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DENR•MCT-1 Promotes Translation Reinitiation Downstream of uORFs to Control Tissue Growth

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Abstract

During cap-dependent eukaryotic translation initiation, ribosomes scan mRNA from the 5′ end to the first AUG start codon with favorable sequence context[1,2]. For many mRNAs this AUG belongs to a short upstream open reading frame (uORF)[3], and translation of the main downstream ORF requires reinitiation, an incompletely understood process[1,4-6]. Reinitiation is thought to involve the same factors as standard initiation[1,5,7]. It is unknown if any factors specifically affect translation reinitiation without affecting standard cap-dependent translation. We uncover here the non-canonical initiation factors Density Regulated Protein (DENR) and Multiple Copies in T-cell Lymphoma-1 (MCT-1) as the first selective regulators of eukaryotic reinitiation. mRNAs containing upstream Open Reading Frames with strong Kozak sequences (stuORFs) selectively require DENR•MCT-1 for their proper translation, yielding a novel class of mRNAs that can be co-regulated and that is enriched for regulatory proteins such as oncogenic kinases. Collectively, our data reveal that cells have a previously unappreciated translational control system with a key role in supporting proliferation and tissue growth.

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Author Contributions S.S. performed most experiments, except as indicated below. K.S. analyzed histoblast proliferation, EcR and InR protein levels and signaling in cells and animals, and performed EcR/InR rescue experiments *in vivo*. P.C.J. assessed proliferation, translation, rRNA and tRNA levels, as well as polysome profiles of DENR-KD S2 cells, and performed *in vitro* translation assays. T.K. established inducible reporter assays in proliferating and quiescent cells (ED Figs. 7a-f, 10c). K.K.M. performed DENR-MCT-1co-IP assays (ED Fig. 10b). K.H. performed DENR RNA-IP assays (ED Fig. 4g). Y.S.C analyzed MCT-1 levels and phosphorylation (ED Fig. 10d'). K.K. established *in vitro* translation assays from S2 cells. A.A.T. performed the bioinformatic analyses.
K.E.D. and A.A.T designed the study and wrote the paper with input from G.S. All authors interpreted data, discussed results, and contributed to writing the manuscript.

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Cellular protein abundance depends mainly on mRNA translation[8]. Little is known about how translation of specific sets of mRNAs can be coordinately regulated[9,10]. mRNAs with uORFs require reinitiation[11-14], whereby ribosomes translate the uORF, terminate, and then restart translating the main ORF[1,4,6,15]. No metazoan *trans*-acting factors have yet been described that selectively affect reinitiation, enabling coordinate regulation of uORF mRNAs. Ligatin/eIF2D and the related DENR•MCT-1 complex are candidate reinitiation regulators. They associate with 40S ribosomal subunits and have domains implicated in RNA-binding and start codon recognition (Extended Data Fig. 1a). *In vitro* they can recycle post-termination complexes, recruit Met-tRNA$_i^{Met}$ to mRNAs containing viral Internal Ribosome Entry Sites[16,17], and affect movement of post-termination 80S complexes to nearby AUG codons[6]. DENR•MCT-1 was not previously implicated in reinitiation, MCT-1 is an oncogene affecting cellular mRNA translation by an unclear mechanism[18-20]. Collectively, these studies suggest DENR•MCT-1 and Ligatin might regulate translation of cancer relevant mRNAs through non-canonical mechanisms.

To study DENR function, we generated DENR/CG9099 knockout *Drosophila* lacking transcript or protein (DENR^{KO}, ED Fig. 1b-d). DENR^{KO} die as pharate adults with a larval-like epidermis (Fig. 1a), due to impaired proliferation of histoblast cells (Fig. 1b, ED Fig. 1e). This is rescued by expressing DENR ubiquitously (Tubulin-Gal4), or specifically in histoblast cells (escargot-GAL4) (X^2–test $p<0.05$, ED Fig. 1f). While DENR is expressed ubiquitously (ED Fig. 1g), quickly proliferating histoblast cells appear more sensitive to DENR loss than non-proliferating tissues. DENR^{KO} also have crooked legs and incorrectly rotated genitals (Fig. 1c, ED Fig. 1h-h'). These phenotypes are not observed in mutants with generally impaired translation (*Minutes*[21]), but are found in flies with reduced cell cycle regulators or Ecdysone Receptor signaling[22,23], suggesting that DENR affects translation of a subset of mRNAs involved in cell proliferation and signaling.

Similar phenotypes were observed in flies expressing RNAi targeting MCT-1/CG5941 (ED Fig. 1i), which like human MCT-1 binds DENR (ED Fig. 1j). Reducing Ligatin gene dosage in DENR^{KO} flies caused fewer animals to reach pupation (X^2–test $p<0.05$, ED Fig. 1k) indicating that DENR^{KO} phenotypes result from loss of DENR•MCT-1 with Ligatin-like activity.

DENR^{KO} larvae and DENR knockdown (DENR-KD) S2 cells grow slowly with reduced protein accumulation rates (Fig. 1d-e, g). Mutant polysome profiles show reduced polysome/monosome (p/m) ratios (Fig. 1f, h, ED Fig. 1l-n'), suggesting defective translation initiation. Despite more ribosomes and initiator tRNA per cell (ED Fig. 1o-p), DENR-KD cells have reduced protein synthesis rates when proliferating (Fig. 1i). When quiescent, DENR-KD cells no longer display these phenotypes, and become enlarged compared to controls (Fig. 1h, i', ED Fig. 1q-r). Thus DENR promotes translation of cellular mRNAs in proliferating but not quiescent cells.

We identified ~100 mRNAs requiring DENR for efficient translation by profiling actively translated mRNAs from 80S and polysome fractions of control and DENR-KD cells and normalizing to total mRNA (Suppl. Table 1). We further analyzed myoblast city (mbc) because it was the second most under-translated mRNA and we could obtain antibody to detect it. Quantitative RT-PCR confirmed that mbc mRNA is under-represented in polysomes of DENR-KD cells (Fig. 2a), leading to reduced mbc protein but not mRNA (Fig. 2b-b'), whereas other proteins were not reduced (ED Fig. 2a). The mbc 5′UTR was sufficient to impart DENR-dependence to an RLuc reporter (ED Fig. 2b). This DENR-dependence requires the 5′ cap and is not accompanied by a drop in general translation (ED Fig. 2c-d', 2c-c'). Combined knockdown of DENR and MCT-1 had no additive effect, as they are a functional complex (ED Fig. 2e). In sum, the DENR•MCT-1 complex selectively promotes cap-dependent translation of mbc via its 5′UTR.

