, whereby the repressor complex was allowed to assemble onto the luciferase reporter RNA prior to start of translation. A pre-incubation of the RNA with *Drosophila* embryo extract for 30 min before the start of translation strongly enhanced the SRE-dependent repression, so that repression was almost complete (Fig. 1B in Jeske *et al.* 2010). The repression efficiency increased with time of pre-incubation but only in the presence of the RNA substrate, indeed confirming a slow repressor complex assembly on the reporter RNA during pre-incubation (Fig. 1C in Jeske *et al.* 2010). Other possibilities to explain the effect of pre-incubation such as decapping or differential RNA degradation were excluded

(Supplement and Supplementary Fig. 1 in Jeske *et al.* 2010). Slow assembly of the repressor complex components was also detected in RNA pull-down assays (Fig. 6B in Jeske *et al.* 2010). Once formed, the repressor complex displayed a high kinetic stability with an estimated half-life of 200 min (Fig. 4 in Jeske *et al.* 2010). Such high complex stability might be advantageous regarding a planned purification of the SRE-bound regulatory complex using methods like RNA affinity chromatography.

Mechanism of repression

According to the current model of SRE-mediated repression, Smaug interferes with the eIF4E - eIF4G interaction (see INTRODUCTION, p. 16). This model is based on the findings that Smaug can associate with the eIF4E-binding protein Cup and repression of SRE-containing luciferase reporters is reduced in embryos lacking functional Cup protein (Nelson *et al.* 2004). Several predictions resulted from this model, which were examined in this doctoral study using the *in vitro* system from *Drosophila* embryos.

Using sucrose gradient analysis it could be shown that the repressed SRE-containing RNA was not associated with the small ribosomal subunit, indicating a block of translation before 48S complex formation (Fig. 5C in Jeske *et al.* 2010). This finding is consistent with the model of Cup-mediated translation inhibition.

An RNA pull-down assay was established that allowed for a more detailed analysis of proteins that bind specifically to the SRE-containing RNA (Fig. 6A in Jeske *et al*. 2010). Analysis of the RNA pull-down assay by Western blotting revealed that Smaug and Cup bind to the RNA in an SRE-dependent manner (Fig. 6C in Jeske *et al.* 2010). Furthermore, eIF4E was part of the complex that assembled on the SRE, whereas eIF4G was excluded. These results strongly support the hypothesis of a repression mechanism that involves Cup and interferes with the eIF4E – eIF4G interaction and show SRE-specific binding of Cup and SRE-specific exclusion of eIF4G for the first time in a direct way. Moreover, the observation that both the cap and the SRE enhance binding of Cup to the RNA supports the model of a repressed closed loop. However, a slight variation of the repression model is conceivable: Since the eIF4F complex is assumed to be a stable unit in the cell (Pestova *et al.* 2007) and Cup colocalizes with eIF4E in *Drosophila* oocytes and can bind to eIF4E independently of RNA (Wilhelm *et al.* 2003; Nelson *et al.* 2004; Nakamura *et al.* 2004), it is likely that a Cup – eIF4E complex competes with the eIF4F complex on the cap structure of the repressed mRNA.

Besides Smaug and Cup, several other proteins were found to associate SRE-dependently with RNA. The catalytically active subunit CAF1 and other components of the CCR4-NOT complex tested, such as CCR4, NOT1, NOT2, and NOT4, were SRE-specifically enriched (Fig. 6B in Jeske *et al.* 2010; data not shown). Binding of the CCR4-NOT complex is consistent with the SRE-mediated deadenylation reaction. It remains to be investigated if Smaug has direct contacts to one or more subunits of the deadenylase complex or if one or more proteins serve to bridge Smaug to the deadenylase. Several 3' UTR-binding proteins have been reported to recruit the CCR4-NOT deadenylase by directly interacting with one of its subunits (Goldstrohm *et al.* 2006; Kadyrova *et al.* 2007; Chicoine *et al.* 2007). Furthermore, it will be interesting to examine the role of the individual subunits of the CCR4-NOT complex in (SRE-mediated) deadenylation.

In addition, two proteins that already have been implicated in translational repression preferentially bound to the SRE-containing RNA (Fig. 6B in Jeske *et al.* 2010). The first was the putative RNA helicase Me31B, which is involved in translational repression of the localizing maternal mRNAs *oskar* and *Bicaudal-D* during *Drosophila* oogenesis (de Valoir *et al.* 1991; Nakamura *et al.* 2001). The yeast Me31B homolog Dhh1 has been shown to function as general repressor of translation (Coller *et al.* 2005). Interestingly, Me31B and its homologs from yeast and trypanosomes have been shown to associate with the CCR4-NOT complex of the respective organism (Schwede *et al.* 2008; Hata *et al.* 1998; Maillet *et al.* 2002; Temme *et al.* 2010). A generally increased mRNA half-life has been observed in yeast *dhh1Δ* mutants, which is however due to a defect in decapping (Coller *et al.* 2001; Fischer *et al.* 2002). Whether binding of Me31B to the SRE RNA is essential for its repression or whether it plays a role in deadenylation remains to be investigated.

The second protein was Trailer Hitch (Tral), which interacts with the C-terminal RecA-like domain of Me31B via its FDF domain (Tritschler *et al.* 2009; Tritschler *et al.* 2008). Interestingly, Tral was shown to co-localize with Cup and to coimmunoprecipitate Cup via its LSm domain (Wilhelm *et al.* 2005; Tritschler *et al.* 2008). However, whether this interaction takes place with Cup bound to SRE-containing RNA and whether this is relevant for repression of *nanos* mRNA remains to be elucidated.

Taken together, it seems possible that Tral can interact simultaneously with Cup and Me31B via its LSm and FDF domain, respectively. Hence, Cup could mediate an indirect recruitment of Me31B to the target mRNA in addition to its interference with the eIF4E – eIF4G association. According to this hypothesis, Me31B

would act downstream of all mentioned repressors, but how it leads to a repressed state of the RNA remains to be examined.

Cap-independent repression

Using the pre-incubation method, efficient SRE-specific translational repression was observed independently of whether the luciferase RNAs used contained or lacked a 5′ m^7G cap structure (Fig. 1 in Jeske *et al.* 2010). In addition, SRE-dependent repression was not sensitive to competition with m^7GpppG cap analog (data not shown). Moreover, the SRE-bound repressor complexes displayed a similar high stability on luciferase RNAs either containing or lacking a cap (Fig. 4B in Jeske *et al.* 2010). These results are not easily explained. In the current model of repression, Cup interferes with the eIF4E – eIF4G association by binding to cap-bound eIF4E (Nelson *et al.* 2004). Thus, binding of eIF4E to the mRNA's cap structure is expected to be required for repression to occur.

Translation initiation on uncapped RNA is poorly characterized on the molecular level. It has been shown to be 5′ end-dependent and to involve scanning (Gunnery *et al.* 1997). Furthermore, there exists evidence that the 43S complex is intrinsically able to associate with mRNA 5′ ends (Pestova *et al.* 2002). However, it is not clear whether eIF4E is essential for translation of uncapped mRNAs or whether it can also bind to uncapped RNA with lower affinity. The fact that translation efficiency of an uncapped mRNA is strongly reduced compared to translation of a capped RNA does not necessarily exclude a role for eIF4E in translation of uncapped mRNA. It might rather reflect differences in the affinities of eIF4E for capped and uncapped RNA.

Assuming that eIF4E is essential for translation of uncapped RNA, strong repression on uncapped RNA could be explained according to the current model that Cup interferes with the eIF4E - eIF4G interaction. In case eIF4E is not essential, repression of uncapped RNA containing an internal poly(A) sequence must be accomplished by a mechanism different from interference between eIF4E – eIF4G interaction (and deadenylation). Taken together, the roles of eIF4E and the eIF4E – eIF4G interaction in translation initiation of uncapped RNAs are not clear. However, unraveling the translation initiation mechanism of uncapped RNAs is a prerequisite to understand the SRE-mediated repression on uncapped RNA.

Results obtained with another approach pointed towards an SRE-specific repression mechanism that is independent of a Cup – eIF4E interaction (Fig. 7 in

Jeske *et al.* 2010). In this experiment (performed by Bodo Moritz) luciferase reporters were used that lacked a cap structure but contained an internal ribosomal entry site (IRES) from the cricket paralysis virus (CrPV). Translation initiation from the CrPV IRES had been reported to act independently of translation initiation factors and hence independently of an eIF4E – eIF4G interaction (Wilson *et al.* 2000; Pestova *et al.* 2003; Deniz *et al.* 2009; Garrey *et al.* 2010). Importantly, SRE-dependent translational repression was still observed using CrPV IRES-containing reporter RNAs. To confirm that translation of the CrPV IRES constructs is indeed independent of translation initiation factors under the *in vitro* translation conditions used, an RNA pull-down assay might be performed that allows to monitor the reduced binding of such factors to IRES-containing RNA compared to capped RNA.

