

# Different modes of translation for *hid*, *grim* and *sickle* mRNAs in *Drosophila*

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Protein synthesis is inhibited during apoptosis. However, the translation of many mRNAs still proceeds driven by internal ribosome entry sites (IRESs). Here we show that the 5'UTR of *hid* and *grim* mRNAs promote translation of uncapped-mRNA reporters in cell-free embryonic extracts and that *hid* and *grim* mRNA 5'UTRs drive IRES-mediated translation. The translation of capped-reporters proceeds in the presence of cap competitor and in extracts where cap-dependent translation is impaired. We show that the endogenous *hid* and *grim* mRNAs are present in polysomes of heat-shocked embryos, indicating that cap recognition is not required for translation. In contrast, *sickle* mRNA is translated in a cap-dependent manner in all these assays. Our results show that IRES-dependent initiation may play a role in the translation of *Drosophila* proapoptotic genes and suggest a variety of regulatory pathways.

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Apoptosis is a cellular process that eliminates cells at risk and is required for organ and tissue development.<sup>1</sup> Apoptosis has been studied in detail in *Drosophila* and the embryonic apoptosis pattern as well as the molecular mechanisms and factors involved in apoptosis are very well known.<sup>1–3</sup> A genetic screen for mutants exhibiting disruption of the pattern of apoptosis led to the identification and posterior characterization of the genes *reaper* (*rpr*), *grim* and *head involution defective* (*hid*) as positive regulators of apoptosis.<sup>4–6</sup> More recently, another gene, *sickle* (*skl*), was also identified as a proapoptotic gene.<sup>7–9</sup> These genes encode early activators of apoptosis that promote cell death by two different mechanisms: they inhibit translation and they inhibit the ability of the inhibitor of apoptosis proteins (IAPs) such as DIAP1 to repress caspase activity.

Translation begins at the initiation step with the recognition of the 5'UTR of an mRNA by proteins that catalyze the association to the 40S ribosomal subunit. One of these factors, the eIF4F complex, is composed of the cap-binding protein eIF4E, the ATPase/RNA helicase eIF4A and the scaffold/adaptor eIF4G, which coordinates the activity of eIF4E, eIF4A, and the further factors such as poly A-binding protein (PABP) and ribosome-associated eIF3.<sup>10</sup> Translation of the majority of eukaryotic mRNAs requires the recognition of the 5' cap structure (m<sup>7</sup>GpppN) by eIF4E. However, in some viral and cellular mRNAs, 5' UTR recognition occurs independently of the cap-structure and is mediated by an internal ribosome entry site (IRES).<sup>11</sup> During apoptosis in mammalian cells, protein synthesis, mostly cap-dependent

initiation, is inhibited by caspase-mediated degradation of translation initiation factors. Affected are eIF4G1, eIF4GII, the p35 subunit of eIF3, eIF4B, eIF2, the poly A-binding protein (PABP) and the eIF4E-binding protein (4E-BP). In addition, changes in the phosphorylation states of eIF4E, eIF4E-BP1 and eIF2 $\alpha$  have been reported.<sup>12</sup> Curiously, a number of proteins that are involved in the regulation and progression of apoptosis, including inhibitors of apoptosis XIAP, cIAP-1 and HIAP2, Bcl-2 and the proapoptotic proteins p97/DAP5/NAT-1 and Apaf-1, are translated in a cap-independent manner by using IRES elements present in their 5' UTRs. In this way, they override the general inhibition of protein synthesis that occur in apoptotic cells.<sup>13</sup>

Most of our knowledge on these events derives from studies in mammalian cells, and few is known about the translation regulation during apoptosis in *Drosophila*, an excellent model to combine genetics and biochemistry to solve many of the remaining questions in the field. Our laboratory has initiated the study of translational control of gene expression of *Drosophila* proapoptotic genes and we have recently reported that the lack of the cap-binding protein eIF4E in mutant embryos results in upregulation of *rpr* transcription and widespread apoptosis.<sup>14</sup> *rpr* mRNA is translated in a cap-independent manner by an IRES<sup>14</sup> and several RNA-binding proteins have been identified as potential translational regulators.<sup>15</sup> Here we studied the mechanism of translation of the proapoptotic genes *grim*, *hid* and *sickle*. We found that *hid* and *grim* can be translated in an IRES-dependent manner, whereas *sickle* is translated in a cap-dependent manner.

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**Abbreviations:** *cad*, *caudal*; eIF, eukaryotic initiation factor; FLuc, Firefly luciferase; *hid*, *head involution defective*; *hsp*, *heat-shock protein*; IRES, internal ribosome entry site; PABP, poly(A)-binding protein; RLuc, Renilla Luciferase; *rpr*, *reaper*; *skl*, *sickle*; UTR, untranslated region

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These results show that IRES-dependent initiation of translation also might play a role in *Drosophila* during apoptosis and suggest a diversity of regulatory mechanisms.

## Results

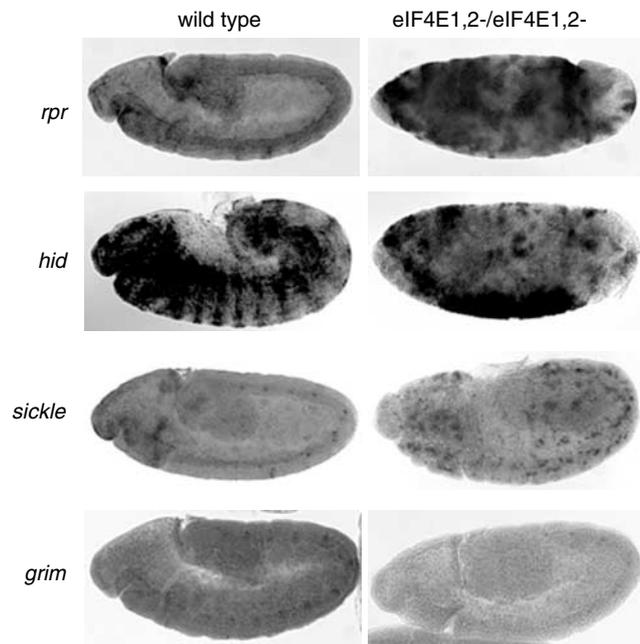
**Transcription of *hid* and *sickle*, but not *grim*, is upregulated in an *eIF4E* mutant.** We have previously shown that *rpr* transcription is upregulated in embryos devoid of *eIF4E*.<sup>14</sup> Thus, before studying the mechanism of translation of the early proapoptotic *Drosophila* genes *hid*, *grim* and *sickle*, we first analyzed their mRNA expression by *in situ* hybridization in embryos of an *eIF4E* mutant, which lacks cap-binding activity and displays widespread apoptosis.<sup>14</sup> We performed double *in situ* hybridization using specific probes for each gene and a balancer-specific probe to identify embryos that contain a wild-type copy of *eIF4E-1,2* from homozygous *eIF4E-1,2* mutant embryos. We found that *rpr*, *hid* and, to a lesser extent, *sickle* mRNA were upregulated and widespread expressed in the homozygous *eIF4E-1,2* mutant embryos (Hernández *et al.*<sup>14</sup> and Figure 1). The pattern of expression and the intensity of *grim* mRNA staining, on the other hand, did not seem to be affected.