Systematic 5′UTR truncations (ED Fig. 2f-h) identified 175-nt necessary and sufficient for DENR-dependence (Fig. 2d, ED Fig. 3a-b), containing 3 uORFs with strong Kozak sequences (stuORFs, red boxes Fig. 2d). Mutating all three stuORF ATGs, or their Kozak sequences, abolished DENR-dependence (Fig. 2e-f, ED Fig. 3c), indicating translation initiation on these stuORFs is necessary for DENR-dependence. No additional *cis*-acting sequences were necessary; removing sequences upstream, downstream, or between the uORFs, or mutating the uORF coding sequences, did not affect DENR-dependence (Fig 2g, ED Fig. 2f-h). Two possible explanations are: 1) DENR promotes bypass of stuORF initiation codons; 2) DENR affects reinitiation after stuORF translation. In model 1, the stuORF stop codon is irrelevant because DENR would act at the stuORF start codon. In model 2 the stuORF stop codon is crucial since translation reinitiation on the main ORF only occurs after termination on the stuORF. Two point mutations removing the stuORF stop codons completely abolished DENR-dependence (Fig. 2h, ED Fig. 3d), indicating that DENR promotes translation reinitiation.

Introducing synthetic stuORFs into a control reporter was sufficient to impart DENR-dependence, with multiple stuORFs acting additively (Fig. 3a-a'). Reinitiation efficiency is reportedly inversely related to uORF length, presumably because initiation factors dissociate from ribosomes as elongation proceeds[7]. Consistently, the ability of DENR to promote reinitiation dropped as uORFs became longer (Fig. 3a'), reaching zero effect on a dicistronic transcript containing a long upstream ORF (not shown). In sum, mRNAs display a continuum of DENR-dependence, depending on the number and length of the uORFs and the strength of their Kozak sequences. A computational search revealed thousands of 5′UTRs containing uORFs (ED Fig. 4a). We generated a predicted "DENR-dependence score" for all transcripts based on the number of uORFs they contain and the strength of their Kozak sequences (ED Fig. 4b-b', Suppl. Table 2). Transcripts with high "DENR-dependence scores" were significantly enriched amongst the mRNAs with reduced translation upon DENR-KD (ED Fig. 4c-d), suggesting a general mechanism. We tested 10 5′UTRs predicted to be DENR-dependent using luciferase assays. Six conferred DENR-dependence (Fig. 3b), and four inhibited reporter translation too strongly to test experimentally. Conversely, 16 5′UTRs without uORFs were not DENR-dependent (Fig. 3c and not shown). Therefore, 5′UTRs with 'stuORFs' are DENR-dependent, identifying a new

class of transcripts whose translation can be co-regulated. Gene Ontology analysis[24] revealed that these genes are enriched for transcriptional regulators and kinases (ED Fig. 4e-f).

Immunoprecipitation of DENR showed that it binds mRNAs containing or lacking stuORFs (ED Fig. 4g), suggesting it interacts generally with initiating ribosomes, but is required on stuORF-containing mRNAs. Since only 15% of genes contain stuORFs, we were surprised to see global effects on polysomes upon DENR-KD (ED Fig. 1l). A DENR-KD timecourse revealed that stuORF-dependent translation drops prior to changes in polysome or ribosome levels (ED Fig. 5), indicating that these are likely secondary consequences.

Since Insulin and Ecdysone receptors (InR and EcR) contain DENR-dependent 5′UTRs (Fig. 3b), we asked if impaired InR and EcR translation contribute to $DENR^{KO}$ phenotypes. Loss of DENR•MCT-1 function in S2 cells or $DENR^{KO}$ animals leads to reduced InR and EcR proteins, but not mRNA levels, and reduced InR and EcR signaling (Fig. 4a-c, ED Fig. 6a-c'). Reconstituting InR/EcR expression in $DENR^{KO}$ animals partially but significantly rescued developmental rate and histoblast proliferation (Fig. 4d-e, ED Fig. 6d). Thus, loss of DENR•MCT-1 causes reduced InR/EcR translation and signaling, and consequently impaired cell proliferation and organismal development.

Data from $DENR^{KO}$ flies and DENR-KD cells suggested that proliferating cells are phenotypically more sensitive to DENR loss-of-function than quiescent cells. One explanation could be that DENR activity is low in quiescent cells, hence its removal has little effect. Using mbc and stuORF reporters, and endogenous mbc translation, as readouts for DENR activity revealed that DENR loss had a larger impact in proliferating compared to quiescent cells (Fig. 2i, ED Fig. 7). Hence DENR•MCT-1 present in quiescent cells is not very active.

To study DENR function *in vivo*, we generated flies carrying fluorescent reporters with or without a stuORF (ED Fig. 8a). These reporters have identical promoters, 5′UTRs and 3′UTRs, and are integrated in exactly the same genomic locus via phiC31-mediated recombination, ensuring their identical transcription. This revealed that DENR promotes stuORF reporter, but not control reporter, expression in animals (ED Fig. 8). Since stuORF-GFP reporter expression is entirely DENR-dependent, it serves as an *in vivo* DENR activity readout. Interestingly, the larval anterior, which contains proliferating tissues like brain and imaginal discs, shows stronger DENR activity (ED Fig. 8b). Inclusion of an RFP normalization control *in trans*, analogous to a dual-luciferase assay setup, revealed high DENR activity (stuORF-GFP/normalization-RFP) in proliferating tissues (brain and imaginal discs), and low activity in tissues with growing, but non-proliferating cells (salivary gland and fat body, ED Fig. 9).

We wondered how DENR•MCT-1 activity is regulated. Neither DENR protein levels nor DENR-MCT-1 binding dropped in quiescent S2 cells (ED Fig. 10ac). Phosphorylation of T82, T125 and a double-phosphorylation on T118/S119 in human and fly MCT-1 have been observed[25]. Using cells where endogenous MCT-1 is knocked-down via its 3′UTR and then reconstituted with MCT-1 versions lacking the endogenous 3′UTR revealed that mutations

blocking T118/S119 phosphorylation abolished MCT-1 activity (ED Fig. 10d-d'). Interestingly, T118/S119 are evolutionarily conserved to humans. Although MCT-1 was observed to be phosphorylatable *in vitro* by Erk and Cdc2[26], we could not observe an effect of Erk, Cdc2, PI3K, Akt or TORC1 inhibition on stuORF reporter expression (ED Fig. 10e-g). Further work will be required to identify upstream kinases regulating DENR•MCT-1.

We have identified a new translational control system regulating an abundant class of mRNAs, featuring: 1) stuORFs as the critical *cis*-element, 2) DENR•MCT-1 as the *trans*-acting factor, and 3) proliferation as an important cellular context. This system differs fundamentally from GCN4/ATF4 paradigms both mechanistically and functionally. Unlike GCN4-type mechanisms[1,2,5,27-30], DENR•MCT-1 functions in non-stressed cells, when general translation is not compromised, and independently of uORF to main-ORF distance (Fig. 2h), to promote proliferation. Importantly, DENR•MCT-1 uncouples translation reinitiation from standard initiation, since it is not required for initiation (Fig. 2c'). In contrast, GCN4-type mechanisms rely on coupling of initiation and reinitiation to antagonistically regulate GCN4/ATF4 versus all other genes (supplemental discussion). Our results suggest that reinitiation can be independently controlled via DENR•MCT-1 to modulate translation of a specific group of mRNAs.

