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eIF4EHP promotes Ldh mRNA translation in and fruit fly adaptation to hypoxia

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Abstract

Hypoxia induces profound modifications in the gene expression program of eukaryotic cells due to lowered ATP supply resulting from the blockade of oxidative phosphorylation. One significant consequence of oxygen deprivation is the massive repression of protein synthesis, leaving a limited set of mRNAs to be translated. *Drosophila melanogaster* is strongly resistant to oxygen fluctuations; however, the mechanisms allowing specific mRNA to be translated into hypoxia are still unknown. Here, we show that *Ldh* mRNA encoding lactate dehydrogenase is highly translated into hypoxia by a mechanism involving a CA-rich motif present in its 3′ untranslated region. Furthermore, we identified the cap-binding protein eIF4EHP as a main factor involved in 3′UTR-dependent translation under hypoxia. In accordance with this observation, we show that eIF4EHP is necessary for *Drosophila* development under low oxygen concentrations and contributes to *Drosophila* mobility after hypoxic challenge. Altogether, our data bring new insight into mechanisms contributing to LDH production and *Drosophila* adaptation to oxygen variations.

Keywords 3′ untranslated region; development; gene expression; lactate dehydrogenase; translation
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Introduction

Adaptation to variations in oxygen concentration is a conserved mechanism in all metazoans, as this process is central for maintaining cell and tissue homeostasis (Samanta *et al*, 2017). At the cellular level, exposure to hypoxia induces a significant reprogramming of gene expression to cope with the energy decrease resulting from the inhibition of oxidative phosphorylation (Nakayama & Kataoka, 2019). Two major conserved processes contribute to hypoxia-induced gene reprogramming. The first relies on the transcriptional activation of gene expression mainly controlled by the Hypoxia Inducible Factors (HIFs), leading to the upregulation of a large panel of genes (Dengler *et al*, 2014). The second one corresponds to a profound modification of the translation program combining a marked decrease in global protein synthesis to limit energy demand with selective production of proteins involved in the hypoxic response (Fähling, 2009). While the mechanisms underlying HIF-dependent transcriptional activation have been well characterized (Lee *et al*, 2020), the ones governing translation reprogramming are only partially understood (Chee *et al*, 2019; Ho *et al*, 2021).

Mechanisms that allow mRNAs to be translated during hypoxia include cap-independent recruitment of ribosomes through Internal Ribosomal Entry Sites (IRES). However, although hypoxia is frequently described as up-regulating hundreds of genes in various cell types, only a limited number of transcripts are known to carry *bona fide* IRES (Morfoisse *et al*, 2014; Webb *et al*, 2015; Hantelys *et al*, 2019). Besides, only few alternative mechanisms mediated by uORF (Barbosa & Romão, 2014) or by selective partitioning of mRNA to the endoplasmic reticulum (ER; Staudacher *et al*, 2015) have been described to permit selective mRNAs translation at low O_2.

Messenger RNA translation initiated by recognition of the 5′cap by eIF4E1 and binding of eIF4G1 and the DEAD box ATP-dependent RNA-helicases eIF4A/B is considered as a reference translational model (Rissland, 2017) while alternative translational initiation complexes have also been described (Borden & Volpon, 2020). Particularly, eIF4E2 and its metazoan homologs are well-described suppressors of cap-dependent mRNA translation but were also shown to mediate mRNA-specific translational activation (Uniacke *et al*, 2012; Jeong *et al*, 2019). In the human renal cell line 786-O, Lee's laboratory has described a RNA-binding complex formed by RBM4, HIF2α, eIF4E2, and eIF4G3 controlling mRNA translation

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under hypoxic conditions through interactions involving mRNA 3′UTR (Uniacke *et al*, 2012). This work has opened new perspectives in understanding the control of protein production in hypoxia. However, HIF2α, a central component of this mechanism, is not conserved in invertebrates such as *Drosophila* (Marygold *et al*, 2017), where hypoxic translation has been described (Deliu *et al*, 2017). Therefore, our understanding of mechanisms promoting hypoxic translation in metazoans remains partial.

Both *Drosophila* larvae and adult flies as well as *Drosophila* S2 cells were shown to be highly resistant to low O_2 concentrations, making this model organism very attractive to study hypoxic translation (Zhou *et al*, 2009; de Toeuf *et al*, 2018). We previously identified *Ldh* encoding lactate dehydrogenase as a highly regulated gene upon oxygen variations in S2 cells (de Toeuf *et al*, 2018). In the present study, we characterized the mechanism promoting translation of *Ldh* mRNA under hypoxic conditions in *Drosophila*. We show that *Ldh* mRNA is efficiently translated under hypoxia despite a strong blockade of protein synthesis. Using a reporter gene approach, we demonstrate that *Ldh* mRNA 3′UTR is sufficient to promote reporter mRNA association with polysomes and translation under hypoxia. We further identified a CA-rich motif as a *cis*-acting element promoting mRNA recruitment on polysomes under hypoxic conditions. We show that the cap-binding translation initiation factor eIF4EHP, the homolog of human eIF4E2 is necessary for the 3′ UTR-dependent translation of *Ldh* mRNA under hypoxia. In accordance with these observations, we found a significant fraction of eIF4EHP to be associated with polysomes and to be excluded from stress granules and P-bodies where most mRNAs are concentrated upon hypoxia. Finally, we demonstrated that eIF4EHP is necessary for *Drosophila* development under hypoxic conditions and strongly influences adult fly mobility upon oxygen recovery.

Altogether, our data reveal a new mechanism promoting mRNA alternative translation upon general inhibition of protein synthesis in *Drosophila* and identify eIF4EHP as a major component of the alternative translation machinery which contributes to *Drosophila* adaptation to oxygen variations.

Results

Ldh mRNA is efficiently translated under hypoxia by a 3′UTR-dependent mechanism

Increased LDH production is a common feature of oxygen-deficient animal cells. LDH catalyzes the reduction of pyruvate to lactate necessary to NAD^+ regeneration and plays a central role in shifting the cellular metabolism from oxidative phosphorylation to lactic glycolysis upon reduction of oxygen availability (Semenza, 2014).

In *D. melanogaster*, *Ldh* (also referred as *ImpL3*) is the unique gene coding for the Lactate Dehydrogenase enzyme. We analyzed the production of LDH in *Drosophila* S2 cells after exposure to variable concentrations of atmospheric oxygen. While hypoxia induces a strong reduction of protein synthesis (Fig 1A and B), it markedly stimulates LDH production (Fig 1C). Polysome profiling analysis confirmed that *Ldh* mRNA is largely found in polysomal fractions under hypoxic conditions in contrast to mRNA encoding ribosomal protein RPL32 whose polysomal abundance is decreased in hypoxia (Fig 1D and E). Therefore, *Ldh* mRNA is efficiently translated into S2 cells exposed to a hypoxic environment while global protein synthesis is strongly repressed under these conditions.

We previously demonstrated that AU-rich sequences localized in *Ldh* mRNA 3′UTR control the stability of this messenger RNA in normoxic conditions (de Toeuf *et al*, 2018). Reporter gene analysis was performed to evaluate the impact of 3′UTR region in controlling *Ldh* expression in hypoxic S2 cells. Constructs containing the Firefly luciferase (Fluc) coding sequence under control of the *Ldh* gene promoter and flanked by different 3′UTR were co-transfected in S2 cells with a Renilla luciferase (Rluc) normalizing reporter plasmid. Quantification of relative luciferase activities after exposure to 1% O_2 for 24 h reveals a significant increase in Fluc production from reporter mRNA containing the *Ldh* 3′UTR as compared to SV40 3′UTR (Fig 2A). Reporter mRNAs accumulate at comparable levels in these cells (Fig 2B), suggesting that *Ldh* 3′UTR promotes mRNA translation in hypoxia.

Ldh mRNA 3′UTR contains a 32 nucleotide-long CA-rich region upstream of the previously identified AU-rich element (Fig 2C). The concomitant presence of AU-rich and CA-rich sequences in 3′UTR was previously described in mammalian *VEGFA* mRNA (Ray & Fox, 2007), the CA-rich element being necessary to promote *VEGFA* expression under hypoxic conditions (Ray *et al*, 2009). We, therefore, analyzed whether the CA-rich motif present in *Ldh* mRNA 3′UTR could play a similar role. As shown in Fig 2A, deletion of the CA-rich motif from the luciferase reporter gene containing the *Ldh* mRNA 3′UTR leads to a decrease of Fluc activity as compared to the full-length 3′UTR. We next analyzed reporter mRNA association with the translation machinery by polysome profiling experiments in stably transfected cells. As shown in Fig 2D and E, while *Fluc* mRNA containing SV40 3′UTR is dissociated from polysomes upon hypoxia, *Fluc* mRNA with *Ldh* 3′UTR remains strongly associated with polysomes in that condition. Removal of the CA-rich sequence from *Ldh* 3′UTR strongly reduces *Fluc* mRNA association to polysomes in hypoxic cells, further confirming the importance of this motif for hypoxic translation. To evaluate whether CA-rich elements are sufficient to induce mRNA recruitment in polysomes under hypoxic conditions, S2 cells were transfected with a luciferase reporter gene containing multiple copies of the CA-rich region from *Ldh* mRNA inserted in SV40 3′UTR. As shown in Fig 2D and E, this reporter mRNA is associated with polysomes in hypoxia. Altogether, these results indicate that the 3′UTR of *Ldh* mRNA counteracts the general translational blockade imposed by hypoxic stress and is sufficient to maintain an efficient translation rate of *Ldh* mRNA under low oxygen concentration. Moreover, the CA-rich region present in *Ldh* mRNA 3′UTR is essential to mediate this effect.

eIF4EHP promotes the 3′UTR-dependent translation of *Ldh* mRNA in hypoxia

The *Drosophila* eIF4E1-related protein eIF4EHP and its mammalian homolog eIF4E2 were primarily described as translational repressors of several mRNAs. However, eIF4E2 has also been reported to specifically activate mRNA translation in different cellular contexts including hypoxia (reviewed in Christie & Igreja, 2021).