***hid* and *grim*, but not *sickle*, 5' UTRs promote cap-independent initiation.** Based on our previous studies on *rpr*, the presence of more mRNA from *hid* and *sickle* in *eIF4E* mutant embryos prompted us to study whether they, and also *grim* mRNA, were translated in the absence of the cap-binding protein. We cloned *sickle*, *hid* and *grim* 5' UTRs into the monocistronic vector containing Firefly luciferase as

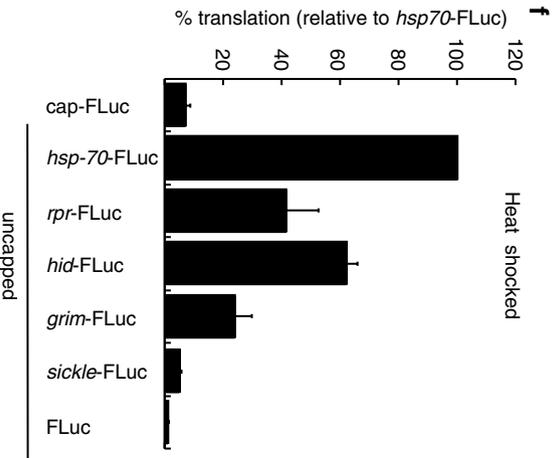
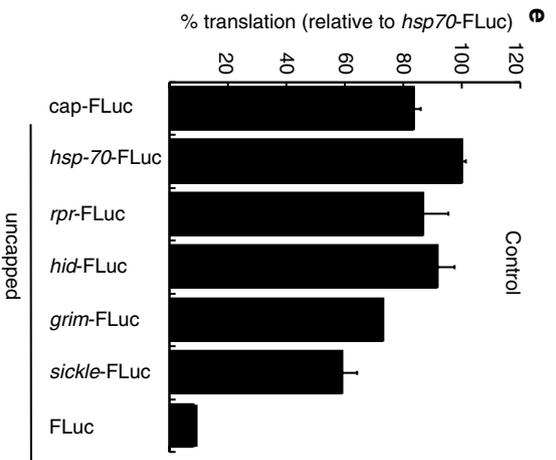
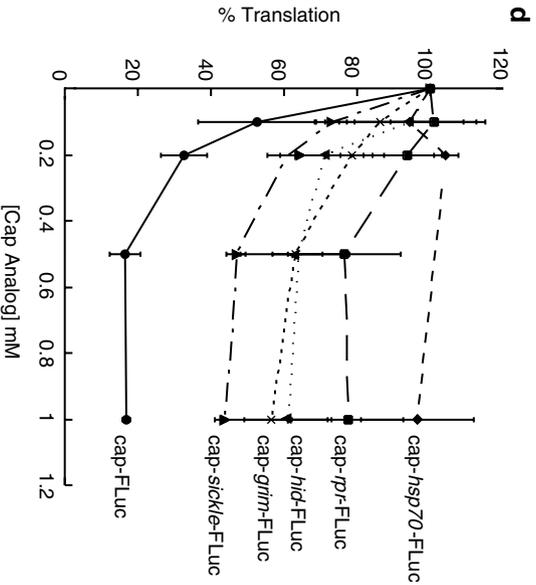
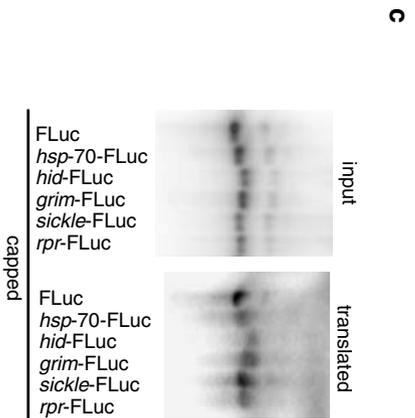
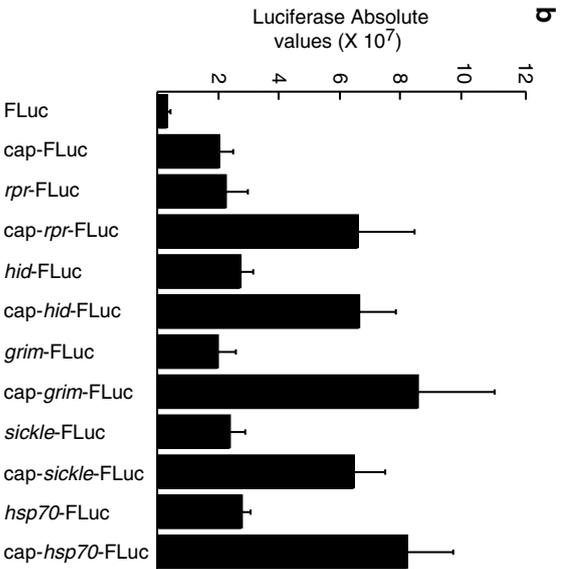
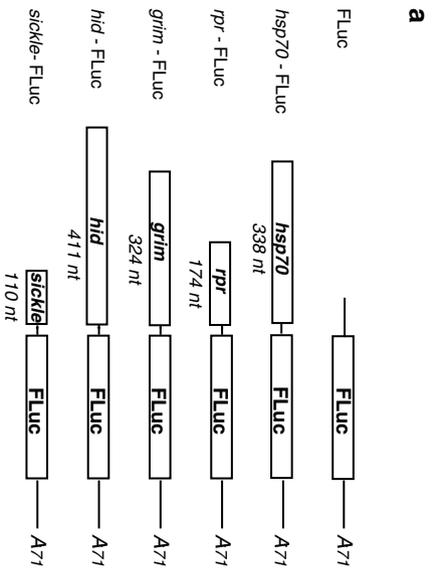
a reporter (FLuc; Figure 2a) and assayed their translation competence in a cell-free *Drosophila*-embryo translation system.<sup>16,17</sup> As expected, the uncapped FLuc control mRNA was not translated efficiently in the extracts, whereas the uncapped reporters containing the 5'UTR of *hid*, *grim* and *sickle* conferred translation to the reporter at levels equivalent to the m<sup>7</sup>GpppG-capped FLuc control vector (cap-FLuc; Figure 2b). The same was observed for the cap-independent *rpr* and *hsp70* mRNAs, which were used as controls in this experiment.<sup>14</sup> m<sup>7</sup>GpppG-capped reporters containing the 5' UTR for proapoptotic genes were translated one to three times more efficiently than their uncapped counterparts (Figure 2b), which is consistent with previous reports on the cap-independent mRNAs from *rpr* and *Ubx*.<sup>14</sup> All capped mRNAs showed the same stability after 90 min of reaction, as measured by the addition of <sup>32</sup>P-labeled transcripts to the reaction (Figure 2c).