Full Methods

Fly stocks

Escargot-GAL4, UAS-nuclear-GFP, UAS-cytoplasmic-GFP (Kyoto DGRC); UAS-MCT-1-dsRNA (Vienna Drosophila RNAi Center).

DENR knockout fly generation

DENR knockout flies were generated by homologous recombination as described [31]. To generate the knockout construct, upstream and downstream genomic flanks were amplified using oligos listed below and cloned into the NotI and AscI sites of pW25 [31] respectively.

Antibodies and immunoblotting

Phospho-dAkt(T342) antibody was developed in collaboration with PhosphoSolutions. Anti-mbc antibody kindly provided by Susan Abmayr[32]. Anti-RpS6, anti-total Akt, anti-phosphoS6K (Cell Signaling); anti-tubulin and anti-EcR (Developmental Studies Hybridoma Bank). Anti-dFOXO[33] and anti-InR[34]. Anti-dDENR and anti-MCT-1 antibodies used in this study were either generated at DKFZ by immunizing guinea pigs with recombinant HIS-tagged full-length dDENR or MCT-1 or at Eurogentec by immunizing rabbits with two DENR-derived peptides selected to be specific and immunogenic (latter are a kind gift of Matthias Hentze). Rabbit polyclonal antibodies to *Drosophila* ligatin/eIF2D were raised to peptides from N- and C-terminal parts of the protein by Eurogentec (Köln, Germany), affinity purified and used 1:200 for western blotting. Mouse anti-tubulin antibody (Sigma; 1:20,000). Goat anti-rabbit-HRP and goat anti-mouse-HRP secondary antibodies from Thermo Fisher Scientific and were used at 1:4000-1:20,000 depending on the primary antibody.

Immunoblotting to nitrocellulose or PVDF was performed either with wet transfer under standard conditions[35] or using an iBlot rapid transfer device (Life Technologies) according to the manufacturer's guidelines. Blots were blocked in 5% milk/TBS-T solution and probed with antibodies diluted as indicated in this solution or in TBS-T without milk. Signals were visualized using Super Signal Dura or Femto reagent (Thermo Fisher) and imaged on a Fujifilm LAS-4000 luminescent image analyzer.

Plasmids, cloning and in vitro transcription

Sequences of all oligos used for cloning are provided in a table below. All constructs were verified by sequencing.

For most plasmid luciferase reporters, 5′UTRs were isolated by PCR using gene-specific primers as PstI-BstBI products (except the 5′UTRs of snoo and rbp6 which were isolated as PstI/ClaI products), and cloned into the respective sites of pAT1152, which contains the hsp70 basal promoter followed by a polylinker, the renilla luciferase (RLuc) ORF and an SV40 polyadenylation signal. This cloning strategy retained the identical Kozak sequence for the RLuc ORF in all constructs. RLuc reporters were cotransfected with an equivalent firefly luciferase (FLuc) reporter containing the same hsp70 basal promoter, an FLuc ORF and the same SV40 polyadenylation signal (pAT1088). Mutations of the uORF ATGs in the mbc 5′UTR were generated by mutating a single nucleotide for each ATG by point mutagenesis (oligo sequenes in table below), in a manner predicted by mfold [36] to not disrupt the secondary structure of the mbc 5′UTR. Likewise, mutagenesis of uORF Kozak sequences, uORF coding sequences and the intervening sequence between uORFs 218/248 and uORF338 were done by site-directed PCR mutagenesis using oligos described in the table below. Introduction of synthetic uORFs into an RLuc reporter containing a control 5′UTR was done as follows. The RLuc reporter containing the CG43674 5′UTR was mutagenized by site-directed PCR mutagenesis to introduce SpeI and AgeI sites into the middle of the 5′UTR. uORFs were then introduced by oligo cloning into the SpeI and AgeI sites. The cloned oligos (sequences in table below) introduce a Kpn21 site at the 5′ end of the insert, which is compatible with AgeI. This allows repeated rounds of oligo clonings using the SpeI and Kpn21 sites to introduce tandem copies of the uORFs.

For stable cell line generation, the full ORF of dMCT-1 was cloned into pMT/V5-HisA vector (Life Technologies) containing a copper-inducible metallothionein promoter, C-terminal V5 epitope tag and polyhistidine affinity tag and SV40 late polyadenylation signal using Kpn I and Xho I sites.

For inducible luciferase reporters, a complete ORF of firefly luciferase was cloned into pMT/V5-HisA (Life Technologies) using EcoR I and Xho I sites. Constructs for inducible synthetic Rluc reporters were generated by subcloning of the CG43674 5′UTR with or without 1aa uORF from corresponding original plasmids in pAT1152 into pTK122 vector (pMT-Renilla with a backbone BstB I site eliminated) using a polylinker EcoR I site and the BstB I site in RLuc.

For luciferase assays with in vitro transcribed mRNA reporters, a synthetic poly(A)$_{72}$ was introduced into the basal FLuc and RLuc reporter plasmids (pAT1088 and pAT1152

respectively) by oligo cloning, followed by the actin 5′UTR, yielding pSS72 and pSS73 - the normalization control plasmid and the negative control plasmid respectively. The actin 5′UTR was replaced with the mbc 5′UTR to generate pAT1337 (oligo seqs below). The mbc ΔATG version of this construct was generated by PCR mutagenesis. Coding sequences + 5′UTR + 3′UTR were PCR amplified from the pAT1152 backbone versions using the same T7 promoter forward primer and a RLuc reverse primer as for the WT Mbc 5′UTR . This fragment was digested with BglII and BstBI and cloned into the respective sites of pMbc WT to generate pCJ1. Plasmids were linearized using a HindIII site directly downstream of the synthetic poly(A) tail for run-off transcription. In vitro transcription reactions to yield capped and A-capped mRNAs were performed as previously described [37].

Cell culture , RNAi , transfection, and reporter assays

S2 cells were grown in flasks at 25°C in either Express-Five serum-free medium (Life Technologies) or Schneider's medium (Life Technologies or Bio&Sell) supplemented with 10% FBS (Life Technologies).

For suspension culture RNAi experiments, S2 cells grown semi-adherently at 25°C were resuspended at 2×10^6 cells/mL in Schneider's Medium without FBS and dsRNA was added to 15μg/mL. Cells were transferred to a T-25 flask and incubated semi-adherently for 1.5 h at 25°C. Subsequently Complete Schneider's medium was added (2 mL per 1 mL of dsRNA-treated culture), and cells were grown semi-adherently for 4 days. Cells were diluted to 0.7×10^6 cells/mL, the surfactant Pluronic F-68 (Life Technologies) was added to a final concentration of 0.1% (v/v) and cells were incubated with gentle agitation on a rocker at 25°C. DENR knockdown was efficient throughout the duration of the experiment, as quantified by immunoblotting (Extended Data Figure 7b). Cells were subsequently collected for quantification of cell density, polysome profiling, or preparation of extracts for in vitro translation assays. Cell numbers were assessed with a hemocytometer and cell viability was assessed in parallel by trypan blue staining.