SRE-specific repression that is independent of translation initiation factors might be explained by the possible involvement of Me31B (see above): Its yeast homolog Dhh1 has been shown to mediate translational repression of CrPV IRES-containing reporters (Coller *et al.* 2005). However, the molecular mechanism of repression by Me31B or Dhh1 is still unclear.

Strong repression is ATP-dependent

To separate the complex assembly on the luciferase reporter RNA from the translation process, an ARS, which is essential for *in vitro* translation, was left out during pre-incubation. However, a significant concentration of ATP was detectable during the pre-incubation time, which was obviously not sufficient to drive translation. To test whether this endogenous level of ATP is essential for repressor complex assembly, repression efficiencies were compared between ATP-depleted or non-treated extract. When ATP was depleted from the pre-incubation, SRE-mediated repression of translation was strongly impaired, indicating that ATP is required for repressor complex formation (Fig. 2 in Jeske *et al.* 2010). The reason for the ATP requirement is not known. Comparison of the proteins assembled on SRE-containing RNA in the presence or absence of ATP suggested that ATP influences two aspects of the repressor complex formation (Fig. 6B in Jeske *et al.* 2010). Firstly, in the presence of ATP, the amount of each protein that bound SRE-specifically increased, namely Smaug, Cup, Tral, Me31B, Caf1, and Not1, either by enhanced complex assembly or by increased complex stability, or both. Secondly, the presence of ATP apparently increased the binding specificity of most of these proteins: They bound to the mutant RNA more efficiently in the absence than in the presence of ATP, thereby reducing

the relative difference between the wild type and the mutant SRE-containing RNA. Taken together, ATP might serve to maintain sequence-specific binding of the regulatory complex. Protein displacement from RNA catalyzed by ATP-dependent helicases has been described, and RNP remodeling events have been discussed in the literature (Jankowsky *et al.* 2001; Fairman *et al.* 2004; Jankowsky *et al.* 2006).

Deadenylation and translational repression can act independently of each other

SRE-dependent deadenylation depends on the presence of the ARS. This fact allowed pre-incubating the deadenylation substrate with extract prior to start of deadenylation by addition of the ARS. However, unlike translational repression, a pre-incubation had no stimulating effect on the deadenylation rate of the SRE-containing RNA (Fig. 3 in Jeske *et al.* 2010). This observation can be explained by at least two possibilities. Firstly, deadenylation complex assembly could be fast and therefore not rate limiting for deadenylation, and the ARS would be required for either complex formation or subsequent deadenylation process, or both. Secondly, the deadenylation complex formation could be slow, but in this case the low level of endogenous ATP would not be sufficient and an ARS would be required for assembly. In the subsequent deadenylation process the ARS would not necessarily be essential. The second case is not very likely, as an obvious lag phase in the deadenylation reaction could not be detected (e.g. Fig. 2A in Jeske *et al.* 2006). However, in either case the deadenylation reaction is different from the translational repression: Assembly of the translational repression complex is slow and facilitated by low levels of endogenous ATP (see above). Upon start of translation by addition of the ARS after pre-incubation, the rate of translation of the SRE-containing RNA is very low from the very beginning and only slightly decreased in the further time course, indicating that presence of ATP during pre-incubation is sufficient to promote complete complex formation (see above). The differences in the ATP requirement and/or rate of complex formation strongly suggest that the deadenylation complex and the repressor complex are not identical, although they share common components like the SRE RNA sequence and Smaug.

The assumption of two distinct regulatory complexes is further supported by the observation that some extract batches displayed deadenylation activity but no translational repression activity, and *vice versa* (data not shown). Furthermore, deadenylation could be observed with high RNA substrate concentrations of 50 nM

and more, which did not allow for detection of translational repression (data not shown). Moreover, the requirement of relatively low RNA concentrations for translational repression suggests involvement of (one or more) factors that are more limited than those of the deadenylation reaction (data not shown).

A similar ATP requirement can be observed for deadenylation and translational repression in *Drosophila* embryo extract mediated by sequences that are targeted by specific miRNAs (Iwasaki *et al.* 2009; Zdanowicz *et al.* 2009): A pre-incubation step is essential to detect translational repression in the subsequent *in vitro* translation, but deadenylation is only observed after pre-incubation, when an ARS has been added to the reaction.

Oskar prevents translational repression and deadenylation of *nanos* mRNA *in vitro*

Smaug recruits both the translational repression and deadenylation complex to the SREs located in the 3' UTR of *nanos* mRNA. Smaug is uniformly distributed in the *Drosophila* embryo, and thus present in the pole plasm where Nanos protein synthesis takes place (Smibert *et al.* 1999). The pole plasm component Oskar is most likely responsible for the loss of repression at the posterior pole, possibly by directly interacting with Smaug (see INTRODUCTION, p. 17 f.). Due to the fact that SRE-dependent repression of *nanos* mRNA can be recapitulated *in vitro*, we sought to test whether this is also possible for Oskar-dependent prevention of repression. *Drosophila* oocytes and embryos contain two Oskar isoforms derived from translation of *oskar* mRNA from two alternative in-frame start codons (Markussen *et al.* 1995). The short isoform was shown to be necessary and sufficient to induce formation of the abdomen and the germ line of *Drosophila* (Markussen *et al.* 1995; Breitwieser *et al.* 1996).

A pull-down assay using a GST fusion of full-length short Oskar mixed with *Drosophila* embryo extract revealed RNase-insensitive binding to Smaug (Fig. 10A in Jeske *et al.* 2010). Furthermore, the presence of Oskar in an RNA pull-down assay with *Drosophila* embryo extract prevented binding of Smaug to the RNA (Fig. 10B in Jeske *et al.* 2010). Oskar – Smaug interaction and prevention of Smaug from binding to the SRE are consistent with published data (Dahanukar *et al.* 1999; Zaessinger *et al.* 2006). Importantly, the presence of Oskar prevented both SRE-dependent deadenylation and translational repression (Fig. 9 in Jeske *et al.* 2010). Addition of Oskar after pre-incubation of the SRE-containing luciferase RNA with *Drosophila*

embryo extract could not relieve the RNA from the repressed state in the analyzed time window of 60 minutes (data not shown). However, this is not necessarily inconsistent with the *in vivo* data: Oskar protein is already present at the pole by late oogenesis (Kim-Ha *et al.* 1995; Markussen *et al.* 1995; Rongo *et al.* 1995), whereas Smaug is not produced until egg deposition (Dahanukar *et al.* 1999; Smibert *et al.* 1999). Taken together, recombinant short Oskar can interfere with SRE/Smaug-dependent regulation *in vitro*, which provides a basis to study the mechanism of deadenylation inhibition and translation activation in more detail in the future.

It will be interesting to examine if Oskar inhibits Smaug binding to the SREs by an allosteric or competitive effect. The group of Robin Wharton reported an interaction of an Oskar fragment with the RNA-binding (SAM) domain of Smaug in yeast two-hybrid analyses and GST pull-down assays (Dahanukar *et al.* 1999). However, mutations in Smaug that strongly reduced its interaction with Oskar *in vitro* without affecting RNA-binding did not impair Smaug function or embryonic patterning *in vivo*, indicating that the Oskar – SAM domain interaction might not be necessary for translation activation of *nanos* mRNA at the pole (unpublished observation of Cary Gardner and Robin Wharton, cited in Dean *et al.* 2002). The protein regions important for the Oskar – Smaug interaction might be conveniently identified, analyzed, and validated using recombinant proteins or domains in combination with the established *in vitro* system of *Drosophila* embryos.

Based on the *in vitro* system, the contribution of other proteins to translation activation of *nanos* mRNA can be investigated as well. In the *Drosophila* embryo, Tudor and Vasa activities are required in addition to Oskar for *nanos* mRNA translation at the pole (see INTRODUCTION, p. 17; Ephrussi *et al.* 1992; Smith *et al.* 1992). While Tudor seems to act only indirectly in *nanos* translation at the pole (Thomson *et al.* 2004), Vasa could be directly involved (Johnstone *et al.* 2004; Gavis *et al.* 1996). Vasa is an ATP-dependent RNA helicase, which interacts with Oskar in yeast two-hybrid and GST pull-down assays (Liang *et al.* 1994; Breitwieser *et al.* 1996; Carrera *et al.* 2000; Hay *et al.* 1988; Lasko *et al.* 1988; Sengoku *et al.* 2006). Besides its function in the pole plasm formation, this interaction could be of importance regarding translation activation of *nanos* mRNA at the posterior pole. Vasa's function in translation stimulation may be related to its association with the translation initiation factor 5B, which is a ribosome-dependent GTPase involved in the ribosomal subunit joining process (see INTRODUCTION, p. 7 ff.; Carrera *et al.* 2000; Johnstone *et al.* 2004; Pestova *et al.* 2000). It will be interesting to test if Vasa specifically stimulates the translation activation of *nanos* mRNA by Oskar protein.