Interestingly, hypoxic treatment induces an increase of eIF4EHP detected in both head (not shown) and entire body extract (Fig 3A) of adult *Drosophila*. We therefore investigated the impact of eIF4EHP on translation in hypoxic conditions.

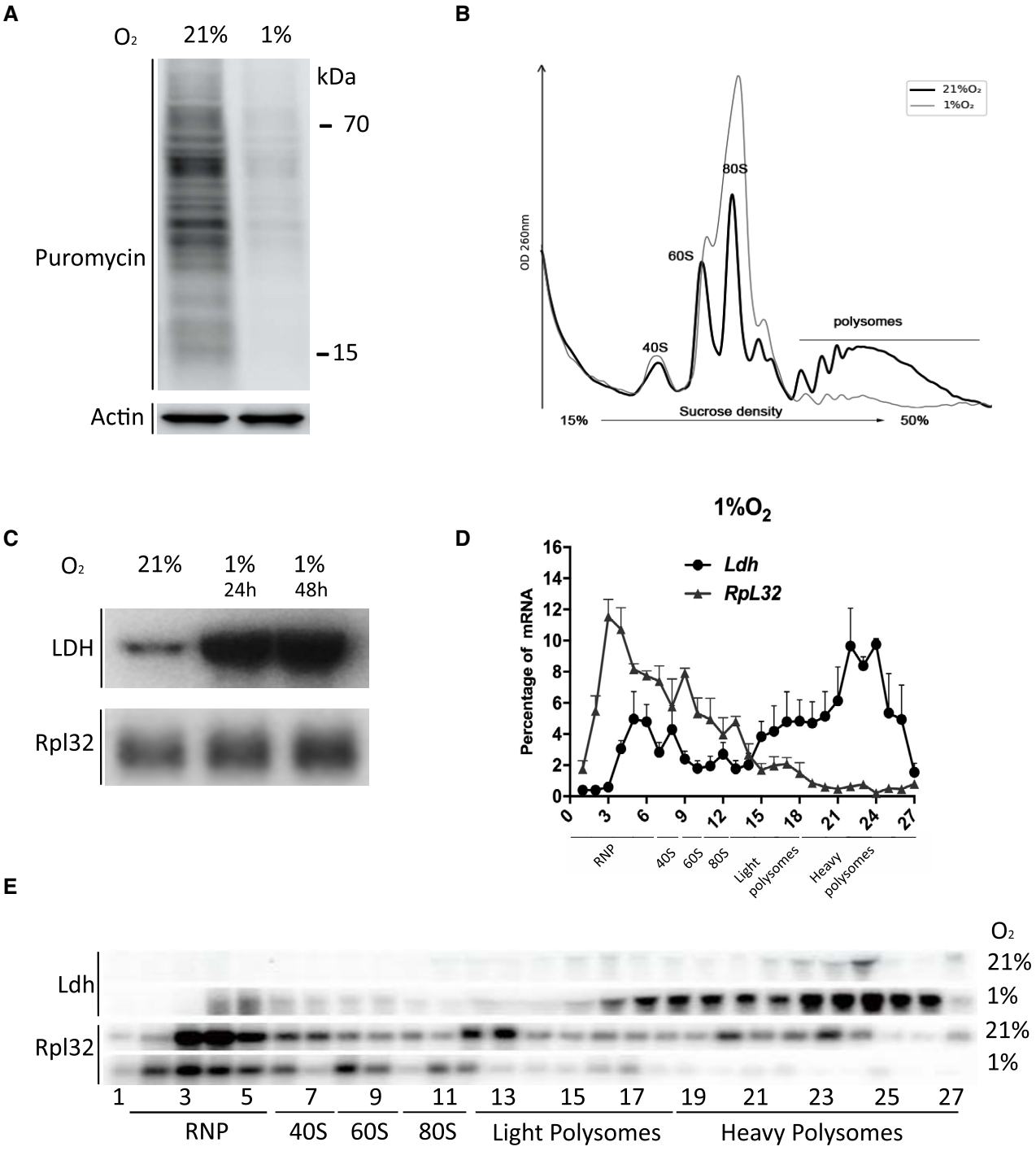

Figure 1. Ldh mRNA is efficiently translated under hypoxia.

S2 cells were incubated at 21 or 1% O_2 concentrations for 24 h.

A Puromycin was added 20 min before cell harvesting. Total puromycin incorporation and Actin level were revealed by western blot.

B Polysome profiles (OD 260 nm) from normoxic (black line) or hypoxic (gray line) cell extracts. Positions of 40S, 60S, 80S, and polysomal peaks are indicated.

C LDH and RpL32 protein levels were detected by western blot in total cell extracts from S2 cells exposed to normoxic conditions or to 1% O_2 environment for 24 and 48 h.

D *Ldh* and *Rpl32* mRNA levels in fractions from polysome profiling experiments were analyzed by northern blotting using ^{32}P-labeled probes. Fractions numbers from top to bottom of the gradient are indicated. Experiment was repeated and quantified; data are shown as mean (n = 3) ± SD.

E One representative experiment performed as in (D) is shown.

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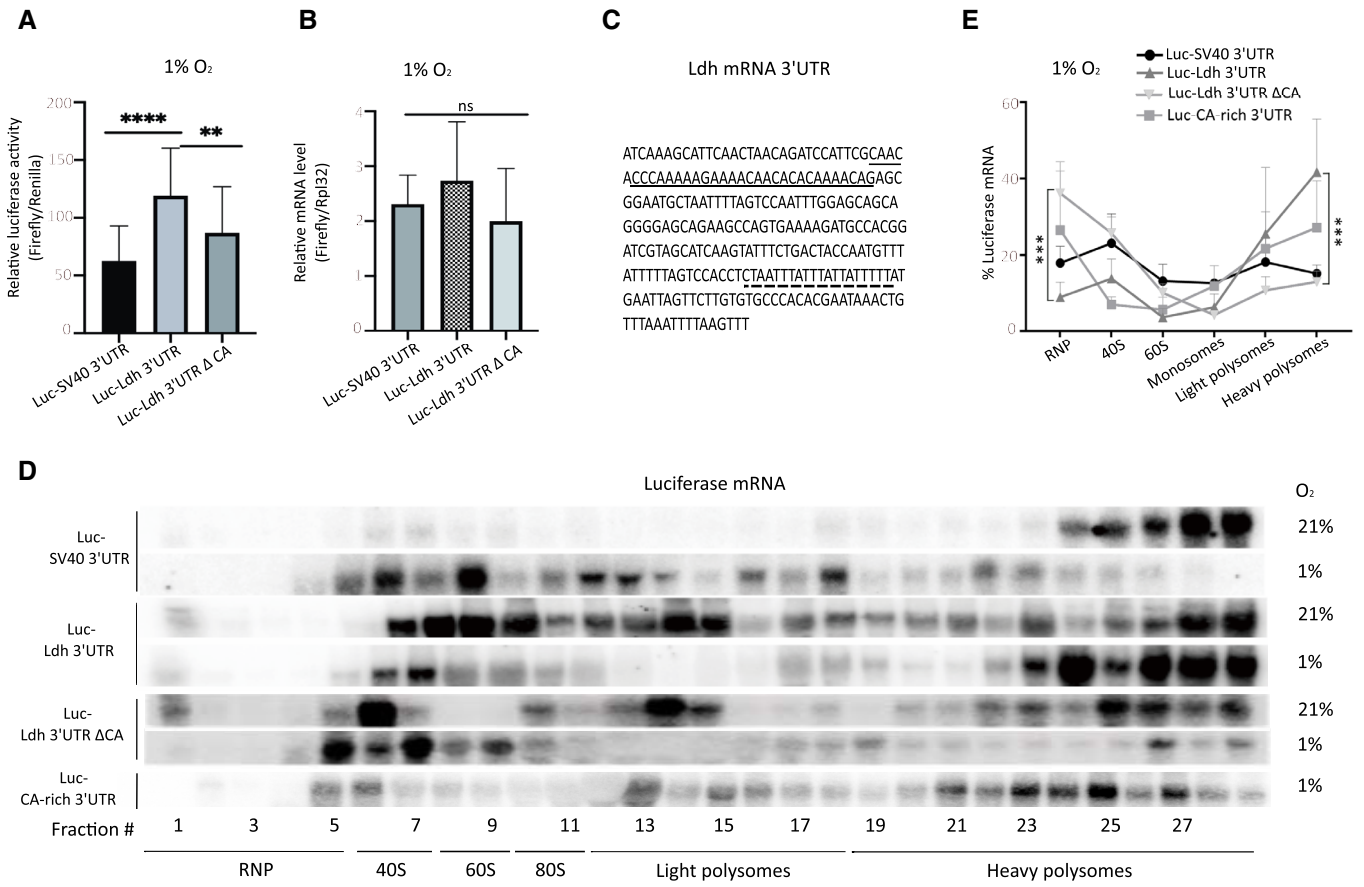

Figure 2. Ldh mRNA 3′UTR and CA-rich motifs enhance translation under hypoxic conditions.

S2 cells were transiently transfected with the indicated constructs and exposed to 1% O_2 for 24 h.

A, B (A) Activities of Firefly (Fluc) and Renilla (Rluc) luciferase, produced from reporter genes under control of the Ldh gene promoter, were measured in total cell extracts by dual luciferase assay, and the ratio of both measures was calculated for each transfection. (B) Relative FLuc and RLuc mRNA levels were measured by RT–qPCR. Values represent mean ± SD, $n = 3$ biological replicates, P-value of one-way ANOVA, $^{****}P \leq 0.001$, $^{**}P \leq 0.01$, $^{ns}P > 0.05$.

C Nucleotide sequence of *Ldh* mRNA 3′UTR. The CA-rich and AU-rich sequences are underlined (solid and dashed line, respectively).

D Indicated stably transfected cell lines were exposed to 21 or 1% O_2 for 24 h before fractionation of cell extracts by ultracentrifugation on linear sucrose gradients (15–50%). The distribution of FLuc mRNA, produced from reporter genes under control of the pMT promoter, was measured by northern blot in each cell types. Fractions numbers from top to bottom of the gradient are indicated.