For standard luciferase reporter assays with DNA or RNA reporters, S2 cells were seeded in 6-well plates and treated with dsRNAs at 12μg/mL. After 3 or 4 days of adherent culture, cells were diluted into a 96-well plate at a fixed low concentration of 90,000 cells/well. After 2 days of incubation, cells were transfected with reporters using Effectene (Qiagen) and 18 hours later they were harvested for luciferase assays.

RNAi, transfection, and reporter assays- proliferative vs. quiescent cells

To obtain proliferating versus quiescent cells in an adherent format for transfection followed by luciferase assays, 5×10^6 S2 cells were treated with 30μg of dsRNA in 2mL of medium in a T-25 flask. After 3 days, they were split into wells of a 24-well dish at a concentration of 0.5×10^6 or 2.0×10^6 cells per well in 1mL of medium and allowed to grow for 3 more days to obtain a state of proliferation or quiescence. For luciferase assays, cells were transfected without replacing the medium 1 day prior to lysis.

For inducible reporter assays, 1 ml of S2 cells at a concentration of $1{,}5 \times 10^6$ per mL was incubated in the presence of 15μg of dsRNA in medium lacking FCS for 1 hour. After that, 4 ml of complete medium was added and the culture was incubated in a T-25 flask for 3 days.

Efficiency of the knockdown was assessed by western blotting. $1,5\times10^6$ S2 cells previously treated with dsRNA were plated onto a 6-well plate at a concentration of $1,5\times10^6$ cells per well in 2mL of medium and grown overnight. On the next day, the cells were transfected with 10 ng of inducible Fluc reporter (transfection control), 20 ng of inducible Rluc reporter with either control or 1aa uORF-containing 5′UTR and 370 ng of a non-specific plasmid using Effectene transfection reagent (Qiagen). For proliferating cells, expression of luciferase reporters was induced by addtition of CuSO4 (FC= 0.1mM) 3-6 hours post-transfection. For quiescent conditions, the transfected cells were first grown for 6 days and then induced with CuSO4 (FC= 0.1mM). In both cases induction was for 18h and the cells were then harvested and lysed with 1x Passive Lysis buffer (Promega). 10 μl of cell extracts were used for dual luciferase assays (Promega) in a multiwell plate luminometer (Perkin-Elmer).

Generation of stable S2 cell lines

For stable line generation, S2 cells grown in complete Schneider's medium (Bio&Sell) supplemented with 10% FBS (Life Technologies) were plated onto a 6-well plate at a concentration of 1×10^6 per well, grown for 18h and transfected with 400 ng of pMT-MCT-1-V5His inducible construct together with 10 ng of pCoBlast plasmid (Life Technologies), which provides resistance to the antibiotic Blasticidin S. Transfection was performed using Effectene reagent (Qiagen) according to the manufacturer's protocol and stable blasticidin resistant clones were selected by incubating of the transfected cells in the presence of decreasing concentrations of Blasticidin S (Life Technologies) from 25 μg/mL to10 μg/mL over several weeks. The resulting polyclonal stable cell lines were confirmed to enable inducible transgene expression by western blotting.

Co-immunoprecipitation from proliferating vs. quiescent cells

Stable S2 cell lines with copper-inducible V5 epitope-tagged MCT-1 were grown under proliferative or quiescent conditions and MCT-1-v5 expression was induced by addition of CuSO4 (FC= 0.5mM) for 48 hours prior to harvesting for immunoprecipitation. For the quiescent state, cells were plated at a density of 10×10^6 per well in a 6-well plate and grown for 4 days prior to induction. For proliferating conditions, the cells were plated at 1×10^6 per well in a 6-well plate and grown for 2 days prior to induction. On day 3 and 4 for quiescent and day 2 for proliferating cells, control cell counts were performed to verify the proliferation/quiescent status of the cells. The cells were harvested, washed with 1xPBS, flash-frozen in liquid nitrogen and kept at −80°C.

Frozen cells were thawed and lysed for 30 min on ice with ES2 lysis buffer (100mM KCl, 20mM Hepes pH7.5, 2.5mM EDTA, 5mM DTT, 0.05% Triton X 100 with protease and phosphatase inhibitors) and spun at 10,000 rcf for 10′ at 4 degrees. Lysate input was normalized by Bradford assay. 50 uL of Protein G Dynabeads were used for each sample. Beads were blocked by washing 5x with 3% BSA in PBS, and were incubated with 20uL of either V5 or Flag antibodies in 1mL PBS-T for 1hr at RT, rotating. Beads were then washed 2x with PBS-T, and 2x with 0.1M Sodium Borate, pH9. Antibodies were coupled to the beads during 2x 30 min incubations rotating at RT in a 1 mL 20mM DMP/0.1M Sodium Borate solution. The reaction was stopped by 2x washes of 50mM glycine, and beads were

washed 3x with PBS-T. Lysate was added to the antibody-coupled beads, and samples were rotated at RT for 10 min followed by washing 3x with lysis buffer. 1X Laemmli loading buffer was added directly to the beads, and beads were boiled for 5 min at 95 degrees. Samples were run on a 15% SDS-PAGE gel, and probed using either V5 or DENR antibody.

Crosslinking and RNA-IP

S2 cells were grown to subconfluency in 10-cm dishes, two dishes were used per IP sample. Cells were washed once with PBS and cellular proteins were cross-linked with 0.5% formaldehyde in PBS for 10 min at room temperature. Further crosslinking was stopped by the addition of 0.25 M glycine (pH 7.0) for 5 min at room temperature. Cells were then scraped on ice in 1ml RNA-IP lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2, 1 mM DTT, 1% NP-40, Mini Complete protease inhibitors EDTA-free (Roche) and 0.2 U/ml RNasin (Promega)). Lysates were cleared by centrifugation (5 min at 4°C and 2,000 rpm in a table-top centrifuge) and split into input (1:5) and IP samples (4:5). The IP samples were precleared with Protein A/G UltraLink Resin (Thermo Pierce) for 1 hr at 4°C and incubated with 2 µl anti-DENR or 3 µl anti-GFP serum for 1h at 4°C. Immuno-complexes were precipitated by incubation with protein A/G beads for 1h at 4°C. Beads were washed three times with low salt RNA IP wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2), followed by 3 washes with high salt RNA-IP wash buffer (50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1.5 mM MgCl2). RNA was eluted from the beads using 1 ml µl TriFast reagent (PeqLab) according to the manufacturer's instructions. 15 µg GlycoBlue (Ambion) was added to the RNA prior to precipitation. 4 µl RNA were subjected to reverse transcription using random hexamer primers and Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions, followed by qPCR analysis.

Quantitative RT-PCR for relative mRNA levels

RNA purification was performed with Trizol reagent according to the manufacturer's recommendations. qRT-PCRs were done as previously described[15]. rp49 was used as a normalization control for experiments analyzing mRNAs. Primer sequences appear in the oligo table below.