It has been shown that Glorund is uniformly distributed in the early *Drosophila* embryo (Kalifa *et al.* 2006). Hence, it will be interesting to test whether Oskar also plays a role in prevention of Glorund-mediated repression of *nanos* translation in the first minutes of the embryonic development, when Smaug has not yet been produced but Nanos protein is detectable at the pole.

4 PUBLICATIONS & ADDITIONAL RESULTS

CHAPTER SIX

CELL-FREE DEADENYLATION ASSAYS WITH *DROSOPHILA* EMBRYO EXTRACTS

Mandy Jeske *and* Elmar Wahle

Contents

Abstract

Deadenylation initiates degradation of most mRNAs in eukaryotes. Regulated deadenylation of an mRNA plays an important role in translation control as well, especially during animal oogenesis and early embryonic development. To investigate the mechanism of sequence-dependent deadenylation, we established an *in vitro* system derived from 0- to 2-h-old *Drosophila* embryos. These extracts faithfully reproduce several aspects of the regulation of *nanos* mRNA: They display translation repression and deadenylation both mediated by the same sequences within the *nanos* 3′ UTR. Here, we describe detailed protocols for preparing *Drosophila* embryo extracts, and their use in deadenylation assays exemplified with exogenous RNA substrates containing the *nanos* 3′ UTR.

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Rapid ATP-dependent Deadenylation of *nanos* mRNA in a Cell-free System from *Drosophila* Embryos*

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Shortening of the poly(A) tail (deadenylation) is the first and often rate-limiting step in the degradation pathway of most eukaryotic mRNAs and is also used as a means of translational repression, in particular in early embryonic development. The *nanos* mRNA is translationally repressed by the protein Smaug in *Drosophila* embryos. The RNA has a short poly(A) tail at steady state and decays gradually during the first 2–3 h of development. Smaug has recently also been implicated in mRNA deadenylation. To study the mechanism of sequence-dependent deadenylation, we have developed a cell-free system from *Drosophila* embryos that displays rapid deadenylation of *nanos* mRNA. The Smaug response elements contained in the *nanos* 3′-untranslated region are necessary and sufficient to induce deadenylation; thus, Smaug is likely to be involved. Unexpectedly, deadenylation requires the presence of an ATP regenerating system. The activity can be pelleted by ultracentrifugation, and both the Smaug protein and the CCR4·NOT complex, a known deadenylase, are enriched in the active fraction. The same extracts show pronounced translational repression mediated by the Smaug response elements. RNAs lacking a poly(A) tail are poorly translated in the extract; therefore, SRE-dependent deadenylation contributes to translational repression. However, repression is strong even with RNAs either bearing a poly(A) tract that cannot be removed or lacking poly(A) altogether; thus, an additional aspect of translational repression functions independently of deadenylation.

Additional results:

A possible role of ATP in the deadenylation process

Introduction

ATP acts as short-term carrier of chemical energy in the cell (Voet *et al.* 1995). Release of its terminal phosphoryl group by hydrolysis or transfer of it to another molecule is connected with the release of free energy, which in turn is used to drive energy-dependent processes. ATP also serves in chemical modification of amino acid residues in proteins. Phosphorylation of proteins is the most abundant regulatory modification within the cell. Phosphorylation can alter the conformation of a protein, which can lead to its activation or inactivation. Furthermore, phosphorylation of proteins can change the affinity for binding partners.

SRE-mediated deadenylation depends on the presence of an ATP-regenerating system (ARS) (see RESULT SUMMARY & DISCUSSION section, p. 25 f.; Jeske *et al.* 2006;). However, why the ARS is essential is unclear, since neither Smaug binding to RNA nor catalysis of poly(A) hydrolysis by the CCR4-NOT complex *per se* is ATP-dependent (Temme *et al.* 2004; Tucker *et al.* 2002; Viswanathan *et al.* 2004; Viswanathan *et al.* 2003; Bianchin *et al.* 2005). Deadenylation in *Drosophila* embryo extract that is mediated by the miRNAs let-7 and miR-2 has also been shown to require an ARS (Iwasaki *et al.* 2009; Zdanowicz *et al.* 2009). Since the miRNA-mediated deadenylation in *Drosophila* has been reported to be catalyzed by the CCR4-NOT complex as well (Behm-Ansmant *et al.* 2006; Eulalio *et al.* 2007), the possibility exists that requirement for the ARS is a general property of the deadenylation process in the extract rather than concerning only one individual sequence-dependent deadenylation.

Is ATP required to remove PABPC from the poly(A) tail?

In the cytoplasm, the poly(A) tail of an mRNA is coated with the cytoplasmic poly(A)-binding protein (PABPC). PABPC has multiple roles in the eukaryotic cell. It stimulates translation initiation and termination, and regulates mRNA stability (Mangus *et al.* 2003; Kühn *et al.* 2004). The function of PABPC in the deadenylation process catalyzed by the CCR4-NOT complex is not well understood and rather

ambivalent. On the one hand, cytoplasmic PABP has been reported to stimulate deadenylation by the CCR4-NOT complex from yeast and humans (Yao *et al.* 2007; Simón *et al.* 2007; Fabian *et al.* 2009). On the other hand, it has been shown to inhibit the yeast activity *in vitro* (Tucker *et al.* 2002; Viswanathan *et al.* 2004). Consistently, SRE-dependent deadenylation in *Drosophila* embryo extract is inhibited by addition of recombinant *Drosophila* PABPC to the reaction (unpublished data from Melinda Diver).

Removal of PABPC from an mRNA's poly(A) tail is likely a prerequisite to the main deadenylation process in the cell. PABPC has a high affinity and specificity for poly(A) RNA. It binds to oligo(A) with a dissociation constant of ~5 nM (Sachs *et al.* 1987; Görlach *et al.* 1994; Deardorff *et al.* 1997; Kühn *et al.* 1996). Since one PABPC molecule covers ~25 nucleotides, a poly(A) tail can associate with several PABPC molecules and cooperative effects even increase the affinity of PABPC for the poly(A) tail (Baer *et al.* 1980; Baer *et al.* 1983; Kühn *et al.* 1996; Melo *et al.* 2003).

It is not known whether deadenylases are able to efficiently hydrolyze a poly(A) tail covered by PABPC or if auxiliary factors are required that promote dissociation of PABPC from the poly(A) tail. Enzymatic activity of purified CCR4-NOT complex components CAF1 and CCR4 was repeatedly observed using RNA substrates that were not covered by proteins (Bianchin *et al.* 2005; Schwede *et al.* 2008; Temme *et al.* 2004; Viswanathan *et al.* 2003; Viswanathan *et al.* 2004; Tucker *et al.* 2002). However, *in vitro* systems derived from HeLa cell culture, *S. cerevisiae*, or Trypanosomes had to be complemented with competitor poly(A) RNA to uncover deadenylation activity on exogenously added RNA substrates (Ford *et al.* 1999; Wilusz *et al.* 2001; Milone *et al.* 2002; Milone *et al.* 2004). The amount of competitor needed to observe deadenylation activity corresponded to the amount necessary to prevent binding of proteins to the substrate's poly(A) tail (Ford *et al.* 1999). Competition with poly(A) RNA was specific, since addition of poly(C) RNA to the *in vitro* systems was not sufficient to induce deadenylation of the exogenous substrate RNAs (Wilusz *et al.* 2001; Milone *et al.* 2004). Interestingly, the activity in the poly(A)-supplemented *in vitro* systems was observed without addition of ATP or an ARS to the extract. For deadenylation to take place in *Drosophila* embryo extracts, an ARS is required, but competitor poly(A) RNA does not have to be added. Moreover, addition of competitor poly(A) RNA does not increase the deadenylation rate in this extract if the ARS is present (data not shown).

Since supplementation of different *in vitro* systems with either poly(A) competitor or an ARS is sufficient to observe deadenylation, these two supplements

might substitute for each other. Thus, the ARS could be required to remove PABPC from the substrate's poly(A) tail, which then allows deadenylation. According to this hypothesis, one would predict deadenylation in *Drosophila* embryo extract to be ATP-independent as soon as PABPC is removed from the system. To test this hypothesis, a few initial experiments were performed, in which the *Drosophila* embryo extract was treated in different ways in order to reduce the free PABPC concentration, and the ATP dependence of the SRE-mediated deadenylation reaction was investigated.