We then performed competition experiments using free cap m<sup>7</sup>GpppG to mimic the absence of *eIF4E* in the extracts<sup>18</sup> (Figure 2d). As expected for an *eIF4E*-independent transcript, the translation of a m<sup>7</sup>GpppG-capped reporter containing the 5'UTR of *hsp70* (cap-*hsp70*-FLuc) was unaffected by the addition of the cap-analog in the translation assay. On the other hand, the translation of the cap-FLuc reporter was reduced more than 80% compared to its translation in control conditions. The translation of m<sup>7</sup>GpppG-capped *rpr* 5'UTR-FLuc reporter mRNA (cap-*rpr*-FLuc) was the least affected among the proapoptotic mRNAs. The translation of m<sup>7</sup>GpppG-capped-*hid* 5'UTR-FLuc mRNA (cap-*hid*-FLuc) and m<sup>7</sup>GpppG-capped- 5'UTR *grim*-FLuc (cap-*grim*-FLuc) reached a plateau at 40% of inhibition. The translation of m<sup>7</sup>GpppG-capped *sickle* 5'UTR-FLuc (cap-*sickle*-FLuc) was the most affected (50% of inhibition) in the presence of free cap (Figure 2d). This experiment showed that the 5'UTRs of the proapoptotic mRNAs confer different levels of cap-independent translation to the reporter as measured by the sensitivity to the addition of free cap.

We then tested the ability of the same reporters to drive translation in extracts derived from heat-shocked embryos, where cap-independent translation is severely impaired and translation of heat-shock proteins mRNAs is favored.<sup>19</sup> In extracts derived from untreated embryos (control), the uncapped reporters bearing *hid*, *grim* and *sickle* 5'UTRs were translated *in vitro* at a level comparable to cap-FLuc, uncapped *rpr*-FLuc and uncapped *hsp70*-FLuc as observed before (Figure 2b and e). This indicates a level of cap-independent translation of all mRNAs. However, in translation extracts derived from heat-shocked embryos, the cap-dependent translation of both cap-FLuc reporter and uncapped *sickle*-FLuc was dramatically reduced (Figure 2f). In contrast, uncapped *rpr*-FLuc, *hid*-FLuc and *grim*-FLuc were able to drive translation in the heat-shocked extracts although with less efficiency than the uncapped *hsp70*-FLuc reporter (Figure 2f). The changes observed in translation efficiency of the different reporters are dependent on the intrinsic translation capabilities of these mRNAs, as we observed that mRNA degradation does not occur in a transcript-specific manner either in heat-shock as well as in untreated extracts (Supplementary Figures 1–2, and Hernández *et al.*<sup>14</sup>). As the translation of cap-*sickle*-FLuc was affected by the presence of free cap competitor (Figure 2d) and



**Figure 1** Expression of proapoptotic mRNAs in *eIF4E-1/2* mutant embryos. Double *in situ* hybridization in homozygous *eIF4E-1/2* embryos<sup>14</sup> with a GFP probe (detectable in wild type embryos, left panels) together with probes for the different proapoptotic genes. *rpr*, *hid* and, to a lesser extent, *sickle* are upregulated. *grim* expression is not affected



largely in heat-shocked lysates (Figure 2f), it indicates that *sickle* mRNA, different than the others, is preferentially translated in a cap-dependent manner.

***hid* and *grim*, but not *sickle*, display IRES activity.** We reported previously that *rpr* and *hsp70* 5'UTRs display IRES activity *in vitro* and *in vivo*.<sup>14</sup> To evaluate whether this mechanism also drives cap-independent translation of the other proapoptotic genes, we inserted the different 5'UTRs as intercistronic fragments in the dicistronic reporters FLuc/hairpin/RLuc and FLuc/*cad*/RLuc that were designed to yield a reduced ribosomal read-through into the second cistron.<sup>14</sup> The reporter constructs are depicted in the Figures 3a and 5a, respectively. The presence of the different 5'UTRs as intercistronic sequences did not significantly affect the expression of the capped first cistron in the *in vitro* translation assays (Figure 3b, FLuc, black bars). In contrast, the presence of *rpr*, *grim* and *hid* 5' UTRs as intercistronic sequences increased the translation efficiency of the second cistron (Figure 3b, RLuc, white bars, and c, see the bars above of the cutoff line). In agreement with the data proving that *sickle* mRNA translation is driven by a cap-dependent mechanism, the presence of *sickle* 5'UTR did not show an effect on the translation of the second cistron when compared with the control FLuc/hairpin/RLuc vector. The insertion of all the 5'UTR sequences in antisense orientation leads to reduction or zero increase of the second cistron expression (Figure 3b and c). The stability and integrity of the transcripts after the translation reaction were not affected (Figure 3d). The same reporters used in Figure 3a were capped with the ApppG analog and used for *in vitro* translation. The graphs showing the absolute values of the first cistron (Figure 4a, FLuc, black bars) and second cistron (Figure 4a, RLuc, white bars) are shown with the same scale as in the corresponding graphs in Figure 3. As expected, the translation of the first cistron was abolished by the presence of the ApppG (Figure 4a), whereas the second cistron was still translated with the same efficiency (Figure 4b). No specific changes in stability and integrity of the transcripts were observed after the translation reaction (Figure 4b).