Quantitative RT-PCR for rRNA and tRNA levels

To quantify 18S, 28S rRNA and initiator tRNA levels in control or DENR-KD cells, total RNA was extracted from an equal number of cells in the presence of a defined amount of spiked in RLuc RNA that had been in vitro transcribed. This RLuc spike-in was then used for normalization in qRT-PCR assays with primers and conditions described previously[14,16]. Primer sequences appear in the oligo table below.

Quantification of de novo protein synthesis by metabolic labeling

Nascent protein synthesis rates of DENR-depleted and control S2 cultures in either the proliferative (>= 4 independent samples) or quiescent (>= 3 independent samples) growth state were assayed using the Click-IT® L-Azidohomoalanine kit (Life Technologies) according to the manufacturer's instructions, but adapted for use with S2 cells. 2 - 6 × 10^6

cells (with or without cycloheximide present as a control) were washed in 25°C PBS , resuspended in 1mL pre-warmed Schneider's Complete *Drosophila* medium lacking methionine (Bio&Sell) transferred to 6-well plates and incubated at 25°C for methionine depletion. After 1 hour, the methionine analog Click-IT® L-Azidohomoalanine (final concentration= 50 μM) was added for nascent protein labeling and cells were subsequently incubated at 25°C for an additional 2 hours. Cells were harvested and lysed in lysis buffer (1% SDS in 50 mM Tris-HCl pH 8.0). Lysates were sonicated, centrifuged briefly to remove debris, and protein concentration in the resulting supernatant was measured by Bradford protein assay (BioRad). Between 0.8 and 7.8 μg of total protein were used for the chemoselective "click" reaction (20 min at RT) between the azide (l-azidohomoalanine) and alkyne (tetramethylrhodamine, TAMRA). Proteins were pelleted by methanol precipitation, solubilized in NuPAGE LDS sample buffer (Life Technologies) and incubated for 10 min at 70°C. Equal amounts of total protein were run on 4-12% Bis-Tris NuPAGE gels (Life Technologies) and TAMRA signals for nascent proteins were detected using the Fujifilm Fluorescent Image Analyzer FLA-9000 system. For quantification of total protein for normalization, gels were subsequently stained with SYPRORuby (Life Technologies) and re-scanned on the FLA-9000. TAMRA and SYPRORuby signals for each sample were quantified using FLA-9000 software and processed in Microsoft Excel.

Polysome profiling from S2 cells

Cells growing in suspension with gentle rocking were first treated with cycloheximide (CHX) to freeze polysomes prior to lysis. CHX (final conc.=100 μg/mL) was added to cultures followed by further incubation for 30 minutes at 25°C on a rocker. Cells were counted and equal cell numbers were subsequently centrifuged at 800 × g for 3 min. The supernatant was discarded and the cell pellet was washed 2 times with 1x PBS, pH=7.4. Cells were resuspended in 175 μL polysome lysis buffer ('PLB': 20 mM Tris, 20 mM NaCl, 3 mM $MgCl_2$, 100 U/mL Recombinant RNasin Ribonuclease Inhibitor (Promega, Mannheim, Germany) and Roche Complete Protease Inhibitor Cocktail (Roche, Grenzach-Wyhlen, Germany). 175 μL of Polysome Extraction Buffer ('PEB': PLB + 1% Triton X-100, 2% Tween-20, and 1% Na-Deoxycholate; all final conc.) was added. Lysates were mixed, incubated on ice for 10 min. and then spun at maximum speed for 10 min. at 4°C in an Eppendorf microcentrifuge. Supernatants were loaded onto 14 × 95 mm Polyclear centrifuge tubes (Seton, Petaluma, CA) containing 17.5 – 50 % sucrose gradients generated using the Gradient Master 108 programmable gradient pourer (Biocomp, New Brunswick, Canada) and centrifuged for 2.5 h at 35,000 rpm in an SW40Ti rotor in a Beckman L7 ultracentrifuge (Beckman Coulter, Krefeld, Germany). After centrifugation gradients were simultaneously fractionated and measured for RNA content using a Piston Gradient Fractionator (Biocomp) attached to a UV monitor (BioRad, Hercules, CA).

For RNA and/or protein isolation after fractionation, Trizol reagent (Life Technologies, Carlsbad, CA) was added to fractions and either protein (followed by TCA precipitation) or RNA (by Pure Link RNA Mini Kit purification (Life Technologies, Calsbad, CA)) was isolated for downstream applications according to the manufacturer's recommendations.

For quantitative analysis of individual elements (i.e. 80s monosomes, polysomes, etc.) from the polysome profiles, all traces were first exported to Microsoft Excel and adjusted as necessary to ensure matching x/y-axis scales. Traces were then exported to Adobe Photoshop and ImageJ for processing, baseline setting, cropping and pixel counting. These pixel counts were then used for determining P/M values, plot generation, and statistical analysis in Microsoft Excel.

For immunoblotting of polysome gradient fractions, proteins in fractions were precipitated with TCA, washed 2x with acetone, and resuspended in protein gel sample buffer prior to electrophoresis and immunoblotting under standard conditions.

Polysome profiling from Drosophila larvae

Polysome profiles from Drosophila larvae were performed by crushing male larvae in lysis buffer (50mM Tris pH7.4,15mM $MgCl_2$,300mM NaCl, 1% Triton-X100, 100μg/mL cycloheximide, 0.1% β-mercaptoethanol, 1x protease inhibitor coctail from Roche, 200 U/mL RiboLock RNAse inhibitor from Fermentas) on ice. Lysates were cleared by centrifugation, equalized in protein concentration and loaded onto a 17.5-50% sucrose gradient and centrifuged for 2.5h at 35000 rpm.

In vitro translation assays with extracts from S2 cells

S2 cells extracts for in vitro translation assays were generated according to a modified version of a protocol previously used for embryo translation extracts [38], adapted for smaller cell volumes. Cells were grown in suspension culture as described above to a density of 5-6 $\times 10^6$ cells/ml, harvested by low–speed centrifugation (5 min, 800×g), and cell pellets were resuspended in ice-cold PBS to wash and then respun. This PBS wash step was repeated a further 2x. After the final wash, pellet volume was carefully estimated and cells were resuspended in precisely 2 pellet volumes of DEIKM Buffer (10mM Hepes pH 7.4, 80mM K Ac 0,5 mM Mg Ac, 5mM DTT, + of Complete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche,1 tablet/10ml)) and then incubated for 30-45 min on ice. Lysis was by ~20 strokes with either a 1ml or 2ml syringe. Extracts were spun for 20min at 20,000×g at 4°C. Supernatants were carefully removed and protein concentrations were checked by Bradford assay (BioRad). Extracts were adjusted to a final concentration of 10ug/ml with DEIKM buffer as necessary and adjusted to a final concentration of 10% glycerol. Extracts with concentrations below 10ug/ml were discarded, as our experience was that they displayed higher variability. Translation extracts were aliquotted, flash-frozen in liquid nitrogen, and stored at −80°C. Translation extracts were used at a final concentration of 40% for in vitro translation reactions, which were performed essentially as described (Gebauer et al 1999). The extracts were verified to be cap and poly(A) tail responsive. We also performed a titration of mRNA concentration for each reporter mRNA and used mRNA concentrations within the linear range of translation for all of the experiments shown.