Results & discussion

Addition of competitor poly(A) RNA leads to ATP-independent deadenylation

In the first experiment, poly(A) competitor served to bind PABPC molecules within the *Drosophila* embryo extract. Poly(A) RNA was added to the extract together with the substrate RNA, and SRE-dependent deadenylation was assayed in the presence and absence of ATP (Fig. 5). In order to achieve complete depletion of ATP, the extract was incubated in the presence of hexokinase and glucose for 5 min before substrate RNA and deadenylation buffer lacking creatine kinase were added (see Jeske *et al.*, 2006). For the control reaction in the presence of ATP, the extract was incubated for 5 min in the absence of hexokinase and glucose and the reaction was started by addition of substrate RNA and deadenylation buffer containing creatine kinase.

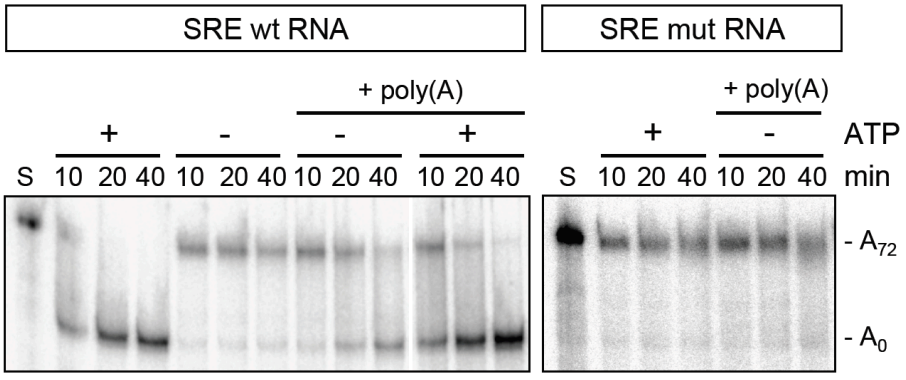

FIGURE 5. **Addition of poly(A) competitor RNA to *Drosophila* embryo extract allows SRE-specific deadenylation in the absence of ATP.**
2 nM of ^{32}P-labeled (capped) SRE wt or SRE mut RNA were incubated with 20% *Drosophila* embryo extract either in the presence of an ATP-regenerating system (+ ATP) or under ATP-depleting conditions (- ATP). Where indicated, 100 ng/µl size-fractionated poly(A) (A_{115}-A_{130}; gift from Sylke Meyer; corresponding to ~1.2 µM PABP-binding sites) were added.

When no poly(A) RNA was added, the deadenylation of the SRE wt RNA was strictly dependent on the presence of ATP as expected. Addition of competitor poly(A) RNA to the reaction did not influence the deadenylation of SRE wt RNA in the presence of ATP. However, an SRE-dependent deadenylation could be observed even in the absence of ATP when poly(A) was present, which is in agreement with the idea that ATP is required to facilitate dissociation of PABPC from the poly(A) tail of the substrate RNA.

To ensure that the observed effect is A-specific the experiment should be repeated including a competition assay with poly(C) RNA. Competition with poly(C) would be a proper specificity control, because PABP does not detectably bind to poly(C) RNA (Burd *et al.* 1991; Kühn *et al.* 1996).

Importantly, the observation of an ARS-independent deadenylation in the presence of poly(A) RNA shows that the deadenylating enzyme *per se* is not ATP-dependent, and indicates that the ARS under normal conditions does not simply serve to prevent an effect that inhibits this enzyme (e.g. accumulation of high AMP levels).

Addition of Paip2 leads to ATP-independent deadenylation with reduced sequence specificity

Paip2 is a protein that specifically interacts with PABPC with a two-to-one stoichiometry (Khaleghpour *et al.* 2001a; Khaleghpour *et al.* 2001b). Paip2 can inhibit translation of polyadenylated mRNAs by decreasing the affinity of PABPC for the poly(A) tail and by competing with eIF4G for binding to PABPC (Khaleghpour *et al.* 2001b; Karim *et al.* 2006). *Drosophila* and human Paip2 are highly similar with an overall amino acid identity of 40%. The PABPC-interacting domains PAM1 and PAM2 display an even higher similarity including a stretch of 11 amino acids in PAM1 that is 100% conserved between human and *Drosophila* (Roy *et al.* 2004). Due to the high conservation between human and *Drosophila* Paip2, human Paip2 is very likely to act on *Drosophila* PABPC (personal communication of Elmar Wahle and Nahum Sonenberg).

Recombinant human Paip2 fused to GST (hPaip2-GST; gift from Nadine Flach and Bodo Moritz) was added to *Drosophila* embryo extract, and the ATP requirement for SRE-dependent deadenylation was investigated (Fig. 6). In the absence of Paip2, deadenylation was SRE-specific and ATP-dependent as expected. In the presence of Paip2, the deadenylation pattern changed dramatically: While deadenylation was

observed in the absence of ATP it was blocked when ATP was present. Furthermore, deadenylation could also proceed on the mutant SRE RNA when ATP was absent. Thus, addition of Paip2 reduced the sequence-specificity of deadenylation. The effects of Paip2 on deadenylation were concentration-dependent, and addition of either GST or buffer had no effect on both ATP and SRE dependence (data not shown).

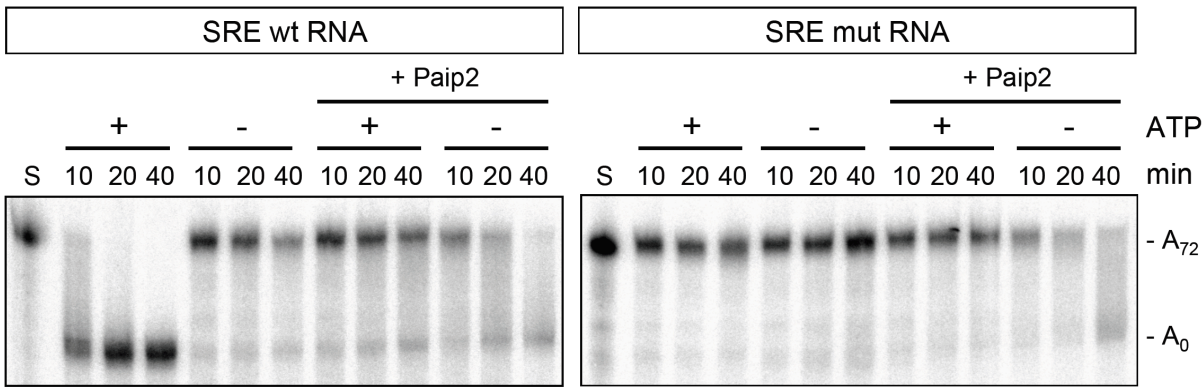

FIGURE 6. **Addition of Paip2 to *Drosophila* embryo extract allows deadenylation in the absence of ATP but with strongly reduced sequence specificity.**
2 nM of ^{32}P-labeled and capped SRE wt or SRE mut RNA were incubated under deadenylation conditions with 20% *Drosophila* embryo extract either in the presence of an ATP-regenerating system (+ ATP) or under ATP-depleting conditions (- ATP) (Jeske *et al.* 2006; Jeske *et al.* 2008). Where indicated, 5 µM GST-Paip2 were present.

The divergent effects of Paip2 on deadenylation are not easily explained. When ATP was absent from the reaction, addition of Paip2 allowed deadenylation, which could be explained by the interference of Paip2 with PABPC binding to poly(A) tail. The ability of Paip2 to interfere with the PABPC – poly(A) interaction has been shown before with recombinant (i.e. unmodified) proteins or protein fragments (Khaleghpour *et al.* 2001; Khaleghpour *et al.* 2001). Release of PABPC from the poly(A) tail could then allow deadenylation in the absence of ATP, which is consistent with the hypothesis of an ATP-dependent PABPC removal from the poly(A) tail.

Presence of Paip2 allowed for deadenylation of the mutant SRE RNA (in the absence of ATP). Assuming that Paip2 removed PABPC from the poly(A) tail, PABPC could be necessary to protect this RNA from unspecific deadenylation under standard deadenylation conditions, and thus might be required for maintaining sequence specificity of the deadenylation. However, upon poly(A) competitor addition, deadenylation of the mutant RNA was not observed (Fig. 5), which might

be explained by a difference of the two competitors in their effective concentration or their affinity for PABPC.

The opposing effect of Paip2 on the deadenylation activity in the presence of ATP might be caused by an ATP-dependent modification (e.g. phosphorylation) of Paip2 itself or its interaction partner PABPC catalyzed by an extract protein. This modification might alter the activity of Paip2, PABPC, or both. Indeed, phosphorylation of recombinant *Drosophila* PABPC and hPaip2 was observed after incubation with *Drosophila* embryo extract in the presence of [γ-^{32}P] ATP (data not shown). However, it is not known if these phosphorylations have any effect on the PABPC – Paip2 and/or the PABPC – poly(A) interaction. There is only one example in the literature describing an effect of phosphorylation on PABP: Phosphorylation of *Arabidopsis* PABPC was shown to reduce the affinity but increase the cooperativity of binding to a poly(A) sequence (Le *et al.* 2000). The modification hypothesis is pure speculation at the moment and might not be true but could serve as a working model to study the effect of Paip2 on PABP and/or deadenylation depending on the ATP status.