We corroborated the IRES activity of *hid* and *grim* by inserting their 5'UTR sequences into the dicistronic reporter FLuc/*cad*/RLuc, which also shows low background of translation of the second cistron<sup>14</sup> (Figure 5a). We used m<sup>7</sup>GpppG-capped dicistronic mRNAs for *in vitro* translation (Figure 5b). The presence of *hid*, *grim* and *rpr* 5'UTR as intercistronic sequences increased the efficiency of translation of the second cistron (RLuc) with respect to the first cistron (FLuc), when compared to the control FLuc/*cad*/RLuc vector. On the contrary, the efficiency of translation of the reporter bearing the *sickle* 5'UTR was comparable to the control vector. We conclude that the proapoptotic genes *rpr*, *hid* and *grim* 5'UTRs

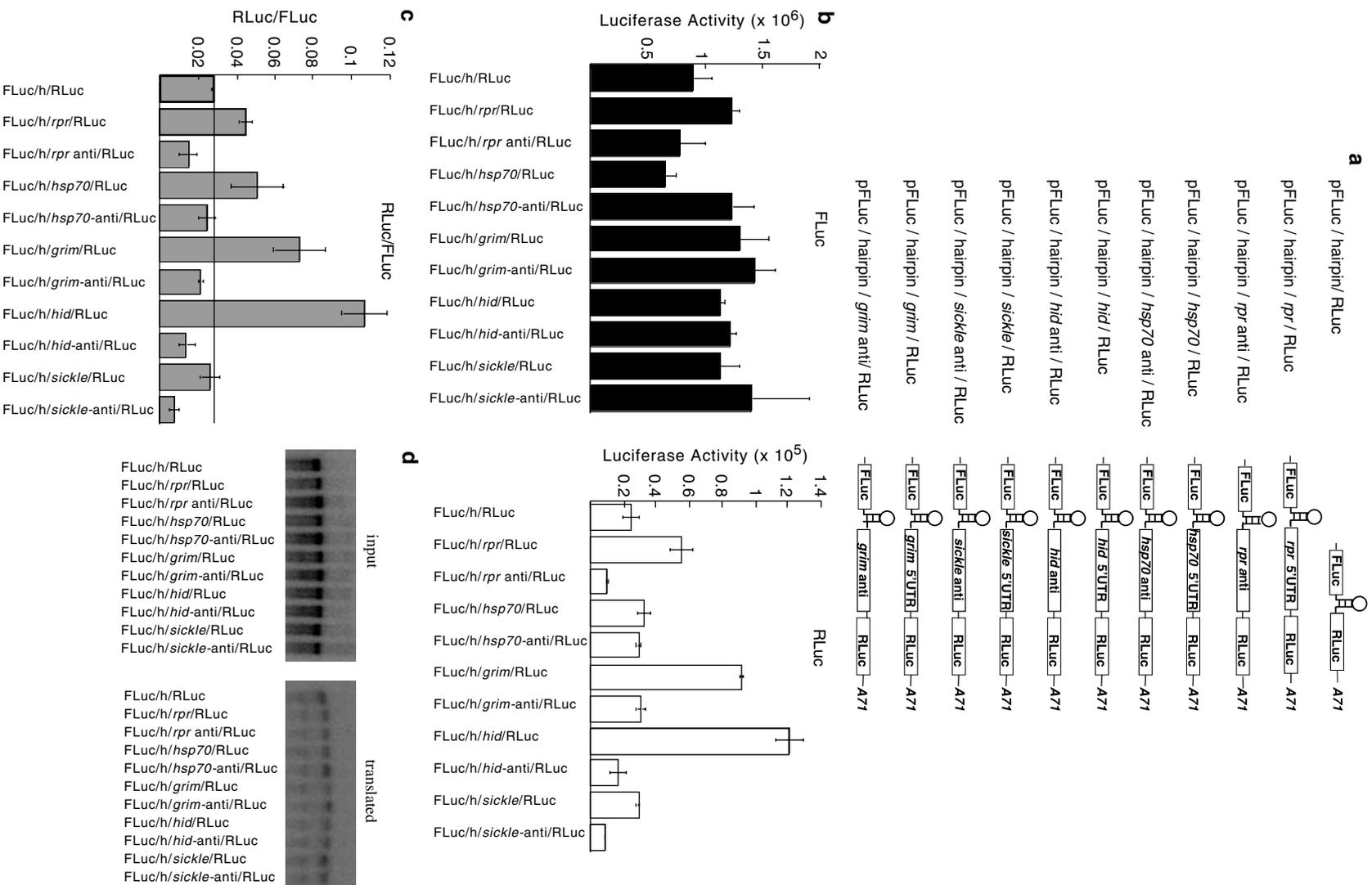
display IRES activity, whereas the *sickle* 5'UTR apparently does not share this mechanism of translation.

***hid* and *grim*, but not *sickle*, are recruited to polysomes at reduced cap-dependent translation.** To validate the *in vitro* data, we determined whether the endogenous mRNAs of *rpr*, *hid*, *grim* and *sickle* are translated in embryos that have been heat-shocked to impair cap-dependent initiation. We isolated ribosomal fractions from extracts derived from 0–12 h-old *Drosophila* embryos grown under normal conditions (Figure 6a) and after heat shock (Figure 6b). By ultracentrifugation in sucrose gradients, we separated those mRNAs not being translated and which are free or associated with 43S, 48S or 80S initiation complexes (U in Figure 6), from those mRNAs that are being actively translated associated with polysomes (P in Figure 6). The integrity of the RNA purified from the different fractions under normal and heat-shock condition was assessed by agarose gel electrophoresis and no degradation was observed (Supplementary Figure 3). In comparison to untreated samples (C in Figure 6c), after heat-shock treatment (HS in Figure 6c), we observed an increase of the total amount of *hsp70* mRNA and a decrease in the levels of *Actin5C*, *rpr*, *hid*, *grim* and *sickle* mRNAs (Figure 6c). This indicated that the heat-shock treatment was efficient. We then quantified by real-time RT-PCR the transcripts corresponding to *hid*, *grim*, *sickle*, *Actin5C*, *hsp70* and *rpr* mRNAs in both untranslated (U) and polysome-associated (P) fractions and plotted the P/U ratio in both conditions. As was observed for the cap-dependent *Actin5C* mRNA, polysome-associated *sickle* mRNAs decreased during heat-shock conditions (P/U 2.2 and 1.5, respectively, in untreated conditions to P/U < 1 during heat-shock conditions). On the contrary, as it was observed for *rpr* and *hsp70* mRNAs, whose cap-independent translation is driven by an IRES element<sup>14</sup> and Figure 6d), *hid* mRNA move from the untranslated fractions in the untreated condition (P/U ratio 1.3) to polysome-associated fractions during heat-shock conditions (P/U 1.9). *grim* mRNA does not change the P/U ratio, indicating that it is still maintained in the polysome fraction despite the inhibition of translation by heat shock. The observation that *sickle* mRNA is released from polysomes, whereas *rpr*, *hid*, *grim* and *hsp70* mRNAs are kept in there upon heat-shock, support the notion of a cap-dependent translation mechanism for *sickle* mRNA and an IRES-dependent translation mechanism for *hid* and *grim* mRNAs.