Bioinformatics

All bioinformatics are based on Flybase release 5.46 [39]. A “DENR-dependence” score was calculated based on the number of ATG start codons in the mRNA 5′UTR and the strength of their Kozaks. To predict Kozak strength, the frequency of each nucleotide found at

positions −4 to −1 relative to the ATG for 'main ORFs' in the genome was quantified, yielding a frequency table (Figure ED4d') and the corresponding consensus of cAaaAUG (Figure ED4d), similar to that of other species and the 'pregenomic' *Drosophila* consensus defined with a much smaller set of genes [40]. A score for the strength of any Kozak in Drosophila was then generated using a multiplicative model whereby the frequencies indicated in Figure ED4d' for each nucleotide position from −4 to −1 relative to the ATG were multiplied together. In this manner, 4-mer sequences frequently observed upstream of main ORF ATGs in the fly genome obtain high scores (maximum of 0.0496) whereas infrequent ones obtain low scores (minimum of 0.00012). The score from all individual ATGs in a 5′UTR were summed to reach the combined score for the 5′UTR.

Oligo Sequences and number of replicates for figure data

Full oligo sequences for all oligos used, as well as information about replicate numbers for each figure panel, are in Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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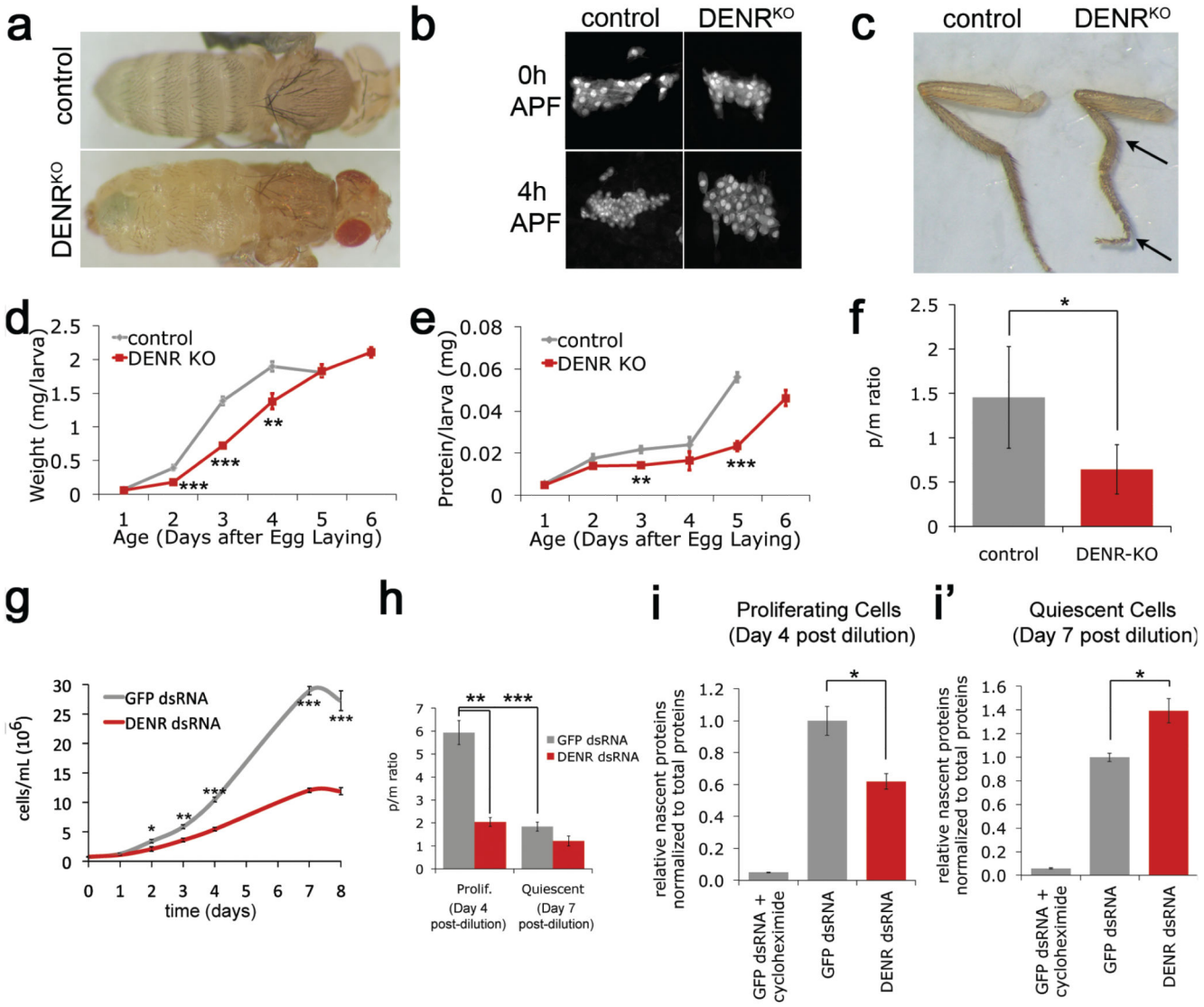

Figure 1. DENR promotes cell proliferation and boosts protein synthesis in proliferating but not quiescent cells

(a) DENR^{KO} die as pharate adults with larval-like abdominal epidermis. **(b)** DENR^{KO} anterior-dorsal histoblast nests have correct cell numbers at onset of pupation (0 h APF), but impaired proliferation the first 4 h of pupal development (4 h APF) (visualized by esgG4>GFP). **(c)** DENR^{KO} have crooked legs. **(d-e)** DENR^{KO} accrue mass (**d**) and protein (**e**) slowly, pupating with 1 day delay (day 6 datapoint, absent in controls). **(f)** Polysome profiles from DENR^{KO} larvae have reduced polysome/monosome ratios. **(g)** DENR knockdown (DENR-KD) cells proliferate slowly and enter quiescence at low cell density. **(h)** Proliferating but not quiescent polysome profiles of DENR-KD cells have reduced p/m ratios. **(i-i')** Proliferating (**i**) but not quiescent (**i'**) DENR-KD cells have reduced *de novo* protein synthesis rates, quantified by metabolic labeling with methionine analog. Error bars: Std dev (d-f) or SEM (g-I'). T-test (d-f) or Mann-Whitney U-Test (g-i') *<0.05, **<0.01, ***<0.001.