To test whether the observed effects of Paip2 on deadenylation are indeed due to its interaction with PABPC, the experiments should be repeated with recombinant PABPC added to Paip2-supplemented reactions. In the case of specificity, PABPC addition is expected to abolish the Paip2 effects on deadenylation.

Centrifugation of the extract results in a pellet fraction that harbors ATP-independent deadenylation activity

Another possibility to reduce the free PABP concentration in *Drosophila* embryo extract might be a centrifugation method. *Drosophila* embryo extract can be fractionated by centrifugation into a clear upper supernatant, a cloudy lower supernatant, and a pellet fraction (Fig. 6 in Jeske *et al*. 2006). The pellet fraction contains the complete SRE-dependent deadenylation activity and proteins known to be involved it this process, such as Smaug and components of the CCR4-NOT complex (Fig. 6 in Jeske *et al.*, 2006). Other proteins also involved in RNA-dependent processes (Me31B and Pacman) are present in the active pellet as well.

The first hint that centrifugation might separate PABPC from the deadenylation activity came from an experiment that was performed to investigate whether sedimentation of the proteins mentioned above depends on the presence of endogenous RNA. For this purpose, the extract was incubated with or without

nuclease prior to ultracentrifugation (Fig. 7): The extract was split and one half was incubated with the calcium-dependent micrococcus nuclease (MN) to degrade endogenous RNA, and the reaction was stopped by addition of the calcium ion chelator ethylene glycol tetra acetic acid (EGTA). The second half of the extract was incubated similarly with the exception of EGTA being present from the very beginning. In this case, MN was present during incubation but not active (pseudo MN treatment, abbreviated as 'Ψ').

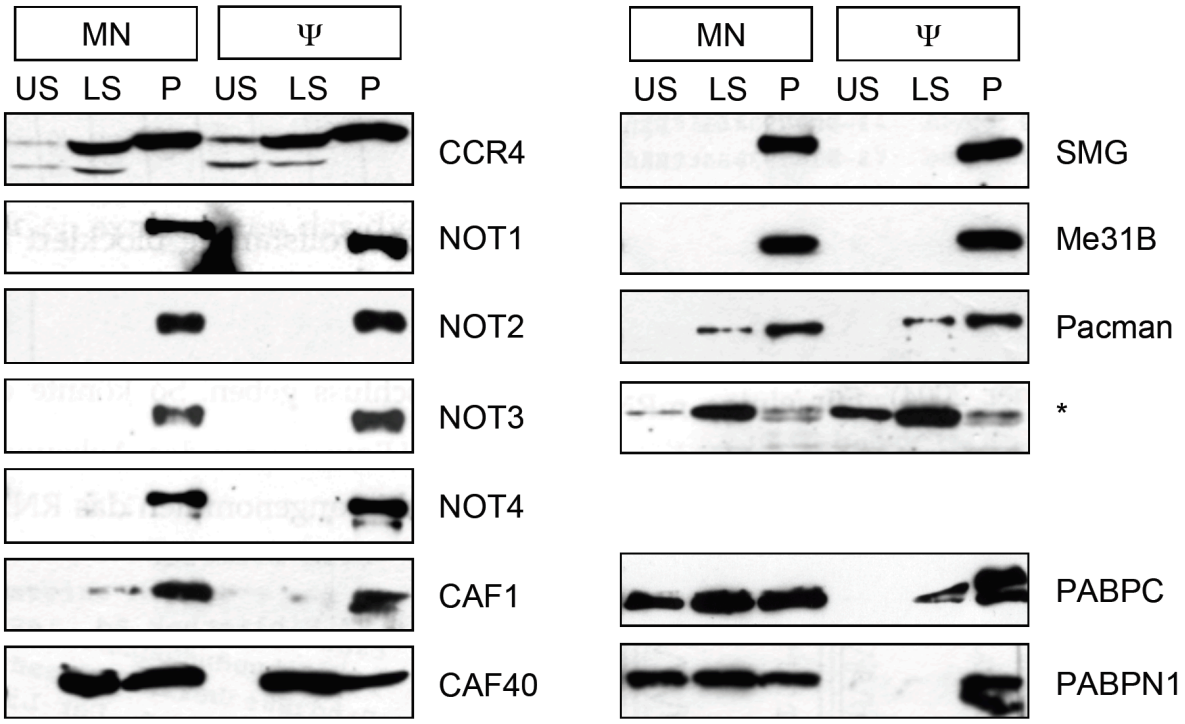

FIGURE 7. **Sedimentation of Smaug, components of the CCR4-NOT complex, and other P body components is not RNase-sensitive.**
Drosophila embryo extract was incubated for 25 min at 15°C with 7.5 u micrococcus nuclease (Fermentas) per µl extract in the presence of 1 mM calcium chloride. The reaction was stopped by addition of EGTA to a final concentration of 2 mM. The treated extract (MN) was centrifuged for 1 h at ~100.000 x g and 4°C to obtain the fractions 'upper supernatant' (US), 'lower supernatant' (LS) and 'pellet' (P). In the control reaction the extract was treated similarly but EGTA was present from the very beginning of incubation so that the nuclease was present but not active ('Ψ'). Comparable fraction volumes were loaded onto SDS polyacrylamide gel and indicated proteins were detected by Western blotting. The asterisk (*) labels a protein from a cross-reaction of the anti NOT4 antibody. The double-bands on the right of the PABP Western blots resulted from a blotting artifact. Western blotting and all antibodies were described in Jeske *et al*., 2006 except anti-PABPC and -PABPN1 (rox2) antibodies (gifts from Nahum Sonenberg and Uwe Kühn, respectively). The experiment was performed in parallel with that shown in Fig. 6B in Jeske *et al*. 2006.

The sedimentation pattern of the aforementioned proteins turned out to be the same, independently of whether the extract was MN-treated, pseudo MN-treated, or untreated (compare Fig. 6 in Jeske *et al.* 2006 and Fig. 7). Thus, neither the incubation

of the extract *per se* nor the presence of RNase had an influence on the sedimentation of these proteins. In contrast, sedimentation of both the cytoplasmic and the nuclear poly(A)-binding protein (PABPC and PABPN1) was sensitive to RNase treatment. While these proteins were almost exclusively found in the pellet fraction in the presence of RNA, more than 50% of both proteins were shifted into the supernatant resulting from centrifugation of the MN-treated extract. Taken together, micrococcus nuclease treatment of *Drosophila* embryo extract and subsequent centrifugation for 1 hr at ~100.000 x g creates a pellet fraction that contains the complete SRE-dependent deadenylation activity, is strongly enriched with Smaug and components of the CCR4-NOT deadenylase, but has a reduced amount of poly(A)-binding proteins.

Slightly changed centrifugation conditions were used to prepare the PABP-depleted pellet fraction: During the sedimentation studies it turned out that centrifugation of the extract for 1 h at 20.800 x g was sufficient to pellet the SRE-specific deadenylation activity (Fig. 8). Recovery of the activity was complete, since the deadenylation rate of the pellet was similar to the rate found in the non-fractionated extract (DEE) and there was no such activity in the supernatant.

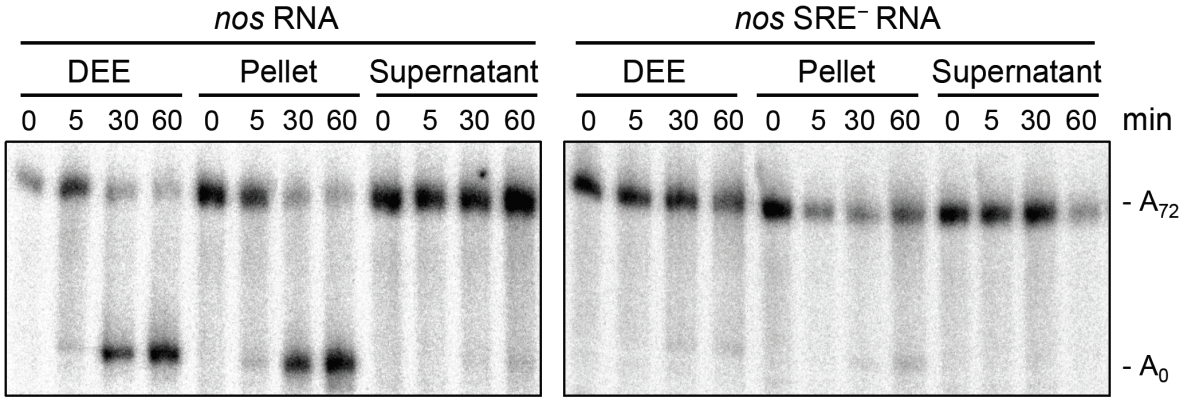

FIGURE 8. **Centrifugation of *Drosophila* embryo extract for 1 h at 20.800 x g results in a pellet fraction containing the complete SRE-dependent deadenylation activity.** 2,5 nM of ^{32}P-labeled *nos* or *nos* SRE$^-$ RNA were incubated with the fractions 'Pellet' or 'Supernatant' under deadenylation conditions (Jeske & Wahle 2008). Activity of untreated *Drosophila* embryo extract ('DEE') used in parallel is shown for comparison. Reactions contained 40% of extract or the respective fractions. Centrifugation was performed without sucrose cushion.