E Signals from three independent experiments performed in 1% O_2 conditions were quantified, and relative signal in each fraction is shown as mean ± SD. P-value of two-way ANOVA, ($^{***}P \leq 0.001$) are shown for *Luc-Ldh* 3′UTR vs *Luc-Ldh* 3′UTR ΔCA samples in RNP and heavy polysome groups.

Source data are available online for this figure.

Drosophila eIF4EHP null mutants are not viable (Valzania *et al*, 2016). We therefore evaluated the impact of eIF4EHP depletion on LDH production under hypoxic stress by RNAi-mediated knock-down (KD). Adult flies ubiquitously expressing an *eIF4EHP*-RNAi-controlled by a Tub-Gal4 (Tub-Gal4/UAS-eIF4EHP_RNAi) driver were exposed to normoxic (21% O_2) or hypoxic (1% O_2) conditions, and LDH was detected by western blot. Control flies (Tm3/UAS-eIF4EHP_RNAi) were exposed to the same conditions. As shown in Fig 3A and B, LDH accumulates after 24 h exposure to 1% O_2 in control flies in the absence of RNAi expression or in flies expressing a mCherry RNAi controlled by a Tub-Gal4 driver (Tub-Gal4/UAS-m-Cherry_RNAi) (not shown). In contrast, LDH level is specifically and strongly reduced in *eIF4EHP RNAi*-expressing flies. We also observed a significant reduction of LDH production in *eIF4EHP*-KO S2 cells as compared to control cells (*Yellow* KO) (Fig 3C and D). A slight reduction in LDH level was also observed in extract from *eIF4EHP* KD flies expose to normoxic environment as compared to controls (Fig 3B). This could result from a basal production of LDH in tissues exposed to suboptimal concentration of oxygen in a normoxic environment. We observed that the association of *Ldh* mRNA to polysomes is markedly reduced in *eIF4EHP*-KO S2 as compared to control cells. Of note, inactivation of *eIF4E6*, another cap-binding factor of the eIF4E family expressed in S2 cells and unable to interact with 4E-BPs and eIF4G1, does not influence *Ldh* mRNA association to polysomes under hypoxia (Fig 3E and F). Finally, we compared puromycin incorporation under normoxic and hypoxic conditions in wild type, *eIF4EHP*-KO cell lines and Cas9 (empty vector) control S2 cells. While under normoxic conditions, similar levels of puromycin incorporation are detected in the 3 cells lines, under hypoxic conditions, puromycin incorporation is

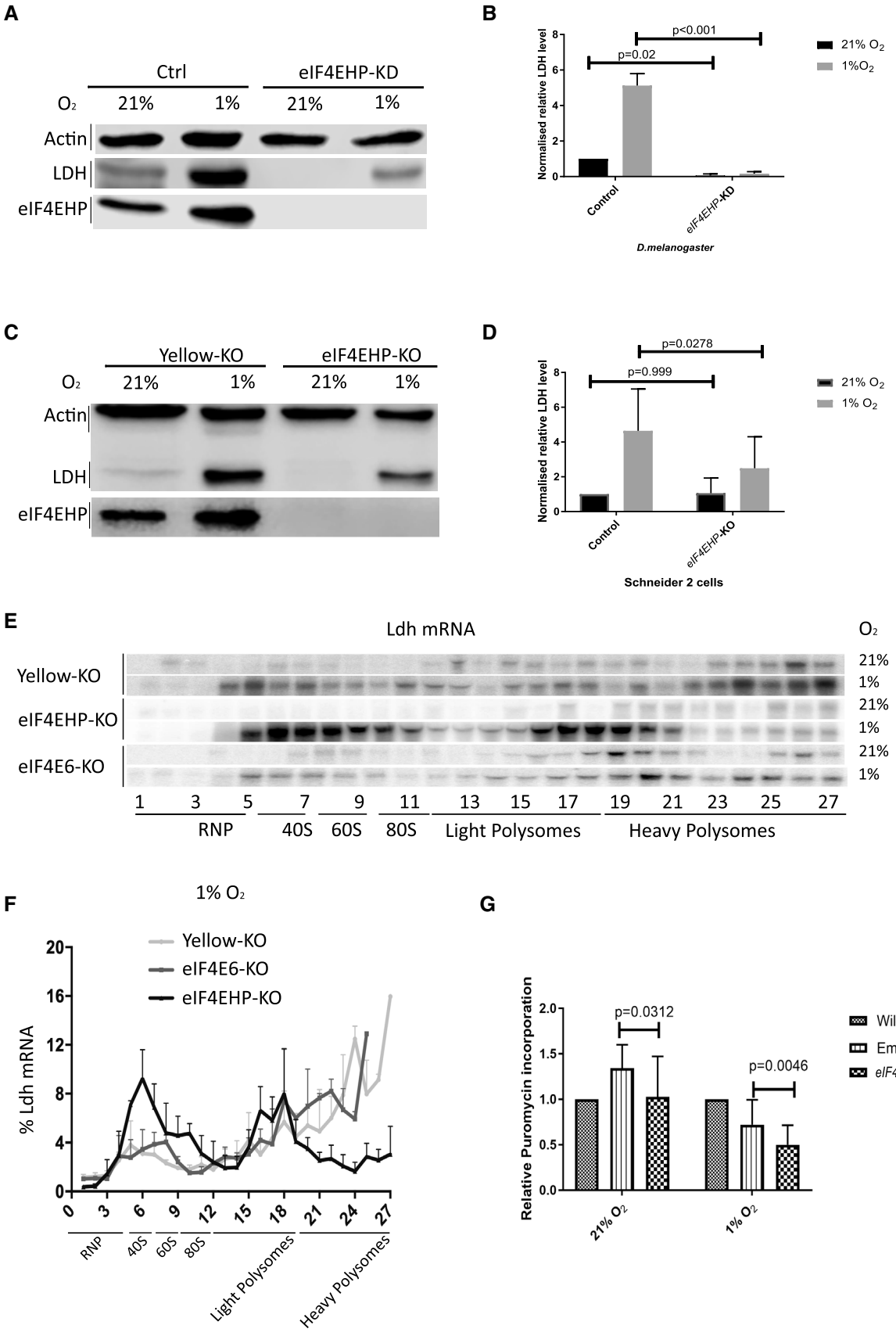

Figure 3.

Figure 3. Translational control of LDH production by eIF4EHP in adult flies and S2 cells under hypoxia.

A Control (TM3 > UAS-eIF4EHP_RNAi) or *eIF4EHP* KD (Tub-GAL4 > UAS-eIF4EHP_RNAi) flies were maintained in either normoxia or hypoxia (1%O_2, 24 h). Protein extracts were prepared from whole flies and eIF4EHP, LDH, and Actin were detected by western blot.

B Quantification of LDH/Actin ratio in three independent experiments performed as in (A). Values represent mean ± SD, $n = 3$ biological replicates, *P*-value of two-way ANOVA.

C S2 cell lines inactivated for *Yellow* or *eIF4EHP* genes were established by a CRISPR-Cas9 strategy as described in the Materials and Methods section. After exposure to normoxic or hypoxic conditions, cells were lysed and Actin, LDH, and eIF4EHP protein levels were detected by western blot.

D Quantification of LDH/Actin ratio in six independent experiments performed as in (C). Values represent mean ± SD, *P*-values of two-way ANOVA.

E Cell extracts from *Yellow*, *eIF4EHP*, or *eIF4E6* KO cells incubated at 21 or 1% O_2 were separated by centrifugation on linear sucrose gradients and *Ldh* mRNA was detected by northern blot in the different fractions. Fractions numbers from top to bottom of the gradient are indicated.

F The mean relative value (± SEM) of *Ldh* mRNA in fractions from three independent experiments performed at 1% O_2 as in (E) is shown. Fractions numbers from top to bottom of the gradient are indicated.

G Indicated S2 cell lines were cultivated at 1% O_2 for 24 h and labeled with puromycin (5 μg/ml) for 20 min before harvesting. Puromycin signal in total cell extracts was quantified by western blot and expressed relative to the level detected in wild-type cells ($n = 6$ biological replicates, *t*-test, two-sided).

Source data are available online for this figure.

significantly reduced in *eIF4EHP*-KO S2 cells as compared to wild type and Cas9 control cells (Fig 3G).

As 3′UTR is required for efficient translation of *Ldh* mRNA in hypoxia, we evaluated whether this 3′UTR-mediated mechanism was dependent on eIF4EHP. As shown in Fig 4A and B, the association to polysomes, under hypoxic conditions, of reporter Fluc mRNAs containing either the *Ldh* 3′UTR or the CA-rich motif is disrupted in *eIF4EHP*-deficient cells where most of the reporter mRNA is shifted in non-polysomal fractions. This observation points to a positive effect of eIF4EHP on 3′UTR-dependent translation under hypoxia mediated by CA-rich motifs. To confirm that eIF4EHP specifically controls the expression of the luciferase reporter gene containing *Ldh* 3′UTR, we overexpressed in WT or *eIF4EHP*-KO S2 cells a construct expressing either the *D. persimilis* eIF4EHP protein or GFP as control. The low degree of conservation between *D. melanogaster* and *D. persimilis eIF4EHP* coding sequences in the region targeted by the sgRNA used to establish the KO cells allows the efficient production of *D. persimilis* eIF4EHP in both KO and control cells (Fig 4C). Cells were transiently co-transfected with a *Fluc-Ldh* 3′UTR reporter plasmid and a *Rluc* normalizing plasmid and were further incubated in hypoxia for 24 h. We observed an increased Fluc activity in presence of *D. persimilis* eIF4EHP as compared to GFP both in *eIF4EHP*-KO and WT S2 cells (Fig 4D). Similar levels of Fluc mRNA were detected in cells expressing GFP or *D. persimilis* eIF4EHP (Fig 4E), thereby confirming a translation activating effect of *D. Persimilis* eIF4EHP on Fluc expression. Taken together, these data demonstrate that translation mediated by *Ldh* 3′ UTR and CA-rich motifs in hypoxia requires eIF4EHP.

eIF4EHP is enriched in cytoplasmic foci distinct from SG and P bodies in hypoxia

Hypoxic stress is an inducer of stress granule (SG) assembly in both human and *Drosophila* cells (Gottschald *et al*, 2010; van der Laan *et al*, 2012). Upon stress, polyadenylated mRNAs accumulate in SG and are mostly excluded from the translational pool (Mateju *et al*, 2020; Riggs *et al*, 2020). Several members of the eIF4E family were previously shown to relocalize to SG upon cellular stress. However, eIF4E2, the mammalian homolog of eIF4EHP, displays a variable subcellular localization depending on cellular stress types (Frydryskova *et al*, 2016). We investigated the subcellular localization of eIF4EHP and mRNA in response to hypoxic stress. Upon hypoxia, polyadenylated mRNAs massively relocalize to cytoplasmic structures compatible with the previously described concentration of mRNA in SG (Fig 5A, right panel). As expected, *Ldh* mRNA detection is increased after hypoxic treatment, but the cytoplasmic distribution of *Ldh* mRNA remains more diffuse as compared to the bulk of polyadenylated mRNAs (Fig 5A, left and middle panels).