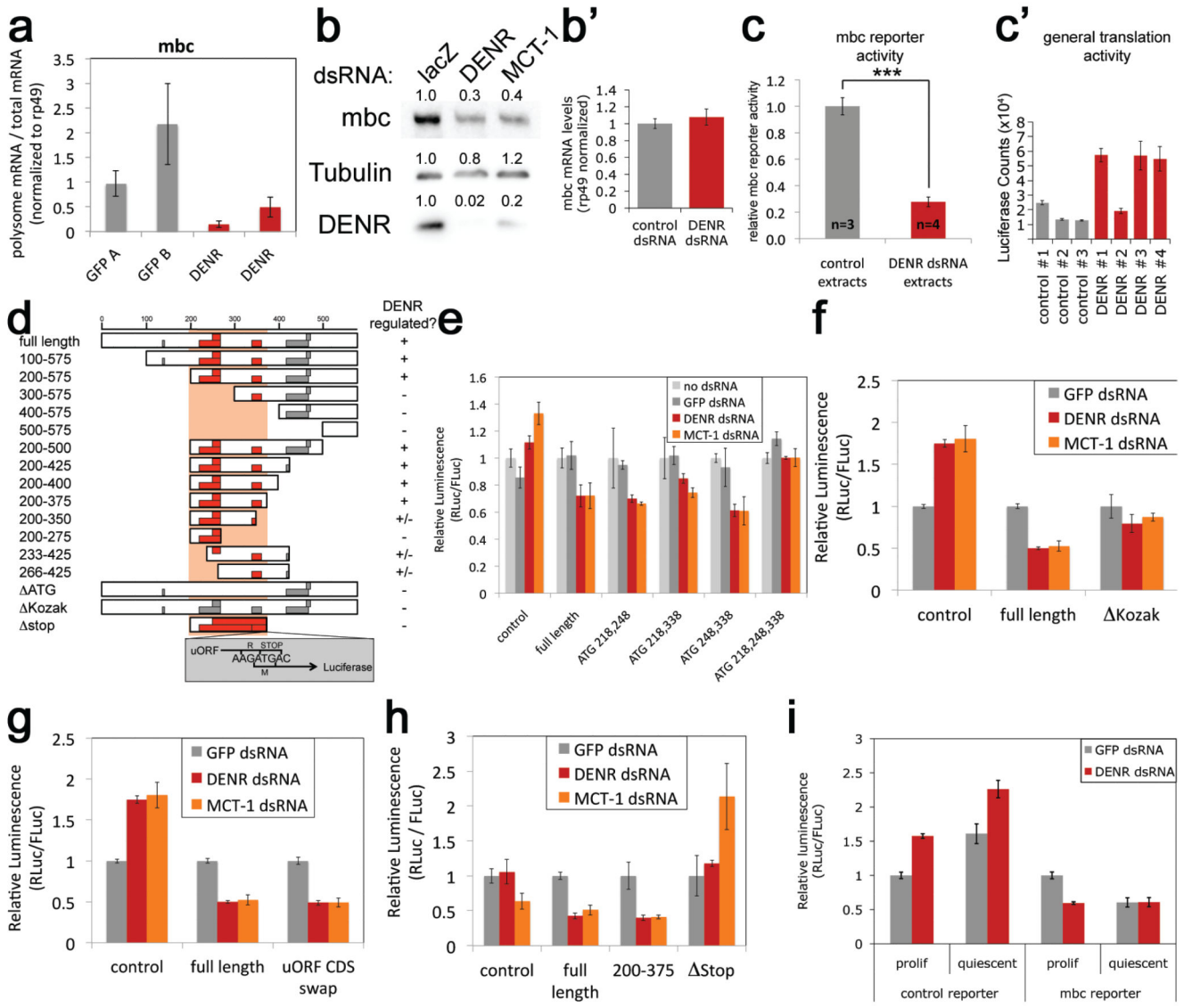

Figure 2. DENR promotes reinitiation of translation downstream of uORFs in the mbc 5′UTR

(a) qRT-PCR validation that the mbc mRNA is preferentially depleted from polysomes in DENR-KD cells (DENR A and B) compared to controls (GFP A and B). **(b-b')** mbc protein (**b**) but not mRNA (**b'**) levels are reduced in DENR and MCT-1 knockdown cells. **(c-c')** Translation extracts from DENR-KD cells are impaired in translating a reporter containing the mbc 5′UTR (**c**) but not in translating a control RLuc reporter mRNA without uORFs in the 5′UTR (**c'**). **(d)** Schematic overview of the mbc 5′UTR and the tested DNA reporter constructs, summarizing results from other panels as well as multiple (≥3) additional replicates on all the luciferase assays, not shown. Details in ED Fig. 3. **(e-f)** Mutating the start codons of the three mbc uORFs with strong Kozak sequences (**e**) or their Kozak sequences to the less functional gtgtATG (**f**) blunts regulation by DENR. **(g)** Mutating the coding sequence of mbc uORFs to poly-glutamine has no effect on DENR regulation. **(h)** Mutation of the stop codons of mbc uORFs 218, 248 and 338, as diagrammed in (d), causing the uORFs to extend past the RLuc ATG, leads to loss of DENR-dependent regulation. **(i)**

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DENR knockdown leads to impaired expression of the mbc 5′UTR RLuc reporter in proliferating but not quiescent S2 cells. Error bars: std dev. *t-test<0.05, ***t-test<0.001