## Discussion

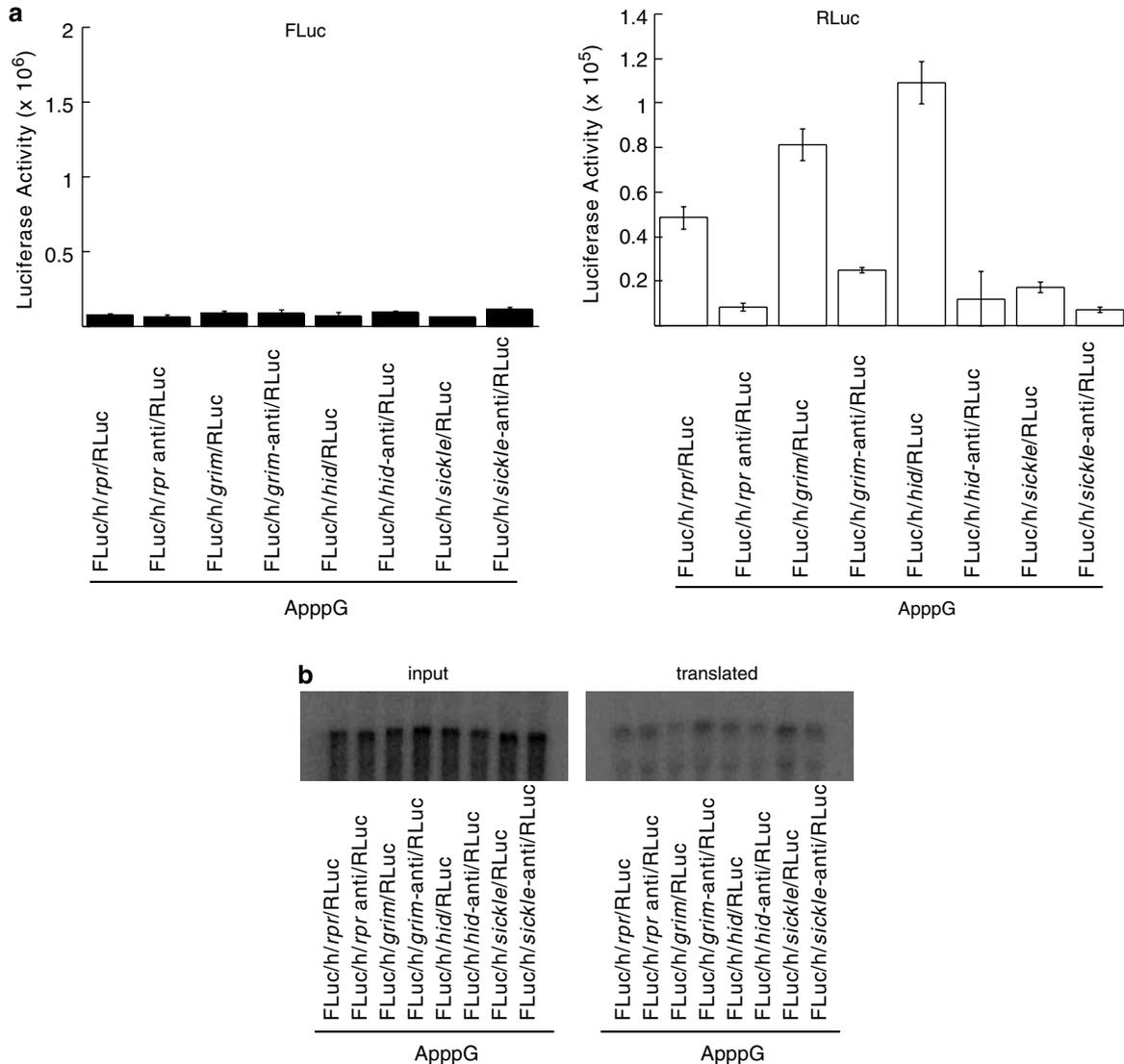
We have recently reported that *Drosophila* I(3)67A<sup>1</sup> is a null allele of *eIF4E*. It displays an embryonic lethal phenotype and shows widespread apoptosis that correlates with extensive

**Figure 2** Analysis for cap-independent translation of different *Drosophila* proapoptotic mRNAs 5'UTRs. (a) Monocistronic reporter mRNAs used in *in vitro* translation assays. (b) *In vitro* translation of capped and uncapped monocistronic reporter transcripts in translation extracts derived from wild-type embryos. (c) Analysis of the stability of the capped transcripts assayed in (b). (d) Competition of cap-dependent translation with increasing concentrations of free cap m<sup>7</sup>GpppG competitor. The efficiency refers to the translation of the reporter mRNA in the absence of the free cap competitor. (e, f) Translation of apoptotic mRNAs 5'UTRs in heat-shocked extracts. (e) Monocistronic mRNA reporters translated in extracts derived from untreated and (f) heat-shocked embryos. All results are normalized to the *hsp70*-FLuc reporter mRNA. Absolute values are not comparable as they were performed with different extracts