In normoxia, eIF4HP has a heterogeneous distribution in the cytoplasm of S2 cells that is further enhanced after exposure to hypoxia (Fig 5B and C). The combined detection of eIF4EHP and polyA$^+$ RNA confirmed that hypoxic treatment increased the granular distribution of eIF4EHP and mRNAs in the cytoplasmic compartment. However, co-localization analyses revealed that these markers remain separately distributed under hypoxia (Fig 5C). To further characterize the nature of the eIF4EHP-containing foci, we performed co-localization experiments with ectopically expressed GFP-Rox8 and GFP-Dcp1 fusion proteins which are *bona fide* markers of stress granules and P-bodies (PB), respectively (Farny *et al*, 2009). As shown in Fig 5D (left panels), GFP-Rox8 is homogeneously distributed in the cytoplasm of S2 cells in normoxia and accumulates in cytoplasmic granules upon hypoxic or arsenite treatments while eIF4EHP remains mostly excluded from these structures. Similarly, PB identified by GFP-Dcp1 labelling displayed very partial co-localization with eIF4EHP in all tested conditions (Fig 5D, right panels). Altogether, these data indicate that in hypoxia, eIF4EHP does not colocalize with the bulk of mRNAs and accumulates in cytoplasmic foci distinct from SG and PB.

eIF4EHP is associated with polysomes and binds to *Ldh* mRNA under hypoxia

To further investigate the function of eIF4EHP in translation under hypoxic conditions, we analyzed its distribution into polysomes by centrifugation on sucrose gradient. As shown in Fig 6A and B (left panels), a large proportion of eIF4EHP co-sediments with light fractions corresponding to ribonucleoprotein particles (RNP), ribosomal subunits (40S/60S), and assembled ribosomes (80S) in extracts from S2 cells and adult fly in normoxia. However, under hypoxia, an increased proportion of eIF4HP is found in polysomes in contrast to ribosomal RpS23 protein which shifts from polysomes to lighter fractions (right panels). These data suggest that eIF4EHP is recruited to the translating machinery under hypoxic conditions. To further demonstrate the direct role of eIF4HP in translation under hypoxia,

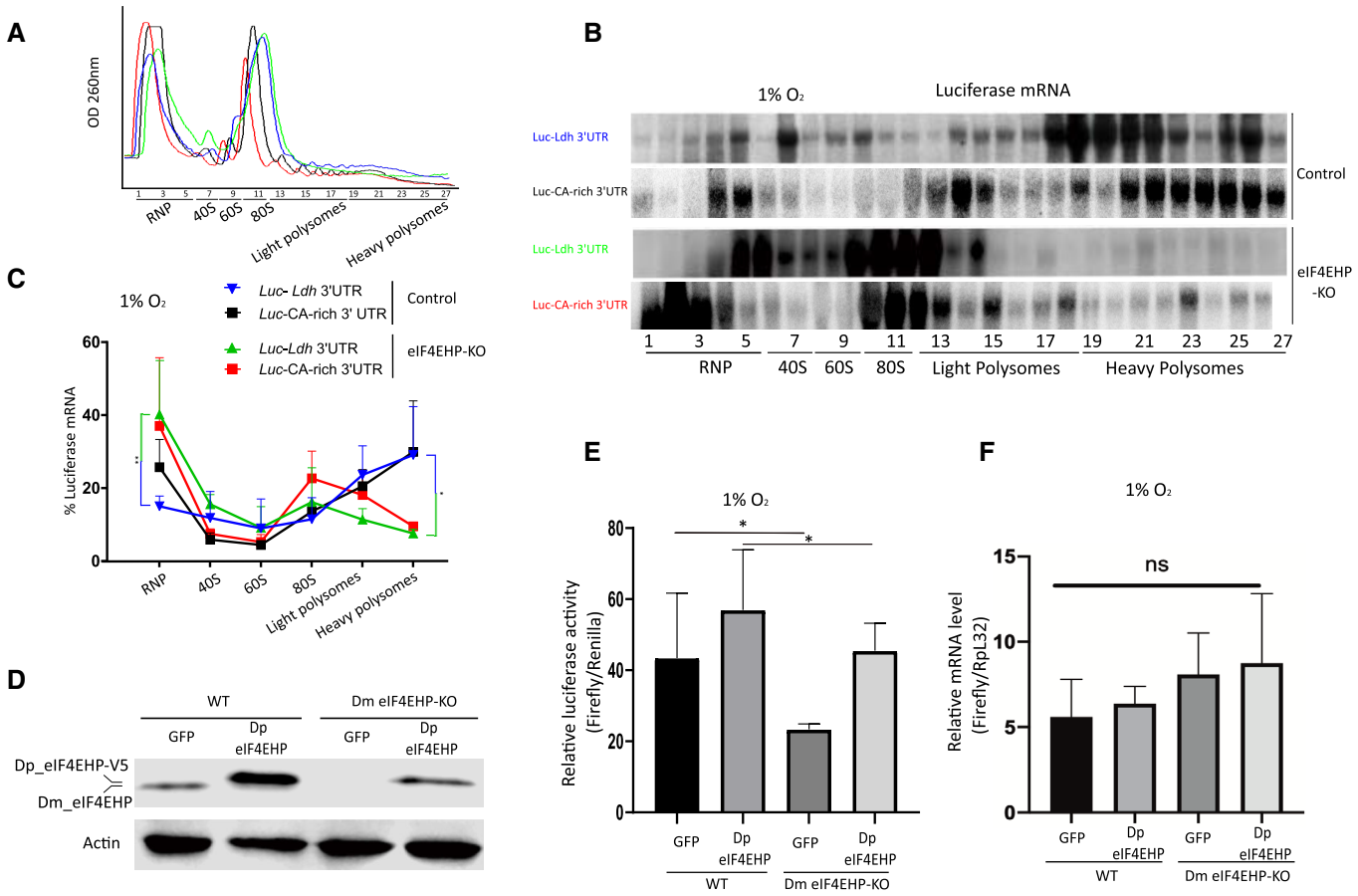

Figure 4. Ldh 3′UTR- and CA-rich-mediated translation under hypoxia requires eIF4EHP.

A Polysome profiles (OD 260 nm) from hypoxic cell extracts used in (B) and (C). Fractions numbers from top to bottom of the gradient and positions of 40S, 60S, 80S, light and heavy polysomes peaks are indicated.

B Polysome profiles of FLuc-Ldh 3′UTR and Fluc-CA-rich 3′UTR reporter mRNA in control (*Yellow* KO) and *eIF4EHP* KO S2 cells in hypoxia.

C Quantification of luciferase mRNA levels from three independent experiments performed at 1% O_2 as in (A). Values represent mean ± SD. *P*-value of two-way ANOVA (**$P \leq 0.01$, *$P \leq 0.05$) are shown for *Luc-Ldh* 3′UTR samples in RNP and heavy polysome groups.

D WT and *eIF4EHP* KO cells were co-transfected with plasmids encoding V5-tagged *D. persimilis* eIF4EHP (Dp eIF4EHP) or GFP, along with FLuc-Ldh3′UTR and RLuc reporters. eIF4EHP proteins were detected by western blot using eIF4EHP antibody (cross-reacting to Dp eIF4EHP).

E, F 24 h after transfection, cells were cultivated in hypoxia (1% O_2) for an additional 24 h before measurement of luciferase activity by dual luciferase reporter assay (E) and luciferase mRNA levels by qPCR (F). Values represent mean ± SD, $n = 3$ biological replicates, *P*-value of one-way ANOVA, *$P \leq 0.05$, $^{ns}P > 0.05$.

Source data are available online for this figure.

we tested its association with *Ldh* mRNA by crosslinking immunoprecipitation (CLIP). Protein extracts from hypoxia-treated WT and *eIF4EHP*-KO cells were immunoprecipitated with agarose anti-eIF4EHP-coated beads after UV crosslinking and the presence of *Ldh* and *Rpl32* (control) mRNA was analyzed in inputs and eluted fractions by RT–qPCR. As shown in Fig 6C, *Ldh* mRNA was enriched in the eluate from WT crosslinked extracts as compared to corresponding fractions of *eIF4EHP*-KO extracts. In contrast, no enrichment of *Rpl32* mRNA was detected in the eluate from immunoprecipitated extracts, indicating that eIF4EHP specifically interacts with *Ldh* mRNA in WT cells under hypoxia.