A further modification was centrifugation of the extract through a 20% (w/v) sucrose cushion, which allows heavy particles to be sedimented but retains lighter particles on top of the cushion.

To create the PABP-depleted pellet fraction, which was to be used for studying the ATP dependence of the SRE-mediated deadenylation reaction, the extract was treated with active or inactive MN, loaded on top of a sucrose cushion and subsequently centrifuged for 1 hr at 20.800 x g. The pellets obtained were resuspended in buffer and assayed for ATP dependence of deadenylation (Fig. 9).

The untreated, unfractionated extract ('DEE') served as control and showed ATP-dependent deadenylation as expected (Fig. 9A). In the presence of ATP, the two pellet fractions obtained from centrifugation of the differentially treated extracts displayed the same deadenylation rates of SRE wt RNA as the control reaction (Fig. 9A). This indicates that the pellets contained the entire deadenylation activity, and sedimentation of the activity was not affected by MN treatment, which is consistent with the result of the Western blot analysis (Fig. 7).

Importantly, the pellet fractions also displayed deadenylation of SRE wt RNA when ATP was absent from the reaction (Fig. 9A). Under these conditions, the deadenylation activity of the pellet derived from MN-treated extract ('Pellet – MN') was somewhat higher than that of the pellet from the mock-treated extract ('Pellet - Ψ') and similar to all activities in the presence of ATP.

Assuming that the PABP amount was reduced in the pellets and the amount of components of the deadenylation activity was constant, the results are consistent with the hypothesis of an ATP-accelerated removal of PABPC from the poly(A) tail. Moreover, the higher ATP-independent activity in the pellet of the MN-treated extract might be explained by a more efficient reduction of PABPC. Unfortunately, detection of PABPC in the individual fractions could not be performed since the antibody was not available at that time.

Supplementing the pellet with supernatant restored the ATP dependence of deadenylation (data not shown). However, restoration of ATP dependence was not possible by the addition of recombinant *Drosophila* PABPC at different concentrations in the range of 2 to 200 nM, because it generally inhibited the activity (in a concentration-dependent manner) in the presence as well as in the absence of ATP (data not shown). The failure of PABPC to restore the ATP dependence can have multiple reasons. Firstly, the hypothesis of an ATP-facilitated removal of PABP from the poly(A) tail may not be true, and separation of the deadenylation activity from PABPC is not the reason for ATP-independent activity of the pellet. Secondly, the recombinant protein may not be functional. Thirdly, the hypothesis could be true and the recombinant protein is functional, but the ATP-dependent factor or any other

component essential for activity in the presence of PABPC has also been separated from the deadenylation activity in the pellet by centrifugation.

Interestingly, deadenylation of the SRE mut RNA was observed using the pellet derived from the MN-treated extract (Fig. 9B). This activity was ATP-independent, as was the case for the SRE wt RNA, but the rate was reduced compared to deadenylation of the latter RNA. Thus, similarly to what was observed in the Paip2 experiment (Fig. 6), conditions that allowed deadenylation in the absence of the ARS reduced the sequence specificity at the same time.

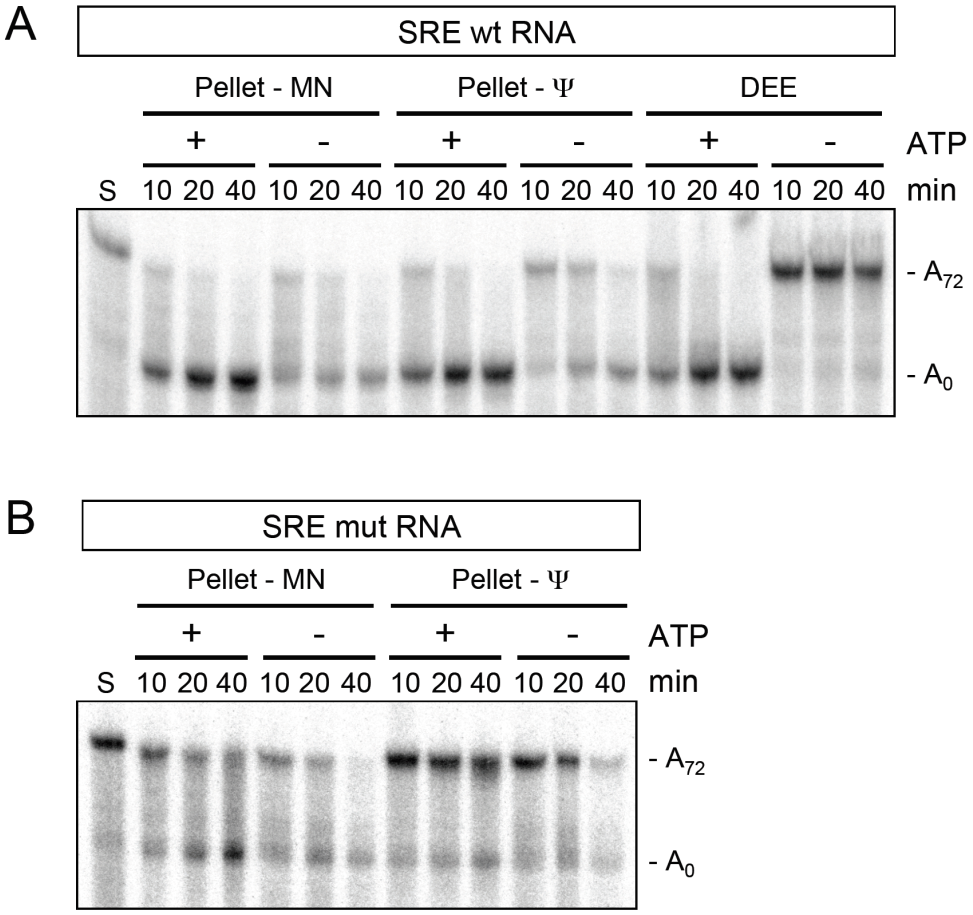

FIGURE 9. **The pellet fraction resulting from centrifugation of *Drosophila* embryo extract contains ATP-independent deadenylation activity.**
Drosophila embryo extract was incubated for 40 min at 15°C in the presence of 60 u MN (Fermentas) per µl extract and 1 mM calcium chloride. The reaction was stopped by addition of 2.5 mM EGTA, the extract was loaded on top of 500 µl of a 20% (w/v in 0.5 x embryo lysis buffer) sucrose cushion and centrifuged in a tabletop centrifuge for 1 h at 20.800 x g and 4°C. The resulting pellet (Pellet – MN) was resuspended in 0.5 x embryo lysis buffer and used in deadenylation assays in the presence of an ATP-regenerating system (+ ATP) or under ATP-depleting conditions (- ATP). Pellet from the mock-treated extract (Pellet - Ψ) was obtained by MN-treatment of the extract and an otherwise identical procedure. The activity of unfractionated extract (DEE) is shown for comparison. The deadenylation assay contained 2 nM ^{32}P-labeled RNA and 20% (v/v) of extract or the respective fraction. Embryo lysis buffer, deadenylation assay, ATP depletion and confirmation have been described (Jeske *et al.*, 2006; Jeske *et al.* 2008).

The correlation between ATP dependence and sequence specificity is supported by another observation from sucrose gradient centrifugation studies. Sucrose gradient analysis is commonly used to separate polysomes, 80S ribosomes, and ribosomal subunits from each other. *Drosophila* embryo extract was separated by centrifugation through a 5 to 25% sucrose gradient, and the resulting fractions were subjected to several analytical procedures to investigate the distribution of the SRE-dependent deadenylation activity and the proteins involved therein (Fig. 10).

The absorption profile monitored at 260 nm showed a separation of the 80S ribosomes, with a peak in fraction 10, from the slowly sedimenting RNP fractions 22 to 26 (Fig. 10A). Protein determination by Bradford assays revealed that most (~50%) of the total protein was present in the lighter fractions (16 to 26) and about one third was present in the pellet fraction 0 of the gradient (Fig. 10B).

Analysis of the collected fractions by deadenylation assays revealed activity ranging from fractions 1 to 16 with a peak in fraction 8 (Fig. 10C & D). Whereas the extract batch used for the experiment harbored SRE-specific deadenylation activity (data not shown), the activity in the gradient fractions was almost equally efficient for SRE wt and SRE mut RNA substrates, with only a faint preference for the wt variant. Additionally, fraction 8 was tested for ATP dependence and displayed complete ATP-independent activity, again showing a correlation between ATP dependence and sequence specificity (data not shown).