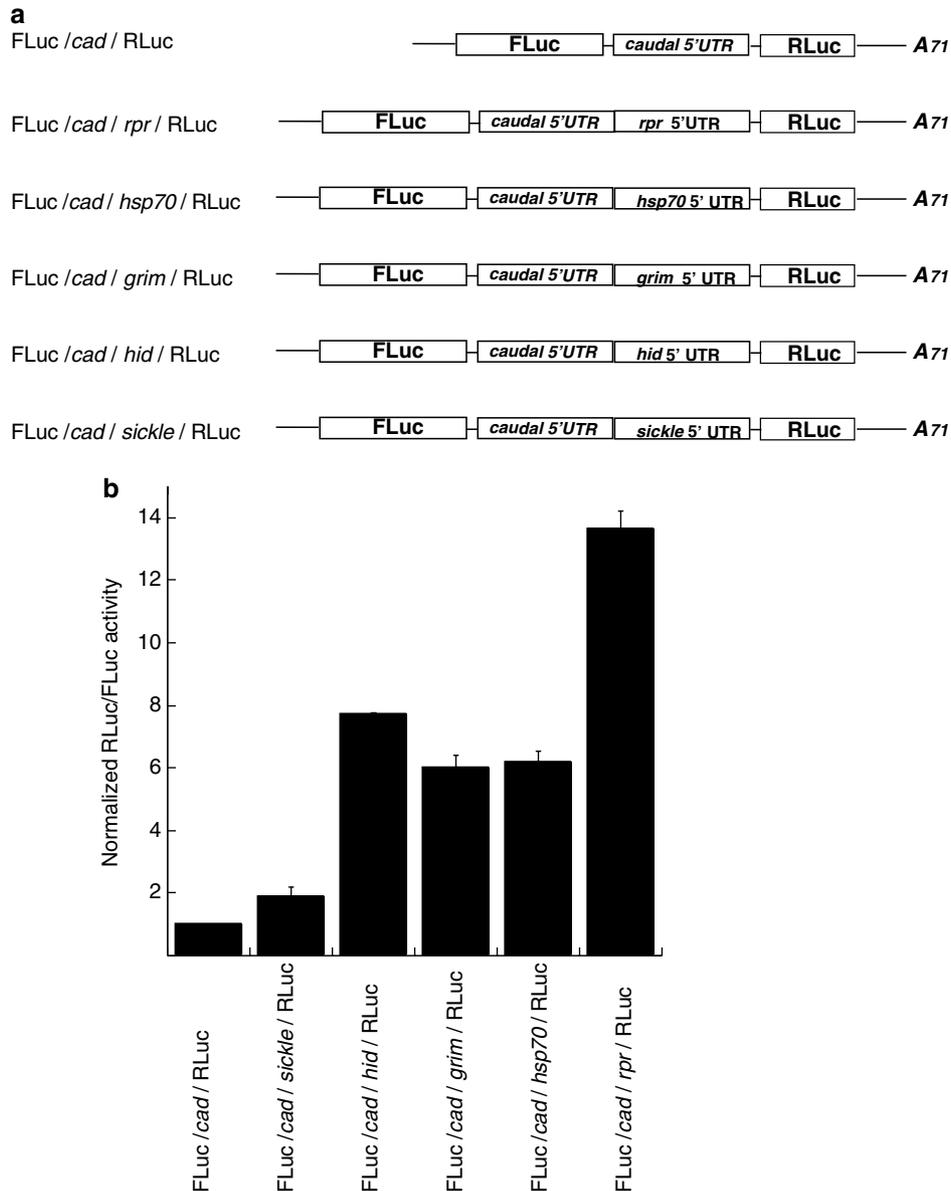
upregulation of *rpr* mRNA transcripts.<sup>14</sup> Here we have found that the early proapoptotic genes, *rpr*, *hid* and *sickle*, but not *grim*, are also upregulated in *eIF4E* mutant embryos. Different transcriptional regulation for proapoptotic genes has also been reported in response to diverse clues. The transcription of *rpr*, but not *sickle*, is upregulated in *crumbs* mutants,<sup>7</sup>

whereas *rpr*, but not the other proapoptotic genes, is activated by the ecdysone stimulus that triggers salivary gland cell death.<sup>20</sup> It also was recently discovered that upon irradiation, the tumor suppressor protein p53 activates *rpr*, *hid* and *sickle* transcription.<sup>21–23</sup> This implies a differential program to activate proapoptotic genes in flies. One of them is the lack



**Figure 4** *rpr*, *hid* and *grim* 5'UTR but not *sickle* 5'UTR ApppG-capped dicistronic transcript show IRES activity. (a) *In vitro* translation of the ApppG-capped dicistronic transcripts containing the different 5'UTRs in sense and antisense orientation. Absolute values of Firefly luciferase activity (FLuc, first cistron, black bars) and values of the Renilla luciferase activity (RLuc, second cistron, white bars) are shown. The translation of the first cistron is reduced and the second cistron is expressed at the same level as was observed by the m<sup>7</sup>GpppG-capped counterparts in Figure 3(b). The same scale as in Figure 3(b) was used. (b) Stability analysis of the dicistronic reporter mRNAs used in (a)

**Figure 3** *rpr*, *hid* and *grim* 5'UTRs, but not *sickle* 5'UTR, show IRES activity in a vector containing a stable hairpin. (a) Reporter dicistronic mRNAs containing a synthetic, stable hairpin that prevents read-through of ribosomes. The different 5' UTR were cloned downstream of the hairpin. (b) *In vitro* translation of the capped dicistronic transcripts containing the different 5'UTRs in sense and antisense orientation. Absolute values of Firefly luciferase activity (FLuc, first cistron, black bars) and values of the Renilla luciferase activity (RLuc, second cistron, white bars) are shown. (c) Efficiency of translation of the second cistron over the first (capped) cistron (RLuc/FLuc values) of the experiment depicted in (b). (d) Stability analysis of the dicistronic reporter mRNAs used in (b) and (c). The minor band observed in the transcripts before and after translation probably is the result of incomplete transcription due to the presence of the stable hairpin that blocks the polymerase activity. As the same amount of this band is observed in all transcripts, it cannot account for the differences in translation of the different reporters tested

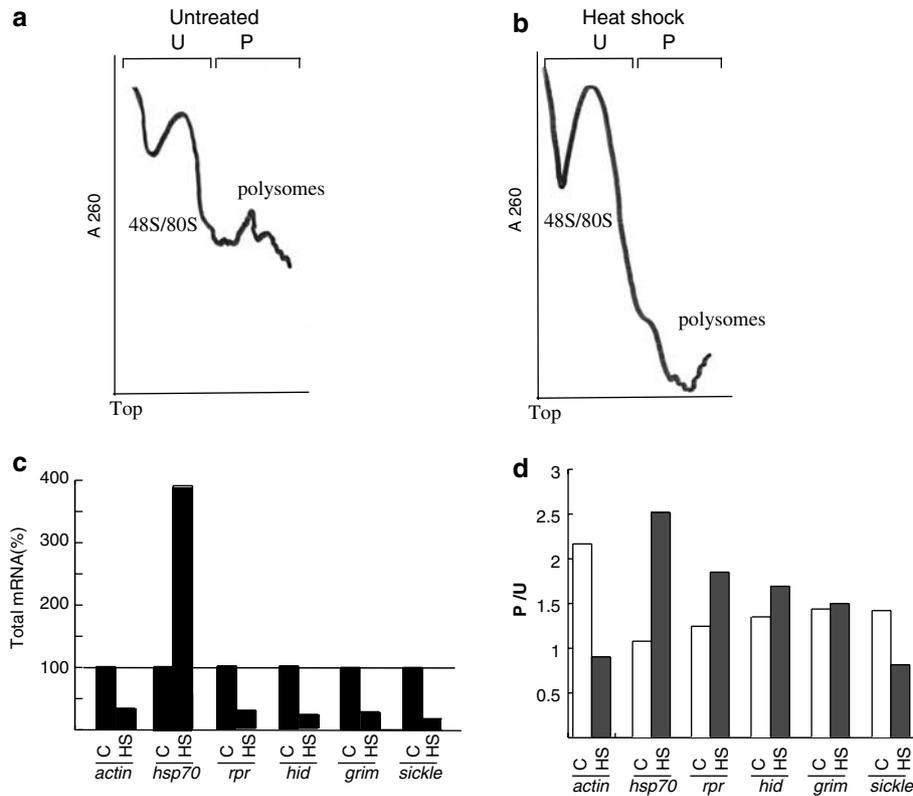


**Figure 5** *rpr*, *hid* and *grim* 5'UTR but not *sickle* 5'UTR show IRES activity in a vector containing the 5'UTR of *maternal caudal* mRNA (*cad*) to prevent read-through. (a) Reporter dicistronic mRNAs used for *in vitro* translation. (b) Translation efficiency (RLuc/FLuc activity) of dicistronic transcripts depicted in (a) normalized to the efficiency in the FLuc / *cad* / RLuc vector