Taken together, these results indicate that eIF4EHP is involved in the direct recruitment of specific mRNA to the translation machinery under conditions where the global translation activity is strongly reduced.

eIF4EHP is required for development under hypoxic environment and for recovery from hypoxic exposure in *D. melanogaster*

Drosophila melanogaster has a high tolerance to oxygen deprivation both during developmental and adult stages (Harrison & Haddad, 2011; Callier *et al*, 2015). To evaluate the importance of eIF4EHP on hypoxia tolerance, flies carrying *UAS-eIF4EHP* or *UAS-mCherry* RNAi cassette were crossed with Tub-Gal4/Tm3. Two hours after egg laying, embryos were kept in normoxia or exposed to 6% O_2 environment until hatching. *eIF4EHP* knock-down (Tub-Gal4/UAS-RNAi) or control individuals (Tm3/UAS-RNAi) were sorted based on the expression of balancer phenotypical markers. In normoxia, the number of *eIF4EHP* KD individuals exceeded the number of control individuals bearing a balancer chromosome. This difference could be explained by the general reduced

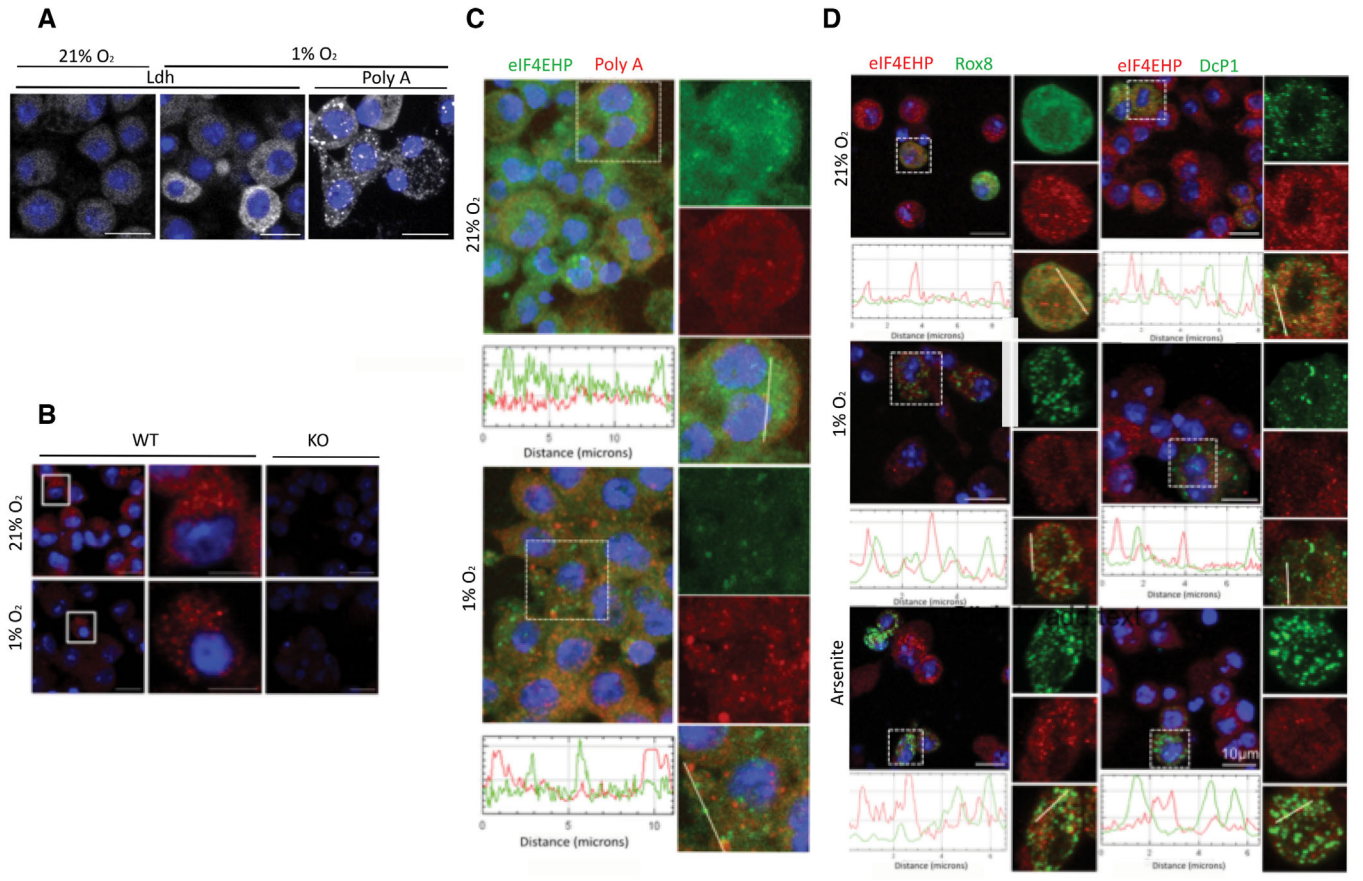

Figure 5. eIF4EHP accumulates in cytoplasmic granules distinct from p-Bodies and stress granules in hypoxia.

A WT S2 cells were cultivated in normoxia (21% O_2) or hypoxia (1%, 24 h) before analysis by single molecule FISH to detect *Ldh* mRNA (left and middle panels) or Oligo dT FISH to detect polyadenylated mRNAs (right panel).

B Immunofluorescent detection of eIF4EHP in WT or *eIF4EHP* KO cells cultivated at 21 or 1% O_2 (24 h) using anti-eIF4EHP monoclonal antibody (see Materials and Methods).

C Combined detection of eIF4EHP by immunofluorescence (green) and polyA mRNA by oligo-dT FISH (red) in normoxic and hypoxic WT S2 cells.

D Combined detection of GFP-Rox8 or GFP-Dcp1 (green) and endogenous eIF4EHP (red) in transfected S2 cells cultivated in normoxia (21% O_2), in hypoxia (1% O_2 for 24 h) or after oxidative stress (250 µM arsenite for 1 h). Representative images of confocal imaging (Z-stack maximum intensity projection) and zooms are shown.

Data information: In all experiments, nuclei were counterstained with DAPI (blue). Red and green signal intensities were quantified with the Zen software (Zeiss).
Source data are available online for this figure.

transmissibility of balancer over WT chromosomes (Ashburner *et al*, 1989). As expected, under hypoxia, a global reduction of eclosion rate is observed independently of the genotype. However, the number of *eIF4EHP* KD individuals was strongly reduced as compared to controls with a more pronounced effect upon maternal transmission of the *eIF4EHP*-RNAi allele for which no *eIF4EHP* KD individuals were detected (Fig 7A).

Exposure to hypoxic conditions alters a large panel of physiological parameters in *Drosophila* (Zhou & Haddad, 2013). Adult flies can survive to strong but transient reduction of O_2 concentration but show impaired mobility during the recovery period (Habib *et al*, 2021). We tested the effect of eIF4EHP depletion on this recovery of mobility after hypoxia. Adult *eIF4EHP*-KD or control flies were exposed to a 1% O_2 environment for 24 h before returning to normoxic conditions. The mobility capacity of flies was assessed by a negative geotaxis test 40 min after return to normoxia. All fly strains show comparable mobility when kept in normoxic conditions (Fig 7B, all panels). *eIF4EHP* KD flies show a strong reduction of mobility during the recovery period with no fly reaching the preset climbing limit during the time of the assay (Fig 7B, upper left panel). This defect in mobility was totally recovered 48 h after the hypoxic period (Fig 7B, upper right panel). Control flies which do not express *eIF4EHP* RNAi have a reduced mobility after 40 min of recovery in normoxia but not to a comparable extent to that observed for *eIF4EHP* KD flies. Finally, expression of a control *mCherry* RNAi does not alter the recovery of the flies from hypoxic exposure as illustrated in Fig 7B (lower panel). Altogether, these data show that eIF4EHP is important for the development of *D. melanogaster* under low oxygen concentrations and for adult flies to recover from hypoxic exposure.