This repeatedly observed correlation suggests the existence of a factor that generally blocks deadenylation and which can be inactivated or removed from the RNA by an ATP-dependent step. Absence or low concentration of this factor might lead to inefficient protection of the poly(A) tail, allowing deadenylation of such RNA substrates that do not specifically recruit a deadenylase. On the other hand, an excess of the protecting factor might be able to block deadenylation activity also in the presence of ATP. Thus, only a proper balance between the deadenylase and the protecting factor might ensure sequence specificity as well as ATP dependence. Imbalance in favor of either the deadenylase or the protecting factor might lead to ATP-independent activity with concurrent loss of sequence specificity or inhibition of deadenylation even in the presence of ATP, respectively.

In Western blot analyses of fractions 1 to 16, which contained the majority of deadenylation activity, only relatively weak signals for the CCR4-NOT components CAF1, CCR4, NOT1, and NOT2 were detected. Most of the signal was present in lighter fractions, which had no deadenylation activity (Fig. 10E).

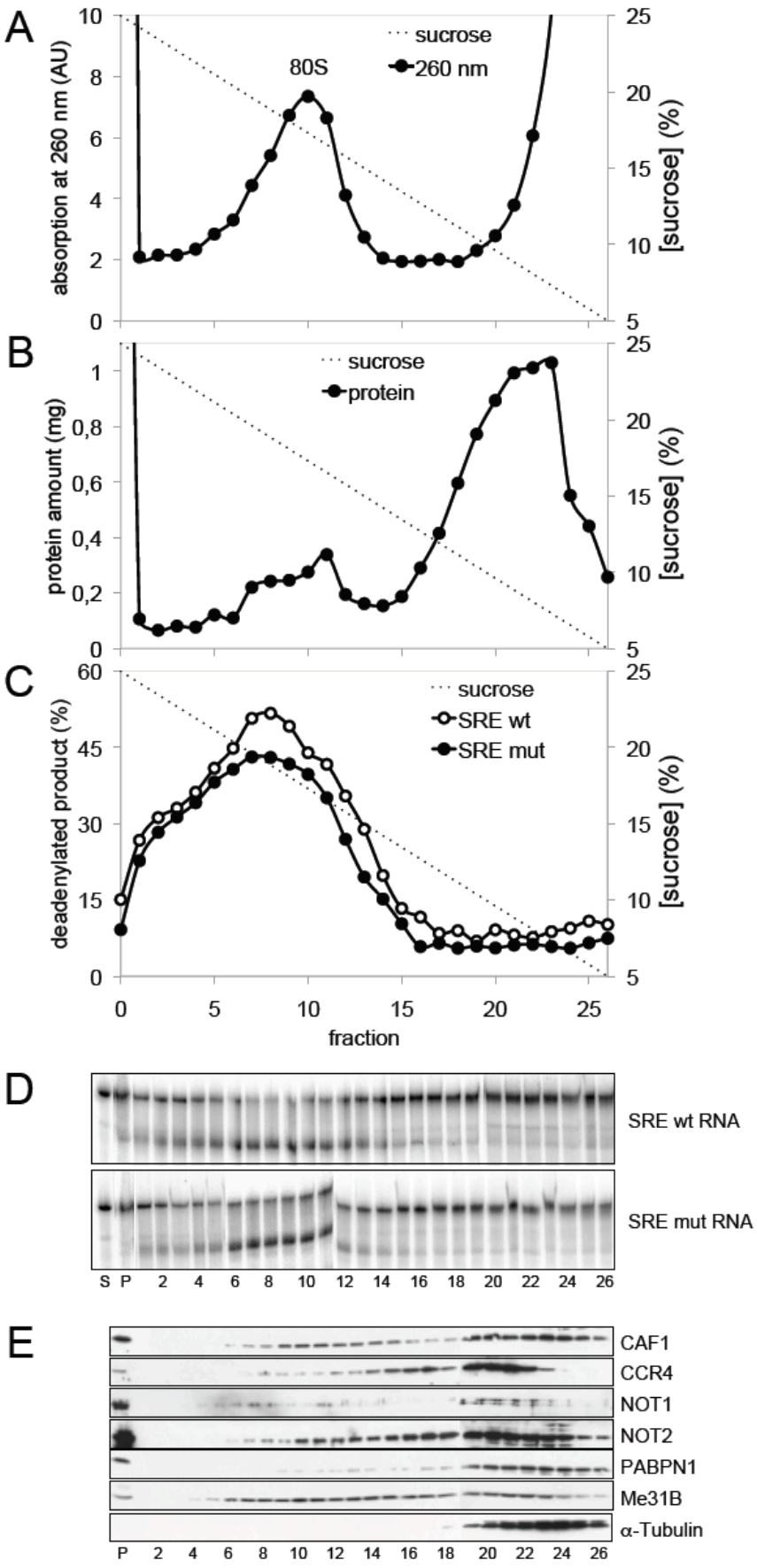

FIGURE 10. **Fractionation of *Drosophila* embryo extract by sucrose gradient centrifugation.** (figure legend see next page)

FIGURE 10. **Fractionation of *Drosophila* embryo extract by sucrose gradient centrifugation.** (figure see previous page)
500 µl of *Drosophila* embryo extract were loaded onto an 11 ml 5 to 25% sucrose gradient and centrifuged for 83 min at 38.000 rpm and 4°C as described (Jeske *et al.* 2010). Fractions of ~500 µl (17 drops) were collected from the bottom (fraction 1) to the top (fraction 26). Fraction 0 is the pellet (P) resuspended in 500 µl of 0.5 x embryo lysis buffer. (**A**) The absorbance at 260 nm was determined and plotted against the fraction number. Absorption units (AU) refer to undiluted samples. The ordinate scaling was chosen to fit the 80S ribosome peak. Values of fractions 0, 23, 24, 25, and 26 were 131.4, 10.1, 18.4, 28.6, and 32.5, respectively. (**B**) Protein concentrations were determined by Bradford assay and total protein amount per fraction was plotted against the fraction number. The pellet contained 4.8 mg protein. (**C**) & (**D**) 9.4 nM of ^{32}P-labeled SRE wt or SRE mut RNA were incubated with 50% of the fractions for 1 hr under deadenylation conditions (Jeske & Wahle 2008). Amount of the deadenylated product plotted against the fraction number is shown in (C). Unreacted substrate RNA (S) is shown for comparison. (**E**) Fractions were analyzed by Western blotting for distribution of the indicated proteins. Western blotting and all antibodies, except the anti-PABPN1 antibody (gift from Uwe Kühn) and the anti-α-Tubulin antibody (T9026, Sigma), have been described (Jeske *et al.*, 2006).

According to the idea presented above, deadenylation inactivity might result from disproportional enrichment of an inhibitory factor in the light fractions. Alternatively, the deadenylase complex might be active only in the form or as a component of the fast-sedimenting particle. Co-migration of deadenylation activity (and significant amounts of CCR4-NOT complex components) with 80S ribosomes suggests the possibility that the deadenylase complex partially associates with ribosomes. However, the P body marker protein Me31B is also found in the heavier fractions, suggesting that the deadenylase might also be part of P bodies, which are assumed to be high-density particles. Whether the deadenylation activity is intimately coupled to the high-density particles remains to be investigated.

Conclusion & outlook

Extracts from different organisms display deadenylation activity if either an ARS or competitor poly(A) RNA is present during the reaction, suggesting a similar net consequence of both reagents, which led to the hypothesis that ATP is required to remove PABPC from the substrate's poly(A) tail (see Introduction, p. 63). Deadenylation in *Drosophila* embryo extract depends on an ARS. Here, initial experiments were performed to test if the requirement for ATP diminishes when the PABPC concentration in the *Drosophila* embryo extract is reduced. Different approaches were used to reduce the PABP concentration in the extract. If either competitor poly(A) or Paip2 were added to the reaction, an SRE-accelerated

deadenylation could be observed under ATP-depleting conditions. Although specificity controls are missing, these results are consistent with the proposed model. In contrast to poly(A) competitor, addition of Paip2 inhibits deadenylation in the presence of ATP, indicating that this inhibition specifically concerns Paip2 or the Paip2 – PABPC interaction. The hypothesis of an ATP-dependent modification (e.g. phosphorylation) of Paip2 and/or PABPC leading to deadenylation inhibition is discussed in the Results & discussion section (p. 68). It might serve as a working model for studying the Paip2 – PABPC interaction with purified proteins that either contain or lack the modification.