of cap-dependent translation in the embryo, which requires further investigation. In this regard, overexpression of the tumor suppressor p53 increases the association of eIF4E with 4E-BP1, thereby reducing the association of eIF4E with eIF4G,<sup>24</sup> a condition that impairs cap-dependent translation. It is interesting to note that in both situations, p53 activation and the lack of *eIF4E*, the mRNAs of the upregulated apoptotic genes must escape the resulting translation inhibition in order to exert their apoptotic function. In this study, we have analyzed the ability of these mRNAs to be translated in the absence of the cap-binding protein eIF4E. *Drosophila rpr* and *hsp70* mRNAs can be both translated in an IRES-dependent manner.<sup>14</sup> Here we have shown that the *Drosophila* pro-apoptotic genes *hid* and *grim*, but not *sickle*, mRNAs are

translated by a cap-independent mechanism as well. Thus, transcriptional activation does not correlate with the mode of translation, as it could have been taken out from the only case of *rpr*.

*hid*, *grim* and *rpr* 5' UTRs display IRES activity and confer translation of a reporter mRNA in the presence of competing free cap and under heat-shock, a situation leading to the impairment of cap-dependent translation. Endogenous *rpr*,<sup>14</sup> as well as *hid* and *grim* mRNAs are present in embryonic polysomal fractions during heat-shock. This agrees with a role of cap-independent translation in the regulation of apoptosis in *Drosophila*. In mammalian cells, several apoptosis-related mRNAs are translated via IRES elements present in their 5' UTRs such as the ones encoding the antiapoptotic proteins



**Figure 6** Recruitment of *grim*, *hid*, but not *sickle*, mRNAs to polysomes upon heat shock. Sedimentation profiles in sucrose gradients from untreated (a) and heat-shocked (b) *Drosophila* embryos. The position of ribosomal complexes 48S and 80S is indicated. U, fraction containing untranslated/initiated mRNAs. P, polysomal fraction. (c, d) Amplification of *hid*, *grim* and *sickle* mRNAs in U and P fractions by real-time quantitative RT-PCR experiments from heat-shocked embryos. (c) Comparison of the total amounts of each mRNA in untreated and heat-shock-treated embryos. The amount of mRNA is calculated as P + U and normalized to untreated embryos. (d) The P/U ratio in the control (white bars) or heat-shock-treated (gray bars) embryos for each mRNA is tested. This value is used as a measure for the translational activity of the mRNA

XIAP, cIAP-1 and HIAP2, Bcl-2 and hsp-70, as well as those encoding the proapoptotic factors p97/DAP5/NAT-1, Apaf-1 and *c-myc*.<sup>25–32</sup> However, *in vivo* evidence of cap-independent translation of endogenous transcripts is still scarce. Based on this evidence and our evidence, one can hypothesize that IRES-dependent translation is an evolutionary conserved feature for the regulation of apoptosis.

Different from picornaviral and cellular IRESs, which possess a predicted complex secondary structure, the IRESs of *rpr*, *hid* and *grim* have no evident secondary structure. Indeed, *Drosophila rpr* and *hsp70* 5' UTRs have a high content of adenines (45–50%);<sup>14,33</sup> as well as the 5'UTR Hsp83 (38%), which suffices to confer efficient translation upon heat-shock by decreasing the likelihood of secondary structure formation.<sup>34</sup> Although no blocks of similarity between *hid* or *grim* with *hsp70* 5'UTR exist, as it the case of *rpr* 5'UTR,<sup>14</sup> they are all rich in adenines. *hid*, *rpr*, *grim* and *sickle* 5'UTR have 50, 45, 37 and 34% adenine content, respectively, and they are translated in this order of efficiency in heat-shocked extracts. This could explain their ability to be translated during apoptosis and under other stress conditions during which cap-dependent translation is impaired. Thus, a correlation seems to link the adenine content of the 5'UTR, and thus a low secondary structure, with the translational efficiency under heat-shock and apoptosis conditions, when no unwinding activity of eIF4A appears to exist. The fact that heat-shock and

proapoptotic mRNAs are translated by a cap-independent mechanism in *Drosophila*, which is correlated with a reduced adenine context in their 5'UTRs, further support the hypothesis that heat-shock response and apoptosis coevolved using common molecular mechanisms in response to cellular stress.<sup>14</sup>

Our data indicate different modes of translation of the proapoptotic genes. Recent evidence suggest that the post-transcriptional regulation of *Drosophila* proapoptotic genes by miRNAs might be very complex.<sup>35–38</sup> The miRNA family miR-2/6/11/13/308 has been shown to downregulate *rpr*, *hid*, *grim* and *sickle* and either different miRNAs could act on different mRNAs or the same mRNA can recognize different miRNAs.<sup>37,38</sup> This combinatorial effect might imply redundant and/or different mechanisms, although no evidence still exists about them. Our results add up to the still unanswered question of whether miRNAs can differentially act on different translation mechanism and, if so, how is the molecular mechanism. These are essential questions on the regulation of gene expression during apoptosis that remains unresolved and which require further investigations.

Translation inhibition during the apoptosis cascade might happen at several levels. In an early response to an apoptotic stimulus, the tumor suppressor p53 is activated. This results in the phosphorylation of eIF4E, increases the association of eIF4E with 4E-BP1, and reduces thereby the interaction

with eIF4G, a condition that diminishes cap-dependent translation.<sup>24</sup> Later on, caspases become active. They selectively cleave several translation factors, such as eIF4G (preventing its association with eIF4E) and PABP, impairing cap-dependent initiation (reviewed by Clemens *et al.*<sup>12</sup>). In later stages, translation is completely shut down by a still unknown mechanism, either involving direct interaction with the small ribosomal subunit as it has been shown for Rpr<sup>39</sup> or other, more general, mechanisms. The ability of different proapoptotic genes to be translated in a cap-dependent or cap-independent manner could reflect the action timing of their products. In this context, one can speculate that *skl* could only be translated when cap-dependent translation has not been impaired, restricting its expression to early stages of the apoptosis pathway.