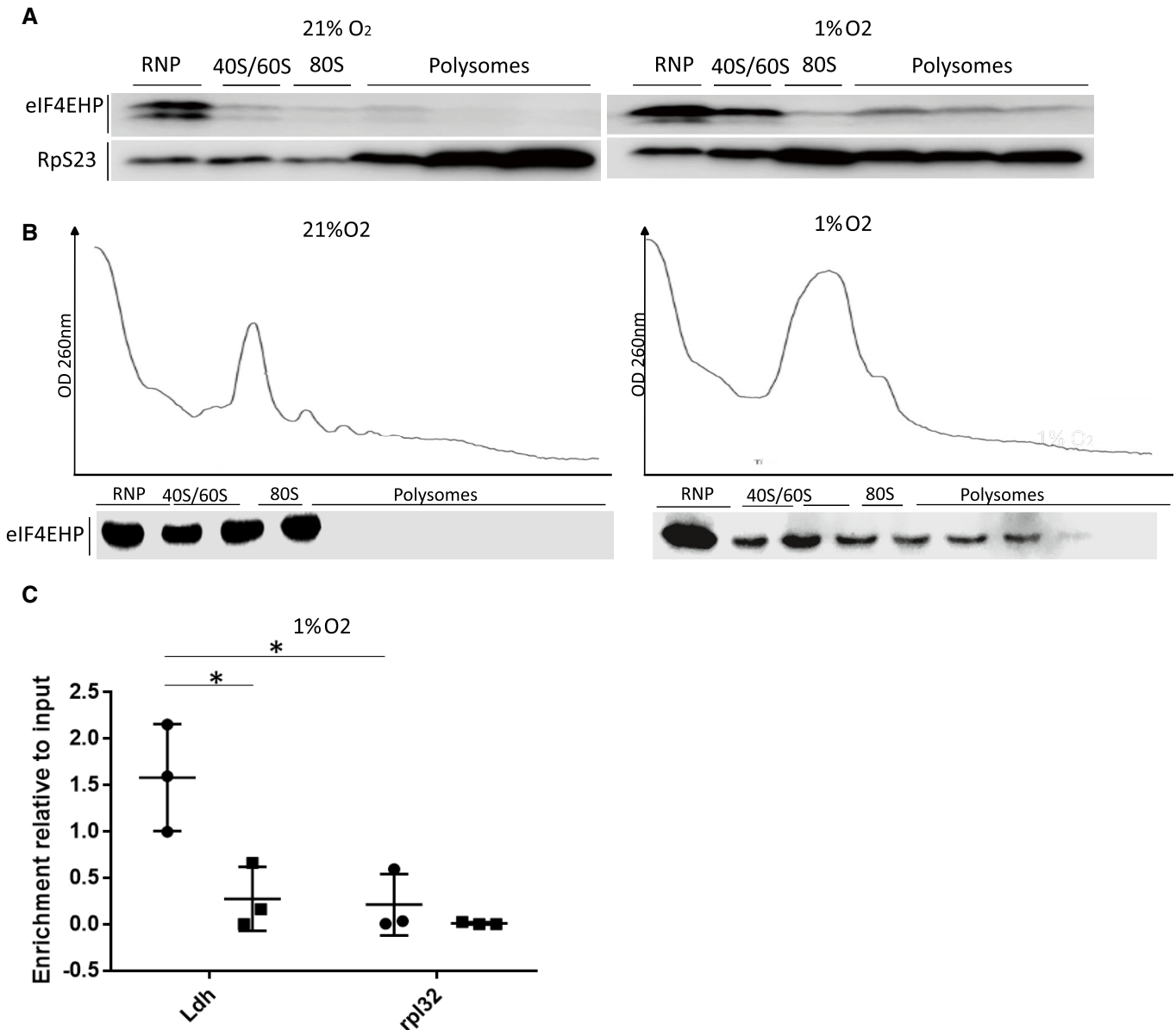

Figure 6. eIF4EHP is recruited to polysomes and interacts with *Ldh* mRNA in hypoxia.

A Cell extracts from normoxic or hypoxic S2 cells were ultracentrifuged as described in Fig 1B, protein extracts from pooled fractions corresponding to the same ribosomal content were analyzed by western blot with anti-eIF4EHP and RpS23 antibodies.

B Adult Canton S flies were kept at 21 or 1% O_2 for 24 h. Total protein extracts were prepared as described in the method section and fractionated by ultracentrifugation on sucrose gradients (15–50%). OD at 260 nm was measured in line during fractionation (upper panels). EIF4EHP was detected by western blot in representative fractions of the gradients.

C Cross-Linking and Immunoprecipitation experiment (CLIP) was performed on cell extracts from normoxic and hypoxic (1% O_2, 24 h) UV irradiated WT or *eIF4EHP* KO S2 cells. eIF4EHP was immunoprecipitated with eIF4EHP antibody-coated protein G sepharose beads before *Ldh* and *Rpl32* mRNA detection by RT-qPCR in total extract (input) or immunoprecipitation eluate. mRNA enrichment levels in eluate as compared to input from three independent experiments were calculated as described in (Fuentes-Iglesias *et al*, 2020). Data represent mean ± SD. Statistical significance was tested by two-way ANOVA followed by Sidak's multiple comparison test, $*P < 0.05$.

Source data are available online for this figure.

Discussion

Oxygen is the final acceptor of the respiratory chain ensuring high yield of ATP production in eukaryotic cells. Upon oxygen reduction, cells adapt to lowered ATP levels by simultaneously reprogramming gene expression and reducing ATP demand. Gene reprogramming enables cells to modulate negatively or positively the expression of genes, several of which sustain the metabolic shift to elevated lactic glycolysis as an alternative ATP-producing pathway. Concomitantly, major energy-consuming processes including protein synthesis are

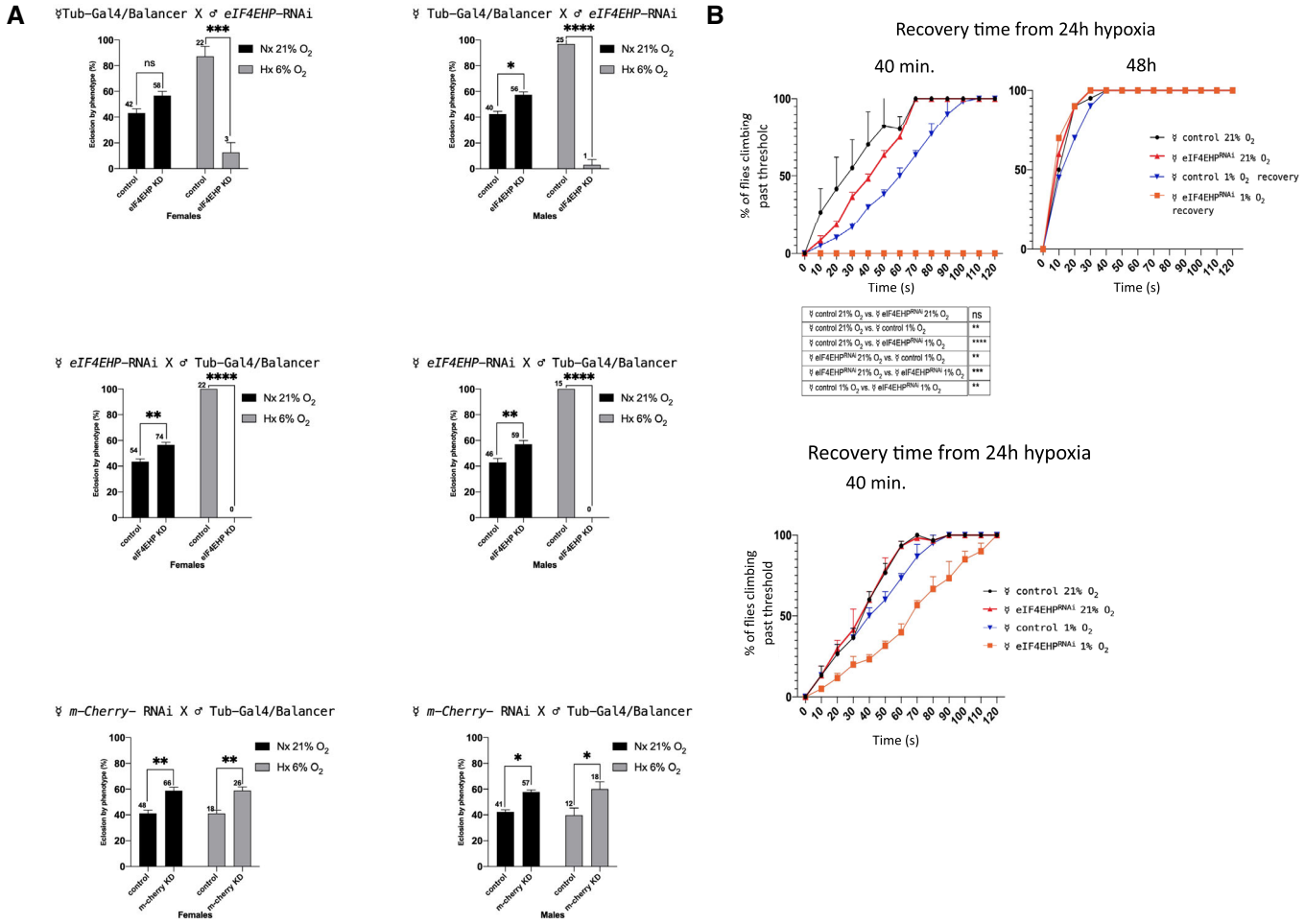

Figure 7. Role of eIF4EHP in development under hypoxia and mobility recovery after hypoxia exposure in *D. melanogaster*.

A Female or male flies with chromosomal insertions of an UAS_eIF4EHP (BDSC 36876) RNAi construct were crossed with male or female Tub > Gal4/Tm3 (BDSC 5138) driver flies. Two hours after mating, embryos were transferred to 6% O_2 (lowest tolerated O_2 concentration) or kept in normoxia until eclosion. Tub-Gal4/UAS-eIF4EHP_RNAi and Tm3/UAS-eIF4EHP_RNAi F1 individuals were counted based on the presence (control) or absence (eIF4EHP KD) of the Tm3 SB marker and results were expressed as % of total eclosions for each condition. Data represent mean ± SD from two independent biological replicates. Statistical significance was tested by two-way ANOVA followed by Sidak's multiple comparisons test. ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

B Mobility of age-matched Tub-Gal4/UAS-eIF4EHP_RNAi (eIF4EHP^{RNAi}), TM3/UAS-eIF4EHP_RNAi (control) (upper panels) or Tub-Gal4/UAS-mCherry_RNAi (m-cherry^{RNAi}) (lower panel) females was assessed by negative geotaxis assay in normoxic conditions (21% O_2) or 40 min and 48 h after an initial exposure to 1% O_2 for 24 h. Flies climbing past threshold within 120 s were counted and expressed as % of total flies. Each assay was performed in triplicate with 20 flies. Data are mean ± SD.

Source data are available online for this figure.

strongly dampened. It has been shown in human cells that a selected set of mRNAs remains associated with the translation machinery under hypoxia and escape translation inhibition (Ho *et al*, 2018). *D. melanogaster* is particularly resistant to oxygen deprivation as it is physiologically exposed to variable oxygen concentrations and all cells of the organism are exposed to the ambient oxygen concentration, making this species the ideal study model to characterize molecular processes underlying cellular adaptation to oxygen variations. In the present study, we show that as in mammalian cells, protein synthesis is strongly reduced in *Drosophila* S2 cells due to a massive disassembly of polysomes. However, the mRNA encoding LDH is actively translated under those conditions, thereby contributing to the overall increased synthesis of LDH in hypoxic conditions. In terrestrial animals, *Ldh* plays an essential role under reduced oxygen concentration by contributing to the metabolic shift to lactic glycolysis. In *Drosophila*, LDH production is essential for a wide range of physiological processes, including cell growth, cell proliferation, and response to infection (Krejčová *et al*, 2019; Li *et al*, 2019) but also contributes to various pathophysiological mechanisms (Eichenlaub *et al*, 2018; Frame *et al*, 2023). *Ldh* expression is strongly induced at the transcriptional level under hypoxia (Li *et al*, 2013). Here, we show that the 3′UTR of *Drosophila Ldh* mRNA is necessary and sufficient to activate translation under hypoxia. While 3′UTR-dependent translation under hypoxia of selected mRNAs has been previously reported in mammalian cells (Uniacke *et al*, 2012), our study points for the first time to the role

of *Ldh* mRNA 3′UTR in translation activation. Within *Ldh* mRNA 3′UTR, we identified a 32 nucleotides CA-rich sequence necessary for translation under hypoxic conditions. Moreover, insertion of multiple CA-rich motifs in a reporter mRNA is sufficient to promote hypoxic translation.