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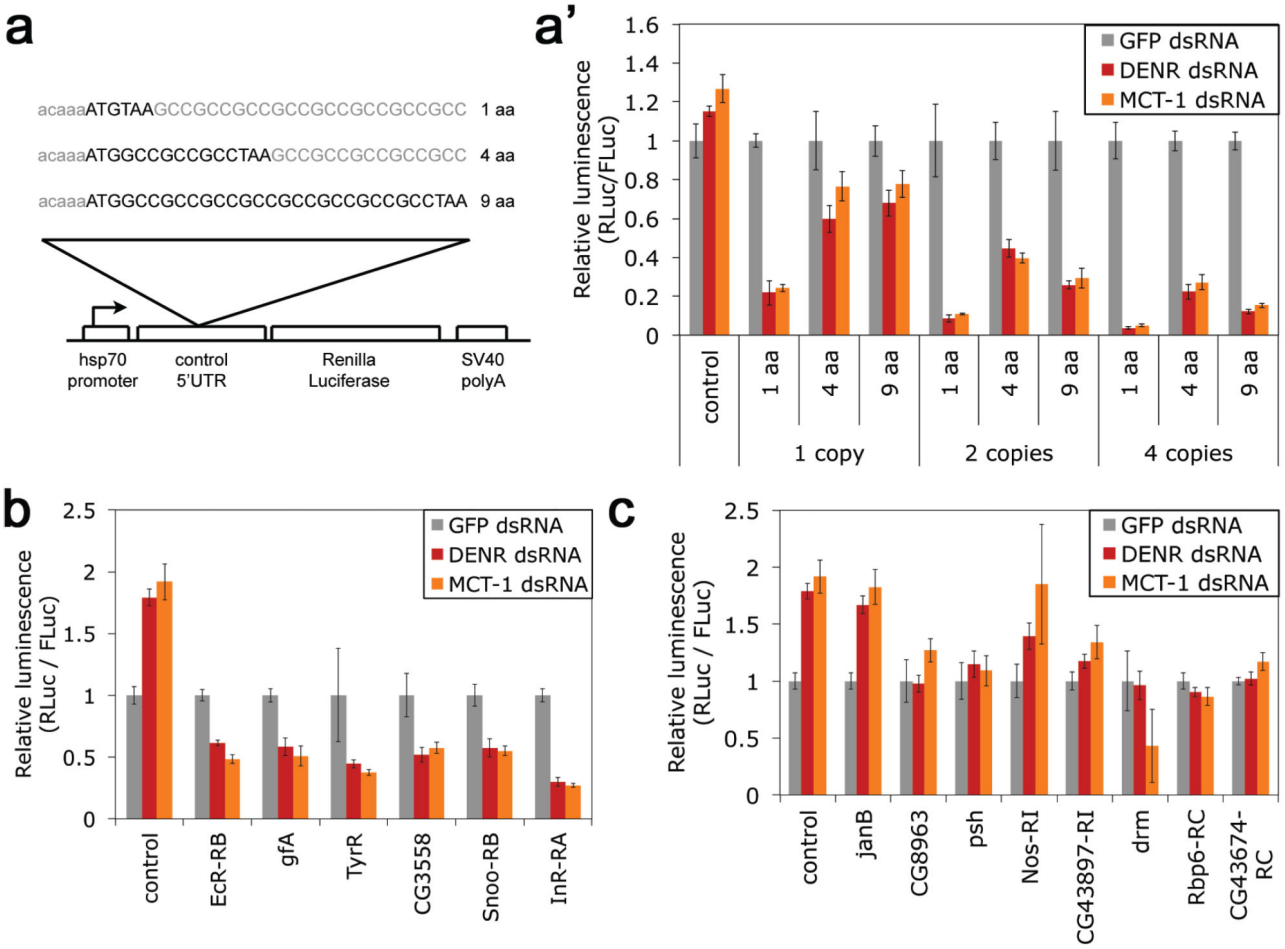

Figure 3. uORFs with strong Kozak sequences (stuORFs) are sufficient to impart DENR-dependent regulation

(a-a') Introduction of synthetic uORFs bearing a 'strong' Kozak into a control 5′UTR imparts DENR-dependent regulation (DNA reporters). **(b-c)** 5′UTRs bearing stuORFs are all DENR-dependent (**b**) whereas 5′UTRs lacking uORFs (**c**) are not. (DNA reporters). Error bars: std. dev.

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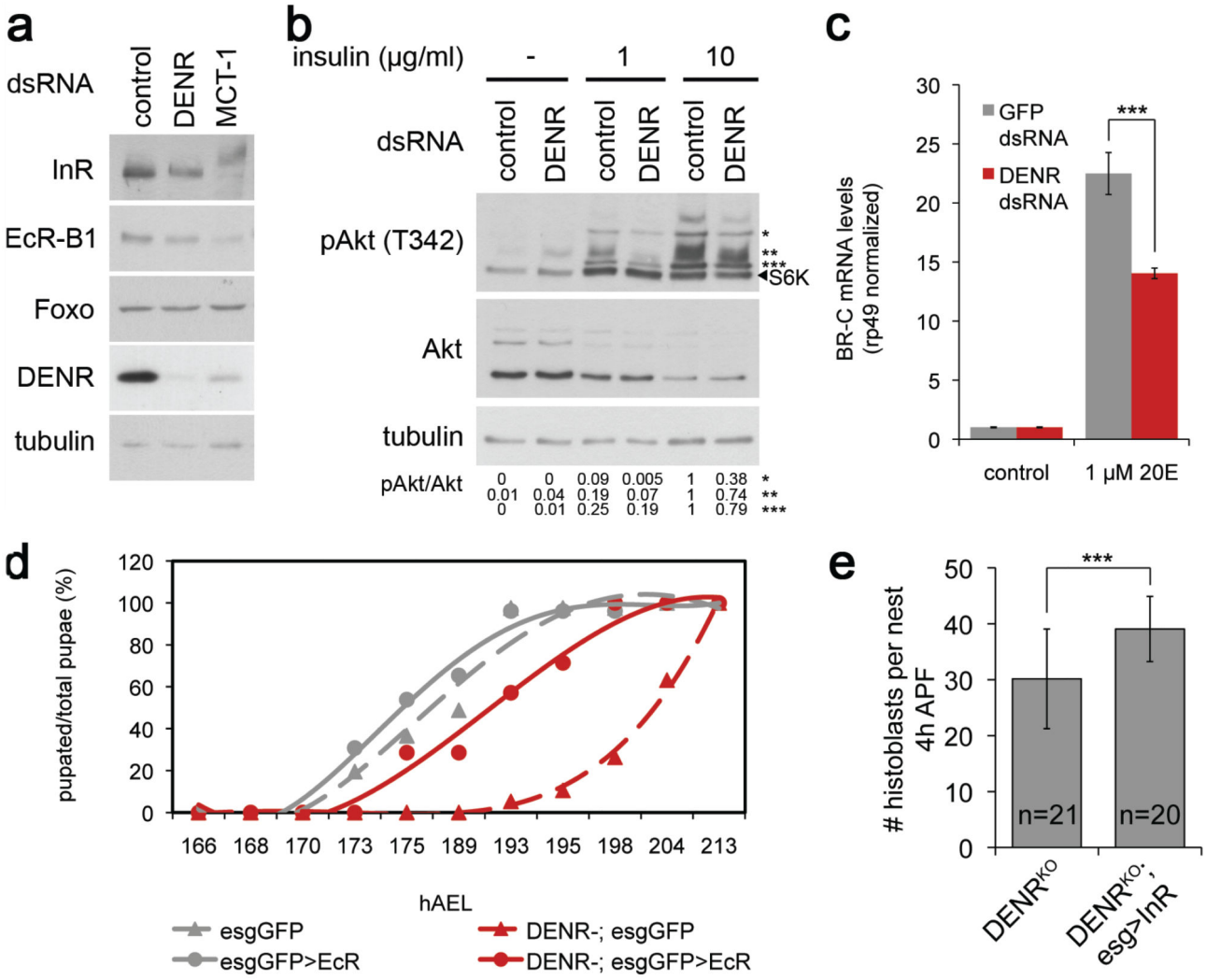

Figure 4. Loss of DENR leads to reduced InR and EcR protein levels and signaling

(a) DENR and MCT-1 knockdown cells have reduced InR and EcR protein levels. **(b-c)** DENR-KD cells are less sensitive to insulin stimulation (1 h) (**b**) and to ecdysone (1 µM, 4 h; **c**). **(d-e)** Expression of EcR (**d**) or InR (**e**) in histoblast cells and imaginal discs of $DENR^{KO}$ using escargot-GAL4 rescues their delayed pupation (**d**) and mildly but significantly their proliferation defect (**e**). Error bars: std dev. ***t-test<0.001