### Materials and Methods

**Fly work.** The *eIF4E-1,2* mutant *l(3)67A<sup>1</sup> r<sup>1</sup> e<sup>4</sup>/TM3, Sb<sup>1</sup> (14)* was obtained from the Mid-America *Drosophila* Stock Center (Bloomington, USA). To identify homozygous mutant embryos, the mutation was balanced over a *TM3, Actin-GFP* chromosome. Homozygous embryos were identified by *in situ* hybridization using a GFP antisense RNA probe.

**Plasmids.** Plasmid SK + II-Grim-short-cDNA was used as template to amplify the *grim* 5' UTR,<sup>6</sup> *hid*<sup>6</sup> and *sickle*<sup>7-9</sup> 5' UTRs were PCR-amplified from a *Drosophila* adult cDNA library. The 5' UTRs were cloned into the *SacI*-*NcoI* site of the pLuc-cassette to create the plasmids *phid*-FLuc, *pgrim*-FLuc, *psickle*-FLuc and into the *Bgl*I site of pFLuc/RLuc, pFLuc/*cad*/RLuc and pFLuc/*hairpin*/RLuc for IRES activity assays. *prpr*-FLuc and *phsp70*-FLuc, pFLuc/RLuc, pFLuc/*cad*/RLuc and pFLuc/*hairpin*/RLuc were described previously.<sup>14</sup> For the synthesis of antisense RNA probes, *hid* and *sickle* ORFs were PCR-amplified from a *Drosophila* cDNA library and cloned into the *EcoRV* site of pBluescript SK(+) to create the plasmid pBS-*hid*-ORF and pBS-*sickle*-ORF.

**Embryo double whole-mount *in situ* hybridization.** Whole-mount *in situ* hybridization of embryos was essentially performed as described.<sup>40</sup> Linearized pBS-*sickle*-ORF, pBS-*hid*-ORF, SK + II-Grim-short-cDNA and pBS-*rpr*-cDNA were used as templates to generate digoxigenin-labeled RNA antisense probes. Linearized pRK27 was used as template to generate an antisense fluorescein-labeled GFP probe. The hybridization was performed with 1  $\mu$ l preheated DIG-labeled probes (either *rpr* or *hid* or *grim* or *sickle*) together with 1  $\mu$ l of preheated fluorescein-labeled probe for GFP. The embryos were first incubated with anti-DIG-AP antibody (Boehringer Mannheim, 1:2000 in PBT). The staining was developed in the dark with FAST BCIP/NBT solution (Sigma, St. Louis, USA). The staining reaction was stopped by washing with PBT. Embryos were then dehydrated in 50, 70 and 100% ethanol and stored at -20°C overnight. Embryos were rehydrated with 50% ethanol, PBT, washed with Glycine-buffer (100 mM Glycine pH 2.2, 0.1% Tween-20) and further washed with PBT. The GFP probe was detected using alkaline phosphatase-coupled anti-fluorescein F(ab) fragment antibody (Boehringer Mannheim, 1:2000 in PBT) and developed with Fast-Red TR/Naphtol AS-MX (Sigma St. Louis, USA). A detailed protocol is available under request. The embryos were mounted in glycerol and images were acquired with an Axioplan Microscope coupled to a Kontron CCD camera.

***In vitro* translation assays.** Translation extracts were prepared from 0 to 12 h-old *Drosophila* embryos as described<sup>16,17</sup> for the indicated times at 25°C. Translation extracts from heat-shocked embryos were prepared from pools of 0–12 h-old embryos that have been treated for 45 min at 37°C and processed without further recovery. Either m<sup>7</sup>GpppG or ApppG-capped transcripts, and uncapped transcripts were synthesized using T3 RNA polymerase (Ampliscribe mRNA transcription kit, Biozym Diagnostics GmbH) and using plasmids linearized with *Xho*I as templates in the presence or absence of m<sup>7</sup>GpppG or ApppG (New England Biolabs). The reaction was digested with DNase I and the transcripts were purified using the RNeasy kit (Qiagen). Reporter gene expression (Firefly and Renilla luciferases) was determined using the Dual-Luciferase reporter assay system (Promega) and detected in a Monolight 2010 Luminometer (Analytical

Luminescence Laboratory). When error bars are shown, they represent the mean of at least two experiments. The possible degradation of the transcripts was assessed using <sup>32</sup>P-labeled reporter RNAs for the translation reaction. After completion of the reaction, the RNA was purified and analyzed by denaturing agarose gel electrophoresis and phosphorimaging.

**Polysome analysis.** *Drosophila melanogaster* Oregon R embryos were collected from population cages in apple juice-agar plates and dechorionated. For heat-shock treatment, 0–12 h-old embryos were heat-shocked for 45 min at 37°C and processed without recovery. Embryos (150 mg) were homogenized on ice in 300  $\mu$ l buffer A (30 mM Hepes pH 7.4, 100 mM K acetate, 2 mM Mg acetate, 5 mM DTT, 50  $\mu$ l/ml RNasin, 2 mg/ml heparin and EDTA-free protease inhibitor cocktail Complete™ (Roche Diagnostics). The homogenate was centrifuged at 14 500  $\times$  g for 20 min. In all, 200  $\mu$ l of the supernatant was layered onto a 10 ml 10–50% sucrose gradient prepared in 15 mM Tris-HCl pH 7.5, 15 mM MgCl<sub>2</sub>, 300 mM NaCl, 1 mg/ml heparin, and centrifuged in a Beckman Ti-SW41 rotor for 2.5 h at 36 K at 4°C. UV absorbance was recorded at 254 nm and 0.5 ml fractions were collected. The fractions corresponding to preinitiation and initiation complexes (40/43/48S and 80S) and the ones corresponding to polysomes were pooled.

**Quantitative real-time RT-PCR.** Pooled fractions of preinitiation/initiation complexes or polysome-bound mRNAs were digested with proteinase K (150  $\mu$ g/ml) in the presence of 1% SDS for 30 min at 37°C. The digestion was adjusted to sodium acetate 0.3 M and the RNA precipitated with ethanol. The RNA pellet was dissolved in H<sub>2</sub>O and digested with RNase-free DNase I to prevent any contamination with genomic DNA, further purified using the RNeasy Mini Kit, and quantified by spectrophotometry. RNA (100 ng) was used for quantitative real-time RT-PCR using the QuantiTect SYBR Green RT-PCR kit (Qiagen) in a DNA Engine Opticon System (M. J. Research Inc.). Sequence-specific 25-mer oligonucleotides for *Drosophila actin5C*, *hsp70*, *hid*, *sickle*, *grim* and *rpr* mRNAs were designed to amplify 100 bp fragments.

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