Interestingly, a CA-rich element was previously identified as a regulator of mRNA translation under hypoxia in the human *VEGFA* mRNA (Ray *et al*, 2009). This element is part of the 3′UTR Hypoxia Stability Region (HSR) which also contains a GAIT-type secondary structure consisting of a stem loop with an asymmetric internal bulge. The HSR sequence of *VEGFA* mRNA undergoes a conformational switch in response to hypoxia mediated by the binding of hnRNPL to the CA-rich region and leads to the sustained expression of *VEGFA* under hypoxic conditions in human monocytes. As CA-rich motifs appear sufficient to promote translation under hypoxia, we can hypothesize that translation of *Ldh* mRNA operates through a different molecular mechanism. Interestingly, CA-rich motifs are found in the 3′UTR of *Ldha* genes from zebrafish, mouse and human, thereby suggesting the evolutionary conservation of these motifs in vertebrates.

Our data identify the translation initiation factor eIF4EHP as an important element of the translation machinery under hypoxia. Hence, eIF4EHP deficiency strongly impairs *Drosophila* development under hypoxia and significantly reduces fly recovery after hypoxic treatment, thereby demonstrating the physiological importance of eIF4EHP in *Drosophila* adaptation to oxygen variations. eIF4EHP belongs to the family of cap-binding eIF4E proteins. In contrast to the canonical cap-binding protein eIF4E1, eIF4EHP displays a reduced affinity to the cap structure and does not interact with eIF4G (Hernández *et al*, 2005). So far, it has been reported to act as a translation repressor by interacting with RNA-binding proteins recruited on mRNA 3′UTR and competing with eIF4E1 binding to the cap structure of these mRNA (Kong & Lasko, 2012; Hernández, 2022 for review). As eIF4EHP does not interact with eIF4G1 to form the active eIF4F cap-binding complex, the recruitment of 43S preinitiation is blocked. In contrast, eIF4EHP is weakly bound by 4E-BP proteins, thereby escaping the 4E-BP-mediated sequestration induced by hypoxia (Zuberek *et al*, 2007). Interestingly, the human homolog of eIF4EHP, eIF4E2, is involved in translation in human cells under hypoxia (Uniacke *et al*, 2012), indicating that the translation activating role of eIF4EHP in hypoxia has been conserved across evolution. In this context, human eIF4E2 can recruit eIF4G3 to promote hypoxic translation (Ho *et al*, 2021). In *Drosophila*, of the three identified members of the eIF4G protein family, eIF4G1 does not interact with eIF4EHP (Hernández *et al*, 2005), eIF4G2 is mainly expressed in testis (Franklin-Dumont *et al*, 2007) and Nat1 does not contain an eIF4E conserved binding domain (Yoshikane *et al*, 2007) suggesting that initiation of *Ldh* mRNA translation may depend on alternative factors. In vertebrates, eIF4E2 can also interact with the threonyl-tRNA synthetase (TRS) to form a cap-dependent alternative translational initiation complex that promotes the specific translation of mRNAs required for vascular development. The latter interaction is vertebrate-specific and therefore cannot account for the translational activity of eIF4EHP described here (Jeong *et al*, 2019). Several eIF4EHP-binding proteins have been identified in *Drosophila*. However, whether acting on global translation or regulating specific mRNA, most of these factors mediate translational repression and contribute to eIF4EHP migration in cytoplasmic granules such as P bodies or stress granules (reviewed in Hernández *et al*, 2005). Our results showing eIF4EHP distribution in cytoplasmic granules distinct from P bodies and stress granules further indicate that eIF4EHP plays a different role in the context of oxygen deprivation. Therefore in *Drosophila*, a putative interaction partner of eIF4EHP mediating 40S subunit recruitment under hypoxia remains to be identified.

The 3′UTR-dependent translation mechanism under hypoxia reported by Uniacke *et al* (2012) in human cells was shown to involve the formation of a complex composed of eIF4E2, eIF4A, HIF2α, and the RNA-binding protein RBM4. The interaction of RBM4 with CGG motifs in mRNA 3′UTR contributes to selective mRNA translation by its ability to recruit the hypoxia-induced HIF2α and form of a translation-competent complex including eIF4E2. However, in mammals, a cluster of RNA-binding proteins also contributes to remodel the translation efficiencies of substantial mRNA populations as part of the hypoxic response (Ho *et al*, 2020). HIF2α belongs to the family of the HIF transcription factors activating the expression of several genes at the transcriptional level upon oxygen deprivation in mammals. It is worth noting that *Drosophila* has no homolog of HIF2α, with the HIF1α protein Sima being a master regulator of hypoxia-induced genes (Graham & Presnell, 2017). While it is found to interact with several components of the translation machinery (Vinayagam *et al*, 2016), Sima has not been identified to interact with eIF4EHP. Here, we show that *Ldh* mRNA is translated by a 3′UTR-dependent mechanism and involves the direct binding of eIF4EHP. At this point, we can hypothesize that eIF4EHP interacts with factor(s) promoting the assembly of a translation initiation complex via the presence of CA-rich motifs present in mRNA 3′UTR. Further work is in progress to characterize this newly identified 3′UTR-dependent mechanism involving eIF4EHP.

While cap-dependent translation relying on the assembly of eIF4F complex including eIF4E1 and eIF4G1 has long been thought to be the unique recruitment mode of the translation 43S preinitiation complex, several non-canonical mechanisms involving alternative cap-binding factors have been described (Uniacke *et al*, 2012; Lee *et al*, 2016; Jeong *et al*, 2019; Weiss *et al*, 2021). These mechanisms play a major role in signaling-induced modifications of the translatome by promoting translation of specific mRNA. Our study shows that such mechanism is essential in *Drosophila* adaptation to oxygen deprivation.

Materials and Methods

Reagents

DNA oligonucleotides were purchased from Sigma-Aldrich. Puromycin, hygromycin, and neomycin were purchased from InvivoGen. pAC-sgRNA-cas9-puro and pAC-y1sgRNA-cas9-puro plasmids (Bassett *et al*, 2014) were obtained from Addgene (# 49330 and 49331). Anti-LDH (H-160) antibody was purchased from Santa Cruz (sc-33781). Anti-LDHA was purchased from Invitrogen (PA5-26531) or from Abcam (Ab130923). Anti-actin (A2066) and anti-puromycin antibodies (clone 12D10, Merck) were purchased from Sigma-Aldrich. eIF4EHP mouse monoclonal antibody was produced as previously described (Twyffels *et al*, 2013). HRP-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Amersham Biosciences and Jackson ImmunoResearch Inc., respectively.

Cell culture and hypoxia treatment

Non-adherent *D. melanogaster* S2 cells were kindly provided by the laboratory of Pr. Neal Silverman (UMass, USA) and grown in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum under normoxia (21% O_2) or hypoxia (1% O_2) induced by N_2 gas flow controlled by an oxygen sensor (CoyLab).

Puromycin labeling

S2 cells (3.10^6 in 2 ml of Schneider medium) were incubated at 21 or 1% O_2 for 24 h. Puromycin (5 μg/ml) was added to cultures and cells were further incubated for 20 min before lysis in EBC buffer (Tris 50 mM pH8, NaCl 125 mM, NP-40 0.5%, Na β-glycerophosphate 50 mM, NaF 50 mM, Na_3VO_4 200 μM, protease inhibitor cocktail (Roche)). Equal amount of protein extracts was analyzed by western blot analysis with anti-puromycin antibody. Actin was measured as loading control.

Protein extraction and Western blot

For S2 cells, protein extracts were prepared by cell lysis in EBC buffer and protein concentration was measured with Bio-Rad protein assay dye reagent concentration. Protein extracts from adult flies were prepared by homogenization in lysis buffer (50 mM Tris, 2% SDS, 1 mM EDTA and protease inhibitor). Proteins were extracted from sucrose fractions by TCA precipitation. Western blots were performed according to standard techniques using the indicated antibodies. Signals were detected using the SuperSignal West Pico Detection Kit (Thermo Fisher), and images were acquired and quantified with a Licor Odyssey FC imaging system.

Generation of stable cell lines

The full-length and ΔCA *Ldh* 3′UTR were cloned into the pMT-luciferase plasmid (de Toeuf *et al*, 2018) or a plasmid containing the Luciferase coding sequence under control of the *Ldh* promoter region and transfected in combination with a neomycin-resistant plasmid (1/20 ratio). *Luc*-CA-rich 3′UTR reporter gene was obtained by inserting three copies of the *Ldh* mRNA CA-rich region (underlined in Fig 2C) downstream of the Firefly Luciferase coding sequence. Cells were selected for at least 3 weeks with neomycin (200 μg/ml). When indicated, cells were induced by $CuSO_4$ (0.5 mM) for 3 h. *eIF4EHP*- and *eIF4E6*-Cas9 and CTRL KO S2 cell lines were generated by transfection of a pAC-Cas9-puro plasmid (Bassett *et al*, 2014) in which the *eIF4EHP* (GGAGCTCCCGGT AGGGCTTCAGG), *eIF4E6* (GGGGATCCGCCGAACAAGGG) or *Yellow* (Addgene #49331) guide sequences were inserted. Cells were selected with puromycin (5 μg/ml) for at least 3 weeks. Subcloned cells were selected by limit dilution. All transfections were performed with FuGENE HD according to the manufacturer's instructions (Promega).