By centrifugation of the extract for 1 h at ~100.000 x g, a pellet was obtained that contained the complete SRE-specific deadenylation activity. When the extract was treated with RNase prior to centrifugation the PABPC concentration in the pellet could be reduced without influencing the sedimentation behavior of the putative components of the SRE-dependent deadenylation machinery. For preparation of pellets that were used for analysis of the ATP dependence, centrifugation conditions were applied (i.e. centrifugation at 20.800 x g through sucrose cushion) that are likely to result in a more efficient PABPC reduction. Consistent with the hypothesis, these pellet fractions displayed deadenylation activity in the absence of ATP, which was even stronger when the extract had been RNase-treated.

The results obtained suggest the existence of a factor, which protects the control RNA from deadenylation and hence serves to maintain sequence specificity. Repeatedly observed correlation between decreasing ATP dependence and decreasing sequence specificity suggests that both properties are conferred by the same factor. Upon 3' UTR-specific recruitment of the deadenylase to the RNA, the protecting factor might be removed by the ATP-dependent step to allow sequence-specific deadenylation. When Paip2 was added to the reaction without ATP, deadenylation of the mutant RNA could be observed, supporting the idea that PABPC could be the protecting factor. Deadenylation of the mutant RNA was also observed when pellets or fractions were used in the assay that resulted either from mild centrifugation of RNase-treated extract or from sucrose gradient centrifugation, respectively. In the latter case, sequence specificity was almost completely lost. However, even in the absence of the protecting factor one would expect accelerated deadenylation of the SRE RNA compared to the mutant, which suggests loss of the *trans*-acting specificity factor Smaug in the fractions obtained after sucrose gradient centrifugation. The distribution of Smaug and PABPC after sucrose gradient centrifugation remains to be examined.

In addition to the described experiments, the possibility to deplete PABPC from the extract by applying the extract to GSH sepharose coupled to GST-Paip2 has been tested in the laboratory. However, it turned out that the deadenylation activity was not able to persist the mock depletion using only GSH sepharose (unpublished observation of Melinda Diver).

All data shown here support the hypothesis of an ATP-dependent removal of PABPC from the substrate's poly(A) tail, but direct evidence is still missing. Since dissociation of PABP from the poly(A) tail seems to be slow (e.g. Simón *et al*. 2007), this process and its potential ARS-dependent acceleration might be visualized by means of an RNA pull-down assay. The RNA substrates should be pre-incubated with extract to allow complex formation and hence PABPC binding. After pre-incubation an excess of competitor poly(A) should be added (to prevent potential re-association of PABPC with the substrate) and the mixture incubated in the presence or absence of the ARS. A time course could reveal if the dissociation of PABPC from the poly(A) tail is accelerated in an SRE- and ATP-dependent manner. To distinguish between active dissociation of PABPC and liberation of PABPC as an indirect consequence of deadenylation, RNA substrates with an internal poly(A) tail should be used. However, use of an internal poly(A) sequence could block PABPC removal of the poly(A) tail in case this process is intimately coupled to the deadenylation process.

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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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6 APPENDIX

List of figures

Abbreviations

4E-BP	eIF4E-binding protein
4E-HP	eIF4E homologous protein
4E-T	eIF4E transporter
A	adenine
ADP	adenosine diphosphate
Ago1	argonaute 1
AMP	adenosine monophosphate
AMP-PCP	adenosine 5′-(β,γ-methylene)triphosphate
AMP-PNP	adenosine 5′-(β,γ-imino)triphosphate
ApppG	adenosine 5′-guanosine-5′-(P^1,P^3-triphosphate)
ARS	ATP-regenerating system
ATP	adenosine triphosphate
ATPγS	adenosine 5′-O-(thiotriphosphate)
bp	base pair
C	cytosine
CAF1	CCR4-associated factor 1
CCR4	carbon catabolite repression 4
cDNA	complementary DNA
CK	creatine kinase
CrPV	cricket paralysis virus
CTP	cytidine triphosphate
D	aspartate
Dcp2	decapping protein 2
DEPC	Diethylpyrocarbonate
Dhh1	DExD/H-box helicase 1
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dT	deoxythymidylate
DTT	dithiothreitol
E.	*Escherichia*
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol tetra acetic acid
eIF	eukaryotic initiation factor
F	phenylalanine

Fig.	figure
g	gravitational force (= 9.81 m/s^2)
glc	glucose
GMP-PNP	guanosine 5′-(β,γ-imino)triphosphate
GST	glutathione S transferase
GTP	guanosine triphosphate
h	hour
HK	hexokinase
Hsp	heat shock protein
IRES	internal ribosomal entry site
LB	Luria broth
LSm	Sm-like (Sm domains/proteins were named after the lupus patient Stephanie Smith)
Luc	luciferase
m^7G	7-methyl guanosine
m^7GpppG	(7-methyl)-guanosine 5′-guanosine-5′-(P^1,P^3-triphosphate)
Me31B	maternal expression at 31B
min	minute
miRNA	micro RNA
M-MLV	Moloney murine leukemia virus
MN	micrococcus nuclease
mRNA	messenger RNA
mRNP	messenger ribonucleoparticle
mut	mutant
n.d.	not determined
NOT	negative on TATA-less
nt	nucleotide
NTP	nucleoside triphosphate
oligo(A)	oligoadenylate
PABP	poly(A)-binding protein
PABPC	cytoplasmic poly(A)-binding protein
PABPN1	nuclear poly(A)-binding protein 1
PAGE	polyacrylamide gel electrophoresis
Paip2	PABP-interacting protein 2
PAM	PABP-interacting motif
PARN	Poly(A)-specific ribonuclease

P body	processing body
PCR	polymerase chain reaction
poly(A)	polyadenylated
RISC	RNA-induced silencing complex
RLU	relative light unit
RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoparticle
RT	reverse transcription
S.	*Saccharomyces*
SAM	sterile a motif
SDS	sodium dodecyl sulphate
Smg	Smaug
SRE	Smaug recognition element
SRE^{-}	SRE with exchange of G3 of loop sequence into C3
T	thymidine
Tral	Trailer hitch
tRNA	transfer RNA
u	units
U	uracil
UTP	uridine triphosphate
UTR	untranslated region
Vts1	vti1-2 suppressor
wt	wild type

Publikationsliste

1. Jeske, M., Meyer, S., Temme, C., Freudenreich, D., and Wahle, E. (2006)
Rapid ATP-dependent deadenylation of *nanos* mRNA in a cell-free system from *Drosophila* embryos.,
The Journal of biological chemistry 281, 25124-33.

2. Jeske, M., and Wahle, E. (2008)
Cell-free deadenylation assays with *Drosophila* embryo extracts.,
Methods in enzymology 448, 107-18.

3. Jeske, M., Moritz, B., Anders, A., and Wahle, E. (2010)
Smaug assembles an ATP-dependent stable complex repressing *nanos* mRNA translation at multiple levels.,
The EMBO Journal, advance online publication 16 November 2010;
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Stand der Veröffentlichungen und Darlegung des Eigenanteils daran

1. Jeske, M., Meyer, S., Temme, C., Freudenreich, D., and Wahle, E. (2006)
Rapid ATP-dependent deadenylation of *nanos* mRNA in a cell-free system from *Drosophila* embryos., *The Journal of biological chemistry 281*, 25124-33.

Eigenanteil: Planung, Durchführung und Auswertung aller Experimente ausgenommen des Experiments in Abb. 5B (Dorian Freudenreich) und der Gewinnung der Antikörper gegen NOT1, NOT2, NOT3 und NOT4 (Dr. Claudia Temme). Anfertigung aller Abbildungen. Anfertigung von Teilen des Manuskripts.

2. Jeske, M., and Wahle, E. (2008)
Cell-free deadenylation assays with *Drosophila* embryo extracts., *Methods in enzymology 448*, 107-18.

Eigenanteil: Planung, Durchführung und Auswertung aller Experimente. Anfertigung der Abbildung und des Manuskripts.

3. Jeske, M., Moritz, B., Anders, A., and Wahle, E. (2010)
Smaug assembles an ATP-dependent stable complex repressing *nanos* mRNA translation at multiple levels., *The EMBO Journal,* advance online publication 16 November 2010; doi:10.1038/emboj.2010.283

Eigenanteil: Planung, Durchführung und Auswertung aller Experimente ausgenommen der Experimente in Abb. 7, 8 und S2 (Bodo Moritz) und der Klonierung und Reinigung des GST-Oskar-Fusionsproteins (Dr. Alexander Anders). Abb. 2B stellt das Ergebnis einer Wiederholung (Bodo Moritz) meines Originalexperiments dar. Anfertigung aller Abbildungen bis auf Abb. 8. Anfertigung von Teilen des Manuskripts.

Erklärung

Hiermit erkläre ich, dass ich meine Dissertationsschrift selbständig und ohne fremde Hilfe verfasst habe. Ich habe keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt. Die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen habe ich als solche kenntlich gemacht.
Mit der vorliegenden Arbeit bewerbe ich mich erstmals um die Erlangung des Doktorgrades.

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Mandy Jeske

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