Sucrose gradient polysome fractionation

Stably transfected S2 cells were cultured at 21% O_2 (60.10^6 cells) or at 1% O_2 (120.10^6 cells) for 24 h. Cells were treated with cycloheximide (10 μg/ml) for 5 min, harvested, and placed on ice for 5 min. Cell pellets were resuspended in 500 μl of polysome lysis buffer (Hepes 25 mM, KCl 100 mM, $MgCl_2$ 5 mM, Nonidet P-40 0.5%, heparin 2 μg/ml, cycloheximide 10 μg/ml, RNaseOUT (Invitrogen) 100 u/ml), incubated on ice for 5 min and centrifuged at 12,000 *g* for 5 min. Lysates were loaded onto linear 15–50% sucrose gradients prepared in polysome lysis buffer and centrifuged at 260,000 *g* using a SW41 rotor for 2 h at 4°C. Fractions were collected with an ISCO Density Gradient Fractionation System (Brandel).

Adult flies were kept in normoxia or exposed to 1% O_2 for 24 h. Approximately 250 flies per conditions were lysed with a Dounce homogenizer (pestle B) in lysis buffer (Tris 10 mM pH7.5, NaCl 150 mM, $MgCl_2$ 10 mM, Igepal CA630 1%, Triton X100 1%, Na deoxycholate 0.5%, DTT 2 mM, cycloheximide 200 μg/ml, DNAse I 2u/ml, RNaseOUT 40u/ml, 1X Protease inhibitor cocktail EDTA free, heparin 10u/ml) and incubated on ice for 20 min. Extracts were cleared by two rounds of centrifugation at 10,000 *g* for 5 min. Supernatants were loaded on 15–50% linear sucrose gradients, ultracentrifuged at 260,000 *g* for 2 h at 4°C. After fractionation, total proteins were recovered by TCA precipitation, rinsed with acetone, neutralized with Tris (1M), and resuspended in Laemmli buffer.

RNA extraction and northern blot

Total RNA and RNA from sucrose gradient fractions were extracted by the Tri-reagent method. RNA from sucrose gradient fractions with heparin (55 u/ml) were extracted with the RNA Purification kit (Sigma RNB100-50RXN). Northern blots were performed as described elsewhere (Lauwers *et al*, 2009). Briefly, blots were hybridized with RNA probes *in vitro* transcribed with ^{32}P-labeled UTP (PerkinElmer Life Sciences). Radioactive signals were detected with a Phosphorimager, and signals were quantified with Quantity ONE software (Bio-Rad).

RNA quantification by real-time PCR (qPCR)

cDNA was synthesized with 1 μg of total RNA using the Prime ScriptTM RT Reagent Kit (Takara) according to manufacturer's instructions. Quantitative PCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems) using SYBR® Premix Ex TaqTM II (Takara) according to manufacturer's instructions. Expression levels were normalized to Rpl32 (ΔΔCT). The sequences of the primers used were Luc: 5′-gcctgaagtctctgattaagt-3′/ 5′-acacctgcgtcg aagatgt-3′; Ldh: 5′-caccgacatcctcaagaacat-3′/5′-gggattggacaccataag ca-3′; Rpl32: 5′-gacgcttcaagggacagtatctg-3′/5′-aaacgcggttctgcatgag-3′; CAT: 5′-tccatgagcaaactgaaacgt-3′/5′-tgtgtagaaactgccggaaatc −3′.

Reporter gene assays

SV40 and full-length *Ldh* 3′UTR sequences were subcloned into a vector expressing the Firefly luciferase coding sequence under control of the *Ldh* promoter. Luc-Ldh3′UTRΔCA reporter gene was generated by cloning a mutated 3′UTR of *Ldh* obtained by a 2-step PCR-based mutagenic procedure. Cells were transiently transfected with these reporter constructs in combination with a Renilla luciferase control plasmid. Twenty-four hours after transfection, cells were exposed to variable O_2 concentrations for another 24 h. Cells were

lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega) according to manufacturer's instructions.

Immunofluorescence and FISH experiments

For immunofluorescence experiments, S2 cells (10^6 in 1 ml) were seeded on microscope slides previously treated with concanavalin A (0.5 μg/ml) for 15 min. After the indicated treatments, cells were fixed and permeabilized as previously described (Twyffels *et al*, 2013). After antibody staining, slides were mounted in fluorescent mounting medium (DAKO, Glostrup, Denmark) supplemented with 100 pg/ml 4′,6′-diamidino-2-phenylindole (DAPI) and sealed with nail polish. For co-localization experiments, cells were transfected with pMT-GFP-Rox8 or pMT-GFP-Dcp1 using FuGENE HD according to the manufacturer's instructions and treated overnight with $CuSO_4$ (0.5 mM) before plating on slides.

For polyA$^+$ RNA FISH experiments, cells were permeabilized with cold methanol after fixation and were subsequently washed 3× with 2XSSC and hybridized with biotinylated oligo dT (1 ng/μl) in hybridization buffer (final composition: dextran 10%, BSA 0.5%, *E. coli* tRNA 1 mg/ml, 2XSSC, Formamide 25%) overnight at 42°C. After wash (3×) with 2XSCC, slides were incubated with Alexa Fluor 594-coupled streptavidin (Thermo Fisher) at 1/1,000 dilution for 1 h at room temperature. Signal amplification was performed by incubation with biotin-coupled anti-streptavidin (1/1,000) in 2xSSC-Tween20 0.2% followed by incubation with Alexa Fluor 594-coupled streptavidin. Slides were washed and mounted in DAKO supplemented with DAPI.

FISH/IF experiments were performed according to the same procedure combining the amplification steps with protein staining in 2xSSC-Tween20 0.2% followed by incubation with.

Alexa Fluor 594-coupled streptavidin and Alexa Fluor 488-coupled donkey anti-mouse IgG (Thermo Fisher) (1/1,000) in 4xSSC-Tween 20 0.2%.

Image acquisition and processing

Images were acquired with a Zeiss LSM710 confocal microscope equipped with a 63×/1.4 oil-immersion Plan-Apochromat objective. Maximum intensity projection images of Z-stacks were generated with Zeiss Zen Blue software. Signal analysis and quantification was performed with ImageJ software. Signal intensity was calculated as the signal mean intensity in individual cells subtracted from signal background. Signal heterogeneity was assessed by calculating the standard deviation of signal intensity in individual cells divided by the square root of the signal intensity. Co-localization of signals was analyzed using RGB Intensity Profile tool in ImageJ on manually drawn lines across imaged cells.

CLIP experiments

Cells cultured in normoxia (21% O_2) or hypoxia (1% O_2, 24 h) were washed once with 1 ml of cold PBS and plated on a new plate before UV crosslinking (254 nm, 150 mJ/cm^2) in a UV Stratalinker 1800 (Stratagene). The cells were collected and lysed in 0.5 ml of lysis buffer (Tris–HCl 50 mM pH 7.5, EDTA 0.5 mM pH 8, NP-40 0.5%, glycerol 10%, NaCl 120 mM, sodium b-glycerophosphate 50 mM, Na_3VO_4 200 μM, NaF 50 mM), protease inhibitor (50× diluted), RiboLock (4,000× diluted), incubated on ice for 10 min and centrifuged at 12,000 *g* for 10 min at 4°C. 50 μl were kept as input and the rest was incubated overnight with 50 μl of protein G Sepharose beads coated with anti-eIF4EHP antibody at 4°C. Beads were washed twice with NT2 buffer (Tris 50 mM pH 7.4, NaCl 150 mM, $MgCl_2$ 1 mM, NP-40 0.05%) before RNA release by proteinase K (0.6 mg/ml) treatment for 30 min at 37°C and 3 min at 95°C. RNA was extracted with 0.5 ml of TRIzol spiked with diluted *in vitro* transcribed CAT mRNA. Total RNA was resuspended in 30 μl of water. 6 μl of each sample were treated with 1 μl of DNase I (Thermo Scientific) for 30 min at 37°C before inactivation in EDTA (5 mM, pH8) for 10 min at 65°C. Reverse transcription and qPCR analysis were performed as described above.

Fly strains and maintenance

The fly strains used in the experiments were obtained from the Bloomington *Drosophila* Stock Center (BDSC), including Canton-S (RRID: BDSC_64349); y^1 v^1 (RRID: BDSC_1509); y^1 sc* v^1 sev^{21}; P{$y^{+t7.7}$ $v^{+t1.8}$ = TRiP.GL01035}attP2 (RRID: BDSC_36876, named as "4EHP RNAi"); P{w[+mC] = tubP-GAL4}LL7/TM3, Sb^1 Ser^1 (RRID: BDSC_5138 named as "Tub-Gal4"); y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8] = VALIUM20-mCherry}attP2; (RRID:BDSC_35785 named as mCherry_RNAi). Flies were maintained on BDSC standard cornmeal medium. All the *in vivo* experiments were carried out at 25°C under indicated O_2 concentrations.

Negative geotaxis assay

Age-matched controls and *eIF4EHP* knock-down female flies were exposed to 1% O_2 environment for 24 h and returned to 21% O_2. Measurement of fly mobility was performed after 40 min and 48 h of recovery time on 20 individuals per condition as previously described (Madabattula *et al*, 2015).

Statistics and graphs

Graphs were made using GraphPad Prism 8. Other statistical tests were performed as indicated in the text.

Data availability

This study includes no data deposited in external repositories.

Expanded View for this article is available online.

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Author contributions

Manfei Liang: Conceptualization; investigation. **Clara Hody:** Conceptualization; investigation. **Vanessa Yammine:** Conceptualization; investigation. **Romuald Soin:** Conceptualization; investigation. **Yuqiu Sun:** Investigation. **Xing Lin:** Investigation. **Xiaoying Tian:** Investigation. **Romane Meurs:** Investigation. **Camille Perdrau:** Investigation. **Nadège Delacourt:** Investigation. **Marina Oumalis:** Investigation. **Fabienne Andris:** Investigation. **Louise Conrard:** Investigation. **Véronique Kruys:** Conceptualization; supervision; funding acquisition; investigation; writing – original draft; project administration; writing – review and editing. **Cyril Gueydan:** Conceptualization; supervision; funding acquisition; investigation; writing – original draft; project administration; writing – review and editing.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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